Oncogene (2012) 31, 3419–3430

www.nature.com/onc

ORIGINAL ARTICLE

Epigenetic inactivation of *paired box gene 5*, a novel tumor suppressor gene, through direct upregulation of p53 is associated with prognosis in gastric cancer patients

X Li[1], KF Cheung[1], X Ma[1,2], L Tian[3], J Zhao[1], MYY Go[1], B Shen[1,4], ASL Cheng[1], J Ying[5], Q Tao[5], JJY Sung[1], H-f Kung[6] and J Yu[1]

[1]*Institute of Digestive Disease and Department of Medicine and Therapeutics, Li Ka Shing Institute of Health Sciences, Prince of Wales Hospital, Chinese University of Hong Kong, Hong Kong;* [2]*Department of Neurology, First Hospital, Hebei Medical University, Shijiazhuang, China;* [3]*School of Public Health and Primary Care, Chinese University of Hong Kong, Hong Kong;* [4]*Department of Gastroenterology, Guangzhou First Municipal People's Hospital, Guangzhou, China;* [5]*Department of Clinical Oncology, Cancer Epigenetics Laboratory, Chinese University of Hong Kong, Hong Kong and* [6]*Stanley Ho Center for Emerging Infectious Diseases, School of Public Health and Primary Care, Chinese University of Hong Kong, Hong Kong*

Using genome-wide methylation screening, we identified that *paired box gene 5* (*PAX5*) is involved in human cancer development. However, the function of *PAX5* in gastric cancer (GC) development is largely unclear. We analyzed its epigenetic inactivation, biological functions and clinical application in GC. *PAX5* was silenced in seven out of eight GC cell lines. A significant down-regulation was also detected in paired gastric tumors compared with adjacent non-cancerous tissues. The down-regulation of *PAX5* was closely linked to the promoter hypermethylation status and could be restored with demethylation treatment. Ectopic expression of *PAX5* in silenced GC cell lines (AGS and BGC823) inhibited colony formation and cell viability, arrested cell cycle, induced apoptosis, suppressed cell migration and invasion and repressed tumorigenicity in nude mice. Consistent with the induction of apoptosis by PAX5 *in vitro*, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) staining showed significantly enhanced apoptotic cells in PAX5-expressed tumors compared with the vector control tumors. On the other hand, knockdown of *PAX5* by *PAX5*–short hairpin RNA increased the cell viability and proliferation. The anti-tumorigenic function of *PAX5* was revealed to be mediated by upregulating downstream targets of tumor *protein 53* (*p53*), *p21*, *BCL2-associated X protein*, *metastasis suppressor 1* and *tissue inhibitors of metalloproteinase 1*, and downregulating *BCL2*, *cyclin D1*, *mesenchymal–epithelial transition factor* (*MET*) and *matrix metalloproteinase 1*. Immunoprecipitation assay demonstrated that PAX5 directly bound to the promoters of *p53* and *MET*. Moreover, *PAX5* hypermethylation was detected in 77% (144 of 187) of primary GCs compared with 10.5% (2/19) of normal gastric tissues ($P<0.0001$). GC patients with *PAX5* methylation had a significant poor survival compared with the unmethylated cases as demonstrated by Cox regression model and log-rank test. In conclusion, *PAX5* is a novel functional tumor suppressor in gastric carcinogenesis. Detection of methylated *PAX5* can be utilized as an independent prognostic factor in GC.

Oncogene (2012) **31,** 3419–3430; doi:10.1038/onc.2011.511; published online 21 November 2011

Keywords: *paired box gene 5*; gastric cancer; tumor suppressor gene; epigenetic alteration; prognosis

Introduction

Although the overall incidence of gastric cancer (GC) is declining in almost every country, it is still a serious health problem and remains the second to fourth most common cancer-related death worldwide. There are marked geographical variations, with China being affected most. Although the mechanism leading to cancer development remains elusive, epigenetic inactivation of tumor suppressor genes by promoter methylation is increasingly recognized to have a crucial role in gastric carcinogenesis. The identification of novel genes targeted by promoter hypermethylation may provide insights into the mechanisms for the inactivation of tumor suppressive pathway and is important for identification of tumor marker in GC (Kang *et al.*, 2003a, b; Lee *et al.*, 2004).

Through methylation-sensitive representational difference analysis, we have recently identified *paired box gene 5 (PAX5)* to be hypermethylated at its promoter in human cancer (Liu *et al.*, 2011). PAX5 was recently characterized as the key nuclear protein in the paired-box-containing family of transcription factors that involved in control of organ development and tissue differentiation (Carotta *et al.*, 2006). PAX5, also known as B-cell-specific activator protein, has an essential role as a transcription factor in early stage of B-cell

Correspondence: Professor J Yu, Institute of Digestive Disease and Department of Medicine and Therapeutics, Li Ka Shing Institute of Health Sciences, Prince of Wales Hospital, Chinese University of Hong Kong, Shatin, New Territories, Hong Kong.
E-mail: junyu@cuhk.edu.hk
Received 21 May 2011; revised 21 August 2011; accepted 29 September 2011; published online 21 November 2011

differentiation, as well as neural development and spermatogenesis (Barberis *et al*., 1990; Adams *et al*., 1992; Nutt *et al*., 1997). PAX5 functions as gene expression activator (Kozmik *et al*., 1992; Zwollo and Desiderio, 1994) or suppressor (Souabni *et al*., 2002; Holmes *et al*., 2006) to control the B-cell commitment (Cobaleda *et al*., 2007). Previous studies have identified that the *PAX5* gene, which is located on chromosome 19p13 (Stapleton *et al*., 1993; Kovac *et al*., 2000), is associated frequently with chromosomal translocations (Busslinger *et al*., 1996). Aberrant *PAX5* expression has been reported in several tumors (Baumann Kubetzko *et al*., 2004; Meza *et al*., 2006; Mhawech-Fauceglia *et al*., 2007; Lazzi *et al*., 2009), thus dysregulated expression of *PAX5* is likely to be involved in carcinogenesis and the malignant progression of human cancers. A recent study demonstrated that the lack of expression of *PAX5* in lymphoid neoplasms is associated with promoter hypermethylation, and cases with *PAX5* silence were characterized by poor clinical outcome (Lazzi *et al*., 2009). In this study, we discovered that the frequent loss of *PAX5* expression in GC is due to promoter methylation. As altered expression of this transcriptional regulator may be the cause of tumor development, we characterized the biological functions of *PAX5* in gastric tumorigenicity both *in vitro* and *in vivo* using loss or gain of PAX5 function in GC cells. The molecular basis and downstream regulating pathways of *PAX5* as a putative tumor suppressor was further generated. The potential clinical application of *PAX5* as a novel biomarker in the outcome of GC was also evaluated in 187 primary GCs.

Results

PAX5 is epigenetically suppressed in GC cell lines

PAX5 transcription was silenced or downregulated in 7/8 (87.5%) of GC cell lines, with KatoIII, MKN28, SNU1, SNU16, AGS and BGC823 being silenced and NCI-N87 being downregulated (Figure 1a). In contrast, mRNA expression could be broadly detected in normal human tissues and fetal tissues (Figure 1b). On the other hand, using methylation-specific PCR and bisulfite genomic sequencing (BGS; Figure 1c), full methylation was detected in five GC cell lines (KatoIII, MKN28, SNU1, AGS and BGC823), and partial methylation was found in one GC cell line (NCI-N87) (Figures 1b and c), whereas unmethylated promoter was detected in expressing cell line (MKN45).

To further confirm whether *PAX5* expression was repressed by promoter methylation, 5-aza-2′-deoxycytidine (5-Aza) was used to pharmacologically interfere with promoter methylation in methylated cell lines. As shown in Figure 1d, this treatment resulted in the restoration of *PAX5* expression in all cell lines examined, conferring that promoter methylation contributes to the epigenetic suppression of *PAX5*.

PAX5 expression is downregulated in primary GCs

The mRNA expression of *PAX5* was evaluated in 18 paired primary GCs using quantitative reverse transcriptase (RT) PCR. *PAX5* was significantly downregulated in gastric tumors compared with their adjacent normal tissues ($P<0.05$; Figure 2).

