CD49f\textsuperscript{high} Cells Retain Sphere-Forming and Tumor-Initiating Activities in Human Gastric Tumors

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Abstract
Identification of gastric tumor-initiating cells (TICs) is essential to explore new therapies for gastric cancer patients. There are reports that gastric TICs can be identified using the cell surface marker CD44 and that they form floating spheres in culture, but we could not obtain consistent results with our patient-derived tumor xenograft (PDTX) cells. We thus searched for another marker for gastric TICs, and found that CD49f\textsuperscript{high} cells from newly-dissected gastric cancers formed tumors with histological features of parental ones while CD49f\textsuperscript{low} cells did not when subcutaneously injected into immunodeficient mice. These results indicate that CD49f, a subunit of laminin receptors, is a promising marker for human gastric TICs. We established a primary culture system for PDTX cells where only CD49f\textsuperscript{high} cells could grow on extracellular matrix (ECM) to form ECM-attaching spheres. When injected into immunodeficient mice, these CD49f\textsuperscript{high} sphere cells formed tumors with histological features of parental ones, indicating that only TICs could grow in the culture system. Using this system, we found that some sphere-forming TICs were more resistant than gastric tumor cell lines to chemotherapeutic agents, including doxorubicin, 5-fluorouracil and doxifluridine. There was a patient-dependent difference in the tumorigenicity of sphere-forming TICs and their response to anti-tumor drugs. These results suggest that ECM plays an essential role for the growth of gastric TICs, and that this culture system will be useful to find new drugs targeting gastric TICs.

Introduction
Gastric adenocarcinomas are the second leading cause of cancer-related mortality in the world [1]. Although early diagnosis by endoscopic screening and surgical treatment give best therapeutic opportunity for gastric cancer patients, 20 to 40% of the tumor have been diagnosed at advanced stages requiring additional systemic treatments. In such cases, tumor heterogeneity including presence of metastatic and/or chemo-resistant subclones is a major obstacle to cure the disease. The cancer stem cell model may give insights and bases to understand the tumor heterogeneity and to establish new strategies to treat them.

Cancer stem cells or tumor-initiating cells (TICs) are cells which possess the capacity to self-renew and to generate heterogeneous lineages of neoplastic cells that constitute the cancer [2]. TICs have been identified in many neoplasms, including tumors in the mammary gland [3], brain [4], prostate gland [5], colon [6,7], pancreas [8], head and neck [9], and liver [10]. These TICs comprise about 1–5% of the whole tumor cells, and can form tumors again even when most cells are eliminated, for example, by chemotherapy. Thus it is important to identify gastric TICs and to characterize them to develop new therapies targeting them. There are several reports on the identification of gastric TICs, mostly using the cell surface marker CD44 [11–14]. A recent study demonstrated that CD44 played an important role in the tumorigenesis [15], but another study showed that CD44 was strongly expressed by both premalignant and malignant gastric epithelial cells, though it was rarely expressed in normal gastric mucosa [16]. Thus it remains to be examined whether CD44 is the best marker for gastric TICs.

In the present study, we could not obtain consistent results that CD44-positive gastric tumor cells were tumorigenic by analyzing patient-derived tumor xenograft (PDTX) cells. We thus looked for another marker for gastric TICs, and found that they strongly expressed CD49f, a subunit of laminin receptors, which has been used to identify TICs in tumors of the prostate gland [17], mammary gland [18], brain [19] and colon [20]. We established a primary culture system for PDTX cells where only CD49f\textsuperscript{high} cells could grow on extracellular matrix (ECM) to form ECM-attaching spheres.
spheres, a feature of stem cells [21]. These CD49\textsuperscript{high} sphere cells formed tumors with histological features of parental ones when injected into immunodeficient mice, indicating that only TICs could grow in culture. We also found that some CD49\textsuperscript{high} sphere-forming TICs were more resistant to chemotherapeutic agents than gastric tumor cell lines, although there was a patient-dependent difference on their response. We thus conclude that CD49f is a promising marker for gastric TICs, and that this culture system will be useful to find new drugs targeting gastric TICs.

