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Galectin-1 is silenced by promoter hypermethylation and its re-expression induces apoptosis in human colorectal cancer cells*

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Abstract

Galectin-1 (gal-1) is an important molecule secreted by many tumors, which induces apoptosis in activated T-cells and promotes tumor angiogenesis, both of which phenomena facilitate successful establishment of tumor in the body. However, little is known about the function of intracellular gal-1 or its transcriptional regulation in colorectal cancer (CRC). Here, we demonstrate that gal-1 expression is epigenetically regulated in CRC through promoter hypermethylation. Intracellular gal-1 induces cell cycle arrest and apoptosis in CRC cells with concomitant down-regulation of Wnt and NF- κ B signaling pathways. Together, these data suggested that gal-1 silencing imparts CRC with the ability to proliferate and escape apoptosis.

Keywords

Colorectal cancer; galectins; galectin-1; *LGALS1*; promoter hypermethylation; apoptosis; Wnt signaling

1. INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide. It is well documented that CRC arises from a series of genetic alterations that include gene silencing, loss of heterozygosity, point mutations and homologous deletions [1]. Gene silencing in CRC is often associated with aberrant hypermethylation of the CpG-rich sequences (CpG islands) in promoter regions of multiple loci of genes [2–4] including *hMLH1*, *CDH1* and *CDKN2A/p16* that are involved in the regulation of cellular processes including proliferation and apoptosis. A large body of evidence indicates that galectins, a family of β -galactoside-binding proteins, participate in a variety of normal cellular functions, and are dysregulated in CRC. Among all the known galectins, galectin-1 (gal-1), encoded by *LGALS1* (*LGALS1* gene; Accession: NM_002305), is well characterized and is a prototype of the galectin family. Gal-1 is both intracellular and secreted protein and

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participates in a variety of biological functions including cell-cell and cell-matrix interactions and cell growth. Gal-1 is dysregulated in cancers and implicated with neoplastic transformation (reviewed in [5]). Perillo, *et al.* [6] have shown previously that extracellular gal-1 induces apoptosis in activated T-cells, suggesting that tumors secrete gal-1 as a tumor immune surveillance mechanism. While tumors secrete a variety of growth factors to induce angiogenesis, recent evidence indicates that tumor-secreted gal-1 also promotes angiogenesis [7]. These studies together highlight the importance of extracellular gal-1 in tumor biology. While the functional role of intracellular gal-1 is beginning to unravel, its role in CRC remains unclear. To better understand the function of gal-1, elucidation of its transcriptional regulation is necessary. Toward this end, we tested the possibility that gal-1 expression is transcriptionally regulated. Utilizing different CRC cell lines, we demonstrate that gal-1 expression is regulated by promoter hypermethylation. Further, we show that intracellular gal-1 regulates cell cycle by arresting at G1 phase, and induces apoptosis in gal-1 negative cells by activating a variety of cellular proteins. Our results suggest that gal-1 regulates cell growth and apoptotic processes, and its down-regulation promotes CRC tumor progression.

2. MATERIALS AND METHODS

2.1. Reagents

Goat polyclonal anti-gal-1 antibody was purchased from R&D Biosystems (Minneapolis, MN). Other antibodies used in this study were purchased from Cell Signaling Technologies (Beverly, MA). Sources of all other reagents and materials were reported previously [8].

2.2. Cell lines and culture conditions

Cell lines used in the study were purchased from American Type Culture Collection, Vanassas, MD, and grown as recommended by the supplier. Colorectal adenocarcinoma cell line, LS-180 was cultured in Eagle's Minimum Essential Medium (MEM). Colorectal carcinoma cell lines, HCT 116 and ATRFLOX, and colorectal adenocarcinoma cell line HT-29 were cultured in McCoy's 5A Modified Medium. Human embryonic kidney cells HEK-293 and the human foreskin fibroblast (HFF-2) cells were cultured in DMEM. Colorectal adenocarcinoma cell line Caco-2 clone (C2Bbe1) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.01 mg/ml human transferrin (MP Biomedicals, OH) and grown in a 37° C incubator with 10% CO₂. All other cell lines were grown in a 37° C incubator with 5% CO₂. All the growth media were supplemented with 10% fetal bovine serum.

