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TNFα induced FOXP3–NFκB interaction dampens the tumor suppressor role of FOXP3 in gastric cancer cells

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ABSTRACT

Controversial roles of FOXP3 in different cancers have been reported previously, while its role in gastric cancer is largely unknown. Here we found that FOXP3 is unexpectedly upregulated in some gastric cancer cells. To test whether increased FOXP3 remains the tumor suppressor role in gastric cancer as seen in other cancers, we test its function in cell proliferation both at basal and TNFα mimicked inflammatory condition. Compared with the proliferation inhibitory role observed in basal condition, FOXP3 is insufficient to inhibit the cell proliferation under TNFα treatment. Molecularly, we found that TNFα induced an interaction between FOXP3 and p65, which in turn drive the FOXP3 away from the promoter of the well known target p21. Our data here suggest that although FOXP3 is upregulated in gastric cancer, its tumor suppressor role has been dampened due to the inflammation environment.

1. Introduction

Transcription factor forkhead box protein 3 (FOXP3) is a member of forkhead transcriptional factor family, and is mainly expressed in regulatory T cells (Tregs). Furthermore, FOXP3 is a key molecule and specific marker for the development and function of these cells [1]. FOXP3 conferred Tregs with the function of immunosuppression and was therefore vital to their function and development [2]. While previous studies mainly focused on Tregs and their role in immunosuppression, recent data indicated that FOXP3 was also expressed by some nonlymphoid cells, in which it repressed various oncogenes [3]. Researchers at the University of Michigan reported that female heterozygous mice harboring FOXP3 mutation are prone to develop breast cancer, due to the downregulation of FOXP3 and overexpression of HER-2 [3]. Simultaneously, the expression of FOXP3 inhibited the expression of SKP2 [4]. Recently, it is reported that FOXP3 played a cancer suppressor role in MCF-7 cells by transcriptionally activating p21 expression, suggesting a tumor suppressor role in breast cancer [5]. In addition, FOXP3 was also found to be upregulated in other cancers, such as ovarian cancers. The increased FOXP3 in turn inhibited cell proliferation, migration and invasion [6]. Although most of the studies suggest a tumor suppressor role for FOXP3, Wang et al. found that elevated FOXP3 expression correlated with increased lymph node metastasis in gastric cancer, suggesting an oncogenic role [7]. The mechanism for the inconsistency remains largely unknown.

The tumor tissue is mainly composed of tumor cells and the tumor microenvironment. The tumor microenvironment is critical for both tumorigenesis and the progression. Particularly, inflammation sustains the growth, proliferation and progression of tumor cells, including the alteration of oncogenes and cancer suppressor genes. Constitutive activation of NF-κB and inflammation are two common features of several tumor cells. While NF-κB plays a role in tumor pathophysiology, the signaling of TNF-α and NF-κB constitutes an important activation pathway in inflammation. The functional role of FOXP3 in inflammatory microenvironment and its interaction with NF-κB in tumor cells have yet to be reported.

The connection between inflammation and tumorigenesis, especially in gastrointestinal cancer, is well-established based on genetic, pharmacological, and epidemiological evidence in the last decade [8]. The inflammatory microenvironment not only promotes the tumorigenesis, but also facilitates the progression of cancer. Up to now, how inflammation influences the FOXP3 function in gastric and other types of cancers remains largely unknown.

Our study here reveals that FOXP3 is surprisingly upregulated in some gastric cancer cells. Although FOXP3 inhibits cell proliferation under basal conditions, it is insufficient to inhibit cell growth under TNF-α treatment. The underlying mechanism is that inflammation induces an interaction between FOXP3 and p65, which in turn reduces the FOXP3 binding with the p21 promoter. Our data

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suggest that although FOXP3 is upregulated in gastric cancer, its tumor suppressor role is downregulated by inflammation. Our findings at least partially explain how FOXP3 loses its tumor suppressor role in gastric cancer.

