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Research Article

Galectin-3 stabilizes heterogeneous nuclear ribonucleoprotein Q to maintain proliferation of human colon cancer cells

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Abstract. Comparative analysis of proteomes using 5-fluorouracil (5-FU)-resistant human colon cancer cell line revealed that decreased galectin-3 expression was significantly associated with retarded proliferation. However, in the presence of 5-FU proliferation rate of cells with suppressed galectin-3 expression did not differ from that of cells with normal galectin-3 expression, even galectin-3 suppression augmented apoptosis. Mechanism by which galectin-3 regulates cancer cell proliferation has been identified in immunoprecipitates of the anti-galectin-3 antibody. Heterogeneous nuclear ribonucleoprotein Q (hnRNP Q) was identified as a protein interacting with galectin-3. Interestingly, while galectin-3 protein was not affected by the hnRNP Q level, its suppression was accompanied by a decrease in hnRNP Q expression. The present study demonstrates that galectin-3 stabilizes hnRNP Q via complex formation, and reduction in the hnRNP Q level leads to slow proliferation and less susceptibility to 5-FU.

Keywords. Galectin-3, hnRNP Q, colon cancer, proliferation, protein stability, 5-fluorouracil.

Introduction

5-Fluorouracil (5-FU) is one of the most widely used chemotherapeutic agents in treating advanced colorectal cancer [1]. However, partial or complete responses of colorectal cancer to 5-FU are generally followed by eventual tumor re-growth [1]. Numerous studies have focused on identifying the mechanisms and key molecules involved in natural or acquired 5-FU resistance. However, conclusive and consistent results have not been demonstrated until now. We have identified proteins associated with 5-FU resistance, and explored their specific roles [2 – 4]. Recent glycoproteome analyses led to the identification of galectin-3 as a protein down-regulated in human colon cancer cell lines with induced 5-FU resistance. Galectin-3, a member of the ß-galactoside-binding proteins, is a multifunctional oncogene [5], which regulates cell growth [6], adhesion [7 – 9], proliferation [8 – 10], angiogenesis [11], and apoptosis [6, 8, 12 – 14]. The present findings provide evidence that down-regulation of galectin-3 leads to diminished human colon cancer cell proliferation via modulation of the hnRNP Q level, resulting in minimized response to 5-FU.

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Materials & Methods

Human colon cancer cell lines and establishment of derivative cell lines by 5-FU adaptation. Human colon cancer cell lines, SNU-61, SNU-81, SNU-769A, SNU-769B, SNU-1033, SNU-C4 and SNU-C5 [15, 16], were obtained from the Korean Cell Line Bank (Seoul, Korea). Three individual cell lines with resistance to the anti-cancer agent, 5-FU (Choongwae Pharma Corporation, Gyeonggi, Korea), were derived from SNU-C5, SNU-769A and SNU-769B cells, as described previously [4]. The relative fold changes in IC_{50} values (IC_{50} of the resistant cell line versus that of the parent cell line) for 5-FU in resistant cell lines from SNU-C5, SNU-769A and SNU-769B were 21.36, 25.30, and 14.12, respectively [4].

Glycoprotein isolation and subcellular fractionation. All procedures were performed at 4°C, unless otherwise indicated. Harvested cells were homogenized with 1 × RIPA containing protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany), and centrifuged at 12 000 × g for 10 min. The supernatant was mixed with lentil lectin sepharose 4B (GE Health Care Life Sciences, Piscataway, NJ), washed five times with 1 × RIPA, and boiled in SDS sample buffer for 5 min for glycoprotein analysis. Subcellular fractionation was performed using mitochondria and nucleus isolation kits (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's instructions.

Western blot analysis. Western blot analysis was performed as described previously [4]. Briefly, cell homogenates containing equivalent amounts of protein were centrifuged at 4000 × g, and the supernatant fractions subjected to SDS-PAGE. Following electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) blocked by incubation for 2 h at 4°C in 1% Tween 20-TBS buffer containing 1.5% non-fat dry milk (Bio-Rad, Hercules, CA) and 1 mM $MgCl_2$. Membranes were incubated for 2 h at room temperature with primary antibodies against galectin-3 (Santa Cruz Biotechnology, Santa Cruz, CA), hnRNP Q (Abcam, Cambridgeshire, UK), poly(-ADP-ribose) polymerase (PARP) (Abcam), lamin B (Oncogene Research. Products, Cambridge, MA), fibrillarin (Abcam), p-ERK (Cell Signaling Technology, Danvers, MA), p-AKT (Cell Sinaling Technology), p-JNK (Cell Signaling Technology), p-4E-binding protein 1 (4EBP1) (Cell Signaling Technology), p-mammalian target of rapamycin (mTOR) (Cell Signaling Technology), p-S6 kinase (S6K) (Cell Signaling Technology) or actin (Sigma). Next, membranes were washed for 3 × 15 min with blocking solution, and incubated with diluted HRP-conjugated secondary antibody (Southern Biotech, Birmingham,UK) for 1 h at room temperature. This was followed by washing with blocking solution (3 × 15 min), incubation with WEST-ZOL® plus chemiluminescence reagent (iNtRON Biotechnology, Gyeonggi, Korea) for 1 min, and exposure to film (Kodak Blue XB-1).

Immunoprecipitation. All procedures were performed at 4°C, unless otherwise specified. Approximately 10^7 cells in 1 ml of cold 1 × RIPA buffer containing protease inhibitors (Roche Diagnostics) were incubated on ice for 30 min with occasional mixing. Cell lysates were centrifuged at 12 000 × g for 10 min, and the supernatant collected carefully without disturbing the pellet. The supernatant was mixed with primary antibody against either galectin-3 (Santa Cruz Biotechnology) or hnRNP Q (abcam), and incubated for 2 h on a rocking platform. Prepared protein G sepharose beads (100 µl) (GE Health Care Life Sciences) were added, and further incubated on ice for 1 h on a rocking platform. The mixture was centrifuged at 10 000 × g for 30 seconds, and the supernatant removed completely. Protein G sepharose beads were washed five times with 1 ml of cold 1 × RIPA to minimize the background. Next, 100 µl of 2 × SDS sample buffer was added to the bead pellet, and heated to 100°C for 10 min. After boiling, immunoprecipitates were centrifuged at 10 000 × g for 5 min, and the supernatant collected for Western blot analysis.

Matrix-assisted laser desorption ionization-mass spectrometry and database searching. SDS-PAGE gels containing proteins of interest were excised, destained with 50% acetonitrile in 0.1 M ammonium bicarbonate, and dried in a SpeedVac evaporator. Dried gel pieces were re-swollen with 30 µl of 25 mM sodium bicarbonate, pH 8.8, containing 50 ng trypsin (Promega, Madison, WI) at 37°C overnight. α–Cyano 4 hydroxycinnamic acid (20 mg) (Bruker Daltonics, Bremen, Germany) was dissolved in 1 ml acetone:ethanol (1:2, v/v), and 0.5 µl of the matrix solution was mixed with an equivalent volume of sample. Analysis was performed using an Ultraflex TOF/TOF system (Bruker Daltonics). The Ultraflex TOF/TOF system was operated in positive ion reflect mode. Each spectrum was the cumulative average of 250–450 laser shots. Mass spectra were first calibrated in the closed external mode using the peptide calibration standard II (Bruker Daltonics), sometimes using the internal statistical mode to achieve maximum calibration mass accuracy, and analyzed with FlexAnalysis software, version 2.4 (Bruker Daltonics). Peptide mass peaks from each spectrum were submitted to

the Mascot peptide mass fingerprinting search form (www.matrixscience.com) for analysis with BioTools software, version 3.0 (Bruker Daltonics). The search included peaks with a signal-to-noise (S/N) ratio greater than 3. The peak list for each sample was sent into and used to query the non-redundant Mass Spectrometry Protein Sequence Database (MSDB) for the protein identification. Standard settings included the following: enzyme, trypsin; missed cleavage, one; fixed modifications, none selected; variable modifications, oxidized methionine; protein mass, blank; mass values, MH^+ (monoisotopic); mass tolerance, varied between 75 and 100 ppm.

