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# High FoxP3 expression in tumour cells predicts better survival in gastric cancer and its role in tumour microenvironment

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**Background:** Forkhead Box P3 (FoxP3) is thought to be a key transcription factor in regulatory T cells (Tregs), and recent data indicate that it is expressed in several tumour cells. However, its precise roles in gastric cancer (GC) and the underlying mechanisms regulating the interaction between GC cells and lymphocytes remain unclear.

**Methods:** FoxP3 expression was examined in tumour cells and Tregs in 150 cases of gastric precancer and cancer, and their prognostic significances were evaluated, respectively, using a tissue microarray containing 135 GC patient samples with a mean 102-month follow-up. FoxP3 involvement in the tumour cells–lymphocytes interaction and its gene function were further investigated.

**Results:** strong cytoplasmic staining of FoxP3 was observed in GC cells. FoxP3 protein expression in tumour cells predicts a good prognosis, whereas high-density Treg predicts a poor prognosis. Moreover, FoxP3 expression in GC cells increased after coculture with peripheral blood mononuclear cells through coculture systems. Upregulation of FoxP3 inhibited tumour growth in tumour-bearing nude mice.

**Conclusions:** High FoxP3 expression in tumour cells predicts better survival in GC, possibility in relation to interaction between tumour cells and lymphocytes in microenvironment. Interfering with FoxP3 expression may open a new therapeutic strategy against tumour progression.

Forkhead Box P3 (FoxP3) is a key transcription factor in regulatory T cells (Tregs), and has important roles in the immunosuppressive functions in Tregs (Hori and Sakaguchi, 2004). Previous studies have documented that an abundance of FoxP3-positive Tregs in tumours was associated with a poor prognosis (Kono *et al*, 2006; Salama *et al*, 2009a; Shen *et al*, 2010). It has recently been demonstrated that tumour cells express FoxP3 (Ebert *et al*, 2008; Karanikas *et al*, 2008) and this expression can improve survival in several cancers (Martin *et al*, 2010; Wang *et al*, 2010a, b; Dimitrakopoulos *et al*, 2011). Its expression in cancer cells is an important mechanism of tumour escape (Hinz *et al*, 2007). We recently observed that FoxP3 expression in gastric cancer (GC) cells inhibits cell proliferation and induces apoptosis (Ma *et al*, 2013). However, the status of FoxP3 protein and mRNA and its involvement in the transformation from precancer (PC) to GC remains poorly understood. Limited data suggest a relationship between FoxP3-positive tumour cells and Treg density and their respective clinical significance regarding GC prognosis.

The tumour microenvironment involves not only the interaction among several cell types, but also the cytokines secreted among them. Both tumour cells and Tregs express FoxP3, making the interactions between them complicated. Tumour cells can

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transform CD4+ T cells into Treg, resulting in immune escape (Liyanage *et al*, 2006; Li *et al*, 2007; Yuan *et al*, 2011). However, it is still unclear whether lymphocytes can affect tumoral FoxP3 expression. If so, how such an interaction occurs and by which mechanism remains unknown. We sought to better understand the potential mechanism by which FoxP3 mediates interaction between tumour cells and lymphocytes.

FoxP3 function and its contribution to GC development remain poorly understood. It is thought to be a tumour suppressor gene that depresses oncogene expression and inhibits tumour growth in breast and prostate cancers *in vitro* and *in vivo* (Liu *et al*, 2009; Zhang and Sun, 2010; Li *et al*, 2011). However, it is either downregulated or not expressed in breast cancer (Zuo *et al*, 2007; Wang *et al*, 2009; Ladoire *et al*, 2011), whereas it is overexpressed in gastrointestinal (Wang *et al*, 2010a, b) and lung cancer (Dimitrakopoulos *et al*, 2011). It is unclear about the differential expression.

On the basis of these results, the aim of the current study was to examine the clinical significance of FoxP3 expression in GC cells and lymphocytes, and to explore the underlying mechanism during interaction between tumour cells and lymphocyte. A better understanding of FoxP3 might open a new avenue for targeted therapeutic strategies against tumour progression.

## MATERIALS AND METHODS

**Patient samples.** A total of 150 cases were included in this study, comprising 30 surgically resected primary GC specimens, 90 neoplastic and cancerous specimens obtained from endoscopic submucosal dissection, and 30 control biopsy samples obtained from normal-appearing gastric mucosa in patients free from neoplastic or inflammatory diseases. Patient samples were as follows: 30 normal tissues (18 males, 12 females; mean age = 49.45 ± 13.11 years, range 38–72 years), 58 PC including low-grade or high-grade intraepithelial neoplasia (40 males, 18 females; mean age = 61.88 ± 10.95 years, range 35–81 years), 32 early GC (EGC) defined as the superficial cancer located in the mucosa and submucosa layers of stomach (21 males, 11 females; mean age = 64.03 ± 12.65 years, range 52–81 years), and 30 advanced GC (AGC) (21 males, 9 females; mean age = 59.48 ± 10.75 years, range 30–70 years). FoxP3 was also detected in tumour and corresponding peritumour tissue microarrays containing 135 samples of GC patients enrolled in Zhongshan Hospital from 2000 to 2005 (90 males, 45 females; mean age = 58.85 ± 11.98 years, range 33–80 years) with a mean follow-up time of 102 months (range 72–132 months). All patients were confirmed by pathological examination. Histological type was assessed according to the World Health Organization classification (Hamilton and Aaltonen, 2000). The study and control groups were demographically similar ($P > 0.05$).

Patients who received radiochemotherapy, suffered from other cancers, or who had a family history of GC were excluded from the study. Informed consent was obtained from all subjects. The project was approved by the Research Ethics Committee of Zhongshan Hospital (Shen *et al*, 2010).