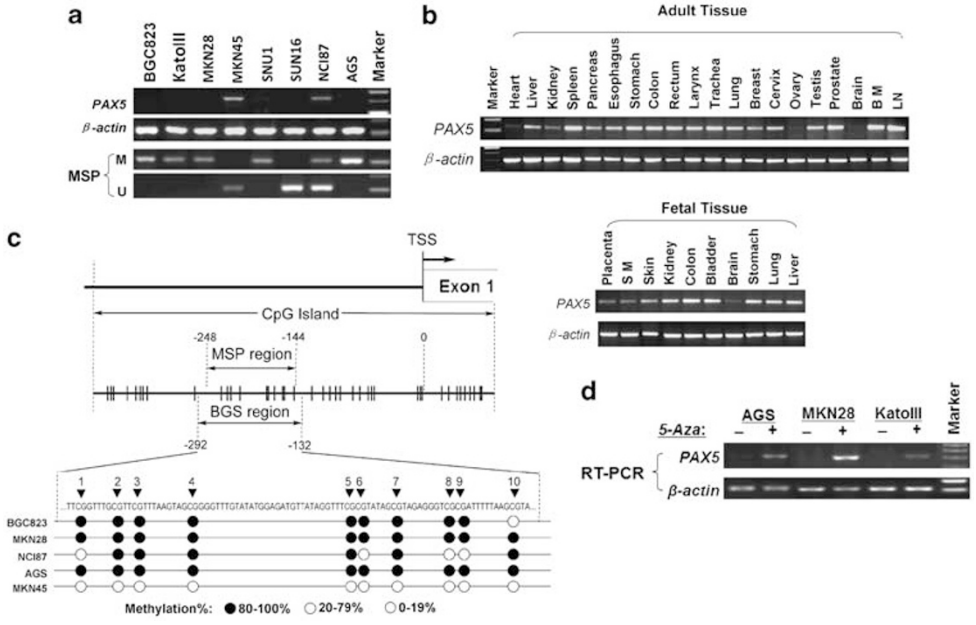

Figure 1 (**a**) *PAX5* mRNA expression and promoter methylaiton status in GC cell lines. M, methylation; U, unmethylation. (**b**) Expression of *PAX5* by semiquantitative RT–PCR, with *β-actin* as an internal control. (**c**) Methylation-specific PCR (MSP) region and BGS region are indicated. (**d**) *PAX5* gene expression was restored after treatment with demethylation reagent 5-Aza in GC cell lines.

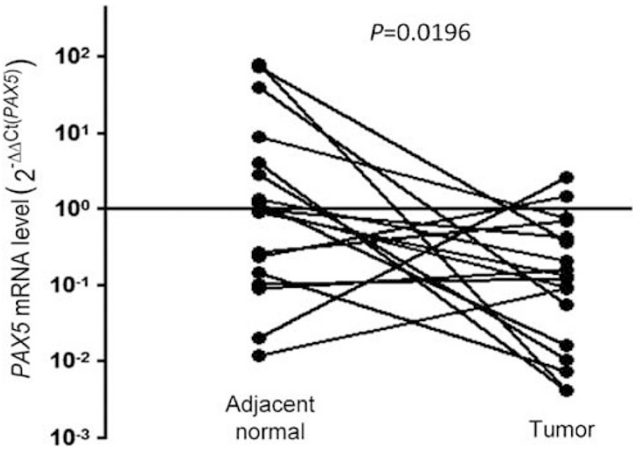

Figure 2 The mRNA expression levels of *PAX5* in paired primary GC tissues as determined by quantitative real-time PCR.

PAX5 inhibits GC cell growth

The frequent silencing of *PAX5* mediated by promoter methylation in GC, but not in normal gastric tissue, suggested that *PAX5* may be a candidate tumor suppressor in gastric carcinogenesis. We thus examined the growth inhibitory effect through ectopic expression of *PAX5* in AGS and BGC823, which showed no *PAX5* expression. Re-expression of *PAX5* mRNA and protein in the transient transfected cell lines was evidenced by RT–PCR and western blot (Figure 3a), which caused a significant decrease in cell viability for 52% in AGS ($P<0.01$) and 59% in BGC823 ($P<0.01$), compared with vector-transfected control cells (Figure 3b). The suppressive effect on cancer cell growth was further confirmed by colony formation assay in stably transfected cell lines. Colony numbers were significantly reduced to 41% in AGS ($P<0.01$), and 42% in BGC823 ($P<0.01$), compared with the control cells (Figure 3c).

PAX5 induces cell apoptosis and causes cell cycle arrest in G_0/G_1 phase

To determine whether the *PAX5*-mediated growth inhibition was the result of apoptosis, cell apoptosis was determined by Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide fluorescence-activated cell sorting (FACS) analysis. Our results showed an increase in the numbers of early apoptotic cells ($5.60\pm0.75\%$ vs $2.87\pm0.38\%$, $P<0.05$) in AGS stably transfected with *PAX5* than those vector-transfected AGS cells (Figure 3d).

We investigated the effect of *PAX5* on cell cycle distribution. FACS analysis of *PAX5*-transfected AGS revealed a significant decrease in the number of cells in the S phase compared with controls ($42.6\pm3.7\%$ vs $35.0\pm1.6\%$, $P<0.05$), conferring the inhibitory effect of *PAX5* on cell proliferation. Concomitant with this inhibition, there was a significant increase in the number of cells accumulating in the G_0/G_1 phase ($49.2\pm1.5\%$ vs $58.6\pm2.6\%$, $P<0.01$; Figure 3e), thus *PAX5* blocks the cell cycle at the G_0/G_1 checkpoint.

PAX5 inhibits GC cell migration and invasion

To investigate the effect of PAX5 in cancer cell migration, the monolayer wound-healing assay was performed. A significant delay in the closure of the wound gaps in AGS cells transfected with *PAX5* as compared with cells transfected with empty vector was observed at both 24 and 48 h (Figure 3f). For the quantitative assessment of cell metastasis and invasiveness, we performed the matrigel invasion assay. The invaded cell number in AGS with PAX5 expression was significantly lower than in control AGS without *PAX5* expression ($P<0.05$; Figure 3g), suggesting that PAX5 inhibits the migration and the invasion of GC cells.

Knockdown of PAX5 promotes cell growth

To further confirm the role of PAX5 in cell growth, the effect of PAX5 was investigated through knockdown *PAX5* with short hairpin RNA (shRNA) in a normal immortalized cell line C2C12. *PAX5* was significantly knocked down by *PAX5*–shRNA (Figure 4a). Knockdown of *PAX5* markedly enhanced cell viability ($P<0.001$; Figure 4b) and colony formation ($P<0.01$; Figure 4c) compared to cells treated with the control shRNA. These data provide evidences that *PAX5* functions as a potential tumor suppressor.

PAX5 inhibits tumor growth in nude mice

In light of the observed anti-proliferative and pro-apoptotic effects of PAX5 on the cell lines *in vitro*, we tested whether PAX5 could alter growth of GC cells *in vivo*. The tumor growth of BGC823 stably transfected with *PAX5* or empty vector (pcDNA3.1) in nude mice was shown in Figure 5a1. The tumor size was significantly lower in *PAX5*-transfected nude mice as compared with the control mice ($P<0.0001$; Figure 5a2). Moreover, the mean tumor weight was significantly lesser in *PAX5*-transfected tumors compared with the control vector-transfected tumors ($P<0.05$; Figure 5a3). Expression of PAX5 protein in isolated tumor cells transfected with *PAX5* was confirmed with PAX5-positive signal in nucleus by immunohistochemistry (Figure 5b).

Terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) staining was performed to validate the apoptosis induced by *PAX5* in the xenograft tumors (Figure 5c1). Consistent with the result obtained in GC cell lines *in vitro*, TUNEL-positive cell was higher in *PAX5*-expressed tumors compared with the vector control tumors ($3.62\pm1.12\%$ vs $1.54\pm0.71\%$, $P<0.01$; Figure 5c2).