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. A colorimetric assay using the tetrazolium salt, MTT, was used to assess cell proliferation after galectin-3 suppression. MTT assays were performed as described in a previous report [4]. Briefly, equal numbers of cells were incubated in each well in 0.18 ml culture medium to which 0.02 ml of 10 × 5-FU (Choongwae Pharma Corporation) or PBS (for untreated 100% survival control) was added. After four days of culture, 0.1 mg MTT was added to each well, and incubated at 37°C for a further 4 h. Plates were centrifuged at 450 × g for 5 min at room temperature and the medium removed. Dimethyl sulfoxide (0.15 ml) was added to each well to solubilize the crystals, and plates immediately read at 540 nm using a scanning multiwell spectrometer (Bio-Tek instruments Inc., Winooski, VT). All experiments were performed three times, and the IC_{50} (μg/ml) values presented as means ± standard deviation.

siRNA synthesis and transfection. The following target sequences were used to generate siRNA (Qiagen, Chatsworth, CA): 5'-CACGGTGAAGCCCAATGCAAA-3' (NM_002306) for galectin-3, 5'-AGGCTGTTAAACTGTATAATA-3' (AY034482) for hnRNP Q, and 5'-AATTCTCCGAACGTGTCACGT-3' for non-silencing control. Transfection of siRNA was performed using HiferFect transfection reagent (Qiagen Hilden, Germany), according to the manufacturer's instructions. Briefly, 2 μl of 20 μM siRNA solution and 20 μl of the transfection reagent were incubated in 100 μl of serum-free RPMI 1640 medium for 10 min to facilitate complex formation. The resulting mixture (final concentration of 5 nM) was added to SNU-81, a human colon cancer cell line (4×10^5), and incubated in a 60 mm tissue culture dish with 4 ml of RPMI 1640.

Construction of galectin-3 expression vector and transfection. The eukaryotic expression vector plasmid, pEGFP–NT-galectin-3, containing the entire coding sequence of human galectin-3 fused in-frame to the N-terminus of GFP, was constructed by PCR amplification of the corresponding cDNA (GenBank accession no. NM_002306) using oligonucleotide primers; sense, 5'-ATGGCAGACAATTTTTCGCT-3', antisense, 5'-TTATATCATGGTATATGAAGCA CTGGT-3'. The fresh PCR fragment was directly cloned into pEGFP-NT vector using TA cloning method (Invitrogen). SNU-C5 cells were transfected with 2 μg of plasmid DNA using lipofectamine reagent according to the manufacturer's instructions (Invitrogen). Following transfection, cells expressing galectin-3 were selected with G418 (Sigma).

MAP kinase (ERK1/2) activity assay. A MAP kinase (ERK1/2) activity assay kit (Chemicon) was employed, as recommended by the manufacturer.

Determination of 5-FU uptake into human colon cancer cells. Galectin-3 siRNA was transfected into the human colon cancer cell line, SNU-81. Transfected cells were incubated in 6-well plates with 0.1 mg/ml 5-FU, a mixture of non-radioisotope 5-FU and ^{3}H-radioisotope labeled 5-FU (Moravek Biochemicals, Inc. Brea, CA) for 48 h. Cells were harvested and lysed in 1% SDS solution. Equal volumes of OPTIPHASE SUPERMIX (Perkin Elmer Life and Analytical Sciences Inc., Boston, MA) were mixed with cell lysates, and radioactivity counted using the 1450 MicroBeta TriLux Microplate Scintillation and Luminescence Counter (Perkin Elmer Life and Analytical Sciences Inc.). The percentage of relative 5-FU uptake was calculated as the radioactivity remaining in the cell lysate, normalized by the total protein amount.

Cell cycle analysis. SNU-81 cells were transfected with siRNA specific for hnRNP Q. The changes in cell cycle regulation were determined using FACS Calibur™ Flow Cytometer (Becton Dickinson, San Jose, Calif, USA) and CellQuest™ software (Becton Dickinson). Propidium iodide-positive cells were quantified as a percentage.

RNA preparation and Affymetrix GeneChip hybridization. Total RNA was extracted using Trizol reagent (Life Technologies, Inc. Carlsbad, CA), according to the manufacturer's instructions. Genes expressed in the chemosensitive and chemoresistant groups were analyzed on a high density oligonucleotide microarray (HG-U133A; Affymetrix, Santa Clara, CA) containing 22 283 transcripts. Target preparation and microarray processing procedures were performed, following the Affymetrix GeneChip Expression Analysis Manual (Affymetrix). Briefly, total RNA extracted was purified with an RNeasy kit (Qiagen). Double-

stranded cDNA was synthesized from total RNA (20 μg) with SuperScript II reverse transcriptase (Life Technologies, Inc. Rockville, MD) and a T7-(dT)24 primer (Metabion, Germany). Biotinylated cRNA was synthesized from double-stranded cDNA using the RNA Transcript Labeling kit (Enzo Life Sciences, Farmingdale, NY) purified and fragmented. Fragmented cRNA was hybridized to the oligonucleotide microarray, which was washed and stained with streptavidin-phycoerythrin. Scanning was performed with an Agilent Microarray Scanner (Agilent Technologies, Santa Clara, CA).

Affymetrix GeneChip Data analysis. GeneChip analysis was performed based on the Affymetrix GeneChip Manual (Affymetrix) with Data Mining Tool (DMT) 2.0 and Microarray Database software. All genes represented on the GeneChip were globally normalized, and scaled to a signal intensity of 500. Fold changes were calculated by comparing transcripts between the control and cells transfected with either galectin-3 or hnRNP Q siRNA. The DMT 2.0 software employed changed calls (increased or decreased) to statistically analyze expression of a particular transcript, and whether it was relatively increased, decreased or unchanged. After filtration through a "present" call ($p < 0.05$), a transcript was considered differentially expressed at a fold change of greater than 2.0.

Semiquantitative RT-PCR. Semiquantitative RT-PCR was performed to confirm the suppression of galectin-3 or hnRNP Q at the transcriptional level before microarray analysis. Single-stranded cDNA was generated from total RNA (5 μg) using the SuperScript Preamplification System for First Strand cDNA Synthesis (Life Technologies, Inc.). The following primer sets were applied for PCR: Galcetin-3, forward 5'-GGCCACTGATTGTGCCTTAT-3', reverse 5'-GAAGCGTGGGTTAAAGTGGA-3'; hnRNP Q, forward 5'-GGGAAACTGGAACGAGTGAA-3', reverse 5'-CTGCTTGCCTCTGAG CTTTT-3'. PCR was performed with 1 μl of cDNA under the following conditions: initial denaturation at 94°C for 5 min, 25 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, followed by final elongation at 72°C for 7 min.

Statistical analyses. Between-group differences were calculated using the non-parametric Mann-Whitney U-test, and within-group correlations with the Spearman rank coefficient. Significance was set at $P < 0.05$.

Results

Galectin-3 is down-regulated in human colon cancer cell lines with 5-FU resistance. Glycoproteins were isolated from the human colon cancer cell line, SNU-769B and its 5-FU-resistant cell line, using lentil lectin sepharose 4B, and subjected to SDS-PAGE. A protein band that stained strongly in the parent cell line (Fig. 1A) was in-gel digested for MALDI-MS analysis, and unambiguously identified as human galectin-3 (Fig. 1B). The level of galectin-3 co-isolated with glycoproteome after lectin chromatography, and the level of galectin-3 in nucleus, cytoplasm, and whole proteome obtained from three pairs of 5-FU-resistant cell lines were determined (Fig. 1C). Galectin-3 was not detected in SNU-C5 and its 5-FU-resistant cell line, but it was detected and down-regulated in all fractions of other adapted cell lines (Fig. 1C). This expression pattern was additionally observed in total proteome and proteomes in the nucleus or cytoplasm of 5-FU-resistant cell lines. Western blot analysis was performed to establish the total levels of galectin-3 in seven human colon cancer cell lines (Fig. 1D, upper panel). Galectin-3 expression was positively correlated with 5-FU sensitivity (Fig. 1D, lower panel).