**Materials and Methods**

**Tumor Tissues and PDX Lines**

Gastric tumor tissues were obtained with informed consent from patients who underwent surgical resection at Tokyo Medical and Dental University Hospital and Asan Medical Center Hospital between 2008 and 2012, and the study was approved by the Medical Research Ethics Committee for Genetic Research of Tokyo Medical and Dental University, and the Institutional Review Board of Asan Medical Center. Written informed consent was obtained from each patient for the use of his/her tumor tissue for this research in both hospitals. Freshly isolated tumor samples were cut into small pieces and transplanted subcutaneously to KSN and BALB/c nude mice at 4–6 weeks old (Japan SCL, Inc., Shizuoka, Japan and Central Lab. Animal Inc, Seoul, Korea, respectively). The animals were housed in specific pathogen-free animal facilities in accordance with the Guideline for Care and Use of Laboratory Animals of the respective Institutional Animal Care and Use Committees, and the research was approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University, and the Institutional Animal Care and Use Committee of Asan Medical Center. When gastric tumors grew in nude mice, some tissues were stored in liquid nitrogen, and were used at early passages (p<6) because tumors grew faster and became more aggressive with the increase in passage number. Gastric tumor tissues just after surgical operation were also used for the analysis.

**Dissociation and Staining of Tumor Cells for FACS Analysis**

Tumor tissues were disaggregated into single cells for FACS (fluorescence-activated cell sorter) analysis. Tissues were cut with scalpels into small fragments, washed thoroughly with CMF-PBS, treated with 0.05% trypsin-0.53mM EDTA with 0.01% DNase I (fluorescence-activated cell sorter) analysis. Tissues were cut with small pieces and transplanted subcutaneously to KSN and BALB/c nude mice at 4–6 weeks old (Japan SCL, Inc., Shizuoka, Japan and Central Lab. Animal Inc, Seoul, Korea, respectively). The animals were housed in specific pathogen-free animal facilities in accordance with the Guideline for Care and Use of Laboratory Animals of the respective Institutional Animal Care and Use Committees, and the research was approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University, and the Institutional Animal Care and Use Committee of Asan Medical Center. When gastric tumors grew in nude mice, some tissues were stored in liquid nitrogen, and were used at early passages (p<6) because tumors grew faster and became more aggressive with the increase in passage number. Gastric tumor tissues just after surgical operation were also used for the analysis.

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**Cell Culture**

Tumor samples were dissociated into single cells as described above. The resulting cells were cultured on rat tail collagen gel or Matrigel in a serum-free condition. We have previously established a primary culture system for rat gastric epithelial cells [22], where we used F-12 medium supplemented with EGF, insulin, hydrocortisone, transferrin, and cholera toxin. We found in the present study that some human gastric tumor cells grew slowly in the medium. We thus compared their growth in various media for normal human epithelial cells provided from Lonza (Basel, Switzerland), and found that cells grew best in REBM medium which contains EGF, insulin, hydrocortisone, transferrin, triiodothyronine, and epinephrine. In the present study, cells were cultured in REBM medium with B27 supplement (Invitrogen, Carlsbad, CA, USA), FGF-2 (ReproCELL, Kanagawa, Japan), Y-27632 ROCK inhibitor (Merck Millipore) and gastrin (Sigma-Aldrich), by modifying the method developed for mouse gastric epithelial cells [23].

**RT-PCR**

The gene expression profiles of gastric cancer cells were examined by RT-PCR. Total RNAs were extracted from cells or tissues by using the Allprep DNA/RNA/Protein Mini kit (Qiagen, Valencia, CA, USA). RNAs were resuspended in RNase-free water and first-strand cDNAs were synthesized by using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen). The primer sequences and PCR conditions are described in Table S1. GAPDH was used as a control to compensate for varying efficiencies of extraction and reverse transcription. The PCR products were separated by electrophoresis on 2.0% agarose gels in 0.5 X TAE buffer.

