Galectin-1 Induces Gastric Cancer Stem Cell Population through the Activation of Wnt/β-Catenin Signaling

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Yeseal Yim

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ABSTRACT

Galectin-1 is a proto-type of lectin that has one carbohydrate recognition domain and it has high affinity for β-galactosides. The different expressions of galectin-1 in normal and tumor tissues of various cancers and the functions of galectin-1 have been documented in several articles. They introduced the intracellular and extracellular role of galectin-1 through the regulation of immune response, tumor progression and metastasis. In gastric cancer, it was reported that the expression level of galectin-1 is higher in malignant tissues than normal tissues of gastric cancer patients, however, the role of galectin-1 is not elucidated, yet.

In this study, we demonstrated that galectin-1 regulates Wnt/β-catenin signaling pathway to induce the number of gastric cancer stem cell population, suggesting that galectin-1 could be a target molecule for gastric cancer therapy.

We conformed that the expression level of galectin-1 is highly expressed in tumor tissues of gastric cancer patients and its expression is reversely correlated to survival probability and first progression rate. Depletion of galectin-1 by its specific siRNAs decreased the cell proliferation, migration, invasion and sphere forming ability of gastric cancer AGS, YCC-2 and SNU-668 cells. On the contrary, over-expression of galectin-1 increased the cell proliferation, migration, invasion and sphere forming ability. We also performed the colony forming assay and Hoechst 33342 dye exclusion assay using gastric cancer MKN-28 cells, and determined that depletion of

galectin-1 reduced the side population of gastric cancer cells. These results suggest that galectin-1 increases the stemness property of gastric cancer cells.

To determine the mechanism, we demonstrated which pathway is regulated by galectin-1 in gastric cancer cells. Interestingly, TOP-Flash luciferase activity, which represents the activation of Wnt/β-catenin signaling, was significantly regulated by galectin-1 without β-catenin transcriptional regulation and nuclear accumulation.

Take these together, galectin-1 regulates Wnt/β-catenin signaling and then increases the stemness property of gastric cancer cells. We suppose here that galectin-1 could be a target molecule to do diagnosis and therapy of gastric cancer.

Key word: Galectin-1, gastric cancer stem cells, Wnt/ β-catenin signaling

I. INTRODUTION

Gastric cancer is one of the common cancers in worldwide and specifically occurs in Eastern Asia.[1] Etiology of gastric cancer is reported as dietary habits, family history and *Helicobacter pylori* infection, but the molecular mechanism of the pathogenesis is still unclear.[2,3] Surgical resection and chemotherapy is demanded to early stage of gastric cancer patients, nevertheless in metastatic cancer case the survival rate markedly decrease. Chemotherapy have limited efficacy to metastatic cancer, so targeted therapy is required[4]. Therefore, it is necessary to clarify the molecular mechanism of gastric cancer progression and metastasis.

Recently, cancer stem cells (CSCs) have been reported as a key target in metastatic cancer and recurrence of cancer.[5] CSCs were first described in hematopoietic tumor[6] and now reported in many solid tumors include breast,[7-9] colon,[10,11] brain,[12] pancreas,[13] prostate.[14] CSCs share properties with normal tissue stem cells, like self-renewal activity, differentiation capacity.[15] In primary tumors, the most major population of cells is non-CSCs and it shows limited self-renewal activity and drug sensitivity. The population of CSCs is minor but it shows unlimited self-renewal activity, drug resistant, multi-differentiation potential and cancer initiating ability.[16]

Identification of the molecular pathway for self-renewal activity of CSCs is an important approach for targeting cancer therapy. Epithelial-to-mesenchymal transition (EMT) is a decisive factor to invasiveness and metastasis, it is also

associated with poor prognosis in various cancers.[17,18] EMT is the capacity of epithelial cells acquire to mesenchymal cells and its invasion into surround tissues and organs. In recent, EMT was reported to induce differentiated cancer cells into CSC-like cells.[8] Moreover, invasive EMT cells are associated with Wnt/β-catenin signaling activation in colon cancer.[19,20] Wnt/β-catenin signaling pathway is known as one of the main pathways that regulate non-neoplastic stem cells and it also reported to involve in CSCs proliferation, differentiation, migration and invasion.[21] In Wnt/β-catenin signaling pathway, β-catenin destruction complex is important to regulate its stability and transcriptional activity of TCF4, T cell factor (TCF) family DNA-binding proteins.[22] Nuclear β-catenin and TCF4 is involved in the control of cancer stem cell property[23] to act as the transcriptional activator[22] and activate Wnt target gene such as c-myc, cyclin D1, CD44, and fibronectin,[24-27] which is necessary for invasive growth.

Galectins are the family of carbohydrate-binding proteins known as S-type lectins. It has high affinity for β-galactosides[28] and it mediates cell to cell or cell to matrix signaling by binding to cell surface glycol-proteins.[29] Accordingly, biological functions of galectins were studied including regulation of immune responses, differentiation, cell migration, tumor progression and apoptosis.[30] Fifteen of galectins were identified and classified into 3 subtypes : proto-type (galectin-1, -2, -5, -7, -10, -11, -13, -14 and -15), chimera-type (galectin-3) and tandem repeat-type (galectin-4, -6, -8, -9 and -12).[28,31] Galectin-1 has one carbohydrate recognition domain which can bind to glycans both as monomer and homo-dimer. The different expressions of

galectin-1 in normal and tumor tissues of various cancers and the functions of galectin-1 have been documented in several articles. They introduced the intracellular and extracellular role of galectin-1 through the immune response, tumor progression and metastasis.[28,32-37]

In gastric cancers, it was reported that the expression level of galectin-1 higher in malignant tissues than normal tissue of gastric cancer patients,[31] but the role of galectin-1 is no elucidated, yet. It was studied that over-expression of galectin-1 induced the cell migration, invasion, tumor progression, and angiogenesis. [34,35,37] Aberrantly activation of Wnt/β-catenin signaling is also studied in gastric cancer, [33,36,38] and it is involved in tumor initiation, tumor growth, and metastasis.[38-40]. These results made us to be interested in whether galectin-1 and Wnt/β-catenin signaling pathway have communicated each other in gastric cancer progression and specifically increasing stemness properties of gastric cancer cells.

In this study, we confirmed that the expression level of galectin-1 is higher in tumor tissues of gastric cancer patients than normal tissues using public GEO database. We performed the Kaplan-Meier plot analysis, and get the results that expression of galectin-1 is reverse correlated to survival probability and first progression rate. These data suggest that galectin-1 is increased and has a role gastric cancer progression. Therefore, we demonstrated the role of galectin-1 in cancer proliferation, and especially in stemness properties of gastric cancer cells. We found out that galectin-1 increased the population of gastric cancer stem cells through the activation of Wnt/β-catenin signaling pathway.

Taken together, in this study, we determined the molecular mechanism how galectin-1 regulates stemness properties of gastric cancer cells and suggested that galectin-1 could be a potent target for gastric cancer therapy.

II. MATERIALS AND METHODS

1. Cell culture

AGS, YCC-2, SNU668 human gastric cancer cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Corning Cellgro, Manassas, VA, USA) and 1% antibiotic-antimycotic (Gibco-BRL, NY, USA). The cells were maintained at 37℃ in a humidified incubator with a 5% CO_2 atmosphere.

2. Knock-down of galectin-1 expression by siRNA

Human galectin-1 siRNAs were 5'-CCAACACCAUCGUGUGCAA-3', 5'-AGGCCAACCUGACCGUCAA-3' purchased from Cosmogenetech (GenePharma Co, Shanghai, China). siRNA (20 nM) were transfected into cells using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA) and cells were harvested after 48 hrs for use in the experiments.

3. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the cells using Isol-RNA Lysis Reagent (5prime, Gaithersburg, MD, USA) according to the manufacturer's protocol. cDNAs were synthesized using 1μg of total RNA by ReverTra Ace® qPCR RT Master Mix (TOYOBO, Tokyo, Japan). PCR was performed using EX-taq DNA Polymerase (TaKaRa, Kyoto, Japan) and the primer sequences as follows:

Galectin-1: 5'-CTCTCGGGTGGAGTCTTCTG-3' (sense) and 5'-ACGAAGCTCTT AGCGTCAGG-3' (anti-sense); β-actin: 5'- AGCCTCGCCTTTGCCGA -3' (sense) and 5'-CTG GTGCCTGGGGCG-3' (anti-sense);

4. Cell proliferation detection assay

The AGS, YCC-2, SNU-668 cells were seeded in 96-well culture plates (3×10^3 cells/well) and incubate 24hrs, cells were transfected with scRNA and galectin-1 siRNA, mock vector and galectin-1 over-expression vector. After 48 hrs of transfection, cell viability was measured using WST based EZ-Cytox (DoGen-bio, Seoul, Korea). WST solution was added to each well, after 1hr of incubation the absorbance was measured on SpectraMAX 250 ELISA reader (Molecular Device Co., Sunnyvale, CA, USA) at 450 nm and is shown as the average of three independent assays.

5. Western blot analysis

Cells were lysed in RIPA buffer (Biosesang, Seoul, Korea) containing a Xpert protease inhibitor cocktail (GenDEPOT, Barker, TX, USA) and Phosphatase inhibitor (NaF, Na_3VO_4), followed by sonication on ice. The cell lysate was centrifuged for 20min at 13,200 rpm at 4℃ and the supernatant was collected. 20 μg of proteins were separated by SDS-PAGE and transferred onto PVDF transfer membranes (Merck Millipore, Darmstadt, Germany). After transfer, the membranes were blocked with 5% skim milk (Becton Dickinson and Company, Sparks, MD,

USA) for 1 hour and incubated with primary antibody dissolved in 5% BSA (Bovagen Biologicals, Victoria, Australia) overnight at 4℃. After that the membranes were incubated with anti-mouse or anti-rabbit secondary antibody conjugated with HRP (Bethyl Laboratories Inc, Montgomery, TX, USA) for 1 hour, followed by protein band were detected by Clarity western ECL substrate (Bio-Rad, Hercules, CA, USA) using LAS 3000. The following antibodies were used: anti-β-actin, anti-galectin-1 (Santa Cruz, Dallas, TX, USA), anti-Flag (Sigma Aldrich, St.Louis, MO, USA).

6. Nuclear fraction

Cells were lysed in Buffer A (10 mM HEPES (pH7.9), 1.5 mM $Mgcl_2$, 10 mM KCl, 1 mM DTT, 0.2 mM PMSF, 0.1 % NP-40) containing a Xpert protease inhibitor cocktail (GenDEPOT, Barker, TX, USA) and Phosphatase inhibitor (NaF, Na_3VO_4), incubation on ice for 15 min. The cell lysate was centrifuged for 10 min at 850 G at 4℃ for 10 min and collect the supernatant (Cytoplasm). Wash the pellet with Buffer A' (Buffer A without NP-40) and centrifuged for 10 min at 1500 G at 4℃ and discard the supernatant. Next, resuspend the pellet with Buffer C (20 mM HEPES (pH7.9), 25 % Glycerol, 0.42 M Nacl, 0.2 mM EDTA, 1.5 mM $Mgcl_2$ 1 mM DTT, 0.2 mM PMSF) and vortex for 15 sec. Incubate the cell lysate for 30 min on ice and vortex every 10 min for 15 sec. After incubation the cell lysate were centrifuged for 10 min at 13,200 rpm at 4℃ and collect the supernatant (Nuclear). Western blot analysis was performed as described above with nuclear fraction protein samples.

7. Immunoprecipitation (IP) assays

Cells were lysed in RIPA buffer, cell lysates (1 mg) were pre-cleared with 20 μl protein-A/G agarose beads (Santa Cruz, Dallas, TX, USA) for 1 hour at 4℃. After incubation, beads were centrifuged for 5 min at 2,500 rpm and the supernatant are transfer to new tube. Pre-cleared cell lysates were incubated overnight with 1 μg anti-FLAG antibody on rotating platform at 4℃, followed by incubation with 20 μl protein-A/G agarose beads for 2 hours. After incubation, beads were washed 3 times in 0.2 % RIPA buffer before dissolved in 2x SDS sample buffer. Western blot analysis was performed as described above with Immunoprecipitated protein samples.

8. Infection of galectin-1 shRNA expressing lenti-viral vectors

shRNA-expressing lentiviral vectors for galectin-1 was purchased from Sigma (Sigma Aldrich, St.Louis, MO, USA). Lentivirus particles were generated using three packaging plasmids, VSVG, RSV-REV, and PMDLg/pPRE, and were co-transfected with Galectin-1 shRNA in HEK293FT cells using lipofectamin2000 (Invitrogen, Carlsbad, CA, USA). Lentiviral particle was transduced to cell lines with polybrene and selected by puromycin and the shRNA sequences as follows:

shGal1 #3; 5'-CCGGACGGTGACTTCAAGATCAAATCTCGAGATTTGATCTTGAAGTCACCGTTTTTTTG-3' , shGal1 #4 ; 5'- CCGGGTGTTGCAGAGGTGTGCATCACTCGAGTGATGCACA CCTCTGCAACACTTTTTTG-3'

9. Sphere forming culture

Cells were grown in Ultra-low attachment plates (Corning Costar, Acton, MA, USA) in Mammary Epithelium Basal Medium (MEBM) (Lonza, Basel, Switzerland) supplemented with B27 (Gibco, Grand island, NY, USA), 20 ng/ml of EGF, and 20 ng/ml of FGF (Peprotech, Rocky Hill, NJ, USA) at a density of 3000 cells/ml. After 15 days, we counted the spheres with diameters >50 μm.

10. Transwell migration and invasion assays

8.0 μm pore transwell chamber (Corning costar, Acton, MA, USA) were used in this assay. Filter were coated with 0.5 mg/ml collagen type I (BD Bioscience, San Jose, CA, USA) for migration assay, and with 1/15 dilution of matrigel (BD Bioscience, San Jose, CA, USA) for invasion assay. AGS, YCC-2, SNU668 cells were transfected with scRNA, galectin-1 siRNA, mock vector and galectin-1 overexpression vector. After 24 hrs, cells were harvest and resuspension with serum free media. 1×10^5 cells were seeded to upper chamber and lower chamber was filled with media containing 10 % FBS and 1 % antibiotics then incubation for 20 hrs. Cells were migrated and invaded into bottom of filter and performed H&E staining. 5 image areas were randomly taken and cell numbers were obtained from three independent experiments average.

11. Hoechst 33342 dye exclusion assay (Side population analysis)

The MKN28 cell was transfected with scRNA and galectin-1 siRNA, after 48 hrs cell were harvest with trypsin and cells (1×10^6cells/tube) were incubation at 1ml of suspension media (HBSS, 2 % FBS, 10 mM HEPES) with Hoechst dye(5 μg) and reserpine(50 μM) at 37℃ for 60 min. Wash the cell with ice suspension media (HBSS, 2 % FBS, 10 mM HEPES) 3 times and treat PI solution (2 μg/ml).

12. Colony formation assay

The GES-1 cells were transfected with mock vector and galectin-1 over-expression vector. After 24 hrs transfection, Cells were seeded in 60mm culture plates (1×10^3cells/well) and incubate 2 wks. Wash the cell with PBS and fix the cells by 1 % Glutaraldehyde for 10 min. After fixation, Wash the cell with PBS and staining with 0.5 % crystal violet for 10 min at RT. Then wash out the crystal violet solution.

13. Luciferase assay

For TOP/FOP reporter assay, cells were transfected with the reporter vectors and β-galactosidase expression vector for normalization transfection efficiency. After 48hrs, the luciferase activity was measured using luciferase assay system (Promega, Fitchburg, WI, USA) according to the manufacturer's instruction.

14. Statistical analysis

Significant differences between the control groups and knockdown or overexpressed galectin-1 groups were determined using the paired t test for multiple samples, if indicated. Differences were considered significant if the P value was less than 0.05. Analysis was performed by Prism 5 software (Graph Pad software, La Jolla, CA, USA)

15. Kaplan-Meier analysis of overall survival and first progression survival

Kaplan-Meier analysis of survival curve was generated using the online resource http://kmplot.com/analysis and gene set for gastric cancer patients.

Gene symbol : LGALS1, Affy ID : 201105_at

III. RESULTS

1. Galectin-1 is highly expressed in gastric tumor tissues and it is reversely correlated with overall survival rate and poor first progression rate.

