Therapeutic potential of human adipose stem cells in a cancer stem cell-like gastric cancer cell model

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Abstract. Cancer stem cells (CSCs) or circulating tumor cells play an important role in tumor initiation, invasion, metastasis and resistance to anticancer therapies. Therapies that target gastric tumor CSCs have potential clinical application for preventing malignant gastric tumor progression and metastasis. We isolated $CD44^+$ gastric cancer cells from the gastric cancer cell line AGS and Hs746T cells and maintained the cells in a novel stem cell culture. The cells were kept in an undifferentiated proliferative state and we characterized their cancer stem cell properties and chemotherapy-resistance behavior. The $CD44^+$ cancer cells were also co-cultured with human adipose stem cells (ADSCs) to determine the chemotherapy-promotion effects of the adipose cells on the $CD44^+$ cancer cells. The $CD44^+$ gastric cancer cell model is a non-adhesion, 3-dimensional, spheroid phenotype. The non-adherent $CD44^+$ cells have cancer stem cell properties and are highly chemo-resistant. However, these cells regained chemo-sensitivity when re-attached to an extracellular matrix-coated attachment surface. The human adipose stem cells significantly promoted the chemo-sensitivity of the non-adherent $CD44^+$ gastric cancer cells. Integrin $\alpha2/\beta2$ and the Wnt signaling pathways are involved in the mechanisms. We concluded that the *in vitro* non-adherent $CD44^+$ gastric cancer cell model resembles the circulating gastric tumor cells *in vivo*. Introduction of an appropriate attachment surface significantly promotes chemo-sensitivity of the non-adherent $CD44^+$ gastric cancer cells. The human adipose stem cells function as a 'living vehicle surface' for such a purpose *in vivo*.

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Abbreviations: 5-FU, 5-fluorouracil; ADSCs, human adipose stem cells; CSCs, cancer stem cells; CTCs, circulating tumor cells

Key words: gastric cancer cells, circulating tumor cells, drug resistance, cell adhesion

Introduction

Metastasis is a major cause of gastric cancer mortality. Metastases begin when cancer cells detach from the original tumor tissue and become circulating tumor cells (CTCs). The CTCs are highly resistant to anticancer drugs and possess the potential to implant in distant organs (1), making the treatment of metastatic gastric cancer very difficult and ineffective. Increasing chemo-sensitivity of the CTCs prior to, during, or after a surgical procedure would be an effective therapeutic strategy. However, the CTCs from body circulation are difficult to isolate and the lack of the knowledge regarding the mechanisms governing the chemo-resistance of CTCs have greatly hindered therapy.

Increasing evidence suggests that gastric cancer is a process in which the persistence of the tumor relies on a small population of tumor-initiating cells, the cancer stem cells (CSCs) (2,3). CSCs are capable of self-renewal and thereby possess the ability for unlimited proliferation. CSC theory proposes that majority of the cancer cells in the cancer tissue do not possess the potential of metastasis to generate new tumors. Thus, only a very small portion of the cancer cells (i.e., the CSCs) are tumorigenic and have the capability to migrate and invade. In contrast to the majority of cancer cells, CSCs persist in tumors as a distinct subpopulation and possess the properties of stem cells such as self-renewal capability, undifferentiated status, expression of stem cell markers and chemo-resistance (4-6). The gastrointestinal cancer stem cells have been isolated from both cancer tissues and cell lines, using colon cancer stem cell marker CD133 and gastric cancer stem cell marker CD44 (2,5,6). Since CTCs are the metastatic cancer cells prior to invasion and plantation to distant tissues, these cells exhibit similar characteristics as CSCs. Indeed, gastric cancer cells cultured in a suspended spheroid phenotype *in vitro* were highly resistant to anticancer drugs (3,7). As non-adherent cells, CTCs lose the expression of the differentiation marker E-cadherin and showed an upregulation of CD44 (3). Thus, examining the role of CTCs or CSCs is a critical point in the therapeutic strategy of the disease. Chemotherapy directed towards the CTCs or CSCs by enhancing their chemotherapy sensitivity would be such an approach.

Cancer cells interact with the microenvironment via complex autocrine and paracrine mechanisms. Disrupting these mechanisms can induce aberrant cell proliferation,

adhesion, function and migration that promote malignant behavior. Adipose tissue, like bone marrow, contains stromal cells called ADSCs (adipose-derived stromal cells). ADSCs are considered a true endocrine tissue since adipose lineage cells display a strong secretory activity. ADSCs have been shown to interfere with the proliferation of tumor cells by altering cell cycle progression (8,9). However, the identification of the mechanisms involved in stromal and cancer cell interactions, particularly phenotypic changes and chemosensitivity of the gastric cancer cells caused by ADSC has not been established.

The effects of ADSCs on cancer cells have been studied previously. For instance, when breast cancer cells obtained *in vivo* are co-injected with ADSCs, initial tumor growth and metastasis were observed (10-13). Crude SVF (stromal vascular fraction) instead of ADSCs were used. SVF fraction is a heterogeneous mixture containing many different cell subsets including native ADSCs, mature endothelial and hematopoietic cells [the latter representing a large portion of this fraction (≤20%)]. The functional properties of SVF are discrepant and may explain why the ability of ADSCs to support or suppress tumor cell proliferation is unclear. Nevertheless, the functional properties of ADSCs are multipotent, providing functional cell support and modulating immuno-inflammatory functions (14).