Genes modulated by PAX5 are generated

To elucidate the molecular basis by which *PAX5* controls GC growth, gene expression profile in *PAX5* stably transfected AGS and HCT116 cell lines were analyzed by complementary DNA (cDNA) expression array. The anti-tumorigenesis effect by *PAX5* was mediated by regulating important genes involved in apoptosis, cell proliferation and metastasis, as derived from both AGS and HCT116 cell lines (Table 1). *PAX5* increased the expression of pro-apoptotic genes

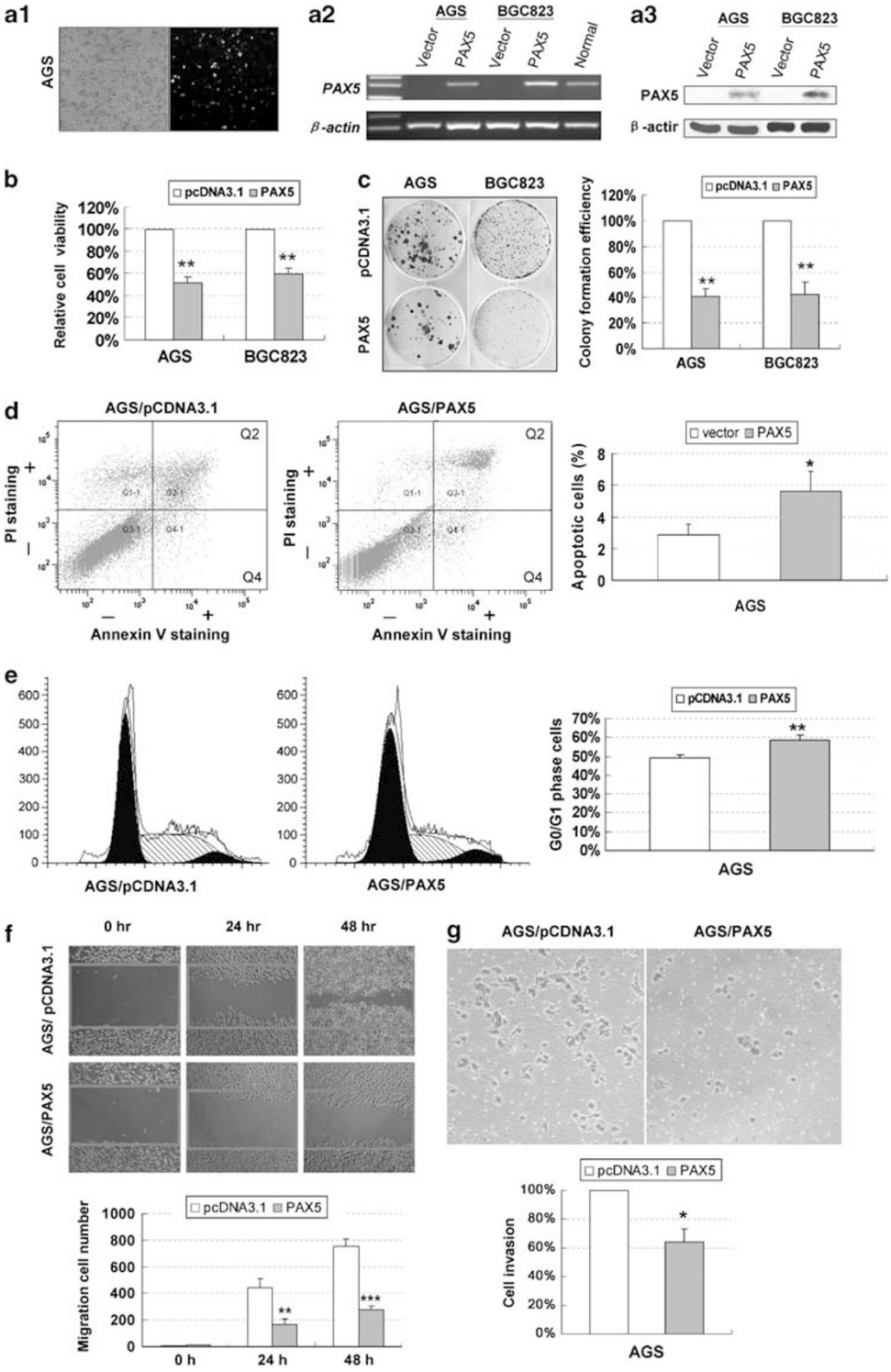

Figure 3 (**a1**) Transfection efficiency was evaluated in AGS transfected with pCDNA3.1–enhanced green fluorescent protein for 48 h. (**a2**) Ectopic expression of *PAX5* mRNA in AGS, BGC823 cell lines and normal tissue was evidenced by RT–PCR. (**a3**) Ectopic expression of PAX5 protein was confirmed by western blot. (**b**) PAX5 significantly suppressed cell viability. (**c**) The effect of *PAX5* on GC cell growth was further confirmed by colony formation assay. Left panel shows the representative images of the colony formation in GC cell transfection with pcDNA3.1/*PAX5* or empty vector (pcDNA3.1). Quantitative analyses of colony numbers are shown in the right panel. (**d**) Flow cytometry assay with Annexin V and propidium iodide double staining. Q2 shows the late apoptotic cells and Q4 shows the early apoptotic cells. (**e**) PAX5 causes cell cycle arrest in G_0/G_1 phase. Left panel shows representative diagram and right panel shows quantities analysis of this assay. (**f**) Wound healing assay. Photographs were taken at 0, 24 and 48 h to determine the different mobility between AGS/vector and AGS/PAX5. Number of cells that entered the scratch region was shown as mean ± s.d. (**g**) Matrigel invasion assay shows invasiveness and metastasis of cells with or without *PAX5*. The data are means ± s.d. of three separate experiments. $*P<0.05$; $**P<0.01$; and $***P<0.001$. A full colour version of this figure is available at the *Oncogene* journal online.

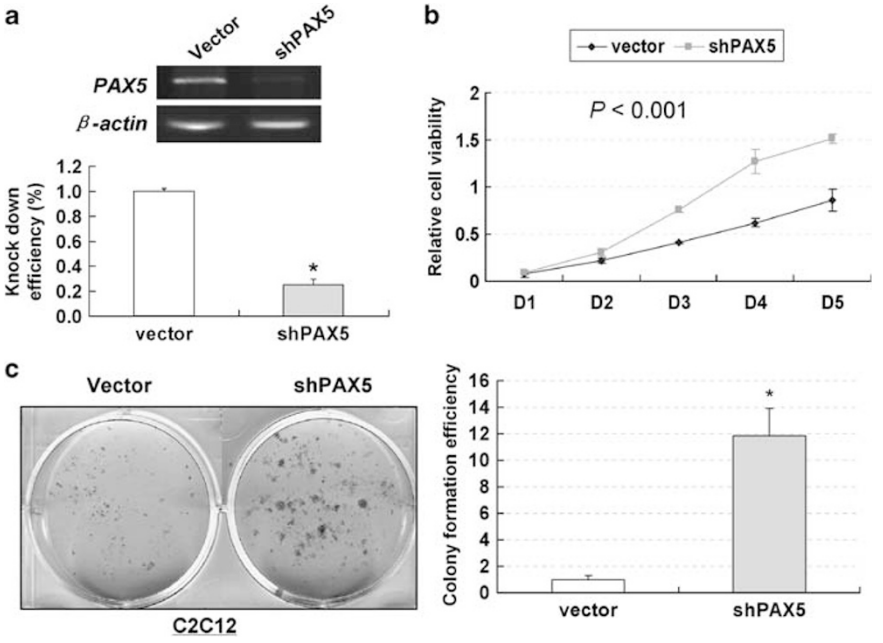

Figure 4 (**a**) Knockdown of *PAX5* in C2C12 by PAX5 shRNA-mediated knockdown was confirmed by semiquantitative RT–PCR (upper panel) and real-time PCR (lower panel). (**b**) shRNA-mediated knockdown of *PAX5* induced cell viability and (**c**) colony formation. The experiments were repeated three times in triplicate. Data are mean ± s.d. $*P<0.01$. A full colour version of this figure is available at the *Oncogene* journal online.

