Knockdown of Arf6 increases drug sensitivity and inhibits proliferation, migration and invasion in gastric cancer SGC-7901 cells

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Abstract. ADP-ribosylation factor 6 (Arf6), a member of the ADP-ribosylation factor family, is overexpressed in different types of cancer cell and promotes invasion, metastasis and drug resistance. However, the potential functions of Arf6 in gastric cancer (GC), and the molecular mechanism underlying these functions, remain to be fully elucidated. In the present study, the results demonstrated that *in vitro* knockdown of Arf6 decreased proliferation, colony formation, migration and invasion in SGC-7901 cells. Arf6 knockdown also markedly decreased the activity of the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway. Furthermore, knockdown of Arf6 was associated with elevated chemosensitivity of SGC-7901 cells to 5-fluorouracil through inactivation of the ERK1/2 signaling pathway. Taken together, these results suggest that Arf6 is involved in regulating proliferation, migration, invasion and drug resistance in GC, and may be a potential therapeutic target for the treatment of GC.

Introduction

In 2013, gastric cancer (GC) had become the third most common cause of cancer-associated mortality globally (1). It is estimated that 951,600 new cases and 723,100 GC-associated mortalities were recorded worldwide in 2012 (2,3). Multistep processes and molecular markers have been confirmed to be involved in the tumorigenesis and invasiveness of GC (4). A combination of surgery and chemotherapy has increased the survival time of patients with GC (5,6). However, a significant number of patients still suffer from relapse due to the resistance of tumor cells to chemotherapeutic agents (5). Therefore, understanding the molecular pathways underlying GC carcinogenesis and progression will assist in improving diagnosis, therapy, and prevention of this disease.

ADP-ribosylation factor 6 (Arf6), a member of the Arf family, has emerged as a critical regulator of membrane traffic, cell polarity and cytoskeletal organization (6,7). There is increasing evidence to confirm that Arf6 is associated with cancer development (8-10). Arf6 is overexpressed in various types of human cancer, including breast cancer, lung adenocarcinoma, clear cell renal cell carcinoma and head and neck squamous cell carcinoma (11-13). In glioblastoma cells, early studies have demonstrated that Arf6 is required for epidermal growth factor (EGF)-induced cell proliferation (14). Arf6 has also been implicated in other cellular processes associated with tumorigenesis, including the epithelial-mesenchymal transition (EMT), migration and invasion (12,15,16). Furthermore, activation of Arf6 is associated with drug resistance in the breast cancer cell lines MDA-MB-231, MDA-MB-453 and Hs578T (17-19). Arf6 has also been demonstrated to mediate EGF-induced EMT in GC cells (20). The EMT phenotype in cancers is associated with migration, invasion and drug resistance (21,22). However, the involvement of Arf6 in the growth, migration, invasion and drug resistance of GC cells remains to be fully elucidated.

The present study was designed to explore the function of Arf6 in the proliferation, migration, invasion and drug resistance in GC cells. Our results demonstrate that Arf6 contributes to the proliferation, migration and invasion of GC cells. Knockdown of Arf6 was demonstrated to increase the sensitivity of GC cells to 5-fluorouracil (5-FU) via inhibition of the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway. Therefore, Arf6 may be a potential target for GC therapy.

Materials and methods

Cell culture. The human GC cell line SGC-7901 was obtained from the Type Culture Collection of the Institute of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in

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Dulbecco's modified Eagle's medium (DMEM; high glucose) supplemented with 10% (v/v) fetal bovine serum (FBS) (both from Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) and antibiotics (100 U/ml streptomycin and 100 μg/ml penicillin; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified incubator at 37°C with 5% CO_2.

