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FOXO1 reduces tumorsphere formation capacity via reciprocal down-regulation of LGR5 in gastric cancer cells

위암세포에서 FOXO1과 LGR5의 상호간 하향조절을 통한 줄기세포능 억제

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Abstract

FOXO1 reduces tumorsphere formation capacity via reciprocal down-regulation of LGR5 in gastric cancer cells

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Background: FOXO1 inactivation is detected in a variety of human cancers, including gastric cancer (GC). Although the role of FOXO1 in cancer stem cells has been reported in pancreatic cancer and glioblastoma, the implication of FOXO1 in GC cell stemness has been elusive. The present study investigated the implication of FOXO1 in GC cell stemness and its association with LGR5.

Methods: The expressions of FOXO1 and LGR5 were modulated in GC

cell lines (SNU-638, MKN45, AGS and MKN28) by stable transfections of their shRNA or gene construct. The effects of transfection on GC stemness were evaluated in vitro and in animal models. In addition, the relationship between FOXO1 and LGR5 was analyzed using GC clinical specimens, cell lines and xenografts.

Results: Tumorspheres obtained from SNU-638 and MKN45 GC cells were cultured under serum-free culture conditions. Western blot showed that the expressions of GC stem cell markers, LGR5, CD44 and CD133, were higher in tertiary tumorsphere cells than those in adherent cells, whereas FOXO1 expression was significantly lower. More importantly, FOXO1 silencing in SUN-638 and MKN45 cells increased tumorsphere formation, whereas FOXO1 overexpression in AGS and MKN28 GC cells produced the opposite results. Additionally, immunohistochemical tissue array analysis showed an inverse relationship between FOXO1 and LGR5 in GC specimens. Further analyses using in vitro and in vivo experiments showed that FOXO1 activity in GC cells negatively controlled and was controlled by LGR5. Also, double knockdown of both FOXO1 and LGR5 in GC cells revealed that LGR5 silencing reversed the FOXO1 shRNA-induced tumorsphere formation capacity even without the FOXO1 restoration. Consistently, FOXO1 silencing did not change cyclin D1 expression in the absence of change in LGR5 expression.

Conclusions: My results suggest that FOXO1 inhibits tumorsphere formation capacity of GC cells through interaction with LGR5. Thus, FOXO1/LGR5 signaling pathway may provide a novel targeted therapy for GC.

Keywords: gastric cancer; cancer stem cell; FOXO1; LGR5

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List of figures

List of Abbreviations

CSC: cancer stem cells

FFPE: formalin fixed paraffin embedded

FHRE: forkhead responsive element

FOXO: Forkhead box O

GC: gastric cancer

LGR5: Leucine-rich repeat-containing G-protein-coupled receptor 5

pAKT: AKT phosphorylated at Ser473

RNA ISH: RNA in situ hybridization

RT-PCR: reverse transcription-polymerase chain reaction

S.D.: standard deviation

SDS: sodium dodecyl sulfate

shRNA: short hairpin RNA

UBC: housekeeping gene ubiquitin C

Contents

Introduction

Gastric cancer (GC) is one of the most common cancers and a major cause of cancer-related death [1]. The heterogeneity and molecular complexity of GC requires many challenges for the development of effective strategies to prevent and treat this disease. Recently, growing evidences support the notion that cancer stem cells (CSCs), a subpopulation of cancer cells, are immortal tumor-initiating cells that have self-renewal capacity, and are responsible for tumor progression, metastasis, drug resistance and recurrence [2]. However, the understanding of molecular regulatory mechanism of GC cell stemness is limited.

Forkhead box O (FOXO) is a subfamily of Forkhead transcription factors, which comprises of four members (FOXO1, FOXO3, FOXO4, and FOXO6) [3]. FOXO is a direct downstream target of PI3K/AKT pathway [4]. Among these, FOXO1 is essential for pleuripotency in embryonic stem cells [5]. Regarding the role of FOXO1 in CSCs, FOXO1 suppressed tumorsphere formation and tumorigenesis of pancreatic cancer [6], but the opposite results were shown in glioblastoma [3]. Thus, implication of FOXO1 in CSCs has been inconsistent according to cancer cell type.

Regarding GC, FOXO1 inactivation and its prognostic significance were shown in GC specimens [7]. Moreover, in vitro experiments showed that FOXO1 down-regulation in GC cells increased cell growth, migration, invasion and epithelial mesenchymal transition [4], and anti-cancer drug resistance [1]. In animal studies, FOXO1 inactivation in xenograft tumors in nude mice promoted tumorigenesis and metastasis of GC cells [4]. However, the implication of FOXO1 in GC cell stemness is uncertain.

Putative CSCs are usually identified based on the expression of specific surface markers [8]. Among the numerous surface markers currently under investigation, CD44 and CD133 have proved to be the most useful for the identification of CSCs in solid tumors [9]. Several groups have reported that CD44 [10,11] and CD133 [12] are GC stem cell markers, but inconsistent results have been shown in GC cells. The expression of CD133 was similar in tumorsphere and adherent cultures of GC cells (HGC-27, MGC-803, MKN45) [13], and it did not increase tumorsphere formation capacity in NCI-N87 cells [14]. Furthermore, purified CD133+ and CD133+/CD44+ cells, obtained from intestinal type GC cases, did not reproduce cancer in mice models [9]. Thus, so far none of these have been confirmed to be a functional CSC marker in GC cells [15].

Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), also known as GPR49, is an established marker of adult stem cells in a number of organs, including the intestine, stomach, liver and hair follicle [16]. In various cancer cells [17,18], high expression of LGR5 has been shown and was associated with CSC properties. In GC, LGR5 protein expression in GC cells was up-regulated in tumorsphere cells compared to adherent cells [15]. Moreover, LGR5 overexpression in GC cells significantly enhanced cell growth, migration and drug resistance as well as tumor initiation [15]. Thus, at the present time, LGR5 appears to be the most useful functional CSC marker for GC [15,16]. However, the underlying molecular mechanism of LGR5 regulation in GC stem cells needs to be further elucidated.

This study investigated the effect of FOXO1 activation on the tumorsphere formation efficiency in vitro after modulation of FOXO1 expression in GC cell lines. Since interactions between transcription factors and stem cell markers are critical for the regulation of stemness of tumor cells [2,3,6,18,19], this study determined the association between FOXO1 and LGR5 in GC samples, cell lines and xenografts.

Materials and methods

Culture of adherent cells, tumorsphere and sub-tumorspheres

GC cell lines with high levels of FOXO1 expression (SNU-638 and MKN45) and low levels of FOXO1 expression (AGS and MKN28) were obtained from Korean Cell Line Bank (Seoul, Korea) were maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies) at 37°C in a humidified 5% CO_2 incubator.

For tumorsphere formation, cells were cultured as described by Liu et al. [20] with slight modifications. Adherent GC cells were seeded into 6-well ultra-low attachment plates (Corning Life Sciences, Lowell, CA, USA) in the serum-free RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10 mM HEPES, 10 ng/mL human recombinant basic fibroblast growth factor (R&D system, Minneapolis, MN, USA), and 20 ng/mL human recombinant epidermal growth factor (Invitrogen). At day 7, tumorspheres were harvested and centrifuged, disaggregated through enzymatic and mechanical dissociation using accutase (Gibco BRL Life Technologies, Grand Island, NY, USA) and pipetting, respectively. Dissociated tumorsphere cells were cultured to permit the regeneration of tumorspheres.

Recovered cells were sub-cultured in the serum-free medium and this processes was repeated every 7 days for secondary and tertiary tumorspheres. At day 21 of culture, pictures were taken under an inverted microscope (Olympus) for morphological examination, and tertiary tumorspheres were collected and dissociated into single cells for Western blot.

