Isolation of cancer progenitor cells from cancer stem cells in gastric cancer

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Abstract. The success of cancer treatment may depend on the complete elimination of cancer stem cells (CSCs). However, data regarding the current characterization of CSCs in different types of tumor are inconsistent, possibly due to the mixture of CSCs with cancer progenitor cells (CPCs). Therefore, it is important to exclude CPCs for the characterization of CSCs. The present study aimed to characterize gastric cancer stem cells (GCSC) by separating GCPC from gastric progenitor cells (GCSC) with flow cytometry. In total, 615 murine gastric cancer (GC) cells were divided into aldehyde dehydrogenase (ALDH)^{high}, ALDH^{low} and ALDH^{neg} groups by flow cytometry according to their ALDH activity. With decreased ALDH activity, the expression levels of stemness-associated markers, CD133^{+}, octamer-binding transcription factory-4 and sex determining region Y-box 2 decreased. The ALDH^{high} and ALDH^{low} cells proliferated and formed tumor spheres in ultra-low adhesion medium without serum, however, the latter formed larger tumor spheres. In mice transplanted with 5,000 cells, the rate of tumor formation in the ALDH^{low} group was significantly higher, compared with that in the ALDH^{high} group. Of note, an increased number of mice developed tumors in the ALDH^{high} group 16 weeks following the injection of 500 cells, whereas tumors appeared at 8 weeks in the ALDH^{low} group. The mice in the ALDH^{neg} group exhibited less tumor formation under these conditions. These results demonstrated that ALDH^{high} cells had characteristics of GCSCs with a high level of self-renewal ability, but were in a relative resting stage. The ALDH^{low} cells had characteristics of GCPCs with limited self-renewal ability, but were in a rapid proliferation stage. These findings suggested that the separation of GCPCs from GCSCs is important for elucidating the biology of GCSCs and identifying strategies to eliminate GCSCs in GC.

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Introduction

Gastric cancer (GC) is one of the most common types of cancer worldwide and its mortality rate is the second highest among all malignancies (1). Recurrence following treatment is the primary cause of GC-associated mortality (2). Therefore, it is important to elucidate the mechanism of drug resistance and identify strategies to prevent the recurrence of GC following treatment. Cancer stem cells (CSCs) are the origin of uncontrolled cancer cell growth. The elimination of CSCs is considered the only way to fully eradicate tumors (3). Thus, identification, isolation and validation of gastric CSCs (GCSCs) may provide novel clues for GC treatment. However, data currently available regarding the isolation, characterization and functional investigations of CSCs are inconsistent and controversial, particularly in GCSCs. For example, Fukuda *et al* (4) obtained GCSCs from MKN-45 cells via side population (SP) cell sorting, whereas Zhang *et al* (5), found that the SP cell sorting method did not apply to all types of GC cell. Takaishi *et al* (6) isolated GCSCs from MKN-45, MKN-74 and N-87 GC cell lines when CD44^{+} was used as a marker, however, no significant differences in tumor formation were found between the SP cells and non-SP cells. Others have reported that CD44^{+} cells show no correlation with the malignancy of GC cells (7). Thus, it is important to isolate pure GCSCs by applying the appropriate methods and markers.

The CSC theory holds that the development of tumors derives from CSCs with unlimited self-renewal ability to generate cancer progenitor cells (CPCs), which have limited self-renewal ability and differentiate into large quantities of regular cancer cells. However, the majority of studies on CSCs do not distinguish between CSCs and CPCs in cell populations with stemness, as CPCs also have self-renewal ability and stemness (8). As CSCs and CPCs may have significantly different biological characteristics, it is important to distinguish between CSCs and CPCs in stem-like cells.

