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Establishing a mouse disease model for future studies regarding gastric anti-cancer therapies

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Abstract

The identification of cancer cell subpopulations that display stem cell-like features may lead to new insights into gastric carcinogenesis and may provide novel treatment strategies. Emerging studies identify a small subpopulation of cells in human cancer capable to initiate tumor growth and responsible for sustain tumor self-renewal. In our study we aimed to identify and isolate gastric cancer stem cell based on $CD44^{+}CD24^{low}$ phenotype, with the goal of establishing a mouse disease model for future studies regarding anti-cancer therapies. For this purpose, $1x10^6$, $1x10^5$, $1x10^4$ CD44 positive cells were injected subcutaneous in severe combined immunodeficient (SCID) mice and the results were compared to those obtained using unseparated cells. The results showed that CD44 positive cells fail to initiate tumors when injected in small number and, therefore, do not possess gastric cancer stem cells properties. Tumor were obtained only when $1x10^6$ NCI-N87 CD44+ cells were injected, the percentage of tumor induction (87.5%) being similar with the one obtained when unseparated cells were used.

In conclusion, CD44 positive cells do not display tumor-initiating properties *in vivo*. Further experiments using two or multiple markers as selection criteria, would be useful to specifically identify and purify gastric cancer stem cells.

Keywords: animal models, cancer, gastric cancer, CD44

1. Introduction

Gastric cancer is a common malignancy and is one of the leading causes of global cancer mortality. In Romania, it is the third cause of cancer death in men and the fifth in women [1]. Despite a declining incidence in recent decades, gastric cancer remains a major health problem with a survival rate of 5 years, below 30% [2].

The origin of human gastric cancer has not been elucidated yet, although there is convincing evidence that most cancers are derived from stem cells [3]. Currently, there are two main theories: the stochastic and the hierarchical models that try to clarify the initiation and progression of malignant tumors. In the stochastic model the cells share the same capabilities to initiate and promote tumor growth, but their behavior depend on both, intrinsic and extrinsic factors that can affect randomly the cells into the tumors [4], [5]. In the hierarchical model, only a small fraction of cells known as cancer stem cells (CSCs) possess self-renewal capacity and tumorigenic potential. These CSC display features of normal stem cells, been able to self-renew and differentiate in different cells type. This stochastic theory suggests that CSC undergo asymmetric division giving rise to progenitors with different tumorigenic potential that form the tumor bulk [6], [7]. Furthermore, CSCs are considered to be responsible for drug resistance, through drug-efflux mechanisms and also play an important role in tumor relapse and metastasis [8], [9].

In solid tumors, the first evidence of the existence of cancer stem cells was provided Al-Hajj and collaborators; they identify and isolate from breast tumor a subpopulation of cells that had tumor initiation capacity *in vivo*, whereas most of the cells of the tumor mass had no ability to form new tumors, even when injected in higher number. The tumor cell population was identified based on CD44, CD24 cell surface markers (CD44 +, CD24-/phenotype). This population could initiate tumors in immunocompromised mice even when a very small number of cells (100 cells) are transplanted in murine model 12629218. Currently, a series of specific markers for tumor stem cells were described in various types of solid tumors (Table 1).

Table 1. Cancer stem cells phenotype in various types of solid tumors

Tumor type	Specific markers	Reference
Breast cancer	$CD44^{+}$ $CD24^{-/low}$	[10]
Prostate cancer	CD44+/alpha2beta1hi/CD133+	[11]
Lung cancer	SP-$C^{pos}$$CCA^{pos}$Lox-K-ras DPCs	[12]
Pancreatic cancer	CD133(+) CXCR4(+); CD44(+)CD24(+)ESA(+)	[13,14]
Cancer gastric	$CD44^{+}$ /SP	[15]
Liver cancer	$CD133^{+}$ / SP	[16]
Brain tumors	CD133+	[17]
Colon cancer	$CD133^{+}$ /	[18]
Ovarian cancer	ALDH+ CD133+	[19]
Melanomas	$CD20^{+}$	[20]

In gastric cancer, recent data supports the existence of tumor stem cells. In their study, Takaishi et al. isolated a subpopulation of CD44+ cells starting from three cell lines: MKN-45, MKN-74, and NCI-N87. The cells identified based on CD44 surface antigen were able to form spheroid colony *in vitro* and have tumorigenic capacity when injected into the SCID immunodeficient mice [15]. In another study, Fuduka K et al., showed that a side population (SP) cells, characterized by the ability to eliminate Hoechst 33342 dye had the properties characteristic of gastric tumor stem cells [21]. Inconsistencies between these two studies require further analysis.