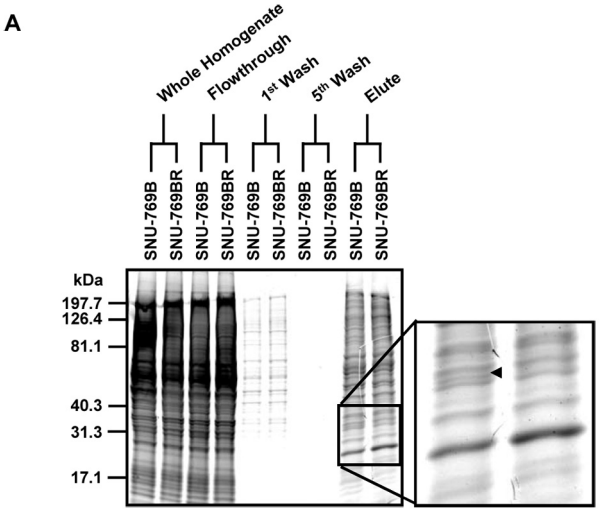
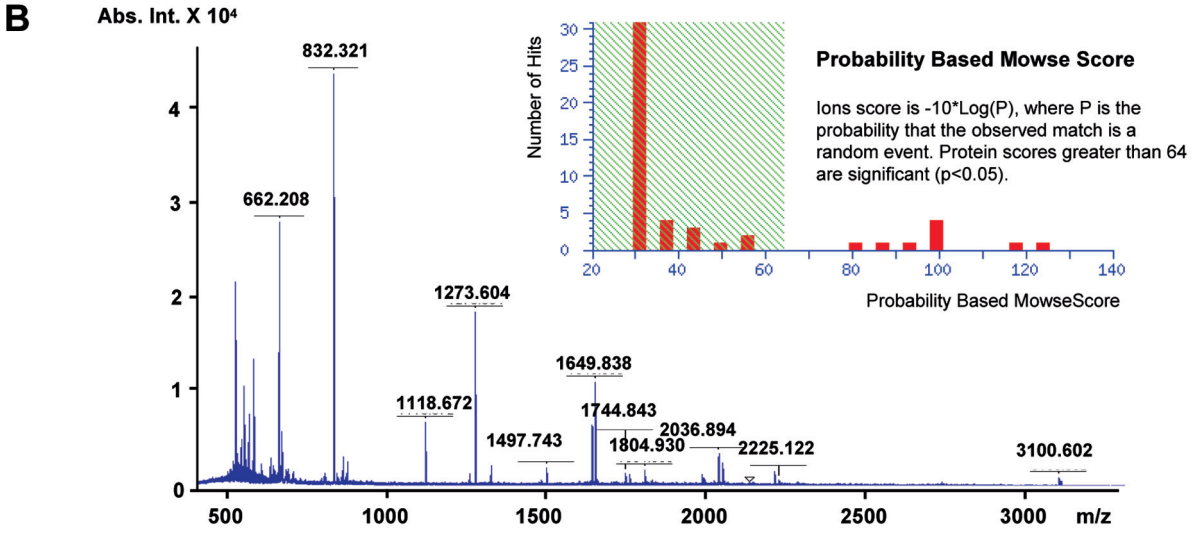


Figure 1. Identification of galectin-3 as a glycoprotein down-regulated in human colon cancer cell lines with induced 5-FU resistance. (*A*) Isolation of glycoproteins from the human colon cancer cell line, SNU-769B, and its 5-FU-resistant cell line, using lentil lectin chromatography. At each isolation step, proteins were subjected to SDS-PAGE. Glycoproteins eluted at the final step were loaded onto the last two lanes of an SDS-PAGE gel. A typical SDS-PAGE pattern after Coomassie blue staining. Proteins displaying a significant decrease in expression in 5-FU-resistant cell lines were selected by comparing the staining patterns of glycoproteins from SNU-769B and its 5-FU-resistant cell line, as depicted in the enlarged partial image.



Rank	Accession	Mass	Score	Description
1.	Q86TY5_HUMAN	13888	124	Full-length cDNA clone CS0DI041YE05 of Placenta of Human
2.	1A3K	15573	119	Galectin-3 carbohydrate recognition domain - Human
3.	LEG3_HUMAN	26041	99	Galectin-3 (Mac-2 antigen) (IgE-binding protein) (35 kDa lectin)
4.	A35820	26172	99	Galectin 3 - Human
5.	AAA88086	26132	99	HUMMAC2 NID: - Homo sapiens
6.	Q6IBA7_HUMAN	26136	99	LGALS3 protein.- Homo sapiens (Human).
7.	Q59FR8_HUMAN	27101	95	LGALS3 protein variant (Fragment).- Homo sapiens (Human).
8.	Q6NVH9_HUMAN	26181	84	Galectin-3.- Homo sapiens (Human).
9.	Q6FGL0_HUMAN	26145	84	LGALS3 protein (Fragment).- Homo sapiens (Human).

1A3K

Mass: 15573
Score: 119
No. of Mass Values Searched: 32
No. of Mass Values Matched: 10
Sequence Coverage: 73%

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
17 - 31	1640.93	1639.93	1639.94	-0.02	0	MLITILGTVKPNANR	
17 - 31	1656.88	1655.88	1655.94	-0.06	0	MLITILGTVKPNANR	Oxidation (M)
32 - 38	862.50	861.50	861.47	0.02	0	IALDFQR	
39 - 49	1273.60	1272.60	1272.60	-0.00	0	GNDVAFHFNPR	
50 - 55	793.39	792.38	792.35	0.03	0	FNENNR	
64 - 70	874.43	873.43	873.41	0.02	0	LDNNWGR	
74 - 86	1497.74	1496.74	1496.77	-0.03	0	QSVFPFESGKPFK	
87 - 97	1324.70	1323.70	1323.72	-0.02	0	IQVLVEPDHFK	
98 - 111	1649.84	1648.83	1648.84	-0.01	0	VAVNDAHLLQYNHR	
121 - 137	1756.87	1755.86	1755.86	0.00	0	LGISGDIDLTSASYTMI	

Figure 1. *(continued)* (*B*) A typical mass spectrum of a MALDI-TOF analysis from a tryptic digest of protein band indicated with an arrow (Fig. 1A). Spectra were processed using FlexAnalysis software (version 2.4), and peptide peaks were submitted to the Mascot peptide mass finger print search engine (www.matrixscience.com) through the BioTools software (version 3.0). The annotated peaks correspond to those peptides that match with the galectin-3 protein. Mascot search results. Mowse score is -10·log(p) where p is the probability that the observed match is a random event. In this analysis, score > 64 indicate identity or at least extensive homology ($p < 0.05$). A probability-based Mowse score > 64 indicated that the identified peptides match the galectin-3 protein sequence with a high degree of certainty. On the histogram of the score distribution, the shaded area represents the score with the greatest statistical uncertainty. The results showed that matching of the tryptic digested peptides of the protein band in Figure 1A with other protein sequences was unsuccessful. MS/MS analysis also confirmed the result of peptide finger printing (data now shown). The peptides matched to galectin-3 protein in the Mascot search. All proteins ranked from 1 to 9 in Figure 1B were either full length or a part of galectin-3 protein. Figure 1B is an example of the Mascot search results showing that the matched peptides isolated from the protein band indicated in Figure 1A accounted for 73 % of the protein sequence of 1A3K.

Galectin-3 suppression affects on human colon cancer cell proliferation and apoptosis. To ascertain the role of galectin-3 for proliferation of human colon cancer cells, SNU-81 cells were transfected with siRNA specific to galectin-3. This cell line was employed, since it expresses significant levels of galectin-3, and displays higher efficacy in siRNA transfection [4]. Galectin-3 expression was markedly decreased up to 96 h after siRNA transfection, regardless of the presence of 5-FU (Fig. 2A). In the absence of 5-FU,

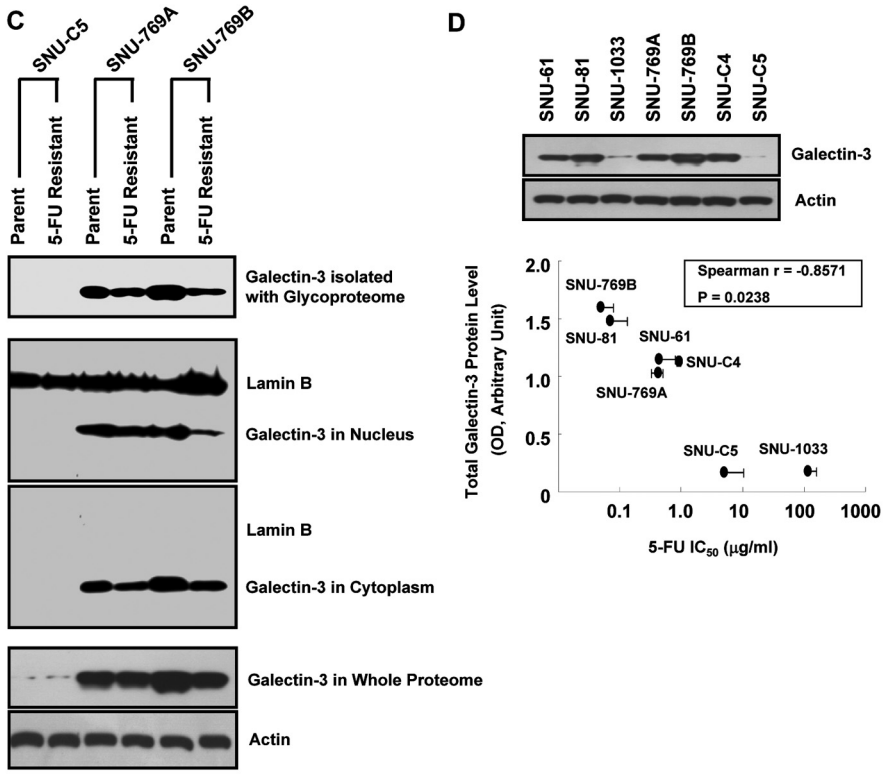


Figure 1. *(continued)* (*C*) Down-regulation of galectin-3 in 5-FU-resistant cell lines and subcellular distribution. Galectin-3 isolated with glycoproteins was down-regulated in 5-FU-resistant cell lines established from SNU-769B and SNU-769A. This pattern was additionally observed in the nucleus and cytoplasmic fractions or total proteomes from 5-FU-resistant SNU-769A and SNU-769B cell lines. (*D*) Positive correlation of the galectin-3 protein level with 5-FU sensitivity of human colon cancer cell lines. Galectin-3 expression in seven individual human colon cancer cell lines. Whole proteomes obtained from human colon cancer cell lines, SNU-61, SNU-81, SNU-1033, SNU-769A, SNU-769B, SNU-C4 and SNU-C5, were subjected to SDS-PAGE, and electro-transferred to PVDF membranes for Western blot analysis. Positive correlation between the galectin-3 level and 5-FU sensitivity. The 5-FU sensitivities of seven human colon cancer cell lines and total galectin-3 protein levels were calculated with the MTT assay, and the optical densities of the galectin-3 immunoreactive signals measured by Western blot analyses, respectively. The correlation between 5-FU sensitivity and galectin-3 protein expression was calculated using the Spearman rank coefficient, P value = 0.0238.