**Effect of Chemotherapeutic Agents on the Growth of TICs in Primary Culture**

HGC-1, HGC-2 and HGC-4 tumors were dissociated into single cells as described above, and seeded on collagen gel in 24-well plates at 50,000 cells per well with REBM-based culture
medium, and their growth was examined after 2 weeks. MKN45 and MKN74 human gastric cancer cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum on plastic, but we found that both cells proliferated rapidly in the RBM-based serum-free condition used for culture of HGC-1, HGC-2 and HGC-4 cells. We thus used both cells as controls. Since MKN45 cells proliferated more rapidly than MKN74 cells, we seeded MKN45 cells more sparsely (1,000 cells per well) than MKN74 cells (3,000 cells per well) to give enough space for the cells to keep growing for 2 weeks.

The cells were plated with 0.5 ml media on day 0, and they were treated with doxorubicin (DXR), 5-fluouracil (5-FU), and doxifluridine (DXF) (Wako Pure Chemical, Osaka, Japan) on day 1, by adding 0.5 ml media containing v2 concentrated chemicals. 5-FU and DXF were dissolved in dimethyl sulfoxide (DMSO), while DXR in water. Thus DMSO (final concentration = 0.1%) was added to each well when cells were treated with 5-FU or DXF. After 2 weeks, cell growth was determined by using 3-(4,5-di-

Statistical Analyses

The results were statistically analyzed using the non-parametric Mann-Whitney’s U test or Student’s t-test. For the analysis of data in tables, Chi-square test was used. A P values of <0.05 were considered statistically significant.

Results

CD49f is a useful Marker for Gastric TICs

We first established PDTX lines by subcutaneously injecting newly-dissected human gastric tumor tissues into nude mice, because such lines have been used to identify TICs in colon [6,25] and lung [26] cancers. We established 5 PDTX lines (HGC-1 to -5, Table 1), and confirmed that histological features of parental tumors were maintained in PDTXs even when tissues were passaged several times (data not shown).

CD44 has been reported as a marker for gastric TICs [11–14]. We thus compared tumorigenicity of CD44-positive and -negative cells in the PDTXs, and found that not only CD44high cells but also CD44low cells were tumorigenic in 3 PDTX lines (Figure S1 and Table S2). Rocco et al. [27] also reported that CD133+ cells were more tumorigenic than CD133- cells, andsorting, the cloning efficiency was usually less than 10%, but CD49f high cells attached to ECM to form spheres in primary cultures [21].

CD49f is Localized at the Epithelial-stromal Interface in the Normal Gastric Mucosa, but is Found on Apical, Lateral and Basal Surfaces in Some Tumor Cells

We immunohistochemically examined the localization of CD49f in the tumor and non-tumor tissues in the stomach, and found CD49f at the epithelial-stromal interface in the normal gastric mucosa (Figure 2A). The result is consistent with the report that CD49f functions as a subunit of receptors for laminins, major proteins in the basal lamina. Gastric mucosa with chronic gastritis showed expression of CD49f along the basal lamina of the gastric foveolae as in normal ones (Figure 2B), and similar expression pattern was found in the intestinal metaplasia (Figure 2C). In tumor cells, however, expression of CD49f was diversified. It was found at the epithelial-stromal interface in some tumor cells (Figure 2G), but it was detected on apical, lateral and basal surfaces in some cells, and some cells only weakly expressed it (Figure 2E). It is interesting that in gastric dysplasias adjacent to carcinoma, CD49f was found on not only basal but also lateral surfaces, as in gastric carcinomas (Figure 2D), indicating that disorganized expression of CD49f might not be a result but a cause of tumorigenesis. A small proportion of tumor cells expressing CD49f all along the cell membrane (Figure 2E) may possibly represent gastric TICs.

CD49fhigh Cells Attach to ECM to form Spheres in Primary Culture

TICs have been reported to grow in serum-free conditions to form spheres in several solid tumors, and sphere formation assay is widely used to detect TICs [21,29]. We thus cultivated gastric tumor cells by modifying single cell culture system for mouse gastric epithelial cells [23]. We found that ECM (collagen gel or Matrigel) was necessary for HGC-1, HGC-2 and HGC-4 PDTX cells to grow, and that they exhibited sphere-formation, a feature of stem cells [21] in culture. We thus used these PDTXs at early passages (P<6) to investigate further the relationship among CD49f expression, sphere formation and tumorigenesis.