**IHC analysis.** FoxP3 protein levels were examined by immunohistochemistry (IHC) in 4-$\mu$m-thick paraffin sections cut from a single selected block containing neoplastic and non-neoplastic gastric tissues. Samples were routinely treated using a standard staining procedure (EnVision Detection Kit, Dako, Carpinteria, CA, USA) and were subsequently incubated with anti-FoxP3 monoclonal antibody clone Ab20034 (dilution 1 : 250; Abcam, Cambridge, UK), also repeated by monoclonal another FoxP3 antibody (Biolegend, San Diego, CA, USA). For antibody-negative controls, the primary antibodies were substituted with normal rabbit serum. Predominantly cytoplasmic staining was observed in tumour cells and nuclear staining in lymphocytes. Tregs in tumour stroma was counted in 10 high-power field (HPF, at ×400 magnification), Treg count of >25 per HPF was defined as high Treg, and Treg counts of <5 per HPF was defined as low Treg. The presence of FoxP3-positive tumour cells and the quantification of infiltrated FoxP3+ Treg cells were, respectively, calculated by two investigators with no knowledge of the clinical grouped data.

**Quantitative real-time PCR.** Total RNA was isolated from biopsy and surgical specimens, or from cultured cells, using Trizol reagent (Invitrogen, San Diego, CA, USA). Complementary DNA was prepared using the Primer Script RT Reagent (TaKaRa, Tokyo, Japan). Expression levels of FoxP3, transforming growth factor (TGF)-$\beta$1 and human epidermal growth factor receptor 2 (HER2) mRNAs were confirmed by SYBR Green II qRT–PCR (quantitative real-time polymerase chain reaction) with two steps, at 95 °C for 30 s then 60 °C for 1 min. All the products were normalised to the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Supplementary Table S1). All qRT–PCR experiments were performed by the same investigator with no knowledge of the corresponding clinical data.

**Cells and cell culture.** The GC cell lines, AGS, and MKN45 were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). They were routinely cultured in DMEM medium (Invitrogen) supplemented with 10% fetal calf serum, 100 U ml$^{-}$ penicillin, and 100 ug ml$^{-1}$ streptomycin (Invitrogen) in 5% $CO_2$ incubator at 37 °C.

**Isolation of PBMCs and coculture model.** Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of GC patients or control blood donors using a method of density gradient centrifugation, as described previously (Martignoni *et al*, 2005; Nowak *et al*, 2010). Part of cells was then cultured for 24 h to allow the attachment of adherent cells, such as dendritic cells. Others were collected for flow cytometry analysis.

The coculture model was established as follows: Transwell plates (Corning, Corning, NY, USA) were used as an indirect coculture model. These plates contain bottom and top chambers with 0.4-$\mu$m membrane filter pores that do not allow GC cells to pass through but allow medium to exchange freely. Co-incubation of the two types of cells together was used as a direct coculture model, whereas single culture of GC cells was defined as a monoculture. GC cells were adjusted to $5 \times 10^5$ cells per ml, seeded in the bottom chambers and incubated for 8 h to allow attachment. Inserts containing $5 \times 10^5$ cells per ml of PBMCs were then transferred to the top chambers and cocultured for another 24 h. As negative controls, inserts with PBMCs were placed on wells in the absence of GC cells, and wells with GC cells were left without inserts. The cell count in the monoculture group was double that in the coculture group to insure approximate cell numbers. GC cells and PBMCs were collected separately for further use.

**Flow cytometry analysis.** The intracellular staining of FoxP3 was performed according to manufacturer's instruction. The isolated PBMCs was fixed and permeabilised with Perm/Fix solution (eBiosicences, San Diego, CA, USA). These cells were stained intracellularly with mouse anti-Human FoxP3 APC antibody (clone 236 A/E7) and mouse isotype IgG1 (both from eBioscience) for 30 min in dark. After washing it twice, they were detected by BD FACS Aria flow cytometer (BD, Franklin Lakes, NJ, USA). Isotype control stained cells were used to adjust parameters of FSC and SSC. Mean fluorescence intensity (MFI) was analysed with FlowJo software (Treestar, Ashland, OR, USA). The details were shown in Supplementary Figure S2.

**Western blotting.** Transfected cells were plated in six-well plates for 48 h and then harvested. The total proteins were separated on

10% SDS–PAGE gels, followed by transferring to polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After incubating with primary monoclonal antibody specific to FoxP3 (1 : 100, Abcam, Cambridge, UK) or GAPDH (1 : 2000, Beyotime, Shanghai, China), proteins were subsequently probed with horseradish peroxidase-conjugated goat anti-mouse IgG (1 : 4000, Santa Cruz, Dallas, TX, USA). Protein bands were visualised by enhanced chemiluminescence according to the manufacturer's instructions (Millipore). The relative intensity was determined using Quantity One Image software (Bio-Rad, Hercules, CA, USA). Each experiment was conducted three times, and data were averaged.

**Selecting a population of cells that stably express FoxP3.** Stable FoxP3- or vector-transfected cells were prepared as described previously (Ma *et al*, 2013). Briefly, pEGFP-FoxP3 or vector shRNA plasmids were transfected into AGS cells using Lipofectamine-2000 (Life Technologies, San Diego, CA, USA), for 24 h without antibiotic selection. Transfected cells were then cultured in medium containing 500 $\mu g\,ml^{-1}$ G-418 to select against non-transfected cells. Antibiotic-resistant cells were subsequently seeded in 96-well plates to form individual clones. After 1 week, the clones were passaged and cultured. PCR and western blotting were used to select high-expressing FoxP3 clones. Selected clones were grown and named as AGS/FoxP3 and AGS/vector, respectively.