galectin-3 siRNA transfection led to decreased cell proliferation (up to ~10%) at 96 h, compared to that of the control, buffer or non-silencing siRNA-transfected cells (Fig. 2B upper panel). However, cell proliferation rate was not altered following galectin-3 siRNA transfection in the presence of 5-FU (Fig. 2B lower panel), while apoptotic cell death was slightly elevated (Fig. 2C upper panel). To determine whether augmented apoptosis by galectin-3 suppression is due to alterations in 5-FU uptake, we measured the uptake of ^{3}H-labeled 5-FU in SNU-81 cells after galectin-3 siRNA transfection. However, galectin-3 suppression did not increase the percent relative 5-FU uptake, compared to controls transfected with buffer only at 96 h after siRNA transfection (Fig. 2C lower panel). The response of SNU-81 to 5-FU was evaluated at 96 h after siRNA transfection. Galectin-3 suppression resulted in a slow proliferation and it caused ~10% increased resistance to 5-FU, compared to the control transfected with buffer only (Fig. 2D). To test the effect of increased expression of galectin-3, expression vector for galectin-3 has been constructed and transfected into human colon cancer cell line SNU-C5 (Fig. 1D, Supplement Fig. 1). The results seem to indicate that 5-FU susceptibility may be increased by induced galectin-3 expression, but the change was not clear. Furthermore, proliferation of SNU-C5 cells were quite affected by expression vector transfection itself (cf. non-silencing siRNA transfection did not affect cell proliferation), and showed very low transfection efficacy [4]. The experimental conditions using SNU-C5 were not exactly fit to prove and explain the role of galectin-3 for 5-FU susceptibility (Supplement Fig. 1).

Galectin-3 suppression decreases phosphorylation of mammalian target of rapamycin (mTOR) and its downstream effectors, 4E-binding protein 1 (4EBP1) and S6 kinase (S6K) in the presence of 5-FU. MAP kinase activity was monitored after suppression to

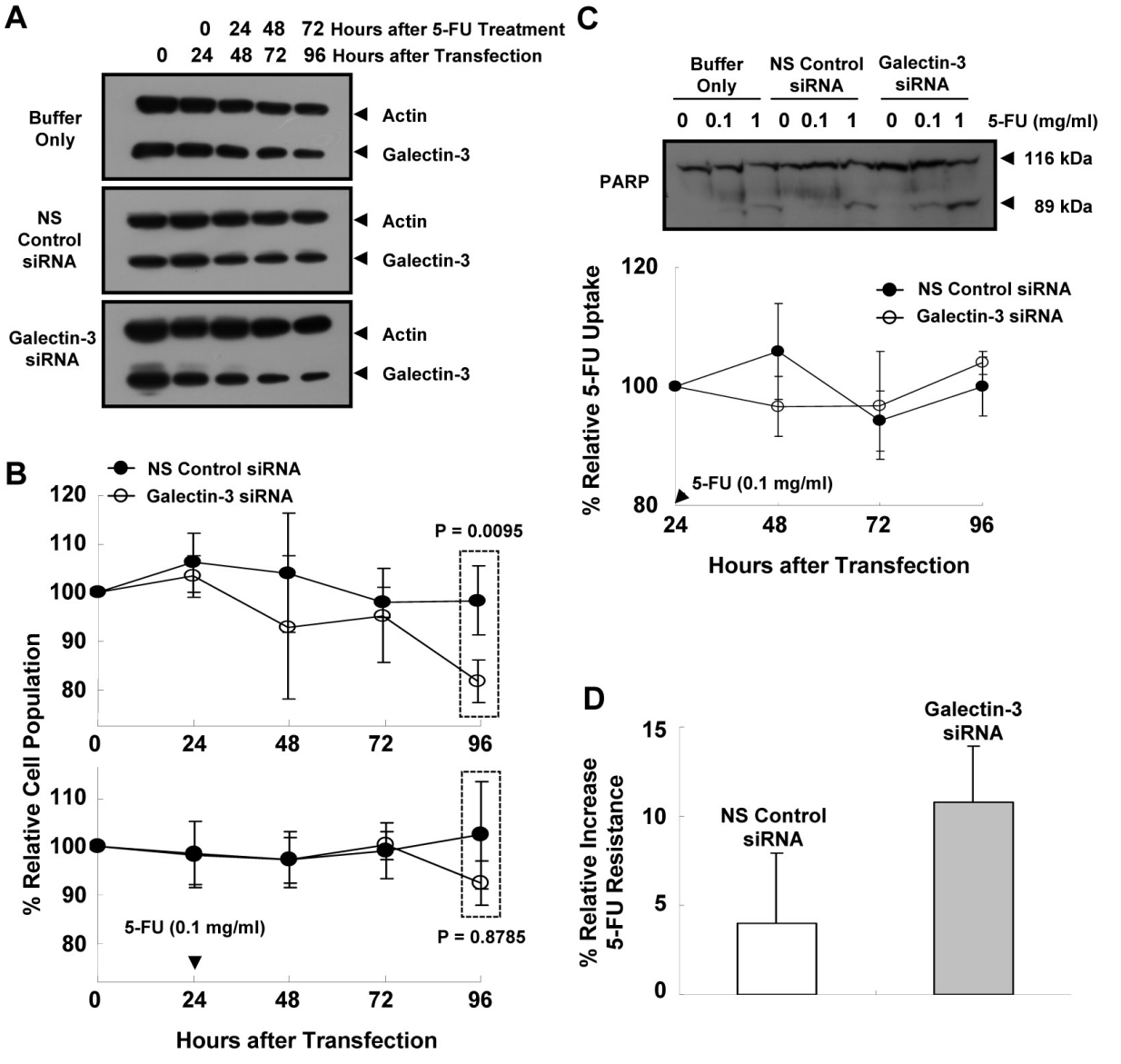


Figure 2. Effects of galectin-3 suppression on proliferation of human colon cancer cells and apoptotic cell death in the presence of 5-FU. (*A*) Galectin-3 suppression by transfection of siRNA into the human colon cancer cell line, SNU-81. Transfection of galectin-3 siRNA in absence of 5-FU led to a decrease in galectin-3 expression up to 96 h. (*B*) Cell proliferation after Galectin-3 siRNA transfection in the absence or presence of 5-FU. Percent relative cell population of SNU-81 was calculated by comparison with the cell population transfected with buffer only. (*C*) Apoptosis and 5-FU uptake after galectin-3 suppression. Apoptosis was determined with the PARP cleavage assay. In the presence of 5-FU, galectin-3 suppression led to a slight elevation in apoptotic cell death of SNU-81 (upper panel). After galectin-3 suppression, SNU-81 cells were cultured in the presence of ^{3}H-labeled 5-FU, harvested and lysated. The cpm value of ^{3}H-labeled 5-FU was determined using 1450 MicroBeta TriLux Microplate Scintillation and Luminescence Counter (Perkin Elmer Life and Analytical Sciences, Inc.), and normalized to total protein in cell lysates. The percent relative 5-FU uptake after galectin-3 suppression was calculated by comparison with 5-FU uptake of cells transfected with buffer only. However, galectin-3 suppression has no effect on 5-FU uptake (lower panel). (*D*) Slight change of 5-FU resistance caused by galectin-3 suppression. % Relative increased resistance of SNU-81 was calculated at 96 h after galectin-3 siRNA transfection as: (% relative population of cells treated with 5-FU after galectin-3 suppression, Figure 2B lower panel) minus (% relative population of non-treated cells after galectin-3 suppression, Figure 2B upper panel).

examine the possibility that galectin-3 regulates cell proliferation via the MEK1/2-ERK1/2 signaling pathway (Fig. 3A). However, we observed no significant changes in MAP kinase activity. In the presence of 5-FU, artificial suppression of galectin-3 reduced activation of mTOR, 4EBP1 and S6K, but the level of phosphorylated AKT, JNK, and ERK were not altered (Fig. 3B).