When HGC-1 gastric tumor cells were cultured after FACSsorting, the cloning efficiency was usually less than 10%, but CD49fhigh cells attached to collagen gel to form spheres (Figure 3A), whereas cell growth was not observed in culture of CD49flow cells (Figure 3B). These spheres were consisted of epithelial cells closely attached to each other with occasional PASC-positive droplets (Figure 3C). It was difficult to cultivate FACS-sorted HGC-2 and HGC-4 cells, and we were not certain whether only CD49fhigh cells could form spheres in their culture. We thus concluded that some gastric TICs proliferated to form spheres in primary culture as has been reported in other TICs.

Only CD49fhigh Sphere-forming TICs can Grow when Unsorted Tumor Cells are Cultured

One possible reason why FACS-sorted HGC-2 and HGC-4 cells did not grow in culture is that they may need autocrine factors to grow, and their concentrations may be less than the threshold value when cells were sparsely cultured (usually we cultured FACS-sorted cells at 10 cells/mm², because of technical reasons). If this hypothesis is correct, cells may grow when cultured
### Table 1. Case description and tumorigenic activity of CD49$^{\text{high}}$ and CD49$^{\text{low}}$ tumor cells.

| Patient number | Age/sex | Site       | Bormann type | Differentiation of tumors | PDTX lines | Ratio of CD49$^{\text{high}}$ cells (%) | Number of cells injected (number of tumors/number of injection) | Differentiation of tumors formed by sorted cells |
|---------------|---------|------------|--------------|--------------------------|------------|----------------------------------------|---------------------------------------------------------------|-------------------------------------------------|
| 1             | 80/M    | Fundic     | II           | Moderate                 | HGC-1      | 2.7                                    | 3,000 (1/1)                                                   | 6,000 (0/1)                                          Moderate                                      |
| 2             | 75/M    | Fundic     | III          | Well                     | HGC-2      | 6.5                                    | 3,000 (3/3)                                                   | 3,000 (1/2)                                          Well                                          |
| 3             | 72/M    | Pyloric    | IV           | Poor                     | HGC-3      | 16.4                                   | 3,000 (1/1)                                                   | 3,000 (0/2)                                          Poor                                         |
| 4             | 71/M    | Fundic     | II           | Moderate                 | HGC-4      | 6.2                                    | 3,000 (1/1)                                                   | 3,000 (0/1)                                          Moderate                                    |
| 5             | 68/F    | Pyloric    | II           | Poor                     | HGC-5      | 10.8                                   | 3,000 (3/3)                                                   | 3,000 (1/4)                                          Poor                                         |
| 6             | 66/M    | Pyloric    | II           | Moderate                 |            | 14.8                                   | 3,000 (1/1)                                                   | 3,000 (0/1)                                          Moderate                                    |
| 7             | 74/M    | Antrum     | III          | Poor                     |            | 20.8                                   | 3,000 (1/1)                                                   | 3,000 (1/1)                                          Poor                                         |
| 8             | 64/M    | Antrum     | II           | Moderate                 |            | 8.8                                    | 3,000 (1/2)                                                   | 3,000 (0/1)                                          Moderate                                    |
| 9             | 40/M    | Fundic     | III          | Poor                     |            | 5.3                                    | 3,000 (1/1)                                                   | 3,000 (0/1)                                          Poor                                         |
| 10            | 72/M    | Antrum     | III          | Poor                     |            | 3.0                                    | 1,000 (1/1)                                                   | 1,000 (0/1)                                          Poor                                         |
| 11            | 70/M    | Fundic     | II           | Moderate                 |            | 10.9                                   | 3,000 (1/1)                                                   | 3,000 (0/1)                                          Moderate                                    |
| 12            | 68/M    | Antrum     | III          | Poor                     |            | 1.1                                    | 3,000 (1/1)                                                   | 3,000 (0/1)                                          Poor                                         |
| 13            | 68/M    | Antrum     | II           | Poor                     |            | 1.6                                    | 3,000 (1/2)                                                   | 3,000 (0/2)                                          Poor                                         |
| 14            | 76/F    | Pyloric    | III          | Poor                     |            | 2.8                                    | 3,000 (2/2)                                                   | 3,000 (0/2)                                          Poor                                         |
| 15            | 71/M    | Pyloric    | III          | Moderate                 |            | 5.5                                    | 3,000 (1/2)                                                   | 3,000 (0/2)                                          Moderate                                    |
| Total         |         |            |              |                          |            | 7.9 ± 6.1                             | 3,000 (20/23)***                                             | 3,000 (3/22)***                                    |