Western blot

Cell lysates were prepared in 100-200 μL of 1x sodium dodecyl sulfate (SDS) lysis buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 0.004% bromophenol blue, and 20% glycerol]. Protein contents were measured using BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Equal amounts of proteins were loaded onto a 10% discontinuous SDS/polyacrylamide gel and electrophoretically transferred to PVDF membranes (Millipore Corporation, Billerica, MA, USA) blocked with 5% nonfat dry milk in phosphate buffered saline-Tween 20 (0.1%, v/v) for 1 h. The membranes were then incubated at 4°C overnight with or without 2 h incubation at room temperature with one of the following primary antibodies: anti-LGR5 (1:1000, Abcam, Cambridge, United Kingdom), anti-CD44 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA,USA), anti-CD133 (1:1000, Cell Applications, San Diego, CA, USA), anti-FOXO1 (1:1000, Cell Signaling Technology, Berverly, MA, USA), anti-pAKT (1:1000,

Cell signaling Technology), anti-AKT (1:1000, Cell signaling Technology), anti-Cyclin D1 (1:1000, Cell signaling Technology) and anti-β-actin (1:1000, Santa Cruz Biotechnology). Horse-radish peroxidase-conjugated anti-rabbit IgG (1:4000, Santa Cruz Biotechnology) or anti-mouse IgG (1:4000, Santa Cruz Biotechnology) was used as a secondary antibody. Enhanced chemiluminescence (Pierce) was used to detect the immunoreactive proteins. Equal protein loading was confirmed by β-actin.

Stable transfection of FOXO1 shRNA and FOXO1A3 mutant gene

FOXO1 expression was modulated by stable transfections as previously reported [1]. GC cells were seeded at 3 x 10^5 cells/well in 6-well plates. For FOXO1 down-regulation, lentivirus-mediated shRNA silencing of FOXO1 was performed. Lentiviral particles containing non-targeting shRNA or FOXO1 shRNA were purchased (Sigma, St. Louis, MO, USA). The sequence of FOXO1 shRNA was 5′-CCGGGCCTGTTATCAATCTGCTAAA CTCGAGTTTAGCAGATTGATAACAGGCTTTTTG-3’. The control non-targeting shRNA particles contain four base-pair mismatches within the short hairpin sequence to any known human or mouse gene. The viral infection was performed by incubating GC cells (SNU-638 and MKN45) in the culture medium containing lentiviral particles for 12 h in the presence of 5 μg/mL Polybrene (Santa Cruz Biotechnology). Pooled puromycin (2 μg/mL)-resistant cells were used for further analyses.

For FOXO1 overexpression in GC cell lines, stable transfection of pcDNA3 containing human FOXO1A3 mutant gene (Addgene plasmid 13508, Addgene Inc., Cambridge, MA, USA) was performed. The plasmid FOXO1A3 encodes a constitutively active FOXO1 containing a threonine-to-alanine substitution at residue 24 and serine-to-alanine substitution at 256 and 319 (three AKT phosporylation sites on FOXO1). Expression plasmid FOXO1A3 (1 μg) or control empty pcDNA3 vector (1 μg) was transfected into GC cells (AGS and MKN28) in 6-well plates using Lipofectamine Plus (Life Technologies) according to the manufacturer's instructions. Twenty-four h after transfection, G418 (3 μg/mL) was added to select stable FOXO1A3 clones and pooled G418-resistant cells were used for further analyses.

Luciferase reporter assay

To determine FOXO1 nuclear DNA-binding activity in GC cells, luciferase reporter assay was performed as previously described [1]. GC cells were seeded in 24-well plates at the density of 3 x 10^4 cells/well and were transiently co-transfected with 0.4 μg forkhead responsive element (FHRE)-luciferase reporter plasmid (reporter construct in which a small region of the Fas ligand promoter contains the three FHREs, Addgene plasmid 1789, Addgene Inc.) and 0.4 mg pSV-b-galactosidase vector (Promega, Madison,

WI, USA), an internal control, using Lipofectamine Plus (Life Technologies). Twenty-four h after transfection, assays for luciferase and β-galactosidase were carried out using a Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was measured on an AutoLumat LB 9505c luminometer (Berthold Analytical Instruments, Nashua, Germany) and was normalised by β-galactosidase activity.

Tumorsphere formation assay

Cells were incubated in anchorage-independent conditions [10]. 50 cells/200 μL were seeded into 96-well ultra-low attachment plates (Corning Life Sciences), and maintained in serum-free RPMI 1640 medium. After 2 weeks, each well was examined under an inverted microscope (Olympus) at x 100 magnification, and tumorsphere formation efficiency was evaluated by total number of wells with tumorspheres. Each experiment was repeated 3 times.

Stable transfection of LGR5 shRNA and LGR5 gene

GC cells were seeded at 3 x 10^5 cells/well in 6-well plates. LGR5 silencing was performed as described by Michelotti et al. [21] with slight modifications. The pRFP-C-RS vectors containing either control non-targeting shRNA or LGR5 shRNA were purchased from OriGene (Rockville, MD, USA). The sequence of LGR5 shRNA was 5′-ATCAGTTACCTAATCTC

CAAGTGCTAGAT-3′. Each vector (1 μg) was transfected into GC cells (AGS and MKN28) using Lipofectamine Plus (Life Technologies) according to the manufacturer's instructions. Twenty-four h after transfection, puromycin (2 μg/mL) was added to select stable LGR5 shRNA clones.

LGR5 overexpression was done as described by Jang et al. [16] with slight modifications. Full-length complementary DNA encoding LGR5 (pEX-LGR5) was purchased from GeneCopoeia (Rockville, MD, USA). GC cells (SNU-638 and MKN45) were seeded at the density of 3 x 10^5 cells/well in 6-well plates and transfected with 1 μg of control empty pEX-EGFP vector or pEX-LGR5 wild type by Lipofectamine Plus (Life Technologies) according to the manufacturer's instructions. Pooled G418 (3 μg/mL)-resistant cells were used for further analyses.

Clinical specimens

GC specimens were collected from GC patients who underwent curative gastrectomy at Seoul National University Hospital, Seoul, Korea, either from 2004 to 2005 (840 cases) or in 2006 (507 cases). Formalin fixed paraffin embedded (FFPE) TMA blocks were prepared as previously described [16]. Briefly, core tissue biopsies (2 mm in diameter) were taken from individual FFPE gastric tumors (donor blocks) and arranged in a new recipient paraffin block (TMA block) using a trephine apparatus

(SuperBioChips Laboratories, Seoul, Korea). Each tissue block was able to contain up to 60 cases. The staining results of the different intratumoral areas of GC in these tissue array blocks showed an excellent agreement. A core was chosen from each case for analysis. This study defined an adequate case as a tumor occupying more than 10% of the core area. Sections of 4 μm thicknesses were cut from each TMA block, deparaffinized, and rehydrated, and then used for immunohistochemistry and RNA in situ hybridization (ISH). This protocol was reviewed and approved by the Institutional Review Board of Seoul National University Hospital (IRB No. H-1209-037-424 for 2004-2005 and C-1309-087-522 for 2006).

Xenograft tumors in nude mice

Subcutaneous GC xenografts in nude mice (BALB/cSlc-n/n) generated in previous study [4] were used. Tumor xenografts derived from SNU-638 GC cells expressing either non-targeting shRNA or FOXO1 shRNA were removed and prepared for immunohistochemical staining or Western blot. All animal procedures were performed in accordance with the procedures described in the Seoul National University Laboratory Animal Maintenance Manual (approval No. SNU-140702-1).

Immunohistochemical staining

Formalin-fixed, paraffin-embedded slides were deparaffinized and rehydrated. Immunohistochemistry was performed on clinical GC specimens (2004 to 2005 and 2006) and xenograft tumors using Ventana BenchMark XT automated immunostainer (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's instructions. The primary antibodies are anti-phospho-FOXO1Ser256 (1:50, Cell Signaling Technology), FOXO1 (1:40, Cell Signaling Technology), LGR5 (1:10, Abcam) and Ki-67 (1:50, DAKO, Glostrup, Denmark). Staining assessment was performed by Jang BG and Kim Y according to the criteria described previously [7,17].

