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ORIGINAL ARTICLE

Suppression of galectin-4 attenuates peritoneal metastasis of poorly differentiated gastric cancer cells

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Abstract

Background Peritoneal dissemination, most often seen in metastatic and/or recurrent gastric cancer, is an inoperable condition that lacks effective treatment. The use of molecular targeted drugs is also limited; therefore, identifying novel therapeutic targets and improving our understanding of this metastatic cancer are an urgent requirement. In this study, we focused on galectin-4, which is specifically expressed in poorly differentiated cells with high potential for peritoneal dissemination.

Methods We knocked out the galectin-4 gene in NUGC4 cells using CRISPR/Cas9-mediated genome editing. Proliferation and peritoneal cancer formation in knockout cells were compared with those in wild-type and galectin-4 re-expressing cells. Western blotting and proximity ligation assays were performed to identify associated molecules affected by the expression of galectin-4. The effect of galectin-4 knockdown on cell proliferation and peritoneal metastasis was studied using a specific siRNA. Expression of galectin-4 in peritoneal metastatic tumors from 10 patients with gastric cancer was examined by immunohistochemistry.

Results Suppression of galectin-4 expression reduced proliferation and peritoneal metastasis of malignant gastric cancer cells. Galectin-4 knockout and knockdown reduced the expression of activated c-MET and CD44. Galectin-4 was found to interact with several proteins on the cell surface, including CD44 and c-MET, via its carbohydrate-binding ability. Immunohistochemistry showed galectin-4 expression in peritoneal metastatic tumor cells in all patients examined.

Conclusions We clarified the role of galectin-4 in the development of peritoneal dissemination of poorly differentiated gastric cancer cells. Our data highlight the diagnostic and therapeutic potential of galectin-4 in the peritoneal dissemination of gastric cancer.

Keywords Galectin-4 · Gastric cancer · Metastasis · Gene knockout techniques · Gene silencing

Introduction

Peritoneal dissemination, most often seen in metastatic and/or recurrent gastric cancer, is an inoperable condition that lacks effective treatment; therefore, it is crucial to investigate the mechanisms and molecules involved. Drugs and immune checkpoint inhibitors that target molecules, such as HER2, VEGFR, and PD-1, are used for gastric cancer, but are not effective in all patients [1]. Thus, expanding our understanding of peritoneal metastasis by identifying the underlying mechanisms and molecules is vital.

During the development of peritoneal metastasis, tumor cells detach from the primary tumor, survive in the abdominal cavity, attach and invade the peritoneal mesothelial cells along with the basement membrane, and then grow via angiogenesis [2]. Galectins are a family of lectins that bind β-galactoside-containing glycans. Their expression is dysregulated in various tumors, although their precise role in cancer is not well understood [3]. Galectin-4 is mainly expressed in epithelial cells of the intestinal tract and has two carbohydrate-binding domains with distinct binding specificities [4]. Therefore, galectin-4 can cross-link and regulate crucial molecules involved in many biological

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processes. Studies have revealed the critical role of galectin-4 in lipid raft stabilization, glycoprotein trafficking, and tumor progression [4, 5].

A previous study using northern blotting showed preferential upregulation of galectin-4 in gastric cancer cell lines prone to peritoneal dissemination [6]; however, further studies were not performed. We observed that the levels of galectin-4-binding MUC1 were markedly high in the peripheral blood of patients with recurrent and/or metastatic breast cancer [7], and that galectin-4 is tyrosine-phosphorylated by members of the Src kinase family in the human gastric adenocarcinoma cell line NUGC4 [8]. Furthermore, Tsai et al. reported that galectin-4 gene expression was specifically increased in highly metastatic cells established by the repeated transplantation of metastatic lesions of human prostate cancer cells into mice [9]. Therefore, we hypothesized that expression of galectin-4 and its binding molecules is strongly correlated with the malignant progression of cancers.

In this study, we sought to clarify the role of galectin-4 in peritoneal dissemination of gastric cancer cells using gene knockout (KO) and knockdown (KD) strategies. We identified molecules associated with galectin-4 that play a critical role in the peritoneal metastasis of gastric cancer.

Materials and methods

A detailed description of the materials and methods is provided in the Electronic Supplementary Material (ESM).

Cell lines and cell culture

The human gastric cancer cell lines MKN7, MKN74, KATO III, MKN45, and NUGC4 were obtained from RIKEN BioResource Center (Tsukuba, Japan). Cells were cultured in RPMI 1640 medium supplemented with 2 mM L-alanyl-L-glutamine solution (FUJIFILM Wako Pure Chemical, Osaka, Japan) and 10% fetal calf serum (FCS).

Growth in low-attachment (GILA) assay

The GILA assay was performed according to the method described by Rotem et al. [10], with some modifications. Briefly, 1.0×10^3 cells were seeded in triplicate 96-well plates and cultured for 5 days in a CO_2 incubator. Tissue culture-treated 96-well clear plates (3595; Corning, Corning, NY, USA) and low-attachment surface plates (3474; Corning) were used for high- and low-attachment growth conditions, respectively. After incubation, the cells were mixed with CellTiter-Glo®2.0, reagents (Promega, Madison, WI, USA), and transferred to a 96-well solid white polystyrene microplate (3362; Corning) to read the luminescence signal for the assay of ATP content. The ratio of the ATP content on the low- to high-attachment surfaces was calculated.

Animal studies

All animal studies were performed at the Tsukuba Labo of Sankyo Labo Service Corporation in accordance with the "Declaration of Helsinki." All protocols were in accordance with the institutional and Japanese governmental guidelines for animal experiments. They were reviewed and approved by the Animal Welfare and Use Committee of the Sankyo Labo Service Corporation. A detailed protocol is provided in the ESM.

Proximity ligation assay (PLA) and selective proteomic proximity labeling assay using tyramide (SPPLAT)

PLA was performed by following the instructions of the Duolink-PLA Kit (DUO92101, Merck, Darmstadt, Germany), and SPPLAT was performed according to the method described by Li et al. [11], with some modifications. A detailed protocol is provided in the ESM.

KD of galectin-4 by RNA interference

We chose the galectin-4 siRNA HSS106035, 5′-CCGGAC AUUGCCAUCAACAGCUGAA-3′ for KD studies, which is reportedly the most effective siRNA among three galectin-4 specific stealth siRNAs [12]. A detailed protocol is provided in the ESM.