2.3. RT-PCR analysis

Isolation and detection of gal-1 transcript from cells were carried out as described previously [9], using the forward primer, 5'-ATGGCTTGTGGTCTGGTCGCC-3' and reverse primer, 5'-TCAGTCAAAGGCCACACATTTGA-3'. The amplified DNA fragments were subsequently sub-cloned into pGEMTeasy vector (Promega, CA) and the insert DNA was sequenced at the DNA sequencing facility of the University of Nebraska, Lincoln, NE. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript was amplified using forward primer, 5'-CAGCCGAGCCACATCG-3' and reverse primer, 5'-TGAGGCTGTTGTCATACTTCTC-3'.

2.4. Methylation-Specific PCR

Methylation-specific PCR primers for gal-1 were designed using the Methprimer algorithm (<http://www.urogene.org/methprimer/>). An unmethylated primer set (U-specific primer set) TATTTTTTGTGTTTGTGTTTGAATGTG (forward) and

CAAAACTACAACACTACTACAACACT (reverse), and a methylated primer set (M-specific set): TATTTTTTGTGTTTGTGTTTGAACGC (forward) and CAAAACTACGACTACTACAACGCT (reverse) were designed based on the positive strand of the bisulfite-converted promoter DNA that spanned the region within the promoter's specific and predicted CpG islands, as indicated in the Results Section. Genomic DNA (2 µg) from each cell line was bisulfite-modified using the EZ DNA methylation kit (Zymo Research, Orange, CA) according to the supplier's instructions. As control, one µg of Universally Methylated DNA, supplied with kit, was treated concurrently with the test samples. After treatment, the resulting bisulfite-modified DNA was collected in 10 µl elution buffer and stored at -20°C. One µl of bisulfite-modified DNA from each sample was amplified independently using the U- and M-specific primers in a 25 µl total reaction volume by PCR.

2.5. 5-Aza-2'-deoxycytidine (5-AzaC) treatment

HCT-116, Caco-2, HT-29 and LS-180 cells were treated with 30 µM 5-AzaC for 6 days, to achieve maximal hypomethylation of DNA. Cells were then harvested and analyzed by western blotting for gal-1 expression.

2.6. Plasmids and transfection

Gal-1 plasmid (pCMV6-XL5/gal-1) and vector control (pCMV6-XL5) were purchased from Origene (Rockville, MD). pEGFP-C1 plasmid was purchased from Clontech, CA. Transfections were carried out using Lipofectamine 2000 reagent (Invitrogen, CA) as described previously [10].

2.7. siRNA directed against gal-1 expression in ATRFLOX cells

Human gal-1 siRNA against *LGALS1* and control siRNA-A (scrambled siRNA sequence) were purchased from Santa Cruz Biotechnology, Inc., CA. Transfection of cells with these siRNA's was carried out using Lipofectamine 2000 reagent.

2.8. Wound healing assay

To determine cell migration, 3×10^5 cells transfected with gal-1 plasmid and vector control were seeded on 35 mm plates and allowed to grow to complete confluence. Cells were serum-starved by growing in 0.02% serum-containing medium for 24 h. Subsequently, a 10 µl plastic pipette tip was used to scratch the cell monolayer to create a cleared area and the loosened cells were removed. Cells were then re-fed with fresh medium containing 10% FBS. Immediately following scratch wounding (0 h) and after incubation of cells at 37 °C for 24, 48 h and 72 h, bright field images (10x fields) of the wound-widths were photographed digitally with an Olympus IX81 microscope. The average width of the entire length of the wound measured on the images obtained at 0 h was set at 100%, and the mean percentage of the wound remaining in the subsequent time points was calculated.

2.9. Cell viability assay

Cell proliferation assay was performed using CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega) according to the supplier protocol. Briefly, 2×10^4 cells were plated per well in 24-well plates in growth media containing 10% fetal bovine serum. MTS assay was carried out at 4, 24 and 48 h by adding 100 µl of the combined MTS/PMS (20:1, v/v) ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]/(phenazine methosulfate) solution into each well containing 500 µl of the culture medium and incubated in 37 °C incubator for 30 min. The absorbance at 490 nm was recorded using ELISA plate reader. Each treatment was performed in hexaplicates and values were averaged. Reagent blank was used as control.

