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KLF14 potentiates oxidative adaptation via modulating HO-1 signaling in castrate-resistant prostate cancer

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Abstract

Insights into the mechanisms by which key factors stimulate cell growth under androgen-depleted conditions is a premise to the development of effective treatments with clinically significant activity in patients with castration-resistant prostate cancer (CRPC). Herein, we report that, the expression of Krüppel-like factor 14 (KLF14), a master transcription factor in the regulation of lipid metabolism, was significantly induced in castration-insensitive PCa cells and tumor tissues from a mouse xenograft model of CRPC. KLF14 upregulation in PCa cells, which was stimulated upstream by oxidative stress, was dependent on multiple pathways including PI3K/AKT, p42/p44 MAPK, AMPK and PKC pathways. By means of ectopic overexpression and genetic inactivation, we further show that KLF14 promoted cell growth via positive regulation of the antioxidant response under androgen-depleted conditions. Mechanistically, KLF14 coupled to p300 and CBP to enhance the transcriptional activation of *HMOX1*, the gene encoding the antioxidative enzyme heme oxygenase-1 (HO-1) that is one of the most important mechanisms of cell adaptation to stress. Transient knockdown of *HMOX1* is sufficient to overcome KLF14 overexpression-potentiated PCa cell growth under androgen-depleted conditions. From a pharmacological standpoint, *in vivo* administration of ZnPIX (a specific inhibitor of HO-1) effectively attenuates castration-resistant progression in the mouse xenograft model, without changing KLF14 level. Together, these results provide comprehensive insight into the KLF14-dependent regulation of antioxidant response and subsequent pathogenesis of castration resistance and indicate that interventions targeting the KLF14/HO-1 adaptive mechanism should be further explored for CRPC treatment.

Key Words

- ▶ Krüppel-like factor 14 (KLF14)
- ▶ CRPC
- ▶ oxidative stress
- ▶ heme oxygenase-1 (HO-1)
- ▶ transcriptional regulation

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Introduction

Worldwide, prostate cancer (PCa) is the most frequently diagnosed male cancer. About one-fourth of PCa patients eventually die from this disease. Because androgen receptor (AR) signaling is crucial for the development

and maintenance of male reproductive organs including the prostate gland, men with PCa that has disseminated distantly usually respond well to androgen deprivation therapy (ADT) (either alone or in combination with

chemotherapy). However, despite initial response rates of ~80–90%, nearly all PCa patients eventually develop progressive disease following ADT. This is referred to as castration-resistant prostate cancer (CRPC), an incurable stage in which ~90% of patients develop metastases with the highest mortality rate (Ferraldeschi *et al.* 2015). Evidence from experimental and clinical studies have identified several potential mechanisms coexisting to confer CRPC, including deregulation of AR signaling (Zuo *et al.* 2016), activation of PI3K/AKT/mTOR pathway (Zhang *et al.* 2014b), perturbed lipid metabolism (Twiddy *et al.* 2011, Qi *et al.* 2013) and unbalanced oxidative stress (Shiota *et al.* 2011, Ning *et al.* 2016). In this scenario, continuous elucidation of the key molecules at different levels should strengthen our mechanistic understanding and provide valuable therapeutic guidance.

The Krüppel-like family of transcription factors (KLFs), containing 17 members and functioning as key modulators in embryogenesis and cell proliferation, differentiation and development, have recently received intensive investigations. KLFs possess highly conserved cysteine and histamine zinc fingers, recognizing and binding exclusively to the CACCC motifs (Guo *et al.* 2015). Despite their structural conservation, most KLFs engage in distinct cooperation or antagonism dependent upon the context (de Assuncao *et al.* 2014). Of particular interest, KLF14 has been identified by genetic studies to be a master trans-regulator of lipid metabolism (Civelek *et al.* 2017). A robust KLF14 expression has been tightly associated with obesity, type 2 diabetes (Bacos *et al.* 2016), breast and colon cancer (Fan *et al.* 2015). KLF14 can regulate lipid-mediated signaling through a distinct epigenetic mechanism (Fan *et al.* 2015). These studies have provided useful information regarding KLF14 function in metabolism. Considering the central involvement of KLF14 in lipid metabolism, we have focused on how KLF14 regulates the gene networks involved in lipid metabolism-related diseases. Thus, the current work aimed on defining a novel function for KLF14, involving transcriptional regulation induced by CRPC to expand our understanding of its role in metabolism regulation.

Through a combination of animal models, genetic tools and pharmacological means, we have uncovered a mechanistic role, reported here, for KLF14 in regulating the antioxidant response in CRPC by modulation of heme oxygenase-1 (HO-1) expression. Furthermore, we provide the functional evidence that *in vivo* administration of a pharmacological inhibitor of HO-1 could attenuate castration-resistant PCa progression. Overall, these findings demonstrate molecular mechanisms by which

the KLF14/HO-1 cascade could serve as a promising therapeutic target for CRPC.

