RPS12-specific shRNA inhibits the proliferation, migration of BGC823 gastric cancer cells with *S100A4* as a downstream effector

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Abstract. Our previous study using suppression subtractive hybridization (SSH), cDNA microarray and semi-quantitative RT-PCR showed that *RPS12* was overexpressed in gastric cancer and it was closely related to metastasis. However, the role of *RPS12* in gastric cancer is not clear, which led us to conduct the current study to further investigate the effects of *RPS12* on the proliferation and migration of gastric cancer cells, and also to explore the underlying molecular mechanisms. RNA interference was used to inhibit the expression of *RPS12*. The expression of *RPS12* and *S100A4* in gastric cancer cells was determined using semi-quantitative RT-PCR and western blot analysis. Cell proliferation and migration were detected by MTT and transwell assay, respectively. In addition, the promoter activity of *S100A4* was measured by a Dual-Luciferase Reporter Assay System. We found that RNAi-mediated *RPS12* downregulation led to reduced proliferation and migration of BGC823 and SGC7901 gastric cancer cells. Further results showed that *RPS12* inhibition led to reduced *S100A4* expression and decreased promoter activity of *S100A4* in BGC823 cells. We demonstrated that ectopic expression of *S100A4* reversed the reduced proliferation and migration ability after *RPS12* inhibition in BGC823 cells. Our findings provide the first demonstration that *RPS12* plays important roles in regulating the proliferation and migration of gastric cancer cells. *S100A4* can mediate the effects of *RPS12* as a downstream effector.

Introduction

Despite its declining incidence, gastric cancer remains a worldwide health problem that is the second leading cause of cancer-related death with little improvement of long-term survival during the past decades. In order to improve patient prognosis and therapy, it is now mandatory to further understand the molecular features involved in the pathogenesis. Although it has been under intensive investigation, the molecular mechanism of gastric cancer is not well understood and needs further exploring.

In an attempt to identify new factors involved in gastric cancer, we previously performed a comprehensive analysis of differentially expressed genes in gastric cancer by SSH (1). *RPS12* was identified as one of the genes overexpressed in gastric cancer compared with the corresponding non-tumorous tissue, which was further confirmed by RT-PCR and DOT blot analysis in more samples. Clinicopathological analysis revealed that the enhanced *RPS12* expression was related to metastasis of gastric cancer (1). Subsequent cDNA microarray studies showed that *RPS12* expression in lymph node metastases was much higher than that in the primary gastric cancers (2). All the above results suggested that *RPS12* might play important roles in the progression and metastasis of gastric cancer, which led us to investigate the function of *RPS12* in gastric cancer cells. Our previous study showed that suppression of *RPS12* expression by RNAi led to increased apoptosis of gastric cancer cells (3). In the current study, we focused on further investigating the effects of *RPS12* on the proliferation and migration of gastric cancer cells, and the underlying mechanisms by exploring the downstream effector of *RPS12*.

Materials and methods

Cell culture. The human gastric cancer cell line BGC823 and SGC7901 were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum in a humidified 37˚C incubator with 5% CO_2.

Construction and transfection of RPS12-specific short hairpin RNA (shRNA) expression vector. Two different *RPS12* hairpin oligonucleotides (oligos) were designed by selecting appropriate sequences from the human *RPS12* mRNA according to the online software of Ambion Inc. (Austin, TX, USA). The

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first one named *RPS12*-shRNA-A oligo (sense: 5'-GATCC**agtg gttggttgcagttgt***TTCAAGAGA***acaactgcaaccaaccacttt**A-3'; antisense: 5'-AGCTT**aaagtggttggttgcagttgt***TCTCTTGAA***acaactgcaaccaaccact**G-3') contained a region specific to bases 386-406 of *RPS12* mRNA (marked in bold); the second one named *RPS12*-shRNA-B oligo (sense: 5'-GATCC**gctgcca aagccttagaca***TTCAAGAGA***tgtctaaggctttggcagctt**A-3'; antisense: 5'-AGCTT**aagctgccaaagccttagaca***TCTCTT GAA***tgtctaaggctttggcagc**G-3') contained a region specific to bases 192-212 of *RPS12* mRNA (marked in bold). The *RPS12*-shRNA-A or *RPS12*-shRNA-B oligo contained a hairpin loop region (italicized), and 5' and 3' linker sequences for subcloning into the *Bam*HI and *Hin*dIII sites of the pSliencer 4.1-CMV neo vectors. Each oligo was annealed with its complementary strand, and the resulting double-stranded shRNA oligo was cloned into the pSilencer 4.1-CMV neo vector (Ambion Inc.) making p*RPS12*-shRNA-A or p*RPS12*-shRNA-B according to the manufacturer's instructions. The control shRNA expression vector (pControl-shRNA) was the same as previously reported (4). Competent JM109 bacteria (Takara, Japan) were transformed with p*RPS12*-shRNA-A/B or pControl-shRNA. The plasmids were prepared from individual bacterial colonies, and confirmed by sequencing analysis.