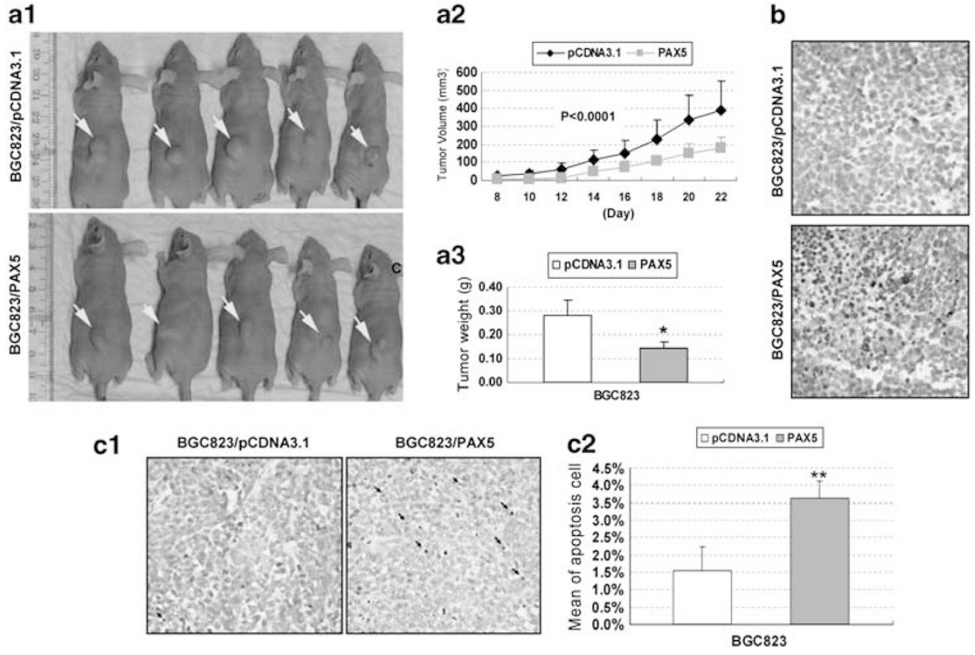

Figure 5 (**a**) PAX5 inhibits growth of tumors derived from BGC823 *in vivo*. (**a1**) Tumor growth in nude mice subcutaneously injected with BGC823/PAX5 or BGC823/vector. (**a2**) Tumor volume curves were plotted against days after treatment ($n=5$/group). (**a3**) Histogram represents mean of the tumor weight from the BGC823/PAX5 and BGC823/vector group. (**b**) Immunostaining of PAX5 in xenograft tumors in nude mice. Brown nuclear signals indicate the PAX5 protein expression. (**c1**) Apoptotic cells in tumor tissues from nude mice are detected by TUNEL staining. The black arrows indicate the apoptotic cell. (**c2**) The histogram represents the overall result of the percentage of apoptosis cell. Data are mean ± s.d. $*P<0.05$ and $**P<0.01$. A full colour version of this figure is available at the *Oncogene* journal online.

including *tumor protein 53* (*p53*), *BCL2-associated X protein* (*BAX*) and downregulated anti-apoptotic gene *BCL2*. *PAX5* also enhanced expression of *p21* (*CDKN1A*), a cell cycle regulator, In addition, inhibition of GC cell migration and invasion by *PAX5* may result from the enhancement of *metastasis suppressor 1*

(*MTSS1*) and *tissue inhibitors of metalloproteinase 1* (*TIMP1*), and depression of *mesenchymal–epithelial transition factor* (*MET*) and *matrix metalloproteinase 1* (*MMP1*). Western immunoblot analysis confirmed that p53 and p21 proteins were expressed at higher levels in the *PAX5*-transfected cells compared with control vector-transfected cells (Figure 6a). *PAX5* also enhanced protein expression of cyclin D1 (Figure 6a), a key G1 phase regulator.

Table 1 The effect of *PAX5* on its downstream gene expression profiles of cancer pathways in both AGS and HCT116 cell lines

Gene name	*Fold change (PAX5/control)*		*Function*
	AGS	*HCT116*	
p53	2.8	3.0	Apoptosis and cell cycle
p21	2.5	1.5	Cell cycle
BAX	2.5	7.3	Apoptosis
BCL2	−2.4	−2.0	Apoptosis
MET	−2.8	−2.4	Migration and invasion
MMP1	−10.6	−4.0	Migration and invasion
MTSS1	2.4	2.1	Migration and invasion
TIMP1	2.2	2.2	Migration and invasion

Abbreviations: BAX, BCL2-associated X protein; MET, mesenchymal-epithelial transition factor; MMP1, matrix metalloproteinase 1; MTSS1, metastasis suppressor 1; PAX5, paired box gene 5; p53, tumor protein 53; p21, tumor protein 21; TIMP1, tissue inhibitors of metalloproteinase 1.

PAX5 directly binds to the promoters of p53 and MET

To evaluate whether the observed PAX5-mediated gene expression was associated with direct promoter binding, chromatin immunoprecipitation (ChIP) assay using specific PAX5 antibody was performed in AGS cells, followed by PCR targeting the promoter regions (Figure 6b). ChIP–quantitative PCR assay indicated that PAX5 binds to the promoters of *p53* and *MET* in AGS cells ($P<0.01$; Figure 6b). However, promoters of *MTSS1* and *TIMP1* showed no physical interaction with PAX5 protein. This finding indicated that *p53* and *MET* genes are the direct targets of *PAX5* in GC cells.

Frequent PAX5 methylation is detected in primary GC patients

PAX5 methylation was evaluated in 187 primary GC tissues and 19 normal gastric biopsy samples by BGS.

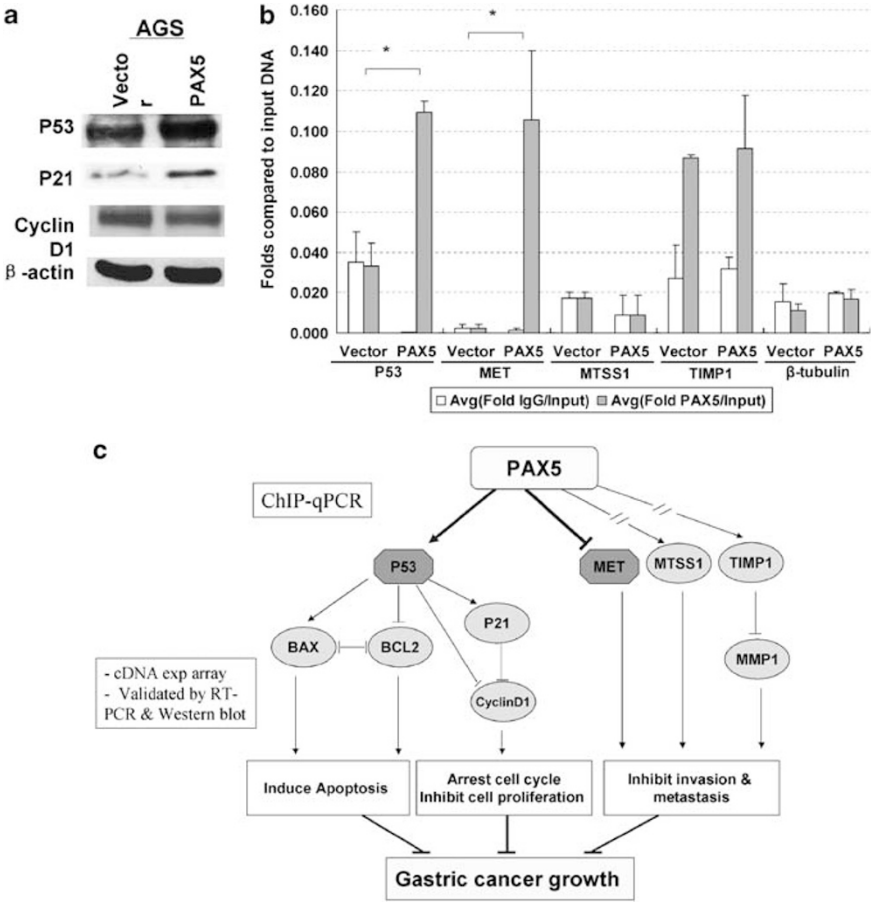

Figure 6 (**a**) Effects of ectopic expression of PAX5 on protein expression of p53, p21 and cyclin D1 using western blotting. β-Actin was used as an internal control. (**b**) ChIP–quantitative PCR (qPCR) was performed to identify direct targets of PAX5 protein. $*P<0.01$. (**c**) Schematic diagram for the mechanisms of anti-tumorigenesis functions of PAX5 deriving from cDNA array, western blot and ChIP–qPCR. $*P<0.05$. A full colour version of this figure is available at the *Oncogene* journal online.

