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TFF3 mediated induction of VEGF via hypoxia in human gastric cancer SGC-7901 cells

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Abstract Increasing evidence indicates that in gastric epithelial cells, induction of TFF3 by hypoxia is mediated by HIF-1. Since VEGF is one of the most important angiogenic factors on cancer progression, we have started to investigate the possible link among HIF-1α, VEGF, and TFF3 in gastric cancer cells. We induced the hypoxic condition in SGC-7901cells using hypoxia-mimetic agent of CoCI2. SGC7901 cells were transfected with pcPUR + U6 plasmid carrying RNAi targeted to human TFF3 and selected puromycin-resistant pools to establish the stable knockdown of TFF3 cells. Our results showed the induction of HIF-1a via hypoxia and consequences of increased expressions of the TFF3 and VEGF in gastric cancer SGC-7901 cells. Over-expression of TFF3 upregulated the mRNA expressions of VEGF and HIF-1a induced by hypoxia, and stable knock-down of TFF3 impaired the mRNA upregulations of VEGF and HIF-1a induced by hypoxia. Furthermore, knockdown of TFF3 reduced the VEGF protein secretion: as VEGF secretion was increased time dependent manner in response to the hypoxia induction in TFF3-WT cells; however, VEGF production was significantly decreased in TFF3-KD cells (621 ± 89 vs. 264 ± 73 at 6 h and 969 ± 97 vs. 508 ± 69 at 12 h, $P < 0.05$). Our data demonstrated the TFF3 mediated regulation of VEGF expression induced by hypoxia, and implicated that TFF3 might be applied as a potential anti-angiogenic target for treatment of gastric cancer.

Keywords TFF3 · VEGF · HIF-1α · Hypoxia

Abbreviations

VEGF	Vascular endothelial growth factor
HIF	Hypoxia-inducible transcription factors
IGF	Insulin-like growth factor
HRE	Hypoxia-response promoter elements
TFF	Trefoil factor
KD	Knockdown
ANOVA	Analysis of variance

Introduction

Growth of solid tumors depends on the induction of new blood vessels; for example, formation of gastric cancer and metastasis. In tumors, angiogenesis is uncontrolled and immature. This is a complicated process and depends on a great variety of angiogenic factors, among them the vascular endothelial growth factor (VEGF) is one of the most important factors [1–4].

VEGF is a mitogenous and mobilizer of endothelial cells, has an angiogenic effect in vivo and secreted by a wide range of cell types including the most tumor cells. It was suggested that formation of blood vessels is triggered

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by reduced supply of oxygen and nutrients, occurring when intercapillary distances exceed 100 μm (i.e., exceeding three to seven cell layers) [5, 6]. Therefore, hypoxia is an important regulator of VEGF expression. Hypoxic regulation of VEGF gene expression is mediated by a family of hypoxia-inducible transcription factors (HIF), which includes HIF-1α, HIF-2α, and HIF-1β [7, 8]. They initiate the response to hypoxia, hypoglycemia, insulin, and insulin-like growth factor (IGF)-1 by binding to hypoxia-response promoter elements (HRE) within a range of physiological oxygen concentrations (0.5–20%) [9]. In addition, hypoxia upregulates expression of VEGF by improving its translation and stabilizing its transcripts [10].

Trefoil factor (TFF) 3 (or intestinal TF) is a member of the TFF-domain peptide family, which is constitutively expressed in mucous epithelial tissues. In the epithelial tissue, TFF3 acts as a motogenic factor and plays an important role during epithelial restitution after wounding and during inflammation [11]. Recent studies also suggest a causal link between local inflammation and occurrence of gastrointestinal tumors. Among the local mediators of inflammatory responses, TFF3 is involved in mucosal protection and repairment of the gastrointestinal tract [12]. The TFF3 peptide is over-expressed during tumor progression and promotes tumor cell invasion and angiogenesis [13, 14].

Hypoxia has been reported as a factor to increase TFF3 mRNA expression in intestinal epithelial cells, which is interpreted as a mechanism for maintenance of barrier function when oxygen levels are low [15, 16]. Increasing evidence indicates that in gastric epithelial cells, induction of TFF1, TFF2 and TFF3 by hypoxia is mediated by hypoxia-inducible factor-1 [17]. Since VEGF is one of the most important angiogenic factors on cancer progression, we have started to investigate the possible link among HIF-1α, VEGF, and TFF3 in gastric cancer cells. In addition, a binding site for HIF-1 has been characterized in the human TFF3 gene promoter [15]. Our data showed the TFF3 mediated regulation of VEGF expression induced by hypoxia, and implicated that TFF3 might be applied as a potential anti-angiogenic target for treatment of gastric cancer.