In gastric cancers, it was reported that the expression level of galectin-1 higher in malignant tissues than normal tissues of gastric cancer patients.[31] To confirmation of these results, we compared the expression level of galectin-1 in gastric cancer tissue with normal tissue using public GEO database. Two GSE gastric patient sets were analyzed (GSE27342, n=80, p=0.002) (GSE65801, n=32, p=0.0004), as below expression level of galectin-1 is highly expressed in tumor tissues of gastric cancer patient tissues in both GSE gastric patients set (Fig.1A).

In other cancer, such as kidney, pancreatic ductal adenocarcinoma, hepatocellular carcinoma, expression level of galectin-1 is reverse correlated with survival rate.[41-43] We performed Kaplan-Meier plot analysis to estimate the prognostic value of galectin-1, the expression level of galectin-1 is reverse correlated to overall survival rate (n=876, p=1.3e-07) (Fig.1B) and first progression rate (n=641, p=3.6e-09) (Fig.1C).

These results represent that the high level of galectin-1 expression might affect gastric cancer patient prognosis.

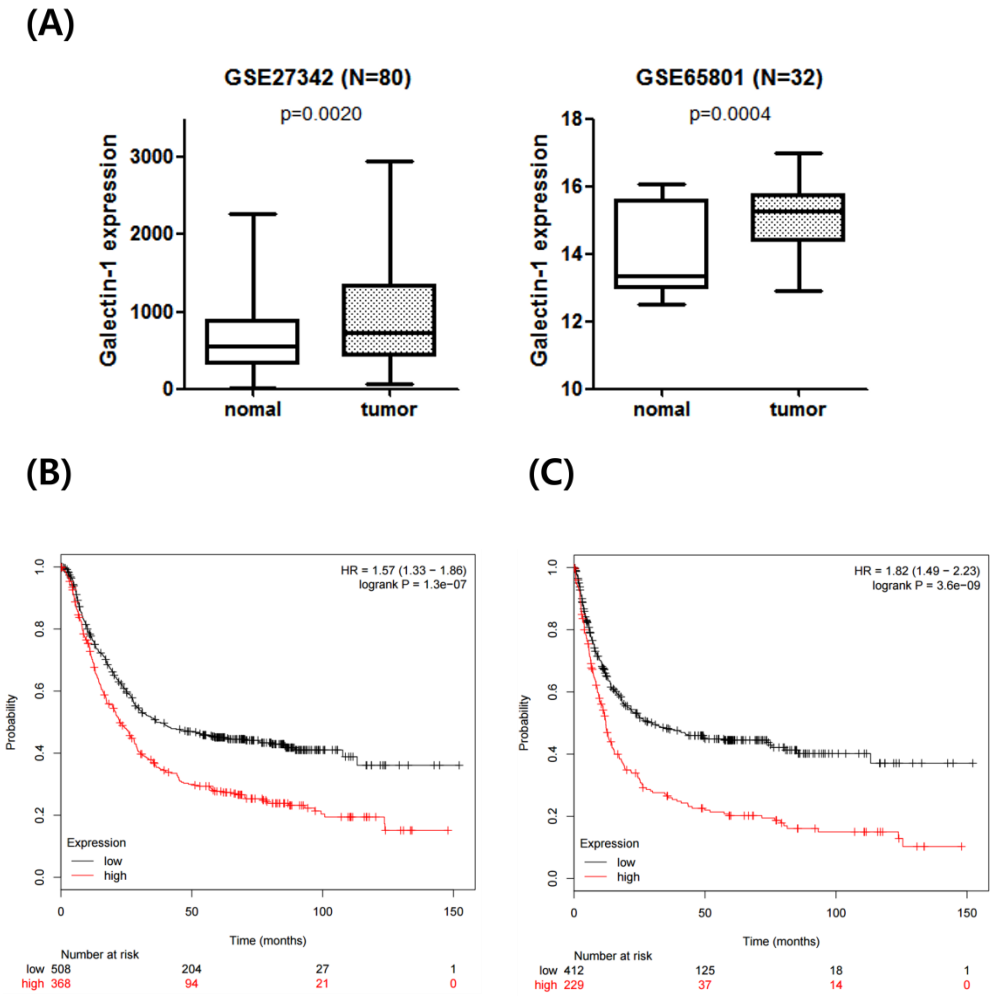


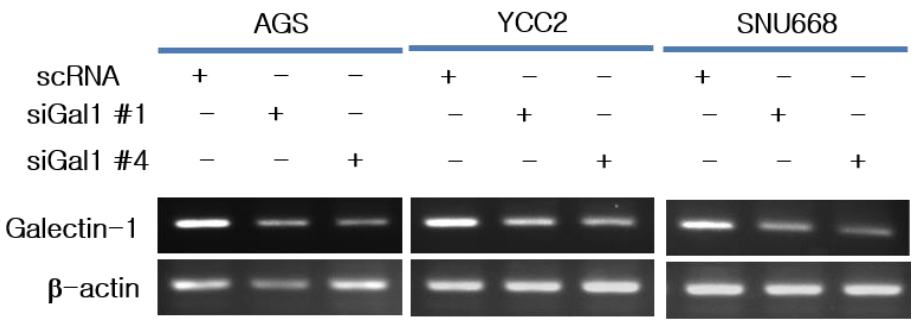
Figure 1. The expression level and survival rate of galectin-1 expression in gastric cancer patients by GEO data set and Kaplan Meier plot analysis. (A) Galetin-1 expression level is compared in normal and tumor tissue. Two sets of gastric cancer patients were used in analysis. [GEO database accession No. GSE27342, GES65801]. (B), (C) Overall survival rate and first progression rate was analyzed with galectin-1 expression level using Kaplan-Meier plot analysis.

2. Knockdown of galectin-1 decreased the stemness property of gastric cancer cells.

Next, we investigated the several effects of galectin-1 depletion in gastric cancer cell lines. Depletion of galectin-1 by its specific siRNAs were transfected in AGS, YCC-2, SNU-668 gastric cell lines, and both siRNAs reduced galectin-1 expression (Fig. 2A). As a result of galectin-1 depletion, both siRNA#1 and siRNA#4 transfected cells significantly decreased the cell proliferation measured by WST assay (Fig. 2B). Also we performed transwell based cell migration and invasion assay, first AGS, YCC-2, SNU668 cells were generated galectin-1 stably knockdown cell lines using galectin-1 shRNA by lentiviral system. Galectin-1 level is detected in stably knockdown cell lines by Western blot (Fig. 2D). Depletion of galectin-1 significantly decreased the migration and invasion in gastric cancer cells (Fig. 2C). These results can affect by cancer stem cell properties, we checked sphere forming ability. Sphere forming assay is known as enrich the cancer stem cells population. To determine whether galectin-1 regulates gastric cancer cell sphere forming ability, generated stable cells (3000 cells/ml) were cultured in 2 weeks as described in ‘materials and methods’. The number and size of sphere were significantly decreased in galectin-1 depleted cell lines (Fig. 2E).

These results represent that depletion of galectin-1 decrease cell proliferation, migration, invasion and sphere forming ability.