Aldehyde dehydrogenase (ALDH) is a marker, which can be used to distinguish between the high degree of stemness of CSCs and the low degree of stemness of CPCs from stem-like

cell populations. ALDH is an enzyme, which detoxifies and is important in stem cell proliferation. Its activity reflects the degree of cell stemness (9-13). Accordingly, several studies have acquired CSCs from $ALDH^+$ tumor cells by assessing ALDH activity (14-19). Although these studies did not distinguish between CSCs and CPCs in acquired stem-like cells, this method can detect the levels expression of ALDH in $ALDH^+$ cell populations. Consequently, the present study hypothesized that pure CSCs are $ALDH^+$ cells with high ALDH activity and CPCs are $ALDH^+$ cells with low ALDH activity. In our previous study, ALDH high ($ALDH^{high}$), low ($ALDH^{low}$) and negative ($ALDH^{neg}$) subgroups we successfully sorted in H22 mouse hepatic cancer cells, and it was found that the characteristics of these cells were similar to those of CSCs, CPCs and regular cancer cells, respectively (20). These results suggested that sorting of $ALDH^{high}$ and $ALDH^{low}$ populations may assist in isolating and characterizing GCSCs and gastric CPCs (GCPCs).

In order to elucidate the causes of the conflicting results in previous studies of gastric cancer stem cells, in the present study $ALDH^{high}$, $ALDH^{low}$ and $ALDH^{neg}$ were successfully sorted from 615 murine GC (MFC) cells using an ALDH assay. It was found that $ALDH^{high}$ and $ALDH^{low}$ cells exhibited characteristics of GCSCs and GCPCs, respectively. These findings suggested that the MFC stem-like cells had two cell subpopulations with distinct characteristics and that CPCs require exclusion for the investigation of CSCs.

Materials and methods

Cell lines and cell culture. MFC cells were purchased from the Chinese Academy of Sciences Typical Culture Preservation Committee Cell Bank (Shanghai, China). The cells were cultured in humidified air at 37˚C with 5% CO_2 in RPMI-1640 (Sigma-Aldrich; Merck Millipore; Darmstadt, Germany) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.).

ALDH assay and cell sorting. ALDH activity was determined using an ALDEFLUOR™ assay (Stemcell Technologies, Inc., Vancouver, BC, Canada) according to the manufacturer's protocol. Briefly, the cells were suspended in ALDEFLUOR™ assay buffer ($2x10^6$ cells/ml). The ALDH reaction substrate, BODIPY-aminoacetaldehyde, was added to the experimental groups, whereas ALDH substrate and the inhibitor, diethylaminobenzaldehyde, were added to the control groups, followed by incubation at 37˚C for 40 min in the dark. An Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to detect ALDH activity, and analyze the proportion of $ALDH^+$ and $ALDH^-$ cells. Cell sorting was performed as previously described (20). Briefly, the 1% of viable cells with the highest fluorescence intensity among the $ALDH^+$ cell population were selected as $ALDH^{high}$ cells, the 1% with the lowest fluorescence intensity among the $ALDH^+$ cell population were selected as the $ALDH^{low}$ cell population and the 1% with the lowest fluorescence intensity among the $ALDH^-$ cell population were selected as $ALDH^{neg}$ cells. Flow cytometry was used to select these cells on a FACS Aria II flow cytometer (BD Biosciences).

Flow cytometry. The cells were suspended in phosphate-buffered saline (PBS; $2x10^6$ cells/ml). Rat anti-CD133-phycoerythrin antibody (clone 13A4; 1:50; cat. no. 12-1331-82; eBioscience, San Diego, CA, USA) and rat anti-CD44-allophycocyanin antibody (clone IM7; 1:50; cat. no. 559250; BD Biosciences) were added to the experimental groups, and the same quantity of normal isotype IgG was added to the control groups. Following incubation at 4˚C for 30 min in the dark, the cells were washed with PBS and subjected to flow cytometric analysis (Accuri C6).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) analysis. RNA was extracted from cells using an RNeasy Total RNA system (Qiagen, Inc, Valencia, CA, USA) according to the manufacturer's protocol. The quantity and purity of the RNA were assessed by measuring the absorbance at 260 and 280 nm. The cDNA was synthesized from total RNA (2 μg) with oligo (dT) primers using an M-MLV reverse transcriptase first strand kit (Invitrogen; Thermo Fisher Scientific, Inc.). A 25 μl PCR reaction contained 4 μl cDNA, 2.5 μl buffer, 1 μl forward primer, 1 μl reverse primer, 1 μl dNTP, 1 μl *Taq* DNA polymerase and 14.5 μl DEPC water. The primers used were as follows: Octamer-binding transcription factor-4 (OCT-4), forward 5'-TGGGCTAGAGAAGGATGTGG-3' and reverse 5'-CTGGGAAAGGTGTCCCTGTA-3'; sex determining region Y-box 2 (SOX-2), forward 5'-GAACGCCTTCATGGTATGGT-3' and reverse 5'-TCTCGGTCTCGGACAAAAGT-3'; GAPDH, forward 5'-GGTTGTCTCCTGCGACTTCA-3' and reverse 5'-TGGTCCAGGGTTTCTTACTCC-3'. The PCR reaction conditions were as follows: 94˚C for 5 min, followed by 30 cycles of 94˚C for 40 sec, 61˚C for 30 sec and 72˚C for 10 min. The PCR products were analyzed on a 2% agarose gel with ethidium bromide. The gel images were analyzed using Image-Pro plus 6.0 software (Media Cybernetics, Inc. Rockville, MD, USA).

