

Epstein-Barr virus–driven promoter hypermethylated genes in gastric cancer

J Yu *, KF To, QY Liang

KEY MESSAGES

1. *Somatostatin receptor 1 (SSTR1)*, *meiotic recombination protein 8 (REC8)*, and *interleukin 15 receptor, alpha (IL15RA)* were identified as novel Epstein-Barr virus (EBV)–driven promoter hypermethylated genes in EBV-positive AGS-EBV, compared with EBV-negative AGS.
2. Transcriptional silence of *SSTR1* and *REC8* in EBV-associated gastric cancers is mediated by promoter methylation. This association was not observed for *IL15RA*.
3. Gain- and loss-of-function experiments revealed

that *SSTR1* and *REC8* acted as tumour suppressors through modulating cell proliferation, cell cycling, apoptosis, and migration.

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J Yu, KF To, QY Liang

Institute of Digestive Disease, Department of Medicine and Therapeutics, The Chinese University of Hong Kong

* Principal applicant and corresponding author: junyu@cuhk.edu.hk

Introduction

Epstein-Barr virus (EBV)–associated gastric cancer accounts for 8% to 10% of all gastric cancers. Its clinicopathological features are distinct from EBV-negative gastric cancer.¹ EBV-associated gastric cancer has been reported to involve promoter hypermethylation of tumour suppressors.^{2,3} We identified *Somatostatin receptor 1 (SSTR1)*, *meiotic recombination protein 8 (REC8)*, and *interleukin 15 receptor, alpha (IL15RA)* to be novel EBV-driven promoter hypermethylated genes in EBV-positive AGS-EBV, compared with EBV-negative AGS.⁴ *SSTR* proteins belong to the G protein-coupled receptor family and are crucial in regulating the growth inhibitory effect of somatostatin and reducing tumour cell growth. The meiotic cohesin *REC8* belongs to the cohesin protein complex, which is essential for correct chromosome disjunction and homologous recombination during mitosis and meiosis. Whether these hypermethylated genes are associated with EBV-associated gastric cancer remains elusive. In this study, we examined the epigenetic regulation, clinical significance, biological function, and molecular mechanism of these genes in gastric cancer.

Methods

Gastric cancer tissue samples were collected at the First Affiliated Hospital of Sun Yat-sen University, Guangzhou from 1999 to 2006 and Prince of Wales Hospital, The Chinese University of Hong Kong from 2005 to 2013. The presence of EBV was determined by detection of EBV-encoded RNA (EBER). This study was approved by the ethics committees of

both The Chinese University of Hong Kong and the Clinical Research Ethics Committee of Sun Yat-sen University. Informed consent was obtained from each subject. The methylation status was evaluated using bisulfite genomic sequencing, combined bisulfite restriction analysis, and pyrosequencing.

Loss- and gain-of-function experiments on *SSTR1* and *REC8* were performed after knock-down by shRNA transfection and overexpression by vector transfection in gastric cancer cell lines. Cell growth was evaluated by cell viability and colony formation assay. The cell cycle distribution and apoptosis were determined by flow cytometry. Migration ability and invasiveness were assessed by wound healing and Matrigel invasion assays. Molecular mechanisms were elucidated by Human Cancer Pathway Array.

Results

SSTR1 mRNA expression was reduced or silenced in 63.4% (7/11) of gastric cancer cell lines, but readily expressed in normal gastric tissues. Promoter hypermethylation of *SSTR1* was detected in cells with *SSTR1* down-regulation (Fig 1). The promoter methylation level of *SSTR1* was higher in EBV-positive than EBV-negative gastric cancers (15.04±8.69% vs 6.93±3.01%, P=0.004). Using receiver operating characteristic (ROC) curve analysis, a cut-off value of 9.675% in *SSTR1* promoter methylation could discriminate EBV-positive from EBV-negative gastric cancers, with 75% sensitivity and 85.7% specificity (area under the ROC curve [AUC]=0.777, 95% confidence interval [CI]=0.579-0.974). *SSTR1* promoter methylation was associated with male gender (P=0.024) and EBER positive staining (P<0.005).