****P<0.001 by Chi-square test.
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closely. We thus tried to cultivate tumor cells much more closely (usually 300 cells/mm²) by seeding them without FACS-sorting. When dissociated HGC-1 and HGC-4 tumor cells were cultured on collagen gel without FACS-sorting, many cells formed spheres, whose diameter increased greatly in 2 weeks (Figure 3D), consistent with the above assumption. When dissociated HGC-2 cells were cultured without FACS-sorting on Matrigel or collagen gel, some HGC-2 cells attached closely to ECM to form a thin layer, and some cells formed spheres on the cell layer. In culture of HGC-2 cells, some flat cells slowly proliferated in the first 2 weeks, but they stopped growth soon, while sphere-forming cells kept growing to form large spheres after 6 to 8 weeks (Figure 3E). Thus the growth pattern of HGC-2 tumor cells was different from that of HGC-1 and HGC-4 cells, though all three tumor cells were similar in that ECM was necessary for the tumor cells to grow to form spheres in culture. We examined features of HGC-2 sphere cells at 8 weeks when pure sphere-forming cells could be easily obtained.

When FACS-sorted CD49fhigh HGC-1 cells were cultured for 2 weeks, the ratio of CD49fhigh cells was decreased to about 50% (Figure 3F), indicating that CD49fhigh cells differentiated into both CD49fhigh and CD49flow cells in vitro. When unsorted HGC-1 cells were cultured, the ratio of CD49fhigh cells was significantly increased about 10-times from 3 to 34%. Also it was increased about 7-times from 6 to 42% in culture of unsorted HGC-4 cells in 2 weeks (Figure 3F). The ratio of CD49fhigh cells in total cells was similar when sorted CD49fhigh HGC-1 cells or unsorted (mostly CD49flow) HGC-1 cells were cultured. This indicates that only CD49fhigh cells could grow to form spheres when unsorted HGC-1 tumor cells were cultured. In culture of unsorted HGC-2 tumor cells, the ratio of CD49fhigh cells was significantly increased more than 10-times from 6 to 88% in 8 weeks. Thus all these tumor cells retain similar features that only sphere-forming CD49fhigh cells could grow in vitro, but their growth pattern differed depending on PDTX lines. These sphere-forming cells as well as PDTXs strongly expressed BMI1, POU5F1 and SOX2 (Figure S3), which are reported to be strongly expressed by stem cells [30].

We then examined tumorigenicity of these sphere-forming CD49fhigh cells. We found that sphere cells obtained by primary culture of unsorted HGC-1 tumor cells were strongly tumorigenic because subcutaneously injection of 3,000 sphere cells always formed tumors and injection of 10 cells sometimes formed a tumor in NOD-SCID mice (Table 2). The tumors exhibited histological features of the parental one (Figure 4), indicating that the CD49fhigh sphere-forming cells retained differentiation potency of TICs in the original tumor. Sphere cells obtained by culture of unsorted HGC-2 and HGC-4 cells also formed tumors with histological features of parental ones (Table 2 and Figure 4), but
tumorigenicity of HGC-4 sphere cells was less than that of HGC-1 sphere cells, and that of HGC-2 sphere cells was at the intermediate between HGC-1 and HGC-4 sphere cells (Table 2). We thus concluded that only CD49fh high sphere-forming TICs could grow when unsorted tumor cells were cultured. These results are consistent with our idea that CD49f is a useful marker for gastric TICs.

Some Sphere-forming TICs are Resistant to Chemotherapeutic Agents

TICs have been reported to be more resistant to chemotherapy than other tumor cells [31]. We thus examined whether the CD49fh high sphere-forming TICs were resistant to anti-tumor drugs. We found that MKN45 and MKN74 human gastric tumor cell lines attached to the collagen gel to grow rapidly in the same condition where unsorted HGC-1, HGC-2 and HGC-4 tumor cells grew to form spheres. We thus used MKN45 and MKN74 cells as controls.