Materials and methods

Cell culture

Human gastric cancer cell line SGC-7901 (provided by the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) and HEK293 cells were cultured in RPMI-1640 medium (Invitrogen Life Technologies Carlsbad, CA, USA) supplied with 10% of FCS, 100 U/ml of penicillin and 100 mg/ml of streptomycin at 37°C humidi-fied atmosphere with 5% of CO2. To induce the hypoxic condition, 2×10^5 of SGC-7901 cells were cultured in 6-well plates and was treated with 200 μM $CoCl_2$ (Wako Pure Chemical Industries, Osaka, Japan) for 3,6, and 12 h, which is one of widely used reagent for mimic hypoxia in vitro and in vivo [18]. Then, cells were harvested and processed for Western blot or RT-PCR analysis as described below.

Real-time quantitative RT-PCR

Total RNA from SGC7901 cells was extracted using TRIzol reagent (Invitrogene, CA, USA), then samples were treated with DNase for 15 min at room temperature, and the RNA was further purified using RNA cleanup kit (Qiagen, CA, USA). The cDNA templates were synthesized using the iScriptTM cDNA synthesis kit and quantitative PCR reactions were performed with iQTM SYBR green PCR supermix (both from Bio-Rad, CA, USA). Quantitative RT-PCR analysis was performed using the ABI 7500 Applied Biosystem, and gene expression levels for each individual sample were normalized to β-actin. Mean relative gene expression was determined and differences were calculated using the $2^{-\Delta C(t)}$ method or agarose gel electrophoresis. The PCR primer sequences were as follows: sense 5′-AAAGGCATGC AGGAGAGAACAGGA-3′ and antisense 5′-GTTTGCACA GCTGCTCTGGATTGT-3′ for TFF3; sense 5′-ACATCTT CAAGCCATCCTGTGTGC-3′ and antisense 5′-TCTGC ATGG TGATGTTGGACTCCT-3′ for VEGF; sense 5′-AG CCTCACCAAACAGAGCAGGAAA-3′ and antisense 5′-AGCGACAGATAACACGTTAGGGCT-3′ for HIF-1α; sense 5′-AGGATGGCAAGGGACTTCCTGTAA-3′ and antisense 5′-GGCACC CAGCACAATGAAGATCAA-3′ for β-actin; sense 5′-AGGCCCAGACAGAGACGTGTACAG and antisense 5′-ACACTCCTCTTCTGGAGGGACGTC for TFF1 and sense 5′-CTGGCGGGGAGTGAGAAAC and antisense 5′-GGAGTCGAAACAGCATCCATT for TFF2.

Establishment of TFF3 expression vector

Full-length of TFF3 was amplified by PCR from cDNA template of SGC7901 cells using upstream (contain Not I site) of 5′-ATAAGAATGCGGCCGCATGCAGGAGA-GAACAGGAGCA and downstream (contain Hind III site) of 5′-CCCAAGCTTGAAGGTGCATTCTGCTTCCTG primers, then fragment was inserted into the Not I and Hind III site of the pCMV-Flag-tag 4C (Stratagen) vector, which constructed the pCMV-TFF3-Flag expression vector and confirmed by sequencing.

Stable knockdown of TFF3

A plasmid carrying RNAi targeted to human TFF3 was constructed as previously described [4, 19–21]. The siTFF3

sequence of the human TFF3 gene, 5′-AGGAAGCAGAAT GCACCTTCTGA-3′, was selected using our original algorithm. SGC7901 cells were transfected with pcPUR + U6-siTFF3 or pcPUR + U6-siRenilla (control) with Lipofectamine™ 2000 (Invitrogen), and selected as 2 μg/ml of puromycin-resistant pools. Then, RT-PCR was performed to confirm the TFF3 mRNA suppression using the sense 5′-AAAGGCATGCAGGAGAGAACAGGA-3′ and antisense 5′-GTTTGCACAGCTGCTCTGGATTGT-3′ preimers followed by agarose gel electrophoresis.