We assumed that the heterogeneous nature of gastric cancer might be an in part explained by the presence of gastric cancer cells with stem-like features. Developing effective anti-cancer therapies requires a deep understanding of the mechanisms of tumor progression and drug resistance. In our study we aimed to identify and isolate gastric cancer stem cell based on $CD44^{+}CD24^{low}$ phenotype, with the goal of establishing a mouse disease model for future studies regarding anti-cancer therapies.

2. Materials and methods

Cell lines and primary cell culture

The cultures used in the experiments were: the primary cell culture GECS59, derived from a patient with gastric adenocarinoma, and two cell lines NCI-N87 (catalogue no. CRL-5822) and AGS (catalogue no. CRL-1739) were purchased from from American Type Culture Collection (USA).

Two gastric cancer cell lines NCI-N87 (catalogue no. CRL-5822) and AGS (catalogue no. CRL-1739) were purchased from American Type Culture Collection (USA). The AGS cells were grown in Ham's F12 medium (Sigma Aldrich), NCI-N87 cells were cultured in RPMI 1640 and the primary cultures were grown in Dulbecco's modified essential medium, all supplemented with 10% fetal bovine serum. The cell cultures were incubated at 37°C and 5% CO2, in a humidified atmosphere.

Flow cytometry

Cells were enzymatically detached with trypsin-EDTA (0.25%) (Gibco), washed twice with phosphate-buffered saline, stain with anti-human monoclonal antibody CD44-PE, EpCAM (CD326)-FITC, CD24-FITC, CD105- FITC, CD133- FITC (BD Pharmingen, USA), and incubated in the dark, at room temperature for 30 minutes. The analyze was performed on Epics Beckman Coulter flow cytometer and the data were analyzed using FlowJo software.

Magnetic activated cell sorting (MACS)

For magnetic separation of CD44 positive cells we used the MACS kit from Milteny Biotech. In this propose, AGS, NCI-N87 and GECS59 cells were labeled with anti-CD44 antibodies coupled with magnetic beads. For positive selection, the CD44 labeled cells coupled with magnetic beads were retained on column that was placed in the magnetic field of a MACS separator. Subsequently, after three consecutive washes with MACS buffer (PBS, 0.5% bovine serum albumin, and 2 mM EDTA) the unlabeled cells were removed and the CD44+ cells were harvested by removing the column from the magnetic field. After isolation, the cells were counted and the purity of the CD44+ fraction was determinate by flow cytometry.

Tumor sphere formation

The CD44 positive and CD44 negative cells were cultured in serum-free RPMI-1640 medium supplemented with 20 ng/mL EGF, 10 ng/mL FGF growth factors and incubated at 37°C with 5 % CO_2. The tumor sphere formation was evaluated for four weeks and the images were acquired with an inverted Zeiss Axio Observer D1 microscope equipped with an image acquisition system.

***In vivo* tumor formation**

The tumorigenic capacity of CD44 positive cells was tested on immunodeficient mice NOD/SCID (NOD.C.B-17Prkdc^{scid}/J), characterized by the absence of functional T cells and B cells. The mice were purchased from the Jackson Laboratory and were maintained in our husbandry under specific pathogen-free conditions. For cell transplantation experiments the six-weeks-old mice were subcutaneously injected into the dorsal flank with 100 µL cell suspension. The mice were divided in several groups (n=8 mice for each lot), and injected with a different number of CD44 positive cells: (1) NCI-N87: $1x10^6$, $1x10^5$, $1x10^4$ cells; (2) AGS: $1x10^6$, $1x10^5$, $1x10^4$ cells and (3) GECS59: $1x10^6$, $1x10^5$, $1x10^4$ cells.

This study was approved by the Ethics Committee of Stefan S. Nicolau Institute of Virology.

