

**SNAIL regulates gastric carcinogenesis through CCN3 and NEFL**

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Keywords:	Gastric Cancer, Cancer Stem Cells, EMT, spheroid

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6 **1 SNAIL regulates gastric carcinogenesis through CCN3 and NEFL**

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8 **2 Role of SNAIL/CCN3/NEFL axis in gastric cancer**

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6 19 **Abstract**  
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8 20 Among cancer cells, there are specific cell populations of whose activities are comparable  
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10 21 to those of stem cells in normal tissues, and for whom the levels of cell dedifferentiation are  
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12 22 reported to correlate with poor prognosis. Information concerning the mechanisms that  
13  
14 23 modulate the stemness like traits of cancer cells is limited. Therefore, we examined five  
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16 24 gastric cancer cell lines and isolated gastric oncospheres from three gastric cancer cell lines.  
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18 25 The gastric cancer cells that expanded in the spheres expressed relatively elevated  
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20 26 proportion of CD44, which is a marker of gastric cancer stem cells, and displayed many  
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22 27 properties of cancer stem cells, for example: chemoresistance, tumorigenicity and  
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24 28 epithelial-mesenchymal transition (EMT) acquisition. SNAIL, which is a key factor in  
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26 29 EMT, was highly expressed in the gastric spheres. Microarray analysis in gastric cancer cell  
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28 30 line HGC27 showed that CCN3 and NEFL displayed the greatest differential expression by  
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30 31 knocking down of SNAIL; the former was up-regulated and the latter down-regulated,  
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32 32 respectively. Down-regulation of CCN3 and up-regulation of NEFL gene expression  
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34 33 impaired the SNAIL-dependent EMT activity: high tumorigenicity, and chemoresistance in  
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36 34 gastric cancer cells. Thus, approach that disrupts SNAIL/CCN3/NEFL axis may be credible  
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38 35 in inhibiting gastric cancer development.

36 **Keywords:** Gastric Cancer; Cancer Stem Cells; EMT; spheroid  
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## 40 Introduction

41 Gastric cancer is one of the most common cancers in East Asia and Eastern Europe  
42 [1]. It is important to critically assess the current advances in our understanding of gastric  
43 cancer and to establish novel and innovative therapeutic strategies. A vast body of literature  
44 has been published on specific aspects of cancer initiating cells and on putative cancer stem  
45 cells (CSCs) which possess properties of stem cells distinct from differentiated progeny  
46 cancer cells [2]. Discovering significant genes and signaling pathways involving gastric  
47 cancer stemness could be helpful approaches to discovering novel therapeutic options.

48 During malignancy transformation, a critical process named the epithelial-  
49 mesenchymal transition (EMT) commonly occurs, and cells usually undergo a rapid change  
50 from differentiated and polarized epithelial state into an invasive mesenchymal composition  
51 [3]. During the development of diverse solid tumors, stem cell like traits were reported to  
52 be related to EMT. For example, after breast cancer cells acquired stem cell like features,  
53 the passaged mammosphere cells manifest with similar features to breast cancer stem cells,  
54 indicating a fundamental link between malignancy propagation and stem cell characteristics  
55 [4-6]. Among all the major EMT transcription factors, SNAIL, a zinc-finger protein, whose  
56 activities in relation to the downregulation of E-cadherin in colon cancer have previously  
57 been reported [7, 8]; binds to the E-boxes in the *CDHI* gene promoter and represses  
58 transcription of the *CDHI* gene [9]. So far SNAIL has been reported to contribute in many  
59 malignancy progression, and its' function in gastric cancer needs to be uncovered further as  
60 well. The precise mechanism of SNAIL-induced cell dedifferentiation and how this gene

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6 61 can provide stem cell like traits in gastric cancer cells remain open to debate and to be  
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8 62 further clarified. The discovery of genes under SNAIL regulation that could also be an  
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10 63 instrumental breakthrough and lead to the establishment of novel therapeutic strategies in  
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12 64 EMT-related stemness and malignancy. In the present study, we extracted CCN3 and NEFL  
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14 65 as targets in the downstream of SNAIL, and determined the association of these two factors  
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17 66 with stem cell like activity in gastric cancer cells.  
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6 79 **Materials and Methods:**

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9 80 **Cell culture, tissue collection and sphere growth**

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11 81 Human gastric cancer cell lines were purchased from RIKEN  
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13 82 (<https://cell.brc.riken.jp/ja/quality/str>), JCRB cell bank  
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15 83 ([https://cellbank.nibiohn.go.jp/about-qc\\_english/](https://cellbank.nibiohn.go.jp/about-qc_english/)), and ATCC  
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17 84 ([https://www.atcc.org/Services/Testing\\_Services/Cell\\_Authentication\\_Testing\\_Service.aspx](https://www.atcc.org/Services/Testing_Services/Cell_Authentication_Testing_Service.aspx)  
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19 85 [x](#)), in which STR analysis is performed in these cell line banks to ensure the authentication  
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21 86 of human cell lines, and were cultured according to the instructions provided by the  
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23 87 manufacturer. Cell lines including human gastric cancer cell lines (HGC27, NCI-N87, GSU,  
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25 88 MKN74, MKN45, NUGC3 and IM95), and embryonic kidney 293T cells were cultured in  
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27 89 DMEM (Nacalai Tesque, Japan) supplemented with 10% fetal bovine serum (HyClone  
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29 90 Defined Fetal Bovine Serum (FBS), USA) and penicillin-streptomycin mixed solution  
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31 91 (10,000u/ml, Nacalai Tesque, Japan). For RNA extraction from each cell line, NucleoSpin  
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33 92 RNA Plus (Takarabio, Japan) was used following the manufacturer's instructions. The  
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35 93 preparation of paraffin-embedded blocks was performed as follows: slices of tumor formed  
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37 94 from each of the cell lines were immersed in 4% paraformaldehyde (Nacalai Tesque, Japan)  
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39 95 to allow the assembling of paraffin-embedded blocks. Cells were cultured in homemade  
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41 96 stem cell medium (DMEM/F12 supplemented with B27 Supplement (ThermoFisher), 10  
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43 97 ng/mL recombinant basic fibroblast growth factor (bFGF, ThermoFisher), 10 ng/mL  
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45 98 epidermal growth factor (EGF, ThermoFisher), and 1% penicillin-streptomycin) to obtain  
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47 99 spheres. A total of  $1 \times 10^4$  cells per milliliter were seeded in culture medium for stem cell  
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5 100 and incubated in ultra-low attachment plates for 5 days. Spheres larger than 80  $\mu\text{m}$  in  
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7 101 diameter were counted using Cell3Imager (InSphero AG and Dainippon SCRREEN, Kyoto,  
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9 102 Japan). TrypLE Express (ThermoFisher) or Trypsin-EDTA (FUJIFILM Wako, Japan) were  
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11 103 used to separate cells from the floating spheres and adherent cells to allow cell counting  
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13 104 and other experiments.  
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### 16 17 105 **In vivo tumorigenicity assay**

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20 106 All procedures involving animals were conducted in accordance with the  
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22 107 Institutional Animal Welfare Guidelines of Kyoto University. NOD/SCID mice were  
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24 108 purchased from the Charles River Laboratories (Yokohama, Japan) and were maintained  
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26 109 according to the Guidelines for Laboratory Animals in the Kyoto University. The  
27  
28 110 tumorigenicity assay was performed by subcutaneous injection of  $1 \times 10^4$  designated cells  
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30 111 into the flanks of 8- to 10-week-old NOD/SCID mice. Mice were sacrificed and examined  
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32 112 for tumor harvest once the tumor had reached pre-determined size (2.5cm maximum).  
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34 113 Tumor size was measured with calipers once a week after the injection.  
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### 40 114 **Lentivirus production, short-hairpin RNA-mediated human SNAIL gene knockdown** 41 42 115 **and stable clone establishment**

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45 116 The lentivirus package system: pMDLg/pRRE (Addgene, plasmid #12251), pRSV-  
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47 117 Rev (Addgene, plasmid #12253) and pMD2.G (Addgene, plasmid #12259) together with  
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49 118 the shRNA plasmid targeting the human SNAIL gene as well as the control vector:  
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51 119 pLKO.1puro (Addgene, plasmid #8453) were co-transfected into 293T cells by  
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53 120 Lipofectamine 3000 (Invitrogen). SNAIL-targeting short-hairpin RNA (MISSION shRNA)  
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6 121 duplex (A: 5-TGCTCCACAAGCACCAAGAGTC-3; B: 5-  
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8 122 CCACTCAGATGTCAAGAAGTAC-3) and NEFL-targeting short-hairpin RNA  
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10 123 (MISSION shRNA) (5-CGACAGCTTGATGGACGAAAT-3) were purchased from  
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12 124 Sigma-Aldrich Co. LCC. (St. Louis, MO, USA). About 48 to 72 hours later, virus  
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14 125 supernatant was collected for concentration. For shRNA knockdown, HGC27 and IM95  
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16 126 cells were seeded onto 6-well plates and infected with optimal virus concentrations  
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18 127 supplemented with 6 µg/mL Polybrene (Sigma, St. Louis, MO, USA), then incubated for  
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20 128 12 hours before replacing with fresh medium. Cells were then selected by puromycin  
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22 129 (INVIVOGEN, Japan) at the concentration of 1.8 µg/ml (HGC27) and 4 µg/ml (IM95) for  
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24 130 2 weeks.

### 131 **Transient transfection**

132 Lipofectamine 3000 reagent was used for the introduction of overexpression vectors  
133 to establish stable lines, including the NEFL (Sino Biol.HG13214-UT), CCN3 (Sino  
134 Biol.HG10264-UT) and SNAIL (Sino Biol.HG16844-UT) overexpression vectors and  
135 matched control vector (Sino Biol.CV011), all of which were purchased from Sino  
136 Biological Inc. (Wayne, PA, USA). Selections were carried out via hygromycin B (Nacalai  
137 Tesque, Japan); resistance and transfection efficiency were verified through real-time qPCR.

### 138 **Microarray data and bioinformatics analysis**

139 Total RNA from each sample was extracted using NucleoSpin RNA Plus kit,  
140 forwarded with Affymetrix Human Genome U133 Plus 2.0 Array (HuGene2.9st, Japan)  
141 analysis. RNA extraction, microarray hybridization, and feature selection were performed



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6 142 according to the manufacturer's protocol. Microarray data can be download from the GEO  
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8 143 database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145867>).

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10 144 Bioinformatics and genetic network construction were performed in R Studio (version  
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12 145 1.2.1335) mainly using RMA [10] from the affy package [11] and the GoPlot package  
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14 146 (<https://CRAN.R-project.org/package=GOplot>) [12]. Treemaps were created using  
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16 147 REVIGO webtool (<http://revigo.irb.hr/index.jsp>) [13]. Gene enrichment analysis was  
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18 148 performed using DAVID 2010 Bioinformatics Resources (<http://david.abcc.ncifcrf.gov/>)  
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20 149 [14].

## 24 150 **Reverse transcription and real-time quantitative PCR**

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27 151 Using the PrimeScript II 1st strand cDNA Synthesis Kit (Takarabio, Japan), 3  
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29 152 micrograms of total RNA were transformed into first-strand complementary DNA synthesis  
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31 153 following directions provided by the manufacturer. Human pre-messenger RNA sequences  
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33 154 were obtained from NCBI gene ([www.ncbi.nlm.nih.gov/gene/](http://www.ncbi.nlm.nih.gov/gene/)) before using NCBI blast  
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35 155 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to design primers in PCR. All the primer  
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37 156 sequences used in this study can be checked in Table S1. Real-time quantitative PCR  
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39 157 (qPCR) was performed to evaluate the expression levels using SYBR Green Master Rox  
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41 158 (Roche, Sigma-Aldrich), and were analyzed using the StepOnePlus real-time system  
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43 159 (Applied Biosystems). The endogenous expression level of GAPDH was used to obtain the  
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45 160 expression levels of other genes via  $\Delta\Delta C_t$  methods.

## 51 161 **Drug resistance and CCK8 assay**

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6 162 Cells were seeded at the concentration of  $1 \times 10^4$  cells/ml in 96-well plates and  
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8 163 incubated overnight before application of various concentration of chemotherapy drugs  
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10 164 ( $1\mu\text{M}$  to  $200\mu\text{M}$  5-FU (KYOWA KIRIN, Japan)). After 96 hours, medium was discarded  
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12 165 and CCK8 assay solution (Dojindo molecular technologies) was added to cells and  
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14 166 incubated for 1 hour at  $37^\circ\text{C}$ . The plate was then read by a microplate reader (OD450;  
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17 167 Infinite F50, TECAN).

### 18 19 20 168 **Western blotting assay**

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23 169 Proteins were extracted from relevant cell lines using ice-cold RIPA buffer (Nacalai  
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25 170 Tesque, Japan) after washing with 1x phosphate-buffered saline (PBS). Later proteins were  
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27 171 separated in 10-20% gels (SuperSep Ace, Fujifilm Wako, Japan) and then transferred onto  
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29 172 PVDF membranes (Immobilon-P, Merck M), and blocked using skimmed milk (Fujifilm  
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32 173 Wako, Japan). The collection of primary antibodies used in this study were Snail (ab53519;  
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34 174 Abcam), CCN3 (ab191425; Abcam), NEFL (ab223343; Abcam), and E-cadherin (24E10,  
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36 175 Cell Signaling Technology), Vimentin (D21H3, Cell Signaling Technology), CD24  
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38 176 (ab199140 Abcam). Proteins were incubated with the primary antibodies over night at  $4^\circ\text{C}$ .  
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41 177 They were then stained with anti-mouse, anti-rabbit or anti-goat secondary antibodies  
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43 178 (Jackson Laboratory) for 60 minutes at room temperature. Thereafter they were incubated  
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45 179 with chemiluminescent HRP substrate (WBKLS0500, Merck M) for 5 minutes.  
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48 180 Chemiluminescence signals were collected via the Fujifilm LAS-3000 (Fuji, Japan) as per  
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50 181 the manufacturer's instructions.