Western blot analysis

SGC7901 cells were lysed in RIPA buffer (50 mmol/l Tris pH 8, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 150 mmol/l NaCl, 1 tablet complete mini protease inhibitor/10 ml, Roche Diagnostics GmbH, Germany) and total protein concentration in extracts was determined with the Pierce BCA protein assay kit (Pierce Chemicals, Boulder, CO, USA) using BSA to generate a standard curve. Membranes were blocked with 5% non-fat dry milk in TBST buffer and incubated overnight with a monoclonal antibody against HIF-1a (dilution rate as 1: 250, BD Biosciences, San Jose, CA, USA); anti-Flag antibodies (dilution rate as 1:2000, Sigma) or anti-actin antibody (dilution rate as 1:4000, Sigma) at 4 degree. Then incubated with HRP-conjugated goat anti-mouse IgG (dilution rate as 1:2500; DakoCytomation, Glostrup, Denmark) at room temperature for 1 h, followed by treatment with supersignal west pico chemiluminescent substrate (Pierce Chemicals) and revealed using LAS-3000 system (Fujifilm, Japan).

Immunofluorescence analysis

SGC-7901 cells were treated with 200 μM $CoCI_2$ for 6 h or untreated cells as control. Then cells were fixed with 4% of paraformaldehyde at 4 degree for 30 min and treated with 0.3% of TritonX-100 for 10 min and blocked with 5% of BSA for 30 min. Then incubated overnight with a monoclonal antibody against HIF-1a (dilution rate as 1:250, BD Biosciences, San Jose, CA, USA) at 4 degree and followed by incubation with anti-mouse (dilution rate as 1:200, Sigma) IgG secondary antibodies. Cell nucleus were stained with 1× DAPI (Sigma), imaging was performed using LSM 510 SYSTEM microscope (Zeiss, Germany), and analyzing was performed using MetaMorph/MetaFluor version 7.0.

Measurement of VEGF using ELISA

VEGF in serum were measured by sandwich enzyme-linked immunosorbent assay (ELISA) as previously described [22]. Briefly, monoclonal antibody against to human VEGF (R&D Systems, Minneapolis, MN, USA) was added to a 96-well plate and incubated at 4° for overnight. After incubating the plate with a blocking solution consisting of PBS containing 1% of bovine serum albumin and 0.05% of Tween 20 for 2 h at room temperature, the samples and the standard recombinant cytokines (R&D Systems) were added to the 96-well plate and incubated at room temperature for 2 h. After washing four times, 200 ng/ml of biotinylated monoclonal antibody to human cytokines (R&D Systems) were added and the reaction was allowed to proceed for 2 h at room temperature. After washing, diluted streptavidin–alkaline-phosphatase (Sigma Bioscience, St. Louis, MO, USA) was added, and the reaction was allowed to proceed for a further 2 h. After the plates were washed four times, 1 mg/ml of *p*-nitrophenylphosphate (Sigma) dissolved in diethanolamine (Sigma) was added to induce the color reaction, which was stopped by adding 1 N NaOH. An automated microplate reader (Vmax, Molecular Devices, Palo Alto, CA, USA) set at 450 nm was used to measure the optical density. The sensitivity limit was 15.6 pg/ml for VEGF. Recombinant human cytokines diluted in culture medium over a concentration range of 10–2,000 pg/ml were used as calibration standards. A standard curve was drawn by plotting optical density versus the log of the concentration of recombinant cytokines.

Statistic analysis

The data is expressed as the mean ± SD. Comparisons between the groups were analyzed using means of one-way ANOVA, LSD test, Spearman rank correlation and Student's *t*-test, $P < 0.05$ was considered statistically significant and SPSS software was employed.

Results

Induction of HIF-1a via hypoxia and increased expressions of the TFF3 and VEGF in gastric cancer SGC-7901 cell

To establish the hypoxic model, SGC-7901cells were treated with the hypoxia-mimetic agent of $CoCI_2$, which has been shown to inhibit the interaction of an E3 ubiquitin ligase von Hippel-Lindau protein with HIF-1α protein [22]. This prevents ubiquitin-dependent hydroxylation and subsequent degradation of HIF-1α protein. We treated the SGC-7901 cells with different concentrations of $CoCI_2$ for 3 h. 200 μM of $CoCI_2$ resulted in marked induction of HIF-1α mRNA expression (Fig. 1a) and employed in the subsequent experiments. To investigate the relationships

