Galectin-9 suppresses the proliferation of gastric cancer cells *in vitro*

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Abstract. Gastric cancer is the second-leading cause of cancer-related mortality worldwide, and the prognosis of advanced gastric cancer remains poor. Galectin-9 (Gal-9) is a tandem-repeat-type galectin that has recently been demonstrated to exert anti-proliferative effects on various types of cancer cells. The aim of our present study was to evaluate the effects of Gal-9 on human gastric cancer cells and the expression levels of microRNAs (miRNAs) associated with the antitumor effects of Gal-9 *in vitro*. In our initial experiments, Gal-9 suppressed the proliferation of gastric cancer cell lines *in vitro*. Our data further revealed that Gal-9 increased caspase-cleaved keratin 18 (CCK18) levels in gastric cancer cells. Additionally, Gal-9 reduced the phosphorylation of vascular endothelial growth factor receptor-3 (VEGFR-3) and insulin-like growth factor-1 receptor (IGF-1R). Furthermore, miRNA expression levels were markedly altered with Gal-9 treatment *in vitro*. In conclusion, Gal-9 suppressed the proliferation of human gastric cancer cells by inducing apoptosis. These findings suggest that Gal-9 could be a potential therapeutic target in the treatment of gastric cancer.

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Introduction

Gastric cancer is the second-leading cause of cancer-related mortality worldwide, and the prognosis of advanced gastric cancer remains poor (1). Despite advances in the treatment of gastric cancer, no standard palliative chemotherapy has been accepted for patients with metastatic gastric cancer (2,3). Thus, there is a strong demand for new treatment options to address advanced stages of gastric cancer.

Galectins are classified according to their carbohydrate-recognition domain (CRD) and are subdivided into three groups: prototype galectins (galectins-1, -2, -7, -10, -13, and -14) and the chimera-type galectin (galectin-3) have a single CRD, while tandem-repeat-type galectins (galectins-4, -8, -9, and -12) have two CRDs joined by a flexible peptide linker (4-6). Galectin-9 (Gal-9) is a tandem-repeat-type galectin that is known for its key roles in eosinophil chemoattraction and activation (7-9). Similar to other galectins, Gal-9 regulates various cellular functions, such as aggregation, adhesion and apoptosis (10,11). In recent studies, Gal-9 has been shown to induce apoptosis and thereby suppress cell proliferation and tumor growth in various hematologic malignancies, such as human melanoma (12,13) and chronic myelogenous leukemia (14). Additionally, our recent studies revealed the antitumor effects of recombinant Gal-9 in various solid malignancies, such as hepatocellular carcinoma (15) and cholangiocarcinoma (16).

However, it is unclear whether Gal-9 suppresses gastric cancer cell proliferation. The aim of this study was to determine the effectiveness of Gal-9 against gastric cancer cell proliferation. Possible mechanisms associated with the antitumor effect of Gal-9 were also explored, including the activation of receptor tyrosine kinases, angiogenesis and microRNAs (miRNAs).

Materials and methods

Reagents and chemicals. Recombinant stable and mutant forms of human Gal-9 lacking the entire linker region were expressed and purified as described in our previous

studies (17). Lactose, sucrose, and fetal bovine serum (FBS) were purchased from Wako Chemicals (Osaka, Japan). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan), and all other chemicals were obtained from Sigma Chemical (Tokyo, Japan).

Cell lines and culture. The human gastric cancer cell lines MKN1, MKN7, MKN45, and MKN74 were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The cells were cultured in RPMI-1640 (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS and penicillin-streptomycin (100 μg/ml; Invitrogen) in a humidified atmosphere with 5% CO_2 at 37˚C.

Cell proliferation assay. We performed cell proliferation assays using a CCK-8 according to the manufacturer's instructions. Samples of each cell line ($1x10^4$ cells/well) were seeded into a 96-well plate and cultured in 100 μl of RPMI-1640 supplemented with 10% FBS. Twenty-four hours after seeding, the cells were treated with 0.01, 0.03, 0.1 or 0.3 μmol/l Gal-9 in the culture medium and cultured for an additional 48 h. To inhibit the galactoside binding of Gal-9, 30 mM lactose was added. Sucrose was added as a control. CCK-8 reagent (10 μl) was then added to each well, and the 96-well plate was incubated at 37˚C for 3 h. The absorbance of each well was measured at 450 nm using an auto-microplate reader.

Enzyme-linked immunosorbent assay (ELISA). Cell apoptosis assays were conducted by measuring the amounts of caspase-cleaved keratin-18 (CCK-18) using an M30 Apoptosense ELISA kit (Previva, Bromma, Sweden) (18). Samples of each cell type ($5x10^3$ cells/well) were seeded into a 96-well plate and cultured in 100 μl of culture medium for 24 h. The seeded cells were then treated with 0.3 μmol/l Gal-9. The remaining steps of the assay were carried out according to the manufacturer's instructions. The amounts of antigen in the controls and samples were calculated by interpolation from a standard curve.

Cell and tissue lysates. The preparation of lysates was conducted according to the methods previously described by us (19). All the steps were carried out at 4˚C. Protein concentrations were measured using a dye-binding protein assay based on the Bradford method (20).

Antibody arrays of phosphorylated receptor tyrosine kinases (phospho-RTKs). Human phospho-RTKs were assayed using human phospho-RTK array kits (R&D Systems) according to the manufacturer's instructions. Briefly, phospho-RTK array membranes were blocked with 5% BSA/TBS (0.01 M Tris-HCl, pH 7.6) for 1 h and incubated with 2 ml of cell line lysates after normalization so that the amounts of proteins were equal. After 3 washes for 10 min each with TBS plus 0.1% v/v Tween-20 and 2 washes for 10 min with TBS alone to remove unbound materials, the membranes were incubated with anti-phospho-tyrosine-HRP antibody for 2 h at room temperature. Unbound HRP-conjugated antibody was washed out with 0.1% Tween-20 in TBS. Finally, each array membrane was exposed to X-ray film using a chemiluminescence detection system (Perkin-Elmer Co.).