### 51 52 53 182 **Immunocytochemistry and fluorescence assay**

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6 183 Cells were seeded onto 8-well culture slides (#354118, Falcon) overnight before  
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8 184 fixation with 4% PFA for 10 minutes at room temperature followed by 1x PBS washes.  
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10 185 Cells were permeabilized with 0.5% Triton X-100/PBS for 5 minutes at room temperature,  
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12 186 washed with 1x PBS, and blocked in 5% BSA (Sigma) in PBS for 60 minutes before  
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14 187 incubating with Snail (20C8, ThermoFisher) primary antibodies followed by the secondary  
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16 188 antibodies Alxea Fluor 594 (ThermoFisher). Slides were mounted in VECTASHIELD  
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18 189 Mounting Medium with DAPI (H-1200; VECTOR Lab, Japan). Fluorescence images were  
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20 190 visualized with Keyence fluorescence microscope.  
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#### 24 191 **Immunohistochemistry staining**

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27 192 Human gastric cancer tissue array (MLB Life Science Japan) and tumor specimens  
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29 193 from mice gastric tumors were deparaffinized, rehydrated and placed in 3% (v/v) H<sub>2</sub>O<sub>2</sub>-  
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31 194 methanol for 15 min at room temperature. The slides were then immersed in blocking  
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33 195 solution (Non-specific Staining Blocking Reagent; Dako-Cytomation, Kyoto, Japan) for 15  
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35 196 min and incubated with the primary antibodies listed below at 4 °C overnight. Antigen-  
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37 197 antibody complexes were detected with a secondary antibody (Histofine Simple Stain  
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39 198 MAX PO (R) for rabbit monoclonal, or (G) for goat polyclonal (Nichirei, Tokyo, Japan)  
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41 199 and visualized using 3,30-diaminobenzidine (0.5 mg/ml in Tris-buffered saline). The list of  
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43 200 primary antibodies and dilution ratios were as follows: 1. Anti-SNAIL antibody (goat  
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45 201 polyclonal, ab53519, Abcam), 1: 1,000; 2. Anti-CCN3 antibody (rabbit monoclonal,  
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47 202 ab191425, Abcam), 1:100; 3. Anti-NEFL antibody (rabbit monoclonal, ab223343, Abcam),  
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5 204 **Flow cytometry**  
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8 205 Incubation buffer was prepared as 1x PBS + 2% FBS. Single cell suspensions were  
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10 206 washed with cooled incubation buffer, and re-suspended in 1x PBS + 2% FBS on ice for 30  
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12 207 minutes for antibody blocking: anti-CD24-FITC (ML5; Bio-legend, San Diego, CA),  
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14 208 CD44-FITC (BJ18; Bio-legend, San Diego, CA) and DAPI (422801, Bio-legend, San  
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16 209 Diego, CA). Cells were suspended in 0.5 mL incubation 1x PBS + 2% FBS to reach a final  
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18 210 concentration of  $10^6$  cells/ml. Data were collected by the BD FACSCanto II or BD  
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20 211 FACSVerse flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) and analyzed with  
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22 212 FlowJo software (TreeStar, San Carlos, CA). Cell debris was excluded from the analysis  
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24 213 based on scatter signals, and fluorescent compensation was adjusted when double stained.  
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30 214 **Statistical Analyses**  
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33 215 Independent sample t tests were performed to compare the continuous variation of  
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35 216 two groups, and the student's test was applied for comparisons of variables.  $P < 0.05$  was  
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37 217 considered significant. All data are reported as mean  $\pm$ SEM.  
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6 223 **Results**

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8 224 **Gastric Spheres Cultured Under Serum-Free Conditions Manifest Stem Cell**

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11 225 **Properties**

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14 226 Self-renewal capability is a major property of stem cells, and can be accurately  
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16 227 assessed via sphere formation [15]. Gastric cancer cell lines produce stem like sphere-  
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18 228 forming cells when cultured under B-27(+) bFGF(+) EGF(+) serum-free medium (CSC  
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20 229 serum-free medium) in ultra-low attachment culture dishes [16]. The original gene  
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22 230 expression profiles and tumor morphologies in cancer cells are well reflected with spheres  
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24 231 cultured in the CSC serum-free condition [17]. We assessed the sphere-forming capacity of  
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26 232 five gastric cancer cell lines for initial culture (cells were collected from attached condition  
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28 233 and seeded in CSC serum-free medium) and passage culture (cells were collected from  
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30 234 formed spheres and seeded in CSC serum-free medium), and found that three cell lines had  
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32 235 the ability to form spheres (Figure 1A). Those spheres all originated from single cell and  
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34 236 not by mere cell herds or aggregations, which was ensured by seeding single cells in 96  
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36 237 well plates and spheres managed to develop after several weeks (data not shown). Sphere-  
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38 238 forming capacity of passaged cells (passage culture) was stronger compared with that of  
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40 239 parental cells (initial culture), when the same number of cells were seeded in CSC serum-  
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42 240 free medium (Figure 1B). To further confirm whether malignant cells possess additional  
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44 241 stemness traits,  $1 \times 10^4$  NCI-N87 cells were transplanted into the flanks of NOD/SCID  
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46 242 mice. Histological analysis of xenografts exhibited an epithelial-like morphology  
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48 243 irrespective of whether they were generated by parental or sphere cells (Figure 1C & S1);  
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6 244 however, sphere cells were more efficient at producing bulky tumors in NOD/SCID mice  
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8 245 compared with parental monolayer cells (Figure 1D).  
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### 12 13 14 247 **Gastric Spheres Express EMT-associated Factors**

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17 248 Acquisition of stemness traits in malignant cells is commonly achieved through  
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19 249 undergoing EMT process, thus tracking the activities of EMT-associated factors will help  
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21 250 uncovering mechanism behind the obtained stemness. Four key EMT factors were  
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23 251 examined in this study, and higher mRNA expression of EMT factors (TWIST1, 2, SNAIL,  
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25 252 and SLUG) were found in spheres compared with parental cells (Figure 2A). HGC27 cells,  
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27 253 which form spheres most efficiently, highly expressed TWIST1 and SNAIL mRNAs  
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30 254 compared with other two sphere-forming cell lines, NCI-N87 and NUGC3. Consistent with  
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32 255 this, immunofluorescence images indicated highest protein expression of SNAIL in HGC27  
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34 256 cells (Figure 2B). The role of TWIST1 has been extensively investigated in previous EMT-  
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36 257 associated researches [18]; therefore, HGC27 and SNAIL were chosen as the target cell line  
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38 258 and molecule pair in the current study.  
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### 44 45 46 260 **SNAIL Regulates Tumorigenicity in Gastric Cancer Cells**

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49 261 In order to investigate whether SNAIL regulates stemness and tumorigenicity in  
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51 262 gastric spheres, we determined phenotypic alteration after lentivirus-mediated short-hairpin  
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53 263 RNA-interfered knockdown of SNAIL (shRNA-SNAIL k.d.). Realtime quantitative PCR  
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5 264 indicated successful knockdown effects using shRNA-SNAIL in HGC27 cell lines and  
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7 265 spheres (Figure 3A). Western blotting showed a similar tendency of 29 Da SNAIL protein  
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9 266 expression (Figure 3B). Epithelial and mesenchymal traits of HGC27 were measured using  
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11 267 antibodies against E-cadherin and Vimentin, representative proteins for epithelial and  
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13 268 mesenchymal phenotypes, respectively. Up-regulation of E-cadherin with down-regulation  
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15 269 of Vimentin by SNAIL knockdown indicated the event of mesenchymal-to-epithelial  
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17 270 transition in HGC27 cell lines (Figure 3B). The most conspicuous phenomenon observed  
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19 271 was that the shape of formed sphere in the CSC serum-free medium switched from having  
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21 272 smooth margins into jagged and sharp edges by the SNAIL knockdown (Figure 3C).  
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24 273 Previous studies reported a CD44<sup>+</sup>/CD24<sup>-</sup> subpopulation of gastric cancer contains gastric  
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26 274 cancer stem cells [19]. As showed in Figure 3D, the fluorescence activated cell sorter  
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28 275 analysis revealed that 91.0% of the HGC27 cell line was CD44<sup>+</sup>/CD24<sup>-</sup>, while knockdown  
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30 276 of SNAIL in this cell line almost completely eliminates this population. This suggested that  
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32 277 gastric cancer stem cells are maintained, at least in part, by the presence of SNAIL.  
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38 278 Stemness such as self-renewability in cancer cells is associated with the capacity of  
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40 279 forming spheres out of the transformed epithelial cells [20]. Therefore, we compared sphere  
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42 280 forming capacity between parental HGC27 and stable SNAIL knockdown HGC27 cells  
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44 281 cultured in monolayer conditions. The number and area of sphere were significantly  
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46 282 decreased by SNAIL knockdown, suggesting a reduced ability to self-renewal (Figure 3E).  
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48 283 Consistent with this, when  $1 \times 10^4$  cells were transplanted into the flanks of NOD/SCID  
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50 284 mice, although histological analysis did not show any significant alterations, stably SNAIL  
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6 285 knockdown HGC27 cells were less efficient at producing bulky tumors compared with  
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8 286 parental HGC27 cells (Figure 3F).  
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11 287 Lower expression of SNAIL was referred to as increased sensitivity upon chemo-  
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13 288 treatment due to diminished self-renewability in malignancy [21]. In our experimental  
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15 289 condition, stable SNAIL knockdown HGC27 cells were more susceptible to 5-fluorouracil  
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17 290 (5-FU) treatment compared with parental HGC27 cells (Figure 3G).  
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21 291 Together, these findings SNAIL regulates tumorigenicity, possibly stemness at least  
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23 292 in part, in gastric cancer cells.  
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### 29 294 **SNAIL-regulating Genes in Gastric Cancer Cells**

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32 295 To unveil the mechanisms underlying phenotypic modifications induced by  
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34 296 knockdown of SNAIL in HGC27 cells, gene expression microarray analysis on a HGC27-  
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36 297 SNAIL knockdown and parental HGC27 cells was performed. Under the alteration of  
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38 298 SNAIL expression, distributed genes movement were shown in Figure 4A. Of those genes,  
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40 299 a total of 1656 and 1832 probe sets ( $|FC| \geq 2$ ) were specifically upregulated or  
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43 300 downregulated in stable HGC27-SNAIL knockdown cells respectively (Figure 4B).  
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46 301 Statistically overrepresented functional processes were obtained through enriched genes  
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48 302 querying in the Gene Ontology (GO) database ( $P < 0.05$ ; Figure 4C). The main processes  
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50 303 enriched in up-/down-regulated genes include those pertaining to multiple binding and  
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52 304 cellular processes. It is suggested that instead of individual activation, genes tend to  
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5 305 collaborate in genetic networks, thus we subjected the enriched GO processes that were  
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7 306 selected as meaningful to REVIGO analysis, which is a useful web tool that summarizes  
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10 307 long lists of GO terms. Several GO terms formed a treemap of the most significant  
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12 308 processes: cell motility, chemotaxis, adhesion, neural regeneration, and cell death, as well  
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14 309 as those involved in EMT (Figure 4D). Within this network, chemotaxis was a focal node  
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16  
17 310 and in the broader group of terms under the chemotaxis title, the regeneration process was  
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19 311 chosen as a target to be studied in greater depth due to the possible role of neuron  
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21 312 development and supervision in malignancy progression. qRT-PCR confirmed down- and  
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23 313 up-regulation of CCN3 (also known as NOV or IGFBP9): Cellular Communication  
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26 314 Network factor 3, and NEFL: Neurofilament Light peptide mRNA expression by SNAIL  
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28 315 knockdown in HGC27cells (Figure 4E).

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### 317 **CCN3 and NEFL Expression in Gastric Cancers**

318 The occurrence of distant metastases and poor survival outcome has been reported  
319 relevant with high SNAIL protein in gastric cancer patients [22]. CCN3 belongs to the  
320 CCN family (cysteine-rich protein 61 (CYR61), connective tissue growth factor (CTGF),  
321 nephroblastoma overexpressed (NOV)). Limited information is known concerning the  
322 functional correlation between EMT with CCN3 and NEFL in cancers. There have been  
323 reports of increased expression of CCN3 in prostate and cervical cancers [23, 24]. CCN3  
324 was demonstrated to promote EMT by activating the FAK/Akt/HIF-1 $\alpha$  pathway in prostate  
325 cancer [25]. NEFL has been implicated in carcinogenesis as a putative suppressor gene in

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5 326 neuron related inhibition of both cell proliferation and invasion in head and neck squamous  
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7 327 cell carcinoma [26]. Using data of transcript level of SNAIL, CCN3 and NEFL from The  
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9 328 Cancer Genome Atlas (TCGA) stomach adenocarcinoma project, each gene expression in  
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11 329 gastric cancers and normal gastric tissues were shown in Figure 5A. There were no  
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13 330 correlations in mRNA expression levels between SNAIL and either CCN3 or NEFL, and  
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15 331 no correlation between CCN3 and NEFL (Figure 5B). Immunohistochemistry analysis was  
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17 332 then performed on a paraffin-embedded human gastric cancer tissue array (n = 30,  
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19 333 purchased from MBL Life Science, Japan). CCN3 expression was detected in 96.7%  
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21 334 (29/30), NEFL expression was positive in 86.7% (26/30) and SNAIL expression occurred  
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23 335 in 26.7% of tumor tissues (8/30). Representative staining with positive and negative images  
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25 336 for SNAIL/CCN3/NEFL are shown in Figure 5C. Compared with Figure S2 of normal  
26  
27 337 gastric tissues, CCN3 antibody only stained positive in some fibroblasts and myofibroblasts  
28  
29 338 besides malignant parts; while NEFL also stained positive in neurofilament as reported  
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31 339 elsewhere.