**Tumour-bearing nude mouse studies.** One month-old BALB/c nude mice were purchased from the Department of Experimental Animals of the Chinese Academy of Sciences (Shanghai, China). Mice were inoculated subcutaneously with FoxP3- or vector-transfected cells ($1 \times 10^6$ cells in 100 $\mu l$ serum-free media) and fed in specific pathogen-free facilities at the Experimental Animal Center of Fudan University. Tumour size was measured with calipers using the formula $V = (a \times b^2)/2$ every 3 days, in which $a$ and $b$ are the largest and the smallest perpendicular diameters, respectively. All mice were killed at 31 days. All animal experiments were conducted in accordance with the current standards of animal care and were approved by the Research Ethics Committee of Zhongshan Hospital (Shanghai, China).

**Statistical analysis.** Statistical analysis was conducted using SPSS 16.0 for Windows (SPSS, Chicago, IL, USA). Data are presented as means ± s.e.m. A chi-squared test was used to analyse the correlation between FoxP3 staining and clinical pathologic features. ANOVA was used to compare values among different groups, and least significant difference tests were used to identify specific differences between the two groups using corrected $\alpha$ values. Paired $t$-tests were performed to compare the mRNA levels in tumoral and peritumoral tissues. The prognostic assessment was performed by Kaplan–Meier survival analysis and log rank tests to identify significance. Bivariate correlation analysis was conducted to examine associations between FoxP3 mRNA status and related molecules.

## RESULTS

**FoxP3 protein is expressed in both lymphocytes and cancer cells.** Strong cytoplasmic staining of FoxP3 was observed in GC cells as determined by IHC in gastric tissues (Figure 1A) and nuclear staining in lymphocytes (Figure 1B). These results are consistent with the staining in our tissue microarray of GC (Supplementary Figure S1) and previous reports showing cytoplasmic FoxP3 staining in GC tissues (Wang *et al*, 2010a). Similar to our previous observation in GC cell lines (Ma *et al*, 2013), we herein confirmed FoxP3 expression in both the nucleus (or perinucleus) and cytoplasm of tumour cells in GC tissues. The involvement of this variable expression remains unclear, and it has been reported that Treg activation may induce subcellular FoxP3 localisation from a cytoplasmic to a more nuclear expression pattern due to post-translational modifications (Chen *et al*, 2006). This suggests that post-translational regulation may also be involved in tumour cell development and that heterogeneous localisation of FoxP3 in GC cells and lymphocytes may be indicative of different roles.

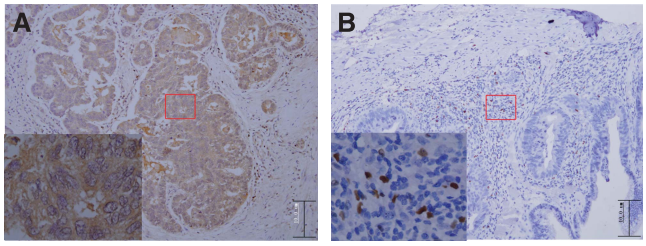


Figure 1. FoxP3 is expressed in both tumour cells and Tregs. (**A**) FoxP3-positive staining in the cytoplasm of tumour cells in an AGC. (**B**) FoxP3-negative staining in tumour cells, but positive stained in the infiltrated Treg in the stroma of an EGC. AB images are showed at × 100, enlarged at × 400.

**FoxP3 expression is increased in the progression from PC to GC.** The positivity rate of cytoplasmic staining of FoxP3 among different groups was 23.3% for control samples, 36.2% for PC, 46.8% for EGC, and 46.2% for AGC samples. It correlated with the severity of disease by linear-by-linear association $\chi^2$-test ($P = 0.014$). Corresponding FoxP3 mRNA levels also increased ($P < 0.01$) (Figure 2A). Furthermore, no mutation of the 12 exons of FoxP3 gene was found in 11 AGC patients with lower FoxP3 expression (data not shown). FoxP3 mRNA levels were significantly higher in tumour than in peritumour ($P = 0.003$) (Figure 2B), which is also positively related with those of TGF-$\beta$1 ($P = 0.020$, $r = 0.351$) and HER2 ($P = 0.002$, $r = 0.443$) (Figure 2C). FoxP3 protein levels were also elevated in tumour relative to peritumour, as confirmed by western blotting (Figure 2D). Surprisingly, FoxP3 expression in the GC cell lines was ∼100-fold lower than in lymphocytes by western blotting (Figure 2E). To further evaluate FoxP3 expression changes in the circulatory system, flow cytometry analysis was used to show that FoxP3 protein levels increased in PBMCs obtained from GC patients relative to controls (Figure 2F). Collectively, these results suggest that alternating FoxP3 profiles in both the local and general environments may account for tumour carcinogenesis and development.

**Tumoral FoxP3 expression correlates with good prognosis, whereas Treg density correlates with poor prognosis.** To assess the role of FoxP3 in tumour prognosis, we analysed the relationship of tumour-infiltrating Treg and tumoral FoxP3 expression with clinical pathological features. First, we found that tumoral FoxP3 expression and Treg density had no significant association with age, gender, TNM stage, or lymph node involvement in 197 GC patients (Table 1). Prognostic analysis showed that patients with FoxP3-positive tumours had a longer survival time and better prognosis, compared with FoxP3-negative patients ($P = 0.042$), whereas patients with elevated Treg counts displayed a shorter survival time and a worse prognosis as determined by tissue microarrays ($P = 0.034$) (Figure 3A and C). Both tumoral FoxP3 and Treg density did not contribute to prognostic assessment in peritumoral tissues (Figure 3B and D). Further sub-analysis revealed that increased Treg counts were associated with poorer overall survival, while the relationship between Treg accumulation and poor prognosis decreased in patients with FoxP3-positive tumours, but increased in tumoral FoxP3-negative patients (Figure 4A and B). The overall survival was better in tumoral FoxP3-positive patients than in FoxP3-negative ones regardless of