To evaluate the effect of galectin-4 siRNA treatment on the peritoneal dissemination of gastric cancer cells, nude mice were injected with MKN45 cells with or without galectin-4 or control siRNA. A detailed protocol is provided in the ESM.

Immunohistochemistry for galectin-4 expression in clinical peritoneal metastatic tumors

Expression and subcellular localization of galectin-4 in peritoneal metastatic tumors from gastric cancer patients was examined using the avidin–biotin–peroxidase complex (ABC) method. A detailed protocol is provided in the ESM.

Statistical analysis

At least two or three experiments were performed to ensure reproducibility. Results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using the

Student's *t* test or Welch's *t* test, and the significance level was set at $P < 0.05$.

Results

Galectin-4 expression in gastric cancer cell lines

We first analyzed galectin-4 protein expression in gastric cancer cell lines with varying peritoneal dissemination potentials. Galectin-4 expression was high in NUGC4 and MKN45 cell lines, which exhibited high peritoneal dissemination potential [13, 14], whereas galectin-3 expression showed little difference (Fig. 1). As several reports have suggested the critical role of epithelial–mesenchymal transition (EMT) in metastasis [15], we examined the expression of EMT-related proteins. E-cadherin, an important molecule in the adherens junction, was expressed in all cell lines, although KATO III, NUGC4, and MKN45 cells showed poor cell–cell adhesion. We could not detect the mesenchymal markers vimentin and ZEB1 in any of the cells, suggesting that these genes were not involved in metastasis.

MKN45 cells showed high expression of c-MET, an important molecule that drives the development of gastric cancer. CD44, a stem cell marker, was expressed in all cells except MKN74 cells, and its high-molecular-weight isoforms were expressed in MKN45 as well as NUGC4 cells. Expression of CD29 (β1-integrin) was observed in all cells, but was relatively low in MKN45 and NUGC4 cells.

Establishment and characterization of galectin-4 KO cells

After puromycin selection, the bulk KO cells obtained were transfected with guide sequences targeting exon 4 and Cas9 expression plasmids (Fig. S1A, lane 4). However, we noticed that a small portion of galectin-4-expressing cells still existed in the bulk KO cells. This may be because a small portion of cells that were transfected with only the Cas9 plasmid were puromycin-resistant but expressed galectin-4. It took a longer time than that required for other galectin-4 expressing cells to obtain sufficient bulk KO cells for freezing the stock. A preliminary proliferation assay also showed growth retardation of bulk KO cells (Fig. S1B). We checked the expression of galectin-4 periodically and observed that galectin-4 expression increased gradually over time. It is reasonable that the ratio of KO cells might decrease when their proliferation rate is lower than that of galectin-4 expressing cells. It is difficult to characterize the cell properties in bulk KO cells, which would decrease the ratio of KO cells.

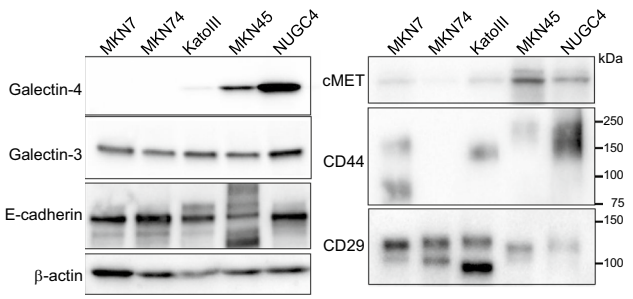

Fig. 1 Profiling of different types of gastric cancer cell lines. **a** Western blot analysis of MKN7, MKN74, KATO III, MKN45, and NUGC4 cells. Following cell lysis, 10 μg protein from each cell line was immunoblotted with antibodies against galectin-4, galectin-3, E-cadherin, β-actin, c-MET, CD44, and CD29 (β1-integrin)

To clarify the role of galectin-4 in gastric cancer cells, we established galectin-4 KO clones with NUGC4 cells. By checking and eliminating clones that expressed galectin-4 by immunostaining the cells, we obtained KO-1a and KO-1b. To obtain clones with different sequences, we transfected them again and selected clones from the final puromycin-surviving cells. KO clones were selected based on galectin-4 mRNA sequence (Fig. S1C) and galectin-4 staining (Fig. S1D). X-ray crystal structure of the N-terminal domain of galectin-4 showed that it is a β-sandwich structure composed of two antiparallel β-sheets. The deleted amino acids form one (KO-2) or two (KO-1a, 1b) β-strands inside the β-sheet of the N-domain. Accordingly, we assume that the protein cannot form a proper structure and may be degraded. We then analyzed the characteristics of clones KO-1a and KO-2 and those of the non-coding crRNA-transfected C1 cells.

The doubling times of KO-1a and KO-2 cells were longer than those of wild-type and C1 cells, suggesting that galectin-4 expression affects cell proliferation (Table S1).

Subsequently, we studied the adhesion of KO cells to the extracellular matrix, which is known to influence the metastatic characteristics of cancer cells [16]. The highest binding of cells was observed for laminin and the lowest for vitronectin. However, no significant differences were observed between KO and galectin-4 expressing cells (Fig. S1E).

Nakashio et al. [13] reported that anti-CD44 monoclonal antibody partially inhibits the binding of NUGC4 cells to mesothelial cells. Because NUGC4 cells express CD44 (Fig. 1), we studied their adhesion to hyaluronate (HA), a known CD44 receptor. Both wild-type and KO-2 cells adhered to the HA-coated wells in a dose-dependent manner; however, their binding abilities toward HA were not significantly different (Fig. S1F). Anchorage-independent growth is a critical factor that influences tumor cell metastasis [17]. We investigated this characteristic using the GILA assay, as growth under this assay condition strongly correlates with colony formation in soft agar assays. Furthermore, the GILA assay can be performed more rapidly and quantitatively, and

