Downregulation of CD24 inhibits invasive growth, facilitates apoptosis and enhances chemosensitivity in gastric cancer AGS cells

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Abstract. – BACKGROUND AND AIM: The human CD24 antigen is a small heavily glycosylated cell surface protein, which is expressed in a large variety of solid tumors, including gastric cancer. Enriched on the surface of many tumor cells, CD24 promotes tumor growth, invasion and metastasis and confers resistance to some chemotherapeutic drugs. In this study, we investigated the possible effect of CD24 suppression on proliferation, apoptosis, migration, invasion and chemosensitivity of gastric cancer (GC) cells.

MATERIALS AND METHODS: We down-regulated CD24 expression by RNA interference and investigated the effects on proliferation, apoptosis, chemosensitivity to doxorubicin, malignant and metastatic potential of a human gastric cancer cell line, AGS, CD24-suppressed clones, AGS-CD24-siRNA-C2, AGS-CD24-siRNA-C4 and AGS-CD24-siRNA-C5 in vitro. We evaluated the effects of CD24 suppression *in vivo* on xenograft tumor growth and metastatic properties following tail iv AGS-CD24-siRNA-C2, AGS-CD24-siRNA-C4 and AGS-CD24-siRNA-C5 clones. We also investigated the effect of CD24-siRNA followed by doxorubicin administration treatment on the xenograft tumor growth.

RESULTS: CD24 suppressed showed significantly decreased proliferation, invasion and increased apoptosis as well as increased chemosensitivity to doxorubicin *in vitro* and *in vivo*.

CONCLUSIONS: CD24 involves in proliferation, invasion and chemosensitivity of human gastric cancer cell line AGS, and that down-regulation of CD24 protein expression decreases the metastatic potential and increases chemosensitivity of gastric cancer (GC) cells. Thus, CD24 may be a promising therapeutic target for gastric cancer.

Key words:

Gastric cancer, CD24, Invasion, Apoptosis, Chemosensitivity.

INTRODUCTION

Despite decreasing incidence, gastric cancer is still the second most common cause of cancer-related death in the world[1]. Even if radical resection is the mainstay of curative treatment of gastric cancer, disease recurrence is very common. Outcome of unresectable or metastatic gastric cancer is still extremely poor, although chemotherapy demonstrated to confer a benefit in terms of survival and quality of life[2]. Therefore, a better understanding of the molecular mechanisms underlying gastric cancer formation and progression should be helpful in developing more effective treatments for this disease.

Historically, CD24 was developed as a cluster of differentiation (CD) marker of hematopoietic lineages that has been found to be overexpressed in many common malignancies and has been associated with the metastatic phenotype[3-7]. Reported functions showed knockdown of CD24 expression by CD24-shRNA significantly inhibited cell viability and induced apoptosis of SKOV3 cells *in vitro*. Administration with CD24-shRNA *in vivo* suppressed tumor volume and increased apoptosis induction[8]. Sagiv et al[9] has recently found targeting CD24 by monoclonal antibodies or small interfering RNA could efficiently inhibited growth and invasion of colorectal and pancreatic cancer. In A549 lung cancer model, the addition of SWA11 mAb (monoclonal antibody) to inhibit CD24 strongly potentiated gemcitabine's anti-cancer efficacy[10]. It demonstrates that targeting of CD24 could be beneficial for the anti-cancer treatment combined with standard chemotherapy regimes.

In gastric cancer, it has observed a relationship between high CD24 expression and lymph node metastasis, venous invasion and lymphatic invasion. CD24 expression tended to be higher in cell lines derived from differentiated gastric carcinoma, in-

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cluding those derived from lymph node metastasis[11]. Duckworth et al[12] has reported CD24 is expressed in gastric parietal cells and could regulates apoptosis. We, therefore, suggested that CD24 could provide a new molecular target for therapeutic strategies.

RNA interference (RNAi) has been recently developed as a powerful tool to suppress expression of specific gene products[8,9]. We used RNAi methods to suppress CD24 expression in AGS cells and show that small interfering RNA (siRNA) suppression of CD24 reduced the proliferative and metastatic capacity of the human gastric cancer cell line AG.S. Furthermore, small interfering RNA (siRNA) suppression of CD24 markedly enhances the sensitivity of cancer cells to doxorubicin treatment *in vitro* and *vivo*.