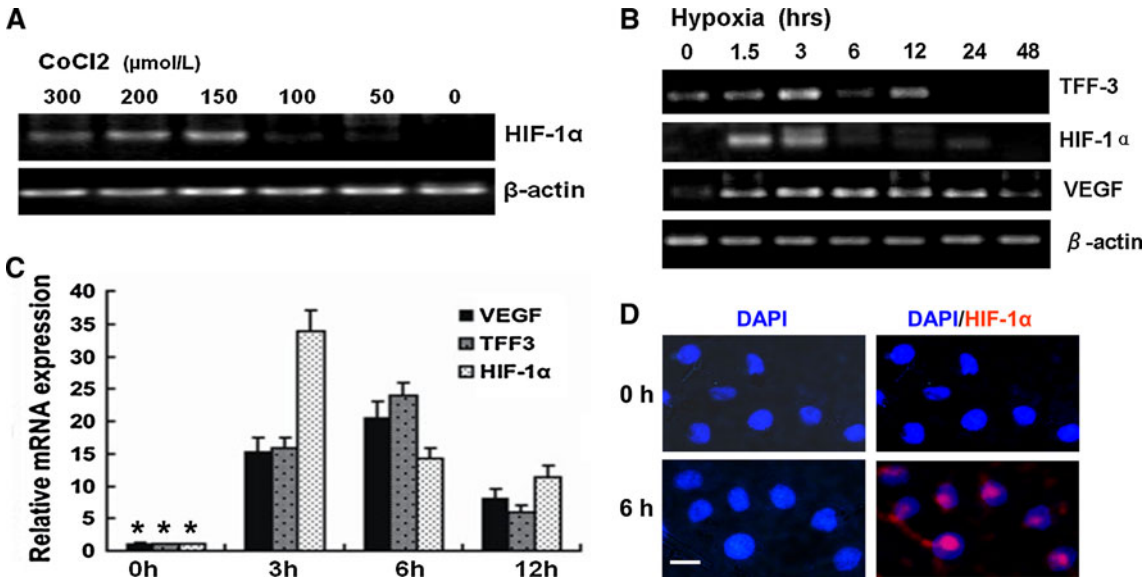

Fig. 1 Induction of hypoxia and expressions analysis of the HIF-1a, TFF3, and VEGF in SGC-7901 cell. **a** 2×10^5 of SGC-7901 cells were cultured in 6-well plates and treated with different concentrations $CoCI_2$ for 3 h and β-actin was used as internal control. **b** SGC-7901 cells were treated with 200 μM of $CoCI_2$ for 0, 1.5, 3, 6, 12, 24, and 48 h, then, analyzed the mRNA expressions of HIF-1α, TFF3, and VEGF using RT-PCR, β-actin as a control. **c** Treated the SGC-7901 cells with $CoCI_2$ for 0, 3, 6, and 12 h, then, analyzed the mRNA expressions of HIF-1α, TFF3 and VEGF using Quantitative RT-PCR, the expression levels of these 3 genes at 0 h were significantly different with the levels at 3, 6, and 12 h ($^*P < 0.05$). **d** Treated the SGC-7901 cells with $CoCI_2$ for 6 h and analyzed using immunofluorescence staining, *red* for HIF-1α protein expression and *blue* color for DAPI positive cell nucleus, *white bar* represent 5 μm