### 340 341 **CCN3 and NEFL are Critical for SNAIL-Induced Stemness**

342 We initially investigated how CCN3 and NEFL function by introducing their  
343 expression vectors into SNAIL knockdown HGC27 cells and parental HGC27 cells  
344 respectively, and those effects were confirmed with western blotting (Figure 6A). Forced  
345 expression of SNAIL or CCN3 in sh-SNAIL knockdown HGC27 cells resulted in  
346 significantly increased number of formed spheres (Figure 6B-a & b); while that of NEFL

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6 347 caused diminished sphere formation ability in parental HGC27 cells (Figure 6B-c).  
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8 348 Furthermore, the chemoresistance against 5-FU of each pair of cells was also measured.  
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10 349 The results were consistent with alterations to sphere formation capacity: the proliferation  
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12 350 rates of HGC27-shSNAIL, HGC27-NEFL, and HGC27-shSNAIL-CCN3 cells were  
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14 351 reduced more rapidly when 5-FU was added in a dose-dependent manner (Figure 6C). The  
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16 352 fact that HGC27-shSNAIL stable knockdown cells produced significantly reduced tumor  
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18 353 masses compared with SNAIL re-introduced rescue cells was proved via xenograft assays  
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20 354 in vivo (data not shown). To further evaluate the role of NEFL molecule during EMT  
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22 355 process, gastric cancer cell line IM95 was selected from several candidate cell lines due to  
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24 356 high NEFL expression level. Several observations can be witnessed after knockdown of  
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26 357 NEFL in IM95: steady knockdown of NEFL using shRNA in IM95 cell was established  
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28 358 and verified (Figure 6D-a & b), with enhanced mesenchymal and decreased epithelial  
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30 359 indexes of vimentin and E-cadherin discovered from knockdown of NEFL respectively  
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32 360 (Figure 6D-b), and increased chemo-resistance effect throughout proliferation assay with  
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34 361 5FU addition as well (Figure 6D-c).  
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6 367 **Discussion**

8 368 In this study, we established three spheres enriched in CD44+ gastric cancer cells.  
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10 369 The spheres displayed EMT phenotype, high tumorigenicity, and chemoresistance against  
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12 370 5-FU treatment. SNAIL, one of the key regulators of EMT, was upregulated in the spheres,  
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14 371 and CCL3 and NEFL were further extracted as downstream targets of SNAIL by  
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16 372 microarray analyses. Re-introduced expression of CCN3 and NEFL partially impaired the  
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18 373 SNAIL-dependent CSC like activity, tumorigenicity, and chemoresistance in HGC-27  
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20 374 gastric cancer cells, respectively.  
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25 375 Previous studies have uncovered that CD44, EPCAM, CD133, CD24, CD166 or  
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27 376 Aldh are CSC markers in certain circumstances and are enriched in spheres generated from  
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29 377 gastric cancer cell lines or clinical tissues [27, 28]. Although it stills opens to be discussed  
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31 378 whether all CD44+ cells are CSCs, the CD44+ subpopulation is reported to prevail in  
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33 379 cancer cells after spheres are formed. In this study, we also showed that the CD44+  
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35 380 subpopulation was enriched in gastric spheres.  
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40 381 In a mammospheres formation assay, expression of EMT-associated factors, such as  
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42 382 TWIST1/2, SNAIL, and SLUG in breast cancer cells are up-regulated and associated with  
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44 383 the acquisition of CSC properties and greater metastatic ability after EMT process [29].  
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46 384 These EMT-associated molecules are involved in multiple signaling pathways in other  
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48 385 types of cancers. In the present study, expression of TWIST1 and SNAIL were significantly  
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50 386 elevated in gastric spheres, especially in HGC27-derived spheres. The role of TWIST1 in  
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52 387 EMT processes during gastric carcinogenesis has been extensively investigated [30, 31],  
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5 388 while that of SNAIL has not been researched as much as TWIST1. Therefore, we focused  
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7 389 on the functional analyses of SNAIL and its downstream targets in gastric carcinogenesis.  
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10 390 Indeed, we showed that knockdown of SNAIL resulted in acquisition of EMT-phenotype  
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12 391 and loss of CD44+ cell population. It also led to the impaired growth of gastric cancer  
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14 392 xenografts and chemoresistance against 5-FU treatment. These data are consistent with  
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16 393 discoveries reported in other solid malignancies [32-34], and indicates crucial roles of  
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18 394 SNAIL in regulating CSC properties.

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22 395 To further explore the underlying mechanisms of SNAIL-mediated gastric  
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24 396 carcinogenesis, we performed microarray analyses which revealed transcriptome alterations  
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26 397 by knockdown of SNAIL. GO terms formed a treemap of the most significant processes:  
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28 398 cell motility, chemotaxis, adhesion, neural regeneration, and cell death, as well as those  
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30 399 involved in EMT. Among the components from the network of chemotaxis, for example,  
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32 400 sox2 has been reported as the key regulator in CSCs and is over expressed in various  
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34 401 tumors. Other significant genes included TGFB1 and NOTCH1; both belong to the Notch  
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36 402 signaling pathway, which plays an important role in angiogenesis and CSC self-renewal  
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38 403 [35]. Indeed, most of the significant GO terms identified are implicated in well-known  
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40 404 signaling processes. Genes from the regeneration process were also included in EMT  
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42 405 process with significant biological functions. Among them, CCN3 is described in prostate  
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44 406 cancer [23]. Meanwhile, NEFL, was found to be expressed in a wide range of malignancies  
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46 407 as a tumor suppressor gene [36]. Based on study interest, CCN3 and NEFL were here  
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48 408 chosen as two possible downstream targets of SNAIL that seem to function in stemness in  
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50 409 gastric cancer.  
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6 410 CCN3 belongs to the three-member family of cysteine-rich regulatory proteins and  
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8 411 has been found in various cancer cells and surrounding tissues, suggesting that in the cancer  
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10 412 microenvironment, it is likely that CCN3 may act as a EMT-regulatory factor [37].  
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12 413 Consistent with this, CCN3 requires its C-terminal domain for bone metastatic function,  
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14 414 and is correlated with aggressive disease progression in prostate cancer [38]. In  
15  
16 415 hepatocellular carcinoma, after being secreted from hepatic cells, CCN3 gained its' activity  
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18 416 in various processes during EMT via HSCs (Hepatic stellate cells) [39]. A previous study  
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20 417 has also shown that increased expression level of CCN3 expression lead to local invasion  
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22 418 and distant metastases in gastric cancer [40, 41]. In this study, we showed that a link  
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24 419 between CCN3 expression and gastric stemness properties, and have further shown that low  
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26 420 levels of CCN3 in HGC27 cells result in reduced stemness and tumorigenicity. Therefore,  
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28 421 approaches that capable of reducing CCN3 expression have the potential to suppress EMT  
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30 422 and to be novel therapies against gastric cancer.  
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36 423 NEFL, which practically maintains the neuronal caliber and functions as a regulator  
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38 424 in intracellular transport to axons and dendrites, has also be implicated in various  
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40 425 carcinogenesis [42]. NEFL acts as a tumor suppressor in non-small cell lung cancer  
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42 426 (NSCLC), inhibiting invasion and metastasis, while methylation may destroy its protective  
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44 427 effect [43]. In NSCLC and breast cancer, patients with higher expression of NEFL mRNA  
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46 428 transcript had a better five-year disease-free survival [36]. In contrast to these reports, our  
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48 429 study showed that NEFL might enhance the tumor development in xenografts. The reason  
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50 430 behind the contrasting outcomes maybe due to the fact that in the advanced stage of  
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52 431 malignancy, cancer cells tend to become much fiercer in metastatic potential and  
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6 432 phenotypes, thus the invasions are nowhere to be hold arrested by merely cytoprotective  
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8 433 genes expression alterations [44]. In either case, NEFL in digestive system cancers such as  
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10 434 gastric cancer may play distinct roles in a context-dependent manner.  
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13 435 In conclusion, we showed that SNAIL regulates the expressions of CCN3 and  
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15 436 NEFL genes in human gastric cancer cells, and that these in turn control the CSC activities.  
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17 437 Strategies that disrupt this possible circle may be possible to treat gastric cancer in future.  
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19 438 Double antagonists targeting both CCN3/NEFL-SNAIL axis may weaken the malignant  
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21 439 progression and dual RNAi study should be considered before RNAi compound  
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23 440 development. Also, protein secretion of the target molecule that plays important roles in  
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25 441 malignancy can be traced and intentionally attacked through clonal antibody. Multiple  
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27 442 channels of further application as therapeutic agents may be helpful for patients with gastric  
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29 443 cancer.  
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42 447 Ltd.  
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For Peer Review



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601 **Legends:**

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9 602 Figure 1. Characteristics of gastric cancer cell lines spheres cultured under serum-free  
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11 603 condition.

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14 604 A. Photographic pictures of sphere morphology.

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17 605 Initial and passage sphere assay were shown in upper and lower lane pattern respectively  
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19 606 with each cell line marked on top.

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22 607 B. Sphere formed per  $2 \times 10^4$  seeded cells as an index of cell renewal capacity.

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25 608 a: sphere count; b: volume summary of sphere formed. Results were expressed as mean  $\pm$   
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27 609 SD, \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

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30 610 C. Enhanced in vivo tumor volume formed from spheres in mice at an injection  
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32 611 concentration of  $4 \times 10^4$  cells.

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35 612 D. Representative H&E slides from gastric cancer cell line NCI-N87 with their sphere  
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37 613 formed tumors. Scale bar= $200\mu\text{m}$ .

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44 615 Figure 2. Stem cell properties and tumor malignancy in gastric cancer spheres.

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47 616 A. Comparison of EMT factors on absolute mRNA expression levels in gastric cancer cell  
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49 617 lines.

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52 618 B. Immunofluorescence of SNAIL expression in HGC27, IM95 and FU97 cell lines.  
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6 619 a. Cells in the gastric cancer cell lines were found to express SNAIL (imaged with red  
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8 620 fluorescent) in the nuclei;  
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11 621 b. Image with DAPI to identify the nuclei of gastric cancer cells;  
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14 622 c. Merged image superimposed on a differential interference contrast background confirms  
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16 623 co-localization. Scale bar=50 $\mu$ m.  
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22 625 Figure 3. SNAIL is sufficient and essential for induction of self-renewal and malignancy in  
23  
24 626 gastric cancer cell line HGC27. (Knockdown is represented as k.d.).

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27 627 A. Real-time quantitative PCR validation of SNAIL expression level in HGC27 and its  
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29 628 stable SNAIL knockdown cell lines.

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32 629 B. Effects of SNAIL knockdown on the expression of E-cadherin and Vimentin in HGC27  
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34 630 cell line.

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37 631 C. Morphology alteration of gastric cancer cell line HGC27 after SNAIL knockdown in  
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39 632 ultra-low attachment condition.

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42 633 D. CD24 and CD44 FACS profiles of HGC27 and HGC27 SNAIL knockdown cell lines.

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45 634 E. Sphere formed per  $2 \times 10^4$  seeded cells from HGC27 and HGC27 SNAIL knockdown cell.

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48 635 F. Diminished in vivo tumorigenicity formed from HGC27 SNAIL knockdown cells in

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51 636 NOD/SCID mice at an injection concentration of  $4 \times 10^4$  cells. H&E staining of

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54 637 representative tumors, scale bar=200 $\mu$ m.  
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5 638 G. Decreased chemoresistance of HGC27 SNAIL knockdown cells compared with HGC27  
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8 639 when treated with 5-FU.  
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14 641 Figure 4. Microarray analysis between HGC27 and its SNAIL knockdown samples.

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17 642 A. Plot analysis of distribution visualization of differentially expressed genes comparing  
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19 643 SNAIL knockdown and control groups.

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22 644 B. Bar plot detailing presented numbers of genes under SNAIL up/down regulation in two  
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24 645 conditions.

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27 646 C. GO terms enrichment analysis of molecular function, cellular component and biological  
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29 647 process of differentially expressed genes (left to right).

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32 648 D. The KEGG enrichment analysis of differentially expressed genes.

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35 649 E. Real-time quantitative PCR validation of CCN3 and NEFL expression level in HGC27  
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37 650 and its stable SNAIL knockdown cell lines.  
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44 652 Figure 5. Elevated expression of SNAIL and CCN3 in patients with stomach  
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46 653 adenocarcinoma.

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49 654 A. mRNA transcript expression levels of SNAIL (a), CCN3 (b) and NEFL (c) are elevated  
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51 655 in patients with gastric cancer from TCGA-STAD database. Box in red: malignant tissue;  
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53 656 box in blue: normal tissue.  
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6 657 B. No correlation existed between CCN3 and NEFL in gastric cancer patients from TCGA-  
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8 658 STAD database.

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11 659 C. SNAIL, CCN3 and NEFL are highly expressed in human gastric cancer tissues.

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14 660 Representative immunohistochemical staining (IHC) images of SNAIL, CCN3 and NEFL  
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16 661 in gastric cancer tissues. Scale bar = 50  $\mu$ m.

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22 663 Figure 6. CCN3 and NEFL correlate with self-renewal and chemoresistance traits in gastric  
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24 664 cancer cells.

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27 665 A. Western blotting analysis of NEFL and CCN3 expression in associated cell lines.

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30 666 B. Sphere formed per  $2 \times 10^4$  seeded cells as an index of cell renewal capacity.

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33 667 a. HGC27 SNAIL knockdown vs. HGC27 SNAIL knockdown with SNAIL re-introduction;

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36 668 b. HGC27 vs. HGC27 with NEFL introduction;

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39 669 c. HGC27 SNAIL knockdown vs. HGC27 SNAIL knockdown with CCN3 re-introduction.

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42 670 C. Chemoresistance alteration of SNAIL (a), CCN3 (b) and NEFL (c) introduction into

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44 671 associated HGC27 cell lines when treated with 5-FU. Results are expressed as mean  $\pm$  SD,

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46 672 \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

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49 673 D. Knockdown of NEFL promoted the mesenchymal traits in gastric cancer cell line IM95.

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52 674 a. Real time qPCR validation of NEFL expression level in IM95 and its stable NEFL

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55 675 knockdown cell lines.

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6 676 b. Effects of NEFL knockdown on the expression of E-cadherin and Vimentin in IM95 cell  
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8 677 line.

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11 678 c. Increased chemoresistance of IM95 NEFL knockdown cells compared with IM95 cells  
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13 679 when treated with 5-FU. Results are expressed as mean  $\pm$  SD, \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

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19 681 Figure S1. Enhanced *in vivo* tumor volume formed from spheres in mice at an injection  
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21 682 concentration of  $4 \times 10^4$  cells of HGC27 and NUGC3 cells. Representative H&E slides  
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23 683 from gastric cancer cell lines with their sphere formed tumors. Scale bar=200 $\mu$ m.

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29 685 Figure S2. Representative immunohistochemical staining (IHC) images of CCN3 and  
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31 686 NEFL in gastric normal tissues. Scale bar = 50  $\mu$ m.