(A)



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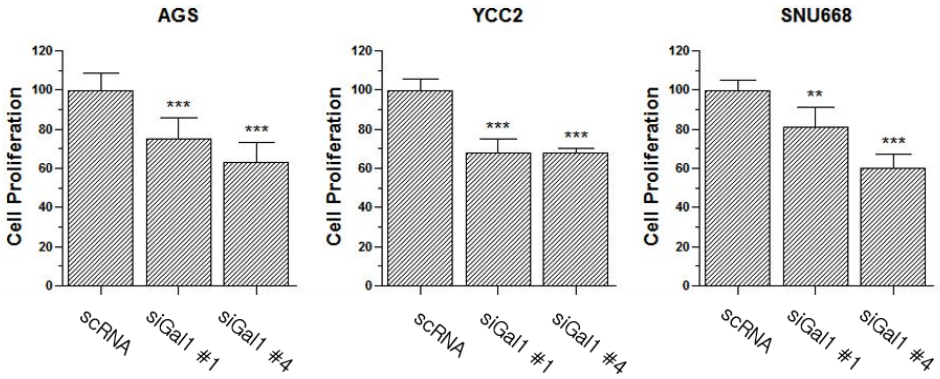

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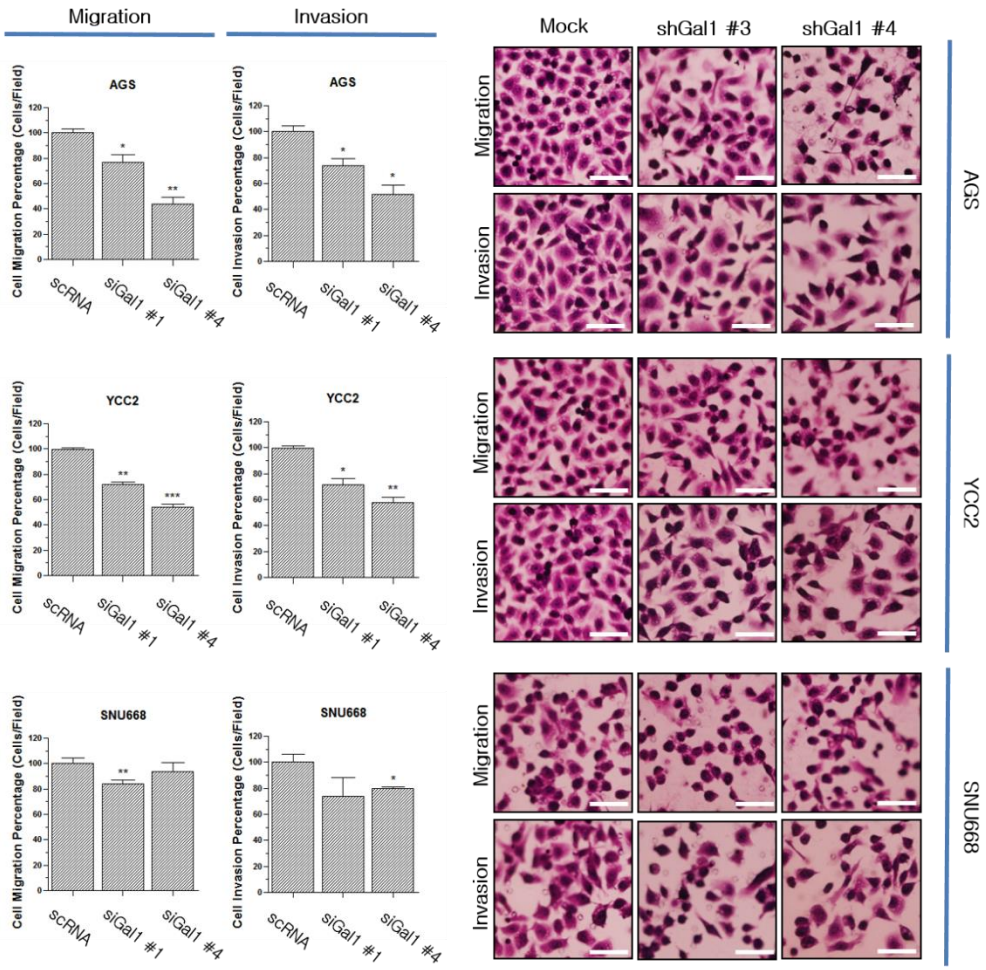
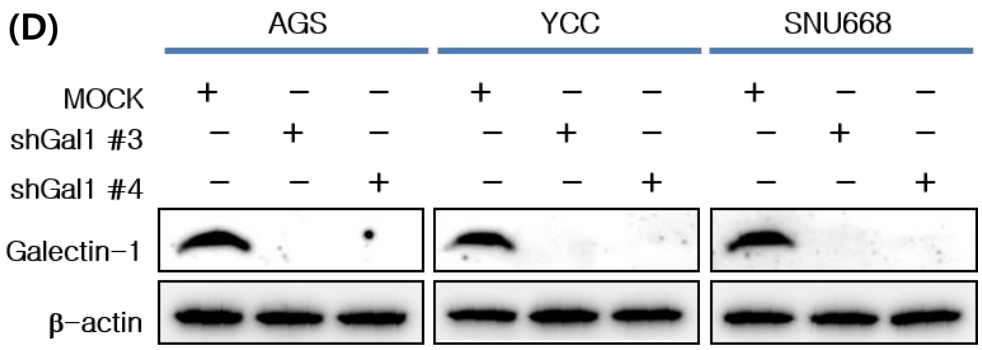




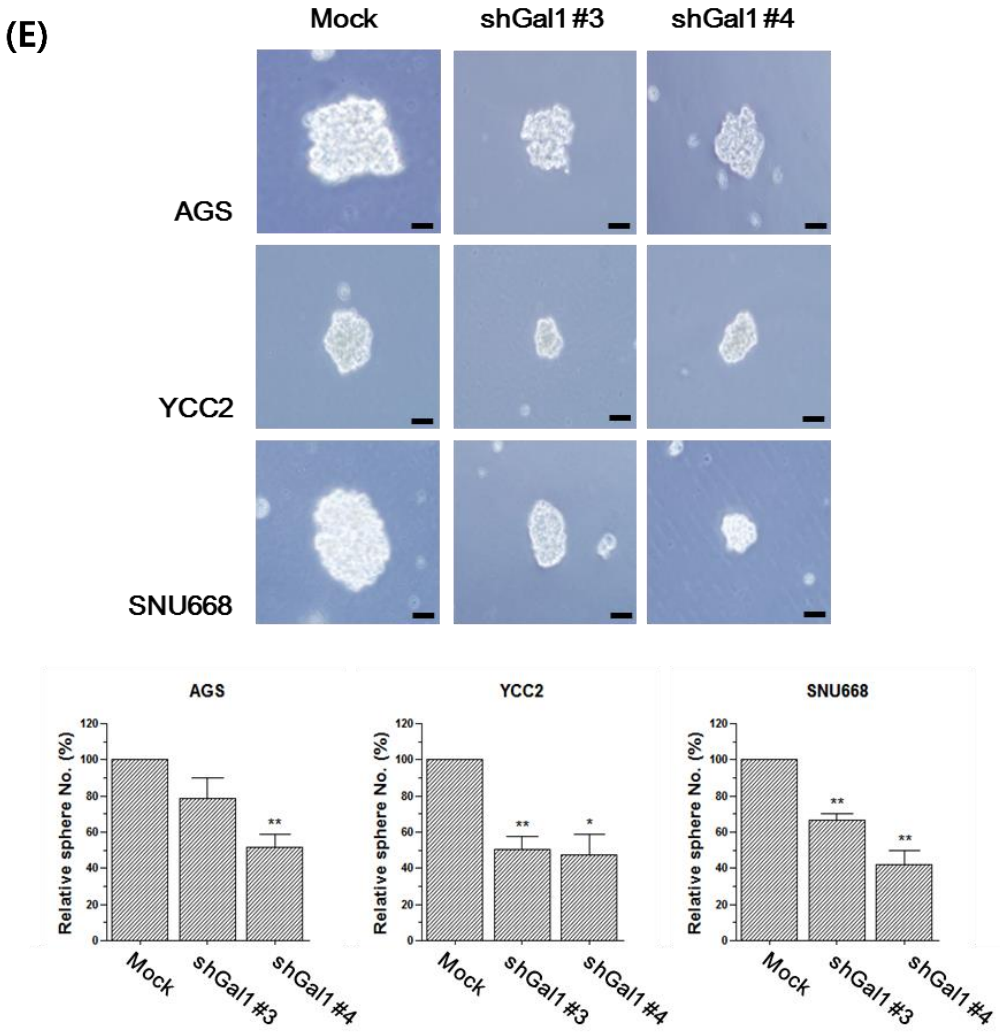


Figure 2. Knockdown of galectin-1 decreased the cell proliferation and stemness phenotype of gastric cancer cells. (A) Galectin-1 specific siRNAs were transfected in AGS, YCC-2, SNU-668 cell lines. Galectin-1 and β-actin expression level was confirmed by RT-PCR. β-actin was used as loading control. (B) Cell proliferation was measured by WST assay in negative control group and galectin-1 knockdown group. (C) Cell migration and invasion assay were performed by transwell based system as described in 'materials and methods'. In each group, 5 images were taken and counted. Scale bar = 5 μm. (D) Galectin-1 lentivirus was infected to generate galectin-1 knockdown stable cell line. Infected cell was selected by puromycin treated and galectin-1 and β-actin expression level were confirmed by western blot analysis. β-actin was used as loading control. (E) The sphere forming ability was measured after 2 weeks of sphere culture as described in 'materials and methods'. Images were taken in whole areas and sphere was counted. Scale bar = 50 μm.