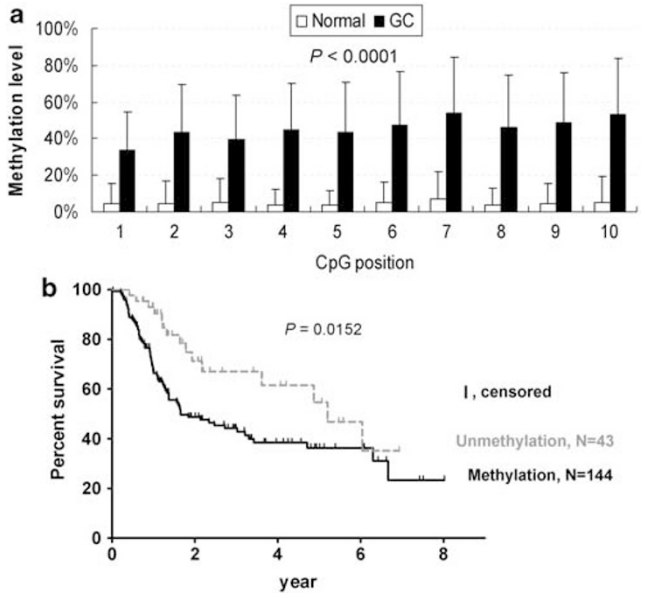

Figure 7 (**a**) Methylation levels in primary GC ($n=187$) and in normal gastric tissue ($n=19$) by BGS. (**b**) Kaplan–Meier survival curves show that GC patients with *PAX5* methylation have poorer survival than others. This difference is statistically significant, based on the log-rank test ($P<0.05$). A full colour version of this figure is available at the *Oncogene* journal online.

The methylation level was significantly higher in GC tissues than in normal controls ($P<0.0001$, Figure 7a), and the methylated *PAX5* was detected in 77% (144 of 187) of primary GCs compared with 10.5% (2/19) of normal gastric tissues ($P<0.0001$). The association between clinicopathological features and *PAX5* methylation in human GCs is listed in Supplementary Table 1. There was no correlation between the methylation of *PAX5* and clinicopathological features, such as age, gender, *Helicobacter pylori* (*H. pylori*) status, histological type, differentiation or pathologic stage.

PAX5 methylation is associated with poor survival of GC patients

The features of GC patients related to the survival status are shown in Table 2. As expected, Tumor-Nodes-Metastases (TNM) stage was a significant prognostic factor ($P<0.0001$). Moreover, *PAX5* methylation was found to be significantly associated with death ($P=0.0009$). Using univariate Cox regression analysis (Supplementary Table 2), *PAX5* methylation was associated with a significantly increased risk of cancer-related death. The relative risk (RR) was 1.963 with 95% confidence interval ranging from 1.127 to 3.418 ($P=0.01$). After the adjustment for potential confounding factors, multivariate Cox regression analysis showed that *PAX5* methylation was a predictor of poorer survival of GC patients (RR, 2.0983; 95% confidence interval, 1.171–3.760); $P=0.01$; Table 3). As shown in the Kaplan–Meier survival curves, GC patients with *PAX5* methylation (median survival of 1.65 years) had poorer survival than others (median survival of 5.20 years; Figure 7b), which showed statistically significant difference based on the log-rank test ($P=0.0152$, hazard ratio = 1.770).

Table 2 Distribution of patient characteristics by survival status

Variable	*Alive* (n = 96)	%	*Dead* (n = 91)	%	P-*value*
Age (years)					
Mean ± s.d.	56.0 ± 11.7		58.2 ± 13.9		0.24
Gender					
M	67	54.0	57	46.0	0.30
F	29	46.0	34	54.0	
H. pylori					
Yes	19	51.4	18	48.6	0.43
No	49	59.0	34	41.0	
Lauren					
Intestinal type	84	53.5	73	46.5	0.11
Diffuse type	10	37.0	17	63.0	
TNM stage					
I	22	81.5	5	18.5	<0.0001
II	19	63.3	11	36.7	
III	37	61.7	23	38.3	
IV	16	26.2	45	73.8	
PAX5 methylation					
No	28	65.1	15	34.9	0.04
Yes	68	47.2	76	52.8	

Abbreviations: F, female; *H. pylori*, *Helicobacter pylori*, M, male; PAX5, paired box gene 5.

Table 3 Multivariate Cox regression analysis of potential poor prognostic factors for gastric cancer patients

Variable	*RR (95% CI)*	P-*value*
Age (years)	1.01 (0.994–1.032)	0.17
Gender		
Male	0.85 (0.547–1.330)	0.48
Female	1.00	
TNM stage		
I	0.10 (0.040–0.258)	<0.0001
II	0.23 (0.125–0.478)	<0.0001
III	0.27 (0.161–0.448)	<0.0001
IV	1.00	
PAX5 methylated		
Yes	2.10 (1.171–3.760)	0.01
No	1.00	

Abbreviations: CI, confidence interval; PAX5, paired box gene 5; RR, relative risk.

Discussion

We demonstrated that *PAX5* is widely expressed in normal human tissues and fetal tissues, but frequently silenced or downregulated in GC cell lines. Down-regulation of *PAX5* was also observed in primary gastric tumors compared with their adjacent non-cancerous tissues, suggesting *PAX5* may be important in gastric

carcinogenesis. The silencing or downregulation of *PAX5* was revealed to be related closely with promoter hypermethylation, as demonstrated by methylation-specific PCR and confirmed by BGS analysis. This was further validated by demethylation treatment with the demethylating reagent 5-Aza; restored expression of *PAX5* in silenced cancer cells was obtained following treatment. These results indicated that promoter hypermethylation of *PAX5* directly mediated the *PAX5* transcriptional silence. One GC cell line SNU16 showed no *PAX5* expression and no promoter methylation, suggesting that other transcription-regulating mechanisms, such as histone modification (Jones *et al*., 1998) or upstream transcriptional regulation (Lee *et al*., 2008), might also contribute to *PAX5* gene silencing. Collectively, our data revealed that the epigenetic regulation mechanism (promoter methylation) on the *PAX5* gene has an important role in its transcriptional silence, which may contribute to the development of GC.

The biological function of PAX5 in human GC was further investigated by gain- and loss-PAX5 function assays. Ectopic expression of PAX5 in silenced cancer cell cells significantly inhibited cell viability and colony formation ability. Cell cycle distribution analysis by flow cytometry revealed a significant decrease in cell proliferation and a proportionate increase of cells in G_0/G_1 phase following *PAX5* transfection. Concomitantly, ectopic expression of PAX5 significantly induced cell apoptosis, decreased cell migration and cell invasion ability in GC cell lines. Conversely, knockdown of PAX5 by shRNA significantly promoted cell growth. Having observed the substantial suppression of GC cell growth by PAX5 *in vitro*, we studied tumor suppressive effect of PAX5 against gastric tumor formation *in vivo*. Our result showed that the tumor growth was significantly retarded in nude mice inoculated with BGC823/*PAX5* compared with those inoculated with BCG823/vector subcutaneously. Together, the consistent results observed *in vitro* and *in vivo* indicated for the first time that PAX5 functions as a tumor suppressor in gastric carcinogenesis. This understanding of the anti-tumor effect PAX5 in GC suggests that restoration of the function of PAX5 could halt or reverse the abnormalities, thus having a potential therapeutic effect.