Materials and methods

Cells treatment

Human PCa cell lines (LNCaP, CWR-R1, 22RV1, PC3 and DU145) and normal human prostate cell RWPE-1 were obtained from American type culture collection (ATCC). All cells used for the current study were authenticated in October 2015 by STR DNA Profiling Analysis (Promega) and had less than 25 passages. RWPE-1 cells were cultured in keratinocyte serum-free medium (Thermo Fisher Scientific) supplemented with 50mg/mL bovine pituitary extract, 5ng/mL epidermal growth factor, 100U/mL penicillin and 100U/mL streptomycin (GIBCO). Other PCa cells were routinely cultured in RPMI-1640 medium supplemented with 10% FBS (GIBCO) in a 37°C, 5% CO₂ incubator. To establish castration-resistant LNCaP sublines (designated as LNCaP-R), LNCaP cells were maintained in media containing 10% charcoal-stripped FBS (FBS-CS, GIBCO) in the presence of 10µM bicalutamide (Sigma-Aldrich) for 4 months, followed by measurement of intracellular prostate-specific antigen (PSA) and AR concentrations using commercially available ELSIA kits (Abcam), as described elsewhere (Ning *et al.* 2016). To study the effects of oxidative stress on KLF14 expression, PCa cells were incubated with H₂O₂ or diamide for various concentrations, in the presence or absence of 20µmol/L diphenyliodonium chloride (Sigma-Aldrich) for different durations, as indicated. To stably knockdown the endogenous expression of KLF14, CWR-R1 and DU145 cells were transfected with pRS-KLF14 shRNA or Scramble shRNA (OriGene, Beijing, China) using ViaFect (Promega). One day after transfection, the transfected cells were selected with 1.0µg/mL puromycin (Sigma-Aldrich) for 1–2 weeks. To overexpress the exogenous KLF14, LNCaP cells were transfected with pcDNA3.1+-DDK-KLF14 or pcDNA3.1+ vector (GenScript, Nanjing, China) using ViaFect, followed by Geneticin selection (200µg/mL; Invitrogen) according to the manufacturer's instructions. To study the signal transduction pathways involved in oxidative stress-dependent KLF14 induction, PC3 cells were incubated with 1mmol/L of H₂O₂ in the presence of different pathway inhibitors as indicated for 15 min.

CRPC patient-derived xenograft model

The study protocol, strictly following the ethical standards of *Helsinki Declaration*, was approved by the Ethics

Committee of Baoji Center Hospital. Written informed consent was obtained from the participant. Local recurrent PCa tumors were obtained from a 62-year-old patient who died of CRPC. The tissues were harvested after radical prostatectomy and were minced into 20–30 mm³ tumor bits, and transplanted subcutaneously into 5-week-old male nude mice, with 50 µL Matrigel (BD Biosciences, Shanghai, China) surrounding the tumors. The xenografts were maintained for about 10 months. Subsequently, the transplantable xenografts were harvested and transplanted to several mice without Matrigel. After the xenografts grew to ~120 mm³, the mice were surgically castrated (Terada *et al.* 2010). Tumor volume changes were recorded every week by two perpendicular diameter measurements.

Cell proliferation

CWR-R1 and DU145 cells with different transfections were plated onto a 96-well plate at 1×10^4 cells/well in media containing either 10% FBS or 10% FBS-CS (Invitrogen). After a 3-day culture, viable cell numbers were determined using Trypan blue staining (Invitrogen) along with hemocytometer count (Beckman Coulter).

Cell apoptosis

PCa cells with different transfections were cultured in media containing either 10% FBS or 10% FBS-CS for 3 days, followed by measurement of apoptosis using an apoptosis ELISA kit (Roche Diagnostics), as per the manufacturer's instructions. Cell apoptotic values were normalized to FBS-treated control cells.

Colony formation assay

PCa cells with different transfections were plated onto a 96-well plate at 400 cells/well and cultured in media containing either 10% FBS or 10% FBS-CS. When cell colonies were large enough to be clearly discerned around 14 days, the colonies were stained by a solution containing 0.5% crystal violet plus 25% methanol for 5 min. The colonies were then scored using a laboratory automatic colony counter (Biobase Biotech, Jinan, China).

In vivo tumor growth assay

Tumor growth in nude mice was performed according to a previously established protocol (Yu *et al.* 2013). PCa cells with different transfections were injected into left flanks of castrated male nude mice. The inoculated

cells were allowed to grow for 6 weeks until the tumors reached a volume of ~100–250 mm³ for CWR-R1 and DU145 cells, and of ~20 mm³ for LNCaP cells, respectively. Tumor volume changes were recorded every week by two perpendicular diameter measurements and were calculated with formula $0.5236 \times r_1^2 \times r_2$ (where $r_1 < r_2$) (Long *et al.* 2000). All procedures involving animals, strictly conformed to the *Guide for the Care and Use of Laboratory Animals*, were approved by the ACUC of Baoji Center Hospital.

Cell viability assay upon oxidative stress

Subconfluent proliferating LNCaP and DU145 cells in 96-well plates were stimulated with different doses of H₂O₂ or paraquat (Sigma-Aldrich) for different time intervals as indicated, followed by measurement of cell viability using CellTiter-Blue Reagent (Promega) and of GSH/GSSG ratio using a commercial GSH/GSSG Ratio Detection Assay Kit (Abcam), according to the manufacturer's instructions. Cell viability values were normalized to 0 dose Ctrl cells.

Promoter reporter assay

For the assessment of *HMOX1* promoter activity, the genomic fragments harboring the putative KLF14-binding sites in human *HMOX1* promoter (~1.8 kb upstream of the translation start site) were subcloned into the pGL4-Luc reporter vector (Promega) using Infusion 2.0 Dry-Down PCR cloning kit (Clontech, Shanghai, China). Promoter activity was further validated by mutation of the putative KLF14-binding site on the promoter at –406/–402 by replacing CACCC with CAaaa using the QuikChange Site-Directed Mutagenesis Kit (Agilent). For reporter assay, 0.5 µg reporter plasmid and pRL-TK Renilla reporter plasmid were co-transfected with pcDNA3.1+DDK-KLF14 or pcDNA3.1+ vector into HeLa cells using FUGENE. 48 h later, cells were treated for 15 min with 1 mmol/L of H₂O₂, 30 mmol/L of diamide or diamide plus 20 µmol/L of diphenyleneiodonium chloride, followed by luciferase activity measurements using a dual luciferase reporter assay kit (Promega).