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6 **1 SNAIL regulates gastric carcinogenesis through CCN3 and NEFL**

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9 **2 Role of SNAIL/CCN3/NEFL axis in gastric cancer**

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3 Ru Chen<sup>1,2</sup>, Kenji Masuo<sup>1,2</sup>, Akitada Yogo<sup>1,3</sup>, Shoko Yokoyama<sup>1</sup>, Aiko Sugiyama<sup>1</sup>, Hiroshi  
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6 19 **Abstract**  
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8 20 Among cancer cells, there are specific cell populations of whose activities are comparable  
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10 21 to those of stem cells in normal tissues, and for whom the levels of cell dedifferentiation are  
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12 22 reported to correlate with poor prognosis. Information concerning the mechanisms that  
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14 23 modulate the stemness-like traits of cancer cells is limited. Therefore, we here examined  
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16 24 five gastric cancer cell lines and isolated gastric oncospheres from three gastric cancer cell  
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18 25 lines. The gastric cancer cells that expanded in the spheres expressed relatively elevated  
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20 26 proportion of CD44, which is a marker of gastric cancer stem cells, and displayed many  
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22 27 properties of cancer stem cells, for example: chemoresistance, tumorigenicity and  
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24 28 epithelial-mesenchymal transition (EMT) acquisition. SNAIL, which is a key factor in  
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26 29 EMT, was highly expressed in the gastric spheres. Microarray analysis in gastric cancer cell  
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28 30 line HGC27 showed that CCN3 and NEFL displayed the greatest differential expression by  
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30 31 knocking down of SNAIL; the former was up-regulated and the latter down-regulated,  
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32 32 respectively. Down-regulation of CCN3 and up-regulation of NEFL gene expression  
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34 33 impaired the SNAIL-dependent EMT activity: high tumorigenicity and chemoresistance in  
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36 34 gastric cancer cells. Thus, approach that disrupts SNAIL/CCN3/NEFL axis may be credible  
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38 35 in inhibiting gastric cancer development.  
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46 36 **Keywords:** Gastric Cancer; Cancer Stem Cells; EMT; spheroid  
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## 38 Introduction

39 Gastric cancer is one of the most common cancers in East Asia and Eastern Europe  
40 [1]. It is important to critically assess the current advances in our understanding of gastric  
41 cancer and to establish novel and innovative therapeutic strategies. A vast body of literature  
42 has been published on specific aspects of cancer initiating cells and on putative cancer stem  
43 cells (CSCs) which possess properties of stem cells distinct from differentiated progeny  
44 cancer cells [2]. Discovering significant genes and signaling pathways involving gastric  
45 cancer stemness could be helpful approaches to discovering novel therapeutic options.

46 During malignancy transformation, a critical process named the epithelial-  
47 mesenchymal transition (EMT) commonly occurs, and cells usually undergo a rapid change  
48 from differentiated and polarized epithelial state into an invasive mesenchymal composition  
49 [3]. During the development of diverse solid tumors, stem cell like traits were reported to  
50 be related to EMT. For example, after breast cancer cells acquired stem cell like features,  
51 the passaged mammosphere cells manifest with similar features to breast cancer stem cells,  
52 indicating a fundamental link between malignancy propagation and stem cell characteristics  
53 [4-6]. Among all the major EMT transcription factors, SNAIL, a zinc-finger protein, whose  
54 activities in relation to the downregulation of E-cadherin in colon cancer have previously  
55 been reported [7, 8]; binds to the E-boxes in the *CDHI* gene promoter and represses  
56 transcription of the *CDHI* gene [9]. So far, SNAIL has been reported to contribute in many  
57 malignancy progression, and its' function in gastric cancer needs to be uncovered further as  
58 well. The precise mechanism of SNAIL-induced cell dedifferentiation and how this gene

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6 59 can provide stem cell like traits in gastric cancer cells remain open to debate and to be  
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8 60 further clarified. The discovery of genes under SNAIL regulation that could also be an  
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10 61 instrumental breakthrough and lead to the establishment of novel therapeutic strategies in  
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12 62 EMT-related stemness and malignancy. In the present study, we extracted CCN3 and NEFL  
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15 63 as targets in the downstream of SNAIL, and determined the association of these two factors  
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17 64 with stem cell like activity in gastric cancer cells.  
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For Peer Review



## 66 **Materials and Methods:**

### 67 **Cell culture, tissue collection and sphere growth**

68 Human gastric cancer cell lines were purchased from RIKEN  
69 (<https://cell.brc.riken.jp/ja/quality/str>), JCRB cell bank  
70 ([https://cellbank.nibiohn.go.jp/about-qc\\_english/](https://cellbank.nibiohn.go.jp/about-qc_english/)), and ATCC  
71 ([https://www.atcc.org/Services/Testing\\_Services/Cell\\_Authentication\\_Testing\\_Service.aspx](https://www.atcc.org/Services/Testing_Services/Cell_Authentication_Testing_Service.aspx)),  
72 in which STR analysis is performed in these cell line banks to ensure the authentication  
73 of human cell lines, and were cultured according to the instructions provided by the  
74 manufacturer. Cell lines including human gastric cancer cell lines (HGC27, NCI-N87, GSU,  
75 MKN74, MKN45 ~~and NUGC3~~, NUGC3 and IM95), and embryonic kidney 293T cells  
76 were cultured in DMEM (Nacalai Tesque, Japan) supplemented with 10% fetal bovine  
77 serum (HyClone Defined Fetal Bovine Serum (FBS), USA) and penicillin-streptomycin  
78 mixed solution (10,000u/ml, Nacalai Tesque, Japan). For RNA extraction from each cell  
79 line, NucleoSpin RNA Plus (Takarabio, Japan) was used following the manufacturer's  
80 instructions. The preparation of paraffin-embedded blocks was performed as follows: slices  
81 of tumor formed from each of the cell lines were immersed in 4% paraformaldehyde  
82 (Nacalai Tesque, Japan) to allow the assembling of paraffin-embedded blocks. Cells were  
83 cultured in homemade stem cell medium (DMEM/F12 supplemented with B27 Supplement  
84 (ThermoFisher), 10 ng/mL recombinant basic fibroblast growth factor (bFGF,  
85 ThermoFisher), 10 ng/mL epidermal growth factor (EGF, ThermoFisher), and 1%  
86 penicillin-streptomycin) to obtain spheres. A total of  $1 \times 10^4$  cells per milliliter were seeded

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6 87 in culture medium for stem cell and incubated in ultra-low attachment plates for 5 days.  
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8 88 Spheres larger than 80  $\mu\text{m}$  in diameter were counted using Cell3Imager (InSphero AG and  
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10 89 Dainippon SCRREEN, Kyoto, Japan). TrypLE Express (ThermoFisher) or Trypsin-EDTA  
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12 90 (FUJIFILM Wako, Japan) were used to separate cells from the floating spheres and  
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15 91 adherent cells to allow cell counting and other experiments.  
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## 17 92 **In vivo tumorigenicity assay**

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20 93 All procedures involving animals were conducted in accordance with the  
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22 94 Institutional Animal Welfare Guidelines of Kyoto University. NOD/SCID mice were  
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24 95 purchased from the Charles River Laboratories (Yokohama, Japan) and were maintained  
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26 96 according to the Guidelines for Laboratory Animals in the Kyoto University. The  
27  
28 97 tumorigenicity assay was performed by subcutaneous injection of  $1 \times 10^4$  designated cells  
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30 98 into the flanks of 8- to 10-week-old NOD/SCID mice. Mice were sacrificed and examined  
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32 99 for tumor harvest once the tumor had reached pre-determined size (2.5cm maximum).  
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37 100 Tumor size was measured with calipers once a week after the injection.  
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## 39 101 **Lentivirus production, short-hairpin RNA-mediated human SNAIL gene knockdown** 40 41 42 102 **and stable clone establishment**

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45 103 The lentivirus package system: pMDLg/pRRE (Addgene, plasmid #12251), pRSV-  
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47 104 Rev (Addgene, plasmid #12253) and pMD2.G (Addgene, plasmid #12259) together with  
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49 105 the shRNA plasmid targeting the human SNAIL gene as well as the control vector:  
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51 106 pLKO.1puro (Addgene, plasmid #8453) were co-transfected into 293T cells by  
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54 107 Lipofectamine 3000 (Invitrogen). SNAIL-targeting short-hairpin RNA (MISSION shRNA)  
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6 108 duplex (A: 5-TGCTCCACAAGCACCAAGAGTC-3; B: 5-  
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8 109 CCACTCAGATGTCAAGAAGTAC-3) and NEFL-targeting short-hairpin RNA  
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10 110 (MISSION shRNA) (5-CGACAGCTTGATGGACGAAAT-3) were was purchased from  
11  
12 111 Sigma-Aldrich Co. LCC. (St. Louis, MO, USA). About 48 to 72 hours later, virus  
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14 112 supernatant was collected for concentration. For shRNA knockdown, HGC27 cells were  
15  
16 113 seeded onto 6-well plates and infected with optimal virus concentrations supplemented with  
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18 114 6 µg/mL Polybrene (Sigma, St. Louis, MO, USA), then incubated for 12 hours before  
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20 115 replacing with fresh medium. Cells were then selected by puromycin (INVIVOGEN, Japan)  
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22 116 at the concentration of 1.8 µg/ml (HGC27) and 4 µg/ml (IM95) for 2 weeks.  
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### 27 117 **Transient transfection**

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30 118 Lipofectamine 3000 reagent was used for the introduction of overexpression vectors  
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32 119 to establish stable lines, including the NEFL (Sino Biol.HG13214-UT), CCN3 (Sino  
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34 120 Biol.HG10264-UT) and SNAIL (Sino Biol.HG16844-UT) overexpression vectors and  
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36 121 matched control vector (Sino Biol.CV011), all of which were purchased from Sino  
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38 122 Biological Inc. (Wayne, PA, USA). Selections were carried out via hygromycin B (Nacalai  
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40 123 Tesque, Japan); resistance and transfection efficiency were verified through real-time qPCR.  
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### 44 124 **Microarray data and bioinformatics analysis**

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47 125 Total RNA from each sample was extracted using NucleoSpin RNA Plus kit,  
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49 126 forwarded with Affymetrix Human Genome U133 Plus 2.0 Array (HuGene2.9st, Japan)  
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51 127 analysis. RNA extraction, microarray hybridization, and feature selection were performed  
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53 128 according to the manufacturer's protocol. Microarray data can be download from the GEO  
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6 129 database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145867>).  
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8 130 Bioinformatics and genetic network construction were performed in R Studio (version  
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10 131 1.2.1335) mainly using RMA [10] from the affy package [11] and the GoPlot package  
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12 132 (<https://CRAN.R-project.org/package=GOplot>) [12]. Treemaps were created using  
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14 133 REVIGO webtool (<http://revigo.irb.hr/index.jsp>) [13]. Gene enrichment analysis was  
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16 134 performed using DAVID 2010 Bioinformatics Resources (<http://david.abcc.ncifcrf.gov/>)  
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19 135 [14].

### 136 **Reverse transcription and real-time quantitative PCR**

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25 137 Using the PrimeScript II 1st strand cDNA Synthesis Kit (Takarabio, Japan), 3  
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27 138 micrograms of total RNA were transformed into first-strand complementary DNA synthesis  
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29 139 following directions provided by the manufacturer. Human pre-messenger RNA sequences  
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31 140 were obtained from NCBI gene ([www.ncbi.nlm.nih.gov/gene/](http://www.ncbi.nlm.nih.gov/gene/)) before using NCBI blast  
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33 141 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to design primers in PCR. All the primer  
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35 142 sequences used in this study can be checked in Table S1. Real-time quantitative PCR  
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37 143 (qPCR) was performed to evaluate the expression levels using SYBR Green Master Rox  
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39 144 (Roche, Sigma-Aldrich), and were analyzed using the StepOnePlus real-time system  
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41 145 (Applied Biosystems). The endogenous expression level of GAPDH was used to obtain the  
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43 146 expression levels of other genes via  $\Delta\Delta C_t$  methods.  
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### 49 **Drug resistance and CCK8 assay**

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52 148 Cells were seeded at the concentration of  $1 \times 10^4$  cells/ml in 96-well plates and  
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54 149 incubated overnight before application of various concentration of chemotherapy drugs  
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5 150 (1 $\mu$ M to 200 $\mu$ M 5-FU (KYOWA KIRIN, Japan)). After 96 hours, medium was discarded  
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8 151 and CCK8 assay solution (Dojindo molecular technologies) was added to cells and  
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10 152 incubated for 1 hour at 37°C. The plate was then read by a microplate reader (OD450;  
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12 153 Infinite F50, TECAN).

#### 154 **Western blotting assay**

155 Proteins were extracted from relevant cell lines using ice-cold RIPA buffer (Nacalai  
156 Tesque, Japan) after washing with 1x phosphate-buffered saline (PBS). Later proteins were  
157 separated in 10-20% gels (SuperSep Ace, Fujifilm Wako, Japan) and then transferred onto  
158 PVDF membranes (Immobilon-P, Merck M), and blocked using skimmed milk (Fujifilm  
159 Wako, Japan). The collection of primary antibodies used in this study were Snail (ab53519;  
160 Abcam), CCN3 (ab191425; Abcam), NEFL (ab223343; Abcam), and E-cadherin (24E10,  
161 Cell Signaling Technology), Vimentin (D21H3, Cell Signaling Technology), CD24  
162 (ab199140 Abcam). Proteins were incubated with the primary antibodies over night at 4°C.  
163 They were then stained with anti-mouse, anti-rabbit or anti-goat secondary antibodies  
164 (Jackson Laboratory) for 60 minutes at room temperature. Thereafter they were incubated  
165 with chemiluminescent HRP substrate (WBKLS0500, Merck M) for 5 minutes.  
166 Chemiluminescence signals were collected via the Fujifilm LAS-3000 (Fuji, Japan) as per  
167 the manufacturer's instructions.

#### 168 **Immunocytochemistry and fluorescence assay**

169 Cells were seeded onto 8-well culture slides (#354118, Falcon) overnight before  
170 fixation with 4% PFA for 10 minutes at room temperature followed by 1x PBS washes.