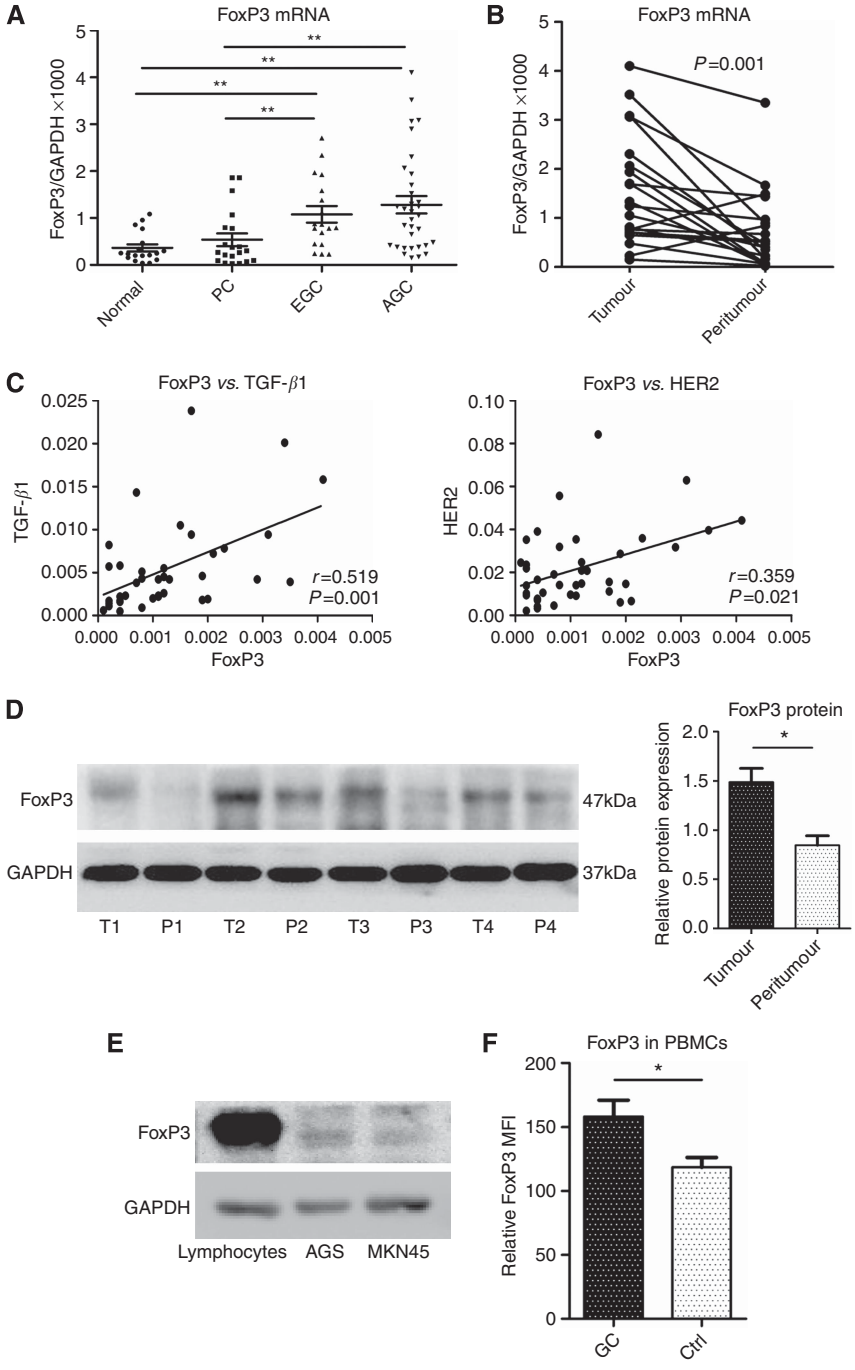


Figure 2. Profiles of FoxP3 in PC and GC cells. (**A**) Relative FoxP3 mRNA levels increased significantly in GC as tissues developed from healthy controls, PC, and to early and AGC as determined by one-way ANOVA analysis. Sub-analysis among different groups was performed by least squared test. (**B**) FoxP3 mRNA levels were significantly higher in tumoral than in peritumoral tissues ($n=20$, paired $t$-test, $P=0.003$). (**C**) FoxP3 mRNA levels show a positive correlation with TGF-$\beta$1 and HER2 in tumours, respectively (bivariate correlation test, $n=37$, $r=0.351$, $P=0.020$; $r=0.443$, $P=0.002$). (**D**) FoxP3 protein levels were higher in tumour than in peritumoral, as determined by western blotting (paired $t$-test, $P=0.024$). (**E**) FoxP3 protein levels in GC cell lines, AGS, and MKN45, were ~100-fold lower than those in lymphocytes as shown by western blotting. (**F**) FoxP3 protein levels shown by MFI were increased in 10 GC patients relative to 10 healthy volunteers, which were calculated by the FlowJo software (Treestar) ($t$-test, $P=0.031$). Each experiment was conducted three times, with each sample point assessed in triplicate, and data averaged. *$P<0.05$; **$P<0.01$.

high or low Treg counts, but with no statistically significant difference (Figure 4C and D). According to the hazard ratio (HR) illustrated in Table 2 by Cox regression analysis, that is, HR > 1 means it is hazard factor, whereas HR < 1 means protection factor, we further concluded that tumoral FoxP3-positive staining was a positive prognostic factor, whereas Treg counts and TNM stages were a negative prognostic factor. However, we speculate that Tregs might make a more significant contribution to prognosis as FoxP3 expression in tumours was ~100-fold lower than in Tregs as observed here and by others (Chen *et al*, 2008).