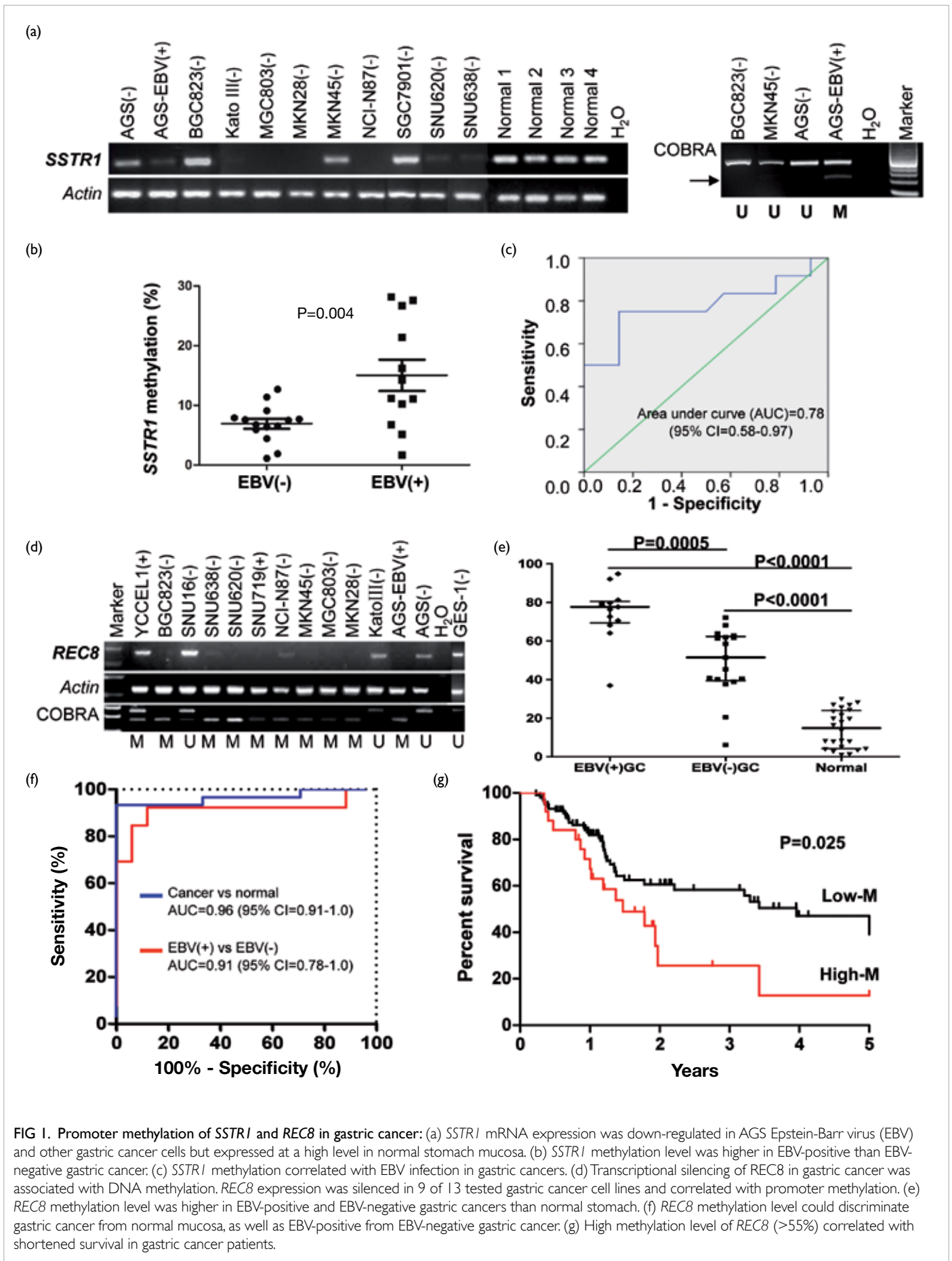


FIG 1. Promoter methylation of *SSTR1* and *REC8* in gastric cancer: (a) *SSTR1* mRNA expression was down-regulated in AGS Epstein-Barr virus (EBV) and other gastric cancer cells but expressed at a high level in normal stomach mucosa. (b) *SSTR1* methylation level was higher in EBV-positive than EBV-negative gastric cancer. (c) *SSTR1* methylation correlated with EBV infection in gastric cancers. (d) Transcriptional silencing of *REC8* in gastric cancer was associated with DNA methylation. *REC8* expression was silenced in 9 of 13 tested gastric cancer cell lines and correlated with promoter methylation. (e) *REC8* methylation level was higher in EBV-positive and EBV-negative gastric cancers than normal stomach. (f) *REC8* methylation level could discriminate gastric cancer from normal mucosa, as well as EBV-positive from EBV-negative gastric cancer. (g) High methylation level of *REC8* (>55%) correlated with shortened survival in gastric cancer patients.