Galectin-3 interacts with hnRNP Q. To establish the mechanism by which galectin-3 regulates the proliferation of human colon cancer cells, interacting

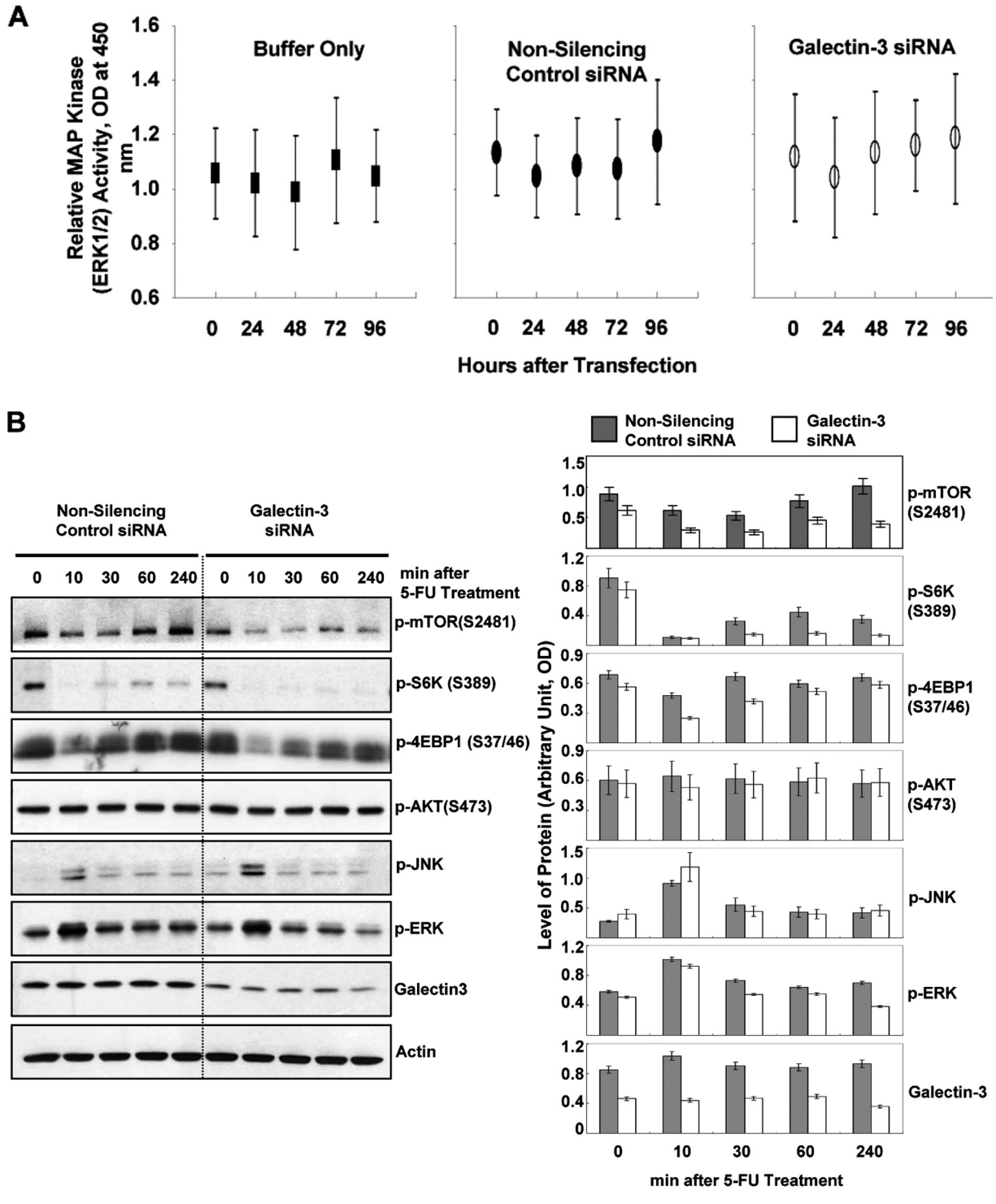


Figure 3. Effect of artificial suppression of galectin-3 in either presence or absence of 5-FU on various signaling pathway. (*A*) Effect of galectin-3 suppression on MAP kinase activity in the absence of 5-FU. As shown in Figure 3A, no significant changes in MAP kinase activity were evident. (*B*) Decreased phosphorylation of mTOR and its downstream effector, 4EBP1 and S6K after galectin-3 suppression in the presence of 5-FU. Various key molecules linked to cell proliferation and anticancer drug response have been checked. In the presence of 5-FU, galectin-3 suppression resulted in decreased phosphorylation of mTOR, 4EBP1 and S6K, but the level of phosphorylated AKT, JUK, and ERK were not changed. Signals from transfection of buffer only did not differ from those of non-silencing control (data not shown).

proteins were screened by MALDI-MS analysis of immunoprecipitates using an anti-galectin-3 antibody (Fig. 4A, B). Immunoprecipitates were fractionated using a salt gradient and acidic pH, and subjected to SDS-PAGE, as shown in Figure 4A. After staining the gel, protein bands in each fraction were excised and in-gel digested to determine peptide mass. The ~60 kDa protein indicated in Figure 4A was successfully identified as human heterogeneous nuclear ribonucleoprotein Q (hnRNP Q) (Fig. 4B). Immunoprecipitates of the anti-galectin-3 antibody was proved by anti-hnRNP Q antibody, and vice versa (Fig. 4C) to confirm interactions between the two proteins. Immunoreactive signals of hnRNP Q and galectin-3 were clearly detected in immunoprecipitates of anti-galectin-3 and anti-hnRNP Q, respectively (Fig. 4C). However, the hnRNP Q level was not associated with response to 5-FU (Fig. 4D).

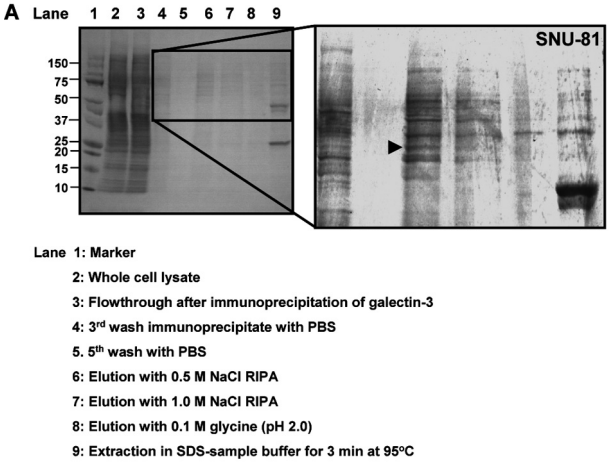


Figure 4. Identification of hnRNP Q as a protein interacting with galectin-3. (*A*) SDS-PAGE of immunoprecipitates using an anti-galectin-3 antibody. Immunoprecipitates of anti-galectin-3 were fractionated using a salt gradient and acidic pH conditions. Each fraction was subjected to SDS-PAGE.

HnRNP Q suppression affects on cell proliferation and 5-FU sensitivity. SNU-81 cells were transfected with siRNA against hnRNP Q, and the rate of cell proliferation assessed, as shown in Figure 6A. At 96 h after transfection, cells displayed a marked decrease in proliferation rate (~50 %), compared to the control (Fig. 5A). However, similar to galectin-3, the cell proliferation rate was not significantly affected upon hnRNP Q suppression in the presence of 5-FU (Fig. 5B). The relative resistance of SNU-81 to 5-FU was significantly increased (up to ~ 44 %) at 96 h after siRNA transfection, compared to control transfected with buffer only (Fig. 5C). However, suppression of hnRNP Q or galectin-3 did not affect the sub-G1 portion relative to the controls transfected with non-silencing siRNA or buffer only (Fig. 5D).