Small interfering RNA (siRNA) transfection. siRNA sequences were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) according to the Arf6 gene sequence [GenBank (23); accession no. NM 001663.3]. The sequences for Arf6 were as follows: siArf6-1, 5'-GUGGCAAAUAAUGAG UAAUTT-3'; siArf6-2, 5'-GCGACCACUAUGAUAAUAU TT-3'; and siArf6-3, 5'-GACGCCAUAAUCCUCAUCUTT-3'. The control siRNA (siCtr) sequence was 5'-UUCUCCGAAC GUGUCACGUTT-3'. SGC-7901 cells were seeded onto 6-well plates at a density of 10^5 cells/well and were incubated for 24 h. Cells were then transfected with control siRNA or Arf6 siRNA with Lipofectamine 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The total siRNA concentration in each well was 100 pmol and untransfected controls were used to demonstrate that there was no significant difference between untransfected controls and cells transfected with control siRNA. Arf6 silencing was confirmed by assessing them mRNA and protein expression levels in the SGC-7901 cells 48 h after transfection, as follows.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. cDNA was synthesized using an RNA to cDNA EcoDry™ Premix (Oligo dT) kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. The following gene-specific primers were used in the present study: Arf6 forward, 5'-CAA GGTCTCATCTTCGTAGTG-3' and reverse, 5'-CATGTG AGCCCCTCATAGAG-3'; GAPDH forward, 5'-TGAACG GGAAGCTCACTGG-3' and reverse, 5'-TCCACCACCCTG TTGCTGTA-3'. PCR analysis was performed using Takara Ex Taq® DNA polymerase (Takara Biotechnology Co., Ltd.) under the following thermocycling conditions: 95°C for 3 min, followed by 28 cycles of 95°C for 30 sec, 57°C (Arf6) or 55°C (GAPDH) for 30 sec, and 72°C for 1 min, with a final extension at 72°C for 6 min. The PCR products were electrophoresed on 1% agarose gels and the bands were visualized by UV fluorescence following staining with ethidium bromide (2.5 g/ml) for 15 min. Data were analyzed by densitometry using Tanon Gel Image System software, version 4.0 (Tanon Science and Technology Co., Ltd., Shanghai, China).

Western blot analysis. Cells were lysed for 5 min on ice in radioimmunoprecipitation assay buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.) containing 1% phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology, Haimen, China). Lysate was then sonicated and centrifuged at 12,000 x g for 10 min at 4°C. The protein concentration was quantified using a bicinchoninic acid assay (Beyotime Institute of Biotechnology). Proteins from each sample (20 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were then blocked with 5% skim milk at room temperature for 1 h and incubated overnight with mouse anti-Arf6 (1:500 dilution, cat. no. sc-7971; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-ERK1/2 (1:1,000 dilution, cat. no. 4695; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-phosphorylated ERK1/2 (pERK1/2) (1:1,000 dilution, cat. no. 4377; Cell Signaling Technology, Inc.), and mouse anti-GAPDH (1:2,000 dilution, cat. no. sc-47724; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Following washing with TBST 3 times, the membranes were incubated with goat anti-rabbit IgG (1:3,000 dilution, cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) or goat anti-mouse IgG (1:3,000 dilution, cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) horseradish peroxidase-conjugated secondary antibodies for 1 h at 37°C, and visualized using enhanced chemiluminescence detection reagents (Thermo Fisher Scientific, Inc.) and were exposed to chemiluminescent film (Thermo Fisher Scientific, Inc.). Data were analyzed using ImageJ software (version 1.6; National Institutes of Health, Bethesda, MD, USA) and were normalized to GAPDH expression.

Cell proliferation assay. Cell proliferation was measured using Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Briefly, control and transfected cells were seeded at a density of $3x10^3$ cells/well in 96-well plates and treated with 0, 1, 10, 25, 50, 100 μg/ml 5-FU for 48 h at 37°C or 10 μM ERK1/2 inhibitor U0126 (both from Sigma-Aldrich; Merck KGaA) for 12 h prior to being treated with 20 μg/ml 5-FU for 48 h at 37°C. CCK-8 (10 μl) was added to each well and incubated for an additional 4 h at 37°C. Optical density (OD) was measured using a microplate reader (Omega Bio-Tek, Inc., Norcross, GA, USA) at 450 nm. Each time-point was repeated in three wells, and the experiment was independently performed three times.

Colony formation assay. Control and transfected cells were seeded at a density of $5x10^2$ cells/well in 6-well plates, and cultured in DMEM in an environment with 5% CO_2 at 37°C for 14 days to allow colonies to form. The plates were stained with 0.5% (w/v) crystal violet in 70% ethanol for 20 min at room temperature, and colonies were counted under a light microscope (TS100; Nikon Corporation, Tokyo, Japan). The experiment was independently performed three times.