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5 171 Cells were permeabilized with 0.5% Triton X-100/PBS for 5 minutes at room temperature,  
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7 172 washed with 1x PBS, and blocked in 5% BSA (Sigma) in PBS for 60 minutes before  
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10 173 incubating with Snail (20C8, ThermoFisher) primary antibodies followed by the secondary  
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12 174 antibodies Alxea Fluor 594 (ThermoFisher). Slides were mounted in VECTASHIELD  
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14 175 Mounting Medium with DAPI (H-1200; VECTOR Lab, Japan). Fluorescence images were  
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16 176 visualized with Keyence fluorescence microscope.  
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### 19 20 177 **Immunohistochemistry staining**

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23 178 Human gastric cancer tissue array (MLB Life Science Japan) and tumor specimens  
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25 179 from mice gastric tumors were deparaffinized, rehydrated and placed in 3% (v/v) H<sub>2</sub>O<sub>2</sub>-  
26  
27 180 methanol for 15 min at room temperature. The slides were then immersed in blocking  
28  
29 181 solution (Non-specific Staining Blocking Reagent; Dako-Cytomation, Kyoto, Japan) for 15  
30  
31 182 min and incubated with the primary antibodies listed below at 4 °C overnight. Antigen-  
32  
33 183 antibody complexes were detected with a secondary antibody (Histofine Simple Stain  
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35 184 MAX PO (R) for rabbit monoclonal, or (G) for goat polyclonal (Nichirei, Tokyo, Japan)  
36  
37 185 and visualized using 3,30-diaminobenzidine (0.5 mg/ml in Tris-buffered saline). The list of  
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39 186 primary antibodies and dilution ratios were as follows: 1. Anti-SNAIL antibody (goat  
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41 187 polyclonal, ab53519, Abcam), 1: 1,000; 2. Anti-CCN3 antibody (rabbit monoclonal,  
42  
43 188 ab191425, Abcam), 1:100; 3. Anti-NEFL antibody (rabbit monoclonal, ab223343, Abcam),  
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45 189 1:400.  
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### 51 190 **Flow cytometry**

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6 191 Incubation buffer was prepared as 1x PBS + 2% FBS. Single cell suspensions were  
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8 192 washed with cooled incubation buffer, and re-suspended in 1x PBS + 2% FBS on ice for 30  
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10 193 minutes for antibody blocking: anti-CD24-FITC (ML5; Bio-legend, San Diego, CA),  
11  
12 194 CD44-FITC (BJ18; Bio-legend, San Diego, CA) and DAPI (422801, Bio-legend, San  
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14 195 Diego, CA). Cells were suspended in 0.5 mL incubation 1x PBS + 2% FBS to reach a final  
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16 196 concentration of  $10^6$  cells/ml. Data were collected by the BD FACSCanto II or BD  
17  
18 197 FACSVerse flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) and analyzed with  
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20 198 FlowJo software (TreeStar, San Carlos, CA). Cell debris was excluded from the analysis  
21  
22 199 based on scatter signals, and fluorescent compensation was adjusted when double stained.  
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## 26 27 200 **Statistical Analyses**

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30 201 Independent sample t tests were performed to compare the continuous variation of  
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32 202 two groups, and the  ~~$\chi^2$  test or Fisher exact student's~~ test was applied for comparisons of  
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34 203 variables.  $P < 0.05$  was considered significant. All data are reported as mean  $\pm$ SEM.  
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## 205 **Results**

### 206 **Gastric Spheres Cultured Under Serum-Free Conditions Manifest Stem Cell**

#### 207 **Properties**

208 Self-renewal capability is a major property of stem cells, and can be accurately  
209 assessed via sphere formation [15]. Gastric cancer cell lines produce stem-like sphere-  
210 forming cells when cultured under B-27 (+) bFGF (+) EGF (+) serum-free medium (CSC  
211 serum-free medium) in ultra-low attachment culture dishes [16]. The original gene  
212 expression profiles and tumor morphologies in cancer cells are well reflected in spheres  
213 cultured in the CSC serum-free condition [17]. We assessed the sphere-forming capacity of  
214 five gastric cancer cell lines for initial culture (cells were collected from attached condition  
215 and seeded in CSC serum-free medium) and passage culture (cells were collected from  
216 formed spheres and seeded in CSC serum-free medium), and found that three cell lines had  
217 the ability to form spheres (Figure 1A). Those spheres all originated from single cell and  
218 not by mere cell herds or aggregations, which was ensured by seeding single cells in 96  
219 well plates and spheres managed to develop after several weeks (data not shown). Sphere-  
220 forming capacity of passaged cells (passage culture) was stronger compared with that of  
221 parental cells (initial culture), when the same number of cells were seeded in CSC serum-  
222 free medium (Figure 1B). To further confirm whether malignant cells possess additional  
223 stemness traits,  $1 \times 10^4$  NCI-N87 cells were transplanted into the flanks of NOD/SCID  
224 mice. Histological analysis of xenografts exhibited an epithelial-like morphology  
225 irrespective of whether they were generated by parental or sphere cells (Figure 1C & S1);



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6 226 however, sphere cells were more efficient at producing bulky tumors in NOD/SCID mice  
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8 227 compared with parental monolayer cells (Figure 1D).  
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### 12 13 14 229 **Gastric Spheres Express EMT-associated Factors**

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17 230 Acquisition of stemness traits in malignant cells is commonly achieved through  
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19 231 undergoing EMT process, thus tracking the activities of EMT-associated factors will help  
20  
21 232 uncovering mechanism behind the obtained stemness. Four key EMT factors were  
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23 233 examined in this study, and higher mRNA expression of EMT factors (TWIST1, 2, SNAIL,  
24  
25 234 and SLUG) were found in spheres compared with parental cells (Figure 2A). HGC27 cells,  
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27 235 which form spheres most efficiently, highly expressed TWIST1 and SNAIL mRNAs  
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29 236 compared with other two sphere-forming cell lines, NCI-N87 and NUGC3. Consistent with  
30  
31 237 this, immunofluorescence images indicated **highest** protein expression of SNAIL in HGC27  
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33 238 cells (Figure 2B). The role of TWIST1 has been extensively investigated in previous EMT-  
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35 239 associated researches [18]; therefore, HGC27 and SNAIL were chosen as the target cell line  
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37 240 and molecule pair in the current study.  
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### 44 45 46 242 **SNAIL Regulates Tumorigenicity in Gastric Cancer Cells**

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49 243 In order to investigate whether SNAIL regulates stemness and tumorigenicity in  
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51 244 gastric spheres, we determined phenotypic alteration after lentivirus-mediated short-hairpin  
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53 245 RNA-interfered knockdown of SNAIL (shRNA-SNAIL k.d.). Realtime quantitative PCR  
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6 246 indicated successful knockdown effects using shRNA-SNAIL in HGC27 cell lines and  
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8 247 spheres (Figure 3A). Western blotting showed a similar tendency of 29 Da SNAIL protein  
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10 248 expression (Figure 3B). Epithelial and mesenchymal traits of HGC27 were measured using  
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12 249 antibodies against E-cadherin and Vimentin, representative proteins for epithelial and  
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14 250 mesenchymal phenotypes, respectively. Up-regulation of E-cadherin with down-regulation  
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16 251 of Vimentin by SNAIL knockdown indicated the event of mesenchymal-to-epithelial  
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18 252 transition in HGC27 cell lines (Figure 3B). The most conspicuous phenomenon observed  
19  
20 253 was that the shape of formed sphere in the CSC serum-free medium switched from having  
21  
22 254 smooth margins into jagged and sharp edges by the SNAIL knockdown (Figure 3C).  
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25 255 Previous studies reported a CD44<sup>+</sup>/CD24<sup>-</sup> subpopulation of gastric cancer contains gastric  
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27 256 cancer stem cells [19]. As shown in Figure 3D, the fluorescence activated cell sorter  
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29 257 analysis revealed that 91.0% of the HGC27 cell line was CD44<sup>+</sup>/CD24<sup>-</sup>, while knockdown  
30  
31 258 of SNAIL in this cell line almost completely eliminates this population. This suggested that  
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33 259 gastric cancer stem cells are maintained, at least in part, by the presence of SNAIL.  
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38 260 Stemness such as self-renewability in cancer cells is associated with the capacity of  
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40 261 forming spheres out of the transformed epithelial cells [20]. Therefore, we compared sphere  
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42 262 forming capacity between parental HGC27 and stably SNAIL knockdown HGC27 cells  
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44 263 cultured in monolayer conditions. The number and area of sphere were significantly  
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46 264 decreased by SNAIL knockdown, suggesting a reduced ability to self-renewal (Figure 3E).  
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48 265 Consistent with this, when  $1 \times 10^4$  cells were transplanted into the flanks of NOD/SCID  
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50 266 mice, although histological analysis did not show any significant alterations, stably  
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6 267 SNAIL knockdown HGC27 cells were less efficient at producing bulky tumors ~~in~~  
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8 268 compared with parental HGC27 cells (Figure 3F).  
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11 269 Lower expression of SNAIL was referred to as increased sensitivity upon chemo-  
12  
13 270 treatment due to diminished self-renewability in malignancy [21]. In our experimental  
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15 271 condition, stable SNAIL knockdown HGC27 cells were more susceptible to 5-fluorouracil  
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17 272 (5-FU) treatment compared with parental HGC27 cells (Figure 3G).  
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21 273 Together, these findings SNAIL regulates tumorigenicity, possibly stemness at least  
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23 274 in part, in gastric cancer cells.  
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### 29 276 **SNAIL-regulating Genes in Gastric Cancer Cells**

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32 277 To unveil the mechanisms underlying phenotypic modifications induced by  
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34 278 knockdown of SNAIL in HGC27 cells, gene expression microarray analysis on a HGC27-  
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36 279 SNAIL knockdown and parental HGC27 cells was performed. Under the alteration of  
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38 280 SNAIL expression, distributed genes movement were shown in Figure 4A. Of those genes,  
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40 281 a total of 1656 and 1832 probe sets ( $|FC| \geq 2$ ) were specifically upregulated or  
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42 282 downregulated in stable HGC27-SNAIL knockdown cells, respectively (Figure 4B).  
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44 283 Statistically overrepresented functional processes were obtained through enriched genes  
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46 284 querying in the Gene Ontology (GO) database ( $P < 0.05$ ; Figure 4C). The main processes  
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48 285 enriched in up-/down-regulated genes include those pertaining to multiple binding and  
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50 286 cellular processes. It is suggested that instead of individual activation, genes tend to  
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5 287 collaborate in genetic networks, thus we subjected the enriched GO processes that were  
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7 288 selected as meaningful to REVIGO analysis, which is a useful web tool that summarizes  
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10 289 long lists of GO terms. Several GO terms formed a treemap of the most significant  
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12 290 processes: cell motility, chemotaxis, adhesion, neural regeneration, and cell death, as well  
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14 291 as those involved in EMT (Figure 4D). Within this network, chemotaxis was a focal node  
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16 292 and in the broader group of terms under the chemotaxis title, the regeneration process was  
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18 293 chosen as a target to be studied in greater depth due to the possible role of neuron  
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21 294 development and supervision in malignancy progression. ~~For example, Sox2 has been~~  
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23 ~~reported as the key regulator in CSCs and is over-expressed in various tumors. Other~~  
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25 295 ~~significant genes included TGFBI and NOTCH1; both belong to the Notch signaling~~  
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27 296 ~~pathway, which plays an important role in angiogenesis and CSC self-renewal [22]. Indeed,~~  
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29 297 ~~most of the significant GO terms identified are implicated in well-known signaling~~  
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31 298 ~~processes. Genes from this regeneration process were also included in EMT process with~~  
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33 299 ~~significant biological functions. Among them, Cellular Communication Network factor 3~~  
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35 300 ~~(CCN3; also known as NOV or IGFBP9) as described in prostate cancer [23]. Meanwhile,~~  
36  
37 301 ~~Neurofilament Light Peptide (NEFL), was found to be expressed in a wide range of~~  
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39 302 ~~malignancies as a tumor suppressor gene [24].~~ qRT-PCR confirmed down- and up-  
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41 303 regulation of CCN3 (also known as NOV or IGFBP9): Cellular Communication Network  
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43 304 factor 3, and NEFL: Neurofilament Light peptide mRNA expression by SNAIL knockdown  
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45 305 in HGC27 cells (Figure 4E).  
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## 308 **CCN3 and NEFL Expression in Gastric Cancers**

309           The occurrence of distant metastases and poor survival outcome have been reported  
310 relevant with high SNAIL expression in gastric cancer patients [25]. CCN3 belongs to the  
311 CCN family (cysteine-rich protein 61 (CYR61)), connective tissue growth factor (CTGF),  
312 nephroblastoma overexpressed (NOV). Limited information is known concerning the  
313 functional correlation between EMT with CCN3 and NEFL in cancers. There have been  
314 reports of increased expression of CCN3 in prostate and cervical cancers [23, 26]. CCN3  
315 was demonstrated to promote EMT by activating the FAK/Akt/HIF-1 $\alpha$  pathway in prostate  
316 cancer [27]. NEFL has been implicated in carcinogenesis as a putative suppressor gene in  
317 neuron related inhibition of both cell proliferation and invasion in head and neck squamous  
318 cell carcinoma [28]. Using data of transcript level of SNAIL, CCN3 and NEFL from The  
319 Cancer Genome Atlas (TCGA) stomach adenocarcinoma project, each gene expression in  
320 gastric cancers and normal gastric tissues were shown in Figure 5A. There were no  
321 correlations in mRNA expression levels between SNAIL and either CCN3 or NEFL, and  
322 no correlation between CCN3 and NEFL (Figure 5B). Immunohistochemistry analysis was  
323 then performed on a paraffin-embedded human gastric cancer tissue array (n = 30,  
324 purchased from MBL Life Science, Japan). CCN3 expression was detected in 96.7%  
325 (29/30), NEFL expression was positive in 86.7% (26/30) and SNAIL expression occurred  
326 in 26.7% of tumor tissues (8/30). Representative staining with positive and negative images  
327 for Snail/CCN3/NEFL are shown in Figure 5C, ~~showing the direct expression distribution~~  
328 ~~in human gastric cancer tissue.~~ Compared with Figure S2 of normal gastric tissues, CCN3

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6 329 antibody only stained positive in some fibroblasts and myofibroblasts besides malignant  
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8 330 parts; while NEFL also stained positive in neurofilament as reported elsewhere.  
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### 12 13 14 332 **CCN3 and NEFL are Critical for SNAIL-Induced Stemness**

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17 333 We initially investigated how CCN3 and NEFL function by introducing their  
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19 334 expression vectors into SNAIL knockdown HGC27 cells and parental HGC27 cells  
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21 335 respectively, and those effects were confirmed with western blotting (Figure 6A). Forced  
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23 336 expression of SNAIL or CCN3 in sh-SNAIL knockdown HGC27 cells resulted in  
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25 337 significantly increased number of formed spheres (Figure 6B-a & b); while that of NEFL  
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27 338 caused diminished sphere formation ability in parental HGC27 cells (Figure 6B-c).  
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30 339 Furthermore, the chemoresistance against 5-FU of each pair of cells was also measured.  
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32 340 The results were consistent with alterations to sphere formation capacity: the proliferation  
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34 341 rates of HGC27-shSNAIL, HGC27-NEFL, and HGC27-shSNAIL-CCN3 cells were  
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36 342 reduced more rapidly when 5-FU was added in a dose-dependent manner (Figure 6C). The  
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38 343 fact that HGC27-shSNAIL stable knockdown cells produced significantly reduced tumor  
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40 344 masses compared with SNAIL re-introduced rescue cells was proved via xenograft assays  
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44 345 in vivo (data not shown). To further evaluate the role of NEFL molecule during EMT  
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46 346 process, gastric cancer cell line IM95 was selected from several candidate cell lines due to  
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48 347 high NEFL expression level. Several observations can be witnessed after knockdown of  
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50 348 NEFL in IM95: steady knockdown of NEFL using shRNA in IM95 cell was established  
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52 349 and verified (Figure 6D-a & b), with enhanced mesenchymal and decreased epithelial  
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6 350 indexes of vimentin and E-cadherin discovered from knockdown of NEFL respectively  
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8 351 (Figure 6D-b), and increased chemo-resistance effect throughout proliferation assay with  
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10 352 5FU addition as well (Figure 6D-c).  
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For Peer Review

## 354 Discussion

355 In this study, we established three spheres enriched in CD44+ gastric cancer cells.  
356 The spheres displayed EMT phenotype, high tumorigenicity, and chemoresistance against  
357 5-FU treatment. SNAIL, one of the key regulators of EMT, was upregulated in the spheres,  
358 and CCL3 and NEFL were further extracted as downstream targets of SNAIL by  
359 microarray analyses. Re-introduction of CCN3 and NEFL partially rescued and impaired  
360 the SNAIL-dependent CSC like activity, tumorigenicity, and chemoresistance in HGC-27  
361 gastric cancer cells, respectively.