We further uncovered the molecular basis of PAX5 exerting the tumor suppressor property in GC using cDNA microarray, western blot and ChIP assays (Figure 6c). The enhanced apoptosis ability by PAX5 was discovered to be mediated at least by the direct upregulation of pro-apoptotic p53 through binding to *p53* promoter and subsequent modulation of p53 downstream targets BAX and BCL2 (Figure 6c). *P53* is known as a tumor suppressor gene that is capable *BCL2* of inducing transcriptional regulation of other anti-tumorigenic molecules (Levine, 1989; Kishimoto *et al*., 1992; Malkin *et al*., 1994; Stuart *et al*., 1995). Induction of p53 had been reported to downregulate *BCL2* in breast cancer (Haldar *et al*., 1994) and upregulate *BAX* in lung cancer (Miyashita and Reed, 1995), two important mediators in the process of program cell death. BAX induces apoptotic cell death by cytokine deprivation and represses activity of BCL2, an apoptotic blocker (Hockenbery *et al*., 1991; Oltvai *et al*., 1993; Ruvolo *et al*., 2001). Therefore, upregulation of *p53* and its downstream targets induced by *PAX5* could explain the effect of *PAX5* in inducing cell apoptosis.

On the basis of the cDNA microarray and western blot analyses, the cell cycle arrest in G_0/G_1 phase caused by *PAX5* was mostly attributable to the upregulation of *p*21 and downregulation of cyclin D1 (Figure 6c). It has been reported that p53 can directly enhance the transcription of *p21* (el-Deiry *et al*., 1993). P21 is a potent inhibitor of cyclin D/Cdk4 and cyclin E/Cdk2, whose kinases govern cell–cycle progression at the restriction and late transition points of G1 (He *et al*., 2005). In addition, inhibition of cyclin D1, a key regulatory subunit of CDK4, could inhibit the cell cycle entry into S phase (Quelle *et al*., 1993; Musgrove *et al*., 1994). P53 was reported to cause G1 cell cycle arrest by indirectly suppressing *cyclin D1* transcription (el-Deiry *et al*., 1993; Rocha *et al*., 2003).

Ectopic expression of PAX5 suppressed the cell migration, as demonstrated by the wound healing assay. This effect was further confirmed by matrigel invasion analysis, suggesting that PAX5 could reduce metastasis and invasiveness of GC cells. Cell migration and invasion are critical events during the cancer progression to metastasis. The anti-migration/invasion effect caused by PAX5 in GC cells was contributed to the upregulation of MTSS1 and TIMP1 and downregulation of MMP1 (Figure 6c). MTSS1 was recently identified as a putative metastasis suppressor, with an important role in inhibiting the metastasis of bladder cancer (Lee *et al*., 2002; Wang *et al*., 2007) and breast cancer (Parr and Jiang, 2009). MMP1 has an important function in cancer progression by promoting invasion and metastasis in prostate and lung cancer (Chambers and Matrisian, 1997; Sauter *et al*., 2008). TIMP1 is an inhibitor of the known MMPs including MMP1 (Visse and Nagase, 2003). Collectively, induction of MTSS1 and TIMP1 and suppression of MMP1 by PAX5 could explain the ability of PAX5 in governing the metastatic nature of GC.

The anti-tumorigenic function of PAX5 was also related to the direct downregulation of *MET*, a well-known proto-oncogene. MET encodes a transmembrane receptor tyrosine kinase (Bottaro *et al*., 1991). The latter can be mutated or overexpressed in a number of epithelial human cancers, including GC, which triggers cell growth, motility and invasiveness (Giordano *et al*., 1993; Benvenuti and Comoglio, 2007; Kanteti *et al*., 2009). We found that PAX5 downregulated *MET* expression by directly binding to the promoter of *MET*, as evidenced by cDNA expression array and ChIP–quantitative PCR assays. Thus, downregulation of *MET* by PAX5 also had a role in the anti-tumorigenic/metastatic properties of *PAX5*.

To investigate the clinical application of *PAX5* in gastric tumorigenesis *in vivo*, we examined the promoter methylation of *PAX5* by BGS in 187 primary GCs and 19 normal controls. *PAX5* gene promoter was found to be commonly methylated in GCs (77%, 144/187) as

compared with normal controls (10.5%, 2/19; $P<0.0001$). Recognizing the tumor suppressor effect of PAX5, the inactivation of this gene by promoter methylation would favor tumor progression and a worse outcome. In this regard, the clinical significance of *PAX5* promoter methylation and its associations with patient outcome were evaluated in 187 primary GC patients. Our results indicated that *PAX5* methylation was significantly associated with a shorter survival independent of patient clinicopathological characteristics (RR, 2.10; 95% confidence interval, 1.171–3.760; $P=0.01$). Our data support an adverse effect of *PAX5* promoter methylation on survival of GC patients. *PAX5* methylation could be regarded as a valuable new prognostic factor for GC.

In conclusion, we have identified a novel functional tumor suppressor gene *PAX5* inactivated by promoter methylation in GC, with important roles in suppressing cell proliferation, inducing apoptosis and inhibiting cell invasion. PAX5 induced cancer cell apoptosis through direct upregulation of p53 and mediating its downstream targets (BAX and BCL2). PAX5 suppressed cell proliferation through upregulation of p21 and downregulation of cyclin D1. PAX5 inhibited cell invasion/metastasis by inducing MTSS1 and TIMP1 and inhibiting MMP1. The anti-tumorigenic function of PAX5 was also mediated by directly downregulating oncogene *MET*. *PAX5* methylation could serve as a putative epigenetic biomarker to predict outcome for GC patients.

Materials and methods

Cell lines

GC cell lines (AGS, Kato III, MKN28, MKN45, N87, SNU1 and SNU16) and immortalized mouse myoblast cell line (C2C12) were purchased from American Type Culture Collection (Manassas, VA, USA). GC cell line BGC823 was kindly provided from Beijing Oncology Hospital, Beijing, China.

Patients and primary human samples

Paired biopsy samples from primary tumor and adjacent non-tumor sites were obtained from 18 GC patients during endoscopy before any therapeutic intervention. Biopsy samples from the adjacent non-tumor areas were subsequently verified by histology to be free of tumor infiltration. The biopsies were snap frozen in liquid nitrogen and stored $-80\,^{\circ}C$ for molecular analyses. The remaining tissue specimens were fixed in 10% formalin and embedded in paraffin for routine histological examination.

A total of 187 primary GC tissues were collected from patients at the time of operation. This included 124 men and 63 women, with average age of 57.1 ± 12.8 years. Tumor was staged according to the Tumor, Nodes, and Metastases (TNM) staging system. Other clinicopathological features, such as *H. pylori* infection and tumor differentiation, were also recorded. In addition, 19 age-matched subjects (average age 51.9 ± 17.2 years) with normal upper gastroscopy were recruited as control. GC patients were being regularly followed up, as the time of diagnosis and the follow-up duration was 0–97.6 months. In total, 91 (48.7 %) patients died in the follow-up period. Informed consent was given by all the patients and controls, and the study protocol was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong.

Semiquantitative RT–PCR and real-time quantitative PCR analyses

Total RNA was extracted from tissues and cell pellets using QIAzol reagent (Qiagen, Valencia, CA, USA). cDNA was synthesized from 2 μg total RNA using Transcriptor Reverse Transcriptase (Roche Applied Sciences, Indianapolis, IN, USA). Semiquantitative RT–PCR and real-time quantitative PCR analyses were performed using primers listed in Supplementary Table 3.

Western blot analysis

Total protein was extracted, and protein concentration was measured by the DC protein assay method of Bradford (Bio-Rad, Hercules, CA, USA). A quantity of 40 μg of protein from each sample was separated by 10% SDS–polyacrylamide gel electrophoresis gel. The protein was transferred to an equilibrated polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK) and then incubated in specific primary antibody at $4\,^{\circ}C$ overnight. After incubation with the secondary antibody, the proteins were detected by enhanced chemiluminescence (Amersham Corporation, Arlington Heights, IL, USA).

Demethylation treatment using 5-Aza

Cells were seeded at a density of 1×10^5 cells/ml in 100-mm dishes and grown for 24 h. Cells were then treated with 2 μM 5-Aza (Sigma-Aldrich, St Louis, MO, USA) for 5 days. 5-Aza was replenished every day.

DNA extraction and methylation-specific PCR

Genomic DNA from cancer cell lines and tissue samples were isolated by using DNA mini kit (Qiagen, Hilden, Germany). One microgram of DNA was modified by sodium metabisulfite as described previously (Tao *et al*., 2002). The bisulfite-modified DNA was amplified by methylation-specific PCR using primer pairs (Supplementary Table 3) that specifically amplify either methylated or unmethylated sequences of the *PAX5* gene.