ISH and interpretation

FFPE GC specimens were collected from 840 patients (2004 to 2005). ISH for LGR5 mRNA was performed on TMA sections using the RNAscope FFPE assay kit (Advanced Cell Diagnostics Inc., Hayward, CA, United States) as described previously [16]. In brief, TMA sections were pretreated with heat and protease digestion followed by hybridization with a LGR5 probe. Then, a horseradish peroxidase-based signal amplification system was hybridized to the LGR5 probe before color development with 3,3'-diaminobenzidine tetrahydrochloride. Positive stain was defined as the presence of brown punctate dots in the nucleus and/or cytoplasm. The housekeeping gene ubiquitin C (UBC) served as a positive control. The

DapB gene, which is derived from a bacterial gene sequence, was used as a negative control. Samples with UBC easily visible under a x 10 objective lens were considered to have adequate quality according to the manufacturer's recommendation. LGR5 staining was graded based on the percentage of tumor cells positive for LGR5 as follows: grade 0, 0–5%; grade 1, 5–10%; grade 2, 10–25%; and grade 3, 25–100%. The sample was considered as positive for LGR5 when expression level of grade 1 or higher was observed. For the discordant cases, a final decision was made by consensus.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed to determine the mRNA level of molecules (LGR5, FOXO1 and β-actin) in GC cells, and the amplification of β-actin transcripts was used as the control to normalize the transcript levels of molecules. Total RNAs were isolated using TRIZOL reagent (Invitrogen), and reverse-transcription was performed to synthesize cDNAs in a 20 μL reaction mixture containing each gene-specific primer, 1 μg of RNA, 2x reaction buffer, 0.4 μL Taq polymerase and 1.2 mM MgCl2. The cDNAs of LGR5 transcripts were amplified for 28 cycles (30 s at 94°C, 30 s at 52°C, and 30 s at 70°C), the cDNAs of FOXO1 transcripts were amplified for 25 cycles (30 s at 94°C, 1 min at 57°C, and 1 min at 72°C), and the cDNAs of β-actin transcripts were amplified for 18 cycles (94°C for 30 s, 52°C for 30 s, and

70°C for 30 s). The PCR cycling numbers had been optimized to avoid the amplification saturation. Five μL RT-PCR product was separated on 1 % agarose gels, which was subsequently stained with ethidium bromide. Primers of LGR5 were: 5'- ACCTCAGTATGAACAACATC-3' (forward) and 5'-GGAGTCCATCAAAGCATTTC-3' (reverse); primers of FOXO1 were: 5'-GCAGATCTACGAGTGGATGG TC-3' (forward) and 5'-AAACTGTGATC CAGGGCTG TC-3' (reverse); primers of β-actin were: 5'-ACACCTTCTAC AATGAGCTG-3' (forward) and 5'-CATGATGGAGTTGAAGGTAG-3' (reverse).

Statistical analysis

Cell culture experiment data were analyzed using Prism version 4.0 (GraphPad Software, San Diego, CA, USA). The significances of the results were determined by the two-tailed Student's t-test. For tissue array analysis, statistical analyses were conducted using SPSS version 11.0 (IBM SPSS, Chicago, IL, USA). The χ^2 test was used to determine the correlations between the expressions of pFOXO1 and LGR5. *P* values < 0.05 were considered statistically significant.

Results

Tumorsphere formation cells showed the up-regulation of LGR5, CD44 and CD133, but the down-regulation of FOXO1

Since the growth of tumorspheres of cancer cells cultured for a few weeks is indicative of self-renewal capacity, tumorsphere cells are the best starting materials for the characterization of CSCs [15]. The present study developed tertiary tumorsphere cells obtained from GC cell lines SNU-638 and MKN45, and then determined the expressions of GC stem cell markers in these cells. Cancer cells were cultured under serum-free, adherent-free conditions as described in the Materials and Methods section. Figure 1A shows photographs of adherent and tumorsphere cultures.

Next, tertiary tumorspheres were dissociated into single cells and Western blot was performed to examine the expressions of previously identified GC stem cell markers (LGR5, CD44 and CD133) in these cells (Figure 1B). Compared to adherent cancer cells, tertiary tumorsphere cells overexpressed GC stem cell markers LGR5, CD44 and CD133. In contrast, FOXO1 was down-regulated in tumorsphere cells compared to adherent cells, suggesting that FOXO1 might be related to self-renewal capacity of GC cells.

FOXO1 inhibited tumorsphere formation in GC cells

To investigate a possible role of FOXO1 in self-renewal capacity of GC cells, FOXO1 expression was modulated and examined the tumorsphere formation efficiency. First, two GC cell lines (SUN-638 and MKN45) with high levels of FOXO1 expression (Figure 2A) were selected. Then, FOXO1 down-regulation was induced by infection of SNU-638 and MKN45 GC cells with lentiviral particles containing FOXO1-targeting short hairpin RNA (shRNA). Western blot and luciferase reporter assay confirmed the down-regulation of FOXO1 expression and activity (Figure 2A) in both cell lines expressing FOXO1 shRNA compared to control shRNA cells. Then, each of control shRNA transfectants and FOXO1 shRNA transfectants were plated into half of 96-well ultra-low attachment plates (48 wells) at a clonal density (50 cells/200 μL). Self-renewal efficiency of GC cells was determined by the number of wells showing tumorspheres. It was found that FOXO1 shRNA transfection increased the number of tumorsphere-positive wells compared to control GC cells (Figure 2A).

Next, FOXO1 up-regulation in AGS and MKN28 GC cells with low levels of FOXO1 expression was induced by permanent transfection of a FOXO1A3 mutant gene (Figure 2B). Consistent with the above results,

FOXO1A3 overexpression in GC cells significantly reduced tumorsphere formation efficiency compared to control GC cells (Figure 2B).

LGR5 expression induced tumorsphere formation in GC cells

To confirm LGR5 effect on the tumorsphere formation capacity in GC cell lines used in this study, GC cell lines was selected with high levels of LGR5 expression (AGS and MKN28) and those with low levels of LGR5 expression (SNU-638 and MKN45) to modulate LGR5 expression. LGR5 was down-regulated by transfection of LGR5 shRNA into AGS and MKN28 GC cells. Western blot (Figure 3A) confirmed the down-regulation of LGR5 expression in both cell lines expressing LGR5 shRNA. Tumorsphere formation assay showed that LGR5 shRNA-expressing GC cells generated lower number of tumorsphere-positive wells than control shRNA-expressing GC cells (Figure 3A). In addition, LGR5 up-regulation in SNU-638 and MKN45 GC cells (Figure 3B) was induced by permanent transfection of LGR5 wild type. Consistent with the above results, LGR5 overexpression significantly increased the number of tumorsphere-positive wells compared to control GC cells (Figure 3B).

An inverse relationship between FOXO1 and LGR5 existed in clinical specimens of GC

Previous studies showed that LGR5 expression in cancer cells is regulated by transcription factors, including Ascl2 [22], GATA6 [23] and Sox9 [24] and vice versa [25]. However, the relationship between FOXO1 and LGR5 in cancer cells remains unknown at the present time.

The present study investigated the association between FOXO1 and LGR5. First, this study examined the expressions of FOXO1 and LGR5 in human GC specimens by immunohistochemical tissue array analysis (Figure 4A). Regarding pFOXO1 expression, an antibody against inactive form of FOXO1 was used, and cells showing distinct cytoplasmic staining, with or without the presence of nuclear staining, were considered to express the inactive form of FOXO1 constitutively [4,7]. Since LGR5 staining was performed using an antibody against cytoplasmic domain of LGR5 (LGR5 aa 800-900), LGR5 was mainly observed in the cytoplasm of gastric carcinoma cells [25]. Figure 4G shows that FOXO1 inactivation (manifested by pFOXO1 expression) is positively correlated with LGR5 protein expression ($P < 0.001$). Additionally, LGR5 mRNA expression in GC cells was detected by RNA ISH performed on GC tissue array slides (Figure 4A). It was found that LGR5 mRNA expression was positively correlated pFOXO1 protein expression ($P = 0.048$) (Figure 4B). Taken together, these results indicate that FOXO1 activation is inversely associated with LGR5 protein and mRNA expressions in GC. Additionally,

the clinical characteristics of the GC patients in relation to the expressions of pFOXO1 and LGR5 are summarized in Table 1. The representativeness of our cohort of GC patients was revealed by univariate analyses. pFOXO1, as well as age, Lauren's classification and TNM stage, was shown to contribute to the outcome of GC patients (Table 2). However, multivariate Cox regression analysis including the TNM stage revealed that either pFOXO1 or LGR5 was not an independent prognostic factor (Table 2).