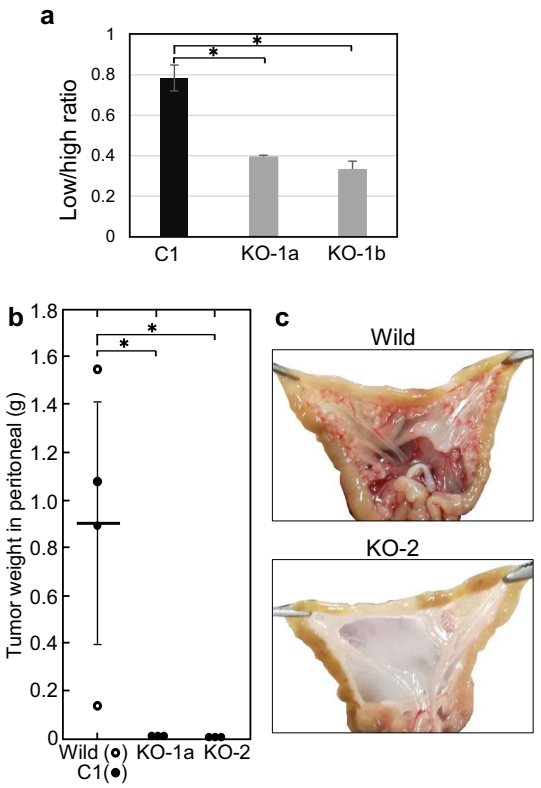

Fig. 2 Analysis of galectin-4 knockout (KO) cells in vitro (**a**) and in vivo (**b**, **c**). **a** GILA assay of NUGC4 control C1 and KO cells. The average low/high ratio of each cell type was acquired from two independent assays. **b** Total tumor weight in the peritoneal cavity. The horizontal line in the middle of the leftmost column shows the average tumor weight of mice inoculated with wild-type (open circle) and control C1 (closed circle) cells, and the accompanying vertical line indicates standard deviation (SD). **c** Macroscopic view of the mesentery of mice inoculated with wild-type and KO-2 cells. $*P < 0.05$

is used to compare cells with different growth rates [10]. We found that KO of galectin-4 significantly suppressed the anchorage-independent growth of NUGC4 cells (Fig. 2a).

KO of galectin-4 attenuates tumor formation of NUGC4 cells

To study whether the expression of galectin-4 affects the ability of peritoneal dissemination in a mouse model, NUGC4 wild-type, C1, KO-1a, and KO-2 cells were intraperitoneally injected into mice. One mouse died on the 38th day after inoculation with C1 cells. Therefore, all the other mice were sacrificed and dissected on day 39. The abdominal cavities of all mice inoculated with galectin-4 KO cells were cancer-free, whereas significant peritoneal dissemination with hemorrhagic ascites was observed in mice inoculated with wild-type and C1 cells expressing galectin-4 (Fig. 2b, c, Table S2). These results strongly suggest that galectin-4 is a critical molecule for tumor formation in NUGC4 cells.

Effect of galectin-4 re-expression on cell proliferation and anchorage-independent growth

To verify the role of galectin-4 in tumor formation, we re-expressed it in KO-2 cells by transfection with a galectin-4 expressing plasmid. Staining with anti-galectin-4 and anti-FLAG-tag antibodies confirmed the re-expression (Fig. S1G). As galectin-4 levels were almost equal in the various clones, we used clone R3 as a rescue cell line for further studies.

Because KO of galectin-4 significantly suppressed the proliferation of NUGC4 cells, we next examined whether re-expression of galectin-4 affected proliferation. The cell doubling time of rescue clone R3 was shorter than that of KO-2 cells; however, it was still longer than that of the wild-type cells (Table S3). The proliferation curves of wild-type, rescue clone R3, and KO-2 cells suggested that the suppression of proliferation in KO cells was partially restored in rescue clone R3 (Fig. 3a).

The GILA assay, which measures anchorage-independent growth, was performed to compare the growth of KO-2 (mock), rescue clone R3, and wild-type cells. KO of galectin-4 suppressed anchorage-independent cell growth up to 28%, whereas rescue of galectin-4 expression in the cells increased growth up to 86% of that of the wild-type cells (Fig. 3b). Results of the GILA assay showed that KO of galectin-4 suppressed the anchorage-independent growth of NUGC4 cells.

Re-expression of galectin-4 partially rescues tumor-forming ability of galectin-4 KO cells

To determine whether re-expression of galectin-4 rescued peritoneal tumor formation in the mouse model, C1, KO-1a, KO-2, and R3 cells were inoculated intraperitoneally. One mouse died on the 27th day after inoculation with C1 cells, and significant peritoneal dissemination was observed in mice inoculated with cells expressing galectin-4 (Fig. 3c, Table S4). All mice inoculated with galectin-4 rescued cells developed tumors; however, their total tumor weight was considerably lower than that of mice inoculated with C1 cells (Fig. 3c).

Identification of molecules differentially expressed between wild-type and KO cells

Next, we sought to identify molecules that were differentially expressed between the wild-type and KO cells.