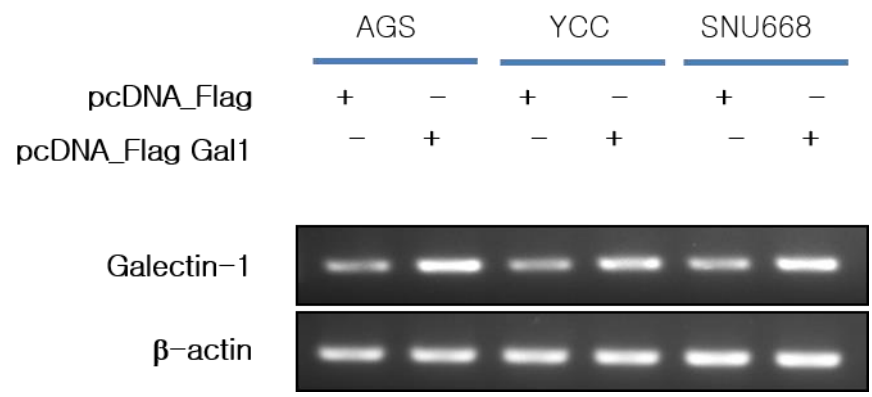
P values<0.05 were considered significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) and calculated using student *t* test.

3. Overexpression of galectin-1 increased the stemness property of in gastric cancer cells

To identify galectin-1 gain of function, AGS, YCC-2, SNU-668 gastric cell lines were transfected with galectin-1 expression vector and detected over expressed galectin-1 by RT-PCR (Fig. 3A). As a result of galectin-1 over expression, galectin-1 over expressed cells significantly increased the cell proliferation then empty vector (mock) measured by WST assay (Fig. 3B). Also we performed transwell based cell migration and invasion assay after generate the galectin-1 stably over expressing cell line. AGS, YCC-2, SNU-668 cells were generated galectin-1 stably over expressing cell line by galectin-1 lentiviral vector. Galectin-1 level is detected in stably over-expressing cell lines by RT-PCR (Fig. 3D). over expression of galectin-1 significantly increase the migration and invasion in gastric cancer cells (Fig. 3C). Next sphere forming assay were performed with generated stable cells (3000 cells/ml). Cells were cultured for 2 weeks, the number and size of sphere were significantly increased in galectin-1 over expressing cell lines (Fig. 3E).

These results represent that over expression of galectin-1 increase cell proliferation, migration, invasion and sphere forming ability. Taken together, we demonstrated that galectin-1 affect cancer stem cell properties, such as cell proliferation, migration, invasion and sphere forming ability in gastric cancer cell lines.

(A)



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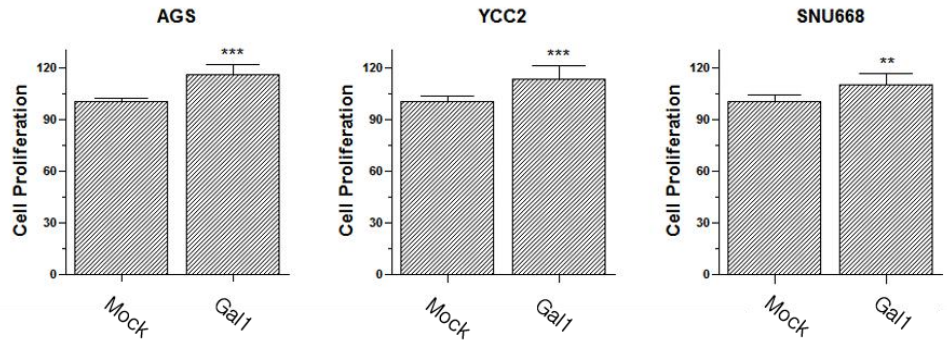

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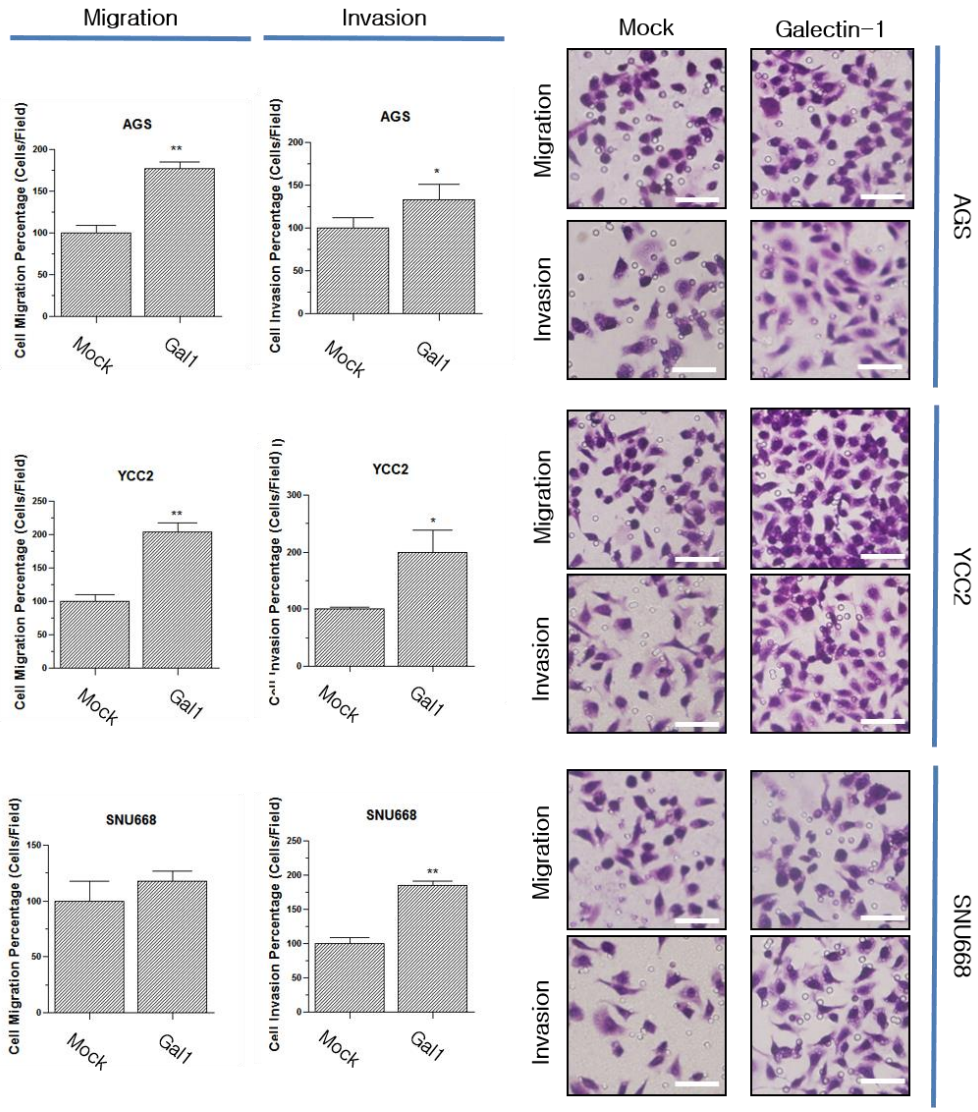