In vitro migration and invasion assays. For the Transwell migration assay, untransfected and transfected SGC-7901 cells in the exponential growth phase were trypsinized with 1X trypsin, washed twice with phosphate-buffered saline, and suspended in DMEM without FBS. Cells ($2x10^4$ cells/well) were seeded into polycarbonate membrane inserts (8-μm pore size) in 24-well Transwell cell culture dishes. Cells were permitted to attach to the membrane for 30 min. The lower chamber was filled with 600 μl DMEM with 10% FBS. Cells were permitted to migrate for 24 h at 37°C. Following incubation, stationary cells were removed from the upper surface of the membranes. The cells that had migrated to the lower surface were fixed with 4% paraformaldehyde at room temperature for 15 min and were stained with 0.1% crystal violet for 15 min at the same temperature. The cells that had migrated
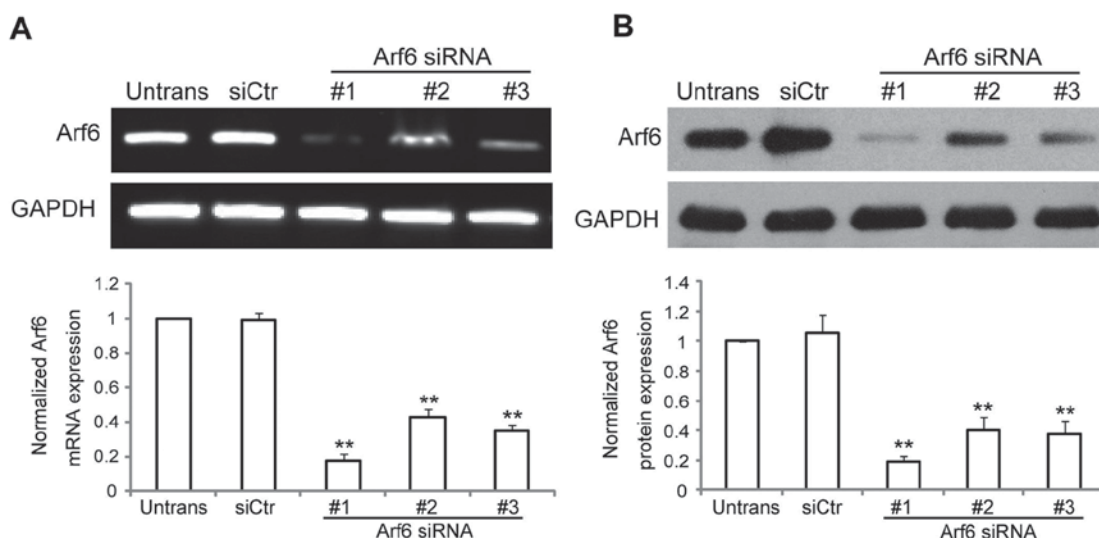
Figure 1. Inhibition of Arf6 expression in SGC-7901 cells by siRNA. (A) Cells were transfected with three siRNA oligonucleotides, and the expression of Arf6 mRNA was analyzed using reverse transcription polymerase chain reaction 48 h following transfection. (B) The expression of Arf6 protein in SGC-7901 cells was analyzed following transfection with the siRNA oligonucleotides by western blot analysis. GAPDH served as a loading control. ** $P<0.01$ vs. siCtr. Arf6, ADP-ribosylation factor 6; siRNA, small interfering RNA; siCtr, control siRNA; Untrans, untransfected.

through the membrane were manually counted at x200 magnification from 5 fields/filter using a light microscope (TS100; Nikon Corporation). The invasion assay was performed using Matrigel-coated chambers from the BioCoat Matrigel Invasion Chamber kit (BD Biosciences, Franklin Lakes, NJ, USA) using the same method as aforementioned for the migration assay.