362 Previous studies have uncovered that CD44, EPCAM, CD133, CD24, CD166 or  
363 Aldh are CSC markers in certain circumstances and are enriched in spheres generated from  
364 gastric cancer cell lines or clinical tissues [29, 30]. Although it stills opens to be discussed  
365 whether all CD44+ cells are CSCs, the CD44+ subpopulation is reported to prevail in  
366 cancer cells after spheres are formed. In this study, we also showed that the CD44+  
367 subpopulation was enriched in gastric spheres.

368 In a mammospheres formation assay, expression of EMT-associated factors, such as  
369 TWIST1/2, SNAIL, and SLUG in breast cancer cells are up-regulated and associated with  
370 the acquisition of CSC properties and greater metastatic ability after EMT process [31].  
371 These EMT-associated molecules are involved in multiple signaling pathways in other  
372 types of cancers. In the present study, expression of TWIST1 and SNAIL were significantly  
373 elevated in gastric spheres, especially in HGC27-derived spheres. The role of TWIST1 in  
374 EMT processes during gastric carcinogenesis has been extensively investigated [32, 33],



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6 375 while that of SNAIL has not been researched as much as TWIST1 remains obscure.  
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8 376 Therefore, we focused on the functional analyses of SNAIL and its downstream targets in  
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10 377 gastric carcinogenesis. Indeed, we showed that knockdown of SNAIL resulted in  
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12 378 acquisition of EMT-phenotype and loss of CD44+ cell population. It also led to the  
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14 379 impaired growth of gastric cancer xenografts and chemoresistance against 5-FU treatment.  
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17 380 These data are consistent with discoveries reported in other solid malignancies [34-36], and  
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19 381 indicates crucial roles of SNAIL in regulating CSC properties.

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22 382 To further explore the underlying mechanisms of SNAIL-mediated gastric  
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24 383 carcinogenesis, we performed microarray analyses which revealed transcriptome alterations  
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26 384 by knockdown of SNAIL. GO terms formed a treemap of the most significant processes:  
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28 385 cell motility, chemotaxis, adhesion, neural regeneration, and cell death, as well as those  
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31 386 involved in EMT. Among the components from the network of chemotaxis, for example,  
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33 387 Sox2 has been reported as the key regulator in CSCs and is over expressed in various  
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35 388 tumors. Other significant genes included TGFB1 and NOTCH1; both belong to the Notch  
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37 389 signaling pathway, which plays an important role in angiogenesis and CSC self-renewal  
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39 390 [22]. Indeed, most of the significant GO terms identified are implicated in well-known  
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41 391 signaling processes. Genes from this regeneration process were also included in EMT  
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43 392 process with significant biological functions. Among them, Cellular Communication  
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45 393 Network factor 3 (CCN3; also known as NOV or IGFBP9) is described in prostate cancer  
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47 394 [23]. Meanwhile, — Neurofilament Light Peptide (NEFL), was found to be expressed in a  
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49 395 wide range of malignancies as a tumor suppressor gene [24]. Based on study interest,  
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6 396 CCN3 and NEFL were here chosen as two possible downstream targets of SNAIL that  
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8 397 seem to function in stemness in gastric cancer.  
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11 398 CCN3 belongs to the three-member family of cysteine-rich regulatory proteins and  
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13 399 has been found in various cancer cells and surrounding tissues, suggesting that in the cancer  
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15 400 microenvironment, it is likely that CCN3 may act as a EMT-regulatory factor [37].  
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17 401 Consistent with this, CCN3 requires its C-terminal domain for bone metastatic function,  
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19 402 and is correlated with aggressive disease progression in prostate cancer [38]. In  
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21 403 hepatocellular carcinoma, after being secreted from hepatic cells, CCN3 gained its' activity  
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23 404 in various processes during EMT via HSCs (Hepatic stellate cells) [39]. A previous study  
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25 405 has also shown that increased expression level of CCN3 expression lead to local invasion  
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27 406 and distant metastases in gastric cancer [40, 44]. In this study, we showed a link between  
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29 407 CCN3 expression and gastric stemness properties, and have further shown that low levels  
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31 408 of CCN3 in HGC27 cells result in reduced stemness and tumorigenicity. Therefore,  
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33 409 approaches capable of reducing CCN3 expression may have the potential to suppress EMT  
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35 410 and to be novel therapies against gastric cancer.  
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41 411 NEFL, which practically maintains the neuronal caliber and functions as a regulator  
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43 412 in intracellular transport to axons and dendrites, has also be implicated in various  
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45 413 carcinogenesis [41]. NEFL acts as a tumor suppressor in non-small cell lung cancer  
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47 414 (NSCLC), inhibiting invasion and metastasis, while methylation may destroy its protective  
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49 415 effect [42]. In NSCLC and breast cancer, patients with higher expression of NEFL mRNA  
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51 416 transcript had a better five-year disease-free survival [24]. In contrast to these reports, our  
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5 417 study showed that NEFL might enhance the tumor development in xenografts. The reason  
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7 418 behind the contrasting outcomes may be due to the fact that in the advanced stage of  
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10 419 malignancy, cancer cells tend to become much fiercer in metastatic potential and  
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12 420 phenotypes, thus the invasions are nowhere to be hold arrested by merely cytoprotective  
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14 421 genes expression alterations [43]. In either case, NEFL in digestive system cancers such as  
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16 422 gastric cancer may play distinct roles in a context-dependent manner.

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20 423 In conclusion, we showed that SNAIL regulates the expression of CCN3 and NEFL  
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22 424 genes in human gastric cancer cells, and that these in turn control the CSC activities.  
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25 425 Strategies that disrupt this possible circle may be possible to treat gastric cancer in future.  
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27 426 Double antagonists targeting both CCN3/NEFL-SNAIL axis may weaken the malignant  
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29 427 progression and dual RNAi study should be considered before RNAi compound  
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31 428 development. Also, protein secretion of the target molecule that plays important roles in  
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33 429 malignancy can be traced and intentionally attacked through clonal antibody. Multiple  
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35 430 channels of further application as therapeutic agents may be helpful for patients with gastric  
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37 431 cancer.  
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44 433 **Competing Interests:**

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47 434 We have received research funds under contract from Sumitomo Danippon Pharma Co.,  
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49 435 Ltd.  
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6 563 **Legends:**

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8 564 Figure 1. Characteristics of gastric cancer cell lines spheres cultured under serum-free  
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14 566 A. Photographic pictures of sphere morphology.

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17 567 Initial and passage sphere assay were shown in upper and lower lane pattern respectively  
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19 568 with each cell line marked on top.

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22 569 B. Sphere formed per  $2 \times 10^4$  seeded cells as an index of cell renewal capacity.

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25 570 a: sphere count; b: volume summary of sphere formed. Results were expressed as mean  $\pm$   
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27 571 SD, \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

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30 572 C. Enhanced in vivo tumor volume formed from spheres in mice at an injection  
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32 573 concentration of  $4 \times 10^4$  cells.

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35 574 D. Representative H&E slides from gastric cancer cell line NCI-N87 with their sphere  
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37 575 formed tumors. Scale bar=200 $\mu$ m.

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44 577 Figure 2. Stem cell properties and tumor malignancy in gastric cancer spheres.

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47 578 A. Comparison of EMT factors on absolute mRNA expression levels in gastric cancer cell  
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49 579 lines.

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52 580 B. Immunofluorescence of SNAIL expression in HGC27, IM95 and FU97 cell lines.  
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6 581 a. Cells in the gastric cancer cell lines HGC27 were found to express SNAIL (imaged with  
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8 582 red fluorescent) in the nuclei;

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10 583 b. Image with DAPI to identify the nuclei of HGC27 gastric cancer cells;

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14 584 c. Merged image superimposed on a differential interference contrast background confirms  
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16 585 co-localization. Scale bar=50 $\mu$ m.

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22 587 Figure 3. SNAIL is sufficient and essential for induction of self-renewal and malignancy in  
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24 588 gastric cancer cell line HGC27. (Knockdown is represented as k.d.).

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27 589 A. Real-time quantitative PCR validation of SNAIL expression level in HGC27 and its  
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29 590 stable SNAIL knockdown cell lines.

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32 591 B. Effects of SNAIL knockdown on the expression of E-cadherin and Vimentin in HGC27  
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34 592 cell line.

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37 593 C. Morphology alteration of gastric cancer cell line HGC27 after SNAIL knockdown in  
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39 594 ultra-low attachment condition.

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42 595 D. CD24 and CD44 FACS profiles of HGC27 and HGC27 SNAIL knockdown cell lines.

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45 596 E. Sphere formed per  $2 \times 10^4$  seeded cells from HGC27 and HGC27 SNAIL knockdown cell.

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48 597 F. Diminished in vivo tumorigenicity formed from HGC27 SNAIL knockdown cells in

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51 598 NOD/SCID mice at an injection concentration of  $4 \times 10^4$  cells. H&E staining of

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54 599 representative tumors, scale bar=200 $\mu$ m.

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6 600 G. Decreased chemoresistance of HGC27 SNAIL knockdown cells compared with HGC27  
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8 601 when treated with 5-FU.  
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14 603 Figure 4. Microarray analysis between HGC27 and its SNAIL knockdown samples.

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17 604 A. Plot analysis of distribution visualization of differentially expressed genes comparing  
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19 605 SNAIL knockdown and control groups.

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22 606 B. Bar plot detailing presented numbers of genes under SNAIL up/down regulation in two  
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27 608 C. GO terms enrichment analysis of molecular function, cellular component and biological  
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29 609 process of differentially expressed genes (left to right).

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32 610 D. The KEGG enrichment analysis of differentially expressed genes.

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35 611 E. Real-time quantitative PCR validation of CCN3 and NEFL expression level in HGC27  
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37 612 and its stable SNAIL knockdown cell lines.

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44 614 Figure 5. Elevated expression of SNAIL and CCN3 in patients with stomach  
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46 615 adenocarcinoma.

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49 616 A. mRNA transcript expression levels of SNAIL (a), CCN3 (b) and NEFL (c) are elevated  
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51 617 in patients with gastric cancer from TCGA-STAD database. Box in red: malignant tissue;  
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54 618 box in gray: normal tissue. \* $p < 0.05$ .

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6 619 B. No correlation existed between CCN3 and NEFL in gastric cancer patients from TCGA-  
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8 620 STAD database.

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10 621 C. SNAIL, CCN3 and NEFL are highly expressed in human gastric cancer tissues.

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14 622 Representative immunohistochemical staining (IHC) images of SNAIL, CCN3 and NEFL  
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16 623 in gastric cancer tissues. Scale bar = 50  $\mu$ m.

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22 625 Figure 6. CCN3 and NEFL correlate with self-renewal and chemoresistance traits in gastric  
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24 626 cancer cells.

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27 627 A. Western blotting analysis of NEFL and CCN3 expression in associated cell lines.

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30 628 B. Sphere formed per  $2 \times 10^4$  seeded cells as an index of cell renewal capacity.

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33 629 a. HGC27 SNAIL knockdown vs. HGC27 SNAIL knockdown with SNAIL re-introduction;

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36 630 b. HGC27 vs. HGC27 with NEFL introduction;

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39 631 c. HGC27 SNAIL knockdown vs. HGC27 SNAIL knockdown with CCN3 re-introduction.

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42 632 C. Chemoresistance alteration of SNAIL (a), CCN3 (b) and NEFL (c) introduction into  
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44 633 associated HGC27 cell lines when treated with 5-FU. Results are expressed as mean  $\pm$  SD,  
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46 634 \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

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50 635 D. Knockdown of NEFL promoted the mesenchymal traits in gastric cancer cell line IM95.

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53 636 a. Real time qPCR validation of NEFL expression level in IM95 and its stable NEFL

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55 637 knockdown cell lines.  
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6 638 b. Effects of NEFL knockdown on the expression of E-cadherin and Vimentin in IM95 cell

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11 640 c. Increased chemoresistance of IM95 NEFL knockdown cells compared with IM95 cells

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13 641 when treated with 5-FU. Results are expressed as mean  $\pm$  SD, \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

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19 643 Figure S1. Enhanced in vivo tumor volume formed from spheres in mice at an injection

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21 644 concentration of  $4 \times 10^4$  cells of HGC27 and NUGC3 cells. Representative H&E slides

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23 645 from gastric cancer cell lines with their sphere formed tumors. Scale bar=200 $\mu$ m.

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29 647 Figure S2. Representative immunohistochemical staining (IHC) images of CCN3 and

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31 648 NEFL in gastric normal tissues. Scale bar = 50  $\mu$ m.