2. Materials and methods

2.1. Cell culture

We used the gastric normal epithelial cell lines (GES-1), and gastric cancer cell lines AGS, MKN45, MKN28 and SGC7901. All of these cells were cultured in 1640 medium (Invitrogen, Carlsbad, CA), supplemented with 2 mmol/L glutamine, 0.06 g/L penicillin, 0.1 g/L streptomycin, and 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Construction and purification of retrovirus

The pCDH-EF1-MCS-3FLAG-T2A-Puro RV plasmid was used for retrovirus production. The Foxp3 sequence was inserted into the RV plasmid for construction of RV-FOXP3 and RV-Vec, and these vectors were confirmed by sequencing. The retrovirus DNA plasmids were then transfected into 293T cells, and the viruses were collected 72 h later. The virus was further purified and concentrated to a titer of 2.37×10^8 IU/mL. These vectors were successfully transfected into AGS cells and the stable cell line that overexpressed FOXP3 was also obtained.

2.3. Knockdown of p21

Knockdown of p21 was achieved by transfection of synthesized RNAi duplexes. The synthesized human p21 siRNA targeting the sequence: 5′-AAUAAUUCUAAUGCCAGAGGC-3′. And the negative control (NC) RNAi duplexes are 5′-UUCUCCGAACGUGUCACGUdtdt-3′ and its complementary reverse sequence.

2.4. Flow cytometry

The effects of FOXP3 on cell cycle both under basal and inflammatory conditions were analyzed by FACS. Cells were plated into six-well plates and treated as indicated. The cells were then collected and washed with PBS without Ca^{2+} and Mg^{2+} and fixed with 70% ethanol. Cells were re-suspended in 1× binding buffer and 5 μL of propidium iodide/10^5 cells, and incubated at room temperature for 15 min. Cells were acquired and the percentage of cells in each stage of the cell cycle was analyzed by flow cytometry.

2.5. MTT

MTT assay was used to assess cell proliferation. Briefly, cells with different treatments were seeded at a density of 1000 cells/well in 96-well plates. For inflammation, cells were treated with 10 ng/mL TNFα. 3-(4,5-Methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was added (100 μg/well) for 4 h at the indicated time. Formazan products were solubilized with DMSO, and the optical densities were measured at 490 nm. All experiments were performed in triplicate.

2.6. Real-time PCR

The total RNA of the cells with different treatment was extracted using TRIzol reagent. After the quantification, 2 μg RNA was reverse transcribed to cDNA. The target gene Foxp3, p21 and internal control gene β-actin were further amplified by real-time PCR. The reaction protocol was as follows: 2 μL cDNA,10 μL 2× SYBR Green Mix, 0.2 μL 10 μmol/L sense primer, 0.2 μL 10 μmol/L antisense primer and 7.6 μL double distilled water. The PCR amplification, fluorescence signal detection and data collection were achieved using 7300, a Real-Time PCR machine (ABI). The other two wells were set for each sample, and the experiments performed in quadruplicate. The Ct mean was calculated and analyzed using ΔΔCt assay. The primer sequences were as follows: hFoxp3: 5′-TTCGAAGAGCCAGAGGACTT-3′ (sense) and 5′-ATGGCACTCAGCTTCTCCTT-3′ (antisense); p21, 5′-ACCATGTGGACCTGTCACTGT-3′ (sense) and 5′-TTAGGGCTTCCTCTTGGAGAA-3′ (antisense); β-actin, 5′-AGAGGGAAATCGTGCGTGAC-3’ (sense) and 5′-CAATAGTGATGACCTGGCCGT-3′ (antisense).

2.7. Western blot

The total protein from cells with different treatment was extracted for Western blot assay. Briefly, the total protein was extracted on ice for 30 min by adding protein lysis buffer and proteinase inhibitor. The mixture was centrifuged at 13,000g for 15 min at low temperature, and the supernatant (total protein) was obtained. The different proteins were quantified using BCA assay, with 80 μg protein loaded into each lane and separated by 120 g/L sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. The proteins were transferred to the NC membrane and further incubated with the indicated antibodies (Anti-Flag antibody from Sigma, Foxp3 Rabbit mAb from Cell Signaling Technology, anti-NF-κB p65 antibody and anti-p21 antibody from Bioworld Technology) at 4 °Covernight. The membrane was then incubated with HRP tagged secondary antibody (1:2000) at room temperature for 2 h before visualized using ECL.