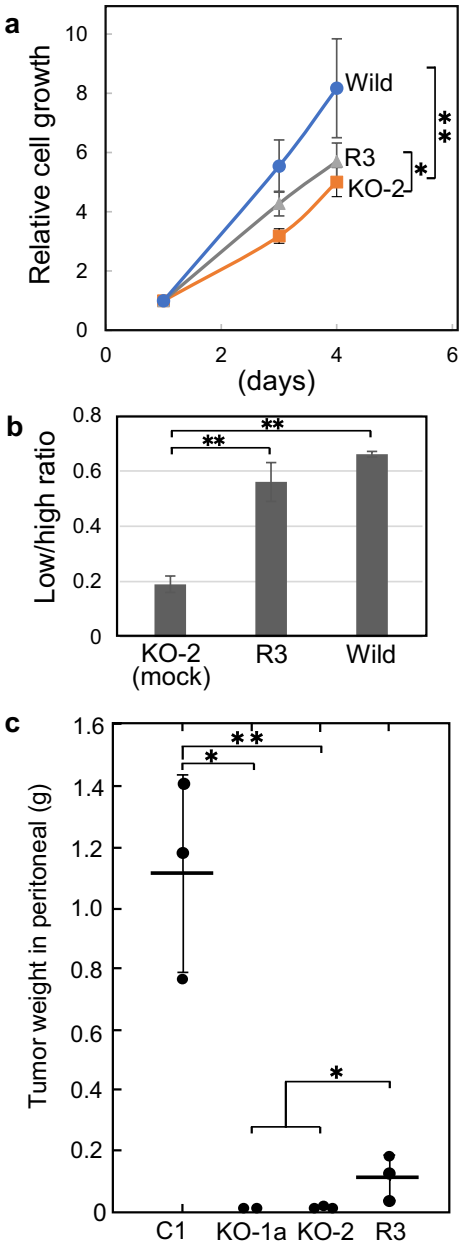

Fig. 3 Analysis of galectin-4 rescue cells in vitro (**a**) and in vivo (**b**). **a** Comparison of growth curves of NUGC4 wild, rescue clone R3, and KO-2 cells. To compare cell proliferation, WST-8 assay was performed on 1, 3, and 4 days after plating of cells. The growth curves were drawn by setting the mean absorption value on day 1 as 1, and values relative to day 1 were plotted. Data are presented as average ± SD of triplicate measurements from three independent experiments. **b** Growth of NUGC4 wild-type, KO-2 (mock), and R3 cells in the low-attachment assay. Bar graphs represent the mean and standard deviation (SD) of the low/high ratios from three independent experiments. **c** Total weight of tumors in the peritoneal cavity. The horizontal line in the middle of the leftmost and rightmost columns shows the average tumor weight of mice inoculated with galectin-4 expressing C1 and R3 cells. The accompanying vertical line indicates SD. $^{*}P < 0.05$, $^{**}P < 0.01$

As treatment with CD44 or β1-integrin antibody [13] and MET inhibitors [18] has been reported to be effective in a mouse model, we compared the expression levels of these molecules in wild-type, rescue, and KO cells.

The expression of c-MET and CD44 was suppressed in KO cells compared to that in galectin-4 expressing cells (Fig. 4a). However, the expression of β1-integrin showed no significant difference between the cells (data available at request). pMET expression was also suppressed in KO cells, and no significant difference was observed in the presence of exogenous HGF (Fig. S1I).

PLA reveals the association of pMET and galectin-4

The expression of pMET (c-MET) has been reported to correlate with the expression of galectin-4. Therefore, we examined whether galectin-4 interacts with pMET (c-MET) using PLA. PLA signals were detected in wild-type (Fig. 4b-1) and control C1 cells (Fig. 4b-2), whereas a large portion of bulk KO cells showed negative non-specific signals (Fig. 4b-3). PLA signals were also detected in a small portion of the bulk KO cells that expressed galectin-4 (Fig. 4b-4).

Strong PLA signals (with pMET antibody) were observed adjacent to the membrane in MKN45 cells (Fig. 4c-1), whereas those with non-immune IgG (control, instead of pMET antibody) were extremely weak (Fig. 4c-2). Incubation of MKN45 cells with NGI-1, an inhibitor of N-glycosylation, resulted in a significant decrease in PLA signals, suggesting that glycosylation is involved in the interaction between pMET and galectin-4 (Fig. 4c-3). Furthermore, PLA signals between galectin-4 and CD44 were detected near the membrane in MKN45 cells (Fig. 4c-4).

Proximity-based labeling of galectin-4 interacting proteins

To identify the molecules that interact with galectin-4 on the cell surface, we labeled MKN45 and NUGC4 cells with SPPLAT (Fig. 5a). Several proteins were biotin-labeled using an anti-galectin-4-HRP antibody. pMET and CD44 were present in the fractions eluted with streptavidin beads, suggesting that both proteins associate with galectin-4 (Fig. 5b). In contrast, the membrane protein CD29 (β1-integrin) was not stained in the eluted fraction. Intracellular proteins, including GAPDH and pAKT, were not specifically stained in the eluted fractions, indicating that the proteins were specifically labeled on the cell surface. More galectin-4 was found in the eluted fraction of MKN45 cells than in that of NUGC4 cells.

When we used galectin-4-HRP instead of anti-galectin-4-HRP, similar proteins (but more) were labeled with biotin. Significantly reduced staining was observed following the incubation of galectin-4-HRP with lactose (Lac) or