2.10. Cell motility assay

Transwell inserts (Corning Costar) with polycarbonate filters containing 8 μm pores were used for the motility assay. Cells (100,000 per insert) in 100–150 μl serum-free medium were plated and allowed to migrate towards complete media for 24 h. Non-migrated cells were removed and nuclei of migrated cells were stained with hematoxylin. Membranes were mounted on glass slides and the hematoxylin-stained cells in the entire membrane were counted under a microscope.

2.11. Cell cycle analysis

Cell cycle analysis was carried by flow cytometry (FACScan Instrument, Becton Dickinson) using the data acquisition CellQuest software, as reported by others [11]. Briefly, subsequent to transfections, cells were washed in PBS and fixed in 70% ethanol. Cells were then washed in PBS and stained with propidium iodide (PI) (5 $\mu\text{g/ml}$) solution, and analyzed for PI fluorescence by flow cytometry.

2.12. Apoptosis assay

Detection of cell apoptosis was carried out using Annexin V-FITC Apoptosis Detection Kit (EMD Biosciences) according to the supplier's recommendations. Cells treated with 5 mM sodium butyrate and, cells transfected with gal-1 plasmid and vector and subsequently treated with 5 μM camptothecin (CPT) for 4 h, were analyzed. Cells transiently expressing gal-1 were incubated in the presence of caspase-3/7 peptide inhibitor I (Calbiochem, Cat. No. 218826) for 24 h and then analyzed. Cells were harvested and treated with annexin V-FITC and PI as recommended by the supplier. Samples were then analyzed by FACScan flow cytometry followed by data analysis using FlowJo software.

2.13. TMRM assay

Transfected cells were washed and resuspended in PBS containing tetramethylrhodamine methyl ester (TMRM) (50 nM) and incubated in dark at 37°C for 30 min. Cells were washed and resuspended in PBS. The mitochondrial membrane potential was measured by FACScan flow cytometry of 10,000 cells and the percent change in TMRM fluorescence intensity was quantified using FlowJo software.

2.14. Other methods

Immunocytochemistry, protein estimation, SDS-PAGE and western blotting analysis were carried as described previously [8].

2.15. Statistical Analysis

Each of the experiments presented in this paper was carried out at least three different times using cells and protein samples obtained from different experiments, with essentially identical results. Statistical analyses between different treatments or groups were determined using t-tests, one-way ANOVA and post hoc multiple range testing as appropriate, using the GraphPad Prism software. Each of the columns represent mean with s.e.m., * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

3. RESULTS

3.1. Differential expression of gal-1 in colorectal cancer cell lines

As a first step toward understanding the function of gal-1, we profiled its expression in different CRC cell lines using RT-PCR and western blotting analyses. Fig. 1A shows the RT-PCR analysis, which indicated that ATRFLOX and HCT-116 cells contained high level

of gal-1 transcript, when compared to HT-29, LS-180 and Caco-2 cells, which contained residual levels (Fig. 1A). Western blot analysis (Fig. 1B) showed that ATRFLOX and HCT-116 cells expressed 14.5 kDa gal-1, whereas, gal-1 was undetectable in LS-180, Caco-2 and HT-29 cells, which corresponded with that of the RT-PCR analysis. HFF-2 cells, previously shown to express gal-1 [8], was used as a positive control. We chose LS-180 cells in most of the further studies as a model cell line as these cells are amenable to high transfection efficiency.

3.2. Butyrate induced gal-1 expression and apoptosis

Lu and Lotan [12] have previously demonstrated that butyrate transactivates the mouse gal-1 transcription by modulating the Sp1 binding to the *LGALS1* promoter. An analysis of the human *LGALS1* promoter using the Web-based Proscan algorithm indicated that the human *LGALS1* promoter contains several Sp1 binding sites, suggesting that butyrate may also upregulate the human gal-1 expression in CRC cells. To test this possibility, LS-180 cells were grown for 48 h in medium supplemented with different concentrations of butyrate and the gal-1 expression was determined by Westernblotting. Fig. 1C shows that cells treated with butyrate displayed *de novo* biosynthesis of gal-1, which was proportionally increased with butyrate concentration. However, we also noticed that the cell viability appeared to be affected as judged by the presence of floaters in the medium in butyrate treated cells. To test if butyrate induced apoptosis, cells were grown in medium containing 5 mM butyrate for 24 h and then analyzed for annexin V positivity. Fig. 1D shows that treatment with butyrate significantly increased the number of cells undergoing apoptosis and necrosis.