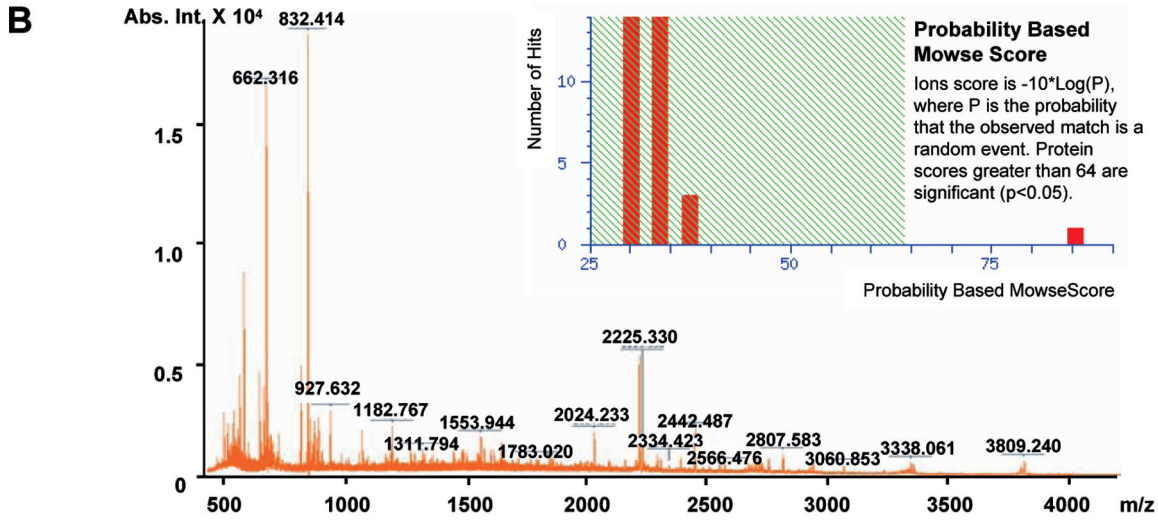
The hnRNP Q protein level is controlled by galectin-3. The hnRNP Q protein level was determined after galectin-3 suppression, with a view to determine the relationship between the two proteins (Fig. 6A). Interestingly, galectin-3 suppression was accompanied by a decrease in the hnRNP Q protein level (Fig. 6A, left upper panel). This finding was specific for hnRNP Q, and for no other hnRNP proteins examined, such as hnRNP M1–4 (Fig. 6A, left lower panel). To examine whether hnRNP Q suppression, in turn, affects the galectin-3 level, SNU-81 cells were transfected with hnRNP Q siRNA, and galectin-3 expression monitored. Interestingly, the galectin-3 protein level was unaffected by hnRNP Q suppression (Fig. 6A, right panels). Transfection of cells with siRNA against either hnRNP Q or galectin-3 led to a decrease in the mRNA (Fig. 6B) and protein levels (Fig. 6A). We further examined the effects of galectin-3 and hnRNP Q suppression on mRNA expression profiling using a high-density oligonucleotide microarray (Supplement Table 1). The observed changes in transcriptional levels after galectin-3 and hnRNP Q suppression are listed in Supplement Table 1.

The subcellular level of hnRNP Q protein is directly affected by the presence of 5-FU. The effects of 5-FU on subcellular galectin-3 and hnRNP Q were examined (Fig. 6C). Without artificial suppression, the immunoreactive signal of hnRNP Q in the cytoplasm of SNU-81 disappeared from 24 h after 5-FU treatment (Fig. 6C upper panel), and additional immunoreactive signals of low molecular weight hnRNP Q appeared in the nucleus from 48 h onwards (Fig. 6D, lower panel).

Discussion

As glycosylation is an important post-translational modification required for the biological functions of proteins in cells, we recently assessed glycoproteome approaches, and found that galectin-3 is down-regulated in human colon cancer cell lines with induced 5-FU resistance (Fig. 1). Even galectin-3 is not a glycoprotein, lectin-binding affinity of galectin-3 resulted in co-isolation of galectin-3 with glycosylated proteins in the lectin chromatography. However, the total intracellular galectin-3 level is also important as the level of galectin-3 co-isolated with glycosylated protein (Fig. 1C).

Numerous reports have shown that galectin-3 is a multifunctional protein involved in important cellular



AAH32643=SYNCRIP
protein=hnRNP Q/NSAP1

Mass: 58927
Score: 85
No. of Mass Values Searched: 93
No. of Mass Values Matched: 18
Sequence Coverage: 36%

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
39 - 60	2442.49	2441.48	2441.24	0.24	1	VAEKLDEIYVAGLVAHSDLDER
43 - 60	2015.24	2014.24	2014.00	0.23	0	LDEIYVAGLVAHSDLDER
61 - 81	2334.42	2333.42	2333.23	0.19	1	AIEALKEFNEDGALAVLQQFK
82 - 100	2152.33	2151.32	2151.01	0.31	1	DSDLSHVQNKSAFLCGVMK Oxidation (M)
124 - 130	842.64	841.63	841.54	0.09	1	IKALLER
131 - 142	1311.79	1310.79	1310.65	0.14	0	TGYTLDVTTGQR
131 - 143	1439.95	1438.95	1438.74	0.20	1	TGYTLDVTTGQRK
143 - 168	2694.57	2693.56	2693.33	0.23	1	KYGGPPPDSVYSGQQPSVGTEIFVGK
144 - 168	2566.48	2565.47	2565.24	0.23	0	YGGPPPDSVYSGQQPSVGTEIFVGK
172 - 184	1593.96	1592.96	1592.80	0.16	0	DLFEDELVPLFEK
185 - 192	927.63	926.62	926.50	0.13	0	AGPIWDLR
222 - 229	1058.67	1057.66	1057.53	0.13	0	LYNNHEIR
255 - 265	1351.91	1350.90	1350.70	0.20	1	TKEQILEEFSK
322 - 331	1182.77	1181.76	1181.62	0.14	1	AFSQFGKLER
335 - 346	1553.94	1552.94	1552.77	0.17	1	LKDYAFIHFDER
337 - 351	1783.02	1782.01	1781.84	0.18	1	DYAFIHFDERDGAVK
444 - 461	2086.18	2085.17	2084.88	0.29	0	GGYEDPYYGYEDFQVGAR
444 - 463	2299.35	2298.34	2298.00	0.35	1	GGYEDPYYGYEDFQVGARGR

Figure 4. *(continued)* (*B*) A typical mass spectrum of a MALDI-TOF analysis from a tryptic digest of protein band indicated with an arrow (Fig. 4A). Spectra processing, peptide mass finger printing and the Mascot search were performed as described in the legend to Figure 1B. A probability-based Mowse score of 85 indicated that the identified peptides match hnRNP Q protein sequence with a high degree of certainty.

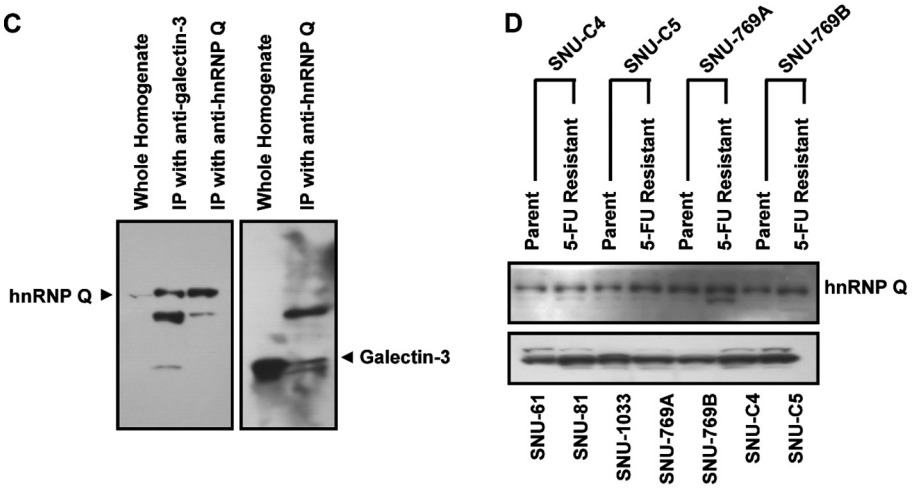


Figure 4. *(continued)* (*C*) Confirmation of interactions between hnRNP Q and galectin-3. The immunoreactive signal of hnRNP Q was detected in immunoprecipitates of galectin-3 and vice versa. (*D*) No correlation was evident between the hnRNP Q protein level and 5-FU sensitivity of human colon cancer cell lines. Immunoreactive signals of hnRNP Q in total proteomes from human colon cancer cell lines and their 5-FU-resistant derivative cell lines were comparable.