Angiogenic profile analysis using an antibody array. A RayBio Human Angiogenesis Antibody Array (RayBiotech, Inc.) was used according to the manufacturer's protocol. This method is a dot-based assay enabling the detection and comparison of 20 angiogenesis-specific cytokines. Each array membrane was exposed to X-ray film using a chemiluminescence detection system (Perkin-Elmer Co.).

Analysis of a miRNA microarray. Samples of each cancer cell line were processed for total RNA extraction with an miRNeasy Mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Using an Agilent 2100 Bioanalyzer (Agilent Technologies), the RNA samples typically had $A_{260/280}$ ratios between 1.9 and 2.1.

After measuring RNA levels with an RNA 6000 Nano kit (Agilent Technologies), the samples were labeled using a miRCURY Hy3/Hy5 Power labeling kit and were hybridized to a human miRNA Oligo chip 10, version 19.0 (Toray Industries, Tokyo, Japan). Scanning was conducted with a 3D-Gene Scanner 3000 (Toray Industries). 3D-Gene Extraction version 1.2 software (Toray Industries) was used to read the raw intensities of the images. To determine changes in miRNA expression between the Gal-9-treated and control samples, the raw data were analyzed via GeneSpring GX version 10.0 (Agilent Technologies). The samples were first normalized relative to 28S RNA and baseline-corrected to the median of all the samples.

Replicate data were consolidated into two groups, i.e., those from the galectin-9-treated cells and those from the control cells, and were organized using the hierarchical clustering and ANOVA functions in GeneSpring software. Hierarchical clustering was performed using the clustering function (condition tree) and the Euclidean correlation as a distance metric. A two-way ANOVA was conducted, and asymptotic p-values (<0.05) were determined with no error corrections to identify the miRNAs that varied most prominently across the different groups. Only changes >50% for at least one of the time-points for each sample were considered significant. All the analyzed data were globally normalized. The statistical significance of differentially expressed miRNAs was analyzed using Student's t-test.

Statistical analysis. All analyses were conducted using JMP8.0 (SAS Institute, Cary, NC, USA). Paired analyses between the groups were conducted using Student's t-test. A p<0.05 was considered to indicate a significant difference between groups.

Results

Gal-9 suppresses the proliferation of human gastric cancer cells. To evaluate the effect of Gal-9 on the *in vitro* growth of human gastric cancer cells, we examined the effect of Gal-9 on the proliferation of 4 gastric cancer cell lines, MKN1, MKN7, MKN45 and MKN74. Cells were grown in 10% FBS and treated with 0.01, 0.03, 0.1 or 0.3 μmol/l Gal-9 or without Gal-9 as a control. The cell proliferation assay was conducted 48 h after the addition of the agents. As depicted in Fig. 1, treatment with Gal-9 led to a strong, dose-dependent inhibition of cell proliferation in the MKN74 and MKN45 cells, which are Gal-9-sensitive gastric cancer cell lines (Fig. 1). However,
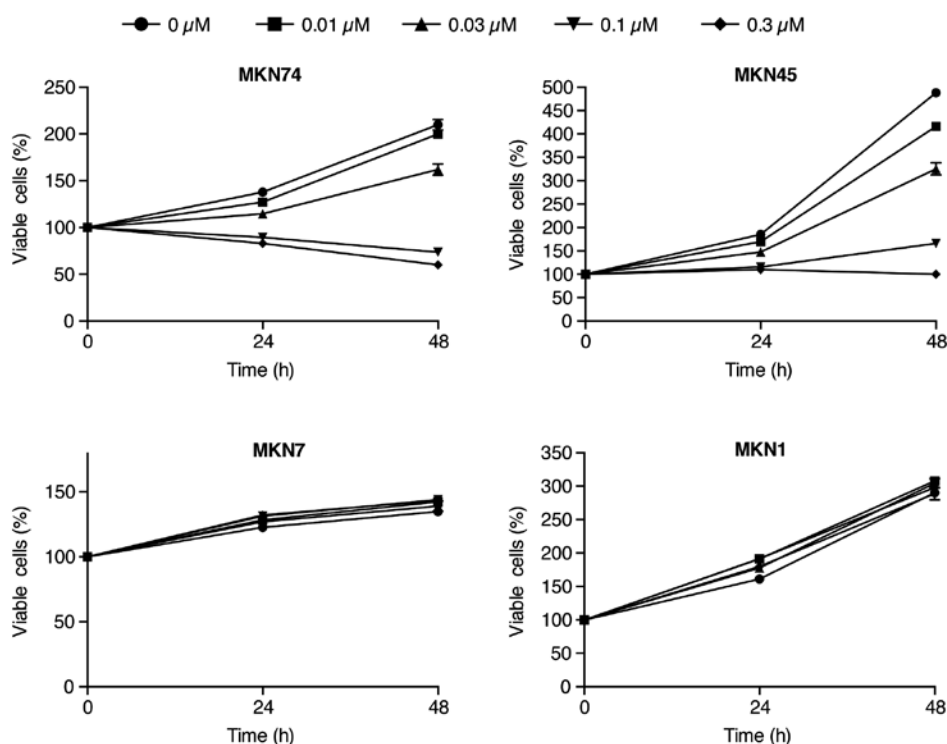
Figure 1. Galectin-9 (Gal-9) inhibits the proliferation of gastric cancer cell lines. MKN45, MKN74, MKN1, and MKN7 cells were seeded at 10,000 cells/well in 96-well plates, and Gal-9 (0.01, 0.03 0.1 or 0.3 μM) was added at time 0 h. A Cell Counting Kit assay was conducted daily from time 0 to 48 h. The mean cell number from three independent cultures is shown. The results are expressed as percentages of viable cells compared with the control. In the MKN45 and MKN74 cells, the conditions at 24 and 48 h in the cells treated with Gal-9 were significantly different compared with those in the control.
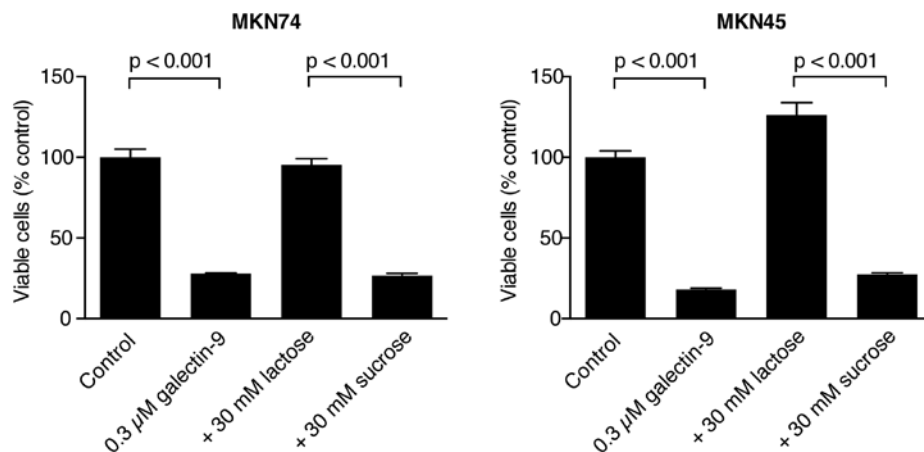