p*RPS12*-shRNA-A/B or pControl-shRNA was transfected into BGC823 cells respectively, using Lipofectamine™ 2000. We selected the more efficient one in inhibiting *RPS12* mRNA expression and renamed it as p*RPS12*-shRNA, this was then used to inhibit *RPS12* expression in BGC823 and SGC7901 cells in the subsequent studies.

The cells transfected with p*RPS12*-shRNA or pControl-shRNA were referred to as BGC823/SGC7901/p*RPS12*-shRNA cells or BGC823/SGC7901/pControl-shRNA cells, respectively.

RNA extraction and semi-quantitative RT-PCR. Total RNA of the cells was extracted using TRIzol reagent (Invitrogen). The reverse transcription reaction was performed using the First-Strand cDNA synthesize kit (Promega, Madison, WI, USA) with 1 μg of RNA in a final volume of 20 μl. The newly synthesized cDNA was amplified by PCR. Primers specific for human *RPS12*, *S100A4* or *β-actin* were designed as follows: *RPS12* sense 5'-ggCTTgggTgCgTTCAAgAT-3', antisense 5'-ggCCT gAgACTCCTTgCCA-3'; *S100A4* sense 5'-gATgT gATggTgTC CACCTT-3', antisense 5'-ATTTCTTCCTgggCTgCTTA-3'; *β-actin* sense 5'-CTCTTCCagCCTTCCTTCCT-3', antisense 5'-CACCTTCACCgTTCCAgTTT-3'. Amplication cycles were: 95˚C for 5 min, then 30 cycles at 95˚C for 30 sec, 58˚C for 30 sec and 72˚C for 30 sec, followed by 72˚C for 10 min. Aliquots of PCR product were checked by electrophoresis on 1.5% agarose gels, and the fragments were visualized by ethidium bromide staining. The experiments were performed three times.

Western blot analysis. Cells were washed with ice-cold PBS after being trypsinized, and then centrifuged at 1,000 rpm for 5 min at 4˚C. The pellet containing 10^6 cells was lysed in 100 μl of lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$ with 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF) and quantified by Bradford method. Total protein (100 μg) was separated by SDS-PAGE (12% polyacrylamide gel) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes were incubated in blocking solution consisting of 5% non-fat milk in TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween-20) at room temperature for 3 h, then immunoblotted with primary antibodies such as anti-RPS12 (1:800 dilution; Proteintech), anti-S100A4 (1:1,000 dilution; Lab Vision) and anti-β-actin (1:1,500 dilution; Santa Cruz) respectively, followed by incubation with a peroxidase-conjugated second antibody (goat anti-rabbit IgG for RPS12, S100A4 and β-actin). The reagent for enhanced chemiluminescence (Amersham, Freiburg, Germany) was used for detection and developed on X-ray film. The experiments were performed three times.

MTT assay. BGC823/SGC7901/p*RPS12*-shRNA cells and BGC823/SGC7901/pControl-shRNA cells at 12 h to 3 days after transfection were plated in 96-well plates at a density of $5x10^3$ cells per well and incubated at 37˚C in 5% CO_2. At different time points (day 3, 4, 5, 6, 7 and 8 after transfection), the proliferation ability was assayed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously reported (4). The absorbance was read at a wavelength of 570 nm in Multiscan MC microplate reader. Each experiment was performed in triplicate and repeated three times.

Transwell assay. For cell migration assays, $5x10^4$ BGC823/SGC7901/p*RPS12*-shRNA cells and BGC823/SGC7901/pControl-shRNA cells at 4th day after transfection were trypsinized, washed, resuspended in serum-free RPMI-1640, and plated in the upper chamber of 8-μm pore transwells (Costar, USA), respectively. The lower chamber was filled with RPMI-1640 supplemented with 10% FBS as a chemoattractant. Cells were incubated at 37˚C for 24 h. Non-migrated cells on the upper surface of the insert membrane were removed with a cotton swab. Migrated cells on the lower surface were washed with PBS, fixed with 10% formaldehyde, stained, photographed and counted under high-power magnification. Five random fields were analyzed for each chamber. The experiments were performed three times.