Spheroid colony formation assay. The cells were inoculated at a density of 5,000 cells/well in ultra-low attachment 6-well plates (Corning Incorporated, Corning, NY, USA). Stem cell culture medium (2 ml; Academy of Military Medical Sciences, Beijing, China) was added to each well. The plate was placed in humidified air at 37˚C with 5% CO_2. Every 2 days, 1 ml stem cell medium was added. The sphere formation of the cells was observed at 7 days under a fluoresence microscope (TE2000-U; Nikon Corporation, Tokyo, Japan).

In vivo tumorigenicity. The $ALDH^{high}$, $ALDH^{low}$ or $ALDH^{neg}$ cells were suspended in PBS following sorting, adjusted to 500 or 5,000 cells per 50 μl, and then mixed with 50 μl Matrigel (BD biosciences). The cells were injected subcutaneously into 6-week-old female 615 mice (Experimental Animal Center of PLA General Hospital, Beijing, China). These mice were maintained under barrier conditions on a 12 h light/dark cycle in a temperature room at 20-24˚C with free access to food and water, and the growth of tumors was observed every week for 16 weeks. The tumor mass was monitored using a caliper and the mice were sacrificed by cervical dislocation at 16 weeks.

Statistical analysis. All data were analyzed using SPSS 19.0 statistical software (IBM SPSS, Armonk, NY, USA). Data

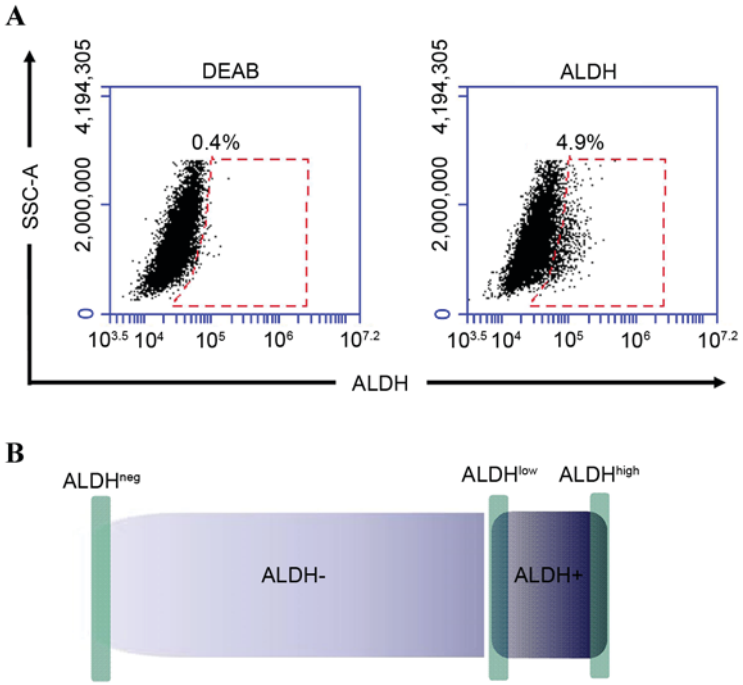

Figure 1. Detection of ALDH activity and cell sorting in MFC cells. (A) Expression of ALDH in MFC cells. (B) Diagrammatic sketch of cell sorting with different ALDH activities in MFC cells. The red lined boxes indicate ALDH^{+} cells and percentages on the graphs indicate the rate of ALDH^{+} cells. ALDH, aldehyde dehydrogenase; DEAB, diethylaminobenzaldehyde; SSC, side scatter.