3. Results and discussion

3.1 Phenotypic characterization of gastric cancer cell lines.

In order to identify and isolate gastric cancer stem cells, we investigate cell surface expression of antigens associated with stem cell phenotype in two gastric cancer cell lines: NCI-N87 and AGS and on the primary culture GECS59 derived from poorly differentiated gastric adenocarcinomas. The AGS cell line is derived from a poorly differentiated primary gastric adenocarcinoma, whereas NCI-N87 was established from a liver metastasis of a well-differentiated gastric carcinoma [22].

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The AGS and NCI-N87 cultures were phenotypic characterized by analysis of cell surface markers CD44, EpCAM, CD24, CD105 and CD133, markers frequently expressed by epithelial tumor stem cells (Figure 1 a, b).

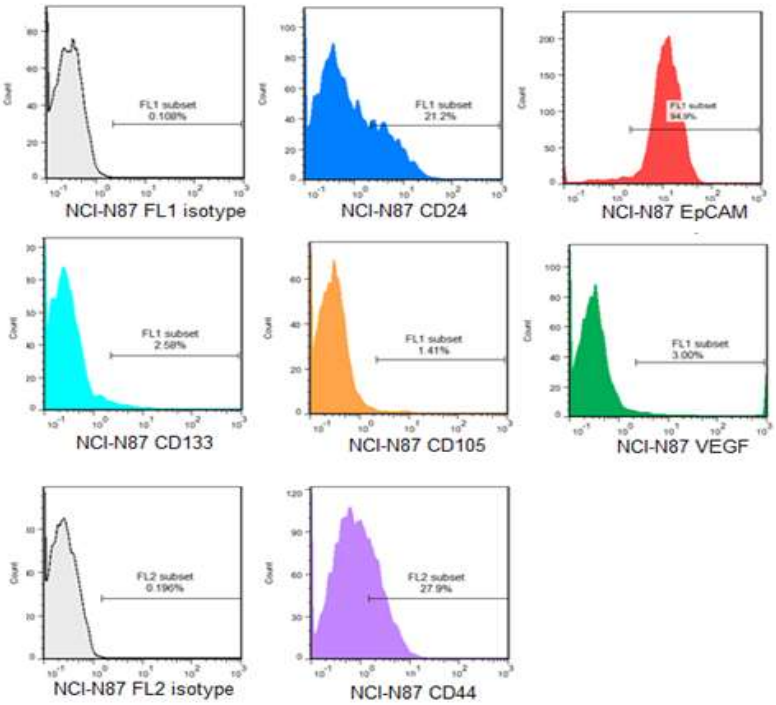

Figure 1a. Flow cytometric analysis of surface expression markers
CD44, EpCAM, CD24, CD105, CD133 in NCI-N87 cell line

Based on the obtained results, the following observations can be made: (1) > 94% of the cells are positive for EpCAM; (2) 21.2% of NCI-N87cells and 54.4% of AGS cells are positive for CD24, and (3) 27.9% of NCI-N87 cells and 14% of AGS cells presents CD44 surface marker. Both cell lines are negative for CD105, CD133 antigens and VEGF.We further investigated the EpCAM, CD24 and CD44 expression in the primary culture GECS59 initiated from well differentiated gastric adenocarcinoma fragments (Figure 2).

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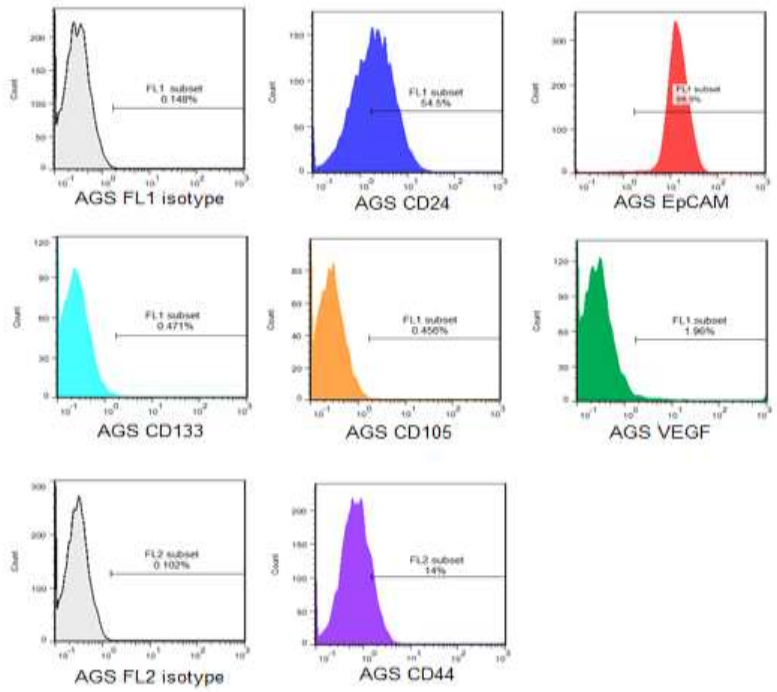