Luciferase reporter assay. The plasmid pGL3-*S100A4*-luc vector containing a regulatory fragment located in the promoter region and the first intron of *S100A4* gene (from -387 to +550), was constructed in a previous study by our group (5). On day 4 after transfection of *RPS12*-specific shRNA, BGC823/*RPS12*-shRNA cells at a density of $1.5x10^4$ cells/well in a 24-well plate were transfected with 0.8 μg of the pGL3-*S100A4*-luc or pGL3-basic-luc constructs, with Renilla luciferase (Promega) as the internal control (5). After incubation for 24 h, the cells were harvested, and luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) and normalized by the internal control values. The experiments were performed three times.

Rescue assay after ectopic expression of S100A4 in BGC823/pRPS12-shRNA cells. To investigate the effect of *S100A4* on gastric cancer cells after *RPS12* suppression, at 48 h after *RPS12*-sepcific shRNA transfection, BGC823/*RPS12*-shRNA cells were transfected with 4 μg pcDNA3.1-*S100A4* (6) using Lipofectamine 2000, with pcDNA3.1-empty vector transfected cells as a control. At different time points, cells were harvested

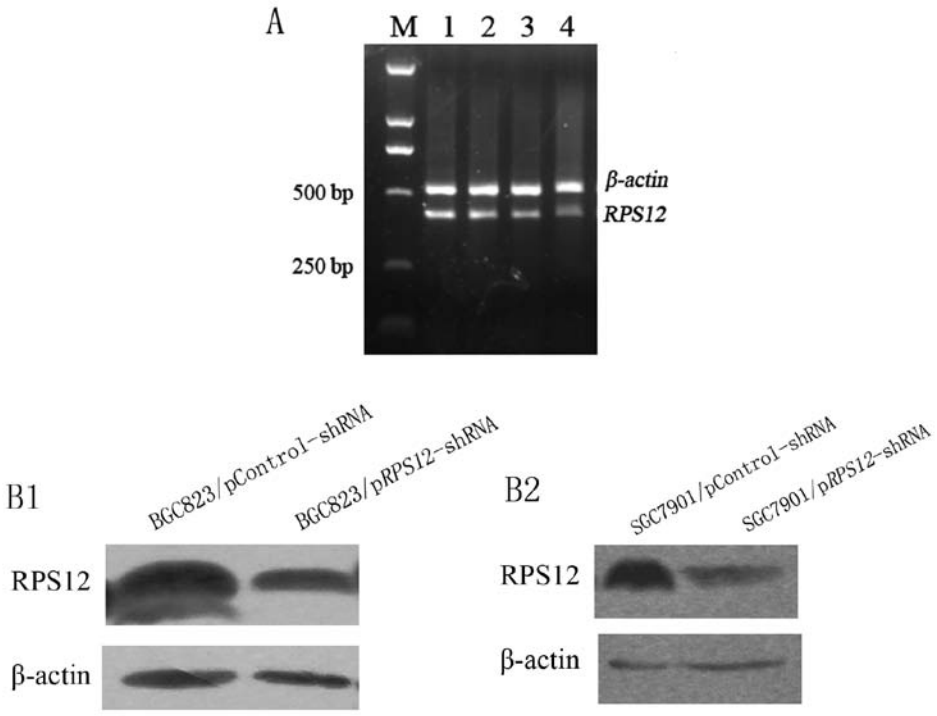

Figure 1. Downregulation of *RPS12* expression in gastric cancer cells by RNAi. (A) Semi-quantitative RT-PCR analysis of *RPS12* mRNA level in BGC823 cells on day 5 after transfection with p*RPS12*-shRNA-A or p*RPS12*-shRNA-B. Lane M, DNA marker-DL2000; lane 1, parental BGC823; lane 2, pControl-shRNA transfection; lanes 3 and 4, p*RPS12*-shRNA-A and p*RPS12*-shRNA-B transfection, respectively. (B) Expression of RPS12 protein in (B1) BGC823 and (B2) SGC7901 cells on day 5 after transfection of p*RPS12*-shRNA was analyzed by western blot analysis as described in Materials and methods.

for determining the proliferation, and migration by MTT and transwell assay, respectively, as described above.