Statistical analysis. Data were statistically analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Data were presented as the mean ± SD of three independent experiments. Differences between experimental groups were analyzed using one way analysis of variance (ANOVA). The Student-Newman-Keuls test was used as a post hoc test following ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Knockdown of Arf6 in SGC-7901 cells by siRNA. To investigate the function of Arf6 in GC cells, our group identified and validated three independent and non-overlapping siRNA sequences to deplete endogenous Arf6 expression in SGC-7901 cells. RT-PCR and western blot analysis were used to evaluate the ability of different Arf6 siRNAs to silence Arf6 expression *in vitro.* As presented in Fig. 1A and B, Arf6 expression was significantly reduced in cells transfected with specific siRNAs against Arf6. Arf6 mRNA expression was reduced by ~82% with siArf6-1, ~57% with siArf6-2 and ~65% with siArf6-3, while protein expression was reduced by ~81% with siArf6-1, ~60% with siArf6-2 and ~62% with siArf6-3, compared with cells transfected with siCtr. Thus, siArf6-1 was selected as the most efficient and specific sequence to silence the expression of Arf6 in the SGC-7901 cells, and was used in all subsequent experiments.

Knockdown of Arf6 inhibits the proliferation of SGC-7901 cells in vitro. The effect of Arf6 knockdown on cell proliferation

and tumorigenesis were assessed using CCK-8 and colony formation assays, respectively. As presented in Fig. 2A, Arf6 knockdown resulted in a significant decrease in the proliferation of SGC-7901 cells at 48, 72, and 96 h. In addition, cells transfected with siArf6 formed fewer and smaller colonies as compared with cells transfected with siCtr (Fig. 2B). Taken together, these data indicate that knockdown of Arf6 resulted in a significant inhibitory effect on cell proliferation and colony formation in SGC-7901 cells *in vitro.*

Knockdown of Arf6 inhibits the migration and invasion of SGC-7901 cells in vitro. The *in vitro* migration and invasion assays were designed to investigate the function of Arf6 in SGC-7901 cell migratory and invasive processes. For the migration assay, untransfected and transfected cells were seeded on Transwell chambers with uncoated filters. In total, 100% of the untransfected SGC-7901 cells were able to migrate to the filters in 24 h, while the migratory percentage of siCtr-transfected cells was 98% and that of siArf6-transfected cells was 38% (Fig. 3A). For the invasion assay, untransfected and transfected cells were seeded on Transwell chambers with Matrigel-coated filters. After 24 h of incubation, the invasion of siArf6 cells was significantly reduced (Fig. 3B). Taken together, these results indicated that silencing Arf6 reduces SGC-7901 cell migration and invasion *in vitro.*

Knockdown of Arf6 decreases activation of the ERK1/2 pathway. According to the results of previous studies, Arf6 regulates the activation of ERK1/2 (24-26). Furthermore, activation of ERK1/2 has been demonstrated to increase cell proliferation, migration and invasion in GC (27-29). Thus, the effect of Arf6 knockdown on the ERK1/2 pathway was investigated in SGC-7901 cells. As presented in Fig. 4A, p-ERK1/2 levels were significantly reduced in the Arf6-knocdown SGC-7901 cells, while total ERK1/2 expression was comparable to that observed in the control cells. This association between Arf6 and p-ERK1/2 expression suggested that Arf6
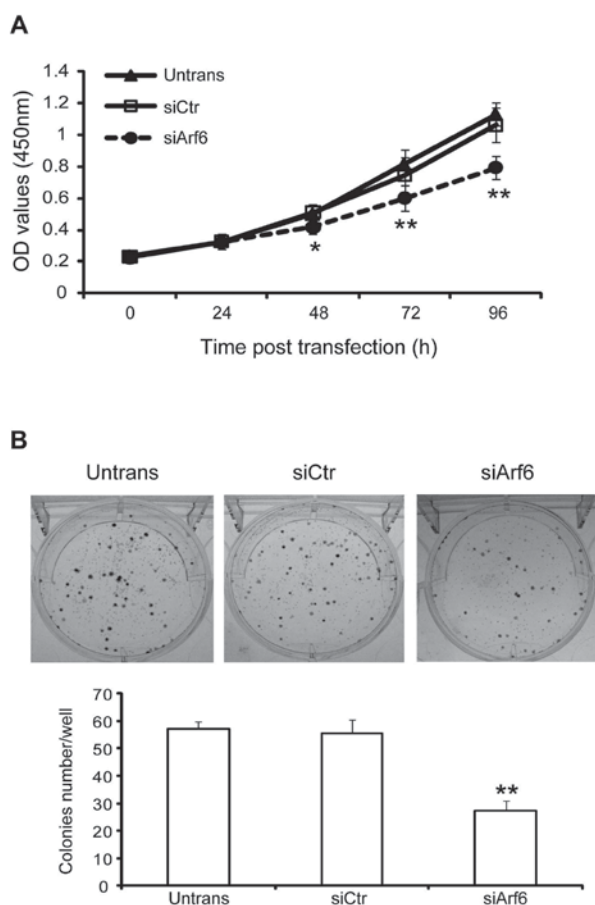
Figure 2. **Knockdown of Arf6 inhibits cell proliferation and colony forma**-tion of SGC-7901 cells *in vitro*. (A) Cell proliferation was analyzed using a Cell Counting Kit-8 assay. (B) Untransfected and transfected SGC-7901 cells were cultured for 14 days to allow colonies to form. Positive colonies were counted under a microscope. The accompanying graph summarizes the colony-forming capacities of the cells. *P<0.05 and **P<0.01 vs. siCtr. Arf6, ADP-ribosylation factor 6; siCtr, control siRNA; Untrans, untransfected; siArf6, siRNA targeting Arf6.