Crosstalk between FOXO1 and LGR5 existed in GC cells

To confirm the relationship between FOXO1 and LGR5, cell culture and animal experiments were performed. Western blot of cultured cells shows that SNU-638 and MKN45 cells with high levels of FOXO1 expression have low levels of LGR5 expression (Figure 5A), whereas AGS and MKN28 cells with low levels of FOXO1 expression showed high levels of LGR5 expression (Figure 5A). Furthermore, FOXO1 silencing in SNU-638 and MKN45 cells by RNA interference increased the expressions of LGR5 protein and mRNA (Figure 5A). Consistently, FOXO1 overexpression in AGS and MKN28 cells by transfection of a FOXO1A3 mutant gene induced the opposite results (Figure 5A). Thus, these results indicate that FOXO1 functions as a negative regulator of LGR5 expression at the transcriptional level in GC cells.

Immunohistochemical analysis showed that xenografts derived from FOXO1 shRNA-expressing SUN-638 cells had higher protein levels of LGR5 and Ki-67 than those derived from control shRNA-expressing cells (Figure 5B). Consistent results were obtained by Western blot (Figure 5C). These results suggest FOXO1 down-regulates LGR5 expression in GC cells in vitro and in vivo.

Next, this study subsequently investigated if LGR5 regulates FOXO1 activation in GC cells (AGS and MKN28 cells) expressing control shRNA or LGR5 shRNA. Western blot and luciferase reporter assay (Figure 6A) demonstrated that LGR5 silencing in AGS and MKN28 cells increased protein expression and activation of FOXO1. In contrast, RT-PCR showed that FOXO1 mRNA expression was not changed (Figure 6A). Consistently, LGR5 overexpression in SNU-638 and MKN45 cell lines induced the opposite results (Figure 6B). Thus, FOXO1 controls and is controlled by LGR5.

Since LGR5 down-regulation decreased AKT phosphorylation in neuroblastoma cells [26], I assessed whether LGR5 regulates AKT activation in GC cells. Western blot showed that LGR5 silencing in AGS and MKN28 cells reduced AKT phosphorylation (manifested by pAKT expression), but not the total AKT expression (Figure 6A). In contrast,

LGR5 overexpression in SNU-638 and MKN45 cells induced the opposite results (Figure 6B).

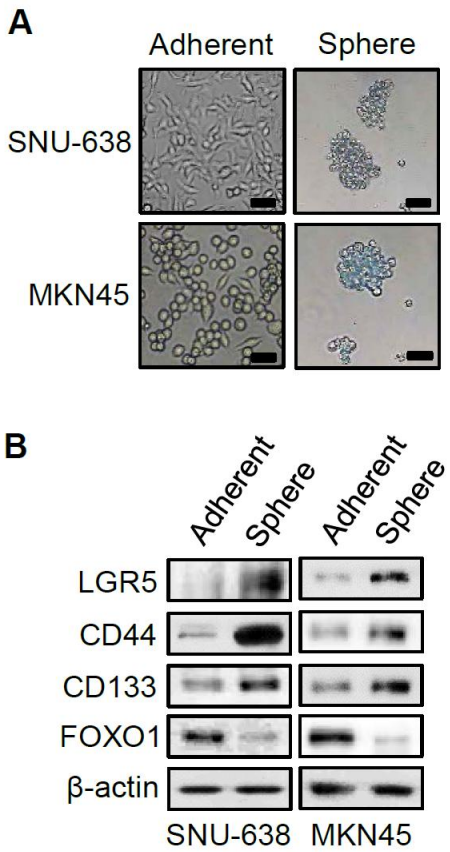
FOXO1 inhibits GC cell tumorsphere formation via suppression of LGR5

The aforementioned results showed that tumorsphere formation capacity of GC cells was decreased by FOXO1 and increased by LGR5. Thus, in order to investigate which one of these two molecules is more important to self-renewal capacity of GC cells, we performed double transfection of FOXO1 and LGR5. Figure 7 showed that FOXO1 down-regulation by RNA interference increased LGR5 expression as well as the number of tumorsphere-positive wells, which were completely suppressed by LGR5 shRNA overexpression in spite of the lack of the restoration of FOXO1.

It has been demonstrated that cyclin D1 were elevated in tumorspheres of a GC cell line AGS [27]. In the present study, FOXO1 inhibition led to increased cyclin D1 expression in SNU638 and MKN45 cells (Figure 8A). Consistently, its overexpression induced the opposite results (Figure 8B). On the other hand, LGR5 silencing in AGS and MKN28 cells decreased cyclin D1 (Figure 8C). In agreement, LGR5 overexpression in SNU-638 and MKN45 cells induced the opposite results (Figure 8D). Thus, double transfection of these two molecules was performed in order to investigate

whether FOXO1 inhibits GC tumorsphere formation directly or indirectly through LGR5. Figure 8E showed that single transfection of LGR5 shRNA into GC cells induced a decrease in cyclin D1 expression as well as an increase in FOXO1 expression. Subsequent FOXO1 silencing did not change cyclin D1 expression as well as LGR5 expression.

Figure 1.


A
Adherent
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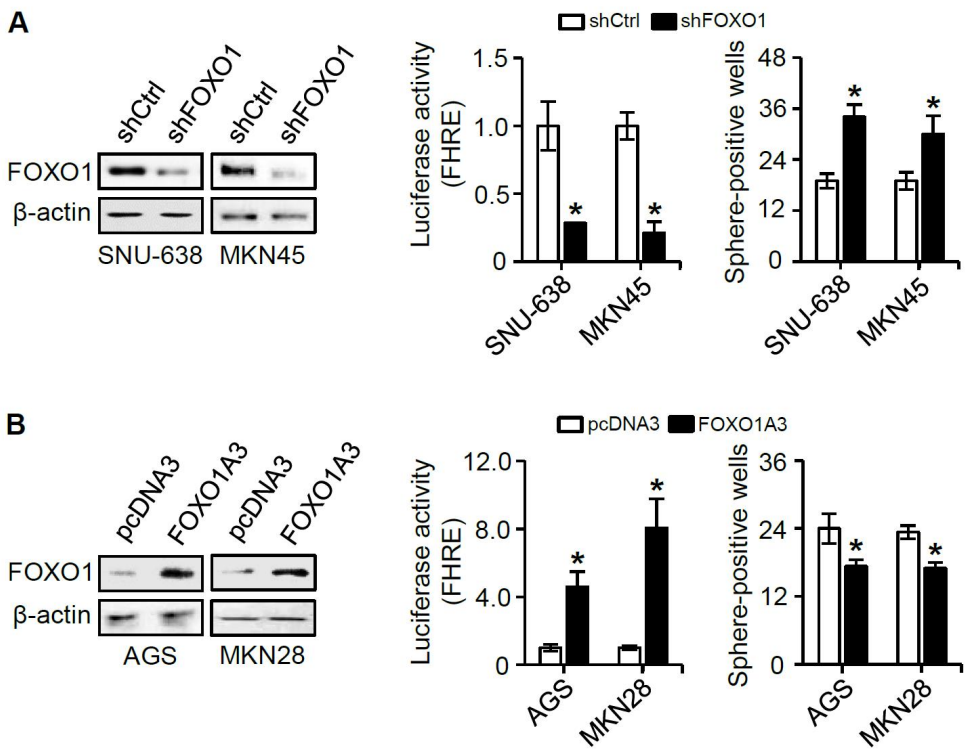
Sphere
LGR5
CD44
CD133
FOXO1
β-actin
SNU-638
MKN45

Figure 1. Expressions of cancer stem cell-related molecules in tertiary tumorsphere cells.

Tumorspheres obtained from SNU-638 and MKN45 GC cells were cultured under serum-free culture conditions and were passaged 3 times. (**A**) Photographs of adherent cells and tertiary tumorspheres were taken at day 21 of culture with an inverted microscope. Scale bars: 100 μm. Original magnification: x 100. (**B**) Protein expressions of LGR5, CD44, CD133 and FOXO1 in adherent and tertiary tumorsphere cells were determined by Western blot.