To study the interactions between ADSC and cancer stem cells, we used purified human ADSC instead of SVF in this study. We hypothesize that a potential attachment surface may be able to adhere the gastric CTCs *in vivo* and therefore promote chemo-sensitivity of the cells to anticancer drugs. We have successfully isolated viable ADSCs in our lab using fluorescent activated cell sorting by $CD34^+$, $CD73^+$ and $CD105^+$ and demonstrated that these type of cells have functional self-renewal and transdifferentiation potential.

In this investigation, we demonstrated that introduction of an appropriate attachment surface significantly promoted chemo-sensitivity of the non-adherent $CD44^+$ gastric cancer cells and that the human adipose stem cells may function as a 'living vehicle surface" for such a purpose *in vivo*.

Materials and methods

Cell culture. Human gastric carcinoma AGS cells were maintained in F-12K medium [Catalog No. 30-2004, American Type Culture Collection (ATCC), Manassas, VA, USA] with addition of 10% non-heat inactivated fetal bovine serum (FBS). Human gastric carcinoma Hs746T cells were maintained in Dulbacco's modified Eagle's medium (DMEM) (Catalog No. 30-2002, ATCC) with addition of 10% FBS. $CD44^+$ gastric carcinoma cells or human adipose stem cells (ADSCs) were maintained in modified StemPro medium (Invitrogen Co., Carlsbad, CA, USA) (1X DMEM/F-12/GlutaMax, 1X StemPro Growth Supplement, 1.8% BSA, 8 ng/ml FGF, 10 ng/ml Nodal, 10 ng/ml Noggin and 0.1 mM 2-mercaptoethanol) in an Ultra Low Attachment Surface (ULAS) flask (polystyrene coated with neutral charged, hydrophilic hydrogel) (Corning Inc.).

Magnetic cell separation. $CD44^+$ gastric cancer cells were labeled with anti-CD44 antibody (Cell Signaling), followed by incubation with magnetic-beads conjugated goat anti-mouse secondary antibody (New England BioLab) and separation of the labeled cells from the unlabeled cell population using a Magnetic Separation Pack (New England BioLab).

Isolation of ADSCs. Human (h) ADSCs were isolated from adipose tissue obtained from 12 female donors during abdominal surgery. At the time of the surgical procedure, adipose tissues were collected into sterile containers and transported to the tissue culture laboratory. Tissues were incubated in a solution containing 33% penicillin/streptomycin and fungizone for 30 min at 4˚C to inhibit bacterial growth. Tissues were then washed with PBS and digested with collagenase (1%) + 0.05% dispase for 2-3 h at 37˚C in a shaking water bath. Collagenase was neutralized with growth media containing 10% FBS and single cells isolated by filtering the suspension through a 70-μm nylon mesh strainer (Falcon). Human adipose cells were pelleted by centrifugation at 1,500 rpm. RBCs were lysed with 160 mM ammonium chloride (Sigma) and ADSCs pelleted as above. Cells were expanded for 3 passages (corresponding to ~3 population doublings per passage) in growth media (DMEM, 10% FBS, 1% penicillin/streptomycin, 4 mM L-glutamine, 1 mM sodium pyruvate) before undergoing further studies. Cultured adipose cells were harvested and subjected to fluorescence-activated cell sorting (FACS) to characterize cell phenotype through $CD34^+$, $CD73^+$, $CD105^+$. A member of the authorized study personnel obtained patient consent and HIPAA authorization; these approved, signed forms will be maintained in the Department of Surgery, Division of Plastic Surgery. For the experiments, cells of the second and third passage were used.

Morphology. Olympus CKX41SF microscope connected with Olympus DP-12 camera (Olympus Co., Japan) were used to analyze and record the morphology of the cells.

Determination of cell viability. The number of viable cells was determined using Vi-Cell™ XR Cell Viability Analyzer (Beckman Coulter, Inc., Fullerton, CA, USA). In brief, the non-adherent $CD44^+$ gastric cancer cells and their adherent parental counterpart gastric cancer cells were treated with 5-FU at the dose and time indicated in the figures and figure legends. The non-adherent $CD44^+$ gastric cancer cells were resuspended in cold PBS buffer. The attached parental gastric cancer cells were trypsinized and resuspended in PBS buffer. Viable cells were counted using the instrument.

Cell lysis preparation and western blot analysis. Western blot analyses were performed as previously described (15,16). Cells were treated and then total cell lysates and membrane proteins were extracted. For total cell lysates, the cells were lysed in lysis buffer. The cell membrane lysates were prepared using Mem-PER Mammalian Membrane Protein Extraction Reagent kit (Thermo Scientific, Rockford, IL, USA). The protein concentration was determined using the Bio-Rad assay system (Bio-Rad, Hercules, CA, USA). Anti-human thymidylate synthase (TS) antibody was purchased from Zymed Laboratories, Inc. (Carlsbad, CA, USA). Anti-human survivin antibody was purchased from R&D Systems, Inc.

(Minneapolis, MN, USA). Anti-human E-cadherin and anti-human β-catenin antibody were purchased from Abcam Inc. (Cambridge, MA, USA). Horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat IgG was used as the secondary antibody and the protein bands were detected using the Fujifilm LAS-3000 system (Fujifilm Life Science, Stamford, CT, USA). The plasma membrane marker α2-integrin was used as a control for equal extraction of cell membrane protein. β-actin was used as internal controls to evaluate the uniformity of total cell lysate protein loading. Antibodies against α2-integrin or β-actin were purchased from Abcam.