RT-qPCR

Total RNA extraction, reverse transcription and PCR were carried out as described previously (Dong *et al.* 2016). Routine DNase (Applied Biosystems/Ambion) treatment (1 unit of DNase I/µg of total RNA) was performed before RT. The primers used were listed in Supplementary Table 1

(see section on [supplementary data](#) given at the end of this article). PCR products were subsequently quantified by SYBR Green intercalation using the MiniOpticon system (Bio-Rad). Relative expression levels from three independent experiments were determined using $2^{-\Delta\Delta Ct}$ method (Zhang *et al.* 2014a).

Immunoblotting

Total protein from PCa cells and xenografts was isolated using Chemicon Total Protein Extraction Kit (Merck Millipore), and protein concentrations were determined by a protein assay kit (Bio-Rad). Immunoblotting was carried out as described previously (Li *et al.* 2014). The antibodies used for the current study were listed in Supplementary Table 2. The specificity and effectiveness of the KLF14 antibody has been discussed in detail in previous work (Argmann *et al.* 2017).

Immunohistochemistry

A Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used for *in vivo* detection of the immunolocalization of AR and KLF14 in formalin-fixed and paraffin-embedded xenograft samples. Briefly, sections were deparaffinized, rehydrated and subjected to antigen retrieval in citrate buffer (95°C, 15 min). Sections were then incubated in 3% H₂O₂ solution in methanol at room temperature for 10 min to block endogenous peroxidase activity, followed by incubation overnight at 4°C with different primary antibodies (Supplementary Table 2). Immunostaining signals were finally developed according to the Vector Laboratories instructions.

Chromatin immunoprecipitation (ChIP) and double ChIP

LNCaP/DDK-KLF14 cells were challenged for 15 min with 1 mmol/L of H₂O₂, and cells were then crosslinked with 1% formaldehyde and sonicated. Soluble chromatin was immunoprecipitated and subjected to ChIP and double ChIP assays, as described in detail in our previous work (Zhang *et al.* 2012). Primers used for ChIP and double ChIP were listed in Supplementary Table 1.

In vivo inhibition of HO-1 activity

LNCaP-R cells were resuspended in PBS and 0.1 mL of cell suspension (1×10^6 cells/mL) and were then injected into left flanks of castrated male nude mice. On the second day

after cell inoculation, zinc protoporphyrin (ZnPP) IX from Sigma-Aldrich was dissolved in 0.25M NaOH and the final concentration was adjusted to 10 µg/µL (Hirai *et al.* 2007). For administration to mice, the stock solutions were finally diluted with PBS by 1% (v/v) for 20 µg/mouse dosage. Nude mice were treated intraperitoneally with ZnPPIX (10 mg/kg/day) or PBS vehicle control on daily basis. The volume changes of tumor xenografts were recorded every week. At the end of 6 weeks after LNCaP-R cells inoculation, mice were killed and the HO-1 activity in tumor xenografts were assayed as described below.

Determination of HO-1 activity

Tumor xenografts were homogenized in ice-cold homogenate buffer (20 mM PBS buffer, 250 mM sucrose, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride and 10 µg/mL leupeptin) as described elsewhere (Araujo *et al.* 2013). After centrifuge, the resultant supernatant was collected and subjected to the measurement of HO-1 activity using Human Heme Oxygenase 1 ELISA Kit (Abcam). Final spectrophotometry was developed in triplicate at 450 nm, as per the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using SPSS 15.0. Statistical comparisons and analyses were performed by either Student's *t* test or one-way ANOVA (Prism 5.00, GraphPad Software Inc.) followed by Tukey *post hoc* analyses. Results are presented as mean $\pm$ S.E.M. and $P < 0.05$ was considered statistically significant.

Results

KLF14 upregulation is associated with the pathogenesis of CRPC

The present study was initially designed to uncover novel mechanisms underlying the potential involvement of KLF14 in CRPC. For this purpose, we established the expression profiles of KLF14 in human PCa cells. Genetic backgrounds of different PCa cells were provided in Fig. 1A. Persistent KLF14 expression was detected in all cell lines by means of immunoblotting. However, KLF14 expression was relatively low in RWPE-1 and LNCaP cells, with higher expression observed in CWR-R1, 22RV1, PC3 and DU145 cells (Fig. 1A). Such a protein expression profile was confirmed by RT-qPCR at the mRNA level (Fig. 1B). Next, we established the castration-resistant sublines (LNCaP-R)

according to a previously validated protocol (Ning *et al.* 2016). Interestingly, when LNCaP became castration resistant, as evidenced by the persistent high production of intracellular PSA and AR in the androgen-depleted medium, LNCaP-R cells showed an evoked level of KLF14 expression (Fig. 1C, D and E). To further provide the *in vivo* evidence for the association of KLF14 upregulation with CRPC pathogenesis, we employed a CRPC patient-derived xenograft model, which progresses with elevated expression levels of PSA and AR (Supplementary Fig. 1), mimicking the clinical behavior of CRPC to the maximum extent (Terada *et al.* 2010). The xenograft tissues were harvested during androgen-dependent growth (AD), castration-induced regression nadir (RN) and castration-resistant regrowth (CR) stages (Fig. 1F). The nuclear expression of AR abated from the AD stage to the RN stage, but fully recovered at the CR stage (Fig. 1G), mimicking the clinical pathogenesis of CRPC. In line with these observations, KLF14 expression was dramatically upregulated with the progression of castration resistance in the xenografts (Fig. 1H and I). Taken together, these results suggest that KLF14 deregulation correlates to CRPC development.