Figure 3. Knockdown of Arf6 inhibits the migration and invasion of SGC-7901 cells *in vitro*. (A) Results of the migration assay (magnifica-tion, x200). (B) Results of the invasion assay (magnification, x200). **P<0.01 vs. siCtr. Arf6, ADP-ribosylation factor 6; siCtr, control siRNA; Untrans, untransfected; siArf6, siRNA targeting Arf6.

may be involved in the regulation of the ERK1/2 signaling pathway.

Knockdown of Arf6 enhances sensitivity of SGC-7901 cells to 5-FU through modulating the ERK1/2 signaling pathway. Previous studies have reported that Arf6 is involved in drug resistance in a variety of types of cancer cell (17-19). The effect of Arf6 knockdown on the sensitivity of SGC-7901 cells to 5-FU was further investigated. To determine the sensitivity of cells to 5-FU, siArf6 or siCtr-transfected SGC-7901 cells were exposed to different concentrations of 5-FU, ranging from 0 to 100 μg/ml, for 48 h. Cell viability was examined using a CCK-8 assay. The cell survival rate appeared to show a dose-dependent decrease in response to 5-FU treatment, and knockdown of Arf6 resulted in increased sensitivity to 5-FU treatment in SGC-7901 cells (Fig. 4B). Next, the present study aimed to determine whether the Arf6 knockdown-enhanced 5-FU sensitivity of SGC-7901 cells was due to inactivation of the ERK1/2 signaling pathway. As presented in Fig. 4C, 5-FU suppressed the phosphorylation of ERK1/2 in SGC-7901 cells. However, knockdown of Arf6 resulted in a smaller reduction of p-ERK1/2 expression, while total ERK1/2 expression was
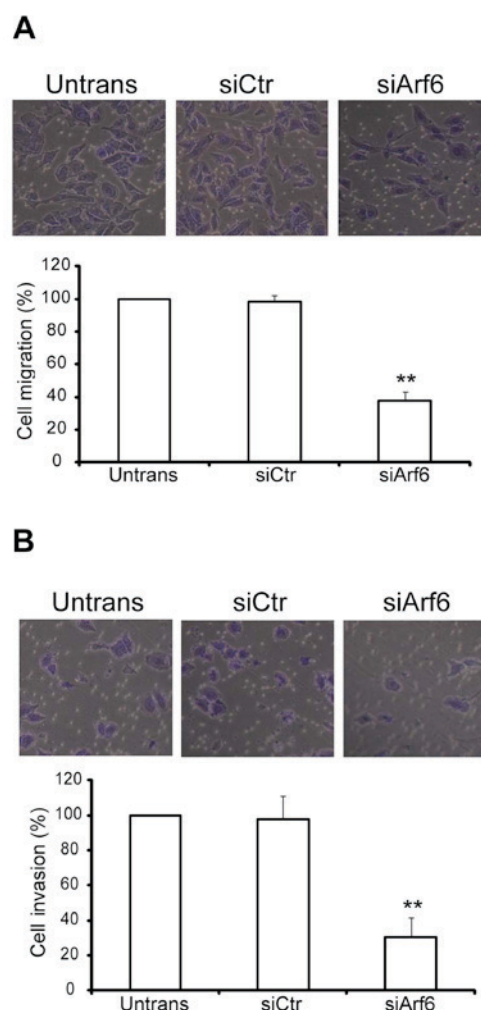
unaffected. The CCK-8 results revealed that U0126 (a specific MEK inhibitor) effectively increased siArf6-mediated 5-FU sensitivity of SGC-7901 cells (Fig. 4D). Collectively, these results indicated that knockdown of Arf6 enhanced the chemosensitivity of SGC-7901 cells to 5-FU by suppressing ERK1/2 activity.