are expressed as the mean ± standard deviation from three independent experiments. Comparisons were made using an independent samples Student's *t* test between two groups and by one-way analysis among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

ALDH assay and cell sorting in MFC cells. The results of the flow cytometric analyses showed that the proportion of ALDH^{+} MFC cells was 5.12±0.91% (Fig. 1A), which was in accordance with the relatively low percentages of CSCs in tumors (21). In order to obtain cancer cells with different differentiation levels, the 1% of the cells with the highest fluorescence in the ALDH^{+} population (ALDH^{high}) were selected and considered to be GCSCs. The 1% of the cells with the lowest fluorescence (ALDH^{low}) were considered to be GCPCs and the 1% with the lowest fluorescence in the ALDH^{-} population (ALDH^{neg}) were considered to be regular cancer cells (Fig. 1B).

Expression of stem cell surface markers in cells with different ALDH activities. In order to confirm the populations, the stem cell surface markers, CD44^{+} and CD133^{+} (22) were detected in the different cell populations using flow cytometry. The results showed that CD44^{+} was expressed in >90% of the total cell subpopulation, indicating that CD44^{+} may not be suitable as a stem cell marker in MFC cells (Fig. 2A). CD133^{+} was significantly higher in the ALDH^{high} (44.07±3.97%) and ALDH^{low} (34.33±3.06%) cells, compared with the ALDH^{neg} (1.60±0.66%) cells (high. vs. neg, P=0.007; low, vs. neg, P=0.006). The expression of CD133^{+} was higher in the ALDH^{high} cells, compared with the ALDH^{low} cells, but this was not significantly different (P=0.09; Fig. 2B and C).

Gene expression in cells with different ALDH activity. In order to investigate the degree of differentiation of the cell subgroups, the relative expression of the stemness-associated genes OCT-4 (23) and SOX-2 (24) were examined using RT-PCR analysis. The results showed that the relative expression levels of OCT-4 were significantly higher in the ALDH^{high} (1.02±0.07) and ALDH^{low} cells (0.93±0.04), compared with the ALDH^{neg} (0.40±0.02) cells; ALDH^{high}, vs. ALDH^{neg}: P=0.001; ALDH^{low}, vs. ALDH^{neg}: P<0.001; Fig. 3A and B). The expression of OCT-4 in the ALDH^{high} cells was higher, compared with that in the ALDH^{low} cells, although this was not significant (P=0.331). A similar trend was observed for the expression of SOX-2. The ALDH^{high} (1.06±0.06) and ALDH^{low} (0.90±0.05) cells had markedly higher expression of SOX-2, compared with the ALDH^{neg} (0.46±0.02) cells (ALDH^{high}, *vs*. ALDH^{neg}: P=0.003; ALDH^{low}, vs. ALDH^{neg}; P=0.003), as shown in Fig. 3B. The expression of SOX-2 in the ALDH^{high} cells was higher, compared with that in the ALDH^{low} cells, although this was not significant (P=0.053).

Sphere formation in cells with differing ALDH activity. To assess the sphere-forming ability of cells with differing ALDH activity, ALDH^{high}, ALDH^{low} or ALDH^{neg} cells were cultured under ultra-low adhesion conditions without serum. The results showed that ALDH^{high} and ALDH^{low} cells formed tumor spheres following 7 days in culture, however, ALDH^{neg} cells did not form spheres. The tumor sphere volumes of the ALDH^{high} cells were significantly lower, compared with those of the ALDH^{low} cells (Fig. 4A). The ALDH activity assays demonstrated that the percentage of ALDH^{+} cells in the spheres formed by ALDH^{low} cells was significantly lower, compared with the percentage in the ALDH^{high} cell spheres (30.5±5.7, vs. 70.1±7.1%, respectively; P<0.001; Fig. 4B and C).