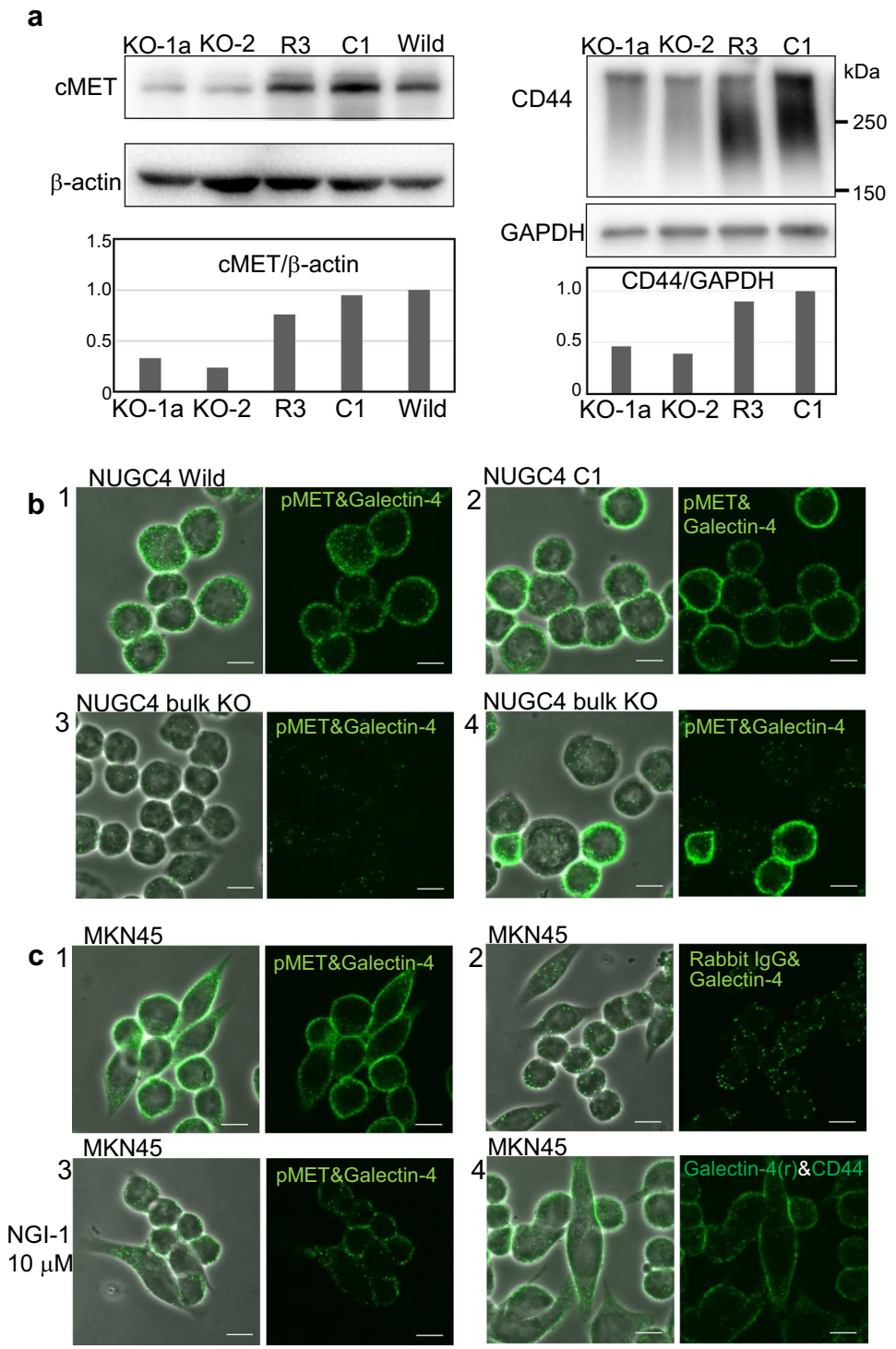

Fig. 4 Profiling of NUGC4 cells with different galectin-4 expression levels. **a** Western blot analysis of KO clones KO-1a, KO-2, rescue clone R3, control C1, and wild-type cells. Following cell lysis, 10 μg of protein from each cell type was immunoblotted with c-MET (left) and CD44 (right) antibodies. Bar graphs represent c-MET and CD44 expression normalized to that of β-actin and GAPDH. **b** PLA in NUGC4 wild-type (1), C1 (2), and bulk KO cells (3,4). A small part of bulk KO cells that showed PLA signals are shown (4). **c** PLA in MKN45 cells (1–4). Positive PLA signals between galectin-4 and pMET are shown in green. The negative control was obtained by substituting the primary pMET antibody with a non-immune rabbit antibody (2). PLA signals in NGI-1-treated cells (3). PLA signals between galectin-4 and CD44 (4). Merged images are also shown (scale bars = 10 μm)

asialofetuin (AF), but not with sucrose (Suc) (Fig. 5c). A small amount of CD44 was still stained in the higher molecular weight fraction in the AF-treated sample. AF binds to galectin-4; however, 25 μM (1 mg/ml) may be insufficient, so faint staining was observed in the AF-treated cells. Interestingly, the higher molecular weight CD44 was still stained in KO clones (Fig. 4a). Higher molecular weight CD44 in MKN45 and NUGC4 cells are