We found that unsorted HGC-1 and HGC-4 tumor cells formed spheres in the presence of anti-tumor drugs. It seemed that not the sphere formation but the growth of spheres was suppressed by the drugs because many small spheres were found when HGC-1 or HGC-4 tumor cells were treated with drugs at higher concentrations (Figure S4). MKN45 and MKN74 cells never formed spheres when cultured in a condition where HGC-1 and HGC-4 cells formed spheres, indicating that sphere formation

Figure 2. Localization of CD49f in gastric tissues. (A–C) CD49f is localized at the epithelial-stromal interface in (A) normal mucosa, (B) chronic gastritis and (C) intestinal metaplasia. (D–G) In (D) gastric dysplasia adjacent to carcinoma and (E–G) gastric tumor tissues, expression of CD49f is diversified: It is localized at the epithelial-stromal interface in some cells (G), but on apical, lateral and basal surfaces in some cells (E, arrows), and expression can hardly be detected in some cells (E, arrowheads). Scale bars represent 50 μm.
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Figure 3. CD49f<sup>high</sup> cells form spheres in primary culture. (A) Phase contrast micrographs showing the growth of spheres in culture of CD49f<sup>high</sup> HGC-1 tumor cells on collagen gel. The same area on days 8, 10 and 14 are shown. It is clear that spheres in the white circles grow rapidly in
could not be induced in these cell lines (Figure S4). The growth of MKN45 and MKN74 cells was severely affected by the drugs, with EC50 (half maximal effective concentration) of 0.02–0.05 μM for DXR, 2–6 μM for 5-FU and 4–10 μM for DXF (Figure 5). In contrast, growth of HGC-4 cells was less affected by them, with EC50 of 0.5 μM for DXR, 30 μM for 5-FU and 40 μM for DXF (Figure 5). This indicates that HGC-4 cells were far more resistant than gastric tumor cell lines to anti-tumor agents. HGC-1 and HGC-2 cells were similar to gastric tumor cell lines concerning their responses to the drugs, though HGC-1 cells were more

Figure 4. Sphere cells form tumors with histological features of parental ones. Light micrographs of tumors formed by subcutaneous injection of sphere cells into NOD-SCID mice (right panels), which are obtained by cultures of (A) unsorted HGC-1, (B) unsorted HGC-2 and (C) unsorted HGC-4 tumor cells. Histological features of tumors formed in mice correspond well with those of parental ones (left panels). Tissue specimens are stained with hematoxylin and eosin. Scale bars represent 50 μm.

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resistant to DXR than tumor cell lines at 0.1 μM. In culture of unsorted HGC-2 cells, flat cells were major in the first 2 weeks (Figure 3E). Thus it was difficult to examine the response of sphere-forming HGC-2 cells to the drugs by examining the cell number at 2 weeks in culture. It is possible that sphere-forming HGC-2 cells may be more resistant to the drugs than gastric tumor cell lines, if another culture system with longer cultivation period (e.g. 6–8 weeks) is used for the analysis. HGC-4 cells were always more resistant to the drugs than HGC-1 and HGC-2 cells, suggesting that there are significant patient-dependent differences between gastric TICs on their responses to chemotherapeutic agents.

Discussion

In the present study, we found that human gastric TICs strongly expressed CD49f on their surface, using 15 primary gastric carcinoma cases. Previously, gastric TICs have been identified by using CD44 [11], CD44 and EpCAM [12], CD44 and CD24 [13], CD44 and CD54 [14], CD90 [32], and aldehyde dehydrogenase 1 [33] as markers, but CD49f has not been used to detect TICs in gastric cancers. This may be the first report showing that CD49f is a promising marker for gastric TICs. We then established a primary serum-free culture system for them, where only CD49fhigh cells could grow to form ECM-attaching spheres with strong tumorigenicity.