3.3. Gal-1 promoter was hypermethylated and demethylation reactivated its expression

To determine if gal-1 expression played a role in the induction of apoptosis in butyrate-treated cells, we examined the human *LGALS1* promoter for the presence of CpG-islands, a target of methylation, by Methprimer Algorithm [13]. The *LGALS1* gene promoter sequence, a 3.0 kb DNA sequence extending from transcription start site (0 bp) to upstream -3.0 kb, was retrieved from the Ensembl genome server and analyzed for the presence of CpG islands (www.ensembl.org/). Although this analysis revealed several CpG-islands, the CpG-rich sequence at -499 to -614 bp region (upstream to transcription start site) was identified as a strong candidate with greater than 60% GC content (observed/expected CpG > 0.6, length of CpG island > 100) (Fig. 2A). To test whether this CpG-rich sequence was hypermethylated, we analyzed its methylation status in representative colon cancer cell lines by methylation-specific PCR as described under Materials and Methods. Fig. 2B shows that PCR amplified the expected sized DNA fragment in the presence of M-specific primer set only in Caco-2 and LS-180 cells, although the amount of PCR amplified DNA was high in the former. A basal amount of unmethylated DNA was amplified with U-specific primer set in LS-180, which was not detectable in Caco-2 cells. Together, these data supported the prediction that the CpG-rich sequence at -499 to -614 bp region in *LGALS1* promoter was methylated. Small amount of unmethylated DNA was amplified with U-specific primer set but not with M-specific primer set, in HCT-116 and ATRFLOX cells, suggesting the unmethylated state of the above CpG region in these cells. In comparison, the gal-1 transcription and expression analyses presented in Figs. 1A and B, these data together suggested that methylation at CpG-rich sequence at -499 to -614 bp region in *LGALS1* promoter played a key role in silencing the *LGALS1* transcription in Caco-2 and LS-180 cells.

To test the above interpretation that promoter methylation was involved in silencing the gal-1 expression, Caco-2 and LS-180 cells were subjected to demethylation using 5-AzaC as described under Materials and Methods and analyzed for gal-1 expression by RT-PCR and western blotting. Fig. 2C shows that treatment with 5-AzaC resulted in an increase in the

level of gal-1 mRNA in both of these cell lines. Fig. 2D shows that originally gal-1 negative Caco-2 and LS-180 cells displayed gal-1 expression following 5-AzaC treatment. Together, these analyses revealed that promoter methylation was involved in silencing the *LGALS1* transcription in these two CRC cell lines.

3.4. Transiently expressed gal-1 was intracellularly located

Although the above experiments involving butyrate and 5-AzaC treatments induced gal-1 expression, it was also possible that these chemical agents altered the expression of a large number of genes, thus precluding in firmly assigning apoptotic function to gal-1. To determine that gal-1 specifically played a role in the induction of apoptosis, LS-180 cells were transfected with gal-1 plasmids as these cells were transfected with high efficiency as judged by the expression of fluorescent GFP in greater than 80% of the cells transfected with pEGFP plasmid (not shown). This high transfection efficiency is suitable to determine the functional role of gal-1. Western blot analysis of LS-180 cells transfected with gal-1 plasmid indicated high level of gal-1 expression (Fig. 3A). To determine the cellular location of transiently expressed gal-1, immunocytochemistry was carried out, which clearly indicated that gal-1 was localized intracellularly (Fig. 3B). To determine if gal-1 was secreted into the extracellular medium and bound to the cell surface, gal-1 in the spent growth medium was immunoprecipitated and analyzed by Western blotting, as described previously [8]. Results of this experiment did not indicate the presence of gal-1 in these immunoprecipitates (not shown), suggesting that the expressed gal-1 was not secreted by these cells.