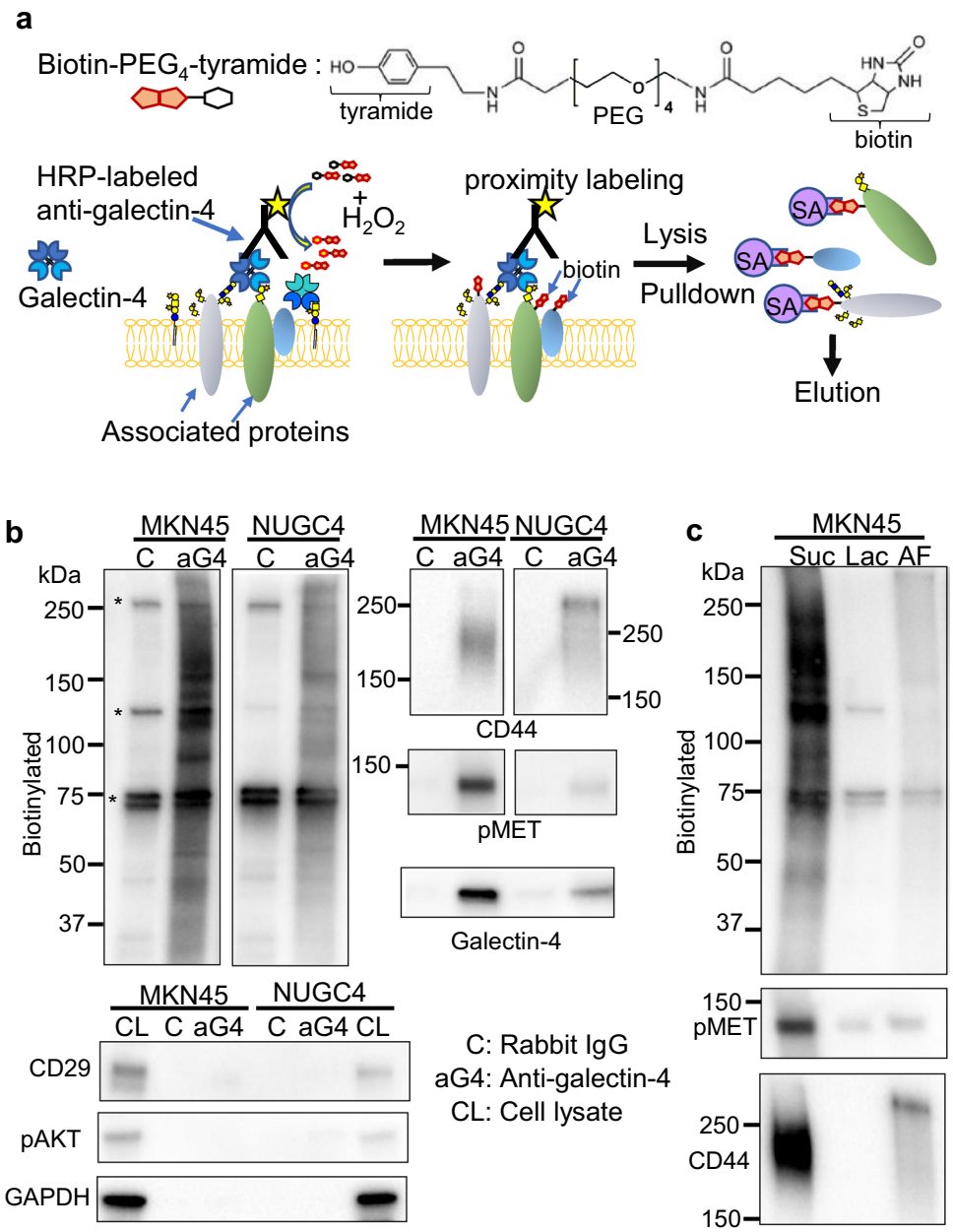

Fig. 5 Analysis of galectin-4-associated proteins. **a** Schema of the SPPLAT protocol. The structure of the biotin-PEG_4-tyramide is shown on top. The anti-galectin-4-HRP enables the proteins in the vicinity of galectin-4 to be biotinylated. Biotinylated proteins were isolated by incubation with streptavidin-Mag-beads (SA). **b** Western blot analysis of MKN45 and NUGC4 cells treated with control rabbit IgG-HRP (C) and anti-galectin-4-HRP (aG4) followed by labeling with biotin-PEG_4-tyramide. The biotinylated proteins were visualized with streptavidin-HRP (upper left panel). A few bands labeled by the control rabbit IgG-HRP (*) may either be authentic avidin-binding proteins or the result of non-specific binding of rabbit IgG. Western blot analysis of biotinylated proteins with pMET (upper right), CD44 (middle right), and galectin-4 antibodies (lower right). Western blot analysis of cell lysates and biotinylated proteins with CD29, pAKT, and GAPDH antibodies (lower panel). **c** Western blot analysis of MKN45 cells treated with galectin-4-HRP plus sucrose (Suc, 50 mM), lactose (Lac, 50 mM), or asialofetuin (AF, 25 μM) followed by labeling with biotin-PEG_4-tyramide. Biotinylated proteins (upper), pMET (middle), and CD44 (lower) were analyzed by western blotting

not necessarily the same; however, they may have a different affinity toward galectin-4 compared to lower molecular weight CD44s.

Galectin-4 KD suppresses the proliferation of gastric cancer cells

We also investigated the effect of galectin-4 KD in MKN45 and NUGC4 cells using siRNA. Galectin-4 siRNA-transfected cells showed decreased levels of galectin-4 protein and suppression of pMET compared with control siRNA-transfected cells (Fig. 6a). The proliferation of galectin-4 siRNA-transfected cells was lower than that of control siRNA-transfected cells, especially in MKN45 cells (Fig. 6b). As shown in Fig. 6c, strong PLA signals were observed adjacent to the membrane in MKN45 cells (Non, non-treated cells). However, decreased PLA signals were observed in galectin-4 siRNA-transfected cells (Gi), indicating that the signals between galectin-4 and pMET are dependent on galectin-4 expression.

Intraperitoneal administration of galectin-4 siRNA suppresses tumor formation by MKN45 cells

Next, we analyzed whether siRNA-mediated KD of galectin-4 suppresses tumor formation in a mouse model.