Immunoprecipitations. Cell lysates containing 200 μg membrane protein were immunoprecipitated by 3 μg of monoclonal antibody against E-cadherin. The complex was then pulled down by Sepharose-conjugated protein G beads (Thermo Fisher Scientific) at 4˚C with gentle tumbling overnight. Immunoprecipitates were washed 4 times, eluted and then analyzed by western blotting with antibodies against E-cadherin and β-catenin.

Integrin assays. Cell surface integrins were identified using the CHEMICON® Alpha/Beta Integrin-Mediated Cell Adhesion Array kit (Chemicon International, Inc., Billerica, MA, USA).

Luciferase reporter assays. The cells were seeded in 24-well plates and grown to 70-80% confluence. Cells were transfected with 0.7 μg of luciferase reporter pTOPFLASH/pFOPFLASH plasmid and 0.1 μg of Renilla luciferase reporter control plasmid per well, using Lipofectamine™ 2000 (Invitrogen Life Technologies, Frederick, MD, USA), as previously described (15-17). T cell factor (TCF) transcriptional activation activity was measured using the luciferase reporter pTOPFLASH/pFOPFLASH plasmids as previously reported (16).

Invasion assay. The invasion assay was performed by using 24-well BD Biocoated Matrigel invasion chambers with 8-μm polycarbonated filters (BD Biosciences, Bedford, MA, USA) (18). In brief, the cells were seeded on Matrigel invasion chamber at 10^5 cells per well. Invasive cells that penetrated through matrigel and migrated to the underside of the membrane were counted under microscopic vision after fixation with 4% formaldehyde in PBS. The average cell number of triplicate wells was determined.

Anchorage-independent growth assay. The soft agar assay testing the anchorage-independent growth *in vitro* was performed (19). Five thousand gastric cancer cells, or 5,000 ADSCs, or a mix of the two (5,000 gastric cancer cells + 5,000 ADSCs) were resuspended with 0.6 ml of 0.3% agarose gel (Invitrogen) in StemPro medium at the absence or presence of 30 μM 5-FU. The cell-agar mixture was immediately seeded into 24-well plates coated with 0.6% agar in StemPro medium at the absence or presence of 30 μM 5-FU. The cultures were maintained in a 37˚C, 5% CO_2 incubator for 2-4 weeks and the cell colonies were scored under microscopic vision. The average colony number of triplicate wells was determined.

Results

The CD44⁺ gastric cancer cells exhibit non-adhesion phenotype. As it is shown in Fig. 1A-a), both the AGS cells and the Hs746T cells demonstrated a monolayer flattened growth pattern when cultured in normal 10% FBS/DMED/F-12 medium and attached to the surface of cell culture flasks. However, the CD44⁺ gastric cancer cells growing in StemPro medium exhibited a non-adherent, spheroid phenotype (Fig. 1A-b). Of interest, the CD44⁺ stem-like cells lost their original morphology and acquired similar spheroid phenotype (compare Fig. 1A-a and -b). Furthermore, the non-adherent CD44⁺ gastric cancer cells were able to re-attach to ECM material vitronectin (Fig. 1A-c), fibronectin (Fig. 1A-d), or laminin (Fig. 1A-e) coated surface and regained the adhesion phenotype.

The non-adherent CD44⁺ gastric cancer cells possess cancer stem cell properties. The non-adherent CD44⁺ gastric cancer cells were almost completely resistant to the chemotherapeutic drug fluorouracil (5-FU) at a dose as high as 300 μM. Alternatively, 5-FU killed more than 80% of the adherent cells in monolayer attachment growth (Fig. 1B). However, the cells regained drug sensitivity when they became attached to the ECM coated surface (Fig. 1B and C). Upon further examination of the cancer stem cell-like CD44⁺ gastric cancer cells, the CD44⁺ non-adherent cancer cells had increased potential of invasion (Fig. 1D) and anchorage-independent colony formation (Fig. 1E). Stem cell markers and molecules involving in chemo-resistance in the CD44⁺ non-adherent cancer cells were also upregulated (gastrointestinal cancer stem cell markers CD44, CD133, embryo stem cell marker Nanog, anti-apoptotic protein survivin and the DNA *de novo* synthase TS (Fig. 2A). The increase in the expression of these molecules was reversed when the cells were attached to ECM material coated surface. Thus, CD44⁺ non-adherent cancer cells exhibited cancer stem cell properties including chemo-resistance, high-grade malignancy and the expression of stem cell markers.

Alteration of integrin and Wnt signaling pathways in the CD44⁺ non-adherent cancer cells. To further investigate the mechanisms underlying the stem cell-like characteristics of the CD44⁺ non-adherent cancer cells, we examined the integrin expression pattern between the CD44⁺ non-adherent cancer cells and the parental adherent cells. Integrin α2/β2 is significantly upregulated in CD44⁺ non-adherent cancer cells. To determine the possible involvement of Wnt signaling in maintaining the cancer stem cell-like phenotype of the CD44⁺ non-adherent cancer cells, we investigated the complex formation of E-cdherin/β-catenin (a cell differentiation marker located in the cell membrane) in the cell models. Our results demonstrate that the expression of E-cadherin was significantly downregulated in CD44⁺ cells compared to their parental cells (Fig. 3A), suggesting the undifferentiated state of the CD44⁺ cells. The complex formation of E-cadherin and β-catenin in the plasmid membrane was also downregulated in CD44⁺ cells (Fig. 3A and B). Accordingly, β-catenin migrated into nuclei where it formed a complex with T cell factor 4 (Tcf-4), initiating Tcf-4 transcriptional activation indicated by