**FoxP3 expression in GC cells increased after coculture with PBMCs.** To further investigate the involvement of FoxP3 during the interaction between tumour and immune cell, a coculture

Table 1. Correlation between FoxP3-positive tumour cells and Treg density with clinical pathology ($n = 197$)

| Features | Tumour FoxP3 + | Tumour FoxP3 − | *P*-value | High Treg | Low Treg | *P*-value |
|---|---|---|---|---|---|---|
| **Age** | | | | | | |
| ⩽50 years | 10 | 10 | 0.755 | 6 | 14 | 0.700 |
| >50 years | 81 | 96 | | 58 | 119 | |
| **Sex** | | | | | | |
| Male | 64 | 66 | 0.310 | 14 | 118 | 0.335 |
| Female | 27 | 40 | | 10 | 55 | |
| **TNM stage (tumour, lymph nodes, metastasis)** | | | | | | |
| Stage 1 | 32 | 40 | 0.859 | 12 | 60 | 0.242 |
| Stage 2 | 16 | 14 | | 3 | 27 | |
| Stage 3 | 39 | 46 | | 9 | 76 | |
| Stage 4 | 4 | 6 | | 0 | 10 | |
| Lymph node (+) | 51 | 60 | 0.923 | 11 | 100 | 0.268 |
| Lymph node (−) | 40 | 46 | | 13 | 73 | |

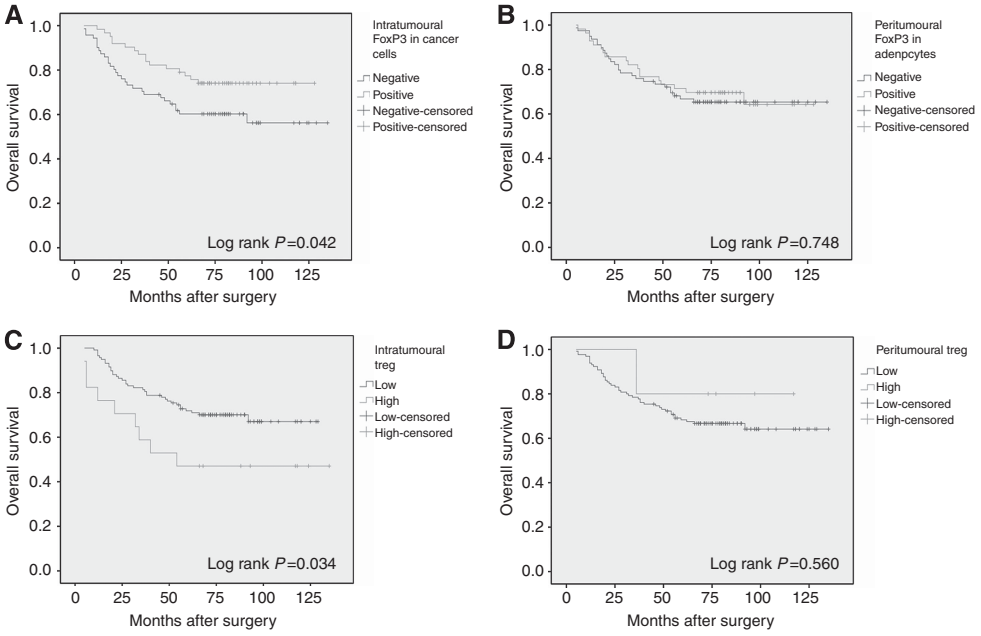


Figure 3. FoxP3 and Treg density predict different prognosis in tumour and peritumour. (**A**) Kaplan–Meier survival analysis indicates that patients with FoxP3-positive tumours have a longer survival time and better prognosis compared with the patients lacking FoxP3 expression in tumour, as determined by tissue microarrays containing 135 cases of GC with a mean of 102 months follow-up time (log rank test, $P = 0.042$). (**B**) FoxP3 expression in adenocytes does not contribute to prognosis in peritumoral tissues. (**C**) Patients with high-density Treg show a shorter survival time and worse prognosis in tumour tissues (log rank test, $P = 0.034$). (**D**) Treg density does not contribute to prognostic assessment in peritumoral tissues.

system was established by co-incubating human GC cell lines with isolated PBMCs. Results show that FoxP3 protein levels in PBMCs was higher in GC patients than in controls (Figure 5A), and that levels increased after coculture (Figure 5B). This may be because tumour cells can transform CD4 + T cells into Tregs and to have an immune-inhibiting function, as observed previously (Hinz *et al*, 2007). We further found FoxP3 were higher in GC cells in direct coculture compared with those in indirect coculture, indicating that a FoxP3-promoting effect depends mainly on direct cell-to-cell contact as assessed both in mRNA (Figure 5C) and protein levels (Figure 5D). These results suggest that direct interaction between GC cell lines and PBMCs might promote FoxP3 expression in a tumour microenvironment. The exact cell subpopulation involved in Foxp3-promoting effect in PBMC will be further investigated.

**FoxP3 gene inhibits tumour growth in tumour-bearing nude mice.** We have showed that FoxP3-overexpressing cells can inhibit cell growth in our previous study (Ma *et al*, 2013). FoxP3-overexpressing cells have an obvious higher FoxP3 expression than vector-transfected cells in mRNA levels (>500-fold) and in protein levels (about 100-fold) (Figure 6A). Herein, we further found that tumour in FoxP3-overexpessing group grew slowly than that in the control group ($n = 5$, *t*-test, $P < 0.05$) (Figure 6B) and tumour size of FoxP3-transfected tumour cells was significantly smaller than those in vector-transfected tumour cells *in vivo* after