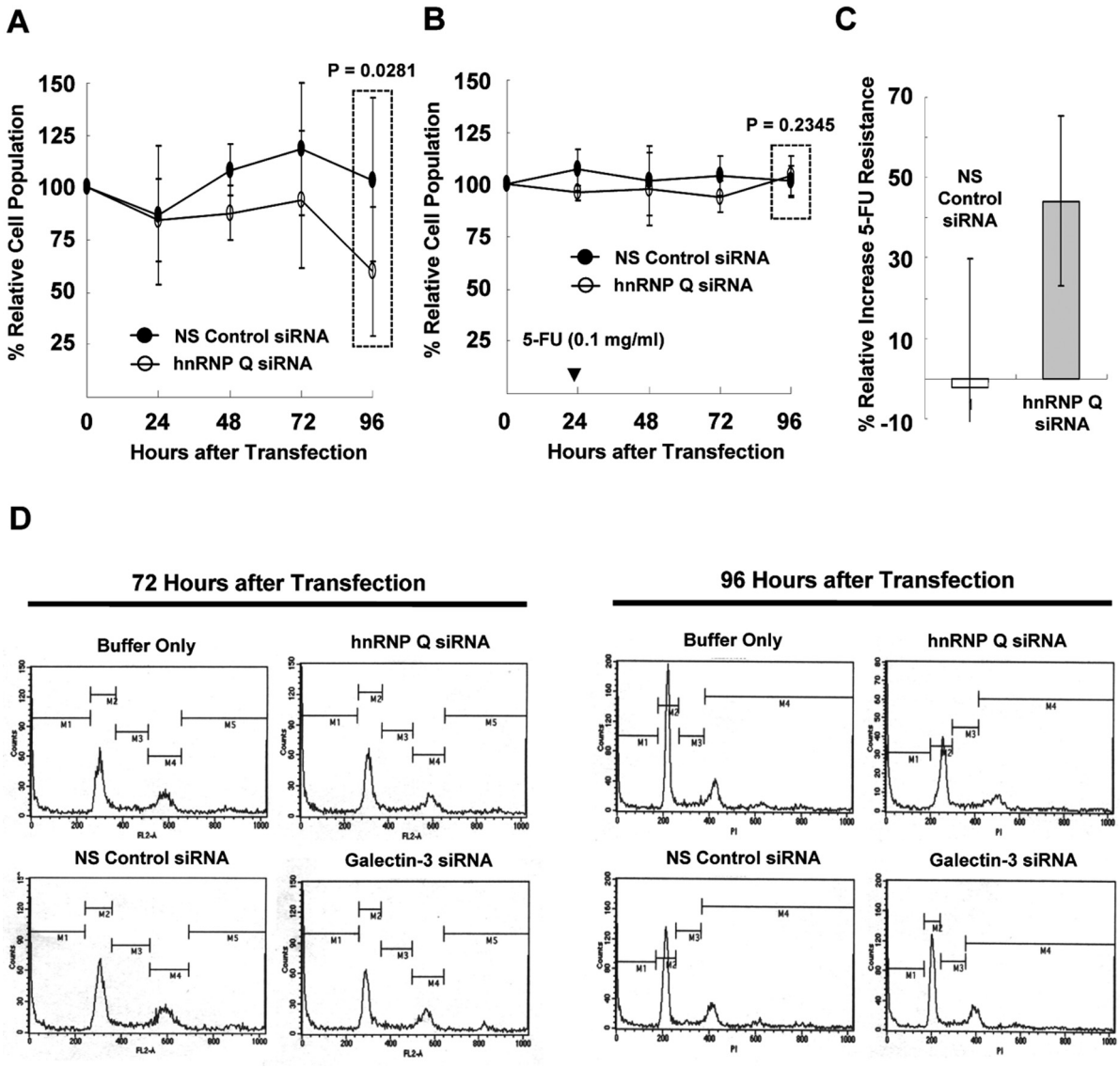


Figure 5. Effects of hnRNP Q suppression on human colon cancer cell proliferation and 5-FU resistance. (*A*) Decreased proliferation of SNU-81 due to hnRNP Q suppression. The percent relative cell population was calculated by comparison with the cell population transfected with buffer only. (*B*) Cell proliferation after hnRNP suppression in the presence of 5-FU. (*C*) Increased 5-FU resistance caused by suppression of hnRNP Q. Percent relative increased resistance of SNU-81 was calculated at 96 h after hnRNP Q siRNA transfection as: (% relative population of cells treated with 5-FU after hnRNP Q suppression, Fig. 6B) minus (% relative population of non-treated cells after hnRNP Q suppression, Figure 6A). (*D*) FACS analysis after hnRNP Q or galectin-3 suppression. Neither hnRNP Q nor galectin-3 induced an increase in the sub-G1 phase.

processes, and plays different roles depending on its subcellular localization. For instance, endogenous galectin-3 inhibits epithelial cell apoptosis induced by chemotherapeutic agents such as cisplatin, genistein, tumor necrosis factor, and nitric oxide [17–19]. This antiapoptotic activity in response to chemotherapeutic drugs is regulated via nuclear export of phosphorylated galectin-3 [20]. However, our current experiments with human colon cancer cell lines do not support a correlation between subcellular localization of galectin-3 and 5-FU response (Fig. 1C). The subcellular level simply reflects total endogenous expression (Fig. 1C). No significant changes in the ratios of subcellular levels in parent and 5-FU-resistant cell lines are evident, following 5-FU treatment (data not shown).

The SNU-81 cell line was employed to clarify the role of galectin-3 in 5-FU resistance, due to higher siRNA transfection efficacy [4] and endogenous galectin-3 levels (Fig. 1D). Alterations in the proliferation rates were investigated after galectin-3 suppression (Fig. 2B). Interestingly, cells transfected with galectin-3 siRNA displayed different patterns of proliferation that were dependent on the presence or absence

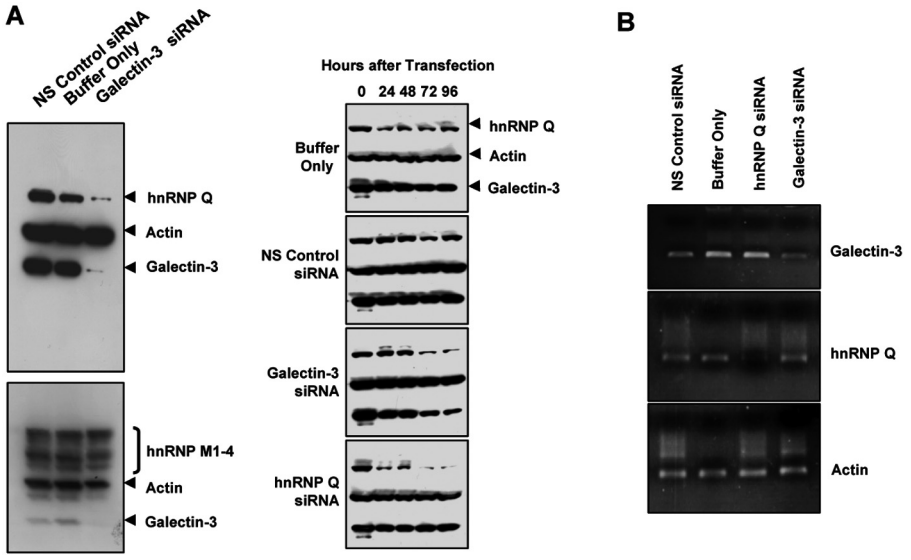


Figure 6. Regulation of hnRNP Q depending on the galectin-3 level. (*A*) Decreased protein level of hnRNP Q after suppression of galectin-3. Galectin-3 suppression resulted in a decreased level of hnRNP Q (left upper panel). However, other hnRNPs, such as hnRNP M1–4, did not display alterations in the protein level after galectin-3 suppression (left lower panel), and hnRNP Q suppression did not affect on the galectin-3 protein level (right panel). (*B*) No changes in the mRNA level of hnRNP Q after galectin-3 suppression. Transfection of siRNA against either galectin-3 or hnRNP Q resulted in a decrease at both the transcriptional and translational levels. However, galectin-3 suppression had no effect on the transcriptional level of hnRNP Q.

of 5-FU (Fig. 2B). Decreased cell proliferation rate is a reported mechanism in cancer cells to escape the toxic effects of anticancer reagents [22, 23]. For instance, increased proliferation is associated with enhanced *in vitro* sensitivity to several anticancer agents in initial acute lymphoblastic leukemia [21], and decreased proliferation, a behavior of clear cell carcinoma of the ovary that contributes to drug resistance [22]. In view of the specific effects of galectin-3 on cell proliferation, we proposed that this protein may alter the responses of human colon cancer cells to 5-FU by modulating the proliferation rate (Fig. 2D).