2.8. Laser scanning confocal microscopy

Cells transduced with RV control or with RV-Flag-Foxp3, were stimulated with TNF-α for 1 h. Cells were washed by precooling PBS (4–8 °C) three times and fixed with 4% paraformaldehyde at 4 °C for 10 min. Then, these cells were treated with 1 mL blocking buffer at 4 °C for 45 min before incubated with Goat Anti-Human FOXP3 antibody (1:200) at 4 °C for 2 h. Then, FITC-tagged anti-Goat

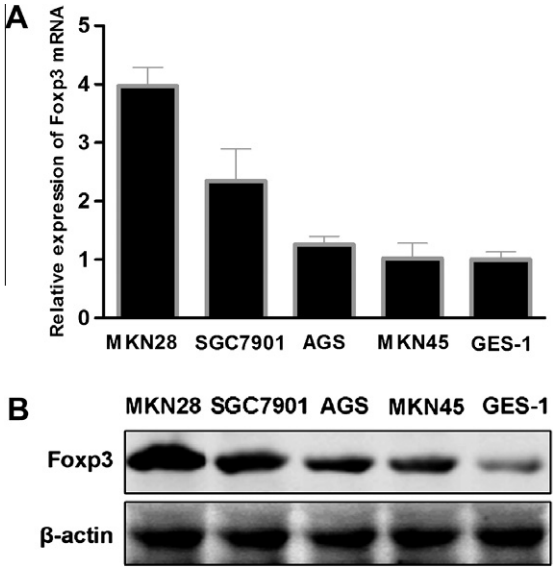

Fig. 1. Expression of FOXP3 in gastric cancer. (A) FOXP3 expression at mRNA level in the available cell lines was detected by qRT-PCR, and β-actin served as an internal reference. (B) FOXP3 expression at protein level in the available cell lines was detected by Western blot, and β-actin served as a loading control.

and PE-tagged anti-Mouse secondary antibodies (1:50) were added into these cells and incubated at 4 °C for 30 min. To visualize the nuclear, DAPI was added at the dose of 10 μg/mL and incubated at room temperature for 5–10 min. After each step, the cells were washed by precooling film breaking buffer. The cells were analyzed using a laser scanning confocal microscope.

2.9. Co-immunoprecipitation (Co-IP)

The IP antibody was added to 200 μL total protein, the mixture gently shaken and incubated at 4 °C overnight. Then the solution was added with 20 μL protein G agarose beads and centrifuged. We then washed the pellet five times with 500 μL lysis buffer, centrifuged at 4 °C for 30 s and discarded the washing buffer. The agarose beads were then resuspended using SDS buffer, vortexed vigorously, and centrifuged the mixture at 4 °C for 30 s. The suspension was then boiled for 5 min and centrifuged at 14,000*g* for 1 min. A 30 μL sample was loaded into a discontinuous SDS–PAGE gradient gel. Finally, Western blot analysis was performed.

2.10. Chip

Chromatin immunoprecipitation was carried out as previous procedure [9]. Briefly, the cells transduced with RV control or with RV-Flag-Foxp3were stimulated with TNF-α for 12 h before fixed with 1% paraformaldehyde. Then the cells were sonicated to produce 200–1000 bp DNA fragments. The anti-Flag antibody and control mouse IgG were used for ChIP assay. The amounts of the interested region in the precipitated DNA fragments were quantitated by real-time PCR and normalized against the Input. The real-time PCR was performed using the following primers: 5′-AGGCACTCAGAGGAGGTGAGA-3′ (sense) and 5′-CAGAAACACCTGTGAACGCA-3′ (antisense).