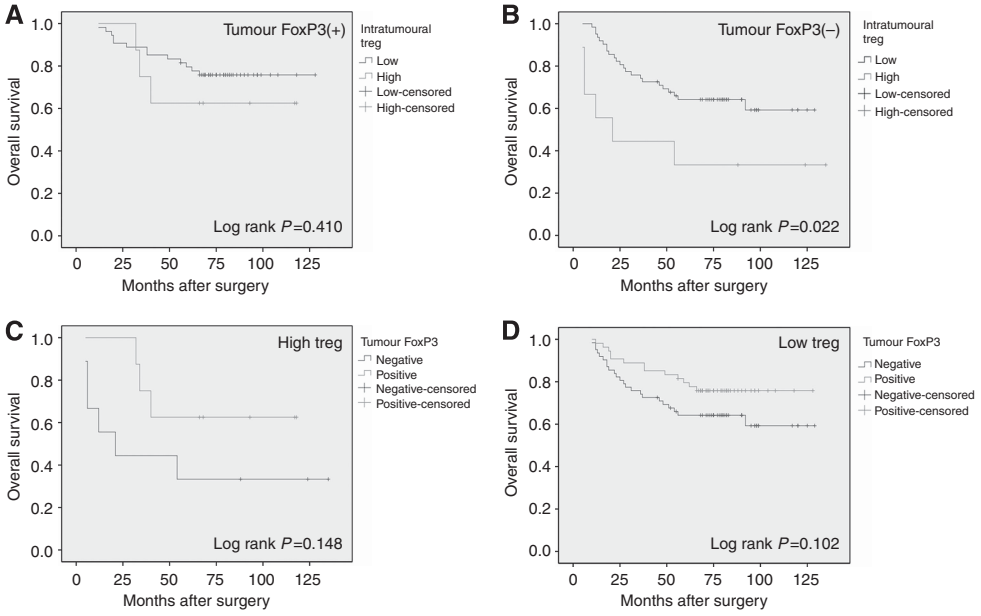


Figure 4. Treg density and FoxP3-positive tumours have different prognostic value. (**A**) The relationship between Treg accumulation and poor prognosis was less pronounced in patients with FoxP3-positive cancer cells. (**B**) An elevated Treg count indicated a worse overall survival rate in patients without tumour FoxP3 expression (log rank test, $P=0.022$). (**C**, **D**) The overall survival rate in patients with FoxP3-positive tumours was better with a mean 102 months follow-up time, although this was not statistically significant.

Table 2. Prognosis factor analysed by Cox regression

| | | | | 95.0% CI for HR | |
|---|---|---|---|---|---|
| | *β* | *P*-value | HR | Lower | Upper |
| TNM stage | 1.104 | 0.000 | 3.016 | 2.035 | 4.471 |
| Tumour FoxP3 | −0.800 | 0.011 | 0.449 | 0.243 | 0.831 |
| No. of Treg/HP | 1.331 | 0.001 | 3.784 | 1.774 | 8.070 |

Abbreviations: *β* = regression coefficient; CI = confidence interval; HR = hazard ratio; No. of Treg /HP = the mean number of Treg in five HPFs (×200); Tumour FoxP3 = positive or negative expression of FoxP3 in tumour cells.

31 days (*t*-test, $P=0.015$) (Figure 6C). These results support the idea that tumours with elevated FoxP3 expression may have better survival because overexpression of FoxP3 could inhibit tumour growth.

## DISCUSSION

Our results indicate that FoxP3-positive staining correlates with a favourable prognosis, whereas Treg counts suggest a poor prognosis. The data also suggest that direct interaction between GC cells and PBMCs promotes FoxP3 expression and cytokine production in a tumour microenvironment. Interestingly, upregulation of the FoxP3 gene inhibits GC cell growth both *in vitro* and *in vivo*. The current study contributes to our understanding of the precise role of FoxP3 in cancer development, and may offer a new perspective for therapeutic strategies against tumour development.

Our study confirmed that Treg density correlated with adverse prognosis, consistent with earlier studies (Kono *et al*, 2006; Lu *et al*, 2011; Tao *et al*, 2012a). However, FoxP3-positive tumours appear to have conflicting clinic significance. Previous studies showed that Treg counts in sentinel lymph nodes were associated with lymph node metastasis (Lee *et al*, 2011), where increased tumour-infiltrating Tregs positively correlated with TNM stage (Yuan *et al*, 2010; Liang *et al*, 2011; Lu *et al*, 2011) and the proportion of Treg/CD4 was associated with GC recurrence (Kim *et al*, 2011). FoxP3-positive cancer cells were associated with pathological differentiation, T stage, and poor prognosis in tongue squamous cell carcinoma (Liang *et al*, 2011) and lymph node metastasis in non-small cell lung cancer (Dimitrakopoulos *et al*, 2011). However, we did not find any difference in Treg counts or tumoral FoxP3 expression in terms of age, gender, TNM stage, or lymph node involvement. Moreover, FoxP3 status in different tumours varied. FoxP3 expression was reported to be reduced in prostate and breast cancer due in part to single somatic hits of the FoxP3 gene (Wang *et al*, 2009). However, no mutation of the 12 exons of FoxP3 was found in 11 patients who have a lower FoxP3 expression during our study observation (data not shown). FoxP3 expression is increased in most tumours, such as in non-small cell lung cancer (Dimitrakopoulos *et al*, 2011), tongue squamous cell carcinoma (Liang *et al*, 2011), liver cancer (Wang *et al*, 2010b), and stomach cancer (Yoshii *et al*, 2012). The result of our current series of case studies showed that FoxP3 mRNA and protein levels were elevated as tissue progresses from PC to cancer. The involvement of this differential expression remains unknown, although it may correlate to tissue specificity. FoxP3 was activated in many other cancers, unlike X-linked FoxP3 which is usually heterozygous in breast and prostate cancers. The reason for this phenomenon needs further investigation.