To identify if gal-1 was bound to the cell surface, flow cytometry was employed. As a positive control, CRC cell line, SW620 was used as it constitutively expresses gal-1 [14]. Fig. 3C shows the fluorescence peak of SW620 cells incubated with goat preimmune serum (solid line, upper panel). Upon incubation with anti-gal-1 antibody (raised in goat, R&D systems), the fluorescence intensity was increased as evidenced by the peak shift to the right, suggesting that binding of anti-gal-1 antibody to the cell surface. This analysis suggested that flow cytometric procedure is suitable to determine the cell surface-bound gal-1. Fig. 3C, middle panel, shows the flow cytometric analysis of LS-180 cells transfected with vector control. The fluorescent intensity obtained with anti-gal-1 antibody was identical to that of preimmune serum, suggesting the absence of surface-bound gal-1. Importantly, LS-180 cells transiently expressing gal-1 did not exhibit any increase in fluorescence intensity, when compared to preimmune serum (Fig. 3C, lower panel). These results suggested that transiently expressed gal-1 was absent at the cell surface, corroborating the above results. Thus, the absence of cell surface-bound gal-1 in LS-180 cells suggested that this cell line is ideal for studying the function of intracellular gal-1.

3.5. Gal-1 retarded cell growth and arrested cell division cycle

We examined cell proliferation of gal-1 transfected LS-180 cells by the cell viability assay as described under Materials and Methods. Fig. 4A shows that cells transiently expressing gal-1 exhibited a significant decrease ($P < 0.001$) in cell proliferation when compared to control. To investigate the mechanism underlying the anti-proliferative effects, we analyzed the cell cycle distribution by flow cytometry. Fig. 4B shows that cells transfected with gal-1 plasmid contained an increased population of cells in G₀/G₁ phase (52%) when compared to vector control (39%). Examination of cell migration by wound healing assay indicated that expression of gal-1 significantly decreased cell migration (Fig. 4C). In addition, there was a significant decrease ($P < 0.001$) in the number of gal-1 transfected LS-180 cells invaded through the membrane filter, when compared to control (Fig. 4D). These results suggested that gal-1 negatively regulates cell cycle, leading to its inhibitory effect on cell proliferation, migration and motility of LS-180 cells.

3.6. Gal-1 expression led to down-regulation of cell proliferative pathways

To determine the mechanisms that were affected by the gal-1 expression, LS-180 cells transiently expressing gal-1 were analyzed for changes in the levels of various cell-signaling proteins by Western blotting. Fig. 5A shows that cells expressing gal-1 contained reduced level of phospho-IKK α/β , a key protein in the NF- κ B signaling. Since phospho-IKK α/β activates p65 through phosphorylation of residue ⁵³⁶S in p65, the level of phospho-p65 was analyzed using phospho-⁵³⁶S antibody. Fig. 5A shows that phospho-p65 was essentially absent in gal-1 expressing cells. There was a slight decrease in the total p65 level in gal-1 transfected cells. These results suggested that gal-1 down-regulated the NF- κ B signaling pathway through inhibition of the IKK α/β and p65 phosphorylation.

Since Wnt signaling is highly active in CRC [15,16]; we also analyzed the effects of gal-1 expression on this pathway. Fig. 5B shows that cells expressing gal-1 contained significantly decreased β -catenin level. Since β -catenin regulates the expression of transcription factors, TCF-1 and TCF-3 [17,18], the levels of these proteins were also analyzed. Fig. 5B shows cells expressing gal-1 contained reduced levels of TCF-1 and TCF-3. Since gal-1 expression led to cell cycle arrest at G₀/G₁ phase, we examined if gal-1 induced changes the phosphorylation of retinoblastoma protein (Rb) and protein levels of cyclin D1 and p21. Fig. 5C shows a significant reduction in phosphorylated Rb (p-Rb) and total cyclin D1, and an increase in the total p21 in cells expressing gal-1. As an alternative approach to establish these effects of gal-1, gal-1 was knocked down with siRNA in ATRFLOX cells, which led to a significant decrease in the levels of p21 and considerable increase in the TCF-1 level, when compared to control cells transfected with siRNA-A (Fig. 5D). Since down-regulation of either cyclin D1 or up-regulation of p21 is known to cause Rb dephosphorylation and growth arrest [19], these results suggested that the cell cycle arrest at G₀/G₁ arrest induced by gal-1 involved dephosphorylation of Rb and increased p21 level.