Materials and Methods

Cell culture

Gastric cancer (GC) cell line AGS was obtained from American Type Culture Collection (ATCC, Shanghai. China). It was cultured in Roswell Park Memorial Institute (RPMI) 1640 (Life Technologies, Inc., Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mg/ml sodium bicarbonate, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.) at 37°C in 5% CO_2.

Reagents

CD24 siRNA (h) (sc-29978) was purchased from Santa Cruz, Shanghai, China. Primary anti-CD24 was from Santa Cruz (1:200) (Santa Cruz, CA, USA).

Transfection and selection of clones

AGS cells were transfected with CD24 siRNA by the Lipofectin method. Briefly, cells were plated in six-well culture plates and grown to 60% confluence.Growth medium was removed, the cells were washed twice in serum-free Opti-MEM, and then incubated 5 hours in 1 mL serum-free Opti-MEM with 10 uL Lipofectin reagent and 2 μg CD24 siRNA with a target or control siRNA as control.At 24 hours after transfection, the medium was replaced with normal growth medium, and after 48 hours transient transfection, each well was passaged into a 10 cm plate with growth medium containing G418 at 600 μg/mL. After 7 to 10 days, the cells were passaged and plated at 30 to 50 per plate.Single colonies were selected and the cloning procedure was repeated.We established six clones of CD24-suppressed cells. The clonal cell lines were maintained in complete medium with G418 at 200 μg/mL.

Western blotting

Following siRNA transfection, AGS cells were harvested by trypsinization (trypsin 0.25% w v^{-1}, 1mM ethylenediaminetetraacetic acid). The cells were lysed in a lysis buffer containing 150mM NaCl, 1% Triton X-100 and 25 mM Tris (pH 7.5). Debris was sedimented by centrifugation for 5 min at 12,000 g, and the supernatants were solubilized for 5 min at 1001°C in Laemmli's sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 100mM dithiothreitol. Protein concentrations of the lysates were determined with a protein quantitation kit (Bio-Rad Laboratories, Hercules, CA, USA), and 50 μg of each sample was separated on a 10% SDS-PAGE gel. Separated polypeptides were then electrophoretically transferred to 0.2-mm nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked for 1 h in a Tris-buffered saline-Tween (25 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween-20) containing 5% (w v^{-1}) nonfat dried milk. The blots were then probed overnight with Anti-CD24 (1:200) and developed using species-specific secondary antibody. Immunoreactive material was detected by the enhanced chemiluminescence technique (Amersham, Little Chalfont, Buckinghamshire, UK).

Cell proliferation assay

Cell proliferation was evaluated by direct evaluation of change in cell number over time.For cell counting assays,triplicate aliquots of 1×10^4 of each clone and a control cell line were seeded on 48-well plates. At 24 hours intervals, cells were harvested by trypsinization and counted on a cell counter.

Apoptosis assay

AGS cells were transfected with CD24 siRNA or control siRNA for 72 hours. Flow cytometric analysis was performed to detect apoptosis according to the manufacturer's protocol.

Invasion assay

The invasive potential of each clone and a control cell line was studied *in vitro* by determining the number of cells that invaded through the Matrigel-coated Transwell polycarbonate membrane inserts according to the manufacturer's protocol. In brief, Transwell inserts with a pore size of 12 μg were

coated with 0.78 mg/mL Matrigel in serum-free medium. Each clone and a control cell line were recovered by trypsinization, washed, and resuspended in serum-free medium and 0.5 mL of cell suspension (0.5×10^6 cells) was added to duplicate wells. After 24 h, the number of cells that passed through the filter was stained using Hema-3 stain kit (Fisher Scientific, Pittsburgh, PA, USA) and counted in 10 random fields under a microscope.

Drug Treatment in vitro

For flow cytometric analysis, 1×10^4 of each clone and a control AGS cell line were treated with the drug vehicle (< 1% dimethyl sulfoxide, DMSO) or 5, 10, 25 uM doxorubicin for 72 h. The concentration and duration of doxorubicin treatment were chosen based on preliminary studies examining its effects on induction of apoptosis. Cell proliferation and apoptosis was deteced as the method above.