Figure 2.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Luciferase activity (FHRE)
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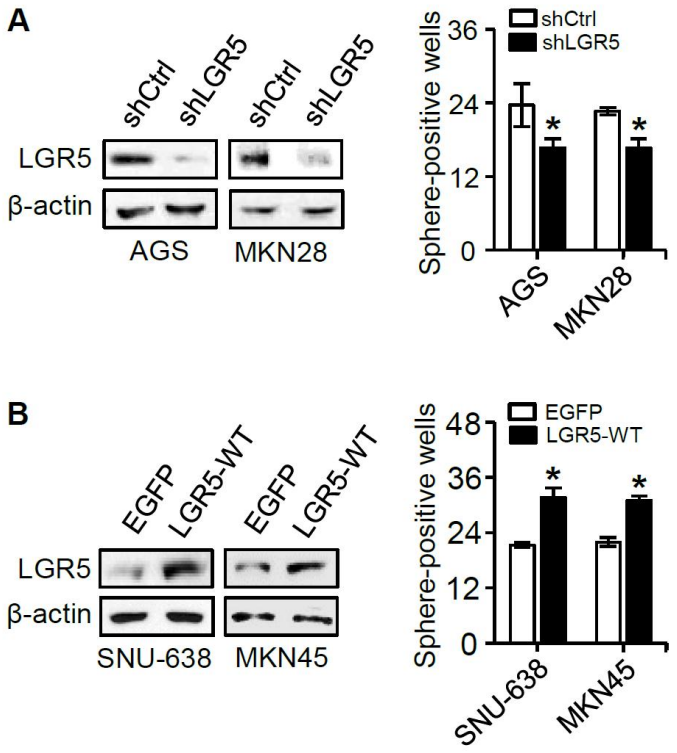
B
pcDNA3
FOXO1A3
pcDNA3
FOXO1A3
FOXO1
β-actin
AGS
MKN28
pcDNA3
FOXO1A3
Luciferase activity (FHRE)
12.0
8.0
4.0
0
AGS
MKN28
Sphere-positive wells
36
24
12
0
AGS
MKN28

Figure 2. Effect of FOXO1 on tumorsphere formation in GC cells. (**A-C**) SNU-638 and MKN45 cells were infected with a lentivirus containing either control shRNA (shCtrl) or FOXO1 shRNA (shFOXO1). (**D-F**) AGS and MKN28 cells were transfected with either pcDNA3 or FOXO1A3. (**A, D**) FOXO1 protein expression was confirmed by Western blot. (**B, E**) FOXO1 transcriptional activity was determined by luciferase reporter assay. (**C, F**) Tumorsphere formation assay was performed as described in Materials and Methods. Each bar is the mean value ± S.D. *$P < 0.05$.

Figure 3.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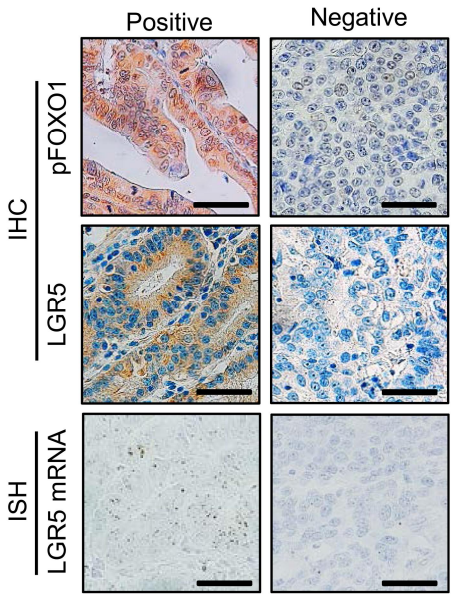
shLGR5
Sphere-positive wells
36
24
12
0
*
*
AGS
MKN28
B
EGFP
LGR5-WT
EGFP
LGR5-WT
LGR5
β-actin
SNU-638
MKN45
EGFP
LGR5-WT
Sphere-positive wells
48
36
24
12
0
*
*
SNU-638
MKN45

Figure 3. Effect of LGR5 expression on tumorsphere formation of GC cells. (**A, B**) AGS and MKN28 cells were transfected with an empty pRFP-C-RS vector (shCtrl) or a LGR5 shRNA vector (shLGR5). (**C, D**) SNU-638 and MKN45 cells were transfected with an empty pEX-EGFP vector (EGFP) or a pEX-LGR5 vector (LGR5-WT). (**A, C**) LGR5 expression was confirmed by Western blot. (**B, D**) Tumorsphere formation assay was performed as described in Materials and Methods. Each bar is the mean value ± S.D. *$P < 0.05$.

Figure 4.

A



B

	pFOXO1 (IHC)		*P* value
	Positive (%)	Negative (%)	
LGR5 (IHC)			
Positive	95 (89)	12 (11)	< 0.001
Negative	251 (72)	98 (28)	
Total	346 (76)	110 (24)	
LGR5 (ISH)			
Positive	17 (38)	28 (62)	0.048
Negative	173 (25)	531 (75)	
Total	190 (25)	559 (75)	

Figure 4. FOXO1 and LGR5 expressions were inversely correlated in GC specimens. (**A**) Representative features of immunohistochemical staining of pFOXO1 and LGR5 (upper and middle) and RNA in situ hybridization of LGR5 (bottom) in human GC specimens. Scale bars: 100 μm. Original magnification: x 400. (**B**) Statistical analysis was performed using χ^2-test. Positive relationships between the expressions of pFOXO1 protein and LGR5 protein ($P < 0.001$) or LGR5 mRNA ($P = 0.048$) are shown.

Table 1

Correlation between the clinicopathologic characteristics and expressions of pFOXO1 and LGR5 in gastric cancer

Variables	Total	pFOXO1 expression		*P* value	LGR5 expression		*P* value
		High	Low		High	Low	
Age (years)							
≤ 60	221	168 (76.0%)	53 (24.0%)	0.946	65(29.4%)	156(70.6%)	0.004
> 60	235	178 (75.7%)	57 (24.3%)		42(17.9%)	193(82.1%)	
Gender							
Male	312	243 (77.9%)	69 (22.1%)	0.140	84(26.9%)	228(73.1%)	0.010
Female	144	103 (71.5%)	41 (28.5%)		23(16.0%)	121(84.0%)	
Lauren's classification							
Intestinal	221	189 (85.5%)	32 (14.5%)	<0.001	74(33.5%)	147(66.5%)	<0.001
Non-intestinal	235	157 (66.8%)	78 (33.2%)		33(14.0%)	202(86.0%)	
Ming's classification							
Infiltrative	401	303 (75.6%)	98 (24.4%)	0.670	88(21.9%)	313(78.1%)	0.039
Expending	55	43 (78.2%)	12 (21.8%)		19(34.5%)	36(65.5%)	
Tumor invasion							
EGC	183	171 (93.4%)	12 (6.6%)	<0.001	54(29.5%)	129(70.5%)	0.013
AGC	273	175 (64.1%)	98 (35.9%)		53(19.4%)	220(80.6%)	
Lymphatic invasion							
Absent	255	219 (85.9%)	36 (14.1%)	<0.001	67(26.3%)	188(73.7%)	0.111
Present	201	127 (63.2%)	74 (36.8%)		40(19.9%)	161(80.1%)	
Venous invasion							
Absent	393	310 (78.9%)	83 (21.1%)	<0.001	99(25.2%)	294(74.8%)	0.030
Present	63	36 (57.1%)	27 (42.9%)		8(12.7%)	55(87.3%)	
Perineural invasion							
Absent	287	249 (86.8%)	38 (13.2%)	<0.001	84(29.3%)	203(70.7%)	<0.001
Present	169	97 (57.4%)	72 (42.6%)		23(13.6%)	146(86.4%)	
T class							
T1	181	169 (93.4%)	12 (6.6%)	<0.001	53(29.3%)	128(70.7%)	0.002
T2	181	131 (72.4%)	50 (27.6%)		45(24.9%)	136(75.1%)	
T3	82	41 (50.0%)	41 (50.0%)		9(11.0%)	73(89.0%)	
T4	12	5 (41.7%)	7 (58.3%)		0(0.0%)	12(100%)	
N class							
N0	246	219 (89.0%)	27 (11.0%)	<0.001	64(26.0%)	182(74.0%)	0.087
N1	128	80 (62.5%)	48 (37.5%)		30(23.4%)	98(76.6%)	
N2	42	25 (59.5%)	17 (40.5%)		10(23.8%)	32(76.2%)	
N3	40	22 (55.0%)	18 (45.0%)		3(7.5%)	37(92.5%)	
M class							
M0	433	336 (77.6%)	97 (22.4%)	<0.001	107(24.7%)	326(75.3%)	0.024
M1	22	9 (40.9%)	13 (59.1%)		0(0.0%)	22(100.0%)	
M2	1	1 (100.0%)	0 (0.0%)		0(0.0%)	1(100.0%)	
TNM stage							
Ⅰ	249	225 (90.4%)	24 (9.6%)	<0.001	71(28.5%)	178(71.5%)	0.002
Ⅱ	87	54 (62.1%)	33 (37.9%)		18(20.7%)	69(79.3%)	
Ⅲ	62	37 (59.7%)	23 (40.3%)		15(24.2%)	47(75.8%)	
Ⅳ	58	30 (51.7%)	28 (48.3%)		3(5.2%)	55(94.8%)	

EGC, early gastric carcinoma; AGC, advanced gastric carcinoma; TNM, Tumor-Node-Metastasis.