Statistical analysis. The data of absorbance in MTT, cell numbers in transwell and luciferase activities in luciferase reporter assay are presented as the means ± SD. Statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA), where $p<0.05$ was considered significant.

Results

Downregulation of RPS12 expression in gastric cancer cells by RNAi. We first evaluated the efficiency of the two shRNAs specific to different regions of *RPS12* in silencing *RPS12* expression on day 5 after transfection by using semi-quantitative RT-PCR (Fig. 1A). The results showed that *RPS12* mRNA expression in p*RPS12*-shRNA-B transfected BGC823 cells was obviously lower than that in p*RPS12*-shRNA-A transfected cells indicating that p*RPS12*-shRNA-B can suppress *RPS12* expression more efficiently than p*RPS12*-shRNA-A in BGC823 cells. Therefore, we selected *RPS12*-shRNA-B (renamed *RPS12*-shRNA) to inhibit *RPS12* expression in BGC823 and SGC7901 cells in the subsequent studies, and observed the cellular effects mainly at/or around day 5 after *RPS12*-shRNA transfection.

The results of western blot analysis showed that RPS12 protein expression in BGC823/SGC7901/p*RPS12*-shRNA cells at day 5 after transfection was obviously lower than that in BGC823/SGC7901/pControl-shRNA cells (Fig. 1B).

Downregulation of ***RPS12 expression leads to decreased proliferation and migration of gastric cancer cells***. We observed the effects of *RPS12* inhibition on the proliferation and migration of BGC823 and SGC7901 cells. The MTT assay showed that BGC823/SGC7901/p*RPS12*-shRNA cells displayed significantly reduced proliferation, as compared to BGC823/SGC7901/pControl-shRNA cells at different time points after transfection of the corresponding shRNA (Fig. 2A). Transwell assays revealed that the number of migrated BGC823/SGC7901/p*RPS12*-shRNA cells (46.2±9.45, 54.68±8.43) was significantly fewer than that of their corresponding pControl-shRNA cells (118.05±15.78, 78.4±27.49) ($p<0.05$) (Fig. 2B and C).

Effect of RPS12 inhibition on S100A4 expression in BGC823 cells. Having demonstrated that *RPS12* plays important roles in regulating proliferation and migration of gastric cancer cells, we attempted to investigate the molecular mechanism through which *RPS12* exerts its functions. It has been demonstrated by our group that *S100A4* plays crucial roles in regulating proliferation of gastric cancer cells (4), and also the migration of the cells (unpublished). We hypothesized that *S100A4* might mediate the effect of *RPS12* as its downstream target. To test our hypothesis, *S100A4* expression was determined by RT-PCR and western blot analysis after *RPS12* inhibition. We found that BGC823/p*RPS12*-shRNA cells displayed an obviously decreased *S100A4* mRNA and protein expression compared with BGC823/pControl-shRNA cells (Fig. 3).

Effect of RPS12 inhibition on the transcriptional activity of S100A4 promoter in BGC823 cells. In order to investigate