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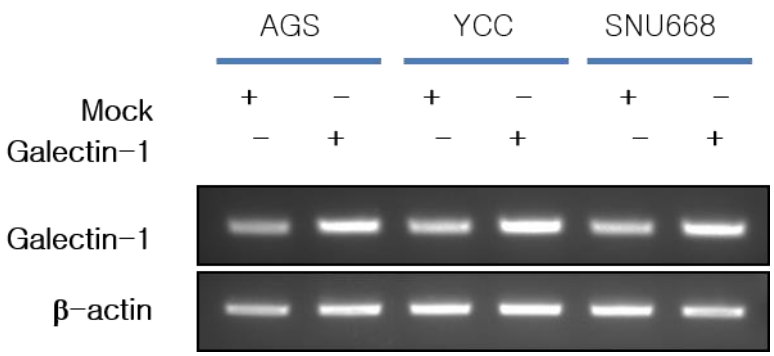
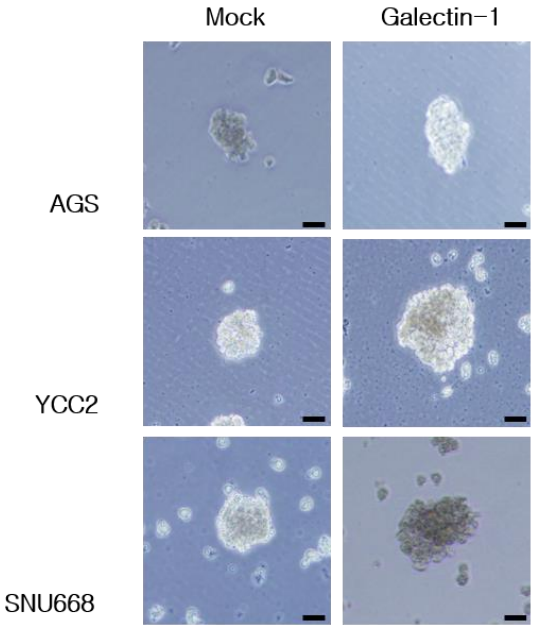


(E)



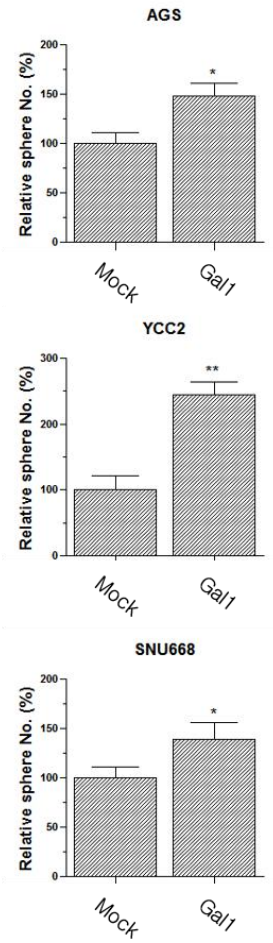


Figure 3. Overexpression of galectin-1 increased the cell proliferation and stemness phenotype of gastric cancer cells. (A) Galectin-1 is over expressed in AGS, YCC-2, SNU668 cell lines using galectin-1 expression vector. Galectin-1 and β-actin expression level was confirmed by RT-PCR. β-actin was used as loading control. (B) Cell proliferation was measured by WST assay in negative control group and galectin-1 over expression group. (C) Cell migration and invasion assay were performed by transwell based system as described in 'materials and methods'. In each group, 5 images were taken and counted. Scale bar = 5 μm (D) Galectin-1 specific lentivirus was infected to generate galectin-1 overexpression stable cell line. Infected cell was selected by puromycin treated and galectin-1 and β-actin expression level were confirmed by RT-PCR. β-actin was used as loading control. (E) The sphere forming ability was measured after 2 weeks of sphere culture as described in 'materials and methods'. Images were taken in whole areas and sphere was counted. Scale bar = 50 μm.

P values<0.05 were considered significant (* p<0.05, ** p<0.01, *** p<0.001) and calculated using student *t* test.

4. Overexpression of galectin-1 increased the tumorigenic cells transformation

To confirm that galectin-1 regulates gastric cancer stemness properties, we performed colony forming assay. Tumorigenic cells transformation can demonstrate by colony forming assay. Galectin-1 expression vector was transfected in GES-1 gastric normal cell line and 1×10^3 cells/well were seeded in culture plate and cultured in 2 weeks. Compared to Mock, galectin-1 over expressed cell has markedly increases of colony forming ability (Fig. 4).

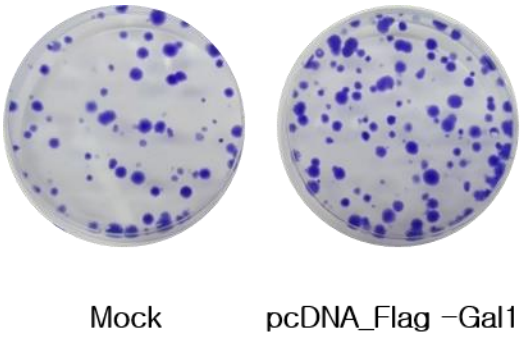


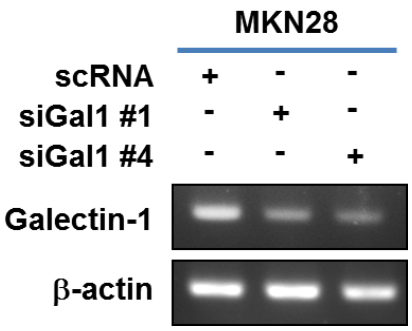
Figure 4. Overexpression of galectin-1 increased tumorigenic cell transformation of gastric normal cells. Colony forming assay for tumorigenic cells transformation analyze in GES-1 gastric normal cell line. GES-1 cell was transfected with galectin-1 expression vector and 1×10^3 cells were seeded after 1 day. Crystal violet was stained after 2 weeks for visible the colony.

5. Knockdown of galectin-1 decreased the population of gastric cancer stem cells

Tumorigenic cells transformation is experimental evidence of cancer stem cell,[5] so next we checked more strong experimental evidence. Side population analysis, as defined by Hoechst 33342 dye exclusion assay, can be used to identify CSCs.[44,45] Side-population cells, which called as CSCs, highly express ATP-binding cassette transporter (ABC-Transporter) which efflux Hoechst 33342 dye[46] and this analysis use basis on cells efflux ability of Hoechst 33342 DNA binding dye to analyze CSCs population. To demonstrate galectin-1 regulate CSCs population, we use MKN-28 cells which has high side population rate relatively higher than other gastric cancer cell lines. MKN-28 was transfected with galectin-1 siRNA and knockdown of galectin-1 is detected by RT-PCR (Fig. 5A), then we analysis side population by flow cytometry as described in ‘materials and methods’. Side population of gastric cancer cells were dramatically decreased in galectin-1 depletion in MKN-28 cells (Fig. 5B) and reserpine treated cells were used as negative control.

These results suggest that galectin-1 increase the stemness property of gastric cancer cells.

(A)



(B)