3.7. Gal-1-induced apoptosis

To determine if gal-1 was involved in the induction of apoptosis, cells transfected with vector and gal-1 plasmids were analyzed by flow cytometry annexin V-FITC positivity as described under Materials and Methods. Fig. 6A shows that LS-180 cells expressing gal-1 contained significantly increased apoptotic cell population ($P < 0.001$) when compared to control. We further investigated whether gal-1 expression results in chemosensitivity to CPT, an agent that is known to induce apoptosis in human gastric cancer cells [20]. Fig. 6B shows that incubation of gal-1 expressing cells with 5 μ M CPT for 4 h increased the percent apoptotic cells by ~ 3 fold. These results suggested that gal-1 expression induced apoptosis and increased susceptibility to CPT-induced apoptosis in LS-180 cells.

3.8. Gal-1-induced apoptosis involved loss of mitochondrial membrane potential (MMP)

Since mitochondrial permeability alterations are closely associated with apoptosis, we investigated the changes in MMP in gal-1 expressing LS-180 cells by TMRM assay as described under Materials and Methods. Fig. 6C shows that cells transfected with vector control contained 4.89% cells exhibiting reduced TMRM fluorescence, whereas, 42.7% cells in gal-1-transfected cells exhibited reduced TMRM fluorescence. Since reduced TMRM fluorescence is an indicator of MMP loss, these data suggested that gal-1 expression was responsible for the loss of MMP. Since MMP loss is associated with altered expression of anti-apoptotic bcl-2 family of proteins [21], we analyzed the status of these proteins. Fig. 6D shows that marked decrease in Bcl_{XL} expression in gal-1 expressing cells. However, the Bcl-2 and Bax levels in gal-1 expressing cells were essentially unaffected.

3.9. Gal-1 activated caspase cascade

To establish that gal-1 induced apoptosis, we examined the activation of the classical caspases in gal-1 expressing cells by Westernblotting. Fig. 6E shows that cells expressing gal-1 contained the 17 kDa cleaved caspase-3 fragment, and 20 kDa cleaved caspase-7 fragment. The 116 kDa poly (ADP-ribose) polymerase-1 (PARP-1) is normally involved in DNA repair and DNA stability, and is cleaved by members of the caspase family during apoptosis, releasing the 89 kDa fragment of PARP-1 [22]. Fig. 6E shows that gal-1 expressing cells contained the 89 kDa PARP fragment. To further determine that caspase activation was responsible for the observed apoptosis, LS-180 cells were transfected with gal-1 for 36 h and then supplemented with caspase-3/7 inhibitor I for additional 24 h. Cells were then analyzed for annexin V-FITC positivity by flow cytometry and the results are shown in Fig. 6F. Percent apoptotic population in gal-1 transfected cells treated with DMSO was considered 100% and the percent of apoptosis in cells treated with caspase-3/7 inhibitor I was normalized. There was a significant decrease in apoptosis in cells treated with caspase-3/7 inhibitor I, suggesting that gal-1 induces apoptosis in LS-180 cells through activation of caspases-3/7.

4. DISCUSSION

An understanding of the molecular mechanisms involved in the CRC onset and progression and the mechanisms by which the body defense controls cancer progression are important requisites in the design of targeted treatment. A large body of evidence indicates that galectins mediate a plethora of cellular functions, making them new molecular targets of cancer therapy. In this regard, gal-1 qualifies as a potential molecular target for therapy [23]. However, the expression or functional role of intracellular gal-1 in CRC is unclear at present. Previous studies showed that gal-1 expression is associated with stroma and the epithelial cells lining the crypts [24], while others have observed that gal-1 is solely restricted to the fibroblasts localized in the stromal regions surrounding the crypts in CRC [24–28]. However, it is possible that the differential gal-1 expression observed in these studies is a reflection of the heterogeneity of the disease itself. On the other hand, the demonstration that fibroblasts localized in the stromal region surrounding the normal as well as CRC tissues express gal-1 abundantly and the fact that gal-1 is a secretory protein together suggests that the extracellular gal-1 influences CRC progression and control. Interestingly, Adams *et al.* [29] have shown that higher concentrations of extracellular gal-1 inhibits cell growth. Importantly, van den Brule *et al.* [30], have shown that gal-1 accumulated in the stromal tissue surrounding carcinomas decreases cell proliferation of ovarian cancer. In addition, tumor-secreted gal-1 selectively induces apoptosis in activated T-cells [6,31]. These observations together raise a possibility that the secreted gal-1 inhibits cell proliferation and induces apoptosis in susceptible cells. Interestingly, not all CRC cells appear to be adversely affected by the secreted gal-1. Horiguchi, *et al.* [32] did not detect any apoptosis in CRC Colo201 cells supplemented with extracellular gal-1. While the secreted gal-1 has been shown to interact with the extracellular glycans of cell-surface proteins including fibronectins, integrins and laminins, it is its interaction with $\alpha 5\beta 1$ fibronectin receptor that determines growth inhibitory and apoptotic functions of gal-1 [33]. It thus appears reasonably clear that tumors have adapted mechanisms to fend off growth inhibitory and apoptotic effects of extracellular gal-1 through elimination of the gal-1 receptor.