The galectin-3 protein stimulates proliferation in various cell types, including rat hepatic stellate cells [23], mesangial cells [24], and fibroblast cells [10]. Several mechanisms are mediated by this protein, depending on the cell type [23]. Recently, Maeda and co-workers [23] demonstrated that galectin-3 promotes the proliferation of rat hepatic stellate cells via the MAP kinase signaling pathway. However, no significant changes in MAP kinase activity were evident upon galectin-3 suppression in the human colon cancer cell line, SNU-81 (Fig. 3A). This finding strongly suggests that galectin-3 is not involved in the MAP kinase signaling pathway to modulate cell proliferation, and that a new galectin-3-regulated proliferation is present in human colon cancer cells. Interestingly, in the presence of 5-FU suppressed galectin-3 resulted in a decrease of phosphorylation of mTOR, 4EBP1, and S6K, but did not affect other survival signals including AKT, JNK, and ERK (Fig. 3B). 5-FU has been reported as an anticancer drug to inhibit the phosphorylation of AKT associated with increased resistance to multiple chemotherapeutic agents (5-FU, adriamycin, mitomycin C and cisplatinum) [24], but it did not affect a series of MAP kinases in activated macrophages [25]. Figure 3B suggests that galectin-3 in the presence of 5-FU is not linked to either AKT or MAP kinase signaling pathway for survival and proliferation of colon cancer cells. Among the signals tested, signals of mTOR and its downstream effectors, 4EBP1 and S6K have been changed by suppression of galectin-3 in the presence of 5-FU (Fig. 3B). Down-regulation of mTOR activity has been recently reported in 5-FU-induced apoptosis of hepatocarcinoma cells [26]. Our finding suggests that suppressed galectin-3 may decrease a rate of proliferation (Fig. 2B) but increase apoptotic cell death (Fig. 2C) by enhancing down-regulation of mTOR activity in the presence of 5-FU (Fig. 3B)

We obtained useful information by investigating the role of galectin-3 on the main signaling pathways to maintain cell proliferation. However, we extended our approaches to the proteomes in the immunoprecipitates of anti-galectin-3 antibody, because the identification of protein(s) interacting with galectin-3 could be a very important clue to understanding the role of galectin-3 at the molecular level. Finally, we found a clear clue that galectin-3 interacts with hnRNP Q (Fig. 4). HnRNPs are abundant multifunctional proteins that play pivotal roles in RNA processing, such as

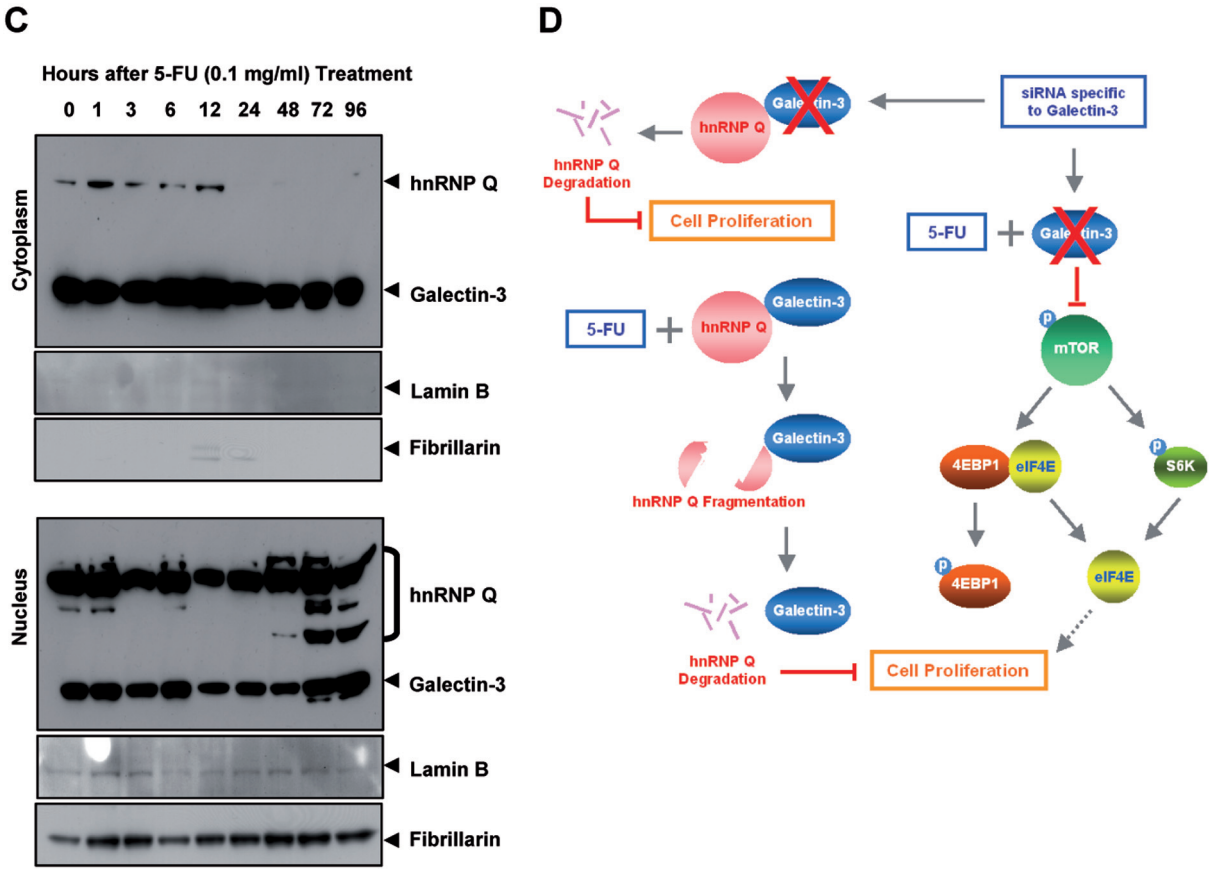


Figure 6. *(continued)*(*C*) Effect of 5-FU on the galectin-3 and hnRNP Q levels. In the presence of 5-FU (0.1 mg/ml), galectin-3 and hnRNP Q protein levels in the nucleus and cytoplasm of SNU-81 were monitored. Galectin-3 did not display any significant changes. However, no immunoreactive signal of hnRNP Q in the cytoplasm was detected from 24 h after 5-FU treatment (upper panel). In the nucleus, additional signals of hnRNP Q appeared from 48 h after 5-FU treatment (lower panel). (*D*) A model for the mechanism of colon cancer cell proliferation controlled by galectin-3 in either presence or absence of 5-FU. Galectin-3 interacts with hnRNP Q, and the stability of hnRNP Q is maintained by forming a complex with galectin-3. Breaking the interaction by suppressed galectin-3 significantly inhibits proliferation of human colon cancer cells. Like galectin-3, a decrease of hnRNP Q itself also significantly interrupted formal proliferation of human colon cancer cells, and 5-FU can induce the fragmentation of hnRNP Q. In addition, phosphorylation of mTOR and its downstream effectors, 4EBP1 and S6K is decreased by suppressed galectin-3 in the presence of 5-FU. The figure suggests several possible mechanisms of galectin-3 to control colon cancer cell proliferation rate, which results in change of response to 5-FU. (mTOR: mammalian target of rapamycin; 4EBP1: 4E-binding protein 1; S6K: S6 kinase; eIF4E: eukaryotic initiation factor 4E).

nascent hnRNA packing, alternative splicing, and mRNA stability [27–29]. Increased hnRNP A2 and B1 expression has been reported in a variety of tumors, including lung [30], and breast [31] cancer, and suppression of hnRNP A1 and A3 significantly reduces the rate of squamous carcinoma cell proliferation [32]. Suppression of hnRNP Q also leads to a considerable decrease in the proliferation rate of human colon cancer cell lines (Fig. 5A). However, similar to galectin-3, hnRNP Q suppression in the presence of 5-FU did not affect the proliferation rate (Fig. 5B).

To further clarify the link between galectin-3 and hnRNP Q, the galectin-3 protein level was evaluated after hnRNP Q suppression and vice versa. Interestingly, the results clearly show that the protein stability of hnRNP Q is regulated by galectin-3 (Figs. 6A and B, Supplement Table 1). Previous data showed that galectin-3 forms a complex with hnRNPs, but the investigators could not assign a role for galectin-3 in either transcriptional regulation or RNA processing [33]. In our current experiments, galectin-3 suppression resulted in decreased hnRNP Q protein, but not mRNA (Fig. 6A, Supplement Table 1). Interestingly, 5-FU induced degradation of cytoplasmic hnRNP Q and fragmentation of nucleic hnRNP Q, but did not affect the galectin-3 level, either with respect to subcellular localization or protein expression (Fig. 6C). Clearly, 5-FU has a direct destabilization effect on hnRNP Q. We propose that the hnRNP Q protein is targeted by the anti-cancer drug, 5-FU, to prevent cancer cell proliferation.

In summary, the present study demonstrates that rate of proliferation of human colon cancer cells can be

decreased by the down-regulation of galectin-3. Galectin-3 interacts with hnRNP Q, and the stability of hnRNP Q is dependent on forming a complex with galectin-3. As with galectin-3, a decrease of hnRNP Q itself also significantly interrupted formal proliferation of human colon cancer cells. Thus, overall findings demonstrate that galectin-3 stabilizes hnRNP Q by cooperative interaction, and breaking this interaction by means of suppressed galectin-3 significantly inhibits proliferation of human colon cancer cells (Fig. 6D).

Electronic supplementary material. Supplementary material is available in the online version of this article at springerlink.com (DOI 10.1007/s00018-009-8562-3) and is accessible for authorized users.

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