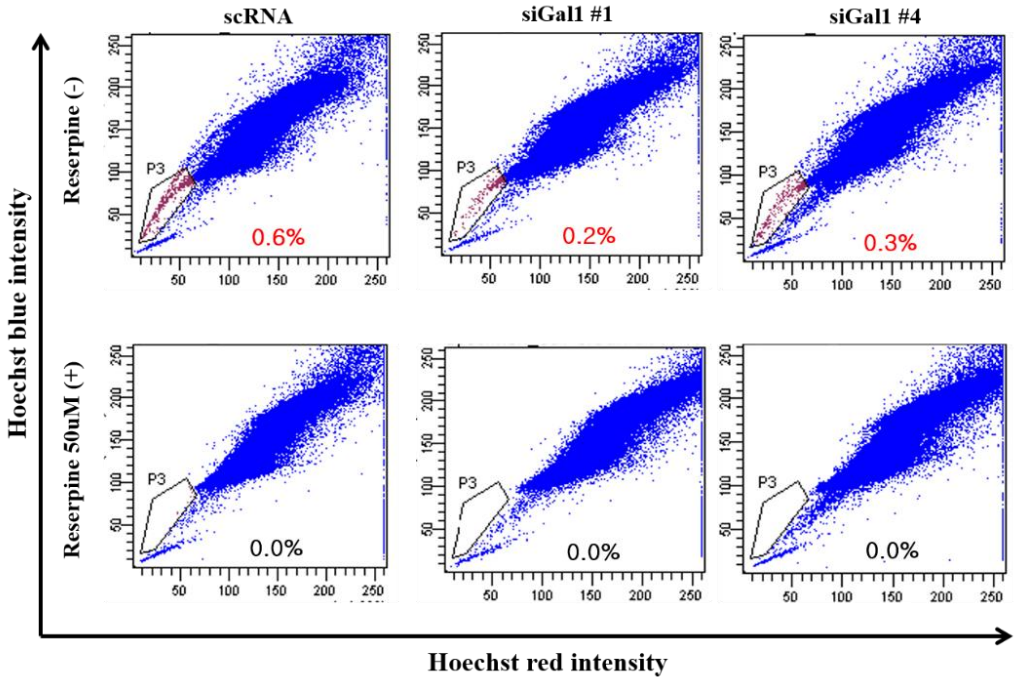


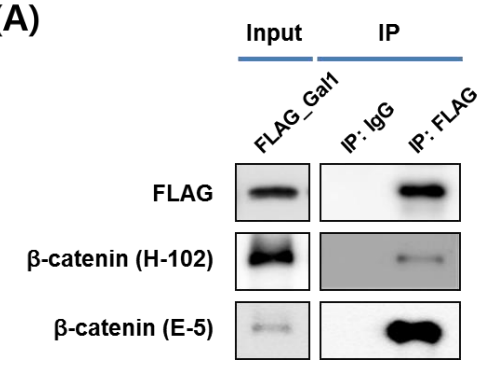
Figure 5. Knockdown of galectin-1 reduced the population of gastric cancer stem cells. (A) Galectin-1 specific siRNA was transfected in MKN-28 and Galectin-1 and β-actin expression level were confirmed by RT-PCR. β-actin was used as loading control. (B) Hoechst 33342 dye exclusion assay (Side population analysis) was performed as described in 'materials and methods'. Galectin-1 siRNA transfected cells were used in analyzed and reserpine treated cells were used as negative control.

6. Direct interaction with galectin-1 and β-catenin was detected in gastric cancer cells

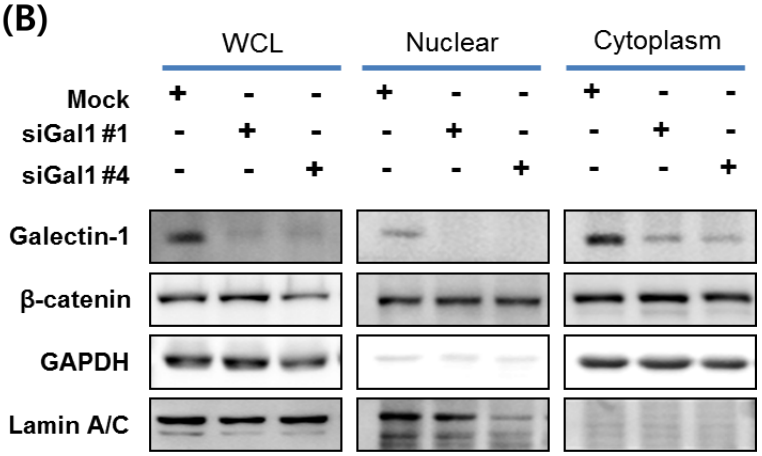
To determine the mechanism, we demonstrated which pathway is regulated by galectin-1 in gastric cancer cells. There are several pathways that control the proliferation, survival, self-renewal in both normal stem cells and cancer stem cells such as Hedgehog, Notch, Wnt and PTEN.[47,48] In gastric cancer, Wnt/β-catenin signaling is reported as aberrantly activated,[33,36] and it also involved in tumor initiation, tumor growth, and metastasis.[21,27,38] Thereby we focused on Wnt/β-catenin signaling in gastric cancer.

First of all, we performed immunoprecipitation (IP) in galectin-1 overexpressed AGS cell line and investigated whether galectin-1 interacted with β-catenin (Fig.6A). We demonstrate galectin-1 interact with β-catenin, so we hypothesis that β-catenin transcriptional level might be regulated by galectin-1. In whole cell lysates, galectin-1 was over expressed and knockdown in AGS cell line, but β-catenin protein level was not changed (Fig. 6B, Fig. 6C left panel). β-catenin is translocated to nuclear and interaction with transcription factor Tcf4 and these complex regulate Wnt target gene. We performed nuclear fractionation to investigate β-catenin nuclear accumulation. In both condition, galectin-1 over expressed and knockdown, β-catenin was not translocated at all (Fig. 6B, Fig. 6C right panel).

(A)



(B)



(C)

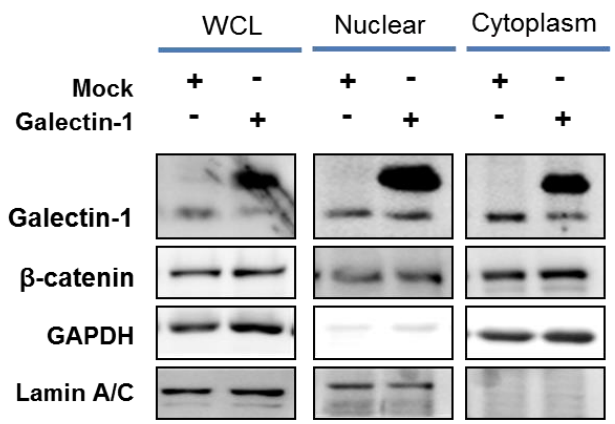


Figure 6. Wnt/β-catenin signaling is activated by galectin-1. (A) Immunoprecipitation (IP) assays were performed in AGS cell lines. Flag tagged galectin-1 is overexpressed and flag was used to immunoprecipitation (IP). Both protein expression level of β-catenin and Flag were detected by Western blot analysis. (B and C) Galectin-1 and β-catenin protein level was detected in AGS WCLs and nuclear fractionation sample. Nuclear fractionation in AGS as described in 'materials and methods'. GAPDH and Lamin A/C were used as nuclear protein fractionation loading control.

7. Transcriptional activation of Wnt/β-catenin signaling was regulated by galectin-1

To clarify Wnt/β-catenin signaling is not involved in gastric cancer stem cells, we performed TOP-Flash assay. TOP-Flash luciferase activity, which represents the activation of Wnt/β-catenin signaling, is widely used. TOP-Flash containing wild type Tcf binding site and FOP-Flash containing mutation type Tcf binding site for negative control. In AGS cell line, TOP-Flash activity was activated by galectin-1 over expression (Fig.7A) and in contrast TOP-Flash activity was inactivated by galectin-1 knockdown (Fig.7B). All TOP-Flash activity data was normalized by FOP-Flash.

These results represent that those stemness properties which regulated by galectin-1 is involved in Wnt/β-catenin signaling. Interestingly, TOP-Flash luciferase activity was significantly regulated by galectin-1 without β-catenin transcriptional regulation and nuclear accumulation. Take these together, galectin-1 regulates Wnt/β-catenin signaling and then increases the stemness property of gastric cancer cells. We suppose here that galectin-1 could be a target molecule to do diagnosis and therapy of gastric cancer.

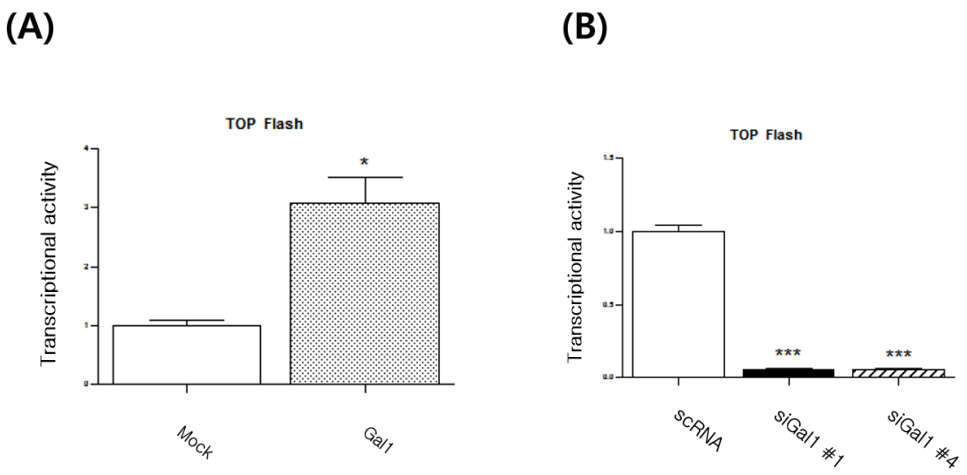


Figure 7. Transcriptional activation of Wnt/β-catenin signaling is regulated by galectin-1. Luciferase reporter assay was performed in AGS cell line. TOP/FOP Flash vector was co-transfected with galectin-1 overexpress vector (A) or galectin-1 specific siRNAs (B). TOP Flash transcriptional activity was normalized with FOP Flash activity described in "Materials and Methods".