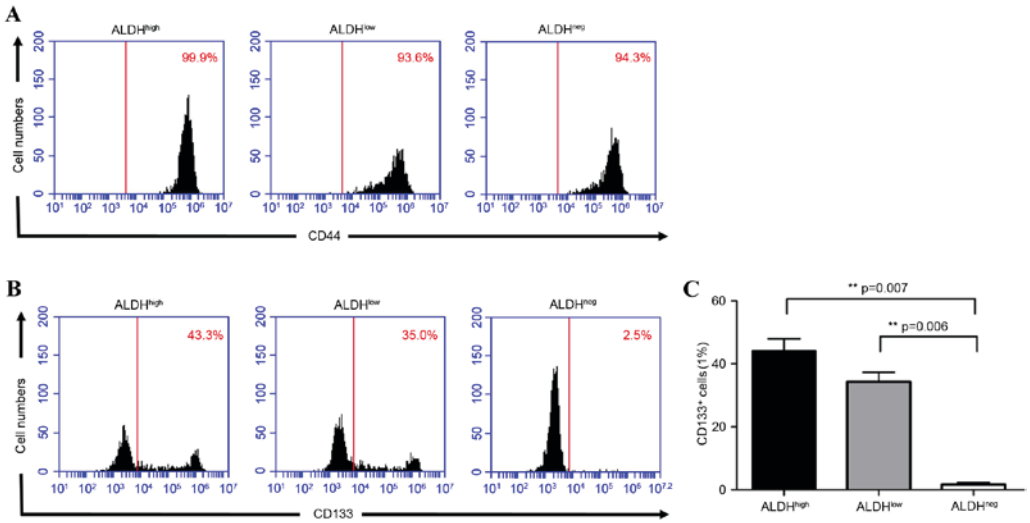

Figure 2. Flow cytometric analysis. Expression levels of (A) CD44$^+$ and (B) CD133$^+$ in cells with different ALDH activity are shown. The percentages in (A) indicate CD44$^+$ cells in the groups of cells with different ALDH activity. The percentages in (B) indicate CD133$^+$ cells in the groups of cells with different ALDH activity. (C) Statistical analysis of the expression of CD133$^+$. ALDH, aldehyde dehydrogenase.

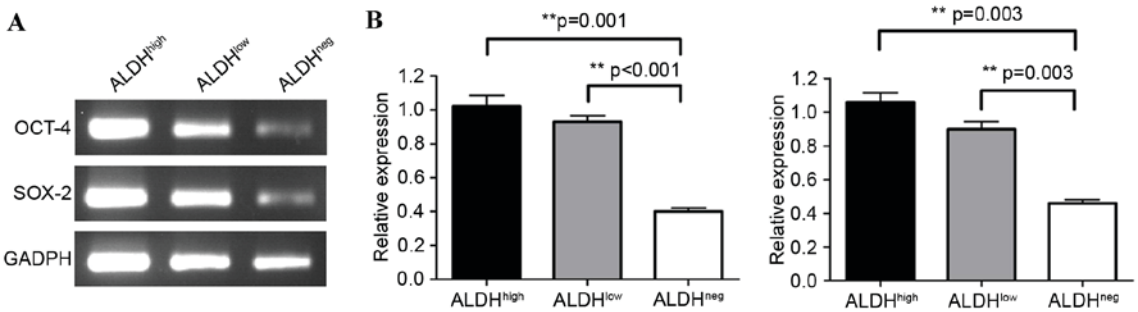

Figure 3. Expression levels of OCT-4 and SOX-2. (A) mRNA expression levels of OCT-4 and SOX-2 in cells with different ALDH activity. (B) Statistical analysis of the relative mRNA expression of OCT-4 (left) and SOX-2 OCT-4 (right). ALDH, aldehyde dehydrogenase; OCT-4 octamer-binding transcription factory-4; SOX-2, sex determining region Y-box 2.