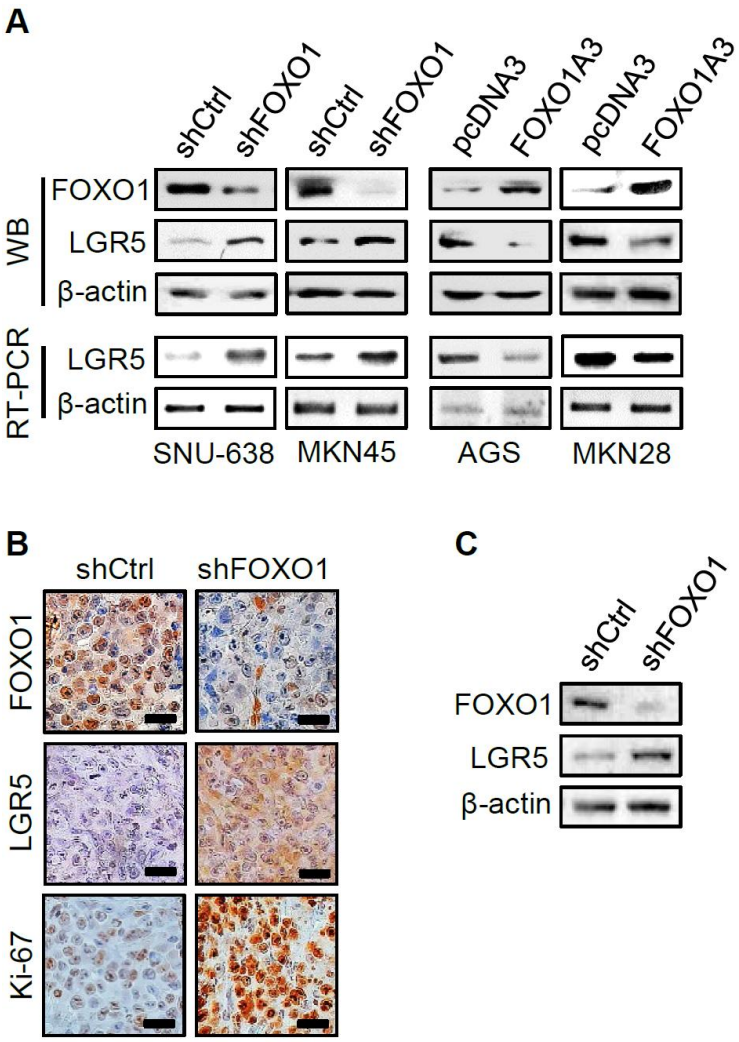
Table 2

Cox proportional hazard analyses for cancer-specific survival in gastric cancer

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (≤ 60years vs.> 60years)	1.899 (1.381–2.613)	<0.001	1.986 (1.410–2.798)	<0.001
Sex (male vs. female)	0.866 (0.614–1.219)	0.866		
Lauren's classification (intestinal vs. non-intestinal)	1.383 (1.008–1.897)	0.045		
TNM stage (Ⅰ vs. Ⅱ–Ⅳ)	8.388 (5.569–12.635)	<0.001	8.129 (5.112–12.927)	<0.001
pFOXO1 expression (low vs. high)	0.368 (0.267–0.508)	<0.001	0.727 (0.510–1.036)	0.078
LGR5 expression (low vs. high)	0.840 (0.562–1.257)	0.397	1.025 (0.679–1.548)	0.907
pFOXO1/LGR5 coexpression				
High/Low	1.017 (0.630–1.643)	0.944		
Low/High	3.045 (1.306–7.101)	0.010		
Low/Low	2.594 (1.580–4.259)	<0.001		
High/High				

HR, hazard ratio; CI, confident interval.

Figure 5.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shCtrl
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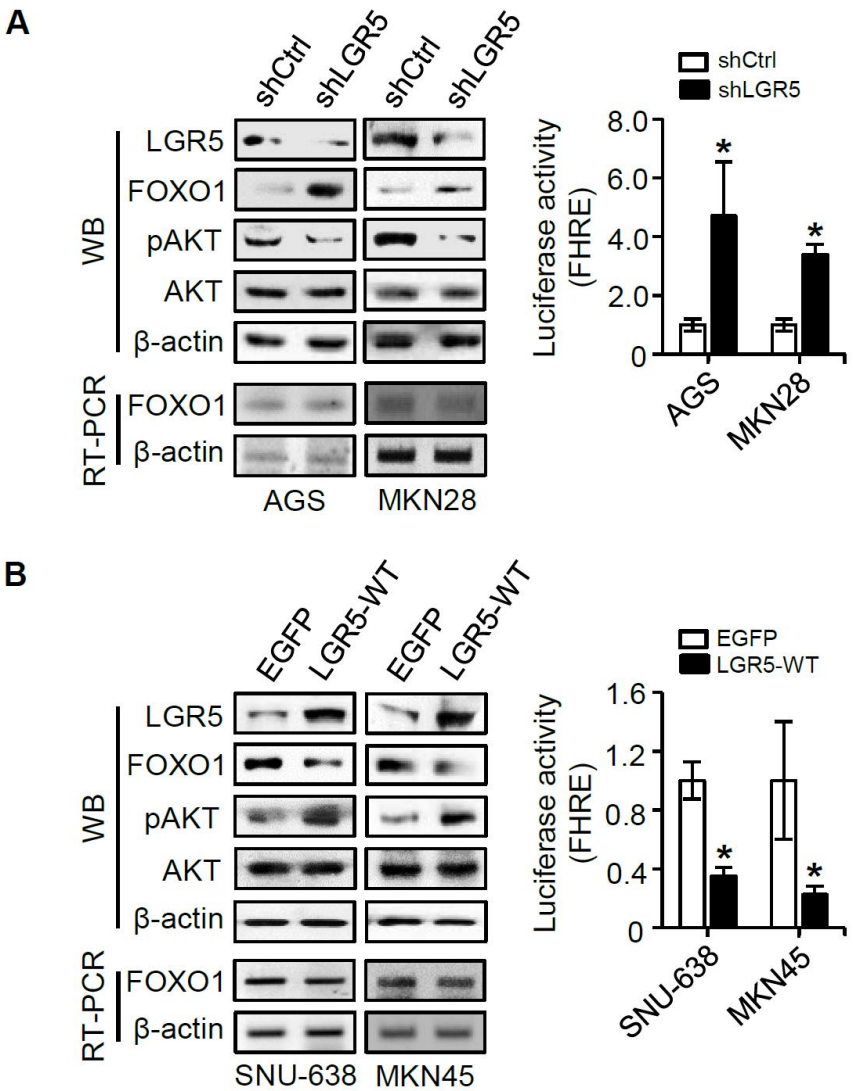
pcDNA3
FOXO1A3
pcDNA3
FOXO1A3
WB
FOXO1
LGR5
β-actin
RT-PCR
LGR5
β-actin
SNU-638
MKN45
AGS
MKN28
B
shCtrl
shFOXO1
FOXO1
LGR5
Ki-67
C
shCtrl
shFOXO1
FOXO1
LGR5
β-actin

Figure 5. Effect of FOXO1 modulation on LGR5 expression in GC cells in vitro and in vivo. Protein expressions of FOXO1 and LGR5 were determined by Western blot (WB) and LGR5 mRNA expression was determined by reverse transcription-polymerase chain reaction (RT-PCR). (**A**) SNU-638 and MKN45 cells were infected with lentivirus particles containing either non-targeting shRNA (shCtrl) or FOXO1 shRNA (shFOXO1). AGS and MKN28 cells were transfected with either pcDNA3 or FOXO1A3. (**B**) Tissue sections were obtained from the xenograft tumors and immunostained for FOXO1, LGR5 and Ki-67. Scale bars: 100 μm. Original magnification: x 400. (**C**) Protein expressions of FOXO1 and LGR5 in xenograft tumors were measured by Western blot.