among HIF-1α, TFF3 and VEGF during hypoxic condition, SGC-7901 cells were treated with $CoCI_2$ for 0, 1.5, 3, 6, 12, 24, and 48 h. Then, we analyzed the mRNA expressions of HIF-1α, TFF3 and VEGF using RT-PCR, β-actin was used as internal control. As shown in the Fig. 1b, $CoCI_2$ induced the mRNA expression of HIF-1α at 1.5–3 h after treatment. As expected, hypoxia induced the increased mRNA expressions of the TFF3 and VEGF in gastric cancer SGC-7901 cell. To further precisely quantify the correlation among expression levels, we treated the cells with $CoCI_2$ for 0, 3, 6, and 12 h, then analyzed the mRNA expressions of HIF-1α, TFF3 and VEGF using Quantitative RT-PCR. As shown in Fig. 1c, $CoCI_2$ induced hypoxia resulted in the fold increases of 34.1 ± 2.1 of HIF-1α, 15.2 ± 0.8 of TFF3 and 15.0 ± 1.3 of VEGF expressions after treatment at 3 h and remained the elevated expressing levels at 6 and 12 h in compare to the baseline expression of untreated cells. One-way ANOVA showed the significant differences among TFF3, VEGF and HIF-1α at 4 of time points ($P < 0.001$); further LSD test revealed the significant differences of three genes at any of two time points ($P < 0.05$); and Spearman rank correlation showed VEGF & TFF3: (r_s: 0.942, $P < 0.01$), VEGF & HIF-1α: (r_s: 0.790, $P < 0.01$), TFF3 & HIF-1α: (r_s: 0.760, $P < 0.01$), correlations are significant at 0.01 level in three groups (2-tailed). On the other hand, to confirm the protein expression of HIF-1α in this model, we treated the cells with $CoCI_2$ for 6 h and analyzed using immunofluorescence staining. And HIF-1α protein (red) was detected in the nucleus compared with the untreated cells (Fig. 1d). Taken together, our results showed the induction of HIF-1a via hypoxia and correlated with increased expressions of the TFF3 and VEGF in gastric cancer SGC-7901 cells.

Overexpression of TFF3 upregulated the mRNA expressions of VEGF and HIF-1a induced by hypoxia in SGC-7901 cells

To investigate whether TFF3 regulates the expressions of VEGF and HIF-1a induced by hypoxia, we cloned the full-length of TFF3 from cDNA of SGC-7901 cell and established the pCMV-TFF3-Flag expression vector. HEK293 cells were transfected with pCMV-Flag and pCMV-TFF3-Flag for 24 h and followed by western blot analysis. As shown in Fig. 2a, around 9 kDa of TFF3-Flag fusion protein is detected on left lane and employed in next experiment, no detection of protein on control lane at right side, actin was employed as an internal control.

SGC-7901 cell was transfected with pCMV-Flag and pCMV-TFF3-Flag for 24 h and treated the cells with $CoCI_2$ for 0, 3 and 6 h, then analyzed the mRNA expressions of VEGF and HIF-1α using Quantitative RT-PCR. As shown in Fig. 2b, in the $CoCI_2$ induced hypoxia condition, overexpression of TFF3 induced the significant increase of VEGF mRNA expression at 3, 6 h (2.6 ± 0.25 and 1.6 ± 0.21 of fold increase, $P < 0.05$, respectively), and

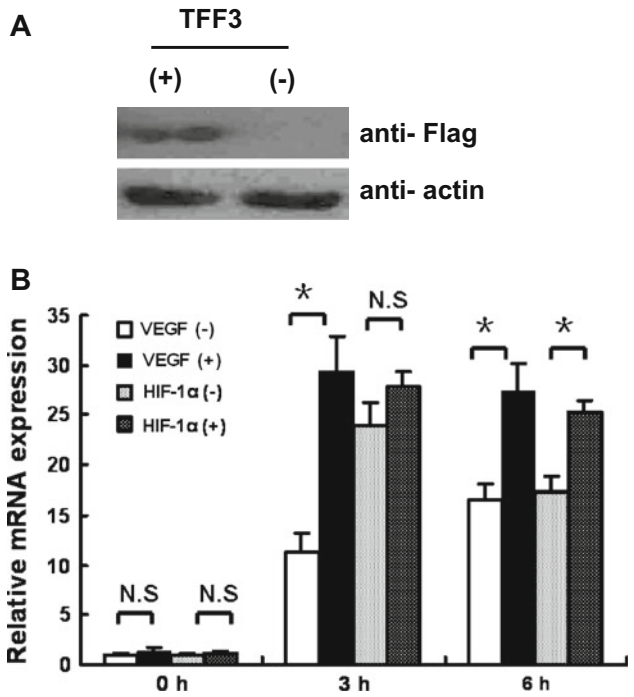

Fig. 2 Establishment of TFF3 overexpressing cell and mRNA expression analysis of VEGF and HIF-1a. **a** HEK293 cells were transfected with pCMV-Flag (−) and pCMV-TFF3-Flag (+) for 24 h and protein expression was detected by western blot using anti-Flag and anti-actin antibodies. **b** SGC-7901 cell was transfected with pCMV-Flag and pCMV-TFF3-Flag for 24 h and treated the cells with $CoCI_2$ for 0, 3, and 6 h, then analyzed the mRNA expressions of VEGF and HIF-1α using Quantitative RT-PCR (*$P < 0.05$)