Oxidative stress induces KLF14 expression

Because castration induces oxidative stress *in vivo*, understanding how reactive oxygen species (ROS) regulates the cellular processes controlling malignant transformation holds a lot of promise in combating CRPC (Zhang *et al.* 2014b). To this end, we treated LNCaP and PC3 cells with H₂O₂ that partially mimic cellular events after castration. H₂O₂ stimulation led to increased levels of KLF14 mRNA in a dose- and time-dependent manner, regardless of cell types (Fig. 2A, B, C and D). We then asked whether oxidative stress other than H₂O₂ can cause KLF14 activation, by treating cells with diamide that oxidizes cellular thiols and provokes a rapid decrease in cellular glutathione, hence causing oxidative stress (Zhang *et al.* 2014b). As expected, diamide treatment also caused KLF14 activation in a dose-dependent manner. In agreement, co-incubation with diphenyleneiodonium chloride (an inhibitor of oxidative stress) effectively abolished diamide-elicited KLF14 activation (Fig. 2E and F). To gain mechanistic insights into the regulation of KLF14 expression, we stimulated PCa cells for 15 min with 1 mmol/L of H₂O₂, in the presence of different pathway inhibitors including LY294002 (PI3K/AKT), U0128 (p42/p44 MAPK), SB203580 (p38 MAPK), SP600125 (JNK), WZ4003 (AMPK) and Go6976 (PKC).

Concomitant incubation with LY294002, U0128, WZ4003 and Go6976 significantly reduced the KLF14 upregulation induced by H₂O₂ (Fig. 2G), suggesting that KLF14 upregulation by oxidative stress may be dependent on PI3K/AKT, p42/p44 MAPK, AMPK and PKC activation.

Endogenous KLF14 protects PCa cells under androgen-depleted conditions

Having established the association between KLF14 expression and CRPC progression, we then determined the malignant status of CWR-R1 and DU145 cells upon inhibition of KLF14 with shRNA. Treatment of CWR-R1 cells with 25 or 50 nmol/L of KLF14 shRNA both led to reduced levels of KLF14 expression (Fig. 3A and B), so we selected 25 nmol/L of KLF14 shRNA for further studies. The androgen-depleted condition (FBS-CS culture)-induced growth inhibition was significantly enhanced in the CWR-R1 and DU145 cells depleted of endogenous KLF14, compared to that in cells transfected with Ctrl shRNA or cells cultured in normal medium (Fig. 3C). Subsequent ELISA methods revealed that KLF14 knockdown in CWR-R1 and DU145 cells led to notable increase of apoptosis in the presence of FBS-CS culture (Fig. 3D). Consistently, lower colony formation efficiency was after FBS-CS incubation in CWR-R1 and DU145 cells stably transfected with KLF14 shRNA (Fig. 3E). Such growth inhibition by KLF14 knockdown was also observed in xenograft models in castrated immunodeficient mice (Fig. 3F). The above-mentioned phenotype was further verified in and DU145 cells (Fig. 3G, H, I, J, K and L). Thus, repression of KLF14 expression compromises the proliferation and clonogenicity of PCa cells under androgen-depleted conditions.

Ectopic overexpression of exogenous KLF14 potentiates androgen-independence in LNCaP cells

To directly ask whether KLF14 contributes to CRPC progression, as inferred from the above-mentioned loss-of-function analysis, we stably overexpressed pcDNA3.1+DDK-KLF14 in LNCaP cells (Fig. 4A) and then cultured these cells in medium containing FBS-CS. FBS-CS-induced growth inhibition was significantly attenuated in LNCaP/DDK-KLF14 cells compared with the pcDNA3.1+vector-transfected cells (Fig. 4B). Conversely, FBS-CS-induced apoptosis was partially but effectively attenuated in LNCaP/DDK-KLF14 cells (Fig. 4C). In agreement, the colony formation efficiency (Fig. 4D) and formation of xenograft tumors in castrated immunodeficient mice

Figure 1

Stimulation of KLF14 expression during the castration-resistant progression of PCa cells. (A) *Left panel*: Genetic backgrounds of PCa cells used in this study. *Right panel*: Relative expression of KLF14 in different PCa cells was determined using immunoblotting. Densitometric scanning of immunoblots was performed in which the level of a target protein was normalized against the protein level in RWPE-1 cells, which was arbitrarily set at 1. (B) KLF14 expression in different PCa cells was evaluated using RT-qPCR. Different superscript letters denote groups that are statistically different ($P < 0.05$). (C) KLF14 expression along the generation of the castration-resistant LNCaP-R cells was assayed by immunoblotting. (D) Intracellular PSA levels of LNCaP-R cells were measured using a commercially available ELSIA kit. Different superscript letters denote groups that are statistically different ($P < 0.05$). (E) Intracellular AR levels of LNCaP-R cells were measured using a commercially available ELSIA kit. Different superscript letters denote groups that are statistically different ($P < 0.05$). (F) Sequential changes in xenograft tumor volume was recorded every week at the AD, castration-induced regression nadir (RN), and CR stages ($n = 4$), respectively. (G) AR immunohistochemistry at AD, RN and CR stages of xenograft tumors. Bar = 25 μm. (H) Immunoblotting analysis of KLF14 expression in different xenograft tumors. (I) KLF14 immunohistochemistry in different xenograft tumors. Bar = 50 μm. A full-colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0383>.

(Fig. 4E) were substantially augmented in LNCaP cells by KLF14 overexpression. Therefore, these data demonstrate that KLF14 directly promotes castration resistance in AD PCa cells.