Whether FoxP3-positive cancer cells are relevant to prognosis is controversial. Some studies suggest that expression is related to poor prognosis in colorectal cancer (Salama *et al*, 2009b) and bladder cancer (Winerdal *et al*, 2011). However, others have demonstrated that tumoral FoxP3 correlated with a good prognosis, such as in HER2-positive breast cancer (Ladoire *et al*, 2011) and in colorectal cancer in clinical phase II (Salama *et al*, 2009b; Ladoire *et al*, 2011). Similarly, in small cell lung cancer, overall survival and recurrence-free survival in patients with FoxP3-positive tumours was better with earlier follow-up (Tao *et al*, 2012b). The relationship between Treg accumulation and poor prognosis was weaker in patients with FoxP3-positive cancer cells. Patients with a high Treg count had significantly worse survival than patients without tumoral FoxP3. Our results also demonstrate that FoxP3-positive cancer cells are a

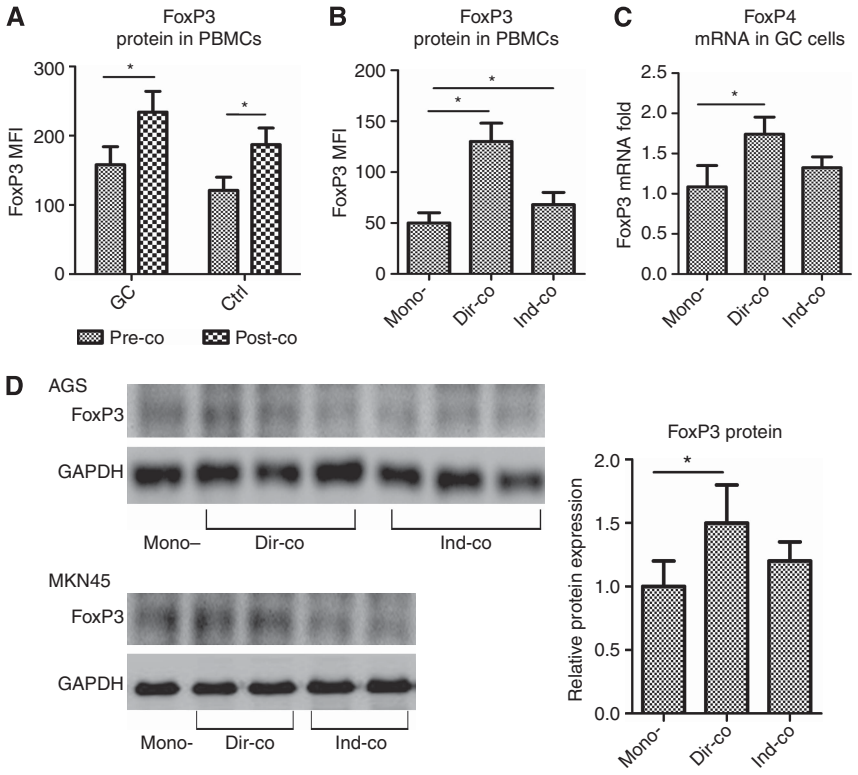


**Figure 5. FoxP3 protein changes after coculture.** (**A**) FoxP3 protein expression in PBMCs increased significantly compared with pre-culture, both in GC and control groups ($n=3$, paired *t*-test, both $P<0.05$). (**B**) FoxP3 protein levels increased in PBMCs in direct coculture compared with those in indirect coculture, although both increased relative to those in monocultures ($n=5$, ANOVA test, both $P<0.05$). (**C**) FoxP3 mRNA levels increased in GC cells in direct coculture compared with those in indirect coculture, although both were higher than those in monoculture ($n=5$, ANOVA test, both $P<0.05$). Data were presented as means ± s.e.m. (**D**) FoxP3 protein levels in GC increased after direct coculture compared with those in indirect coculture, by western blotting. The experiment was conducted three times; one experiment was showed in left graph. The pooled results in AGS cells were showed in the right graph (ANOVA test, $P=0.035$). *$P<0.05$.

good prognostic factor, whereas high-density Treg is a poor prognostic factor. We speculate that increased expression of FoxP3 in advanced tumours might be a compensatory result: the later the tumour stage, the more the FoxP3 expression. However, the contribution of Foxp3-positive tumour cells to prognosis appears slight compared with the effect of Treg.

GC is a disease caused by multiple genes and etiologies. The relationship between FoxP3 expression and prognostic prediction is variable. Apart from the differences in experimental design and methods, it may be correlated with a variety of tumour microenvironments, especially the interaction between tumour cells and various immune cells. Our previous study demonstrated that higher intratumour infiltrating Treg numbers and FoxP3 + / CD8 + ratios are associated with adverse prognosis in resectable GC tissues (Shen *et al*, 2010). Others revealed that tumour cells could inhibit IL-6 synthesis and enhance Treg function, resulting in a good prognosis (Tao *et al*, 2012b). GC cells can produce large amounts of TGF-$\beta$1 to drive the transformation of naive CD4 + T cells into Treg (Lu *et al*, 2011). The same effect also exists in ovarian (12) and pancreatic cancers (Liyanage *et al*, 2006; Hinz *et al*, 2007), and stimulated Tregs are thought to be an important mechanism of tumour escape. We presumed that FoxP3-positive cancer cells might alter Treg counts, as FoxP3 mRNA and protein levels increased in PBMCs, resulting in further immune escape and ultimately determining the prognosis. Besides, our previous study showed that the concentration of TGF-$\beta$1 in the cell supernatant were increased after GC cells cocultured with PBMCs and its levels increased more in the direct coculture group than in the indirect group (Ma *et al*, 2013). As PBMCs contain various cell populations, such as T, NK, and B cells, coculturing with each lymphocyte population in PBMCs can better interpret the involvement of responsible cell types. Previous studies have showed that coculture of Foxp3-expressing pancreatic carcinoma cells and melanoma cells with naive CD4( + )CD25( − ) T cells completely inhibited T-cell proliferation, but not activation (Hinz *et al*, 2007; Niu *et al*, 2011). Melanoma tumour cells may directly drive Treg activation and expansion in tumour microenvironment (Martin-Orozco *et al*, 2010). Besides, GC cells induce human Treg cells through the production of TGF-$\beta$1 (Yuan *et al*, 2011). Our present coculture system was a real-time stimulation, which showed on the other way that FoxP3 expression was increased in direct interaction more than in indirect one.