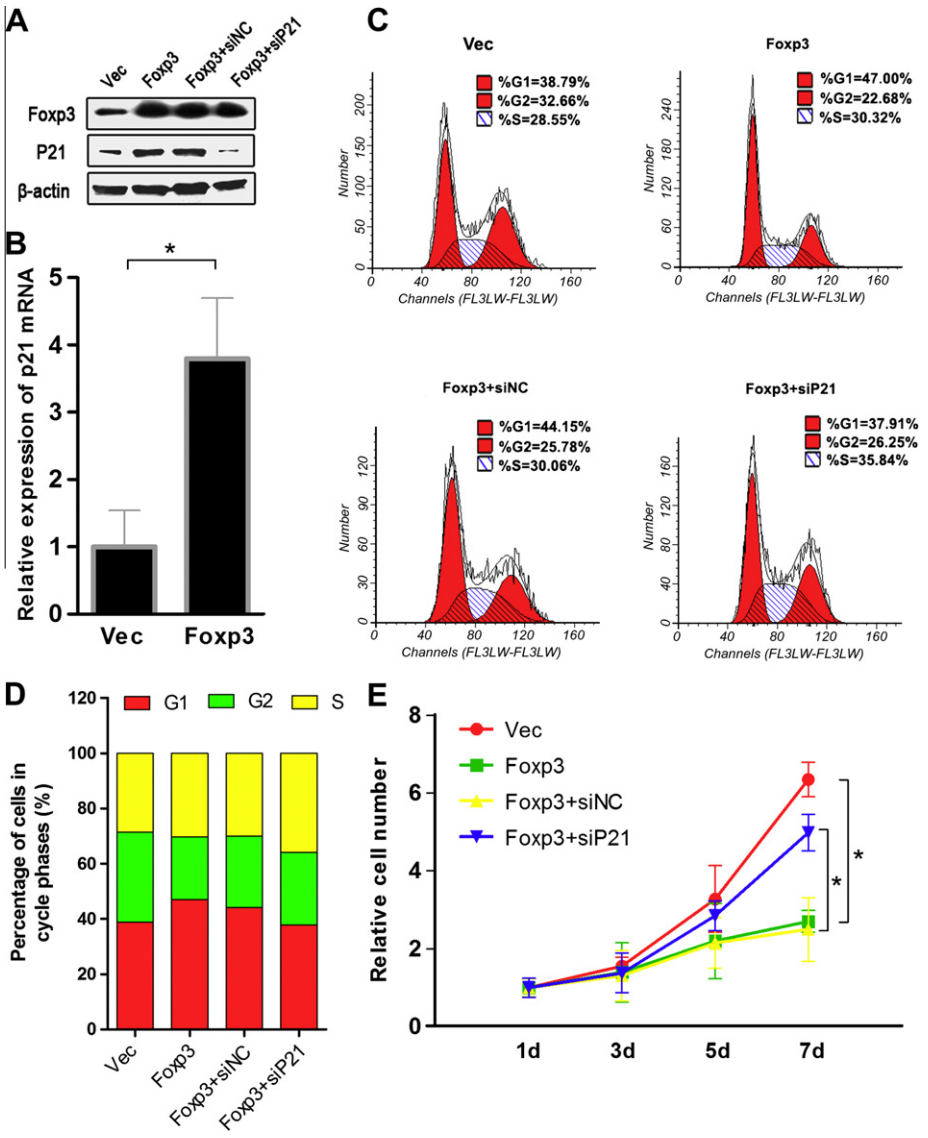

Fig. 2. FOXP3 inhibits cell proliferation in a p21-dependent manner under basal condition. (A) Expression of FOXP3 and knockdown efficiency of p21. AGS cells were transfected with FOXP3 expression retrovirus vector (Foxp3) with or without co-transfection of p21 RNAi duplexes. Both FOXP3 and p21 expression were analyzed by Western blot. (B) Expression of p21 upon FOXP3 overexpression. AGS cells were transfected with empty vector (Vec) or FOXP3 vector (Foxp3) for 24 h and p21 expression was analyzed by qRT-PCR. β-actin served as an internal reference and relative expression of p21 was calculated by 2-ddCt. $^{*}P < 0.05$. (C) AGS cells were transfected with empty vector (Vec), FOXP3 vector (Foxp3) or FOXP3 in combination with Negative Control RNAi (siNC) or p21-siRNA (siP21) for 48 h before harvested for cell cycle analysis. (D) Quantification of data in Fig. 2C. (E) Cells were treated same as above and MTT assay was done for cell proliferation analysis. All the experiments were done in triplicates ($P < 0.05$).

2.11. Statistical analysis

The data were represented as mean ± SD. The non-paired *t*-test was used to compare the significance of difference in the means between different groups. $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Increased expression of FOXP3 in gastric cancer

To explore the potential role of FOXP3 in gastric cancers, we first analyzed its expression in both normal and cancerous cell lines by both qPCR and Western blot (Fig. 1A and B). All the cancer cell lines demonstrate higher expression levels of FOXP3 than the normal GES-1 cells. Especially, FOXP3 was highly expressed in some poor differentiated cell lines (Fig. 1A and B).