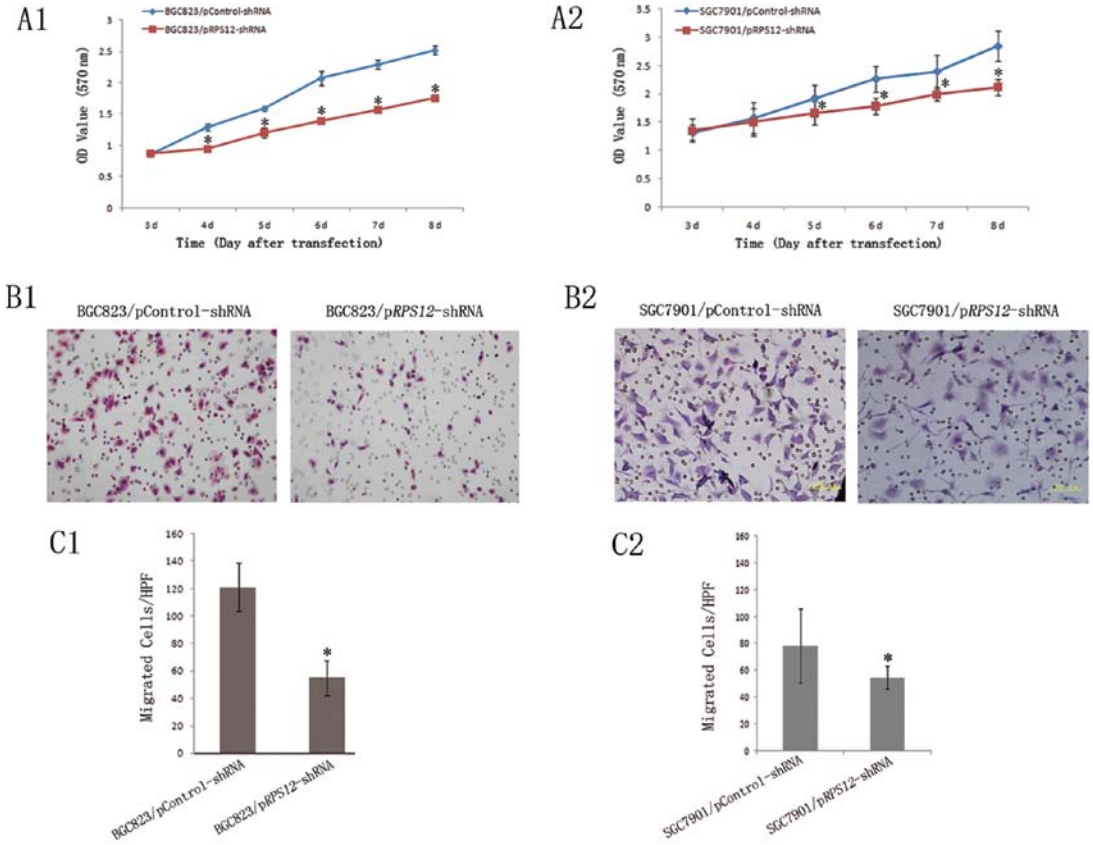

Figure 2. Downregulation of *RPS12* expression inhibits proliferation and migration of gastric cancer cell BGC823 and SGC7901. (A) Proliferation of BGC823/SGC7901/pRPS12-shRNA and BGC823/SGC7901/pControl-shRNA cells was analyzed by (A1-A2) MTT assay as described in Materials and methods. On day 3, 4, 5, 6, 7 and 8 after transfection. *p<0.05 vs the corresponding pControl-shRNA cells. (B) The representative pictures of transwell assays for BGC823/SGC7901/p*RPS12*-shRNA and BGC823/SGC7901/pControl-shRNA cells (B1 and B2, BGC823 and SGC7901, respectively), as described in Materials and methods. (C) The numbers of migrated cells were plotted as mean from triplicate experiments; *p<0.05 vs the corresponding pControl-shRNA cells (C1 and C2, BGC823 and SGC7901, respectively). Bars indicate SD. HPF, high power field (x200).

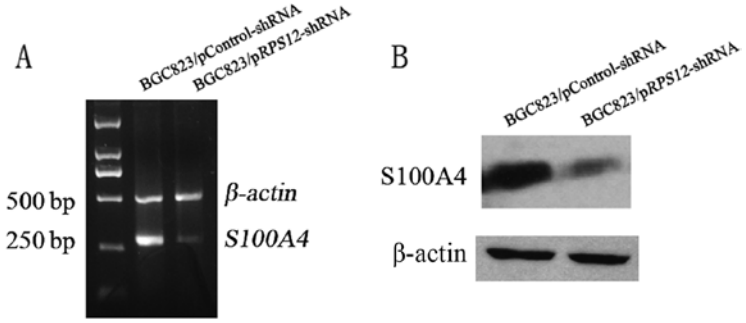

Figure 3. *RPS12* inhibition leads to a decrease in *S100A4* expression. BGC823/p*RPS12*-shRNA or BGC823/pControl-shRNA cells were grown under normal culture conditions. RNA and protein were extracted for (A) RT-PCR and (B) western blot analysis on day 5 after p*RPS12*-shRNA or pControl-shRNA transfection as described in Materials and methods.

whether *RPS12* regulates the transcriptional activity of *S100A4* promoter, pGL3-*S100A4*-luc plasmid was transfected into BGC823/p*RPS12*-shRNA cells or BGC823/pControl-shRNA cell, respectively. The results of Dual-Luciferase Reporter Assay showed that luciferase activity of pGL3-*S100A4*-luc plasmid in BGC823/p*RPS12*-shRNA cells was significantly lower than that in BGC823/pControl-shRNA cells (Fig. 4).