Tumor formation in cells with differing ALDH activity in mice. In order to assess the tumor-forming ability of cells with differing ALDH activity, the sorted cells were subcutaneously injected in mice at different concentrations (500 and 5,000 cells per 50 μl PBS) to observe tumor formation. The results are shown in Table I. Tumors appeared 4 weeks following the injection of 5,000 cells and tumor formation was observed in all mice at 8 weeks in the ALDH^{low} group. Of the six transplanted mice, four developed tumors 16 weeks following the injection of 5,000 cells in the ALDH^{high} group, and only one mouse developed tumors 16 weeks following injection of 5,000 cells in the ALDH^{neg} group. However, when the injected number of cells was decreased to 500, tumors first appeared in the ALDH^{low} group 8 weeks following injection. The rate of tumor formation in the ALDH^{high} group was higher, compared with that in the ALDH^{low} group with extended duration, and no tumors formed in the ALDH^{neg} group.

Table I. Tumor formation in mice injected with cells of differing ALDH activity.

		Mice with tumors (n)			
Cell type	Cells (n)	4 weeks	8 weeks	12 weeks	16 weeks
ALDH^{high}	5,000	0	1	2	4
ALDH^{low}	5,000	3	6	6	6
ALDH^{neg}	5,000	0	1	1	1
ALDH^{high}	500	0	0	1	3
ALDH^{low}	500	0	1	1	1
ALDH^{neg}	500	0	0	0	0

n=6 mice per group. ALDH, aldehyde dehydrogenase.

Discussion

In the present study, it was shown that the expression of the stemness-associated markers, CD133$^+$, OCT-4 and SOX-2, decreased with a decrease in ALDH activity in MFC cells. The ALDH^{high} and ALDH^{low} cells formed tumor spheres, however the ALDH^{low} cells formed larger tumor spheres. In mice transplanted with 5,000 cells, the rate of tumor formation in the ALDH^{low} group was significantly higher, compared