3.2. FOXP3 inhibits cell proliferation in p21-dependent manner under basal condition

Inflammation is a characteristic of gastric cancer, especially those with advanced TNM status. In addition, FOXP3 was found to inhibit breast cancer by upregulating p21 [5]. Based on these and the above data, we hypothesize that FOXP3 might function differently under basal and inflammatory conditions. Firstly, we transfected cells with FOXP3 expression retrovirus vector, which increased the FOXP3 expression both at mRNA level and protein levels significantly (Fig. 2A and B). As expected, increased the p21 mRNA level by about 3.8-fold under the basal condition (Fig. 2B). Consistently, FOXP3 transfection increased cells in the G1 phase by almost 9% (Fig. 2C and D). MTT assay further confirmed that FOXP3 significantly decreased the proliferation of AGS cells ($P < 0.05$) (Fig. 2E). Knockdown of p21 by RNAi (Fig. 2B) significantly reversed the tumor suppressor role of FOXP3, as seen by the cell cycle distribution and MTT assay (Fig. 2C–E). The findings suggest that FOXP3 functions as a tumor suppressor by upregulating p21 transcription.

3.3. TNF-α treatment blocks the tumor suppressor role of FOXP3

Since inflammation is one of the most common features of gastric cancer progression, we asked whether the tumor suppressor role of FOXP3 persisted under inflammation. The gastric cancer cell line AGS transfected with either control or FOXP3 was further treated with vehicle or TNF-α. As shown in Fig. 3A and B, no significant difference was seen in cell cycle distribution between FOXP3 transfection and control groups under TNF-α treatment. Similar results were obtained from the MTT assay (Fig. 3C). Accordingly, TNF-α