Figure 2. The antagonistic effect of lactose against galectin-9 (Gal-9) was assessed using a Cell Counting Kit assay. Cells were incubated with or without 30 mM lactose in addition to 0.3 μM Gal-9 for 48 h. The effect of Gal-9 was antagonized by lactose, suggesting that the β-galactoside-binding nature of Gal-9 is essential for the activity. The p-values were calculated relative to the control.

no anti-proliferative effects of Gal-9 were detected in the MKN7 and MKN1 cells (Fig. 1).

The anti-proliferative effect of Gal-9 is inhibited by lactose in Gal-9-sensitive gastric cancer cells. The growth of the MKN74 and MKN45 cells was inhibited by Gal-9 with 30 mM sucrose but not with 30 mM lactose (Fig. 2). These data suggest that the binding of β-galactoside is essential for Gal-9 to inhibit the proliferation of the MKN74 and MKN45 cells, which are Gal-9-sensitive gastric cancer cells.

Gal-9 induces apoptosis in Gal-9-sensitive gastric cancer cells (MKN74 cells) but not Gal-9-resistant cells (MKN7 cells). To determine the mechanism(s) of the Gal-9 anti-proliferative effects, the level of caspase-cleaved cytokeratin 18 (CCK18), which is specifically produced during apoptosis, was measured using ELISAs in gastric cancer cells treated with 0.3 μM Gal-9. Remarkably, Gal-9 increased the levels of CCK18 in Gal-9-sensitive cells (MKN74 cells) but not in Gal-9-resistant cells (MKN7 cells) (Fig. 3). This result suggests that apoptosis is involved in Gal-9-induced cytostasis in the Gal-9-sensitive cells.
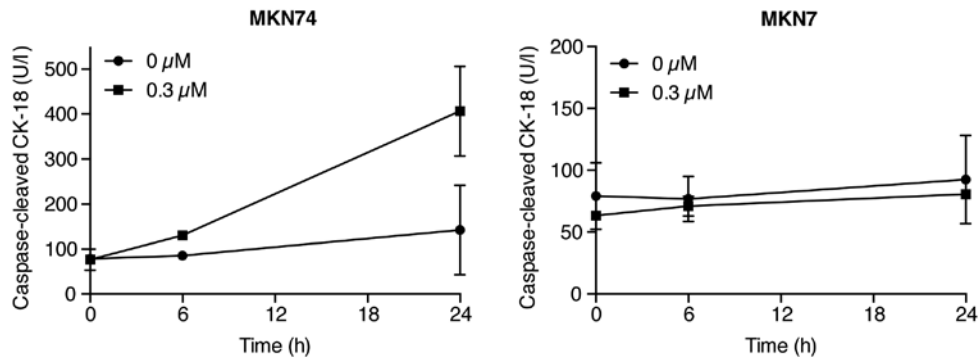
Figure 3. Galectin-9 (Gal-9) induced apoptosis in the MKN74 cells. Caspase-cleaved keratin 18 (CCK18) produced specifically in apoptosis was determined with an enzyme-linked immunosorbent assay (ELISA). Cells were incubated with or without 0.3 μM Gal-9. Culture supernatants were used for the assay. The study was performed in triplicate.
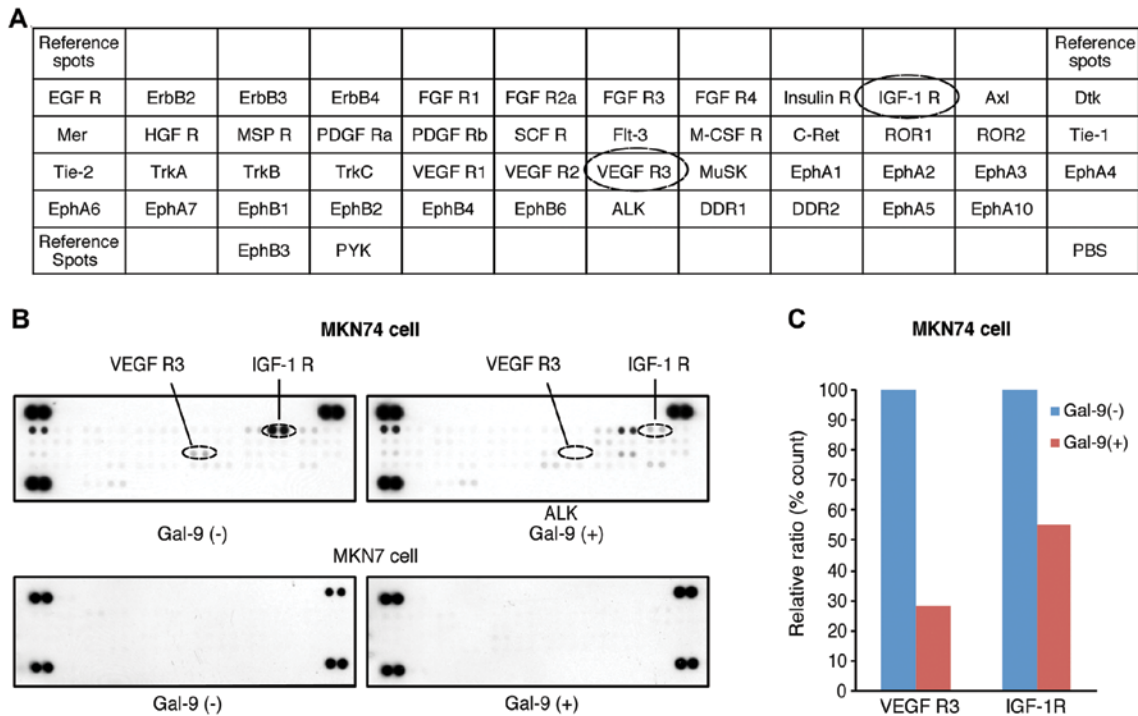