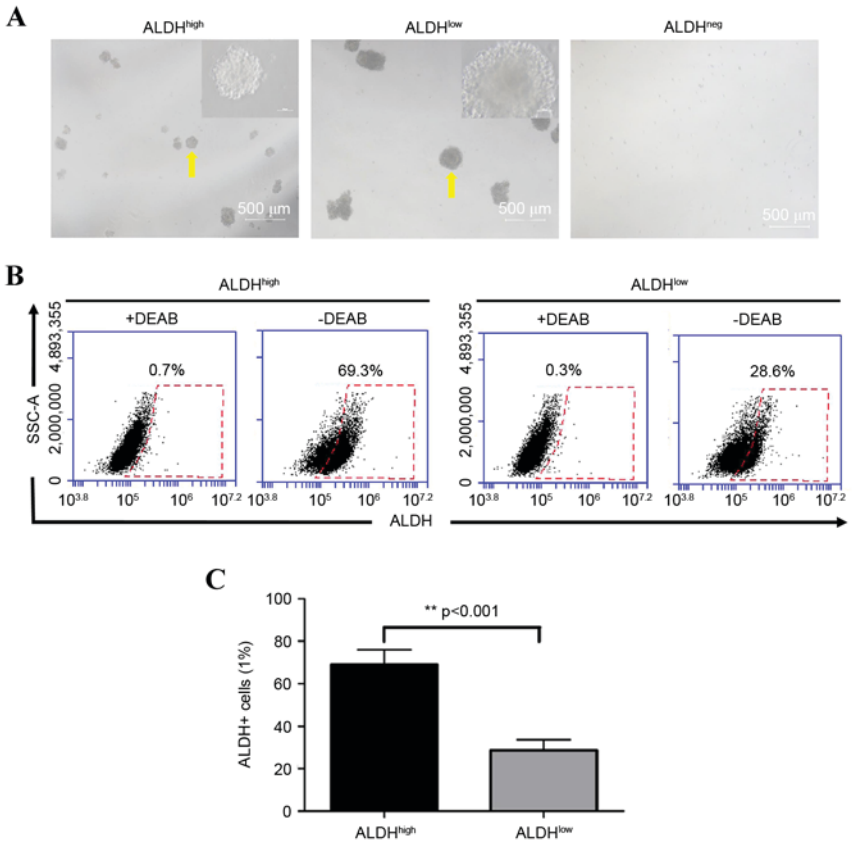

Figure 4. Sphere formation *in vitro*. (A) Sphere formation of cells with different ALDH activities. The arrows indicate the enlarged tumor spheres. Overall magnification, x40; local magnification, x400. (B) Detection of ALDH activity in the spheres and (C) quantification. The red lined boxes indicate ALDH$^+$ cells and percentages on the graphs indicate the rate of ALDH$^+$ cells. ALDH, aldehyde dehydrogenase; DEAB, diethylaminobenzaldehyde; SSC, side scatter.

with that in the ALDH^{high} group. On injection of the mice with 500 cells, tumor development was delayed, however, more mice developed tumors in the ALDH^{high} group, compared with the ALDH^{low} group. These results demonstrated that ALDH^{high} cells had the characteristics of GCSCs and ALDH^{low} cells had the characteristics of GCPCs. These findings suggested that the separation of GCPCs from GCSCs may be important to elucidate the biology of GCSCs and for developing strategies to eliminate GCSCs for the treatment of patients with GC.

The theory of CSCs suggests that tumor cells have a differentiation level similar to that of stem cells (8). Undifferentiated CSCs initially generate CPCs and then further differentiate into regular cancer cells. Although CPCs are important in tumor cells, few studies have systematically investigated CPCs, and there are no reports distinguishing CSCs from CPCs in GC. To the best of our knowledge, only the study by Beier *et al* (25) and our previous study have sorted CPCs. Our previous study sorted CPCs in H22 cells using an ALDH activity assay (20). Beier *et al* (25) reported the successful isolation of CD133$^-$ cerebral glioma CPCs from CD133$^+$ cerebral glioma CSCs under conditions of stem cell cultivation. However, they did not show the percentage of CSCs in the CD133$^+$ cells, therefore, it is possible that CD133$^-$ cerebral glioma CPCs also contain regular cancer cells. In our previous study stemness-like cells were distinguished according to CSC sorting methods including, stemness-associated marker sorting, sphere enrichment (21), SP sorting (4) and ALDH activity sorting (26). Although stemness-assocated marker sorting is the most common way to isolate CSCs, it is not able to further distinguish CPCs from the selected CSCs. Sphere enrichment is a useful method by itself, however, the cells in the formed sphere may contain regular cancer cells (27). The SP method is another way to sort CSCs, however it requires the chemical, Hoechst 33342, which is cytotoxic and may affect the reliability of the data. ALDH is important in stem cell differentiation and proliferation. ALDH activity reflects the degree of stemness of stem cells and has been used as a functional stem cell marker in sorting various types of CSC (14-19). Consistent with the findings of the present study, Katsuno *et al* (28) found that the tumor formation of ALDH$^+$ GC cells is more marked, compared with that of ALDH$^-$ GC cells in GC tissues and cell lines. In addition, Zhi *et al* (29) successfully acquired a GC stem-like cell population via ALDH activity detection. These findings indicate that ALDH may be a reliable marker for the acquisition of GCSCs.

The results of the present study showed that ~5% of the MFC cells were ALDH$^+$ in the total cell population, which is in accordance with the low percentage of CSCs in solid tumors (21). Consequently, ALDH$^+$ cells were defined as stem-like cells and ALDH$^-$ cells were defined as non-stemess cells. In order to isolate GCSCs and GCPCs from the stem-like cell population, our previously reported ALDH activity assay was used (20). The 1% of the ALDH$^+$ cells with the highest activity were selected and considered to be GCSCs. Although a subset of GCSCs in the ALDH$^+$ cells may be missed in the low

proportion selection method, cells acquired from the selection are more accurate and the data are more reliable. In addition, the 1% of $ALDH^+$ cells with the lowest activity were selected and considered to be GCPCs, as its degree of stemness was the weakest of the $ALDH^+$ cells and close to non-stemness cells ($ALDH^-$) at the differentiated stage of CPCs (30). Finally, the 1% of the $ALDH^-$ cells with the lowest activity were selected and defined as regular cancer cells.