CD49f or integrin α6 (ITGA6) is a 150 kDa transmembrane protein, expressed mainly on T cells, monocytes, and epithelial and endothelial cells [34]. CD49f associates with integrin β1 chain (CD29) to form VLA-6, and with integrin β4 chain (CD104) to form the α6β4 complex, both of which are known to function as the laminin receptor [35]. An emerging consensus is that α6β4 complex synergizes with specific molecules such as ErbB2, EGFR, Ron, Fyn, c-Met, protein kinase C, CD151, and CD9 to activate key signaling pathways for the invasion and migration of carcinoma cells via activation of signaling molecules such as PI3K [36,37]. In previous experiments with mammary and brain tumor cells [18,19], CD49fhigh cells were cultured in non-adherent conditions to induce sphere formation. Thus attachment to ECM may not be necessary for some CD49fhigh cells to exhibit tumorigenic activity while other CD49fhigh cells were cultured just in the presence of ECM [17,38]. In previous studies with gastric TICs, cells were cultured on non-adherent substrata to form floating spheres [12–14,32], and substratum was used to induce their differentiation into non-tumorigenic cells. In the present study, we found that CD49fhigh cells could not grow on non-adherent substrata, and they could survive only in the presence of ECM in all three cases analyzed (data not shown). This suggests that characteristics may be different between TICs identified in the present study and those reported in previous

Table 2. Tumorigenicity of sphere cells obtained by culture of unsorted cells (number of tumors formed in NOD-SCID mice/number of injection of tumor cells).

| PDTX line | Number of injected cells | 3,000 | 1,000 | 300 | 100 | 30 | 10 |
|-----------|--------------------------|-------|-------|-----|-----|----|----|
| HGC-1     |                          | 8/8** | 4/8   | 5/10 | 1/6 | 0/2 | 1/2 |
| HGC-2     |                          | 3/4   | 3/4   | 2/4  | 0/2 | 0/2 | 0/2 |
| HGC-4     |                          | 2/6** | 1/6   | 0/6* | 0/6 | 0/4 | –  |

**P<0.01;  *P<0.05 by Chi-square test;  –not determined.

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Figure 5. Some TICs are more resistant to anti-tumor drugs than gastric tumor cell lines and other TICs. HGC-1, HGC-2 and HGC-4 tumor cells, and MKN45 and MKN74 tumor cell lines are seeded on day 0, treated with various concentrations of (A) doxorubicin, (B) 5-fluorouracil, and (C) doxifluridine from days 1 to 14, and cell numbers on day 14 are determined by MTT assay. Black and blue asterisks show that HGC-1 and/or HGC-4 cells are significantly (**, P<0.01; *, P<0.05 by Student’s t-test) more resistant to the drugs than MKN45 and MKN74 cell lines, respectively, while pink and brown asterisks indicate that HGC-4 cells are significantly (**, P<0.01; *, P<0.05 by Student’s t-test) more resistant to the drugs than HGC-1 and HGC-2 cells, respectively.

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ones though both formed spheres in vitro and exhibited strong tumorigenicity in vivo.

It is well known that growth, differentiation and progression of cancer cells are severely affected by ECM [39]. The role of laminin on the progression of tumors has been intensively investigated. Woo Ho Kim, one of co-authors, has repeatedly reported in collaboration with Hynda Kleinman that laminin-adherent human colon cancer cells exhibited strong tumorigenicity, increased growth and decreased apoptosis [40,41], and similar results have been reported in pancreatic cancers [42]. Consistent with these, gastric carcinomas have been reported to use 

\[ \text{integrin and newly deposited laminins to adhere to surrounding tissues during the invasion} \]

[43], and Koike et al. [44] showed that invasive behavior of gastric cancer cells was inhibited by treatment with anti-\( \alpha \)-6 integrin antibody. These results strongly indicate that CD49f plays an important role in regulating invasiveness of human gastric cancers, but its molecular mechanism remains to be solved. Recently, Yu et al. reported that OCT4 and SOX2 directly bind to the \( ITG46 \) promoter to induce the expression of CD49f, and that CD49f plays a pivotal role in maintaining cellular pluripotency through the PI3K/AKT/p53 pathway in mesenchymal stem cells [45]. Consistent with this, we found that SOX2, POU5F1 (a gene encoding OCT4) and \( ITG46 \) were all strongly expressed in human gastric tumors examined, except that SOX2 was not expressed by MKN74 cell line (Figure S3). Our culture system for CD49\( ^{\text{high}} \)-gastric TICs will be useful for further analysis on the role of CD49f in gastric tumorigenesis.