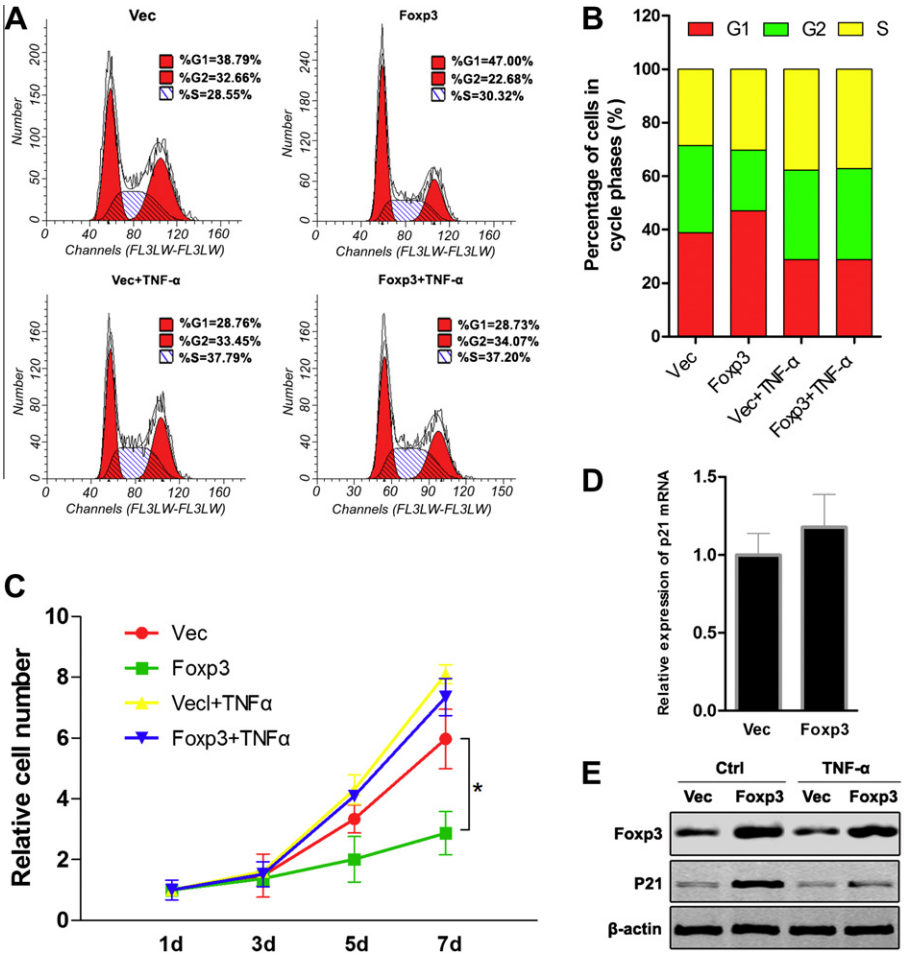

Fig. 3. TNF-α dampens the tumor suppressor role of FOXP3. (A) AGS cells transfected with empty vector (Vec) or FOXP3 vector (Foxp3) were further treated with control or 10 ng/mL TNF-α for 48 h before harvested for cell cycle analysis by FACS assay. (B) Quantification of data in Fig. 3A. (C) Cells were treated same as above and MTT assay was done for cell proliferation analysis. All the experiments were done in triplicates ($P < 0.05$). (D) AGS cells were transfected with empty vector (Vec) or FOXP3 vector (Foxp3) with TNF-α (10 ng/mL) for 24 h and expression of p21 was analyzed by qRT-PCR. β-actin served as an internal control and relative expression of p21 was calculated by 2-ddCt. No significant induction of p21 by FOXP3 was found upon TNF-α treatment. (E) AGS cells of stably expressed FOXP3 (Foxp3) and its corresponding empty vector (Vec) were served as control (Ctrl) and further treated with 10 ng/mL TNF-α (TNF-α) for 24 h. p21 expression was analyzed by Western blot. β-Actin served as an internal control.

treatment nearly blocked the induction of p21 mRNA by FOXP3 (Fig. 3D). Expression of p21 at protein level induced by FOXP3 was also significantly blocked under TNF-α simulation (Fig. 3E).