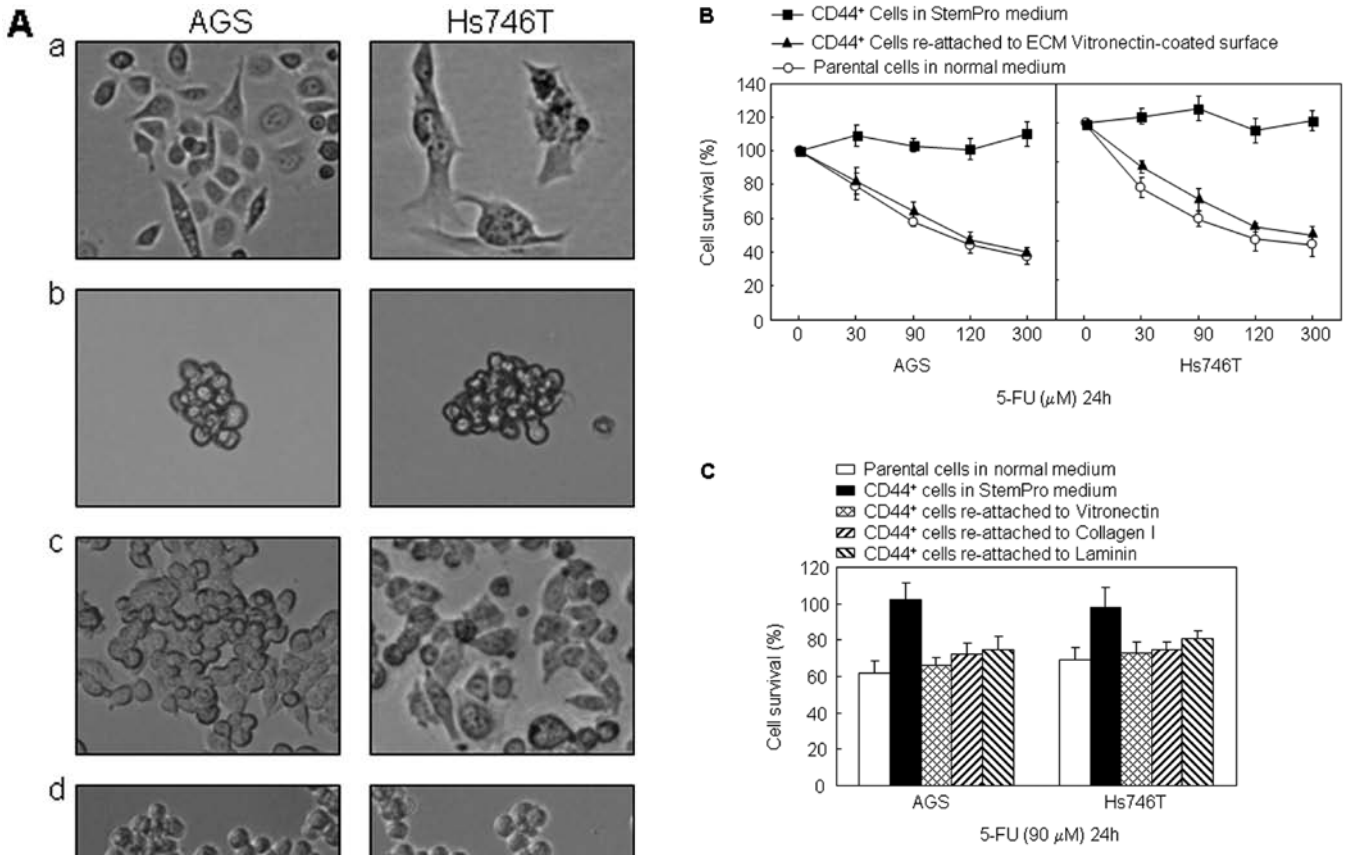
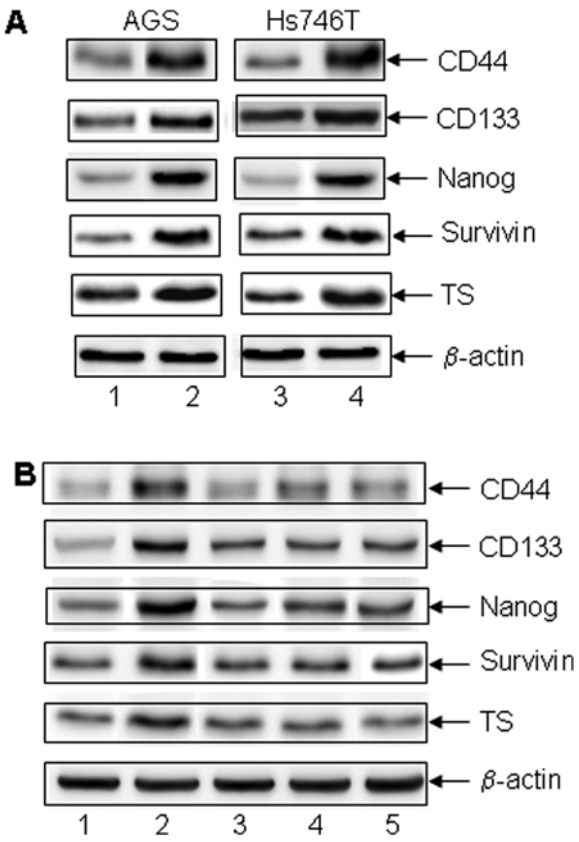