In vivo therapeutic experiments

All animal procedures and care were approved by The Affiliated Hospital of Medical College, Qingdao University. Each clone and a control cell line ($1 \times 10^6/0.1$ mL /mouse) were injected s.c. into the flank of 6-week-old female severe combined immunodeficient (SCID) mice (n = 20/experiment) and growth was measured twice weekly; after establishment of palpable lesions, mice (~5 mm in average size; 7 days after AGS cell injection and 15 days after CD24 siRNA clone injection) were assigned to two treatment groups (10/group): (a) control (vehicles only), (b) doxorubicin (1.6 mg/kg biweekly i.p.; low-dose schedul results in minimal side effect). Mice were followed for tumor size and were sacrificed after doxorubicin treat for 21 days. Tumor size was measured with calipers in two dimensions, and size was calculated using the following formula: $a \times b^2/2$, where "a" is the tumor length and "b" is the diameter.

An experimental lung metastasis AGS model was used to evaluate metastases. CD24 siRNA clone or AGS ($1 \times 10^6/0.1$ mL/mouse) were injected into the tail vein of female SCID mice. Metastases development were evaluated 14 days after injection. During necropsy in lungs were excised, visible metastasis was counted, and the lungs were fixed in formalin and paraffin embedded for IHC studies.

Mouse xenograft tumor immuno-histochemistry and TUNEL staining

Immunohistochemistry was performed on tumors. 1:200 CD24 primary antibody dilutions were used.Staining distribution and intensity was evaluated and scored by at least two independent reviewers blinded to the therapeutic group to which each slide belonged.

TUNEL staining was performed according to the manufacturer's protocol. The slides were stained with 3,3′-diaminobenzidine reagent and counterstained with hematoxylin. For TUNEL scoring, the average number of positive nuclei was calculated in five high power microscopic fields (×400) selected from a central region in viable tumor areas avoiding areas containing necrosis.

Statistical analysis

Results are expressed as the mean ± SD of at least 3 independent experiments. Statistical analysis was carried out by one or two-way ANOVA or by Student's t test (where appropriate). Significance was set at $p \leq 0.05$. All statistical analyses were carried out using the SPSS 11.0 software (SPSS Inc., Chicago, IL, USA).

Results

Suppression of CD24 expression by siRNA

We established six independent cloned cell lines of AGS cells in which CD24 was suppressed, which were named Clones AGS-CD24-siRNA-C1, AGS-CD24-siRNA-C2, AGS- CD24-siRNA-C3, AGS-CD24-siRNA-C4, AGS-CD24-siRNA-C5 and AGS-CD24-siRNA -C6.Western blot analysis was done to verify suppression of CD24 in AGS by siRNA. Expression of CD24 was significantly decreased in all CD24-suppressed cell clones (Figure 1). Clones AGS-CD24-siRNA-C1 and AGS-CD24-siRNA-C3 expressed low levels of intracellular CD24.
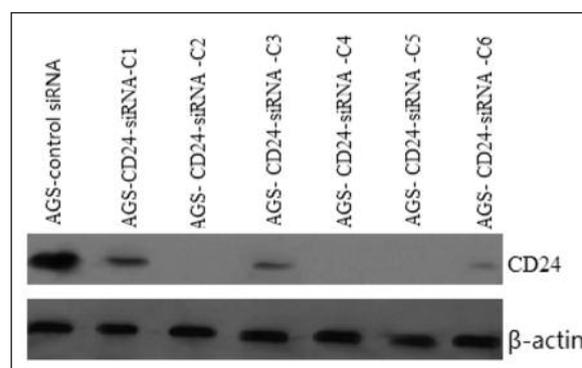