Ectopic expression of ***S100A4 reverses the effects of RPS12 inhibition on the proliferation, migration of BGC823 cells***. Having found that *S100A4* is a molecular target of *RPS12*, we further determined whether *S100A4* could mediate the cellular effects of *RPS12* by rescue assay. As shown in Fig. 5, transfection of pcDNA3.1-*S100A4* into BGC823/p*RPS12*-shRNA cells led to increased *S100A4* expression in the cells at both mRNA and protein level compared with pcDNA3.1-empty vector trans-

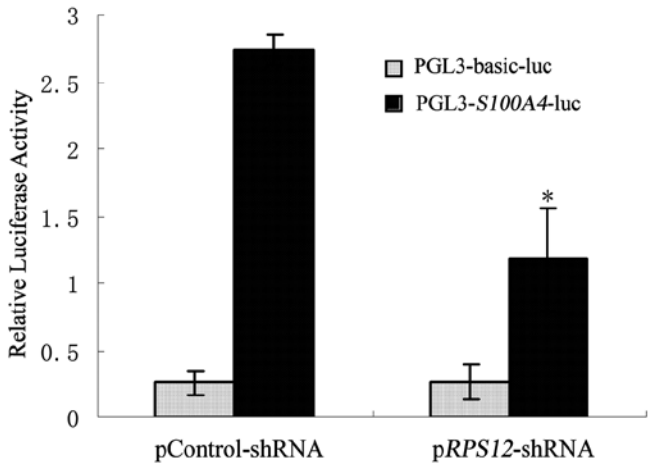

Figure 4. Effect of *RPS12* knockdown on the transcriptional activity of *S100A4* promoter in BGC823 cells. The relative luciferase activity is represented by the ratio of luciferase activity of firefly (from the various pGL3 vectors) to that of Renilla (from pRL-TK construct, internal control). The data represent the mean ± SD from three experiments (*p<0.05).

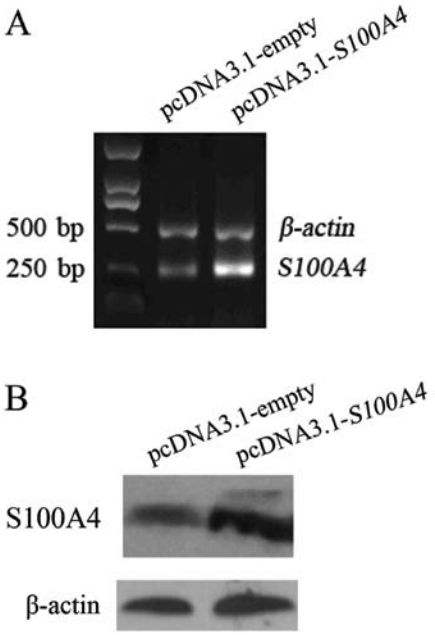

Figure 5. pcDNA3.1-*S100A4* transfection enhances *S100A4* expression in BGC823/p*RPS12*-shRNA cells. Expression of *S100A4* in BGC823/p*RPS12*-shRNA cells transfected with pcDNA3.1-*S100A4* or pcDNA3.1-empty vector was analyzed by (A) RT-PCR and (B) western blot analysis, respectively, as described in Materials and methods.

fection. We then found the following cellular effects. At day 3, 4 and 5 after pcDNA3.1-*S100A4* transfection, the proliferation of BGC823/p*RPS12*-shRNA cells was significantly higher than that after pcDNA3.1-empty vector transfection (p<0.05) (Fig. 6A). The number of migrated cells was significantly increased in BGC823/p*RPS12*-shRNA cells after pcDNA3.1-*S100A4* transfection (97±11.37), as compared to that in BGC823/p*RPS12*-shRNA cells after pcDNA3.1-empty vector transfection (49±8.73) (p<0.05) (Fig. 6B).