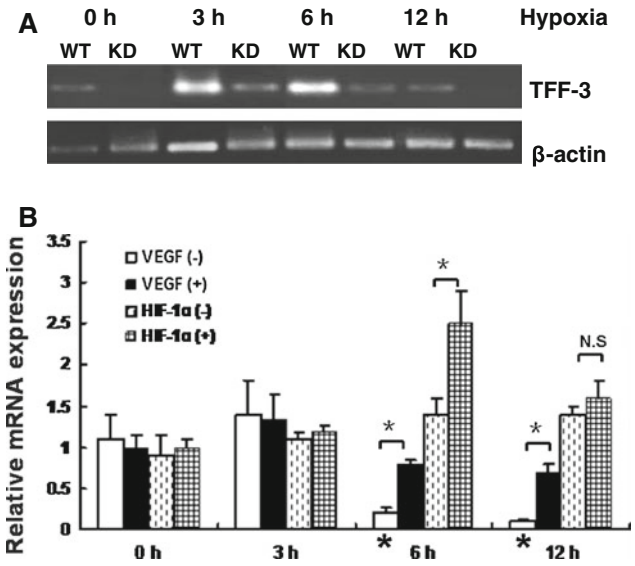

Fig. 3 Establishment of TFF3 knockdown cell line and mRNA expression analysis of VEGF and HIF-1a via hypoxia in SGC-7901 cells. **a** The SGC7901 cells were transfected with pcPUR + U6-siTFF3 or pcPUR + U6-siRenilla (control) and selected as puromycin-resistant pools. Then, TFF3-WT and TFF3-KD cell pools were grown in 6-well plates and treated with $CoCI_2$ for 0, 3, 6, and 12 h, and RT-PCR was performed to confirm the TFF3 mRNA suppression. **b** TFF3-KD cell pools were grown in 6-well plates and transfected with pCMV-Flag and pCMV-TFF3-Flag for 24 h and treated the cells with $CoCI_2$ for 0, 3, 6, and 12 h, then analyzed the mRNA expressions of VEGF and HIF-1α using Quantitative RT-PCR (*$P < 0.05$ and N.S *$P > 0.05$)

HIF-1α expression at 3, 6 h (2.6 ± 0.25, $P > 0.05$ and 1.5 ± 0.12, $P < 0.05$, respectively) in compare to the control cells. One-way ANOVA showed the significant differences among VEGF (−), VEGF (+), HIF-1α (−) and HIF-1α (+) at 3 of time points ($P < 0.05$). Taken together, our results showed the overexpression of TFF3 upregulated the mRNA expressions of VEGF and HIF-1a induced by hypoxia in SGC-7901 cells by time dependent manner.

Stable knockdown of TFF3 impaired the mRNA upregulations of VEGF and HIF-1a induced by hypoxia in SGC-7901 cells

To investigate the role of TFF3 on induction of HIF-1α and VEGF via hypoxia, we established the stable TFF3 knockdown (KD) SGC-7901 cells as followed: The siTFF3 sequence of the human TFF3 gene was selected using our original algorithm. SGC7901 cells were transfected with pcPUR + U6-siTFF3 or pcPUR + U6-siRenilla (control) and selected as puromycin-resistant pools. Then, TFF3-WT and TFF3-KD cell pools were grown in 6-well plates and treated with $CoCI_2$ for 0, 3, 6 and 12 h, and RT-PCR was performed to confirm the TFF3 mRNA suppression. As shown in Fig. 3a, both baseline and upregulation of TFF3 mRNA expressions induced by hypoxia were suppressed in TFF3-KD cells compared with the TFF3-WT cells. Recently, it has been suggested that siRNA creoss-reacts with nontargeted gene that contain sequences homolog to the siRNA. To exclude this type of phenomenon, we used Quantitative RT-PCR to examine the expression of the TFF1 and TFF2 genes, which has similar sequences to the siRNA that was targeted to the TFF3 gene. The WT and TFF3-KD cells showed similar low levels of TFF1 and TFF2 expressions (data not shown and employed primers are listed at Materials and methods part), which confirms the specificity of the TFF3-KD in the TFF3-KD cells. Then, to determine the role of TFF3 on induction of HIF-1α and VEGF and whether transfection of the TFF3 rescue the phenotype in TFF3-KD cell, this cell was employed in following experiments (Fig. 3b). TFF3-KD cell pools were grown in 6-well plates and transfected with pCMV-Flag and pCMV-TFF3-Flag for 24 h and treated the cells with $CoCI_2$ for 0, 3, 6, and 12 h, then analyzed the mRNA expressions of VEGF and HIF-1α using Quantitative RT-PCR. Knockdown of the TFF3 did not show the significant difference on VEGF and HIF-1α expressions at 3 h after hypoxia induction in TFF3-KD cells transfected with