Figure 1. The non-adherent CD44+ gastric cancer stem-like cells were chemo-resistant. (A)-(a) The monolayer attachment growth pattern of the parental gastric cancer cells in normal culture medium as described in Materials and methods; (b) the isolated CD44+ gastric cancer cells maintained in StemPro medium showing a non-adherent, three-dimensional, spheroid phenotype; (c-e) the non-adherent CD44+ gastric cancer stem-like cells from (b) were re-attached to vitronectin (c), fibronectin (d), or collagen I (e) coated surfaces respectively. (B) The different cell groups as indicated were treated with 5-FU at the doses indicated for 24 h. Viable cells were counted as described in Materials and methods. The data represent the mean and standard error of the mean of triplicate experiments. (C) The different cell groups as indicated were treated with 90 μM of 5-FU for 24 h. Viable cells were counted as described in Materials and methods. The data represent the mean and standard error of the mean of triplicate experiments. (D) The non-adherent CD44+ gastric cancer stem-like cells possessed higher potential in cellular invasion compared to their parental counterpart cells in adherent states. The assay was performed as described in Materials and methods. Error bars represent the mean and standard error of the mean of triplicate experiments. (E) The non-adherent CD44+ gastric cancer stem-like cells possessed higher potential in anchorage-independent growth compared to their parental counterpart cells in adherent states. The assay was performed as described in Materials and methods. Error bars represent the mean and standard error of the mean of triplicate experiments.

the luciferase reporter pTOPFLASH/pFOPFLASH activity (Fig. 3C).

ADSCs promotes chemo-sensitivity of the CD44+ non-adherent cancer cells. Interestingly, the CD44+ non-adherent cancer cells re-acquired chemo-sensitivity when they were attached to ECM material coated surface. However, in the *in vivo* environment, no such attachment surface can be introduced into the circulation to adhere the CTCs. Therefore, we explored ADSCs for such a purpose. We found that ADSCs possessed two distinct phenotypes, i.e., the monolayer flattened attachment pattern and the non-adherent, three-dimensional,



spheroid pattern (Fig. 4A). Cloning of a single ADSC using limit delusion method showed that a single ADSC was able to divide and proliferate to a full clone (Fig. 4A-a-f). Co-culture of ADSCs with CD44$^+$ non-adherent cancer cells resulted in significant downregulation of the markers of cancer cell proliferation. Since the co-culture device only allowed the communication between the two cell culture media, but not between the cells, the results suggest that molecules in the medium produced by the ADSCs had inhibitory effects on the gastric cancer cells. As expected, ADSCs as non-malignant cells did not form colonies in soft agar gel whereas CD44$^+$ cancer cells did (Fig. 4C). When the two cells were mixed, the cancer cells formed less colonies (Fig. 4D). In the presence of 5-FU on the soft agar gel, the colony formation by the cancer cells in the cell mix group was significantly reduced to almost zero compared to the group of cancer cells alone (Fig. 4D). These results suggest that ADSCs promoted chemo-sensitivity of the CD44$^+$ cancer cells.

Discussion

We report a novel CD44$^+$ cancer stem cell-like cell model prepared from the human gastric cancer AGS and Hs746T cell lines. CD44$^+$ cells exhibited cancer stem cell properties including a non-adherent, spheroid phenotype and high