Discussion

Arf6 is a member of the Arf family which exhibits pleio-tropic biological functions (7,30-33). Arf6 has been reported to be upregulated in multiple types of tumor, and has been demonstrated to be involved in a number of biological processes, including cancer cell growth, EMT, cell adhesion, migration, invasion, angiogenesis, malignant transformation and resistance to chemotherapy (18,24,34-36). However, the biological functions of Arf6 in GC remain to be fully elucidated.

To investigate the potential associations between Arf6 expression and the biological features of GC cells, Arf6 expression was knocked down in GC cells using three siRNA
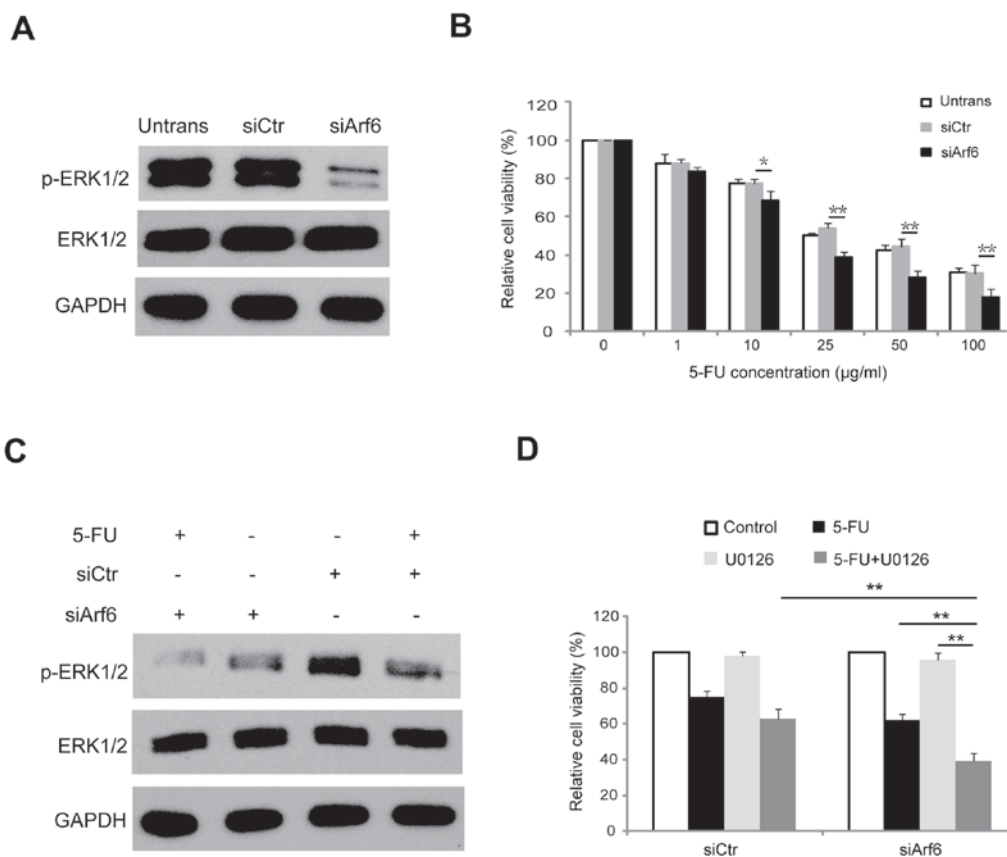
Figure 4. Knockdown of Arf6 increases the sensitivity of SGC-7901 cells to 5-FU through modulation of ERK1/2 signaling. (A) SGC-7901 cells were transfected with siCtr or siArf6, and 48 h following transfection proteins were extracted and subjected to western blot analysis. (B) Dose-dependent CCK-8 assay was employed to investigate the effect of Arf6-knockdown on the viability of SGC-7901 cells. (C) Western blot analysis of ERK1/2 and p-ERK1/2 in SGC-7901 cells transfected with siCtr or siArf6 and treated with 20 μg/ml 5-FU for 48 h. (D) SGC-7901 cells transfected with siCtr or siArf6 were treated with 10 μM U0126 for 12 h prior to being treated with 5-FU, and cell viability was determined using a CCK-8 assay. *P<0.05 and **P<0.01 vs. siCtr. Arf6, ADP-ribosylation factor 6; 5-FU, 5-fluorouracil; siCtr, control siRNA; siArf6, siRNA targeting Arf6; CCK-8, Cell Counting Kit-8; ERK1/2, extracellular signal-regulated kinase 1/2; p, phosphorylated; Untrans, untransfected.