Figure 1. Western blot assay. CD24 expression in suppressed cells (AGS clones C1, C2, C3, C4, C5, and C6) and control cells (AGS-control siRNA) were verified by Western blotting assay.
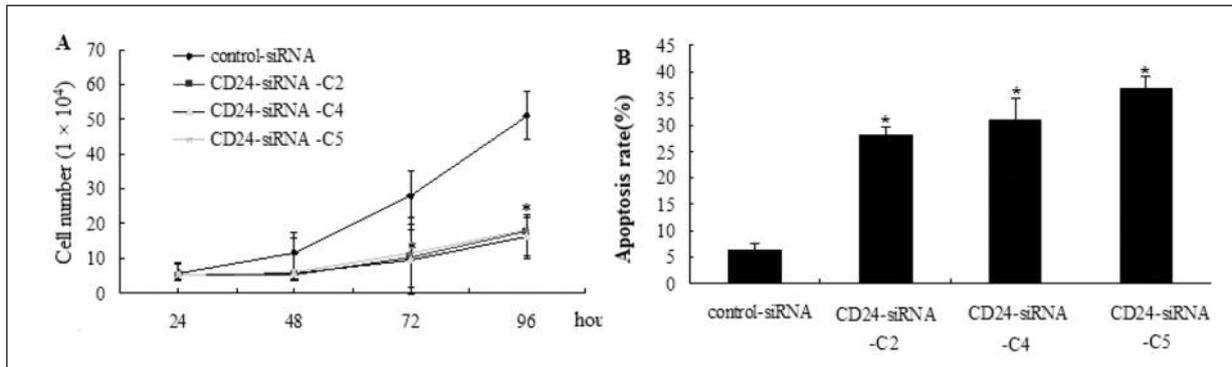
Figure 2. A, Cellular proliferation assays. Triplicates containing 1×10^4 of AGS -CD24-siRNA-C2, C4, C5, and siRNA control cells were evaluated for proliferation at 24-hour intervals by direct counting. Points, mean; bars, SE. **B,** Cellular apoptosis assays. Triplicates containing 1×10^4 of AGS-CD24-siRNA-C2, C4, C5, and siRNA control cells were evaluated for apoptosis at 72-hours by flow cytometric analysis. Points, mean; bars, SE. (vs control $^*p < 0.05$).

Clones AGS-CD24-siRNA-C6 expressed a very small quantity of intracellular CD24; and clones AGS-CD24-siRNA-C2, AGS-CD24-siRNA-C4 and AGS-CD24-siRNA-C5 did not express detectable CD24. We selected C2,C4 and C5 for further investigation.

Knockdown of CD24 expression inhibits cell proliferation in vitro

CD24-siRNA-C2, C4 and C5 clones showed proliferation rates *in vitro* that were significantly less (<50%) than control cells (AGS-control siRNA) (Figure 2A). Analysis indicated a significant difference between CD24-siRNA-C2, -C4 and -C5 compared with AGS-control siRNA ($p < 0.05$).

Knockdown of CD24 expression promotes cell apoptosis in vitro

CD24 siRNA or control siRNA were transiently transfected into AGS cells for 72 hours.

Western blot analysis was done to verify suppression of CD24 in AGS by siRNA. Expression of CD24 was significantly decreased in the AGS-CD24-siRNA cells (data not shown). Flow cytometric analysis was performed to detect apoptosis after siRNA transfection. As shown in Figure 2B, the apoptosis rate was significantly increased after CD24-siRNA transfection.

Knockdown of CD24 expression inhibits cell invasion in vitro

In this study, we assessed the ability of CD24 suppression to inhibit invasion of AGS cells in vitro. AGS-CD24-siRNA-C2,C4,C5, and siRNA control cells were seeded in Matrigel-coated invasion chambers. After 24 h, cells that migrated through the Matrigel barrier were stained and counted. Invasion was significantly inhibited in the AGS-CD24-siRNA-C2, C4, C5 cells (Figure 3).
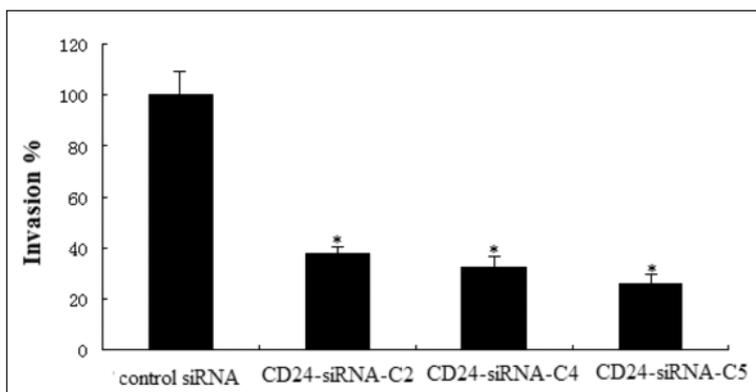