We found in the present investigation that CD49\( ^{\text{high}} \) gastric TICs formed ECM-attaching spheres. There are reports showing that substrata are essential to induce sphere-formation by TICs in brain [46,47] and prostate gland [17,38,48] tumors while others show that TICs can form spheres in non-adherent conditions, as described above. This indicates that TICs may be divided into two types, depending on whether ECM is needed for their growth or not. It is well known that embryonic stem cells and induced pluripotent stem cells need feeder cells/ECM including laminins for their survival and growth in vitro [49]. We have also demonstrated that normal gastric epithelial cells can survive only in the presence of ECM [50]. Thus gastric TICs identified in the present study seem to retain some characteristics of normal stem cells, and TICs which can grow in the non-adherent condition may be derived from those which need ECM to grow. Our culture system may be useful to analyze how ECM-independent TICs emerge from ECM-dependent ones, and how it is related to the progression of cancer.

It is interesting that HGC-1 cells were more tumorigenic than HGC-4 cells (Table 2), while the latter was more resistant to chemotherapeutic agents than the former (Figure 5), indicating that chemo-resistance and tumorigenicity are independent features of TICs. Recently several drugs have been identified which demonstrated that normal gastric epithelial cells can survive only for their survival and growth in vitro [49]. We have also noted that embryonic stem cells–perspectives on current status and future directions. Cancer Res 66: 9339–9344.

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Supporting Information

Figure S1 CD44-negative, CD133-negative cells form tumors with histological features of parental ones. HGC-2 and HGC-5 PDTXs were dissociated into single cells, and their tumorigenicity was analyzed by injecting sorted cells into immunodeficient mice. CD44-negative, CD133-negative cell fractions (shown by red squares in FACS analyses) formed tumors with histological features of parental ones while CD44-positive cells (shown by black squares) were not tumorigenic. Tissue specimens are stained with Alcian blue-PAS-hematoxylin. Scale bars represent 50 µm.

Figure S2 Cell surface antigen profiles are maintained in tumors formed by injection of CD49\( ^{\text{high}} \) cells. Cell surface antigen profiles of HGC-3 and HGC-4 PDTX cells differed greatly, but these profiles of secondary tumors (right panels) formed by injection of CD49\( ^{\text{high}} \) cells (shown by red squares in FACS analyses) into immunodeficient mice were similar to those of primary tumors (left panels).

Figure S3 Gene expression profiles of human gastric tumor cell lines, PDTXs and sphere-forming TICs. MKN74 and MKN45 human gastric tumor cell lines, HGC-1 and HGC-4 PDTXs, and HGC-1 and HGC-4 sphere cells formed by culture of unsorted cells expressed stem cell–related genes including \( \text{BM}41, \text{NANOG, POU5F1, SOX2 and } ITG46 \) at similar levels though MKN74 cells did not express SOX2, and HGC-4 sphere cells expressed \( \text{NANOG} \) strongly.

Figure S4 Phase contrast micrographs of doxorubicin (DXR)-treated HGC-1 and HGC-4 tumor cells, MKN45 and MKN74 tumor cell lines on day 14 in vitro. Their growth was quantified by MTT assay and results are shown in Figure S4. Scale bars represent 200 µm.

Table S1 Primer sequences and PCR conditions.

Table S2 Case description and tumorigenic activity of CD44\( ^{\text{high}} \) and CD44\( ^{\text{low}} \) gastric cancer cells.

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Author Contributions

Conceived and designed the experiments: HF WHK SJJ YY. Performed the experiments: HF HSS SS CF KB. Analyzed the data: HF HSS SJJ. Contributed reagents/materials/analysis tools: JHK YSP MJK K. Kato MI HK JHY YE K. Kojima. Wrote the paper: HF SJJ YY.
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