Further analyses of the stemness-associated markers revealed that $CD44^+$ was expressed at high levels in all three cell subpopulations, suggesting that $CD44^+$ was not a suitable marker for MFC cells; however, the levels of $CD133^+$, OCT-4 and SOX-2 decreased with a decrease in ALDH activity, and were lowest in the $ALDH^{neg}$ cells. These results suggested that the activity of ALDH was positively correlated with the degree of stemness in the MFC cells, with the $ALDH^{high}$ and $ALDH^{low}$ cells being stem-like cells, and $ALDH^{neg}$ cells being regular cancer cells.

A sphere formation assay is one of the classic methods for detecting CSCs (6). The present study found that $ALDH^{high}$ and $ALDH^{low}$ cells formed tumor spheres, which suggested that the two types of cells have a certain degree of self-renewal ability. However, the volumes of the spheres were considerably lower for the $ALDH^{high}$ cells, compared with the $ALDH^{low}$ cells. In addition, the percentage of $ALDH^+$ cells in the spheres formed by the $ALDH^{low}$ cells was significantly lower, compared with the spheres formed by the $ALDH^{high}$ cells. These results suggested that $ALDH^{low}$ cells formred larger tumor spheres with a large number of $ALDH^-$ cells, consistent with the lower self-renewal but rapid differentiation abilities of CPCs. By contrast, the $ALDH^{high}$ sphere cells were comprised predominantly of $ALDH^+$ cells, suggesting that tumor spheres are generated by self-renewal. In addition, the volumes of the spheres were relatively low, indicating stable and slow proliferation of $ALDH^{high}$ cells, a typical characteristic of CSCs (21). However, $ALDH^{high}$ and $ALDH^{low}$ cells formed spheres under certain conditions, providing evidence that the purity of CSCs is low by sphere enrichment.

Various studies have suggested that CSCs have higher tumor-forming abilities, compared with other cell subpopulations (4,6,21). However, other studies have reported opposite results. Read *et al* (31) found that tumor-forming cells express markers of neural progenitor cells rather than stem cell markers in a mouse model of medulloblastoma . Ucar *et al* (17) found that the time for *in vivo* tumor formation of H522 cells with high ALDH activity is significantly longer, compared with that of cells with low ALDH activity.

Combined with the results of the present study, several novel perspectives have been suggested. The proportions of CSCs and CPCs are low in solid tumors, and CSCs in a resting state do not exhibit tumor formation ability in a short duration (21). However, tumors with pathological significance require numerous regular cancer cells to obtain a certain volume. Consequently, if the observational period is not long enough, it is possibly to falsely conclude that CSCs do not have stemness-associated properties. Although CPCs have limited self-renewal ability, they proliferate rapidly (27). When certain numbers of CPCs (5,000 $ALDH^{low}$ cells) were injected into mice, they showed high tumor-forming ability, as CPCs generate large numbers of regular cancer cells. When fewer tumor cells (500 $ALDH^{high}$ cells) were injected into mice, CSCs demonstrated a high tumor-forming ability when the observation period was long enough. However, mice injected with CPCs showed weaker tumor formation ability, compared with the CSC group, although tumors formed at an early stage in the CPC group, possibly due to the limited self-renewal ability of CPCs. The characteristics of $ALDH^{neg}$ cells were in accordance with those of regular cancer cells, with no self-renewal ability and limited proliferative ability (27). Therefore, tumor formation ability was significantly lower, compared with that of $ALDH^{high}$ and $ALDH^{low}$ cells. Thus, it was hypothesized that CSCs, CPCs and regular cancer cells are cell subpopulations with differing differentiation stages and different proliferative abilities in tumor tissues.

In conclusion, the results of the present study suggested that GCSCs and GCPCs are two stem-like subgroups with different characteristics, and these two subgroups exist in the stem-like cells of MFC cells. Excluding GCPCs from stem-like cells to achieve a higher purity of GCSCs may benefit future investigations of GCSCs and CSCs.

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