Figure 1b. Flow cytometric analysis of surface expression markers CD44, EpCAM, CD24, CD105, CD133 in AGS cell line.

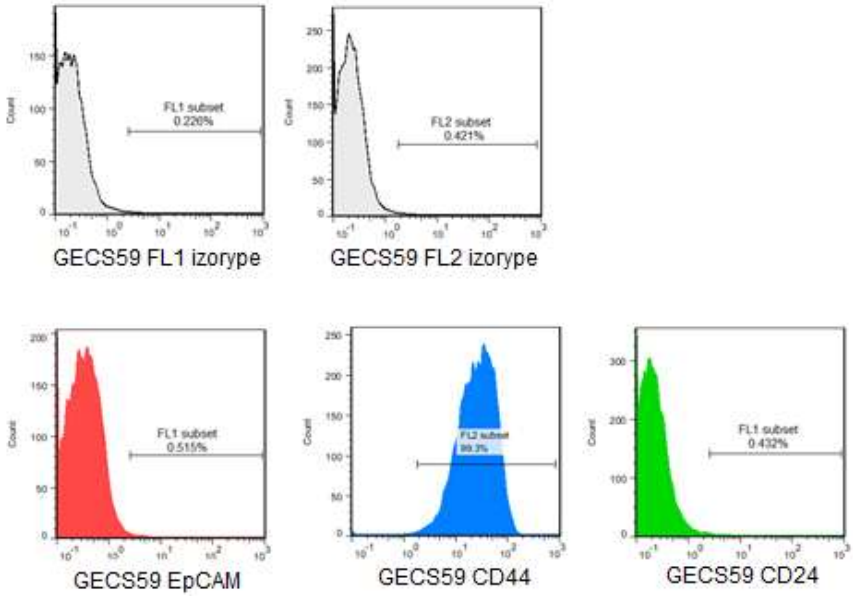

Figure 2. Flow cytometry analysis of surface expression markers CD44, EpCAM, CD24 in primery cultures GECS59 p4.

3.2 Isolation of putative gastric tumor stem cells using MACS technology

To investigate the tumorigenic potential of CD44 positive gastric cancer cells, we performed a magnetic separation using MACS technology.

CD44 has been identified as a potential marker for putative gastric cancer stem cells, been able of self-renewal and to induce tumor formation into immunodeficient mice. Also, the CD44+ gastric cancer cells displayed an increased resistance to chemo- or radiotherapy [15]. CD44 antigen is a surface glycoprotein involved in cell to cell interactions, cell adhesion and migration. CD44 is a receptor for hyaluronic acid, but can also interact with other ligands such as osteopontin, collagen and matrix metalloproteinase (MMPs). The CD44 gene transcript is at least partially activated by beta-catenin and the Wnt signaling pathway (also involved in tumor progression). CD44+ gastric tumor cells have self-renwal capacity and produce differentiated progenitors [15]. This protein is involved in a series of cellular functions, including cell growth, survival and differentiation [23] lymphocyte activation [24] and tumor metastasis [25].

After phenotypic characterization, the AGS and NCI-N87 cells were magnetically separated based on CD44 marker. Cells were subjected to two successive round of magnetic separation using CD44 antibodies. Following positive selection, the selected population had a purity of 58.5% (exemplified for AGS in figure 3). To increase purity, the cells were transferred to a second column, and the fraction purity increased to 94.3% following this procedure.