KLF14 protects PCa cells from oxidative stress via restoring cellular redox balance

Because castration dramatically increases oxidative stress of the prostate, and KLF14 elevation leads to constitutive resistance against castration-induced cell death, we asked whether oxidative stress and KLF14 signaling functions in a reciprocal manner. To this end, we measured the effects of KLF14 alteration on the cellular sensitivity to two different oxidizing agents, namely H_2O_2 and paraquat. As shown in cell viability assays,

the pcDNA3.1+-DDK-KLF14/LNCaP cells were significantly more resistant to H_2O_2 insult, compared to the pcDNA3.1+ vector-transfected LNCaP cells. This protective effect appeared in a dose- and time-dependent manner (Fig. 5A and B). In contrast, KLF14 knockdown by stable shRNA transfection enhanced H_2O_2 -impaired cell viability in DU145 cells over a wide range of doses (Fig. 5C) and stimulation durations (Fig. 5D). Paraquat induces the excessive generation of superoxide ions (O_2^-), which are detoxified by a mechanism distinct from H_2O_2 (Bae *et al.* 2004). Interestingly, ectopic KLF14 overexpression led to more paraquat resistance in LNCaP cells (Fig. 5E), whereas KLF14 inhibition caused a significant reduction in cell viability in paraquat-treated DU145 cells (Fig. 5F). Thus, both exogenous and endogenous KLF14 protects PCa cells against distinct forms of oxidative stress. To rule out the

possibility that the protective effects of KLF14 are limited to long-term assays, we tested the effect of transient expression of pcDNA3.1+-DDK-KLF14 on the response of LNCaP cells to H_2O_2 . In accordance with the preceding phenotype in stable transfections, at H_2O_2 doses of 400–500 nmol/L, KLF14 transfection exhibited significant protective effects (Fig. 5G). Apparently, overexpression of KLF14 (by either stable or transient transfection) protects and inactivation of KLF14 by knockdown sensitizes PCa cells against oxidative stress. Because cellular response to oxidative stress depends essentially upon the redox balance, we explored the effect of KLF14 overexpression on the redox balance of LNCaP cells after treatment with different doses of H_2O_2 for 24 h. In vector-transfected LNCaP cells, H_2O_2 induced a dose-dependent shift in the redox state to increased GSSG and decreased GSH levels. Contrarily, pcDNA3.1+-DDK-KLF14/LNCaP cells were able to maintain significantly higher ratios of GSH/GSSG over a wide range of H_2O_2 doses (Fig. 5H), indicating that

KLF14 may enhance the antioxidant response upon oxidative stress.

KLF14 regulates HO-1 expression at the transcriptional level

To dissect the molecular mechanisms underlying the promoting effect of KLF14 on the antioxidant response, we examined the expression levels of a range of antioxidant genes in pcDNA3.1+-DDK-KLF14/LNCaP cells using RT-qPCR. These experiments revealed that KLF14 induced a ~5.3-fold increase in expression of *HMOX1* (Fig. 6A), the gene encoding heme oxygenase 1 (HO-1), a rate-limiting enzyme conferring cytoprotection against oxidative stress (Nitti *et al.* 2017). We confirmed the parallel upregulation of KLF14 and HO-1 expression in the CRPC patient-derived xenograft model using immunoblotting analysis (Fig. 6B). More importantly, KLF14 overexpression resulted in a significant induction of HO-1 expression,

Figure 2

Regulation of KLF14 expression by oxidative stress. (A) LNCaP cells were incubated with different concentrations of H_2O_2 for 15 min, followed by RT-qPCR analysis of *KLF14* expression. Different superscript letters denote groups that are statistically different ($P < 0.05$). (B) PC3 cells were incubated with different concentrations of H_2O_2 for 15 min, followed by RT-qPCR analysis of *KLF14* expression. Different superscript letters denote groups that are statistically different ($P < 0.05$). (C) LNCaP cells were incubated with 1 mmol/L of H_2O_2 for different durations, followed by RT-qPCR analysis of *KLF14* expression. Different superscript letters denote groups that are statistically different ($P < 0.05$). (D) PC3 cells were incubated with 1 mmol/L of H_2O_2 for different durations, followed by RT-qPCR analysis of *KLF14* expression. Different superscript letters denote groups that are statistically different ($P < 0.05$). (E) LNCaP cells were incubated with different concentrations of diamide for 15 min in the presence or absence of 20 μ mol/L of diphenyleneiodonium chloride, followed by RT-qPCR analysis of *KLF14* expression. Different superscript letters denote groups that are statistically different ($P < 0.05$). (F) PC3 cells were incubated with different concentrations of diamide for 15 min in the presence or absence of 20 μ mol/L of diphenyleneiodonium chloride, followed by RT-qPCR analysis of *KLF14* expression. Different superscript letters denote groups that are statistically different ($P < 0.05$). (G) RT-qPCR showing KLF14 expression in PC3 cells induced with H_2O_2 (1 mmol/L) for 15 min, in the presence of different pathway inhibitors. Different superscript letters denote groups that are statistically different ($P < 0.05$). A full-colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0383>.

Figure 3

Repression of the castration-resistant progression in PCa cells by KLF14 inhibition. (A) KLF14 stable knockdown in CWR-R1 cells by shRNA treatment was validated using RT-qPCR. Different superscript letters denote groups that are statistically different ($P < 0.05$). (B) KLF14 stable knockdown in CWR-R1 cells by shRNA treatment was validated using immunoblotting. (C) CWR-R1 cells with different transfections were cultured for 3 days in media containing either 10% FBS or 10% FBS-CS, followed by Trypan blue staining and viable cells count. Different superscript letters denote groups that are statistically different ($P < 0.05$). (D) CWR-R1 cells with different transfections were cultured for 3 days in media containing either 10% FBS or 10% FBS-CS, followed by apoptosis ELISA measurement. Different superscript letters denote groups that are statistically different ($P < 0.05$). (E) Anchorage-dependent clonogenic ability of CWR-R1 cells with different transfections was assessed using colony formation assay. (F) CWR-R1 cells with different transfections were injected into left flanks of castrated male nude mice. The inoculated cells were allowed to grow for 6 weeks until the tumors reached a volume of ~100–250 mm³. Tumor volume changes were recorded every week accordingly ($n = 5/\text{group}$, $*P < 0.05$, $**P < 0.01$). Inserted is the immunohistochemical staining of KLF14 expression in xenografts at the end of 6 weeks after cell inoculation. (G) KLF14 stable knockdown in DU145 cells by shRNA treatment was validated using RT-qPCR. Different superscript letters denote groups that are statistically different ($P < 0.05$). (H) KLF14 stable knockdown in DU145 cells by shRNA treatment was validated using immunoblotting. (I) DU145 cells with different transfections were cultured for 3 days in media containing either 10% FBS or 10% FBS-CS, followed by Trypan blue staining and viable cells count. Different superscript letters denote groups that are statistically different ($P < 0.05$). (J) DU145 cells with different transfections were cultured for 3 days in media containing either 10% FBS or 10% FBS-CS, followed by apoptosis ELISA measurement. Different superscript letters denote groups that are statistically different ($P < 0.05$). (K) Anchorage-dependent clonogenic ability of DU145 cells with different transfections was assessed using colony formation assay. (L) DU145 cells with different transfections were injected into left flanks of castrated male nude mice. Tumor growth was examined as described above ($n = 5/\text{group}$, $*P < 0.05$, $**P < 0.01$). Inserted is the immunohistochemical staining of KLF14 expression in xenografts at the end of 6 weeks after cell inoculation. A full-colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0383>.