Figure 4. Effects of galectin-9 (Gal-9) on phospho-RTKs in MKN74 and MKN7 cells. (A) Template showing the location of tyrosine kinase antibody spotted onto human Phospho-RTK array. (B) Representative expression levels of various phospho-RTKs in MKN7 and MKN74 cells treated with or without Gal-9. Reduced expression levels of VEGFR and IGF-1R were detected in the MKN74 cells treated with Gal-9. (C) Densitometry analysis indicated that the ratios of the p-VEGFR-3 and p-IGF-1R spots of the Gal-9-treated cells to those of the untreated cells were 28.2 and 55.1%, respectively.

Differences in phospho-RTKs in Gal-9-sensitive versus Gal-9-resistant gastric cancer cells treated with and without Gal-9. We next used a phospho-RTK array system to identify the key receptor tyrosine kinases (RTKs) associated with the anti-proliferative effects of Gal-9. Using this antibody array (Fig. 4A), we simultaneously screened the expression levels of 42 different RTKs activated in MKN74 and MKN7 cells with or without 0.3 μM Gal-9. Gal-9 reduced the expression levels of vascular endothelial growth factor receptor-3 (VEGFR-3) and phosphorylated insulin-like-growth factor-1 receptor (IGF-1R) in the MKN74 cells (Fig. 4B). In contrast, no activated RTKs were upregulated in the Gal-9-resistant MKN1 cells.

Densitometric analyses revealed that the ratios of the p-VEGFR-3 and p-IGF-1R spots of the Gal-9-treated cells to those of the untreated cells were 28.2 and 55.1%, respectively (Fig. 4C).

Effects of Gal-9 on angiogenesis in Gal-9-sensitive versus Gal-9-resistant gastric cancer cells. To examine the relationship between angiogenesis and Gal-9, an angiogenesis antibody array analysis was conducted regarding the anti-tumor effects of Gal-9 (Fig. 5A). Using the antibody array, we simultaneously screened the expression levels of 20 different angiogenesis-related proteins in MKN7 and MKN74 cells with or without Gal-9. The expression levels of interleukin-8 (IL-8), tissue inhibitor of metalloproteinase-1 (TIMP-1), TIMP-2, growth-related oncogene (GRO) and regulated-on-activation normal-T-cell-expressed and secreted (RANTES) were induced by Gal-9 treatment in the MKN74 cells as detected by
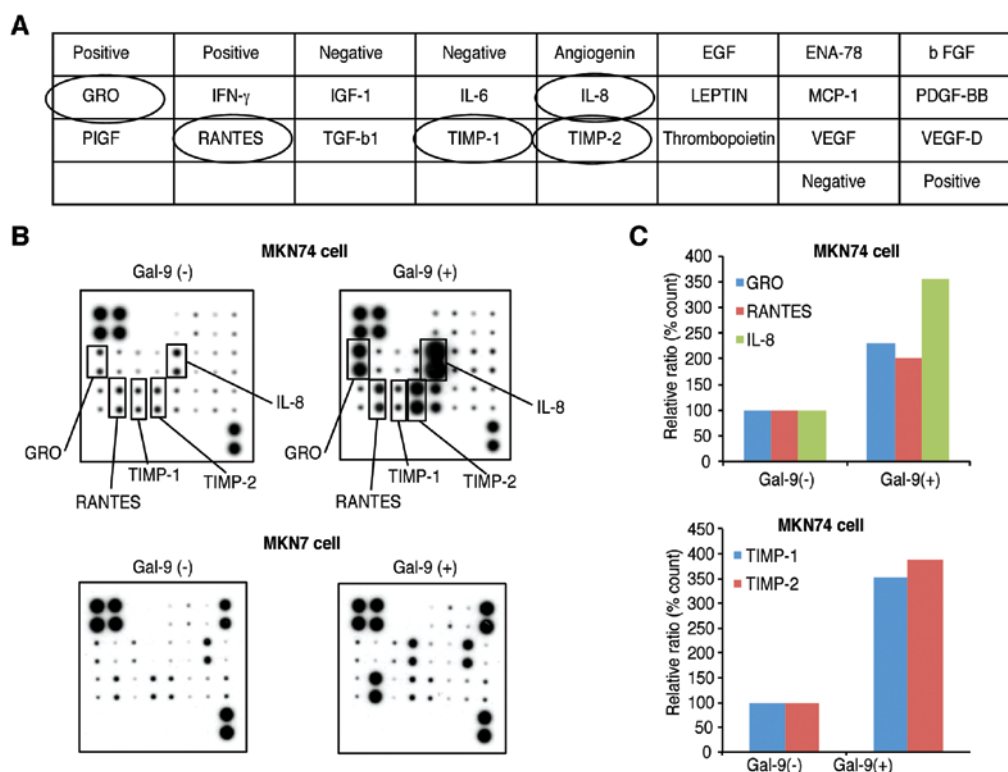
Figure 5. Effects of galectin-9 (Gal-9) on angiogenesis in the MKN74 and MKN7 cells. (A) Template depicting the location of antibodies for angiogenesis-related proteins spotted onto a human angiogenesis array. (B) Representative expression levels of various antibodies for angiogenesis-related proteins in the MKN7 and MKN74 cells treated with or without Gal-9. Increased expression levels of IL-8, TIMP-1, TIMP-2, GRO, and RANTES were detected in the MKN74 cells treated with Gal-9. (C) Densitometry analysis indicated that the ratios of the IL-8, TIMP-1, TIMP-2, GRO, and RANTES spots of the Gal-9-treated cells to those of the untreated cells were 356.1, 355.3, 389.36, 231.1 and 201.0%, respectively.

the protein array (Fig. 5B). In the Gal-9-resistant MKN1 cells, no altered expression of angiogenesis molecules was detected with Gal-9 treatment.