pCMV-Flag. As expected, mRNA expression of VEGF was dramatically suppressed at 6 and 12 h (data not shown and TFF3-WT cells transfected with pCMV-Flag showed the same results with Fig. 1c). Of interest, transfection of the pCMV-TFF3-Flag partially rescued the downregulation of VEGF at 6, 12 h ($P < 0.05$) and HIF-1α at 12 h ($P < 0.05$), respectively. One-way ANOVA showed the significant differences between VEGF and HIF-1α at 4 of time points ($P < 0.001$). These results indicate that knockdown of TFF3 impaired the mRNA upregulations of VEGF and HIF-1a induced by hypoxia and transfection of the TFF3 partially rescued this phenotype in transcriptional level.

Stable knockdown of TFF3 reduced the VEGF protein secretion induced by hypoxia in SGC-7901 cells

To determine the role of TFF3 on regulation of VEGF and HIF-1α protein expressions, TFF3-WT and TFF3-KD cells were grown in 12-well plates and treated with $CoCl_2$ for 0, 3, 6, and 12 h. Then, the VEGF production in supernatants was measured using ELISA and equal amounts of total cell lysates were used for western blot analysis of HIF-1α. As expected, VEGF secretion was increased time dependent manner in regards to the hypoxia induction in TFF3-WT cells; however, VEGF production was significantly decreased in TFF3-KD cells (621 ± 89 vs. 264 ± 73 at 6 h and 969 ± 97 vs. 508 ± 69 at 12 h, $P < 0.05$) (Fig. 4a). On the other hand, western blot analysis showed that HIF-1α protein expression was decreased in TFF3-KD cells at 3 h, but no difference was observed between 6 and 12 h after induction of hypoxia (Fig. 4b). These results showed that knockdown of TFF3 reduced the VEGF protein secretion and suggesting the TFF3-mediated regulation of VEGF protein expression induced by hypoxia.

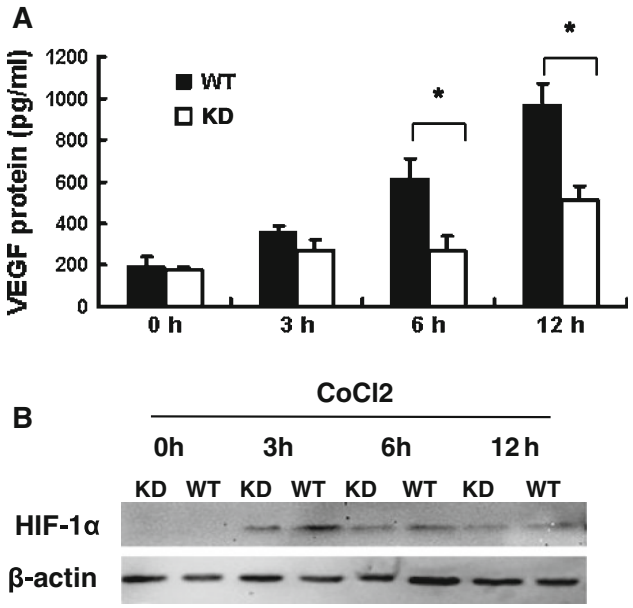

Fig. 4 Analysis of VEGF and HIF-1α protein expressions in TFF3 knocked down in SGC-7901 cells via hypoxia. **a** TFF3-WT and TFF3-KD cells were grown in 12-well plates and treated with $CoCl_2$ for 0, 3, 6, and 12 h. Then, the VEGF content of the culture supernatants was measured using ELISA (*$P < 0.05$). **b** Equal amounts of total cell lysates collected from same experiment with VEGF measurement were used for western blot analysis of HIF-1α, β-actin was employed as internal control