Figure 6.


A
shCtrl
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shCtrl
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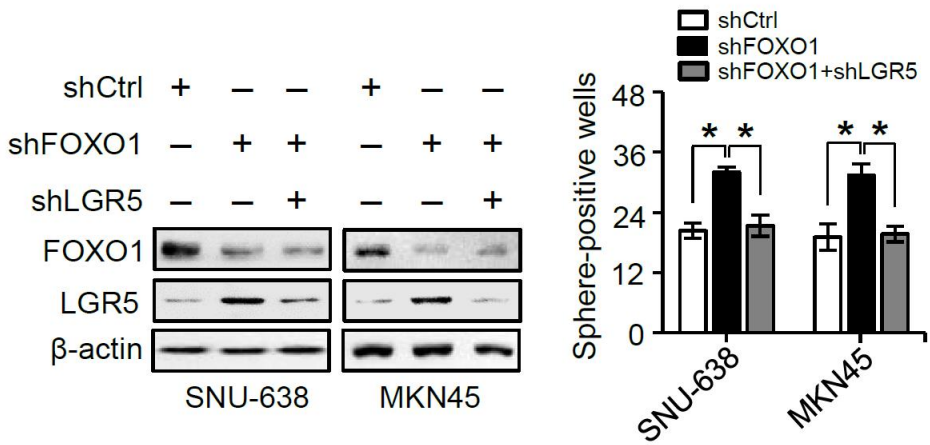
WB
LGR5
FOXO1
pAKT
AKT
β-actin
RT-PCR
FOXO1
β-actin
AGS
MKN28
shCtrl
shLGR5
Luciferase activity (FHRE)
8.0
6.0
4.0
2.0
0
AGS
MKN28
B
EGFP
LGR5-WT
EGFP
LGR5-WT
WB
LGR5
FOXO1
pAKT
AKT
β-actin
RT-PCR
FOXO1
β-actin
SNU-638
MKN45
EGFP
LGR5-WT
Luciferase activity (FHRE)
1.6
1.2
0.8
0.4
0
SNU-638
MKN45

Figure 6. Effect of LGR5 modulation on FOXO1 in GC cells. Protein expressions of LGR5, FOXO1, pAKT and total AKT were determined by Western blot (WB) and FOXO1 mRNA expression was determined by reverse transcription-polymerase chain reaction (RT-PCR). FOXO1 transcriptional activity was determined by the luciferase reporter assay and was normalized by β-galactosidase activity. (**A**) AGS and MKN28 cells were transfected with an empty pRFP-C-RS vector (shCtrl) or a LGR5 shRNA vector (shLGR5). (**B**) SNU-638 and MKN45 cells were transfected with an empty pEX-EGFP vector (EGFP) or a pEX-LGR5 vector (LGR5-WT).

Figure 7.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FOXO1
LGR5
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shFOXO1
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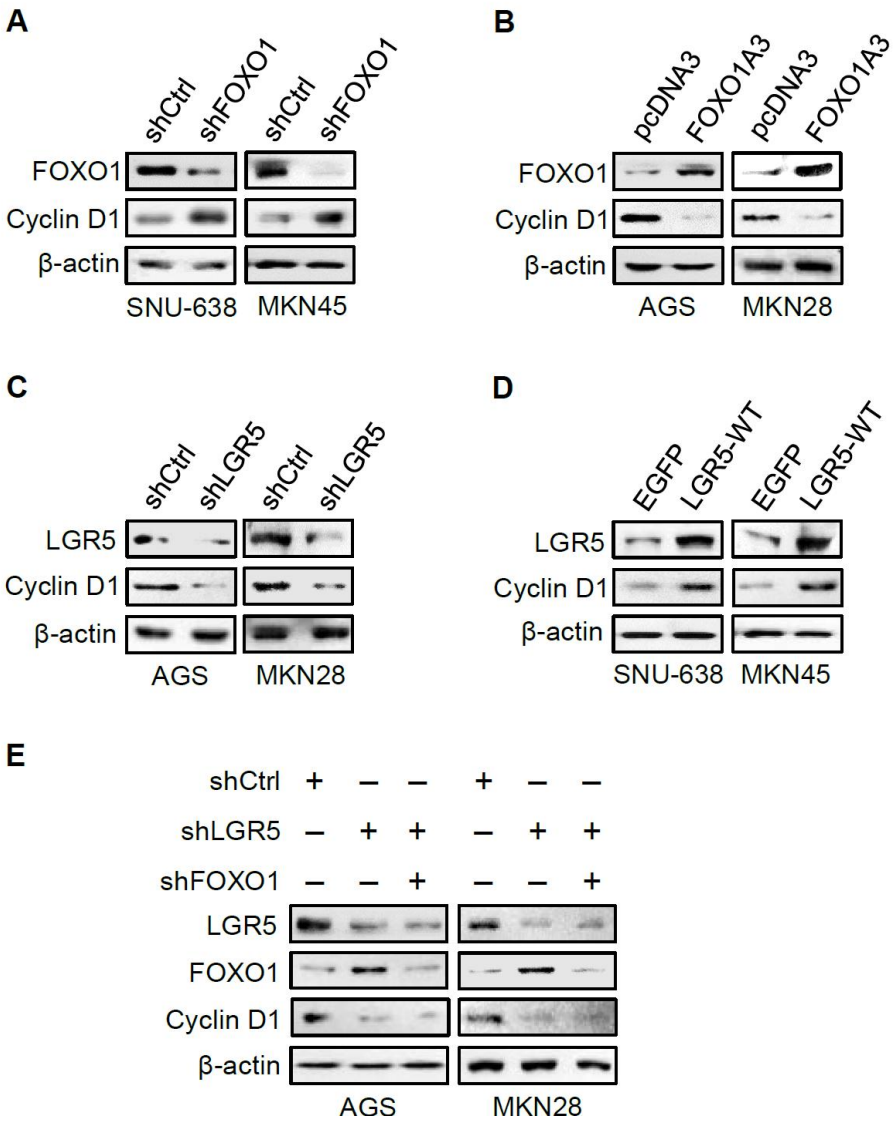
MKN45

Figure 7. Effect of co-transfection of FOXO1 shRNA and LGR5 shRNA on tumorsphere formation of GC cells. SNU-638 and MKN45 cells were transfected with FOXO1 shRNA (shFOXO1), LGR5 shRNA (shLGR5) or both shRNAs. Protein expressions of FOXO1 and LGR5 were determined by Western blot. Tumorsphere formation assay was performed as described in Materials and Methods. Each bar is the mean value ± S.D. *$P < 0.05$.

Figure 8.


A
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FOXO1
Cyclin D1
β-actin
AGS
MKN28
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shLGR5
shCtrl
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LGR5
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LGR5
FOXO1
Cyclin D1
β-actin
AGS
MKN28

Figure 8. Effects of FOXO1 and LGR5 on cyclin D1 expression in GC cells. Protein expressions of FOXO1, LGR5 and cyclin D1 were determined by Western blot using GC cell showing high expression of FOXO1 (SNU-638 and MKN45) or LGR5 (AGS and MKN28). (**A**) FOXO1 was down-regulated by lentiviral infection with non-targeting shRNA (shCtrl) or FOXO1 shRNA (shFOXO1). (**B**) FOXO1 was up-regulated by transfection with pcDNA3 vector or expression plasmid FOXO1A3. (**C**) LGR5 expression was silenced by transfection with a pRFP-C-RS vector (shCtrl) or a LGR5 shRNA vector (shLGR5). (**D**) LGR5 expression was overexpressed by transfection with a pEX-EGFP vector (EGFP) or a pEX-LGR5 vector (LGR5-WT). (**E**) Cells were co-transfected with LGR5 shRNA (shLGR5), FOXO1 shRNA (shFOXO1) or both shRNAs.

Discussion

There are accumulating evidences that self-renewal property of CSCs is implicated in cellular heterogeneity [28]. Since GC is characterized by its heterogeneity and molecular complexity, the elucidation of pathways that regulate GC stem cells may provide new targets for therapeutic development. The present study reports, for the first time, the function and underlying molecular mechanism of FOXO1 in GC cells in relation to CSC properties.