Somatic mutations, deletions, and epigenetic inactivation of FoxP3 are reportedly widespread between human breast and prostate cancers (Liu *et al*, 2009; Zhang and Sun, 2010; Li *et al*, 2011), whereas no mutation was found in GC in this study. FoxP3 inhibits cell growth, cell proliferation, migration, and invasion in a lot of cancer cells (Liu *et al*, 2009; Zhang and Sun, 2010; Li *et al*, 2011), and inhibits breast and prostate cancer growth by transcriptionally repressing oncogenes HER2 (Zuo *et al*, 2007), c-Myc (Wang *et al*, 2009), Skp2 (Zuo *et al*, 2007), and increasing tumour suppressor gene p21 (Liu *et al*, 2009). Mice with heterozygous FoxP3 mutations succumbed to spontaneous mammary tumours (Zuo *et al*, 2007), whereas those with prostate-specific deletions of FoxP3 developed prostate intraepithelial neoplasia (Wang *et al*, 2009). Our previous and current results show that FoxP3 inhibits GC cell proliferation *in vitro* and upregulated FoxP3 inhibited tumour growth in tumour-bearing nude mice. Therefore, FoxP3 gene functions in GC cells can further explain its roles in prognostic assessment.

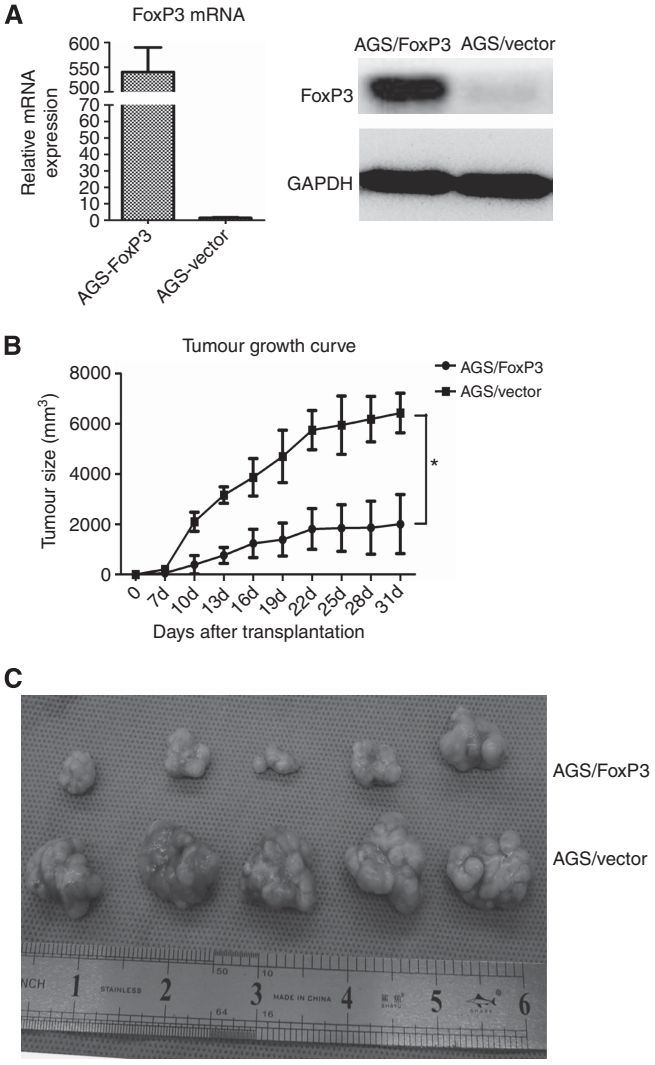


Figure 6. FoxP3 upregulation inhibits GC cell growth *in vivo*. (**A**) FoxP3-transfected cells have a obvious higher FoxP3 expression than vector-transfected cells in mRNA levels (>500-fold, left) and in protein levels (about 100-fold, right). (**B**) FoxP3- or vector-transfected cells were transplanted subcutaneously into nude mice. Tumour size was measured with calipers using the formula $V=(a\times b^2)/2$ every 3 days, in which *a* and *b* are the largest and the smallest perpendicular diameters, respectively. Tumour in FoxP3-overexpessing group grew slowly than that in the control group ($n=5$, *t*-test, $P<0.05$). (**C**) Tumour size of FoxP3-transfected cells was significantly smaller than those in vector-transfected cells *in vivo* after 31 days (*t*-test, $P=0.015$). *$P<0.05$.

In summary, our observations are novel in that FoxP3 expressed in GC cells suggests a better prognosis, which correlated with the interaction between tumour cells and immune cells, and with a variety of molecules, such as TGF-$\beta$ and HER2, in a complex tumour microenvironment, together with contributions from FoxP3 growth-inhibiting effects. FoxP3 expression in cancer may represent an example of molecular mimicry and reveals an important immunoregulatory function between cancer cells and lymphocytes. Given the function of FoxP3, we speculate that it confers a survival advantage on FoxP3-positive patients. With the crucial roles of FoxP3, these findings might open new strategies for immunotherapeutic treatment of tumour progression. More studies are needed to further explore which immune cells have key roles during this interaction, especially in light of the current anticancer efforts to interfere with Foxp3 expression.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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