As a first step toward understanding the function of intracellular gal-1, we have undertaken task of profiling the gal-1 expression in five different CRC cell lines, the results of which were in agreement with the observations of Lahm and co-workers [14], who have reported that CRC cells differentially express gal-1. Early studies carried out in Lotan's laboratory have shown that butyrate is an inhibitor of cell proliferation, and subsequently demonstrated

that butyrate modulates Sp1 binding to the mouse gal-1 promoter and induces gal-1 expression [12]. Interestingly, Ruemmele et al.[34] have shown that butyrate induces apoptosis in CRC Caco-2 cells through disruption of mitochondrial integrity and caspase activation. Although these studies did not directly implicate gal-1 in the induction of apoptosis, here we demonstrated that gal-1 induces apoptosis. We further demonstrated that the gal-1-induced apoptosis involves MMP collapse, decreased Bcl_{XL} and activated caspases. The data that gal-1 expression confers the cells with increased sensitivity to apoptotic agents, raises a distinct possibility that gal-1-expressing tumors can be treated with apoptotic agents. Together, these data support the view that gal-1 is a key player in the induction of apoptosis.

Data presented here indicates that gal-1 interferes with cell proliferation by affecting the NF- κ B and Wnt signaling pathways, which are frequently dysregulated in CRC [15,35]. The observations that gal-1 expression led to the loss of activated IKK α/β and p65 and, the loss of TCF-1 and -3 transcription factors together suggest that gal-1 is a negative regulator of the NF- κ B and Wnt signaling. The mechanism of gal-1 induced cell cycle arrest involves down-regulation of cyclin D1 and upregulation of CDK inhibitor p21, causing inhibition of CDK activity and dephosphorylation of Rb. The growth inhibition observed upon gal-1 expression extends support to the view that this lectin is a negative regulator of the NF- κ B and Wnt signaling pathways. However, it is unclear as to the mechanisms by which these signaling pathways are regulated in CRC cells expressing endogenous gal-1. It is possible that the negative regulation of these signaling pathways require higher amounts of gal-1. It is equally possible that these cells have adapted compensatory mechanisms to overcome the negative effects of endogenous gal-1.

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Figure 1. Gal-1 expression analysis

(A). *RT-PCR analysis*. Five μ g of total RNA isolated from each cell line indicated in the figure, was analyzed for the presence of gal-1 transcript by RT-PCR. Equal volumes of these PCR mixtures were separated on 1% agarose gels containing ethidium bromide. GAPDH was amplified as an internal control. (B). *Western blot analysis*. Equal amount of total cell lysates (20 μ g) obtained from the indicated cell lines was separated on 4–20% acrylamide gradient SDS-PAGE gels followed by immunoblotting using anti-gal-1 antibody. Gal-1 and β -actin were identified with arrows. Standard protein markers were loaded in the far right lane. (C). *Effect of butyrate on gal-1 expression*. LS-180 cells were treated with the indicated concentrations of sodium butyrate prepared in PBS (untreated was indicated with C) for 48 h and analyzed for gal-1 expression in equal amount of cell lysates (20 μ g). β -actin was stained to indicate equal protein loading. (D). *Analysis of butyrate induced apoptosis*.

Cells treated with 0 (control) and 5 mM butyrate were subjected to apoptosis assay by flow cytometry as described in the Materials and Methods section. The *ordinates* show the degree of staining with propidium iodide (FL2-H), indicative of necrosis or late apoptosis, whereas the *abscissas* show the fluorescence intensity due to annexin V-FITC binding (FL1-H), indicative of apoptosis. The percentages of cells in quadrants, Q1 (necrosis), Q2 (late apoptosis), Q3 (early apoptosis) and Q4 (unstained/live cells) were shown.