Although several transcription factors regulate stemness of cancer cells [2,3,6,22-25], FOXO1 effect on CSC property is cancer cell-type specific [3,6]. FOXO1-negative cells are cancer stem-like cells in pancreatic cancer [6]. In contrast, FOXO1 contributes to maintain stemness of glioblastoma stem cells [3]. In the present study, tertiary tumorspheres were generated from GC cell lines (SNU-638 and MKN45). Western blot showed that tertiary tumorsphere cells had higher expressions of CSC markers (LGR5, CD44 and CD133), but lower FOXO1 expression than adherent cells. These findings suggested the possible role of FOXO1 as a regulator of the self-renewal capacity of GC cells. Furthermore, FOXO1 expression was modulated to investigate the effect of FOXO1 on GC tumorsphere formation.

It was found that FOXO1 silencing by RNA interference increased tumorsphere formation and FOXO1 overexpression by transfection of FOXO1A3 mutant gene decreased tumorsphere formation. These results indicate that FOXO1 inhibits self-renewal capacity of GC cells.

In previous studies, CSC identification has been performed using two different approaches: tumorsphere formation in vitro and implantation of candidate CSCs into immunodeficient mice [6]. In general, these two approaches provided similar results in evaluating candidate CSCs for many solid tumors. However, Barrett et al. [29] recently reported that self-renewal does not always match with tumor growth potential in mouse models of high-grade glioma. Thus, I modulated FOXO1 expression and identified its effect on self-renewal capacity of GC cell lines by serum-free, adherent-free tumorsphere culture.

In the present study, I generated tertiary tumorspheres derived from GC cell lines (SNU-638 and MKN45), and found that tumorsphere cells had higher expressions of CSC markers (LGR5, CD44 and CD133), but lower FOXO1 expression than adherent cells. These findings suggested the possible role of FOXO1 as a regulator of the self-renewal capacity of GC cells. Further experiments showed that FOXO1 silencing increased tumorsphere formation and FOXO1 overexpression decreased

tumorsphere formation. Thus, these results indicate that FOXO1 inhibits self-renewal capacity of GC cells.

Transcription factor/stem cell marker pathways are critical for the regulation of cancer cell stemness [11,19,22-25]. Transcription factors regulate CSC markers in various cancers. In colon cancer cells, Ascl2 silencing suppressed the expressions of CD133, Sox2, Oct4 and LGR5 [22]. GATA6 enhanced LGR5 expression in colorectal cancer cells [23]. Sox9 down-regulation resulted in decreased LGR5 expression in glioblastoma [24]. In turn, regulation of transcription factors by stem cell markers has been also shown [11,25]. Inhibition of LGR5 resulted in a significant decrease in β-catenin level in GC cells [25]. CD44 knockdown down-regulated the expression of Oct4 in GC cells. [11]. Additionally, positive crosstalk between STAT3 and CD44 was shown in breast cancer cells [19]. However, the relationship between FOXO1 and LGR5 remains unknown.

Although LGR5 appears to be the most promising GC stem cell marker [15,16,25], the molecular mechanisms underlying the regulation of LGR5 in GC cells need to be further elucidated. In the present study, immunohistochemical results showed a positive relationship between FOXO1 inactivation (manifested by pFOXO1 expression) and LGR5 protein/mRNA expression in GC specimens. In cell culture experiments,

FOXO1 down-regulation increased LGR5 expression in GC cells at the transcriptional level, whereas FOXO1 overexpression induced the opposite results. Consistently, GC xenografts derived from FOXO1 shRNA transfectants showed higher LGR5 protein expression than those derived from control shRNA transfectants. In turn, further in vitro analysis showed that LGR5 down-regulation increased FOXO1 protein expression and transcriptional activity in GC cells. Taken together, my results indicate that a negative reciprocal regulatory loop between FOXO1 and LGR5 exists in GC.

Previous study demonstrated that the activations of AKT and FOXO1 were inversely associated in GC specimens [7]. Furthermore, treatment of GC cells with a PI3K inhibitor LY294002 or an AKT inhibitor IV resulted in FOXO1 activation [4]. In the present study, LGR5 knockdown up-regulated FOXO1 activation, but down-regulated AKT activation. Thus, it seems that LGR5 inhibited FOXO1 activation in GC cells via PI3K/AKT signaling.

The present study showed that FOXO1 and LGR5 induced opposite effects on self-renewal capacity of GC cells, and that negative crosstalk between these two molecules existed. Thus, I compared the importance of these two molecules in the regulation of self-renewal capacity of GC cells using co-transfection of FOXO1 shRNA and LGR5 shRNA. It was found that LGR5 expression and tumorsphere formation capacity of GC cells were

increased by FOXO1 shRNA transfection, but these changes were reversed after the subsequent transfection of LGR5 shRNA in spite of the lack of the restoration of FOXO1. Since consistent results obtained by LGR5 down-regulation followed by FOXO1 down-regulation, it appears that dysregulation of FOXO1 may increase self-renewal capacity of GC cells through the altered expression of LGR5 and that LGR5 expression, rather than FOXO1 expression, is more critical to the induction of GC cell tumorsphere formation.

In conclusion, the present study demonstrates, for the first time, that FOXO1 down-regulation enhances self-renewal capacity of GC cells manifested by tumorsphere formation. Additionally, my results demonstrated FOXO1/LGR5 pathway which is a novel molecular mechanism for targeted GC therapy.

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국 문 초 록

목적: 여러 종류의 암에서 전사인자인 forkhead box O 1 (FOXO1)의 불활성화가 일어나지만, 암줄기세포에 미치는 FOXO1 의 영향은 암의 종류에 따라 상반되게 나타날 수 있음이 보고되었다. 위암의 경우에는 FOXO1 이 종양억제유전자임이 밝혀졌으나 위암줄기세포에 미치는 영향은 알려져 있지 않으므로, 본 연구는 이를 규명하고 위암의 대표적인 줄기세포표지자인 LGR5 가 관련되어 있는지를 관찰하였다.

실험방법: 위암의 줄기세포에서 FOXO1 의 역할을 평가하기 위해서, 자가재생을 유지하기 위한 능력을 소유하고 있는 암줄기세포 유사 세포주 (tumorsphere cells)를 확립하였다. 위암 세포주 중에서 FOXO1 의 발현이 높은 SNU-638 과 MKN45 와 LGR5 의 발현이 높은 AGS 와 MKN28 을 선택하고, 과발현 DNA 와 shRNA 를 이용한 유전자이입 방법을 통하여 FOXO1 과 LGR5 의 발현을 변화시켰다. FOXO1 의 형질전환이 자가재생능 (self-renewal capacity)에 미치는 영향을 평가하기 위하여 배양상태에서 위암세포의 tumorsphere 형성을 관찰하였고, FOXO1 과 LGR 의 관련성을 확인하기 위하여 위암환자의 조직과

이종이식 마우스 모델에서 생성한 이형종양조직을 사용하여 면역조직화학염색과 제자리부합법 (in situ hybridization)을 시행하였다.

결과: 위암의 암줄기세포 유사세포주에서 줄기세포 표지자들 (LGR5, CD44 그리고 CD133)의 발현은 증가하였고, 반면 FOXO1 의 발현은 감소하였다. FOXO1 발현 억제는 위암 세포의 tumorsphere 형성능을 증가시켰지만, FOXO1 발현 증가는 반대의 결과들을 초래하였다. 그리고 위암 환자의 조직, 위암 세포주, 이형 종양조직들에서 FOXO1 과 LGR5 의 역 상관관계가 존재하고, 이 두 분자가 negative crosstalk 를 하고 있음이 관찰되었다. 또한, 위암 세포에서 FOXO1 과 LGR5 의 double knockdown 은 FOXO1 shRNA 에 의해 증가된 tumorsphere 형성능이 LGR5 의 유전자 억제에 의해 파기되고, 이는 FOXO1 의 복원이 없이 이루어짐을 밝혔다.

결론: 본 연구의 결과로부터, FOXO1 와 LGR5 가 상호적인 negative crosstalk 를 통하여 위암의 자가재생능을 조절하는데 중추적인 역할을 수행함을 관찰하였다. 따라서, 이 결과는 FOXO1/LGR5 pathway 가 표적치료제의 개발에 유용한 정보를 제공할 것으로 사료된다.

주요어: 위암; 암줄기세포; FOXO1; LGR5

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