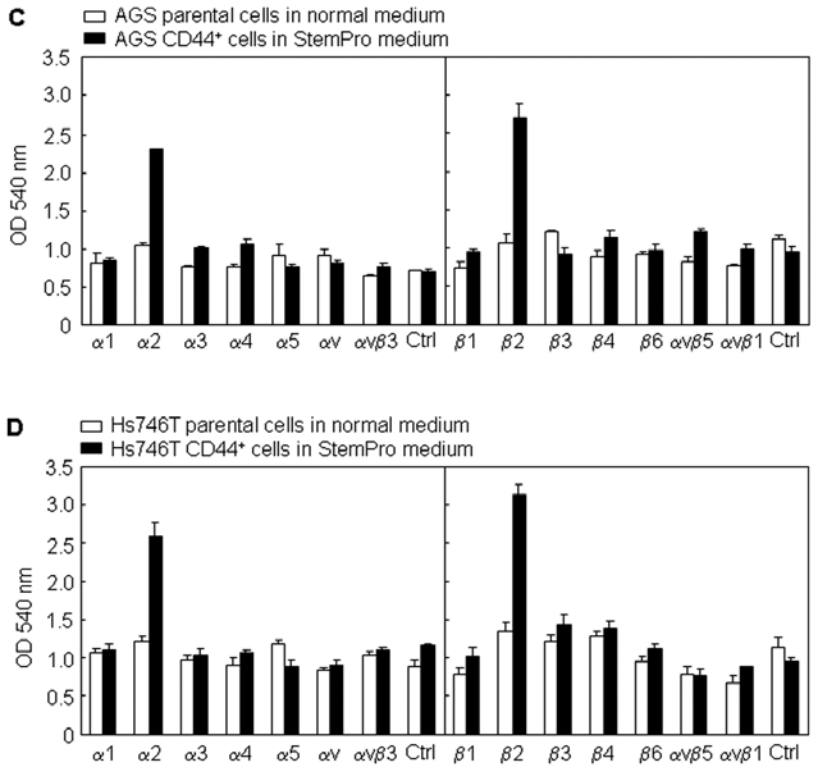


Figure 2. The cancer stem cell markers are upregulated in the non-adherent CD44$^+$ gastric cancer stem-like cells. (A) The expression levels of the proteins indicated were determined by western immunoblot analyses as described in Materials and methods. The expression of β-actin was used as control for equal protein loading. Lanes 1 and 3, the adherent parental gastric cancer cells; lanes 2 and 4, the non-adherent CD44$^+$ gastric cancer stem-like cells. (B) Western immunoblot analyses were performed as in (A). Lane 1, the adherent parental gastric cancer cells; lane 2, the non-adherent CD44$^+$ gastric cancer stem-like cells; lane 3, the non-adherent CD44$^+$ gastric cancer stem-like cells re-attached to vitronectin-coated surface; lane 4, the non-adherent CD44$^+$ gastric cancer stem-like cells re-attached to fibronectin-coated surface; lane 5, the non-adherent CD44$^+$ gastric cancer stem-like cells re-attached to collagen I-coated surface. (C and D) The cell surface expression of integrins/subunits in AGS cells (C) and Hs746T cells (D) was determined using Alpha/Beta Integrin-Mediated Cell Adhesion Array kit as described in Materials and methods. The expression of human α and β subunits are indicated in the figures. Open bars, the adherent parental gastric cancer cells. Solid bars, the non-adherent CD44$^+$ gastric cancer stem-like cells.

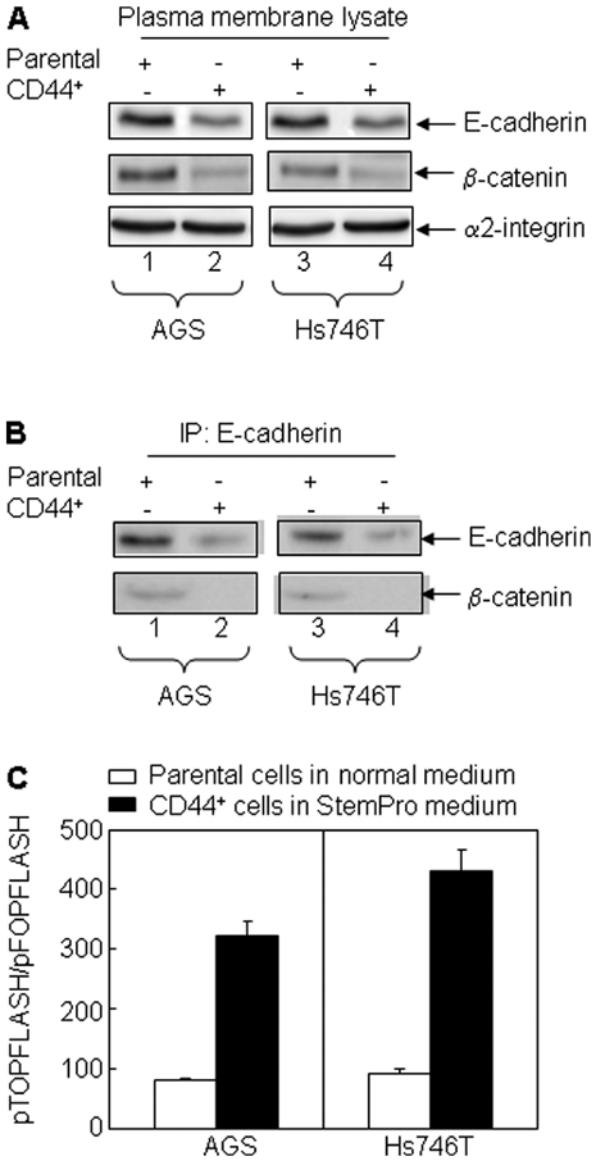


Figure 3. Wnt signaling β-catenin/TCF signaling pathway was upregulated in the non-adherent CD44+ gastric cancer stem-like cells. (A) The cell membrane localization of E-cadherin and β-catenin was downregulated in the non-adherent CD44+ gastric cancer stem-like cells. The plasma membrane lysates were extracted and western blot analyses for E-cadherin and β-catenin were performed as described in Materials and methods. Lanes 1 and 3, the adherent parental gastric cancer cells; lanes 2 and 4, the non-adherent CD44+ gastric cancer stem-like cells. α2-integrin (a plasma membrane marker) was used as a control for equal extraction of cell membrane proteins. (B) The plasma membrane lysates were immunoprecipitated (IP) with antibody against E-cadherin and the immunoprecipitates were then analyzed by western blotting for E-cadherin and β-catenin. Lanes 1 and 3, the adherent parental gastric cancer cells; lanes 2 and 4, the non-adherent CD44+ gastric cancer stem-like cells. (C) TCF4 gene transcriptional activities were upregulated in the non-adherent CD44+ gastric cancer stem-like cells. TCF4 luciferase reporter pTOPFLASH and control pFOPFLASH plasmids were prepared and the assays of TCF4 gene transcriptional activities were performed as described in Materials and methods. The error bars represent the standard error of the mean of triplicate experiments. Open bars, the adherent parental gastric cancer cells. Solid bars, the non-adherent CD44+ gastric cancer stem-like cells.

chemo-resistance. More importantly, this is the first study to demonstrate that the cells regained chemo-sensitivity after they were re-attached to an extracellular matrix coated surface. Mixed co-culturing CD44+ cancer stem cell-like cell with ADSCs from different donors inhibited cancer cell viability, proliferation and phenotype and promoted chemo-sensitivity of the CD44+ cancer cells.