sequences, and siRNA targeting of Arf6 in SGC-7901 cells *in vitro* resulted in efficient, specific inhibition of endogenous Arf6 mRNA and protein. Further experiments demonstrated that knockdown of Arf6 in SGC-7901 cells significantly inhibited the migration and invasion of SGC-7901 cells *in vitro*. These results indicated that Arf6 expression is associated with pro-metastatic events in SGC-7901 cells. These data are consistent with previous results in other tumor cell lines, including breast cancer cells (37) and lung cancer cells (13). Furthermore, Arf6 has also been implicated in the modulation of cancer cell growth and the tumorigenic phenotype of cancer cells in pancreatic and lung cancer (10,35). The present study also demonstrated that Arf6-knockdown SGC-7901 cells had reduced proliferation and a reduced ability to form colonies. Taken together, these results suggest that Arf6 expression is associated with migration, invasion, proliferation and tumorigenicity in SGC-7901 cells.

Previous studies have demonstrated the presence of an association between Arf6 and ERK1/2 signaling in several cancer cell lines, and this association has been implicated in cancer progression (20,24,25). Furthermore, ERK1/2 signaling has been demonstrated to mediate cell proliferation, migration and invasion in various types of tumor cell, including GC cells (27-29). In the present study, the effect of Arf6 knockdown on ERK1/2 activation was investigated in SGC-7901

cells. Phosphorylation of ERK1/2 was markedly reduced in Arf6 siRNA-transfected cells compared with the control cells, indicating that the migration, invasion, proliferation and tumorigenicity of SGC-7901 cells are regulated via the ERK1/2 pathway. However, the precise mechanisms by which Arf6 knockdown inhibits tumor growth, migration and invasion require further study.

Previous studies have demonstrated that Arf6 confers resistance to multiple chemotherapy agents, including gemcitabine, fluorouracil and temsirolimus (17-19). However, whether Arf6 is involved in chemoresistance in GC cells specifically remains unclear. In the present study, knockdown of Arf6 was revealed to sensitize SGC-7901cells to 5-FU *in vitro*, suggesting that Arf6 induces 5-FU resistance in GC cells. Inhibition of the ERK1/2 pathway has been reported to increase 5-FU efficacy in multiple cancer cell lines, including GC cell lines. Furthermore, the results of the present study demonstrated that Arf6 knockdown significantly decreased ERK1/2 signaling pathway activity. Thus, whether Arf6 regulates chemosensitivity to 5-FU by modulating ERK1/2 in SGC-7901 cells was investigated. The results revealed that the specific ERK1/2 inhibitor U0126 effectively increased Arf6 siRNA-mediated 5-FU sensitivity. These results indicated that Arf6 may regulate chemosensitivity to 5-FU through the ERK1/2 signaling pathway in SGC-7901 cells.

In conclusion, the results of the present study demonstrated that knockdown of Arf6 inhibits SGC-7901 cell proliferation, migration and invasion, and increases the sensitivity of SGC-7901 cells to 5-FU, with the increasing drug sensitivity potentially associated with the inhibition of ERK1/2 signals. Understanding the mechanisms underlying these effects may provide novel strategies for GC treatment. Combining Arf6 gene therapy with traditional chemotherapy may be an effective anti-GC strategy in the future.

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