3.4. Interaction between p65 and FOXP3 inhibits p21 transcription activation

Next, we asked how inflammatory signaling affected the regulatory effect of FOXP3 on p21 promoter. P65 plays a central role in inflammation. Similar as the TNF-α treatment, p65 transfection also decreased the induction of p21 by FOXP3 both at mRNA and protein level (Supplementary Fig. 1A and B). Laser scanning confocal microscope revealed ectopic FOXP3 was mainly expressed in the nuclei, with only a small proportion in the cytoplasms both under normal and TNF-α simulated inflammatory condition (Fig. 4A), while p65 enters the nuclei specifically under inflammatory condition. As illustrated in Fig 4A, FOXP3 and NF-κB co-localized in the nuclei of AGS cells under TNF-α simulated inflammatory condition, suggesting that there might be an interaction between FOXP3 and p65 under inflammatory condition. Further study revealed that p65 interacted with FOXP3 in tumor cells when cells were treated with TNF-α by using Co-IP analysis, while there was no detectable interaction between FOXP3 and p65 without TNF-α treatment (Fig. 4B). Consistent with the interaction of FOXP3 and p65, ChIP assay revealed that reduced occupation of FOXP3 on p21 promoter under TNF-α treatment (Fig. 4C). All of these suggest that the nuclear accumulation of NF-κB and thus the interaction between NF-κB and FOXP3 might drive the FOXP3 away from the p21 promoter.

4. Discussion

Our study reveals that FOXP3 was upregulated in some gastric cancer cells. Unlike the tumor suppressor role of FOXP3 reported in most previous studies, we found that FOXP3 was insufficient to inhibit the cell cycle under TNF-α treatment. The molecular mechanism study suggested that inflammation induced an interaction between FOXP3 and p65, which in turn suppressed the FOXP3 binding with the p21promoter target.

FOXP3 was universally expressed in tumor cells, such as gastric cancer, melanoma, lung cancer, colon cancer and so on [10]. Previous studies suggest that FOXP3 functions as a tumor suppressor in breast cancer and other malignancies [3,4,6]. However, our study suggests that FOXP3 does not act as a tumor suppressor in most gastric cancers, in which inflammation is prevalent. It is well known that the tumor microenvironment is critical for the tumorigenesis and development. Inflammation provides an important niche for the growth, proliferation and progression of tumor cells, including the alteration of oncogenes and cancer suppressor genes [11,12]. During chronic inflammation, pro-inflammatory molecules, such as cytokines, inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), and NF-κB are upregulated [13]. Together, these processes provide a favorable microenvironment for the exponential growth of malignant cells. Our study suggests that