To establish our gastric cancer stem cell-like model, we isolated CD44 positive cells from human gastric carcinoma cell lines (AGS and Hs746T cells). The isolated-cells were cultured in a stem-cell culture medium (StemPro) to retain the cells in an undifferentiated proliferation status. The isolated CD44+ cancer cells growing in the StemPro exhibited a suspended, non-adherent, 3-dimentional spherical growth phenotype compared to their adherent counterparts grown in the regular medium which showed monolayer attachment to the surface of tissue culture plate. Remarkably, the non-adherent CD44+ cancer stem-like cells were highly resistant to 5-FU, a chemotherapeutic agent used clinically to treat gastrointestinal malignancies. As shown in Fig. 1B, the non-adherent cancer cells tolerated 5-FU in a concentration as high as 300 μM. In humans, this would not be achievable at therapeutic doses of 400 mg/m^2/day. We suggest that this may be a reason why chemotherapeutic drugs fail to kill the circulating gastric tumor cells *in vivo*. Moreover, when the non-adherent CD44+ gastric cancer cells were placed on ECM material coated surface, the cells quickly reacquired attachment phenotype. Thus, the re-attached cancer cells re-gained chemo-sensitivity similar to the adherent cells.

The mechanism of how CD44+ non-adherent cancer cells remain highly resistant to 5-FU is not clear. Other investigators have reported that the non-adherent cancer cells expressed a high level of cancer stem cell markers such as CD44 and CD133 compared to the adherent cells (4,5,20). Furthermore, Nanog, a transcription factor functioning in maintaining embryo stem cells in the undifferentiated state (21), survivin, an anti-apoptotic molecule (22,23) and thymidylate synthase (TS), a key enzyme involved in the *de novo* synthesis of DNA which circumvents the efficacy of 5-FU (24,25) are also significantly upregulated in the CD44+ non-adherent cells. These results suggest that the CD44+ non-adherent cancer cells possess CSC-like properties which cause drug resistance of these cells. CSCs are defined as a distinct subpopulation of cancer-initiating cells that constitute a small percentage of the tumor bulk. It is believed that in the general cancer cell population, only CSCs possess the stem cell-like characteristics including undifferentiated status, drug resistance, tumorigenicity, expression of stem cell markers, self-renewal and metastasis (26-29).

The CD44+ non-adherent cancer cells *in vitro* resembled metastatic gastric CTCs *in vivo* in that both cell types survived in an anchorage-independent, non-adherent manner and both possessed the potential of re-attachment. Like the CD44+ non-adherent cells that are more resistant than their adherent counterparts, CTCs are also resistant to anticancer drugs (1). Converting CTCs from a non-adherent to an adherent phenotype may increase the sensitivity of the cells to anticancer drugs. In the cell culture condition, cell attachment proteins bind to the negatively charged, hydrophilic surface of the polystyrene surface of the tissue culture plates to retain the cells in an attachment state. In intact tissue, the structural framework formed by fibroblasts and their synthesized extracellular matrix and collagen provides surface for cells to attach and grow. Accordingly, we hypothesize that introduction of a