REC8 mRNA expression was silenced in 9 of 13 tested gastric cancer cell lines, and this was correlated with promoter methylation (Fig 1). Promoter methylation level of *REC8* was higher in EBV-positive than EBV-negative gastric cancers ($74.8 \pm 3.9\%$ vs $48.9 \pm 4.3\%$, $P=0.0005$), and in both gastric cancers than normal stomach mucosa ($14.4 \pm 2.0\%$, both $P < 0.0001$). *REC8* promoter methylation was associated with EBER positive staining ($r=23.73$, 95% CI=10.72-36.74, $P=0.001$) but not with other clinicopathological features. Using ROC curve analysis, a cut-off value of 33.4% in *REC8* promoter methylation could discriminate gastric cancer from normal mucosa, with 93.3% sensitivity and 100% specificity (AUC=0.96, 95% CI=0.91-1.0). A cut-off value of 68.1% could discriminate EBV-positive from EBV-negative gastric cancer, with 84.6% sensitivity and 94.1% specificity (AUC=0.91, 95% CI=0.78-1.0). *REC8* was down-regulated by promoter methylation in gastric cancer, especially in the EBV-positive subtype. In patients with EBV-negative gastric cancer, a high level of *REC8* promoter methylation (>55%) predicted shortened survival ($P=0.025$).

IL15RA was down-regulated via promoter methylation specifically in AGS-EBV but this was not a common phenomenon in gastric cancer (data not shown).

To investigate the function of *SSTR1* in gastric cancer, *SSTR1* expression was knocked down by shRNA transfection in BGC823 cells as evidenced by western blot (Fig 2). *SSTR1* knock-down increased cell viability as indicated by MTS assay ($P < 0.05$) and clonogenicity of BGC823 as indicated by colony assay ($P < 0.05$). Cell cycle progression was promoted, with decreased cells in G1 phase ($P < 0.01$) and increased cells in S phase ($P < 0.001$). *SSTR1* knock-down also increased migration ability ($P < 0.01$) and invasiveness ($P < 0.01$) of BGC823 cells. Similar effects were also observed in AGS cells (data not shown). In gain-of-function experiments, ectopic expression of *SSTR1* in MGC803 cells was evidenced by western blot. *SSTR1* inhibited clonogenicity of MGC803 cells ($P < 0.01$) and growth of xenograft tumours derived from MGC803 *in vivo* ($n=9$ /group). The molecular basis of *SSTR1* was revealed by pathway cDNA array and western blot. *SSTR1* functioned as a tumour suppressor through regulating cell cycle progression, inhibiting proliferation, inducing apoptosis, and suppressing migration/invasion.

We overexpressed *REC8* in AGS-EBV cells for gain-of-function experiments. Ectopic expression of *REC8* was evidenced by western blot (Fig 3). Cell viability was decreased as indicated by MTS assay ($P < 0.05$). *REC8* also inhibited clonogenicity of AGS-EBV cells ($P < 0.01$) and cell cycle progression, with increased cells in G1 phase ($P < 0.05$) and decreased cells in S phase ($P < 0.001$). *REC8* also

induced apoptosis and suppressed the migration ability of AGS-EBV cells. Similar effects were also observed in BGC823 cells (data not shown). When *REC8* expression was knocked down in GES-1 cells, cell growth was promoted as evidenced by increased cell viability and clonogenicity. *REC8* knock-down also increased cell migration ability. *REC8* also functioned as a tumour suppressor through regulating cell cycle progression, inhibiting proliferation, inducing apoptosis, and suppressing migration/invasion.

Discussion

SSTR1 promoter methylation was associated with EBV infection and may play a role in the development of EBV-associated gastric cancer. EBV infection was the only factor associated with a high *REC8* promoter methylation level. Higher *REC8* promoter methylation correlated with shortened survival in gastric cancer patients. Promoter methylation-mediated silencing of *REC8* may play a role in gastric carcinogenesis.