Bisulfite genomic sequencing

A total volume of 2 μl of bisulfite-treated DNA was amplified by primers of BGS (Supplementary Table 3). Sequencing was performed using the BigDye Terminator Cycle Sequencing kit version 1.0 (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed by using SeqScape software (Applied Biosystems) and Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Methylation percentage of each CpG site was calculated according to the formula: methylation% $=H_C/(H_C+H_T)\times100\%$ (H_C = height of peak C and H_T = height of peak T).

Construction of PAX5 expression vector and PAX5 shRNA vector

The expression vector (pcDNA3.1–*PAX5*) encoding the full-length open reading frame of human *PAX5* gene was constructed by PCR cloning. Briefly, total RNA from human stomach (Ambion, Austin, TX, USA) was reverse transcribed into cDNA. Sequence corresponding to the open reading frame of *PAX5* was amplified by PCR and verified by DNA sequencing. PCR-amplified insert was subcloned into the pCDNA3.1 TOPO TA expression vector (Invitrogen, Carlsbad, CA, USA). The shRNA vector pGFP-V-RS-shPAX5 and its

control vector were purchased from OriGene (Rockville, MD, USA).

Colony formation assay
Cancer cells (AGS, HTC116 and BGC823; 2×10^5/well) were plated in six-well plates and transfected with pcDNA3.1–*PAX5* or empty vector using Lipofectamine 2000 (Invitrogen). Two days after transfection, cells were subsequently split at 1:20 ratio in six-well plates with 0.5 mg/ml neomycin (G418; Invitrogen). On the other hand, C2C12 cells (5×10^5/well) were plated in a 6-well plate and transfected with 2 μg/ml pGFP-V-RS-shPAX5 or control shRNA vector using Lipofectamine 2000. Two days after transfection, cells were subsequently split at 1:20 ratio in 6-well plates with 0.5 μg/ml puromycin (Invitrogen). After 14–18 days of selection to establish stable clones, cells were fixed with 70% ethanol and stained with crystal violet solution. Colony with more than 50 cells/colony was counted. The experiment was conducted in three independent triplicates.

Cell viability assay
Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay using CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA).

Cell migration assay
Cell migration was assessed using a scratch wound assay. Briefly, pcDNA3.1–*PAX5* or empty vector stably transfected AGS cells were cultured in six-well plates (5×105 cells/well). When the cells grew up to 90% confluence, three scratch wounds across each well were made using a P-200 pipette tip (Axygen, Union City, CA, USA). Images of the wound closure areas were taken at 0, 24 and 48 h.

Invasion assay
Matrigel invasion assay was performed on pcDNA3.1–*PAX5* or empty vector stably transfected AGS using the 24-well matrigel-biocoated invasion chamber (BD Biosciences, Bedford, MA, USA) as described previously (Ying *et al*., 2006).

Flow cytometry
The stably transfected AGS cells with pcDNA3.1–*PAX5*-expressing or pcDNA3.1 empty vector were fixed in 70% ethanol and stained with 50 μg/ml propidium iodide (BD Pharmingen, San Jose, CA, USA). The cells were then sorted by FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) and cell cycle profiles were analyzed by ModFit 3.0 software (Verity Software House, Topsham, ME, USA). Apoptosis was determined by dual staining with Annexin V-FITC (Invitrogen) and propidium iodide. The Annexin V-positive cells were counted as apoptotic cells.

In vivo *tumorigenicity*
BGC823 cells (1×10^6 cells in 0.1 ml phosphate-buffered saline) transfected with *PAX5* or pcDNA3.1 were injected subcutaneously into the dorsal left flank of 5-week-old male Balb/c nude mice as described previously (Yu *et al*., 2010). All experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

Immunostaining and in situ *DNA nick end labeling*
Immunohistochemical staining was performed using PAX5-specific antibody as described previously (Yu *et al*., 2010). TUNEL assay was performed with Dead End TM Colorimetric TUNEL System (Promega). The apoptotic cell ratio was calculated as percentage of positive cells in at least 1000 cells.

cDNA expression array
Gene expression profiles in both AGS and HCT116 stably transfected with pcDNA3.1–*PAX5* or pcDNA3.1 empty vector were analyzed by the Human Cancer PathwayFinder RT^2 Profiler PCR Array (SABiosciences, Frederick, MD, USA; http://www.sabiosciences.com) according to the protocol. Genes with fold changes more than or less than 1.5 were considered to be of biological significance.

ChIP assay
ChIP analysis was performed by using Red ChIP Kit (Diagenode, Liège, Belgium). The immunoprecipitated and input DNA in AGS/vector and AGS/*PAX5* cells was used as a template for quantitative PCR analysis using the primers listed in Supplementary Table 3.

Statistical analysis
Mann–Whitney U test was performed to compare the pathological variables of the two sample groups in the functional assay. The difference in tumor growth rate between the two groups of nude mice was determined by repeated measures analysis of variance. The Fisher's exact test was used for analysis of patient features. RRs of death associated with *PAX5* methylation status and other predictors were estimated by univariate Cox proportional hazards model. Multivariate Cox models were also constructed to estimate the RR for *PAX5* methylation. Kaplan–Meier survival curve and log-rank test were used to evaluate overall survival data corresponding to *PAX5* methylation status. Data were considered statistically significant when P-value is <0.05.

Abbreviations

5-Aza, 5-aza-2′-deoxycytidine; BAX, BCL2-associated X protein; BGS, bisulfite genomic sequencing; ChIP, chromatin immunoprecipitation; CI, confidence interval; GC, gastric cancer; *H. pylori*, *Helicobacter pylori*; MET, mesenchymal epithelial transition factor; MMP1, matrix metalloproteinase 1; MSP, methylation specific PCR; MTSS1, metastasis suppressor 1; ORF, open reading frame; p53, tumor protein 53; *PAX5*, *paired box gene 5*; PBS, phosphate buffered saline; PI, propidium iodide; qPCR, quantitative polymerase chain reaction; RR, relative risk; TIMP1, tissue inhibitors of metalloproteinase 1; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labelling.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This study was supported by research grants of National Basic Research Program of China (973 Program, 2010CB529305), Hong Kong General Research Fund (473008), RFCID (11100022, 10090942), CUHK Group Research Scheme (3110043) and Scheme C.

References

Adams B, Dorfler P, Aguzzi A, Kozmik Z, Urbanek P, Maurer-Fogy I *et al*. (1992). Pax-5 encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. *Genes Dev* **6**: 1589–1607.

Barberis A, Widenhorn K, Vitelli L, Busslinger M. (1990). A novel B-cell lineage-specific transcription factor present at early but not late stages of differentiation. *Genes Dev* **4**: 849–859.

Baumann Kubetzko FB, Di Paolo C, Maag C, Meier R, Schafer BW, Betts DR *et al*. (2004). The PAX5 oncogene is expressed in N-type neuroblastoma cells and increases tumorigenicity of a S-type cell line. *Carcinogenesis* **25**: 1839–1846.

Benvenuti S, Comoglio PM. (2007). The MET receptor tyrosine kinase in invasion and metastasis. *J Cell Physiol* **213**: 316–325.

Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF *et al*. (1991). Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science* **251**: 802–804.

Busslinger M, Klix N, Pfeffer P, Graninger PG, Kozmik Z. (1996). Deregulation of PAX-5 by translocation of the Emu enhancer of the IgH locus adjacent to two alternative PAX-5 promoters in a diffuse large-cell lymphoma. *Proc Natl Acad Sci USA* **93**: 6129–6134.

Carotta S, Holmes ML, Pridans C, Nutt SL. (2006). Pax5 maintains cellular identity by repressing gene expression throughout B cell differentiation. *Cell Cycle* **5**: 2452–2456.

Chambers AF, Matrisian LM. (1997). Changing views of the role of matrix metalloproteinases in metastasis. *J Natl Cancer Inst* **89**: 1260–1270.

Cobaleda C, Schebesta A, Delogu A, Busslinger M. (2007). Pax5: the guardian of B cell identity and function. *Nat Immunol* **8**: 463–470.

el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM *et al*. (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**: 817–825.