Densitometric analyses indicated that the ratios of the IL-8, TIMP-1, TIMP-2, GRO, and RANTES spots of the Gal-9-treated cells to those of the untreated cells were 356.1, 355.3, 389.36, 231.1 and 201.0%, respectively (Fig. 5C).

miRNA profiles of the cell lines treated in vitro with or without Gal-9. Using a custom microarray platform, we analyzed the *in vitro* expression levels of 2,555 human miRNA probes in gastric cancer cell lines that were treated with or without Gal-9. As presented in Table I, when the expression levels of miRNAs were measured in the MKN74 cells treated *in vitro* with or without 0.3 μmol/l Gal-9, 153 miRNAs were significantly upregulated 24 h after the Gal-9 treatment, whereas 18 miRNAs were downregulated.

An unsupervised hierarchical clustering analysis and a Pearson's correlation analysis indicated that MKN74 cells treated *in vitro* with Gal-9 clustered together and separately from the untreated MKN74 cells (Fig. 6).

Discussion

Gastric cancer is a very common disease worldwide and is the second most frequent cause of cancer-related deaths (21,22). Although the overall incidence and mortality have dramatically declined over the past few decades, gastric cancer remains a major health problem (23). More than half of gastric

cancer patients have lymph node metastasis when initially diagnosed or after surgical resection, which results in a poor prognosis (24-26). Patients with advanced stages of gastric cancer are treated with chemotherapy and radiation, either singly or in combination. However, none of these therapies is fully curative. Therefore, new therapeutic strategies are urgently required for the treatment of advanced gastric cancer. In recent studies, higher levels of Gal-9 expression were observed in patients without lymph-vascular invasion, lymph node metastases or distant metastases, and Gal-9 expression is closely associated with better survival rates in gastric cancer (27). Additionally, the downregulation of Gal-9 mRNA levels was observed in gastric cancer tissues; therefore, the loss of Gal-9 expression may be involved in the progression of gastric cancers (28). In the present study, we found that Gal-9 inhibited the proliferation of gastric cancer cell lines. In addition, we identified miRNAs associated with the antitumor effect of Gal-9 in gastric cancer.

Recombinant Gal-9 inhibits proliferation in various cancers such as melanoma (13) and chronic myelogenous leukemia (14) by inducing apoptosis. Our present results revealed that Gal-9 suppresses the proliferation of human gastric cancer cell lines *in vitro*. Gal-9 strongly inhibited cell proliferation in a dose-dependent manner in MKN45 and MKN74 cells but not in MKN1 and MKN7 cells. Notably, the histological type of the MKN74 and MKN7 cell lines is intestinal, while the MKN45 cells are a diffuse type, and the MKN1 cells are an adenosquamous carcinoma (29). Furthermore, the MKN7 and MKN1 cells were established from metastatic foci in the

Table I. Changes in expression compared with untreated cells and chromosomal locations of miRNAs in MKN74 cells treated with galectin-9.

miRNA	Fold (treated/control) means ± SD	P-value	Chromosomal localization
Upregulated			
hsa-miR-204-3p	12.41±3.011	0.00023	9q21.12
hsa-miR-4688	11.27±2.983	0.00032	11
hsa-miR-4731-5p	10.05±4.236	0.00091	17
hsa-miR-4459	8.36±2.17	0.00016	5
hsa-miR-4730	7.78±2.432	0.00406	17
hsa-miR-4294	7.54±3.654	0.00164	10
hsa-miR-3196	7.39±2.829	0.00191	20
hsa-miR-3648	7.32±1.06	0.00049	21
hsa-miR-4687-3p	6.74±1.938	0.00011	11
hsa-miR-3197	6.46±1.153	0.00408	21
hsa-miR-4530	6.35±0.738	0.00058	19
hsa-miR-638	6.33±1.676	0.0023	19p13.2
hsa-miR-1908	6.31±1.595	0.00116	11
hsa-miR-4442	6.28±1.781	0.00281	3
hsa-miR-4488	6.19±1.839	0.00057	11
hsa-miR-6125	6.16±0.699	0.00048	12
hsa-miR-4787-5p	5.98±1.567	0.00031	3
hsa-miR-6088	5.79±1.124	0.00091	19
hsa-miR-4466	5.78±1.562	0.00633	6
hsa-miR-3621	5.6±1.271	0.0008	9
hsa-miR-1237-5p	5.6±1.902	0.00142	11
hsa-miR-3940-5p	5.54±1.068	0.00106	19
hsa-miR-1227-5p	5.52±1.785	0.01023	19
hsa-miR-4792	5.49±1.065	0.0012	3
hsa-miR-4508	5.41±0.595	0.00019	15
hsa-miR-3665	5.4±1.682	0.00066	13
hsa-miR-2861	5.38±1.946	0.00057	9
hsa-miR-6126	5.36±1.517	0.00735	16
hsa-miR-5787	5.29±0.798	0.00049	3
hsa-miR-3656	5.15±1.083	0.00053	11
hsa-miR-6075	5.14±1.757	0.00036	5
hsa-miR-1246	5.08±1.896	0.03458	2q31.1
hsa-miR-1229-5p	4.92±1.854	0.00022	5
hsa-miR-4667-5p	4.84±0.891	0.00294	9
hsa-miR-4756-5p	4.68±4.77	0.00762	20
hsa-miR-6132	4.63±0.546	0.00712	7
hsa-miR-1268a	4.63±0.402	0.00071	15q11.2
hsa-miR-6085	4.28±1.456	0.00273	15
hsa-miR-4484	4.17±1.354	0.01331	10
hsa-miR-652-5p	4.15±0.76	0.001	Xq23
hsa-miR-4800-5p	4.1±0.03	0.02576	4
hsa-miR-6131	4.08±0.446	0.02093	5
hsa-miR-6724-5p	4.07±1.875	0.0041	21
hsa-miR-4745-5p	4.04±0.883	0.01705	19
hsa-miR-642a-3p	4±0.901	0.0114	19q13.32
hsa-miR-1973	3.97±0.422	0.00364	4
hsa-miR-663a	3.88±0.336	0.00185	20p11.1