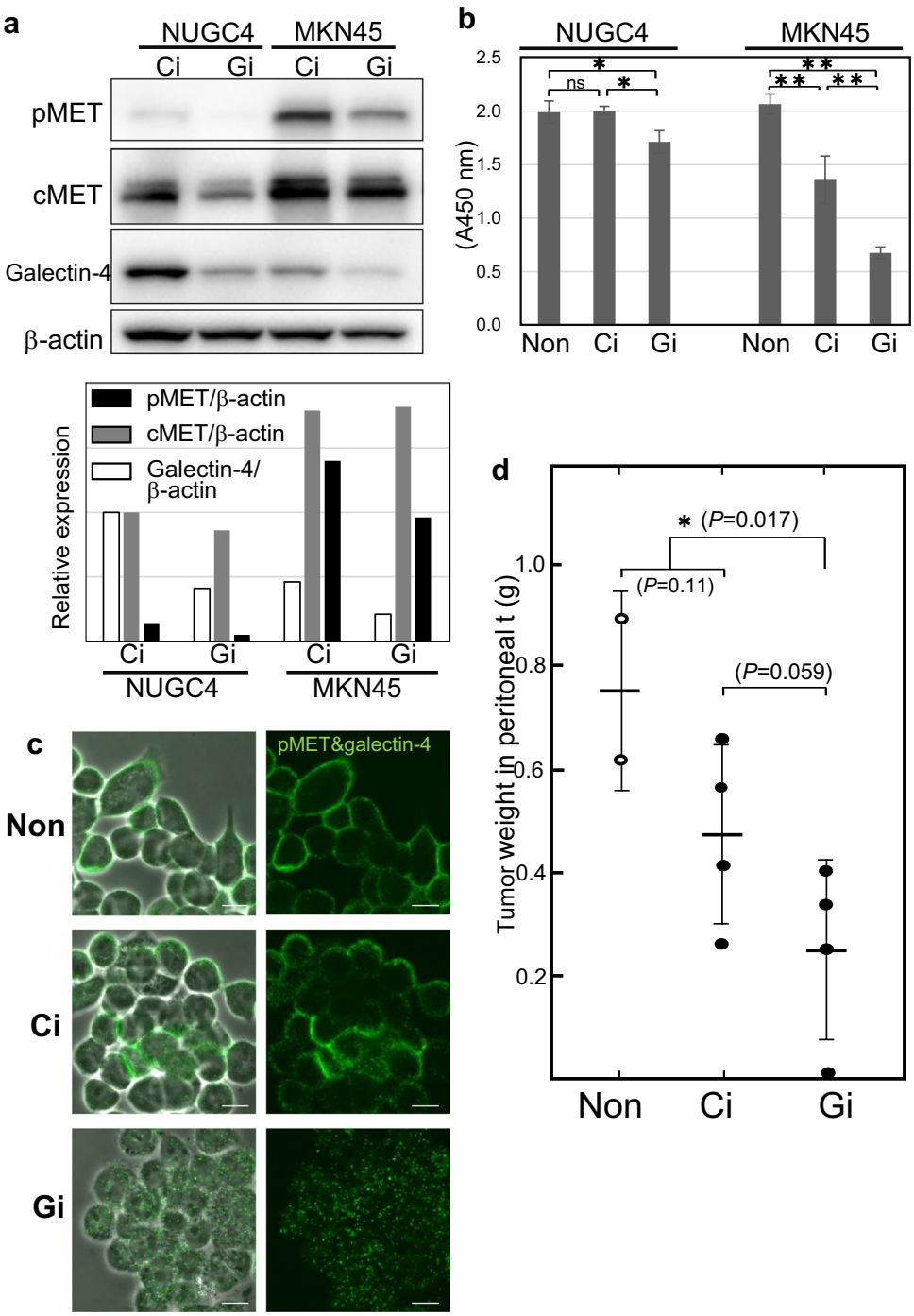

Fig. 6 Analysis of galectin-4 knockdown cells in vitro (**a–c**) and in vivo (**d**). **a** Western blot analysis of siRNA-treated NUGC4 and MKN45 cells with pMET, c-MET, galectin-4, and β-actin antibodies. Bar graphs represent pMET, c-MET and galectin-4 expression normalized to that of β-actin. **b** Effect of galectin-4 knockdown on the proliferation rate of NUGC4 and MKN45 cells. The proliferation of cells was determined by WST-8 assay at 72 h following transfection. **c** PLA of siRNA-treated MKN45 cells. Positive PLA signals between galectin-4 and pMET are shown in green. Reduced PLA signals of galectin-4/pMET complex in galectin-4 siRNA-treated cells (Gi) compared to that in control siRNA-treated (Ci) and non-treated (Non) cells. Merged images are also shown (Scale bars = 10 μm). **d** Galectin-4 siRNA suppresses tumor formation of MKN45 cells. All mice were inoculated with MKN45 cells followed by no treatment (Non), treatment with control siRNA (Ci), or treatment with galectin-4 siRNA (Gi). The horizontal line in the middle of each column represents the average tumor weight of each group, and the accompanying vertical line indicates standard deviation (SD). $*P < 0.05$, $**P < 0.01$

The average tumor weight of mice treated with galectin-4 siRNA (Gi) was significantly lower than that of non-treated (Non) and control siRNA-treated (Ci) mice ($P = 0.017$; Fig. 6d). Although the difference of tumor weight between galectin-4 siRNA-treated (Gi) and control siRNA-treated (Ci) cells was not statistically significant ($P = 0.056$), galectin-4 siRNA treatment tended to suppress tumor weight in the peritoneal cavities.

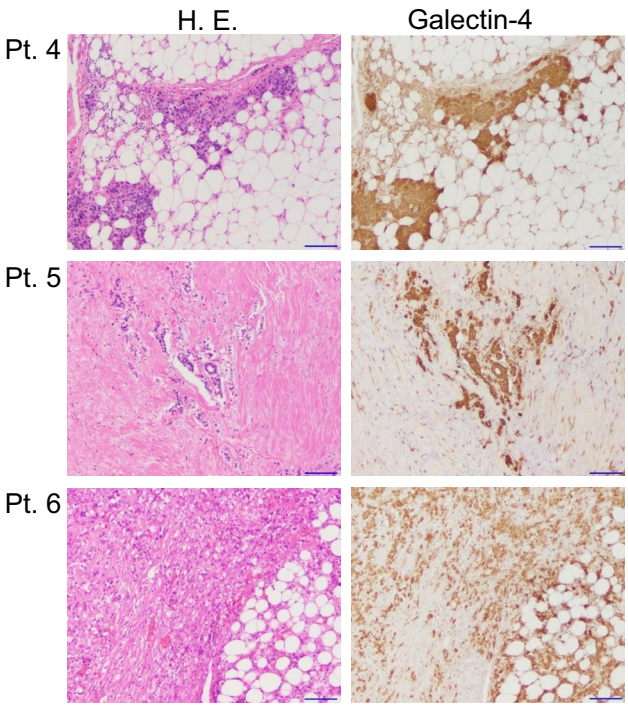

Fig. 7 Representative histological findings (H. E.) and immunohistochemical expression of galectin-4 in the serial sections of peritoneal metastatic tumors from three patients with gastric cancer. Each patient's number (Pt. 4, Pt. 5, and Pt. 6) was according to supplementary Table S5. A scale bar in each panel indicates 100 μm in length

Galectin-4 was expressed in peritoneal metastatic tumors from gastric cancer patients

Expression and subcellular localization of galectin-4 in peritoneal metastatic tumors from 10 patients with gastric cancer (Table S5) were examined by immunohistochemistry. Marked galectin-4 expression was observed in both cytoplasm and nucleus of tumor cells in patients (Fig. 7), suggesting its potential involvement in development of peritoneal metastasis.

Discussion

Using KO and KD approaches, we identified galectin-4 as a critical molecule for the peritoneal dissemination of gastric cancer cells with high metastatic potential. Furthermore, we observed that the expression of galectin-4 affects the proliferation of gastric cancer cells.

The galectin-4 expression does not always correlate with malignancy in different tissues or cancer cell types; for example, the expression of galectin-4 enhanced lymph-node metastasis in lung adenocarcinoma [19] but reduced metastasis in pancreatic adenocarcinoma [20]. Galectin-4 functions as a tumor suppressor in colorectal cancer [12]. The migration and invasion of hepatocellular carcinoma HCCLM3 cells were reduced following overexpression of galectin-4. Conversely, it was upregulated following the KD of galectin-4 in vitro [21]. However, the same group reported that overexpression of galectin-4 promotes the proliferation of HCCLM3 cells in vitro and in vivo [22]. These contradictory results may be partly attributable to differences in the surrounding environmental factors [23] and interacting molecules. Therefore, we studied the biological role of galectin-4 by analyzing its localization and its associated molecules in gastric cancer cells.

Peritoneal dissemination is a multistep process, beginning with the detachment of cancer cells from the primary tumor, transmigration to the abdominal cavity, survival in the cavity prior to attachment to the distant peritoneum, invasion of subperitoneal tissue, and proliferation via neoangiogenesis [24, 25]. All five analyzed gastric cell lines expressed E-cadherin (Fig. 1a). However, cell–cell adhesion through homophilic interactions was not observed in Kato III, NUGC4, and MKN45 cells. Homophilic interactions and attachment to the extracellular matrix and hyaluronate were the same in the wild-type and KO cells (Figs. S1E, F). Our preliminary experiments revealed that NUGC4 cells that express elevated levels of galectin-4 have a lower ability to invade the Matrigel. In hepatocellular carcinoma HCCLM3 cells, overexpression of galectin-4 reduced their ability to invade the Matrigel [21]. Therefore, the ability to invade and adhere to the extracellular matrix in vitro experiments does not always reflect the in vivo behavior of certain cancer cell types and may not be related to galectin-4 expression.