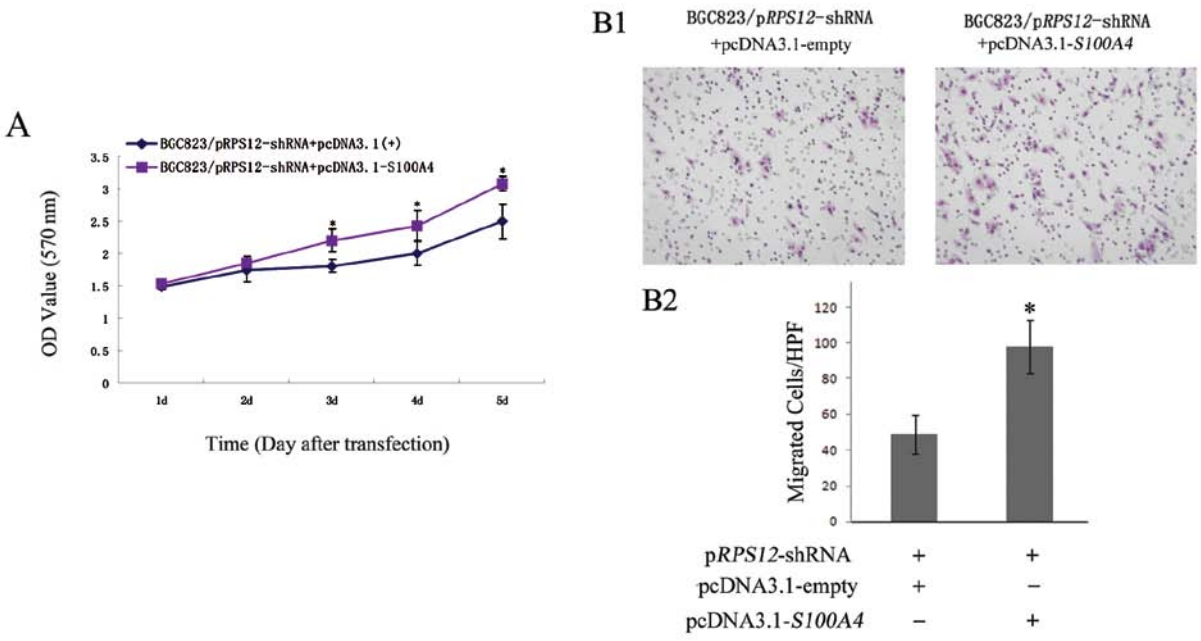

Figure 6. Ectopic expression of *S100A4* reverses the effects of *RPS12* inhibition on the proliferation, migration of BGC823 cells. (A) Proliferation of BGC823/p*RPS12*-shRNA cells transfected with pcDNA3.1-*S100A4* or pcDNA3.1-empty was analyzed by MTT assay as described in Materials and methods. On day 3, 4 and 5 after transfection of pcDNA3.1-S100A4. *p<0.05 vs BGC823/p*RPS12*-shRNA cells transfected with pcDNA3.1-empty. (B) Migration of BGC823/p*RPS12*-shRNA cells transfected with pcDNA3.1-*S100A4* or pcDNA3.1-empty was analyzed by transwell assays as described in Materials and methods. (B1) Representative pictures of transwell assays. (B2) The number of migrated cells was plotted as mean from triplicate experiments; *p<0.05 vs BGC823/p*RPS12*-shRNA cells transfected with pcDNA3.1-empty; HPF, high power field (x200).

Discussion

The ribosome is an organelle essential for protein synthesis in cells, which is composed of ribosomal RNAs and ribosomal proteins (RPs). At present, approximately 80 different ribosomal proteins are found in eukaryotic ribosomes (7). It is very interesting that the reports showing the involvements of RPs in various tumors are emerging (8-14). Kasai *et al* (15) reported decreased expression of 10 RP genes and increased expression of 2 RP genes in colorectal cancer compared with normal epithelia. Other reports have also shown the overexpression of RP genes in different types of cancers, such as *S8*, *L12*, *L23a*, *L27* and *L30* mRNAs in human hepatocellular carcinoma (16), *RPL13* in gastric cancer (9), and *RPL19* in colorectal cancer (10). Additionally, transcriptional loss of *RPL14* was observed in lung and oral cancers (17). Reports on the association of abnormal expression of RPs with tumorigenesis are still increasing. *RPS12* gene, located in 6q23.2, encoding a ribosomal protein of 132 amino acids as a component of the 40S subunit of ribosome, was isolated as a clone overexpressed in gastric cancer in our previous studies by SSH. Subsequent experiments showed that its overexpression was associated with lymph node metastasis of gastric cancer (1,2). The enhanced expression of *RPS12* gene has also been reported in colorectal cancer and squamous cell carcinoma of the uterine cervix when compared with normal tissues (11,18). O'Donohue *et al* reported depletion of *RPS12* causes delayed processing of 18S ribosomal RNA, although a less severe phenotype is seen for *RPS12* than for other small subunit r-proteins (19). However, whether or how the overexpression of *RPS12* contributes to the development and progression of gastric cancers is not clear.