Figure 3. siRNA for CD24 inhibits invasion of AGS cells. siRNA for CD24 inhibits invasion of human AGC cells in Matrigel-coated invasion chambers. AGS-control siRNA, AGS-CD24-siRNA-C2, C4 and C5 cells were seeded in the upper chamber in medium supplemented with 5% FCS. After 24 h, cells migrated in the lower chamber were stained and counted. In the lower chamber, medium supplemented with 10% FCS was used as chemoattractant. Invasion of the AGS-control siRNA cells was set to 100. Results are reported as percent migration ± SD compared with AGS-control siRNA cells. Experiments were carried out twice in triplicate (vs control,$^*p < 0.05$).
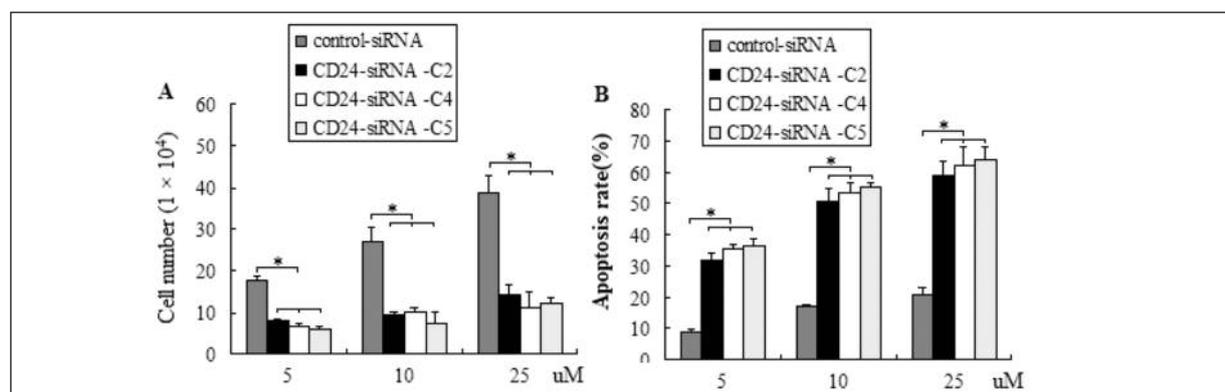
Figure 4. Down-regulation of CD24 enhances doxorubicin-mediated cell death. CD24-siRNA-C2, -C4, -C5 and siRNA control clones were cultured for 72 h in the presence of 5, 10, 25 μM doxorubicin. Then the cells were evaluated for proliferation by direct counting (**A**) and for apoptosis at by flow cytometric analysis (**B**). Points, mean; bars, SE (vs control *$p < 0.05$).

Down-regulation of CD24 enhances doxorubicin-induced cell death

To investigate the role of down-regulation of CD24 on the doxorubicin mediated cellular response, we attempted to down-regulate its expression by employing siRNAs targeting the CD24.AGS-CD24-siRNA-C2, C4, C5, and siRNA control cells were treated with 5, 10, 25 μM doxorubicin for 72 h. Doxorubicin treatment did not affect the CD24 levels measured in the control experiment (data not shown). Doxorubicin treatment in CD24-siRNA-C2, C4 and C5 clones showed proliferation rates that were significantly less (< 20%) than control cells (AGS-control siRNA + doxorubicin) ($p < 0.05$) (Figure 4A). Flow cytometric analysis shown in Figure 3B, the apoptosis rate was significantly increased after CD24-siRNA transfection followed by doxorubicin treatment than the controls ($p < 0.05$).

Effect of suppression of CD24 on tumor growth in vivo

We evaluated tumor growth *in vivo* by s.c. implantation of 10^6 tumor cells into nude mice. CD24-siRNA-C2, -C4 and -C5 tumors showed significantly slower growth rates than control cells (control siRNA) at all time points (Figure 5A). There were no significant differences between CD24-siRNA-C2, -C4 and -C5 tumors. Suppression of CD24 expression was verified by western blot on samples of each tumor (data not shown). We analyzed whether CD24 levels affect tumor growth in nude mice by affecting apoptosis. TUNEL assay shown that the TUNEL positive cells was significantly increased in CD24-siRNA-C2, -C4 and -C5 tumors compared with their respective controls (Figure 5 B).These results are consistent with the *in vitro* results indicating that CD24 affect apoptosis of gastric cancer cells.

Next, we evaluated the effect of CD24 on the doxorubicin mediated response *in vivo*. As shown in Figure 5 A, the size of tumors were significantly different between the CD24-siRNA-C2, -C4, -C5 and control siRNA groups ($p < 0.05$). Combinatorial therapy of CD24 down-regulation and doxorubicin efficiently suppressed tumor growth in the mouse. TUNEL assay shown that the TUNEL positive cells was significantly increased in combinatorial therapy groups compared with doxorubicin groups alone (Figure 5 B).