In contrast, galectin-4 KO suppressed cell proliferation, including anchorage-independent proliferation (Table S1A; Figs. 2a, 3a and b). The KD of galectin-4 using siRNA also reduced the proliferation of NUGC4 and MKN45 cells (Fig. 6b), although the same siRNA increased the proliferation of HT29 colon cancer cells [12]. These results suggest that understanding the biological role of galectin-4 is essential to study its expression levels and associated molecules. Our results revealed that galectin-4 KD reduced c-MET activation (Fig. 6a). Furthermore, galectin-4 siRNA treatment decreased the growth of MKN45 cells to a greater extent than that of NUGC4 cells (Fig. 6b). This could be explained by the proliferation of MKN45 cells being highly dependent on the c-MET signaling pathway, as PHA-665752, a selective inhibitor of c-MET signaling, effectively suppressed the proliferation of MKN45 cells, but not that of NUGC4 cells (Fig. S1J).

In this study, we identified c-MET and CD44 as proteins associated with galectin-4, and found that their expression was affected by that of galectin-4. Both proteins are transmembrane glycoprotein receptors associated with diverse cellular functions and tumorigenesis. c-MET-mediated signaling pathways play vital roles in the pathogenesis of

scirrhous gastric cancer [18]. In addition, cancer stem-like cells, which express CD44 at the tumor invasive front, are significantly associated with poor survival in patients with gastric cancer [26]. Both proteins are therapeutic targets that have been extensively studied [27, 28]. c-MET is regulated at various steps, including activation, internalization, and degradation by many molecules [29]. Glycosylation is an essential factor for the regulation of this protein [30]. CD44 is modified by N- and O-linked glycans and has a complex regulatory mechanism [31, 32]. Targeting multiple molecules is an effective strategy in cancer therapy. As galectin-4 affects the expression of several critical molecules as a master regulator in the upstream signaling pathway, suppressing galectin-4 may be effective in treating peritoneal metastasis. Molecules other than c-MET and CD44 may also associate with galectin-4, because several of them were labeled by galectin-4 (Fig. 5b, c). Therefore, we intend to identify these molecules by proteomic analysis in future studies.

Ohtsubo et al. [33] reported that galectin-9 interacts with and stabilizes Glut-2, a glucose transporter, by binding to its glycan portion and regulating its expression. We observed that galectin-4 also interacted with its associated proteins in a carbohydrate-dependent manner (Figs. 4c-3, 5c). Increased galectin-4 staining observed in the eluted fraction of MKN45 cells can be explained by the presence of galectin-4 on the cell surface of MKN45 cells. Therefore, more proteins of MKN45 cells were labeled with anti-galectin-4 than that of NUGC4 cells, although the amount of galectin-4 in cell lysate of MKN45 was less than that in NUGC4 cell lysate (Fig. 1).

Cancer cells acquire abnormal proliferative and metastatic potentials, and changes in cell surface sugar chains occur simultaneously. Therefore, many types of cancer-associated modifications of glycans are used to diagnose cancer [34, 35]. As galectin-4 regulates the trafficking of glycoproteins in enterocyte-like cells [36, 37], we are currently profiling N- and O-linked glycans on the cell surface by comparing gastric cancer cells with varying levels of galectin-4 expression to elucidate the precise mechanism. As galectin-4 specifically binds to certain types of glycosphingolipids [38], we also found a difference between wild-type and KO cells (study in progress).

In this study, we have shown a regulatory role of galectin-4 on c-MET expression in poorly differentiated gastric cancer cells. Using clinical specimens, Ma et al. [39] have shown an association of c-MET with larger tumor size, deeper tumor invasion, presence of lymph-node metastasis, venous invasion, and distant metastasis. Hayashi et al. [19] have reported that galectin-4 is a novel predictor of lymph-node metastasis, using a proteomic approach and immunohistochemical analysis of surgically resected lung adenocarcinoma specimens. Therefore, it is possible that galectin-4 may be involved in lymph-node and hematological metastasis as well as peritoneal metastasis. Importantly, levels of circulating galectins-2, -4, and -8 were increased in patients with colon and breast cancer, particularly in those with metastasis. These galectins' ability to promote cancer cell adhesion to vascular endothelial cells has been shown in vitro, suggesting their metastasis-promoting effect [40]. Similarly, serum levels of galectin-4 were significantly higher in patients with advanced gastric adenocarcinoma [41]. Here, we provide the first demonstration of abundant expression of galectin-4 in the peritoneal metastatic tumors from patients with gastric cancer. Immunostaining reveals that galectin-4 is also localized to the nucleus in metastatic tumor cells. Nuclear localization has also been found in lung adenocarcinomas with lymph-node metastasis [19], suggesting that galectin-4 function in cancer progression may depend on its subcellular localization similar to galectin-3 [42]. Further studies are needed to assess galectin-4 expression in clinical specimens, including metastatic lymph nodes, and hepatic and peritoneal specimens to clarify these issues.

In vivo experiments with KO (Fig. 2b–c, 3b) and KD of galectin-4 (Fig. 6d) demonstrated that galectin-4 is a potential therapeutic target for peritoneal dissemination. However, more effective protocols and/or reagents for suppressing galectin-4 are required to improve its therapeutic effect. In addition, we are investigating whether inhibition of galectin-4 binding is effective for treating peritoneal metastasis of gastric cancer cells.

In conclusion, our results demonstrated that galectin-4 participates in the peritoneal dissemination of malignant gastric cancer cells by promoting cell proliferation and other unknown mechanisms by interacting with several molecules, including c-MET and CD44. This suggests that galectin-4 is a novel upstream regulator of cancer cell signaling. More detailed analyses are needed to understand the precise mechanisms involved in the galectin-4-mediated regulation of associated molecules, especially with respect to glycosylation. Our findings will offer a novel therapeutic approach targeting several molecules in patients with peritoneal dissemination.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All institutional and national guidelines for the care and use of laboratory animals were followed in this study. The Kanazawa University Human Genome and Gene Analysis Research Ethics Committee (Project No. 181) and the Noguchi Institute Research Ethics Committee (Project No. 2201) approved the design and protocols for studying the gastric cancer patients with peritoneal metastasis.

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