Discussion

This study demonstrated that a TFF3 mediated induction of VEGF expression via hypoxia in gastric cancer cells. The TFF3 and VEGF expression were detected in the gastric SGC7901 cancer cells, and induction of HIF-1a in hypoxia was consequenced with increased mRNA expression of the TFF3 and VEGF. Overexpression of TFF3 upregulated the mRNA expressions of VEGF and HIF-1a induced by hypoxia in SGC-7901 cells. Furthermore, Knockdown of TFF3 impaired the mRNA upregulations of VEGF, HIF-1a and decreased the VEGF protein secretion induced by hypoxia; and transfection of the TFF3 partially rescued mRNA downregulations of VEGF and HIF-1a in TFF3-KD cell on transcriptional level. However, xogenous TFF3 (data not shown) could not rescue the phenotypes in TFF3-KD cells in regards to VEGF protein secretion at Fig. 4a, which agreement with the direct regulation of VEGF protein expression depend upon broad spectrum of signaling pathways [7, 8].

TFF3 is a member of the TFF-domain peptide family, which is constitutively expressed in mucous epithelial tissues, where it acts as a monogenic factor and plays an important role during epithelial restitution after wounding and during inflammation. In contrast to these beneficial functions, TFF3 was also reported to be involved in cell scattering and tumor invasion via autocrine loops and may serve as potential targets in the control of gastrointestinal cancer progression [23].

The pathogenesis of gastric cancer is remaining not well understood. This common type of cancer is generally believed to occur in a multistep process which involves alterations of various tumor suppressor genes and oncogenes during the progression through benign lesions towards carcinoma. The TFF3 and tumor angiogenesis regulator, VEGF activates STAT3 signaling through Tyr (705) phosphorylation of STAT3α and STAT3β isoforms [14]. Blocking of STAT3 signaling abrogates TFF3 and VEGF-induced cellular invasion and reduces the growth of HCT8/S11 tumor xenografts in athymic mice. STAT3 exerts anti-apoptotic and mitogenic effects [24]. Since VEGF has been reported to be critically involved in the

development and differentiation of gastric cancer, we explored TFF3 regulates the expression of VEGF in a gastric epithelial cell line SGC-7901. We detected the upregulation of endogenous TFF3 and VEGF expression in SGC-7901 cells induced by hypoxia. Baseline mRNA expression of TFF3 was detected in gastric SGC-7901 cells; overexpression of TFF3 upregulated the mRNA expressions of VEGF-induced by hypoxia, and the VEGF expression was significantly down-regulated both in transcription and protein levels in stably TFF3 silenced SGC-7901 cells. These results showed the TFF3 mediated regulation of VEGF expression induced by hypoxia.

Although, HIF-1α and HIF-2α subunits are highly conservative at protein level, share similar domain structure, heterodimerize with HIF-1β, their effect on the expression of some genes may vary [25]. The HIF-1 plays a major role in controlling the ubiquitous transcriptional response to hypoxia, it is clear that a number of other transcription factors are also activated either directly or indirectly [26]. Subsequently, it was determined that HIF-1 is widely expressed and that consensus HIF-1-binding sequences exist in a number of genes and are termed HRE. An increased upregulation of TFF3 and VEGF mRNA expression via hypoxia was detected and these results indicated a link between HIF-1α and these two genes. The immediate region of the HIF-1consensus site also contains consensus binding sites for the transcription factors GATA-1, GATA-2, and MyoD. They have been implicated in either induction or repression of genes in hypoxia [27–29] [30]. A previous study also observed the up-regulation of TFFs mediated by HIF-1 in hypoxia and induction of TFF3 was regulated by HIF-1a in hypoxia, as the same HRE consensus sequence are present in the rat TFF3 promoter (3/−13, relative to the transcription start site), which suggest that HIF-1 may act as a transcription factor in the up-regulation of TFF3 gene [15, 17]. Our data showed the higher mRNA upregulation of HIF-1α expression in SGC-7901 cells after induction of hypoxia; however, this upregulation was suppressed to baseline level in TFF3-KD cells. No significant difference of HIF-1α protein expression was observed at 6 and 12 h after induction of hypoxia in TFF3-KD cells. Taken together, TFF3 is a downstream of HIF-1a mediated signaling cascade; on the other hand, TFF3 might be a potential upstream regulator of the HIF-1a during hypoxic condition in some extent.

Conclusion

Our data demonstrated the TFF3 mediated regulation of VEGF expression induced by hypoxia, and implicated that TFF3 might be applied as a potential anti-angiogenic target for treatment of gastric cancer.

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