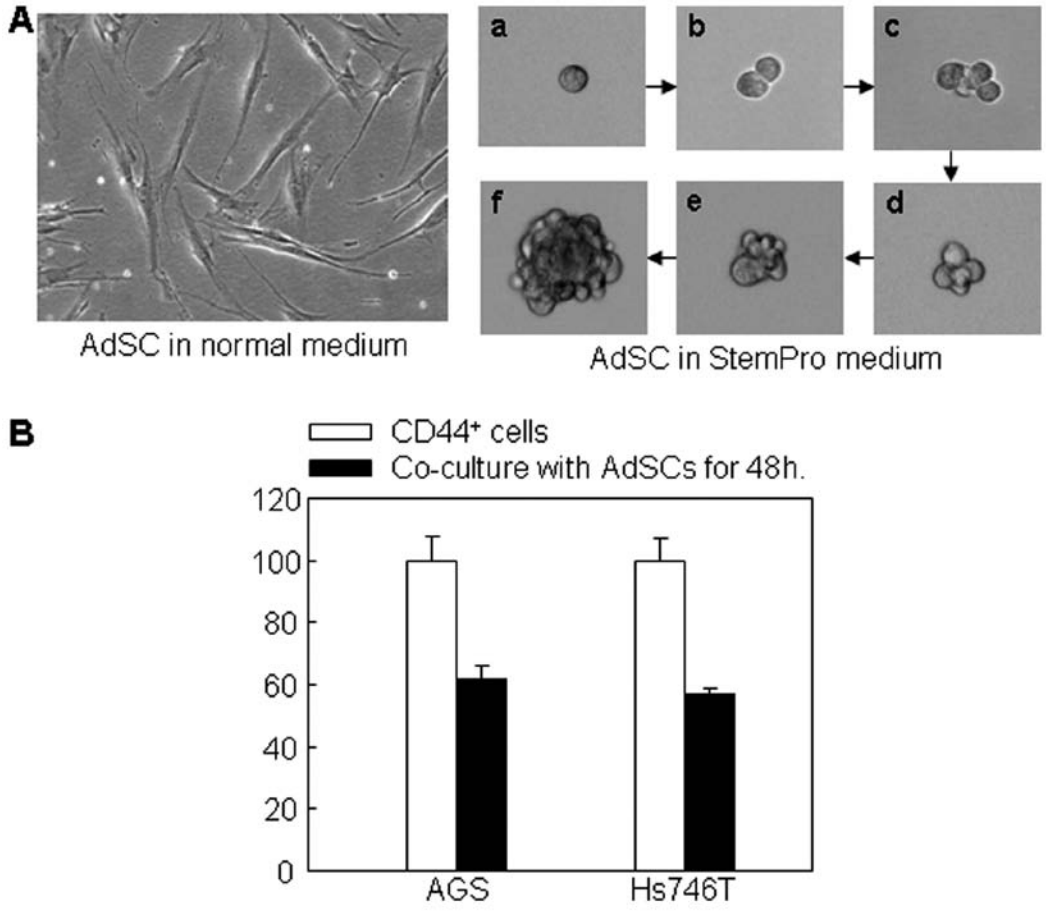


Figure 4. Mixed co-culture of the ADSCs and the non-adherent CD44+ gastric cancer stem-like cells promoted chemo-sensitivity of the cancer cells. (A) Morphology of ADSCs. Left panel, ADSCs in normal medium; right panel, limiting dilution cloning of the ADSCs. The clone of the cells was maintained in StemPro medium in an ultra low attachment surface flask, as described in Materials and methods. (B) Co-culture of the two cell groups was performed using Transwell culture plates as described in Materials and methods. Open bars, the non-adherent CD44+ gastric cancer stem-like cells. Solid bars, the non-adherent CD44+ gastric cancer stem-like cells co-cultured with ADSCs for 48 h. (C) Anchorage-independent assay for mixed co-culture of the ADSCs and the non-adherent CD44+ gastric cancer stem-like cells promoted chemo-sensitivity of the cancer cells. Five thousand of the non-adherent CD44+ cancer stem-like AGS cells (left column) or 5,000 of the ADSCs (right column), or a mix of the two groups of cells (middle column) were re-suspended with 0.3% soft agarose gel in StemPro medium in the absence (upper panel) or presence (lower panel) of 30 μM of 5-FU, as described in Materials and methods. Similar results were obtained on Hs746T cells (data not shown). (D) Colony counting of (C). The number of the colony formation per 1,000 cells that were originally seeded was counted under a microscope. The data represent the mean and standard error of triplicate experiments.

surface coating with the necessary attachment materials would allow the CTCs to adhere *in vivo*. Thus, the adherent cancer cells would be much more sensitive to anticancer drugs and standard doses of chemotherapeutic agent could then be used to kill the attached CTCs. Hypothetically, circulating cells would not be attached to the attachment surface since blood cells are naturally anchorage-independent and do not possess the potential of adhesion.

We tested our hypothesis that converting CTCs from a non-adherent to an adherent phenotype may increase the sensitivity of the gastric cancer cells to chemotherapeutic agents by providing

the ADSCs as surface vehicles. ADSCs are an active component of ECM producing tissues. Therefore ADSCs have the potential to adhere to $CD44^+$ non-adherent cells and trigger the adhesion signals in the cells (i.e., living vehicle surface). ADSCs are easy to acquire from the waste of body fat tissues and the cells do not induce immune rejection reaction from the host. Since isolated ADSCs no longer possess properties of adipose cells, they would not accumulate materials that may cause fat embolism. ADSCs are pluripotent adult stem cells that can be manipulated into the cell type that is desired for the aforementioned purpose *in vivo*. Reports regarding the effects of ADSCs against cancer are mixed. Both pro- or anti-breast cancer by ADSCs have been recorded (10-13). The effects of ADSCs on gastrointestinal cancers are not established. A recent study reported that ADSCs provoked pancreatic cancer cell death both *in vitro* and *in vivo* (30). ADSCs have also been manipulated to act as a ligand delivery vehicle for cancer therapy (31). In these prior studies, crude SVF (stromal vascular fraction) instead of isolated ADSCs were used. Since the SVF fraction is heterogeneous and contains many cell subsets including native ADSC, mature endothelial and hematopoietic cells (the latter representing a large portion of this fraction, ≤20%), the functional properties of SVF are variable. Thus, prior studies have not been able to demonstrate the ability of ADSCs to support or suppress tumor cell proliferation when using SVF. In our studies, we used purified human ADSCs instead of SVF using fluorescent activated cell sorting by $CD34^+$, $CD73^+$ and $CD105^+$. Our results suggest that purified ADSCs suppressed the growth of the non-adherent cancer stem cell-like $CD44^+$ cells.

We used ADSCs as a living vehicle surface to adhere CTCs in the circulation. The presence of the ADSCs did promote the chemo-sensitivity of the cancer stem cell-like $CD44^+$ cells to 5-FU, as indicated by soft agar assay. Application of ADSCs for such a purpose has not been previously reported.

In conclusion, we have established a gastric cancer stem cell-like model which can be used as an *in vitro* tool for the study of CTCs and CSCs. The non-adherent cancer cell model may also be applied in high-throughput screening of agents targeting CSCs or resistant cancer cells. In principle, the application of automated screening technologies could facilitate the identification of agents that kill CSCs or resistant cancer cells. However, the screening depends on the ability to propagate stable, highly enriched populations of CSCs *in vitro*, which is not currently possible for the CSCs of solid tumors (32). The non-adherent cancer cells possess CSC characteristics of high drug resistance and they are easy to prepare and maintain. Thus, the non-adherent cancer cell model may be useful for such purposes.

Our work represents the first study using non-engineered cells (ADSCs) to treat gastric cancer stem cells in a non-adherent cancer cell model. This effect of ADSCs on promoting chemo-sensitivity on gastric cancer stem cell-like $CD44^+$ cells is mediated at least in part by ADSC acting as a living vehicle surface and cell-cell interaction. We speculate that *in vivo* ADSC may modify the microenvironment of the tumor and thus promote its sensitivity to chemotherapeutic agents and inhibit its proliferation.

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