Giordano S, Zhen Z, Medico E, Gaudino G, Galimi F, Comoglio PM. (1993). Transfer of motogenic and invasive response to scatter factor/hepatocyte growth factor by transfection of human MET protooncogene. *Proc Natl Acad Sci USA* **90**: 649–653.

Haldar S, Negrini M, Monne M, Sabbioni S, Croce CM. (1994). Down-regulation of bcl-2 by p53 in breast cancer cells. *Cancer Res* **54**: 2095–2097.

He G, Siddik ZH, Huang Z, Wang R, Koomen J, Kobayashi R *et al*. (2005). Induction of p21 by p53 following DNA damage inhibits both Cdk4 and Cdk2 activities. *Oncogene* **24**: 2929–2943.

Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsmeyer SJ. (1991). BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proc Natl Acad Sci USA* **88**: 6961–6965.

Holmes ML, Carotta S, Corcoran LM, Nutt SL. (2006). Repression of Flt3 by Pax5 is crucial for B-cell lineage commitment. *Genes Dev* **20**: 933–938.

Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N *et al*. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet* **19**: 187–191.

Kang GH, Lee HJ, Hwang KS, Lee S, Kim JH, Kim JS. (2003a). Aberrant CpG island hypermethylation of chronic gastritis, in relation to aging, gender, intestinal metaplasia, and chronic inflammation. *Am J Pathol* **163**: 1551–1556.

Kang GH, Lee S, Kim JS, Jung HY. (2003b). Profile of aberrant CpG island methylation along multistep gastric carcinogenesis. *Lab Invest* **83**: 519–526.

Kanteti R, Nallasura V, Loganathan S, Tretiakova M, Kroll T, Krishnaswamy S *et al*. (2009). PAX5 is expressed in small-cell lung cancer and positively regulates c-Met transcription. *Lab Invest* **89**: 301–314.

Kishimoto Y, Murakami Y, Shiraishi M, Hayashi K, Sekiya T. (1992). Aberrations of the p53 tumor suppressor gene in human non-small cell carcinomas of the lung. *Cancer Res* **52**: 4799–4804.

Kovac CR, Emelyanov A, Singh M, Ashouian N, Birshtein BK. (2000). BSAP (Pax5)-importin alpha 1 (Rch1) interaction identifies a nuclear localization sequence. *J Biol Chem* **275**: 16752–16757.

Kozmik Z, Wang S, Dorfler P, Adams B, Busslinger M. (1992). The promoter of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. *Mol Cell Biol* **12**: 2662–2672.

Lazzi S, Bellan C, Onnis A, De Falco G, Sayed S, Kostopoulos I *et al*. (2009). Rare lymphoid neoplasms coexpressing B- and T-cell antigens. The role of PAX-5 gene methylation in their pathogenesis. *Hum Pathol* **40**: 1252–1261.

Lee BL, Kim WH, Jung J, Cho SJ, Park JW, Kim J *et al*. (2008). A hypoxia-independent up-regulation of hypoxia-inducible factor-1 by AKT contributes to angiogenesis in human gastric cancer. *Carcinogenesis* **29**: 44–51.

Lee JH, Park SJ, Abraham SC, Seo JS, Nam JH, Choi C *et al*. (2004). Frequent CpG island methylation in precursor lesions and early gastric adenocarcinomas. *Oncogene* **23**: 4646–4654.

Lee YG, Macoska JA, Korenchuk S, Pienta KJ. (2002). MIM, a potential metastasis suppressor gene in bladder cancer. *Neoplasia* **4**: 291–294.

Levine AJ. (1989). The p53 tumor suppressor gene and gene product. *Princess Takamatsu Symp* **20**: 221–230.

Liu W, Li X, Chu E, Go M, Xu L, Zhao G *et al*. (2011). Paired box gene 5 is a novel tumor suppressor in hepatocellular carcinoma through interaction with p53 signaling pathway. *Hepatology* **53**: 843–853.

Malkin D, Sexsmith E, Yeger H, Williams BR, Coppes MJ. (1994). Mutations of the p53 tumor suppressor gene occur infrequently in Wilms' tumor. *Cancer Res* **54**: 2077–2079.

Meza JL, Anderson J, Pappo AS, Meyer WH. (2006). Analysis of prognostic factors in patients with nonmetastatic rhabdomyosarcoma treated on intergroup rhabdomyosarcoma studies III and IV: the Children's Oncology Group. *J Clin Oncol* **24**: 3844–3851.

Mhawech-Fauceglia P, Saxena R, Zhang S, Terracciano L, Sauter G, Chadhuri A *et al*. (2007). Pax-5 immunoexpression in various types of benign and malignant tumours: a high-throughput tissue microarray analysis. *J Clin Pathol* **60**: 709–714.

Miyashita T, Reed JC. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80**: 293–299.

Musgrove EA, Lee CS, Buckley MF, Sutherland RL. (1994). Cyclin D1 induction in breast cancer cells shortens G1 and is sufficient for cells arrested in G1 to complete the cell cycle. *Proc Natl Acad Sci USA* **91**: 8022–8026.

Nutt SL, Urbanek P, Rolink A, Busslinger M. (1997). Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. *Genes Dev* **11**: 476–491.

Oltvai ZN, Milliman CL, Korsmeyer SJ. (1993). Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**: 609–619.

Parr C, Jiang WG. (2009). Metastasis suppressor 1 (MTSS1) demonstrates prognostic value and anti-metastatic properties in breast cancer. *Eur J Cancer* **45**: 1673–1683.

Quelle DE, Ashmun RA, Shurtleff SA, Kato JY, Bar-Sagi D, Roussel MF *et al*. (1993). Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes Dev* **7**: 1559–1571.

Rocha S, Martin AM, Meek DW, Perkins ND. (2003). p53 represses cyclin D1 transcription through down regulation of Bcl-3 and inducing increased association of the p52 NF-kappaB subunit with histone deacetylase 1. *Mol Cell Biol* **23**: 4713–4727.

Ruvolo PP, Deng X, May WS. (2001). Phosphorylation of Bcl2 and regulation of apoptosis. *Leukemia* **15**: 515–522.

Sauter W, Rosenberger A, Beckmann L, Kropp S, Mittelstrass K, Timofeeva M *et al*. (2008). Matrix metalloproteinase 1 (MMP1) is associated with early-onset lung cancer. *Cancer Epidemiol Biomarkers Prev* **17**: 1127–1135.

Souabni A, Cobaleda C, Schebesta M, Busslinger M. (2002). Pax5 promotes B lymphopoiesis and blocks T cell development by repressing Notch1. *Immunity* **17**: 781–793.

Stapleton P, Weith A, Urbanek P, Kozmik Z, Busslinger M. (1993). Chromosomal localization of seven PAX genes and cloning of a novel family member, PAX-9. *Nat Genet* **3**: 292–298.

Stuart ET, Haffner R, Oren M, Gruss P. (1995). Loss of p53 function through PAX-mediated transcriptional repression. *EMBO J* **14**: 5638–5645.

Tao Q, Huang H, Geiman TM, Lim CY, Fu L, Qiu GH *et al*. (2002). Defective *de novo* methylation of viral and cellular DNA sequences in ICF syndrome cells. *Hum Mol Genet* **11**: 2091–2102.

Visse R, Nagase H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* **92**: 827–839.

Wang Y, Liu J, Smith E, Zhou K, Liao J, Yang GY *et al*. (2007). Downregulation of missing in metastasis gene (MIM) is associated with the progression of bladder transitional carcinomas. *Cancer Invest* **25**: 79–86.

Ying J, Li H, Seng TJ, Langford C, Srivastava G, Tsao SW *et al*. (2006). Functional epigenetics identifies a protocadherin PCDH10 as a candidate tumor suppressor for nasopharyngeal, esophageal and multiple other carcinomas with frequent methylation. *Oncogene* **25**: 1070–1080.

Yu J, Chu ES, Wang R, Wang S, Wu CW, Wong VW *et al*. (2010). Heme oxygenase-1 protects against steatohepatitis in both cultured hepatocytes and mice. *Gastroenterology* **138**: 694–704.

Zwollo P, Desiderio S. (1994). Specific recognition of the blk promoter by the B-lymphoid transcription factor B-cell-specific activator protein. *J Biol Chem* **269**: 15310–15317.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)