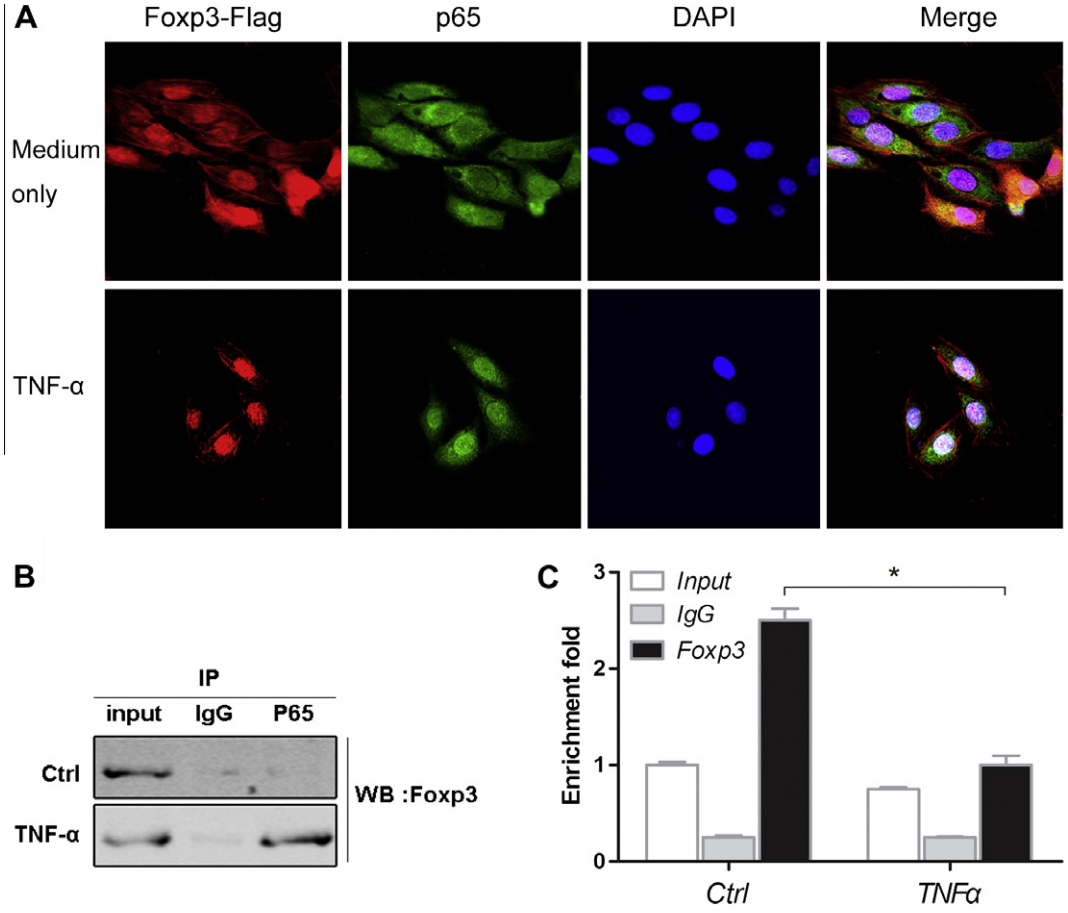

Fig. 4. Interaction between p65 and FOXP3 inhibits the transcriptional activation of p21. (A) Cells were cultured with or without TNF-α treatment. Total FOXP3 and endogenous p65 localization were analyzed by laser scanning confocal microscopy. FOXP3 and NF-κB co-localized in the nuclei of AGS cells under TNF-α treatment for 1 h. (B) Cells were cultured under basal and TNF-α treated conditions. Co-IP analysis revealed that p65 co-immunoprecipitated with FOXP3 in tumor cells when treated with TNF-α for 1 h. (C) Cells were cultured under both basal and TNF-α treated conditions. ChIP assay revealed reduced the occupation of FOXP3 on p21 promoter under TNF-α treatment.

inflammation not only promotes the cancer progression but also downregulates the tumor suppressor genes, such as FOXP3.

We found that inflammation blocks the tumor suppressor role of FOXP3 mainly through the nuclear localized p65. Nuclear localized p65 interacts with FOXP3, and thus dissociates the FOXP3 away from the p21 promoter. FOXP3 has also been found to directly interact with c-Rel through its N-terminal region to repress the NF-κB pathway in mature Treg cells [14]. In fact, FOXP3 has many other binding partners. FOXP3 is a key transcription factor required for the suppressive function of Treg cells. Upon the induction of FOXP3 expression in Treg cells, a number of inflammatory cytokines, such as IL-2 and IFN-γ, are downregulated, whereas IL-10, CTLA-4, glucocorticoid-induced tumor necrosis factor receptor (TNFR)-related protein (GITR) and CD25 are upregulated [15]. It is well known that transcriptional activation is driven by the interaction between transcription factor and the components of the basal transcription apparatus such as TATA-binding proteins [16–19], p300 [20] and cyclic-AMP-response element (CREB)-binding protein (CBP) [20,21]. An attractive hypothesis would be that p65 competes with FOXP3 for binding such factors, which results in inhibition of transcription. In this regard, it may be interesting to test whether p65 target genes are also repressed.

It has been proposed that Foxp3 forms high molecular weight, multimeric transcriptional complex [22]. Although our study reveals that interaction between FOXP3 and p65 reduces the FOXP3 enrichment on p21 promoter, the underlying mechanism remains largely unknown. Further clarifying the detailed complex would be informative for the understanding of the detailed mechanism.

As FOXP3 plays a central role in Treg induction and function [1,2], our study might also have implications for Treg function. It may therefore be interesting to investigate whether p65-FOXP3 interaction explains Treg induction deficiency in serious inflammation [23,24].

In summary, our data here suggest that although FOXP3 is upregulated in gastric cancer, its tumor suppressor role has been dampened due to the inflammation environment. Our findings here at least partially explain why FOXP3 lose its tumor suppressor role in gastric cancer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.039.

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