SSTR1 might exert its tumour suppressive function through proliferation and apoptosis regulators, inducing *MDM2*, *AKT*, *PI3KR1*, *BCL-XL*, and *MET*. The increased G1/S phase transition by *SSTR1* knock-down might be associated with downregulation of cyclin-dependent kinase inhibitors p15, p16, p21, and p27 and upregulation of *MYC* and *CDC25A*. The suppressive effect of *SSTR1* on migration and invasion ability might be due to the down-regulation of integrin family members (*ITGA1*, *ITGA2*, *ITGA3*, and *ITGB5* subunits), and other important migration/invasion-related genes *MMP1*, *PLAUR*, and *IL8*.

The anti-growth effect of *REC8* might be mediated by inhibiting the cell proliferation regulators (*G6PD* and *SLC2A1*) and apoptosis inhibitor (*NOL3*), while inducing expression of the apoptosis regulator (*GADD45G*) and tumour suppressors (*PinX1*, *IGFBP3*, and *ETS2*). *REC8* caused cell-cycle arrest at G1/S transition through inhibiting *MCM2* and increasing *PinX1*. The anti-migration function of *REC8* may be mediated through inhibiting EMT promoters *SNAI1* and *SNAI2* (that are involved in generating de-differentiated cells) and inducing the migration inhibitor *LDHA*. Whether these cancer-associated genes modulated by altered expression of *SSTR1* or *REC8* are direct downstream targets requires further investigation.

Conclusion

Both *SSTR1* and *REC8* are novel EBV-associated promoter hypermethylated genes in gastric cancer. They play a role in suppressing gastric cancer through modulating the expression of important effectors involved in the regulation of cell proliferation,