Table I. Continued.

miRNA	Fold (treated/control) means ± SD	P-value	Chromosomal localization
Upregulated			
hsa-miR-4749-5p	3.87±0.722	0.00053	19
hsa-miR-4741	3.8±0.168	0.00361	18
hsa-miR-1469	3.8±0.652	0.00034	15q26.2
hsa-miR-1228-5p	3.8±0.564	0.00047	12
hsa-miR-4463	3.78±0.131	0.00074	6
hsa-miR-4505	3.73±0.418	0.00081	14
hsa-miR-1915-3p	3.72±1.571	0.00219	10p12.31
hsa-miR-4763-3p	3.7±0.411	0.00186	22
hsa-miR-491-5p	3.69±0.866	0.00014	9p21.3
hsa-miR-4651	3.66±0.884	0.00136	7
hsa-miR-4435	3.62±1.308	0.0397	2
hsa-miR-1275	3.62±1.031	0.00237	6
hsa-miR-3180	3.53±1.989	0.00998	16
hsa-miR-31-5p	3.44±1.15	0.00019	9p21.3
hsa-miR-1909-3p	3.34±0.825	0.00498	19p13.3
hsa-miR-3620-5p	3.3±0.472	0.00182	1
hsa-miR-149-3p	3.21±0.307	0.00355	2q37.3
hsa-miR-4725-3p	3.18±0.711	0.00036	17
hsa-miR-132-3p	3.17±2.048	0.00242	17p13.3
hsa-miR-1587	3.13±0.557	0.0024	Xp11.4
hsa-miR-4327	3.12±0.843	0.0008	21
hsa-miR-4726-5p	3.12±0.454	0.00084	17
hsa-miR-1247-3p	3.11±1.069	0.02297	14q32.31
hsa-miR-486-3p	3.1±0.267	0.01475	8p11.21
hsa-miR-1268b	3.1±0.561	0.00049	17
hsa-miR-4497	3.08±0.395	0.00092	12
hsa-miR-642b-3p	3.06±0.565	0.00667	19
hsa-miR-937-5p	3.06±0.337	0.005	8q24.3
hsa-miR-4728-5p	3.03±1.161	0.04874	17
hsa-miR-365a-5p	3.03±0.216	0.00296	16p13.12
hsa-miR-3180-3p	3.01±0.606	0.00032	16
hsa-miR-675-5p	2.97±1.027	0.02744	11p15.5
hsa-miR-4656	2.89±0.275	0.00358	7
hsa-miR-4270	2.83±0.615	0.01617	3
hsa-miR-5001-5p	2.83±0.452	0.01792	2
hsa-miR-4257	2.81±0.263	0.00051	1
hsa-miR-4450	2.79±1.073	0.01547	4
hsa-miR-4507	2.79±0.356	0.01068	14
hsa-miR-3937	2.77±1.063	0.00578	X
hsa-miR-513a-5p	2.74±0.442	0.023	Xq27.3
hsa-miR-1202	2.7±0.082	0.00084	6
hsa-miR-760	2.69±0.634	0.0243	1p22.1
hsa-miR-4638-3p	2.67±0.394	0.01986	5
hsa-miR-5585-3p	2.67±1.341	0.00679	1p35.2
hsa-miR-671-5p	2.66±0.892	0.00623	7q36.1
hsa-miR-4655-5p	2.65±0.38	0.00198	7
hsa-miR-212-3p	2.64±0.448	0.02006	17p13.3
hsa-miR-4299	2.56±1.727	0.01568	11
hsa-miR-4706	2.54±0.781	0.00115	14

Table I. Continued.

miRNA	Fold (treated/control) means ± SD	P-value	Chromosomal localization
Upregulated			
hsa-miR-132-5p	2.52±0.469	0.01826	17p13.3
hsa-miR-4722-5p	2.49±0.116	0.01955	16
hsa-miR-3135b	2.48±0.096	0.00061	6
hsa-miR-4732-5p	2.48±0.216	0.00737	17
hsa-miR-4758-5p	2.46±0.487	0.0002	20
hsa-miR-2392	2.4±0.749	0.01092	14
hsa-miR-4449	2.37±0.582	0.04587	4
hsa-miR-29a-3p	2.36±0.685	0.00438	7q32.3
hsa-miR-6722-3p	2.34±0.282	0.00175	9
hsa-miR-3714	2.31±0.186	0.03306	3
hsa-miR-744-5p	2.3±1.104	0.02676	17p12
hsa-miR-5006-5p	2.23±0.694	0.01224	13
hsa-miR-3141	2.22±0.677	0.00299	5
hsa-miR-1185-1-3p	2.21±0.698	0.03128	14
hsa-miR-4436b-5p	2.19±0.712	0.01295	2
hsa-miR-4417	2.14±0.536	0.00643	1
hsa-miR-5196-5p	2.13±0.42	0.00448	19
hsa-miR-296-5p	2.12±0.383	0.00725	20q13.32
hsa-miR-6722-5p	2.11±0.255	0.01874	9
hsa-miR-614	2.09±0.597	0.00179	12p13.1
hsa-miR-3678-3p	2.08±0.805	0.04442	17
hsa-miR-1914-3p	2.03±0.474	0.02587	20q13.33
hsa-miR-4640-5p	2.03±0.371	0.03783	6
hsa-miR-4498	2.03±0.522	0.04462	12
hsa-miR-194-5p	1.97±0.588	0.00849	1q41
hsa-miR-29b-1-5p	1.97±0.34	0.00444	7q32.3
hsa-miR-4695-5p	1.95±0.352	0.00097	1
hsa-miR-4707-5p	1.93±0.2	0.04286	14
hsa-miR-4690-5p	1.92±0.098	0.01814	11
hsa-miR-92b-5p	1.91±0.499	0.00537	1q22
hsa-miR-4465	1.9±0.148	0.00753	6
hsa-miR-3679-3p	1.88±0.185	0.00101	2
hsa-miR-711	1.87±0.536	0.0124	3
hsa-miR-3185	1.84±0.161	0.01036	17
hsa-miR-192-5p	1.83±0.464	0.02096	11q13.1
hsa-miR-370	1.82±0.547	0.02131	14q32.2
hsa-miR-658	1.81±0.577	0.03598	22q13.1
hsa-miR-222-3p	1.76±0.614	0.01237	Xp11.3
hsa-miR-3195	1.71±0.131	0.02378	20
hsa-miR-557	1.67±0.415	0.01678	1q24.2
hsa-miR-221-3p	1.59±0.406	0.03976	Xp11.3
hsa-miR-4634	1.54±0.367	0.04868	5
hsa-miR-1225-5p	1.52±0.189	0.03676	16p13.3
hsa-miR-3609	1.52±0.002	0.0084	7
hsa-miR-30c-1-3p	1.48±0.065	0.00171	1p34.2
hsa-miR-6515-3p	1.45±0.106	0.0161	19
hsa-miR-21-3p	1.44±0.343	0.02759	17q23.1
hsa-miR-3144-5p	1.44±0.124	0.00824	6
hsa-miR-22-3p	1.41±0.028	0.01301	17p13.3