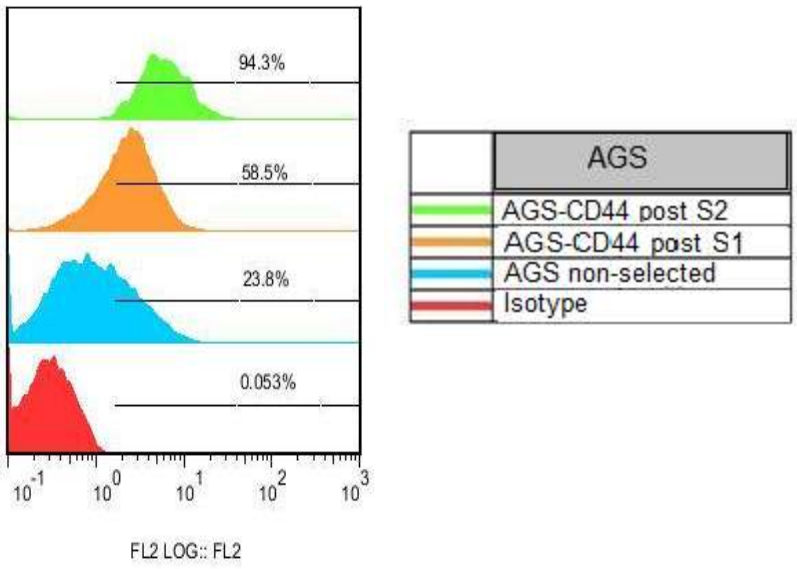

Figure 3. Purity of the CD44 cell fraction after two rounds of magnetic separation.

After magnetic separation, the phenotype of separated cells was tested using surface markers associated with stem cells. Our results indicate that isolated cells were >99% positive for CD44 and present variable percentage of CD24 expression, between 6.25 and 57.1% in NCI-N87 and respectively AGS cells (figure 4).

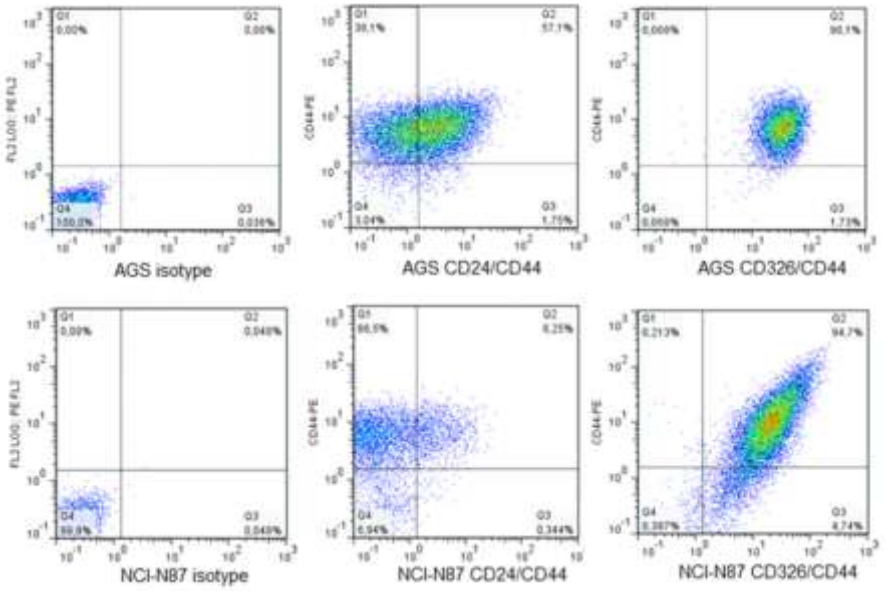

Figure 4. Phenotype of separated CD44+ form AGS and NCI-N87 gastric cell lines.

3.3 Tumorigenic potential of presumptive gastric cancer stem cells.

The identification of cell populations that harbors cancer stem cells characteristics may be challenging. Different approaches have been used to highlight the cancer stem cells – the most commonly used *in vitro* methods implies sphere formation assays, serial colony-forming unit (CFU) assays or label-retention assays. *In vivo* assay, based on serial transplantation experiments in animal models remain the gold standard method for identifying stem cells.

All this methods are based on the unique properties of stem cells to undergo self-renewal and to eliminate Hoechst 33342. For the *in vitro* method we used sphere formation assays that involve the culture of selected cell fraction under non-adherent conditions, in a serum-free medium supplied growth factors EGF and FGF.