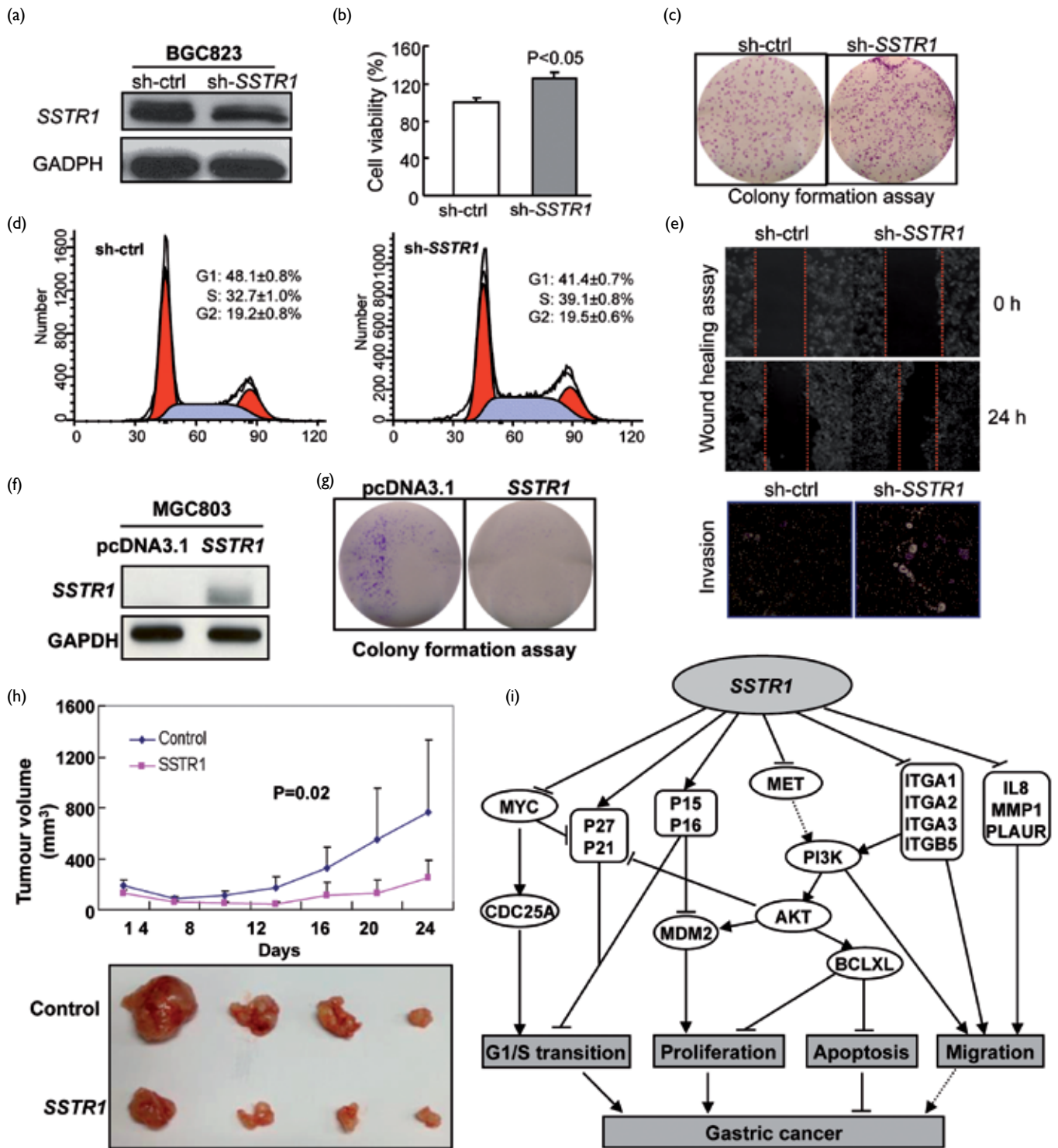


FIG 2. *SSTR1* as a tumour suppressor in gastric cancer: (a) *SSTR1* expression was knocked down by shRNA transfection. (b) *SSTR1* knock-down increased cell viability. (c) *SSTR1* knock-down increased clonogenicity in BGC823 ($52.6 \pm 9.1\%$ increase, $P < 0.05$). (d) *SSTR1* knock-down promoted cell cycle progression (G1 phase, $P < 0.01$; S phase, $P < 0.001$). (e) *SSTR1* knock-down increased the migration ability (1.2 ± 0.0 fold, $P < 0.01$) and invasiveness (1.9 ± 0.4 fold, $P < 0.01$) of BGC823. (f) Ectopic expression of *SSTR1* in MGC803 cells. (g) *SSTR1* inhibited tumour cell clonogenicity of MGC803 ($68.8 \pm 10.9\%$ decrease, $P < 0.01$). (h) *SSTR1* inhibited growth of xenograft tumours derived from MGC803 in vivo ($n = 9/\text{group}$). (i) Schematic diagram of the molecular events for *SSTR1* function as a tumour suppressor through regulating cell cycle progression, inhibiting proliferation, inducing apoptosis and suppressing migration/invasion.