Table I. Continued.

miRNA	Fold (treated/control) means ± SD	P-value	Chromosomal localization
Upregulated			
hsa-miR-622	1.38±0.072	0.01576	13q31.3
hsa-miR-4650-3p	1.38±0.096	0.00266	7q11.21
hsa-miR-877-5p	1.38±0.156	0.02297	6p21.33
hsa-miR-660-5p	1.3±0.247	0.03583	Xp11.23
hsa-miR-3907	1.25±0.128	0.01567	7
hsa-miR-3161	1.24±0.009	0.00212	11
hsa-miR-4714-3p	1.24±0.04	0.04315	15
hsa-miR-532-5p	1.22±0.135	0.01241	Xp11.23
Downregulated			
hsa-miR-30d-3p	0.36±0.137	0.01586	8q24.22
hsa-miR-4785	0.44±0.144	0.04552	2
hsa-miR-4521	0.46±0.185	0.02384	17
hsa-miR-4722-3p	0.5±0.171	0.03034	16
hsa-miR-4683	0.56±0.089	0.01651	10
hsa-miR-200b-5p	0.58±0.137	0.01679	1p36.33
hsa-miR-345-5p	0.6±0.19	0.04389	14q32.2
hsa-miR-590-5p	0.6±0.077	0.00326	7q11.23
hsa-miR-103a-2-5p	0.61±0.116	0.0355	20p13
hsa-miR-761	0.65±0.017	0.0427	1
hsa-miR-5692c	0.65±0.132	0.01502	5
hsa-miR-299-3p	0.68±0.02	0.03763	14q32.31
hsa-miR-126-5p	0.69±0.079	0.00758	9q34.3
hsa-miR-4301	0.7±0.152	0.03201	11
hsa-miR-4522	0.72±0.161	0.03506	17
hsa-miR-627	0.74±0.034	0.00686	15q15.1
hsa-miR-1323	0.75±0.066	0.04283	19q13.42
hsa-miR-2681-5p	0.76±0.055	0.03387	13

miRNAs, microRNAs; SD, standard deviation.

lymph nodes (29,30), whereas the MKN74 and MKN45 cells were derived from liver metastases (29,30). Accordingly, these data suggest that the liver metastatic type of gastric cancer cells (MKN74 and MKN45 cells) are more sensitive to Gal-9 treatment compared with the lymph node metastatic type of cells (MKN7 and MKN1 cells), regardless of their respective histological differentiation. These results suggest that Gal-9 may be a potential targeting moiety useful in distant metastasis in the advanced stages of gastric cancer.