Figure 2. Analysis of LGALS1 promoter methylation

(A). *MethPrimer* analysis of *LGALS1* promoter. Promoter region of *LGALS1* was analyzed by *MethPrimer* algorithm as described in Results. The shaded areas represent the CpG clusters. **(B).** *Methylation-specific PCR*. Bisulfite-treated genomic DNA from the indicated cell lines was amplified using Methylated- (M) and Unmethylated- (U) specific primers of *LGALS1* promoter, as described under Materials and Methods section. The universal unmethylated and methylated human DNA standards were used as control. **(C and D).** *Effect of 5-AzaC on gal-1 expression*. LS-180 and Caco-2 cells were treated with 0 (C) and 30 (T) μ M 5-AzaC and analyzed by RT-PCR (*Panel C*) and western blotting (*Panel D*) for gal-1 expression. GAPDH was used as loading control for RT-PCR. β -Actin was developed to indicate equal protein loading in the western blot.

Figure 3. Gal-1 expression in LS-180 cells. (A). Transient expression of gal-1

LS-180 cells were transfected with vector (C) or gal-1 plasmid (Gal-1) and analyzed by Western blotting at the indicated time intervals for gal-1 expression. (B).

Immunocytochemistry of gal-1 in LS-180 cells. The cells were stained with anti-gal-1 antibody followed by Alexa Fluor-488 coupled secondary antibody. The nuclei were stained with propidium iodide (PI). Images were obtained using Leica Confocal Laser Microscope. The scale bar represents 10 microns. (C). *Characterization of cell surface-bound gal-1.* Cell surface-bound gal-1 in SW620 (upper panel), and LS-180 (middle and lower panels) cells was analyzed by flow cytometric analysis as described under Materials and Methods section. Fluorescence peaks obtained with preimmune serum (solid line) and anti-gal-1 antibody (dotted line) were shown.

Figure 4. Effects of gal-1 on cellular processes

(A). *Cell proliferation assay*. Vector and gal-1 plasmid transfected cells were subjected to MTS proliferation assay as described under Materials and Methods. (B). *Cell cycle analysis*. The transfected cells were subjected to cell cycle analysis by flow cytometry and the G₁, S and G₂/M phase distribution of cells was quantified using FlowJo software. (C). *Wound healing assay*. The transiently transfected cells were used in the wound healing assay as described under Materials and Methods. (D). *Cell motility assay*. The transiently transfected cells were used in motility assay as described under Materials and Methods. * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$.

Figure 5. Effects of gal-1 on cell signaling

LS-180 cells were transiently transfected with vector (C) and gal-1 plasmid (Gal-1) for 24 h. Cell lysates (20 µg each) were analyzed for: **Panel: A.** NF-κB signaling molecules - p65, P-p65 and P-IKK α/β; **Panel: B.** Wnt signaling molecules - β-catenin (β-cat), TCF-1 and TCF-3 and **Panel: C.** Cell cycle molecules – cyclin D1, phospho-Rb (p-Rb) and p21. **(D).** Effect of gal-1 knockdown on cell signaling. ATRFLOX cells were transfected either with gal-1 siRNA (siRNA) or control siRNA-A (C) for 48 h and analyzed for the expression of gal-1, p21 and TCF-1. β-actin was stained to indicate equal protein loading.

Figure 6. Analysis of gal-1 induced apoptosis in LS-180 cells

(A). *Apoptosis assay*. Cells transiently transfected with gal-1 plasmid and vector for up to 48 h and then subjected to apoptosis assay as described in the Materials and Methods section. (B). *The effect of CPT on gal-1 induced apoptosis*. Cells transfected as above were subjected to apoptosis assay in the presence of 5 μ M CPT or DMSO. (C). *TMRM assay*. Transfected cells were subjected to TMRM assay. Percent change in the TMRM fluorescence in the cells was analyzed using flow cytometry. (D and E). *Western blot analysis of different cellular proteins*. Lysates obtained from cells transfected with vector (C) or gal-1 plasmid (Gal-1) were subjected to Western blotting for detecting the indicated proteins. (F). *Effect of caspase inhibitors on gal-1-induced apoptosis*. Apoptosis assay was carried out on gal-1 plasmid-transfected cells in the presence of DMSO (vehicle control) and 60 and 170 nM caspase-3/7 inhibitor I. ** indicates $P < 0.01$ and *** indicates $P < 0.001$.