Figure 4

Potentiation of castration-resistant PCa cell growth by ectopic KLF14 overexpression. (A) Generation of LNCaP/DDK-KLF14 cells was validated using immunoblotting. (B) LNCaP cells with different transfections were cultured for 3 days in media containing either 10% FBS or 10% FBS-CS, followed by Trypan blue staining and viable cells count. Different superscript letters denote groups that are statistically different ($P < 0.05$). (C) LNCaP cells with different transfections were cultured for 3 days in media containing either 10% FBS or 10% FBS-CS, followed by apoptosis ELISA measurement. Different superscript letters denote groups that are statistically different ($P < 0.05$). (D) Anchorage-dependent clonogenic ability of LNCaP cells with different transfections was assessed using colony formation assay. (E) LNCaP cells with different transfections were injected into left flanks of castrated male nude mice. Tumor growth was examined as described above ($n = 5/\text{group}$, $*P < 0.05$, $**P < 0.01$). Inserted is the immunohistochemical staining of KLF14 expression in xenografts at the end of 6 weeks after cell inoculation. A full-colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0383>.

at both translational (Fig. 6C) and transcriptional levels (Fig. 6D). These results led us to investigate whether KLF14 functions as a transcriptional regulator of *HMOX1*. As revealed by luciferase assays, transfection with pcDNA3.1+-DDK-KLF14 significantly increased the *HMOX1* reporter activity relative to control vector. This stimulatory effect was notably amplified by co-incubation with H_2O_2 or diamide. In good contrast, simultaneous treatment with diphenyleneiodonium chloride successfully abolished diamide-enhanced *HMOX1* transactivation by KLF14 (Fig. 6E). Interestingly, subsequent evidence derived from analysis of the 5' flanking regions of human *HMOX1* identified four sequences containing the conserved binding site of KLF14 (CACCC box, Fig. 6F). We then tested the hypothesis that KLF14 functions as a regulator of the putative binding sites in the *HMOX1* promoter. ChIP assay revealed that KLF14 could bind the promoter region that harbors the proximal CACCC box (−645/−641) (Fig. 6G), demonstrating that this is a functional KLF14-binding site in the human *HMOX1* promoter. Consistently, this region (−645/−641) exhibited increased reporter activity relative to control vector and this stimulatory effect was significantly amplified by co-incubation with H_2O_2 . Interestingly, mutation of the nt −645/−641 site greatly reduced promoter activity (Fig. 6H and I). To extend our mechanistic understanding of how H_2O_2 regulates the transcription of *HMOX1* through KLF14, we performed a double ChIP

assay. Initial ChIP was done with anti-MTA2 antibody to immunoprecipitate the KLF14-bound DNA sequences, and the second ChIP was done with the anti-CBP or anti-p300 antibodies. We observed simultaneous co-association of KLF14 and CBP or p300 with the *HMOX1* chromatin, and this recruitment was substantially augmented when H_2O_2 was added (Fig. 6J). Thus, together, these data demonstrate that KLF14 transcription factor is involved in the positive regulation of *HMOX1* transcription.

HO-1 inhibition counteracts KLF14-induced castration resistance

The preceding data have shown that upregulation of KLF14 expression induces HO-1 expression and resistance to oxidative stress, underscoring a fundamental role for KLF14 in the regulation of the antioxidant response under androgen-depleted conditions. In this regard, we finally initiated efforts toward evaluating the effect of HO-1 inhibition on KLF14-induced castration resistance. At the *in vitro* level, we probed the role of KLF14 targeting HO-1 in LNCaP cells stably expressing pcDNA3.1+-DDK-KLF14. The transfection efficiency of *HMOX1* shRNA in DDK-KLF14/LNCaP cells was over 80% but HO-1 silence exerted no effects on KLF14 expression level (Fig. 7A). Worthy to note, HO-1 knockdown successfully abolished KLF14 overexpression-induced cell proliferation (Fig. 7B)

Figure 5

Conferring of PCa resistance to oxidative stress by KLF14. (A) LNCaP cells with different transfections were treated with different concentrations of H₂O₂ for 24 h, followed by viability measurement (**P* < 0.05, ***P* < 0.01). (B) LNCaP cells with different transfections were treated with 10 nM of H₂O₂ for different durations, followed by viability measurement. (C) DU145 cells with different transfections were treated with different concentrations of H₂O₂ for 24 h, followed by viability measurement. (D) DU145 cells with different transfections were treated with 10 nM of H₂O₂ for different durations, followed by viability measurement. (E) LNCaP cells with different transfections were treated with different concentrations of paraquat for 24 h, followed by viability measurement. (F) DU145 cells with different transfections were treated with different concentrations of paraquat for 24 h, followed by viability measurement. (G) LNCaP cells were transiently transfected with pcDNA3.1+DDK-KLF14 or Ctrl vector as described in the Materials and methods section. 48 h later, cells were incubated with different concentrations of H₂O₂ for 24 h, followed by viability measurement. (H) LNCaP cells were transiently transfected with pcDNA3.1+DDK-KLF14 or Ctrl vector as described above. 48 h later, cells were incubated with different concentrations of H₂O₂ for 24 h, followed by measurement of GSH/GSSG ratio using a commercial kit. A full-colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0383>.