Effect of CD24 silencing in an in vivo pseudometastatic model

The next step for the characterization of CD24 function in gastric cancer was to test the effect of CD24 knockdown in animal models. Severe combined immunodeficient mice were injected in the tail vein with 3×10^6 control siRNA, CD24-siRNA -C2,C4 and C5 (10 animals). Animals were sacrificed at day 14 and autopsy was carried out to remove lungs and detect macrometastases. Autopsy showed a reduction of tumor cell burden in lungs in the CD24-siRNA-C2, C4 and C5 tumors. The major site for metastases was a pararenal area involved in 100% of the animals in control siRNA groups. Involvement of this pararenal area was reduced in CD24-siRNA-C2, C4 and C5 groups. We analyzed the percentage of metastatic invasion in the lung of each animal. From each group, histologic specimens were prepared and the largest sagittal section of lung was evaluated using an image analysis software to measure the extent of tumor involvement. We plotted the percentage of the in-
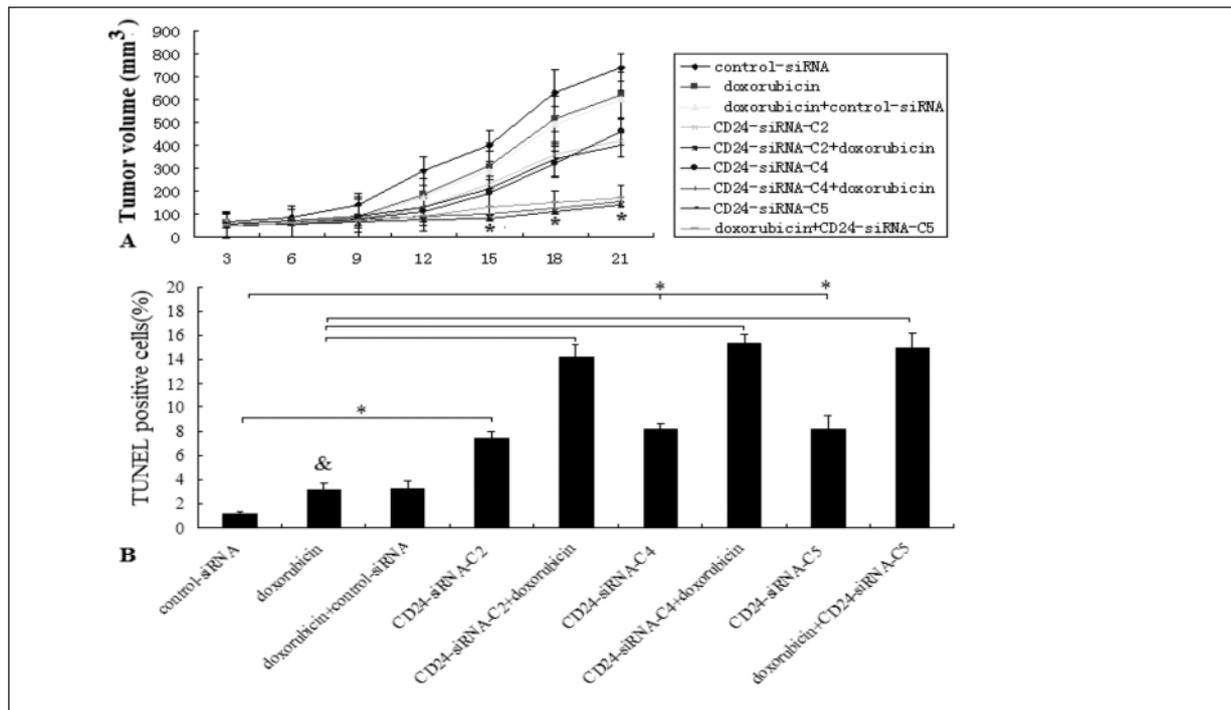
Figure 5. A, tumor growth curves. Mice received 1×10^6 viable cells by s.c. injection. These curves show tumor growth until day 21. Points, mean; bars, SE. vs control, $^*p < 0.05$. **B,** Cell apoptosis was detected by TUNEL staining in the tissues at day 21., Points, mean; bars, SE. vs control, $^*p < 0.05$. $^\&p < 0.05$.

vaded areas to estimate the metastatic burden in each group of animals (Figure 6). These findings show that silencing of CD24 significantly reduces the metastatic burden in a pseudometastatic model of gastric cancer.