***In vitro* sphere formation assays.** For AGS and NCI-N87cells lines, the CD44+ cells were obtained through magnetic separation, whereas primary culture GECS59 was used without magnetic separation due to the high percentage of CD44 positive cells (99%).

The tumorigenic ability of the selected cells was tested *in vitro* by cultivating the cells in RPMI-1640 serum free medium, supplemented with EGF and FGF growth factors. The tumor sphere formation was evaluated for four weeks and the images were acquired with an inverted Zeiss Axio Observer D1 microscope equipped with an image acquisition system.

The AGS and GECS59 cells presented an enhanced colony formation capacity compare with CD44+ cells isolated from NCI-N87 cell line (Figure 5). The CD44 negative fraction did not show the ability to form spherical colonies. Our results showed that CD44+ cells from poorly differentiated carcinomas (AGS, and GECS59) have a higher capacity to form spherical colonies compared to those isolated from well-differentiated carcinomas (NCI-N87).

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Figure 5. Spherical colonies formed by CD44 + cells separated from primary; microscopy images: visible, 63X.

***In vivo* xenograft assay**

To evaluate *in vivo* tumor formation capability of selected population, $1x10^6$, $1x10^5$, $1x10^4$ CD44 positive cells were injected subcutaneous in severe combined immunodeficient (SCID) mice and the results were compared to those obtained using in unseparated cells.

Tumor progression was monitored weekly by palpation and measurements performed by the same specialist. Tumors were obtained only when $1x10^6$ NCI-N87 CD44+ cells were injected (Table 2). The percentage of tumor induction (87.5%) was similar with the one obtained when unseparated cells were used. In conclusion, CD44 positive cells do not display tumor-initiating properties *in vivo.*

Table 2. Tumorigenic capacity of CD44 positive and unseparated gastric cancer cells at 10 weeks post-inoculation.

Cells		$1x10^6$	$1x10^5$	$1x10^4$
NCI-N87	**CD44 positive**	7/8	2/8	0/8
	unseparated cells	7/8	2/8	0/8
AGS	**CD44 positive**	0/8	0/8	0/8
	unseparated cells	7/8	7/8	0/8
GECS59	**CD44 positive**	1/8	0/8	0/8
		NA	NA	NA

Regarding the tumorigenicity of the injected cell lines, it is observed that both cell lines form tumors from the first two weeks. Xenografts formed by injection of NCI-N87 are small, compact and well-defined, unlike those obtained by injection of AGS that are larger, well-vascularized and diffuse. In terms of the number of cells to be used for tumor induction *in vivo*, the data in the literature are contradictory. Most authors use a large number of cells, around 10^7, to get tumors quickly in 3-5 weeks [26], [27]. Takaishi S. and colab. were the first who reported the use of a small number (200 cells) of CD44+ tumor cell subtype with increased autoregeneration and proliferation properties in order to generate tumors in immunodeficient animals [15], [28], [29]. However, our results indicated that $CD44^+CD24^{low}$ positive cells fail to induce tumors in immunodeficient mice even when injected in higher number. Our findings are consistent with the ones achieved by researchers from the

University of Naples which sustained that CD44 is not a marker of gastric tumor stem cells and demonstrated this by injecting mice with purified $CD44^+CD133^+$ / $CD44^+CD133^-$ and unsorted cells. They used two animal models for this purpose: NOD/SCID and Nude mice injected subcutaneously or intraperitoneally. Their results showed that animals injected with $CD44^+CD133^+$ / $CD44^+CD133^-$ cells (between $3x10^4$ and $1.5x10^5$) did not produce tumors, but mice injected with unseparated cells ($5x10^5$-$1.5x10^6$) developed tumors [30].

4. Conclusion

Our experiments have demonstrated that less than $1x10^6$ cells, even CD44 positive, do not induce tumor formation in immunodeficient laboratory mice than probably in a very long time. Consequnetly, establishing a mouse disease model for future studies regarding gastric anti-cancer therapies, will have to be based on injection of higher number of cells or CSC isolated using two or multiple markers as selection criteria. Further experiments would be useful for the identification of molecules expressed in gastric CSC that would allow the purification of the subpopulation of cancer cells that can induce tumor formation.

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