P values<0.05 were considered significant (* p<0.05, ** p<0.01, *** p<0.001) and calculated using student *t* test.

IV. DISCUSSION

In gastric cancer, gastric cancer stem cells study has not been documented well, but its phenotypes are similar to various solid cancer stem cells. We propose to demonstrate the factors which involved in gastric cancer stem cell differentiation. Over expressed factors which have been reported in gastric cancer, we identified the galectin-1 as a factor related with cancer stem cells. In this study, we tried to examine the function of galectin-1 in gastric cancer stem cells. Firstly, we confirmed that the expression level of galectin-1 is highly expressed in gastric tumor tissue then gastric normal tissue. Also, gastric cancer patients with high level of galectin-1 expression represent poor survival rate and poor first progression rate through analyzed correlation with galectin-1 and survival rate (Fig.1). These data showed, the over-expression of galectin-1 in gastric cancer has a significant effect on the proliferation of gastric cancer cells. Furthermore, to investigate the several effects of galectin-1 depletion and overexpression, we performed cell proliferation assay, cell migration, invasion assay and sphere forming assay. Inhibition of galectin-1 led to decrease the cell proliferation, migration, invasion and sphere forming ability (Fig.2). On the contrary to this, over expression of galectin-1 were increased cell proliferation, cell migration, invasion and sphere forming ability (Fig.3). These data suggest that galectin-1 regulates cancer stemness phenotype with cell proliferation and cell motility in gastric cancer cells.

To confirmed of expression of galectin-1 effect on the gastric cancer stem cell, we were performed that two main experimental evidence of cancer stem cells[5], one is colony forming assay to detect tumorigenic cell transformation and the other is Hoechst 33342 dye exclusion assay to detect CSCs population. We demonstrated that galectin-1 over expression markedly induced tumorigenic cells transformation, in result induced colony forming ability. Hoechst 33342 dye exclusion assay was performed in MKN-28 cell line which has high side population rate relatively higher than other gastric cancer cell lines. The side population rate was dramatically decreased in galectin-1 knockdown cell line. These results taken together, galectin-1 regulate cell proliferation, migration, invasion and sphere forming ability and that results were followed by the change in the population of gastric cancer stem cells.

To determine the mechanism, we demonstrated which pathway is regulated by galectin-1 in gastric cancer cells. There are several pathways that control the proliferation, survival, self-renewal in both normal stem cells and cancer stem cells such as Hedgehog, Notch, Wnt and PTEN[47,48]. In gastric cancer, Wnt/β-catenin signaling is reported as aberrantly activated in gastric cancer[33,36], and it also involved in tumor initiation, tumor growth, and metastasis[21,27,38]. Thereby we focused on Wnt/β-catenin signaling in gastric cancer. At first, we were performed TOP-Flash luciferase activity, which represents the activation of Wnt/β-catenin signaling. Interestingly, TOP-Flash luciferase activity was significantly activated by galectin-1 exists. Also, we confirmed galectin-1 was binding with β-catenin but its transcriptional level and nuclear accumulation was not changed at all through the

galectin-1 expression. As a result, we demonstrated that the galectin-1 has an important role in Wnt/β-catenin signaling activation and confirmed its gastric cancer stem cell property. It is necessary to investigate the factors which affect the Wnt/β-catenin signaling activation by galectin-1, independent from β-catenin.

Especially, galectin-1 is abundantly expressed in nuclear, cytoplasm and extracellular matrix and the location of galectin-1 seems to be important in inducing its gastric cancer stem cell property. Though we did not clarify the molecular mechanism of galectin-1 in gastric cancer stem cells regulation, nevertheless we verified that gastric cancer stem cells property is significantly regulated by galectin-1 and Wnt/β-catenin signaling.

Therefore, take these results together, our study represents that galectin-1 and Wnt/β-catenin signaling were increased the stemness property in gastric cancer cells. Therefore, we suggest here that galectin-1 could be a target molecule to do diagnosis and therapy of gastric cancer.

V. CONCLUSION

This study demonstrated that galectin-1 affects the cell proliferation, cell migration and invasion in gastric cancer cells. It also increases the gastric cancer stem cell properties via Wnt/β-catenin signaling activation. Therefore, we suggest here that galectin-1 could be a target molecule to do diagnosis and therapy of gastric cancer.

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ABSTRACT (IN KOREA)

갈렉틴-1 은 한 개의 당을 인식하는 도메인을 가지는 렉틴으로 베타-갈락토사이드에 높은 친화력을 가지는 단백질이다. 갈렉틴-1 은 세포 내외부에 풍부히 존재하고 있고, 특히, 다양한 암 종의 정상조직에 비해 종양조직에서 과 발현되고 있음이 보고된다. 세포 내부와 외부의 풍부하게 존재하는 갈렉틴-1 은 다양한 역할을 가지는 것이 보고되고 있는데, 특히, 면역반응과 종양의 진행 및 전이 등에 관련된 연구가 다수를 이루고 있다. 위암의 경우, 위암 환자의 정상조직보다 악성종양조직에서 갈렉틴-1 이 높게 발현한다고 보고되고 있고, 위암의 발병에 큰 역할을 담당하는 헬리코박터 균에 의해 발현이 증가됨이 보고된 바가 있다. 그러나 발현양상이 종양 세포에서 증가된 것을 보고한 것에 비해, 갈렉틴-1 이 위암에서 작용하는 여러 기전에 대한 연구는 많이 진행이 되고 있지 않다. 본 연구는 위암에서 과 발현 되어 있는 갈렉틴-1 이 위암의 증식과 전이에 관여하고, 특히, 윈트/베타-카테닌 신호전달 과정을 통하여 위암줄기세포의 수를 증가시킨다는 것을 입증하고, 갈렉틴-1 이 위암의 분자표적치료에 이용될 수 있는 가능성을 제시하였다. 먼저, 환자 데이터를 통해 위암조직에서 갈렉틴-1 이 과발현 되어있고, 발현이 높을수록 암 환자의 생존율이 좋지 않은 것을 확인하였다. 위암 세포인 AGS, YCC-2, SNU-668 세포를 이용하여, 갈렉틴-

1 을 억제하면 세포의 증식과 이동 및 위암 줄기세포의 구 형성능이 감소되는 것을 관찰하였고, 반대로 갈렉틴-1 을 과 발현하면 이러한 현상이 증가되는 것을 관찰하였다. 또한 갈렉틴-1 의 발현에 의한 콜로니 형성 능력을 분석하고, Hoechst33342 배출 분석 실험을 통하여 갈렉틴-1 이 세포에서 억제되면 위암 줄기세포의 수가 감소되는 것을 통하여, 갈렉틴-1 이 위암 줄기세포의 특성을 가지는데 주요한 인자임을 확인하였다. 이러한 갈렉틴-1 의 위암 줄기 세포능을 조절하는 기전을 찾기 위해, 기존에 알려져 있는 다양한 신호전달과의 관계를 확인하였고, 특히 위암의 경우 윈트/베타-카테닌의 활성이 증가되어 있는 것을 확인하고, 윈트/베타-카테닌 신호전달 활성 정도를 갈렉틴-1 의 발현과 더불어 확인한 결과, 흥미롭게도 베타-카테닌의 발현조절과 핵 내 이동 없이도, 갈렉틴-1 에 의하여 윈트 신호전달 활성이 증가한 것을 확인할 수 있었다. 이 같은 결과를 토대로 본 연구에서 갈렉틴-1 이 윈트/베타-카테닌 신호전달 활성을 조절하여 위암의 줄기 세포능을 조절한다는 것을 확인하였고, 이러한 중요한 역할을 담당하고 있는 갈렉틴-1 을 표적으로 한 위암의 분자 표적 치료의 가능성을 확인하였다.

핵심되는 말: 갈렉틴-1, 위암줄기세포, 윈트/베타카테닌 신호전달