and compromised KLF14-stimulated colony formation efficiency under androgen-depleted conditions (Fig. 7C). We then tried to solid this observation by examining the *in vivo* antitumor effect of ZnPPiX, a specific inhibitor of HO-1 (Fig. 7D) (Hirai *et al.* 2007). i.p. injection of ZnPPiX (30 mg/kg; once a day) suppressed the castration-resistant growth of LNCaP-R xenograft tumors, especially during the late stage of tumor development (Fig. 7E), which correlated well to a decrease of HO-1 activity in xenograft tumors (Fig. 7F). Notably, *in vivo* application of ZnPPiX effectively suppressed HO-1 expression but exerted no effects on KLF14 level in xenograft tumors (Supplementary Fig. 2). Thus, HO-1 antagonism with ZnPPiX might be an effective and achievable treatment modality for CRPC, in which KLF14 overexpression-induced castration-resistant progression.

Discussion

On the basis of the available data, we concluded that KLF14 expression is stimulated in castration-resistant PCa

cells and xenograft tissues, which appears to be irrelevant to the AR pathway and may contribute to the oxidative stress-specific lineage characteristic of CRPC pathogenesis. Functionally, KLF14 upregulation potentiates the antioxidant response and cell growth under androgen-depleted conditions. Mechanistically, in cooperation with CBP and p300, KLF14 positively regulates the antioxidant response in PCa cells by directly targeting *HMOX1* transactivation (Fig. 8).

Known as an essential 'conductor of the metabolic syndrome orchestra', KLF14 involvement in cancer biology has just started to emerge. To this end, whether KLF14 functions as an oncogene or tumor suppressor gene has attracted much attention. Fan *et al.* have reported that KLF14 expression is significantly reduced in breast and colon cancer. By using KLF14 KO mice, they also demonstrate that KLF14 suppression serves as a potent mechanism leading to centrosome amplification and tumorigenesis. Thus, KLF14 functions fundamentally as a tumor suppressor in breast and colon cancer (Fan *et al.*

Figure 6

Positive regulation of HMOX1 transcription by KLF14. (A) Relative expression levels of different antioxidant genes in LNCaP/DDK-KLF14 and LNCaP/vector cells were evaluated using RT-qPCR (* $P < 0.05$ and ** $P < 0.01$ compared to the values in LNCaP/vector cells). (B) Immunoblotting analysis of KLF14 and HO-1 expression in CRPC patient-derived xenograft model. (C) Immunoblotting analysis of KLF14 and HO-1 expression in LNCaP/DDK-KLF14 and LNCaP/vector cells. (D) RT-qPCR analysis of *KLF14* and *HMOX1* levels in LNCaP/DDK-KLF14 and LNCaP/vector cells. Different superscript letters denote groups that are statistically different ($P < 0.05$). (E) The pGL4-Luc-HMOX1 reporter plasmid and pRL-TK Renilla reporter plasmid were co-transfected with pcDNA3.1+DDK-KLF14 or pcDNA3.1+ vector into HeLa cells using FUGENE. 48 h later, cells were treated for 15 min with 1 mmol/L of H_2O_2 , 30 mmol/L of diamide or diamide plus 20 μ mol/L of diphenyleneiodonium chloride, followed by luciferase activity measurements. Different superscript letters denote groups that are statistically different ($P < 0.05$). (F) Schematic of predicted KLF14 binding sites on the *HMOX1* promoter region. (G) ChIP analysis showing recruitment of KLF14 onto a specific region of the *HMOX1* promoter. (H) Schematic of the mutated KLF14 binding site on the *HMOX1* promoter region. (I) The mutation of the putative KLF14-binding site on the promoter at -645/-641 was carried out by replacing CACCC with CAaaa using the QuikChange Site-Directed Mutagenesis Kit. The WT or mutated pGL4-Luc-HMOX1 reporter plasmid and pRL-TK Renilla reporter plasmid were co-transfected with pcDNA3.1+DDK-KLF14 or pcDNA3.1+ vector into HeLa cells using FUGENE. 48 h later, cells were treated for 15 min with 1 mmol/L of H_2O_2 . Relative luciferase activity was measured using a dual luciferase reporter assay kit. (J) Double ChIP analysis of KLF14-CBP/p300 complex onto the *HMOX1* promoter in LNCaP/DDK-KLF14 cells upon challenge for 15 min with 1 mmol/L of H_2O_2 . A full-colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0383>.

Figure 7

Therapeutic effectiveness of *in vivo* application of HO-1 inhibitor against castration-resistant PCa cell growth. (A) LNCaP/DDK-KLF14 cells were transiently transfected with HMOX1 shRNA or Ctrl shRNA. 48 h later, cells were harvested and subjected to immunoblotting analysis. (B) LNCaP cells with different transfections were cultured for 3 days in media containing either 10% FBS or 10% FBS-CS, followed by Trypan blue staining and viable cells count. Different superscript letters denote groups that are statistically different ($P < 0.05$). (C) Anchorage-dependent clonogenic ability of LNCaP cells with different transfections was assessed using colony formation assay. (D) Schematic representation of the experimental procedure for *in vivo* application of HO-1 inhibitor (ZnPPiX) in the LNCaP-R cells-derived tumor xenograft model. (E) LNCaP-R cells-derived tumor xenograft model was established as described in 'Materials and methods' section. Nude mice were treated intraperitoneally with ZnPPiX (10 mg/kg/day), or PBS vehicle control on daily basis. The volume changes of tumor xenografts were recorded every week ($*P < 0.05$). (F) Relative HO-1 activity was measured in LNCaP-R cells-derived tumor xenografts using a commercial ELISA kit. Different superscript letters denote groups that are statistically different ($P < 0.05$). A full-colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0383>.