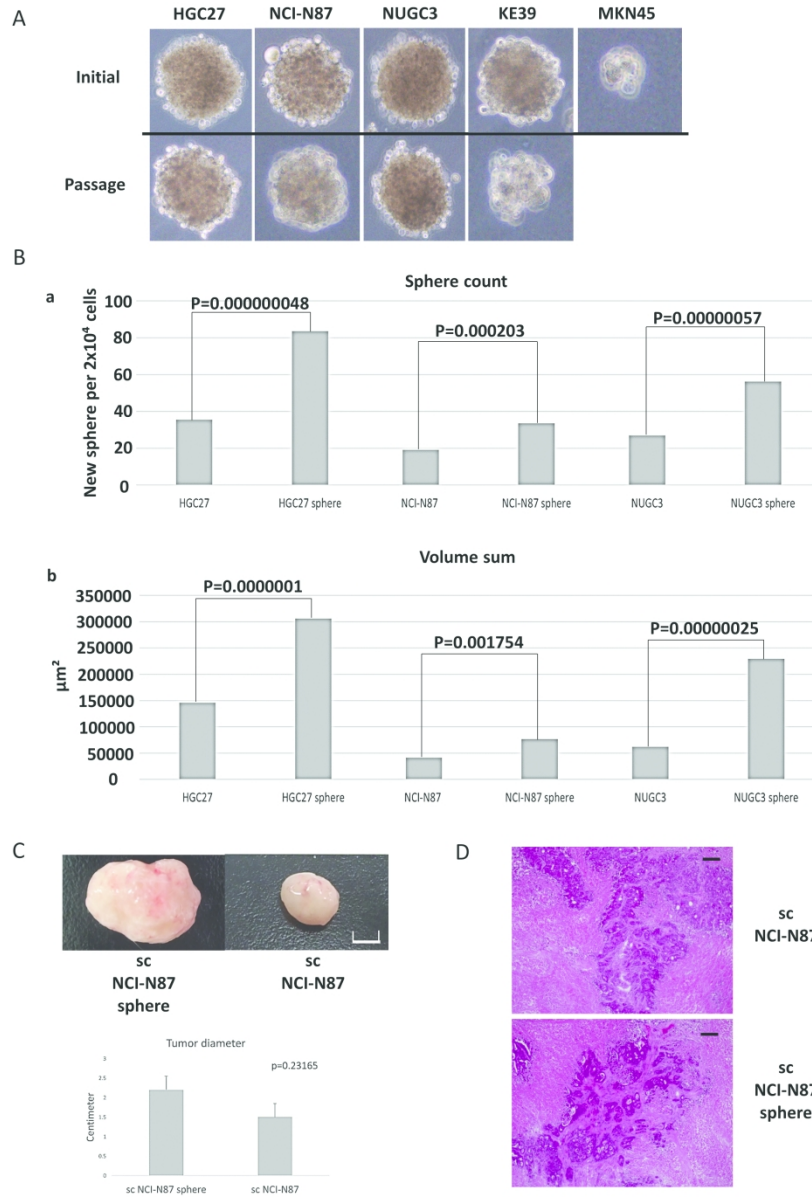
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Table S1. Primer sequences

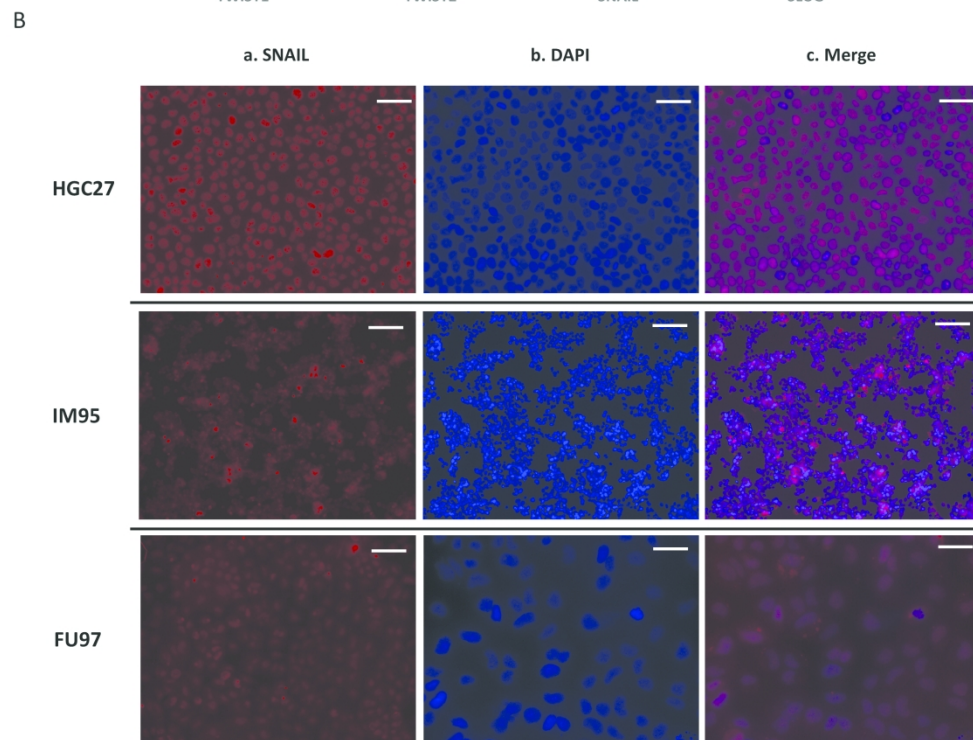
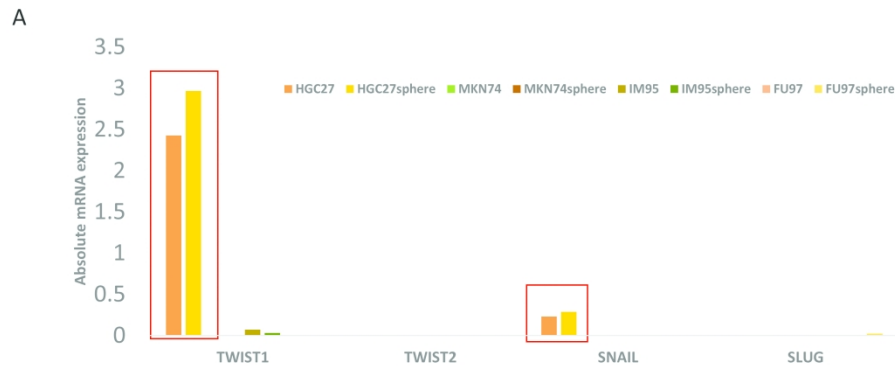
GAPDH	forward	5' -GAC ATC AAG AAG GTG GTG AAG C- 3'
	reverse	5' -GTC CAC CAC CCT GTT GCT GTA G- 3'
TWIST1	forward	5' -CTC AGC AGG GCC GGA GAC CT- 3'
	reverse	5' -CCC CAC GCC CTG TTT CTT TGA- 3'
TWIST2	forward	5' -CTC AGC AGG GCC GGA GAC CT- 3'
	reverse	5' -CCC CAC GCC CTG TTT CTT TGA- 3'
SNAIL	forward	5' -TCT CTA GGC CCT GGC TGC TAC A- 3'
	reverse	5' -CGC CTG GCA CTG GTA CTT CTT G- 3'
SLUG	forward	5' -GAA GAT GCA TAT TCG GAC CCA CAC- 3'
	reverse	5' -TTG ACC TGT CTG CAA ATG CTC TGT- 3'

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Characteristics of gastric cancer cell lines spheres cultured under serum-free condition. A. Photographic pictures of sphere morphology. Initial and passage sphere assay were shown in upper and lower lane pattern respectively with each cell line marked on top. B. Sphere formed per 2x10<sup>4</sup> seeded cells as an index of cell renewal capacity. a: sphere count; b: volume summary of sphere formed. Results were expressed as mean ± SD, \* p<0.05; \*\* p<0.01. C. Enhanced in vivo tumor volume formed from spheres in mice at an injection concentration of 4 x 10<sup>4</sup> cells. D. Representative H&E slides from gastric cancer cell line NCI-N87 with their sphere formed tumors. Scale bar=200μm.



43 Stem cell properties and tumor malignancy in gastric cancer spheres.

44 A. Comparison of EMT factors on absolute mRNA expression levels in gastric cancer cell lines.

45 B. Immunofluorescence of SNAIL expression in HGC27, IM95 and FU97 cell lines.

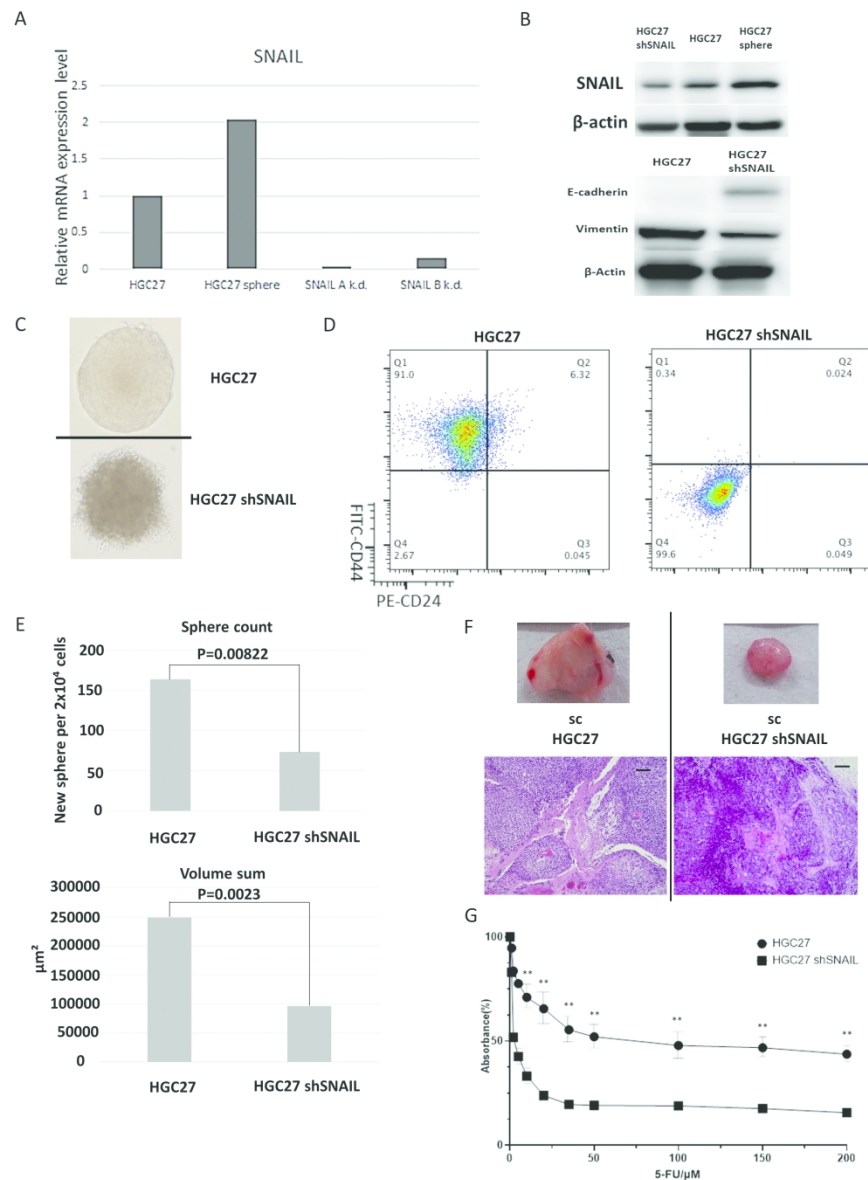
46 a. Cells in the gastric cancer cell lines were found to express SNAIL (imaged with red fluorescent) in the nuclei;

47 b. Image with DAPI to identify the nuclei of gastric cancer cells;

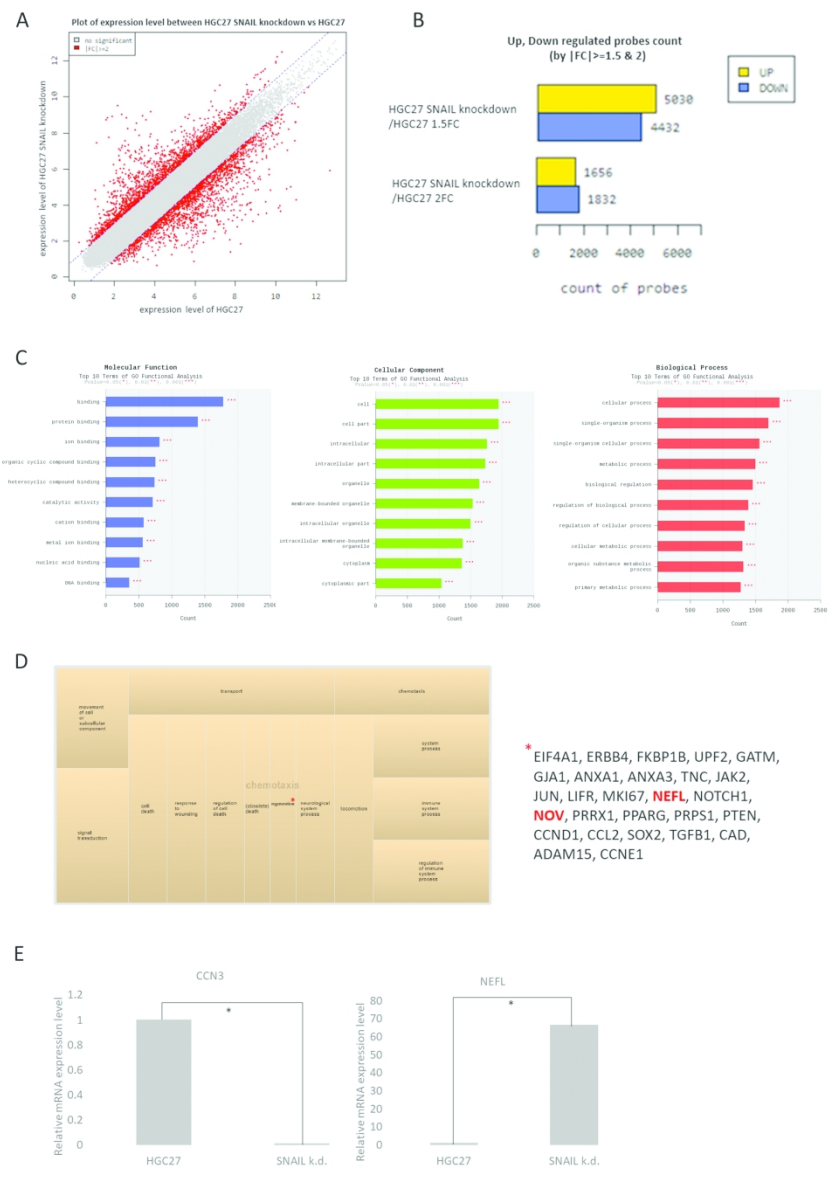
48 c. Merged image superimposed on a differential interference contrast background confirms co-localization.