We carried out the current study to investigate the function of *RPS12* in gastric cancer and demonstrated that *RPS12* suppression by RNAi led to reduced proliferation and migration in gastric cancer cells BGC823 and SGC7901. The findings indicate that *RPS12* may take part in the development, progression and metastasis of gastric cancer or prognosis of the patients by regulating proliferation, migration of gastric cancer cells. Similar to our findings on the cellular effects of *RPS12*, it has been reported that inhibition of *RPL15* expression suppressed the proliferation of gastric cancer cells (20), while *RPL44* enhanced colony formation and cell proliferation of hepatocellular carcinoma cells (13). Taken together, the findings above suggest that aberrant expression of certain ribosomal proteins contributes to tumorigenesis and progression of certain types of cancers by regulating biological behavior of the cancer cells.

Based on the important roles of *RPS12* in gastric cancer cells as described above, we try to decipher the mechanisms through which *RPS12* performs the functions. Previous studies indicated that ribosomal proteins may exert their roles by regulating the expression of downstream genes. Khanna *et al* (21) demonstrated that ribosomal protein *S29* induced apoptosis in non-small cell lung cancer cells by downregulating *BCL-2*, *BCL-XL* and *survivin*, and also upregulating *P53* and *BAX* expression. It was also reported that *RPL6* inhibited cell cycle progression through downregulation of cyclin E (22). Since we found that *RPS12* affected many biological characteristics of gastric cancer cells, we investigated whether *RPS12* might exert its functions by regulating a multifunctional downstream target gene. *S100A4*, also known as metastatin-1 (Mts-1), is a member of the S100 family calcium-binding proteins. It is overexpressed in gastric cancer and its expression correlates with poor prognosis of the patients (23-25). It has also been shown that *S100A4* inhibition leads to decreased *in vitro* and *in vivo* growth of gastric cancer BGC823 cells (4). We previously found that *S100A4* suppression leads to decreased migration of BGC823 cells (data not shown). These findings suggest that *S100A4* can regulate many biological characteristics of gastric cancer cells. We speculated that *S100A4* may be a downstream effector mediating the role of *RPS12*. We examined and found that *RPS12* inhibition led to decreased expression of *S100A4* at both mRNA and protein level in BGC823 cells, which indicated that *S100A4* might be a downstream target of *RPS12*. Our further study on luciferase reporter assay showed that *RPS12* suppression significantly reduced the activity of *S100A4* promoter, suggesting the involvement of *RPS12* in the transcriptional regulation of *S100A4* gene. How *RPS12* regulates the transcription of the *S100A4* needs to be clarified. Other reports have proved that some ribosomal proteins are gene transcription regulators. An example of RPs as a gene transcription regulator is the mammalian *RPL11*. It binds to the Myc oncoprotein and inhibits Myc-mediated transcriptional activation of target genes (26). L5, L23 and S7 were shown to bind to MDM2 and inhibit MDM2-mediated p53 ubiquitination and degradation, leading to p53 activation (27-29). Based on these studies, we hypothesize that *RPS12* may interact with or affect the stability of certain transcription factors regulating *S100A4* expression. In what way *RPS12* control *S100A4* gene transcription is under investigation by our group.

In order to further investigate whether *S100A4* mediates the biological effects of *RPS12* in gastric cancer cells, we carried out rescue assays. The results showed that ectopic expression of *S100A4* reversed the reduced migration and proliferation ability, which suggested that as a downstream effector, *S100A4* could partly mediate the roles of *RPS12* in BGC823 cells.

In summary, the current study investigated the effects of *RPS12* on gastric cancer cells and the underlying mechanisms. We demonstrated for the first time that *RPS12* suppression was able to inhibit the proliferation and migration of gastric cancer cells. *RPS12* inhibition reduces the activity of *S100A4* promoter and leads to the downregualtion of *S100A4* expression. Ectopic expression of *S100A4* is able to reverse the reduced proliferation, and migration by *RPS12* inhibition. Collectively, the evidence suggests that *RPS12* may take part in the development and progression of gastric cancer by affecting the proliferation and migration, and the effects may be at least partly mediated by *S100A4*. *RPS12* might be a new potential target for diagnosis and therapy of gastric cancer.

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