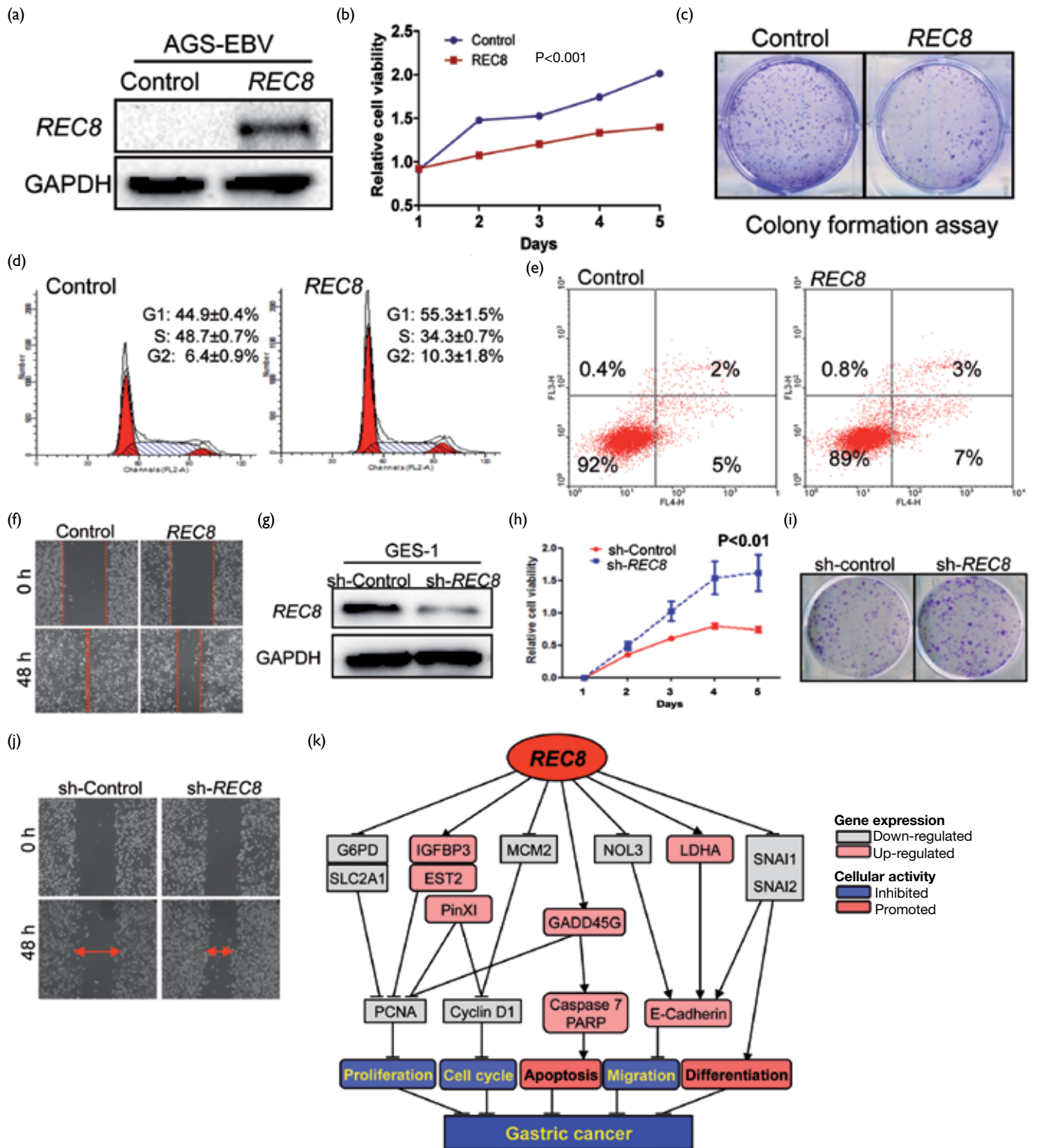


FIG 3. REC8 as a tumour suppressor in gastric cancer: (a) ectopic expression of REC8 in AGS Epstein-Barr virus (EBV) cells. (b) REC8 suppressed cell viability in both AGS-EBV cells. (c) REC8 reduced clonogenicity in both AGS-EBV cells ($66.7 \pm 6.0\%$, $P < 0.05$). (d) REC8 inhibited cell cycle progression by increasing cells in G1 phase ($P < 0.05$) and reducing cells in S phase ($P < 0.001$). (e) REC8 induced cell apoptosis in AGS-EBV cells. (f) REC8 inhibited the migration ability of AGS-EBV ($60.9 \pm 6.2\%$, $P < 0.01$). (g) REC8 was knocked down in GES-1. (h) REC8 knock-down increased cell viability in GES-1 cells. (i) REC8 knock-down increased clonogenicity in GES-1 cells. (j) REC8 knock-down increased the migration ability of GES-1 cells ($P < 0.01$). (k) Schematic diagram of the molecular events for REC8 function as a tumour suppressor through inhibiting proliferation, regulating cell cycle progression, inducing apoptosis, and suppressing cell migration.

cell cycle, apoptosis, and cell migration/invasion. Epigenetic silencing of *REC8* or *SSTR1* by EBV infection may contribute to the pathogenesis of EBV-associated gastric cancer.

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Results of this study have been published in:

- (1) Yu J, Liang Q, Wang J, et al. *REC8* functions as a tumor suppressor and is epigenetically downregulated in gastric cancer, especially in EBV-positive subtype. *Oncogene* 2017;36:182-93.
- (2) Zhao J, Liang Q, Cheung KF, et al. Somatostatin receptor 1, a novel EBV-associated CpG

hypermethylated gene, contributes to the pathogenesis of EBV-associated gastric cancer. *Br J Cancer* 2013;108:2557-64.

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