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SNAIL is sufficient and essential for induction of self-renewal and malignancy in gastric cancer cell line HGC27. (Knockdown is represented as k.d.). A. Real-time quantitative PCR validation of SNAIL expression level in HGC27 and its stable SNAIL knockdown cell lines. B. Effects of SNAIL knockdown on the expression of E-cadherin and Vimentin in HGC27 cell line. C. Morphology alteration of gastric cancer cell line HGC27 after SNAIL knockdown in ultra-low attachment condition. D. CD24 and CD44 FACS profiles of HGC27 and HGC27 SNAIL knockdown cell lines. E. Sphere formed per  $2 \times 10^4$  seeded cells from HGC27 and HGC27 SNAIL knockdown cell. F. Diminished in vivo tumorigenicity formed from HGC27 SNAIL knockdown cells in NOD/SCID mice at an injection concentration of  $4 \times 10^4$  cells. H&E staining of representative tumors, scale bar =  $200 \mu\text{m}$ . G. Decreased chemoresistance of HGC27 SNAIL knockdown cells compared with HGC27 when treated with 5-FU.



Microarray analysis between HGC27 and its SNAIL knockdown samples.

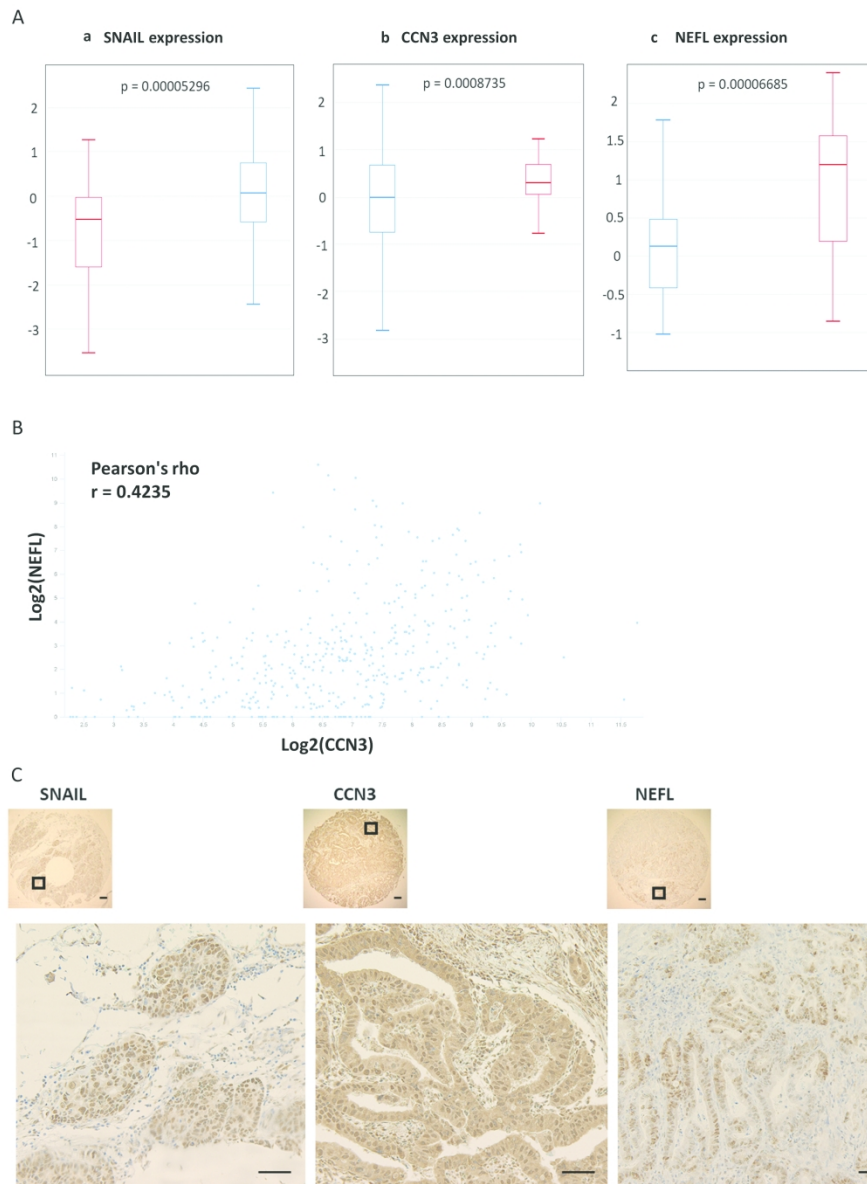
A. Plot analysis of distribution visualization of differentially expressed genes comparing SNAIL knockdown and control groups.

B. Bar plot detailing presented numbers of genes under SNAIL up/down regulation in two conditions.

C. GO terms enrichment analysis of molecular function, cellular component and biological process of differentially expressed genes (left to right).

D. The KEGG enrichment analysis of differentially expressed genes.

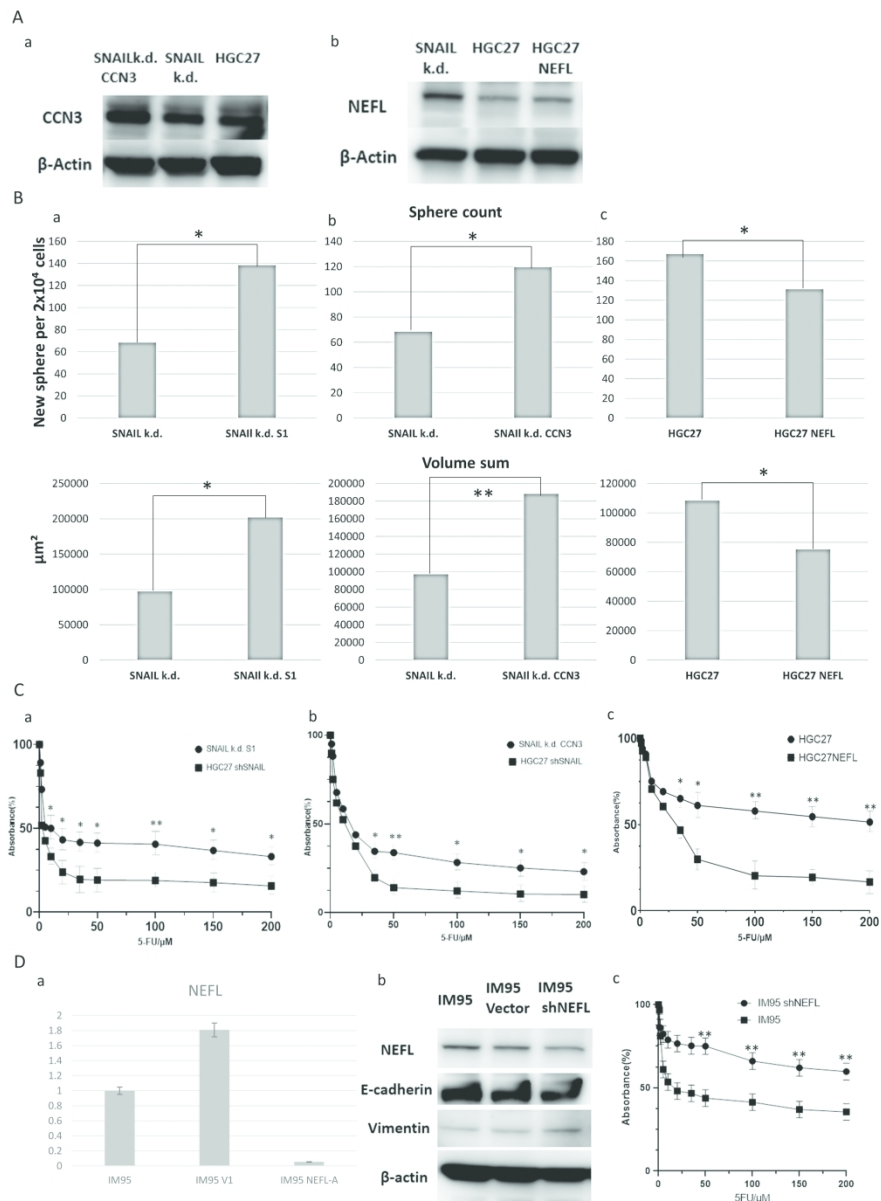
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45 Elevated expression of SNAIL and CCN3 in patients with stomach adenocarcinoma. A. mRNA transcript  
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CCN3 and NEFL correlate with self-renewal and chemoresistance traits in gastric cancer cells.

A. Western blotting analysis of NEFL and CCN3 expression in associated cell lines.

B. Sphere formed per  $2 \times 10^4$  seeded cells as an index of cell renewal capacity.

a. HGC27 SNAIL knockdown vs. HGC27 SNAIL knockdown with SNAIL re-introduction;

b. HGC27 vs. HGC27 with NEFL introduction;

c. HGC27 SNAIL knockdown vs. HGC27 SNAIL knockdown with CCN3 re-introduction.

C. Chemoresistance alteration of SNAIL (a), CCN3 (b) and NEFL (c) introduction into associated HGC27 cell lines when treated with 5-FU. Results are expressed as mean  $\pm$  SD, \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

D. Knockdown of NEFL promoted the mesenchymal traits in gastric cancer cell line IM95.

a. Real time qPCR validation of NEFL expression level in IM95 and its stable NEFL knockdown cell lines.

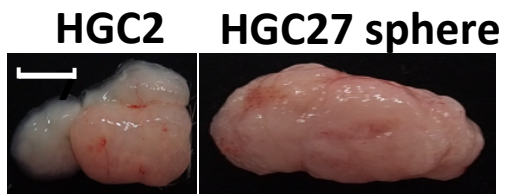
b. Effects of NEFL knockdown on the expression of E-cadherin and Vimentin in IM95 cell line.

c. Increased chemoresistance of IM95 NEFL knockdown cells compared with IM95 cells when treated with 5-FU. Results are expressed as mean  $\pm$  SD, \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

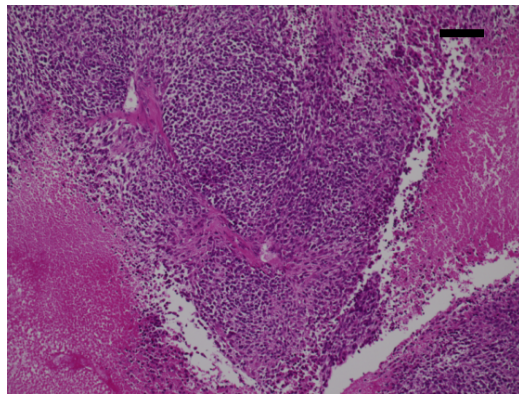
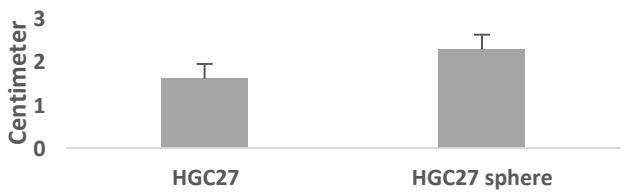
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# Figure S1

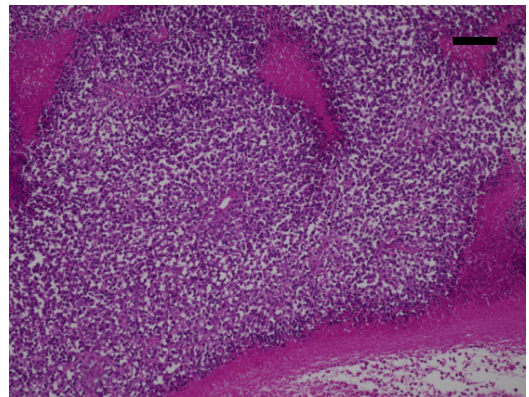
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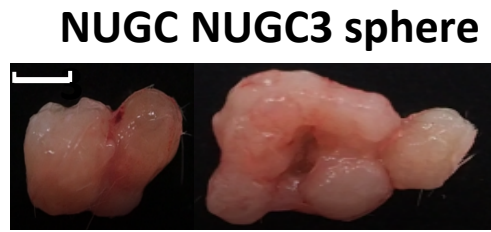
**HGC2 HGC27 sphere**  
Tumor diameter P=0.195751



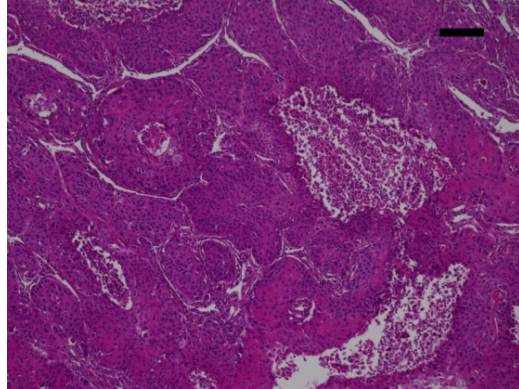
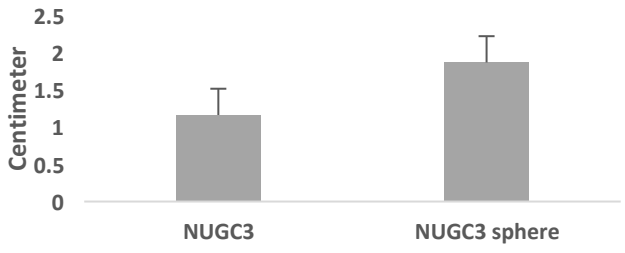
**sc  
HGC27**



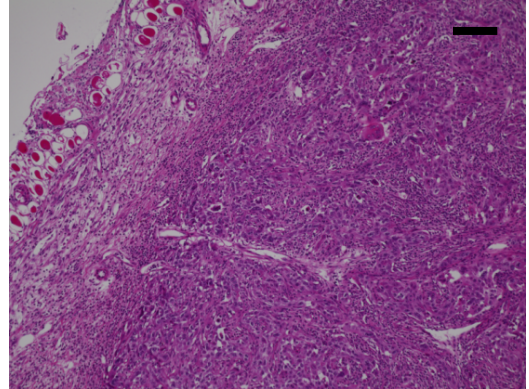
**sc HGC27  
sphere**



**NUGC NUGC3 sphere**  
Tumor diameter P=0.010501



**sc  
NUGC3**



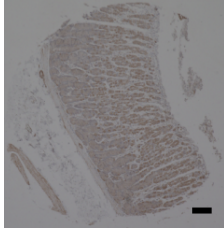
**sc NUGC3  
sphere**



# Figure S2

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**CCN3**



**NEFL**