Metastatic lesions from lung were evaluated for CD24 expression by western blot. Metastatic lesions in lungs of CD24-siRNA-C2, C4 and C5 groups exhibited reduced CD24 expression than the CD24 expression in the control groups (data not shown).

Discussion

In this study, we established stable clones of the AGS gastric cancer cell line in which CD24 expression was completely suppressed by siRNA, and investigated the effects of eliminating CD24 on the invasive growth, apoptosis, chemosensitivity, malignant and metastatic potential of AGS cells. It has reported overexpression of CD24 in gastric cancer is associated with poor prognosis11. Thus, suppressing expression of CD24 in gastric tumor cells would be predicted to affect different aspects of the malignant and metastatic properties of cancer, including proliferation, invasion, metastasis, or differentiation status.

One notable effect of reducing or eliminating CD24 mRNA and protein in AGS cells was a decrease in the proliferation rate and a increase in the apoptosis rate *in vitro* and a reduction in the rate of tumor growth *in vivo*.

These results suggest that CD24 expression influences proliferation rates in AGS cells.

A second significant finding of this study is that downregulation of CD24 decreased the metastatic capacity of the AGS cell line *in vivo* and *vitro*. *In vitro* assays showed that downregulation of CD24 was indeed able to reduce significantly tumor invasiveness. *In vivo*, we observed suppression of metastasis to lung in CD24-suppressed cells. There are at least two explanations for the deceleration of metastasis or invasion by CD24 suppression: one possibility is that CD24 contributes to the cell surface properties of tumor cells and influences their ability to adhere, migrate, and/or survive in different organ environments; a second possibility is that the decreased tumor size over time that results from a decrease in the proliferation rate yields fewer tumor cells that are capable of seeding metastases.

With respect to tumor size, we found that CD24-suppressed tumor grew slowly, and progress to large volumes at the end-stage. TUNEL staining

Figure 6. Effect of CD24 inhibition in a pseudometastatic model of gastric cancer *in vivo*. Major sagittal section of lungs of each animal was evaluated for the extent of metastatic invasion using an image analysis program. Data are presented as percent of invaded area ±SE. Significant differences in metastatic burden compared with the control siRNA group are indicated ($p < 0.05$).
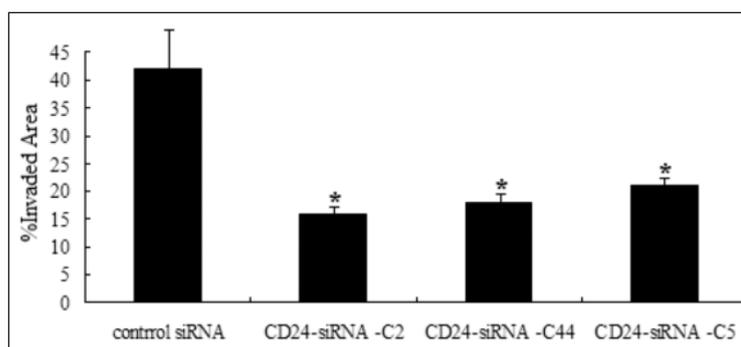
showed more apoptosis cells was found in the CD24-suppressed tumor, which suggests that decreased tumor growth rates are associated with increased apoptosis rate in CD24-suppressed tumor.

A third significant finding of this study is that downregulation of CD24 enhances the chemosensitivity in gastric cancer AGS cells *in vitro* and *in vivo*. We found that downregulation of CD24 enhance apoptosis induced by doxorubicin in innately drug-resistant human gastric cancer cells. Downregulation of CD24 have therapeutic potential when used in combination with doxorubicin in reversing drug resistance in gastric cancer cells.

Conclusions

Our data have shown for the first time the relevant role of downregulation of CD24 in apoptosis, invasion, metastasis and chemosensitivity of gastric cancer cells *in vitro* and *in vivo*. Its inhibition significantly decreases metastasis formation, inhibits growth and increases chemosensitivity to doxorubicin *in vitro* and *in vivo*. Furthermore, the present findings suggest that the combination of CD downregulation alone or with doxorubicin administration may represent a novel targeted therapy for metastatic gastric cancer.

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Conflict of Interest

None.

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