2015). In contrast, in thyroid cancer, KLF14 expression has been shown to be upregulated compared with normal thyroid tissue. Knockdown of KLF14 expression results in reduced cell proliferation, G1-phase arrest, and increased apoptosis. Therefore, thyroid-originated KLF14 is an oncogene essential for cancer cell survival (Boot *et al.*

2016). These conflicting results strongly suggest that KLF14 may exhibit a dual function in response to distinct cell context depending upon the state of cell malignancy.

Evidence from both experimental and clinical studies have confirmed that castration, the major approach for treatment of late stage PCa, notably enhances oxidative

Figure 8

Summary diagram of the possible mechanisms related to KLF14 function contributing to promotion of the antioxidant response via transcriptional regulation of HO-1 expression during castration-resistant PCa progression. A full-colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0383>.

stress in prostatic cells. The latter in turn contribute tremendously to the development and progression of CRPC (Shiota *et al.* 2010, 2011). Therefore, it is logical that an impaired antioxidative capacity should be expected along the pathogenesis of CRPC. Nevertheless, the expression of many antioxidant genes has so far been found to be upregulated in CRPC. For instance, ADT-induced ROS enhances the expression and activity of NF-E2 related factor 1 and 2 (namely Nrf1 and Nrf2), two essential transcriptional factors governing the expression of numerous antioxidant genes. The resultant upregulation in selective antioxidant protein expression is believed to be associated with increased tumor survival under androgen-deprived conditions, thus promoting CRPC progression (Schultz *et al.* 2010). Similarly, expression of the antioxidant proteins Prx-1 and Txn-1 has been shown to be significantly stimulated in castration-resistant C4-2B cells than in AD LNCaP cells (Schultz *et al.* 2010).

It is reasoned that in concert with oxidative stress, differential antioxidant signaling pathways also help to regulate the aggressive behavior of CRPC cells. Our findings extend these knowledge by identifying KLF14 as a potent protector against oxidant toxicity by augmenting the antioxidant defenses (e.g., elevated expression of HO-1 and enhanced production of GSH). Given that ROS is detrimental to both normal and cancer cells, advanced PCa cells must develop mechanisms to get adapted to hypoxia and conditions of high oxidative stress. In favor of this hypothesis, intracellular ROS levels, along with GSTM3 expression (an enzyme functions in the detoxification of products of oxidative stress by conjugation with glutathione), have been reported to be induced simultaneously in castration-resistant and enzalutamide-resistant PCa cells (Shiota *et al.* 2017). These findings together suggest that a proper balance between oxidative stress and antioxidant pathways is absolutely required for CRPC pathogenesis. In this regard, the increased expression of KLF14 may entitle the castration-resistant PCa cells more equipped for constitutive modification of antioxidant signaling, thereby enhancing the aggressive phenotype of these cells.

Another relevant intriguing question addressed by the current study was how KLF14 regulates oxidative stress-elicited antioxidant response. We show that KLF14 activates *HMOX1* transcription by directly binding its chromatin and transient knockdown of *HMOX1* is sufficient to overcome KLF14 overexpression-potentiated PCa cell growth under androgen-depleted conditions, indicating that KLF14 upregulation may promote uncontrolled HO-1-directed antioxidant response and thus promotes castration resistance. The upregulation of HO-1 is one of the most important mechanisms of cell adaptation to stress. HO-1 induction has been frequently correlated with tumor growth, aggressiveness, metastatic and poor prognosis in lung, pancreatic, prostate, colorectal, ovarian cancer, melanoma, and hepatoma (Loboda *et al.* 2015). In particular, anticancer treatments such as chemo-, radio-, and photodynamic therapy all increase HO-1 expression (Nitti *et al.* 2017). Through the antioxidant and antiapoptotic properties of its metabolic products, HO-1 plays a key role in maintaining redox homeostasis during cancer progression (Loboda *et al.* 2016). Nevertheless, the transcriptional or posttranscriptional mechanisms responsible for controlling HO-1 expression in response to cytotoxic stress are poorly understood. Our findings extend these understanding by identifying KLF14 as a potent coactivator of *HMOX1* transcription. Of note, multiple putative *cis*-regulatory elements have

been previously identified in the *HMOX1* promoter region including –146 to –37bp for CREB (Astort *et al.* 2016), –44bp for Nrf2 (Potteti *et al.* 2016) and –1036 to +1bp for Sp1 and p53 (Tung *et al.* 2015). In contrast, the promoter region of *HMOX1* bound by KLF14 (–645 to –641bp) was not included in the above-mentioned *cis*-acting elements. These results collectively broaden our understanding of the diversity of the transcriptional regulation sites of *HMOX1* expression.

Previous study has shown that the HO-1-specific antagonist, ZnPPiX, inhibits tumor growth of LL/2 lung cancer in C57BL mice (Hirai *et al.* 2007). The present study is the first report demonstrating that ZnPPiX reduces the castration-resistant progression of PCa cells induced without changing the high level of KLF14 expression. Given that LNCaP-R cells are AR-positive PCa cells with high KLF14 expression, our data indicate that HO-1 antagonism might have significant antitumor effects against CRPC.

In summary, we have demonstrated that KLF14 directly regulates *HMOX1* transactivation and potentiates resistance against androgen-depleted conditions by enhancing antioxidant response in PCa cells. Furthermore, we show that ZnPPiX, a pharmacological inhibitor of HO-1, attenuates castration-resistant progression. Overall, the work presented here identified a critical role of KLF14/HO-1 cascade, both as a target and as a modifier of the oxidative stress signaling in conferring an optimal adaptive mechanism during the pathogenesis of CRPC.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0383>.

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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