The cleavage of cytokeratin by caspase (CCK18) occurs as an early event during apoptosis following the activation of apoptosis executioners, particularly effector caspases. However, cytokeratin remains intact during other types of cell death, such as autophagy or necrosis (31). In the present study, Gal-9 increased the levels of CCK18 in the MKN74 cells, which are sensitive to Gal-9, but not the MKN1 cells, which are resistant to Gal-9. These data indicate that Gal-9
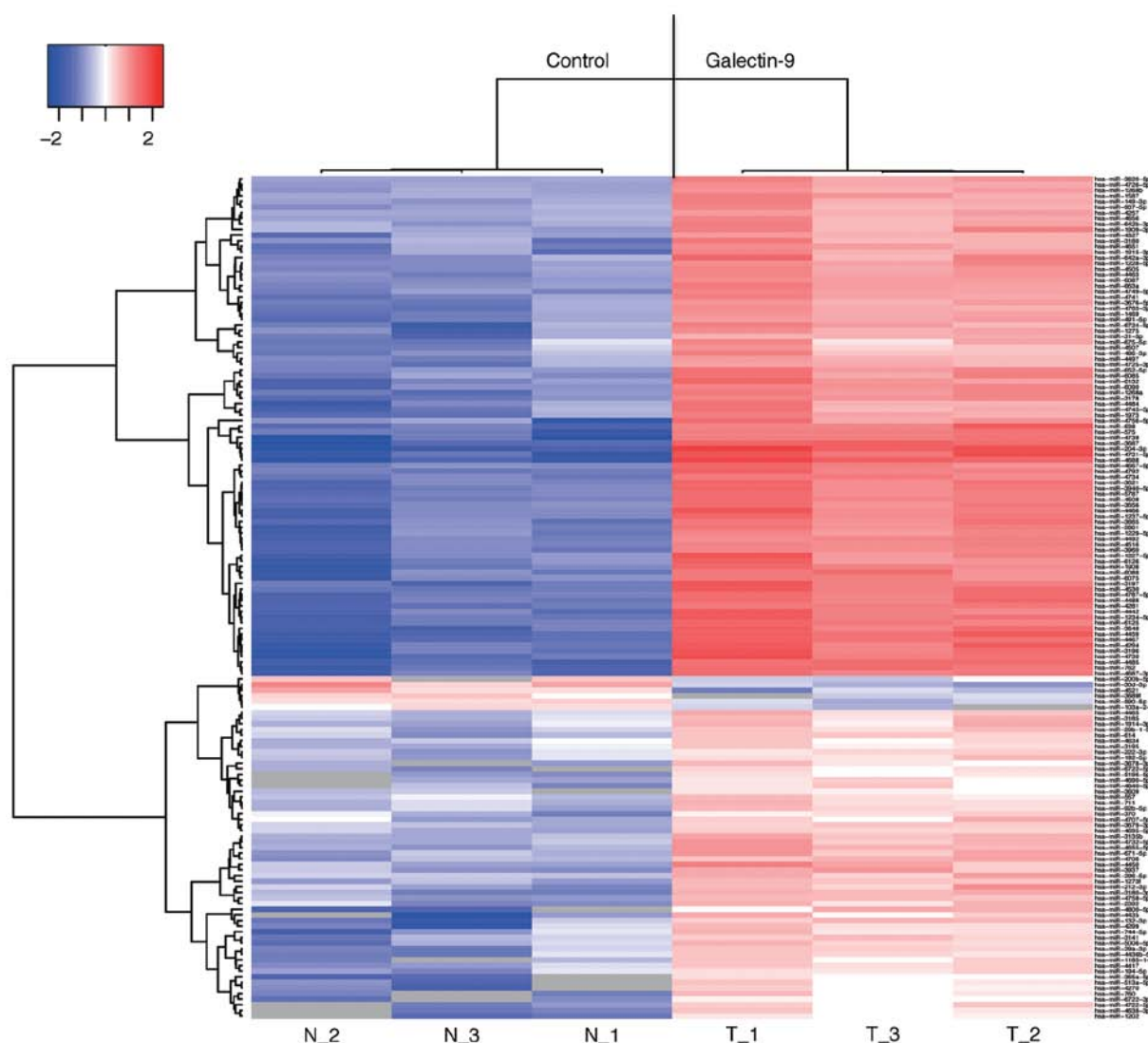
Figure 6. Hierarchical clustering of the MKN74 cells with or without galectin-9 (Gal-9) treatment. MKN74 cells were clustered according to the expression profiles of 171 miRNAs that were differentially expressed between the treated and untreated MKN74 cells. The analyzed samples are in columns, and the miRNAs are presented in rows. The miRNA clustering tree is shown on the left, and the sample-clustering tree is on the top. The color scale on the top illustrates the relative expression levels of miRNAs; red represents a high expression level, and blue represents a low expression level.

suppresses the proliferation of Gal-9-sensitive gastric cancer cells by inducing apoptosis.

Since the discovery of these proteins, RTKs have been investigated as key regulators of the proliferation, differentiation, and metastasis of gastric cancer cells (32). Our present study used phospho-RTK arrays to demonstrate that Gal-9 treatment reduced the expression levels of IGF-1R and VEGFR-3 in Gal-9-sensitive cells (MKN74) when compared with those in Gal-9-resistant cells (MKN7). IGF-1R is involved in cell proliferation and the prevention of apoptosis (32,33). Galamb *et al* found via immunohistochemistry that IGF-1R was overexpressed in gastric cancer tissues when compared with normal mucosa (34). Additionally, the mRNA expression levels tended to be higher in cancer tissues than in normal mucosa (35). The overexpression of IGF-1R is associated with a poor response to chemotherapy and poor outcomes in stage I-IV gastric cancers (36). Gal-9 may reduce the expression levels of phospho-IGF-1R in gastric cancer and thereby improve chemosensitivity and the disease prognosis.

Galectins are important regulators of tumor progression that influence tumor cell transformation, immune escape and angiogenesis (4-6). Using angiogenesis-related protein arrays, we observed that IL-8, GRO, TIMP-1, TIMP-2 and RANTES were enhanced in Gal-9-sensitive gastric cancer cells. The enhanced expression of IL-8, in particular, may be associated with a poor prognosis, as determined by stage and histology, and indicate a more aggressive gastric cancer (37-39). These data suggest that Gal-9-sensitive gastric cancer cells immune to the antitumor effect of Gal-9 may produce angiogenesis-related proteins and thereby regulate angiogenesis in the *in vivo* microenvironment of gastric cancer tissues.

MicroRNAs, small non-coding RNA sequences, have been shown to regulate the development and progression of various cancers (40). To identify the miRNAs associated with the antitumor effects of Gal-9, we used miRNA expression arrays to determine variations in the miRNA profiles of the MKN74 cell line in cultures treated with or without Gal-9. The cluster-based analysis clearly demonstrated that Gal-9

treatment affected the expression of numerous miRNAs. In the analysis, we selected sets of miRNAs with significantly altered expression levels with or without Gal-9 treatment. These altered miRNAs may provide clues to the molecular basis for the anticancer effects of Gal-9 in gastric cancer. Our data revealed that miR-204-3p and miR-1246 were upregulated in MKN74 cells treated with Gal-9. miR-204 targets Bcl-2, brain-derived neurotrophic factor (BDNF), SIRT1 and IGFBP5 expression and leads to apoptosis and cell cycle arrest in gastric cancer, neuroblastoma, ovarian cancer and papillary thyroid carcinoma. Furthermore, miR-204-3p has also been reported to act on fibronectin, inhibit the proliferation of hepatocellular carcinoma (HCC) endothelial cells and promote apoptosis (41). In contrast, miR-1246 has been reported to be a target of the p53 family, to inhibit Down syndrome-associated DYRK1A, and consequently activate NFTA1c and induce apoptosis (39). Our previous studies have shown that Gal-9 treatment upregulated miR-1246 in hepatocellular carcinoma (15) and cholangiocarcinoma (16), suggesting that miR-1246 may be associated with the antiproliferative effects of Gal-9 in various cancer cells.

In conclusion, our results revealed that Gal-9 suppresses human gastric cancer cell proliferation, possibly by inducing apoptosis, regulating RTK pathways and angiogenesis-related molecules, and altering miRNA expression profiles. These findings suggest that Gal-9 may be a novel therapeutic target in the treatment of gastric cancer.

Acknowledgements

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