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# Galectin-3 binding protein promotes cell motility in colon cancer by stimulating the shedding of protein tyrosine phosphatase kappa by proprotein convertase 5

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ABSTRACT

It has previously been reported that shedding of the PTPκ ectodomain drives enhanced motility of colon cancer cells. Herein, we provide mechanism underlying the regulation of PTPκ shedding by galectin-3 binding protein. PTPκ was inarguably scissored by the processed form of proprotein convertase 5 (subtilisin/kexin type 5), and galectin-3 binding protein which is over-produced in colon cancer cells and tissues contributed to increased cancer cell motility by acting as a negative regulator of galectin-3 at the cell surface. The high expression ratio of galectin-3 binding protein to galectin-3 was clinically correlated to lymphatic invasion. These results suggest that galectin-3 binding protein may be a potential therapeutic target for treatment of, at least, colon cancer patients with high expression of galectin-3 binding protein.


## 1. Introduction

Cancer is accompanied by multiple features that are advantageous to the proliferation and survival of cells, including cell invasion, migration, immune avoidance, and anti-apoptosis. Although often overlooked, the proteolytic cleavage of cell-surface proteins is one common feature of cancers, and contributes to tumor progression by several mechanisms [1]. Some useful biomarkers are, in fact, the cleavage products of membrane proteins, such as the soluble form of Her2/neu [2]. Many lines of evidence indicate that a myriad of protease family members, including a disintegrin and metalloproteinases (ADAMs) and matrix metalloproteinases (MMPs), are actively secreted, resulting in the proteolytic cleavages of membrane proteins, termed protein shedding. Protein shedding is not only responsible for proper function of precursor proteins following the cleavage of an N- or C-terminal region, but the degradative process also leads to pathological conditions; for example, the increased availability of growth factors and/or decreased adhesion forces of cells [3,4]. Protein tyrosine phosphatase kappa (PTPκ) is one of the proteins known to be cleaved from the plasma membrane to facilitate cancer cell migration during cancer progression [5]. However, the mechanism underlying the shedding process has not been fully elucidated, especially in terms of the proteases and regulators implicated.

Galectin-3 binding protein, also known as Mac-2-binding protein, is a secreted glycoprotein. Increased levels of galectin-3 binding protein have been observed in cancer tissues and sera, and have been demonstrated to be associated with poor prognosis [6]. Despite the enhanced secretion of the protein by tumor cells, the role of galectin-3 binding protein in cancer progression has not been fully elucidated, although some reports have implicated the protein in cell adhesion [7] and in secretion of proteases, including proMMP-7 [8]. In addition, despite the fact that galectin-3 binding protein is known to bind to galectin-3, little is known about the interaction or the effect of this interaction on cancer progression [9].

Herein, we report that PTPκ is cleaved by a processed form of proprotein convertase 5 (PC5A) and that galectin-3 binding protein and galectin-3 emulate each other for PTPκ cleavage independent of PC5A expression. Given the cancer-specific expression of galectin-3 binding protein, it may represent an attractive target for molecularly-targeted therapy in colon cancer.

*Abbreviations:* ADAM, a disintegrin and metalloproteinase; LacNAc, N-acetyllactosamine; LGALS3, galectin-3; LGALS3BP, galectin-3 binding protein; MMP, matrix metalloproteinase; PC5, proprotein convertase 5; PTPκ, protein tyrosine phosphatase kappa.

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## 2. Materials and methods

### 2.1. Cell culture

WiDr, a derivative of the human colonic adenocarcinoma cell line HT-29, was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% $CO_2$.

### 2.2. Clinical samples

Tissue samples were obtained from colorectal cancer patients at Our Lady of Mercy Hospital at The Catholic University of Korea (Inchon, Korea) and from healthy volunteers at KRIBB (Daejeon, Korea), with agreement to participate obtained from all subjects. Tissues were ground with a mortar and pestle in the presence of sea sand, and clear protein preparations were obtained following centrifugation and filtration through a 0.45-μm syringe filter.

### 2.3. Stable transfectants

The human PC5A gene was cloned into the pcDNA 3.1 hygro(+) plasmid vector (Invitrogen), and the galectin-3 and galectin-3 binding protein genes were cloned into the pcDNA 3.3 Topo vector (Invitrogen). Stable transfectants of cells with silenced expression of the proteins were also established by transfecting the cells with shRNA vectors (Sigma). The inserted DNA sequences for gene silencing are as follows: PC5A, 5′- CCGGCCTGTGAAGATGGACGGT ATTCTCGAGAA TACCGTCCATCTTCACAGGTTTTTG-3′ and 5′-CGGGCCCAGTCTAC CTA TTTCAATCTCGAGATTGAAATAGGTAGACTGGGCTTTTTG-3′; galectin-3, 5′-CC GGCTCACAGTAACCCTTTCACCACTCGAGTGGTGAAAGG GTTACTGTGAGTTTTTTG -3′ ; galectin-3 binding protein, 5′-CCGGCC TGGA CACCAACAGCTCGAACTCGAGT TCGAGCTGTTGGTGTCCAGG TTTTT-3′, 5′-CCGG GTCAGTCAAGTGCTTCCACAAC TCGAGTTGTGG AAGCACTTGACTGACTTTTT-3′ and 5′-CCGGCGGAAGTCACAACT GGT CTATCTCGAGATAGACCAGTTGTGACTTCCGTTTTT-3′. The recombinant and control vectors were transfected using an electroporator according to the manufacturer's instructions (NeonTM, Invitrogen). Each of the stable transfectants was selected by RT-PCR and immune-blot analysis.

### 2.4. Immuno-blot analyses

Proteins were resolved on 10–15% SDS–PAGE gels and transferred electrically onto PVDF membranes (Immobilon-P, Millipore). The membranes were blocked in 0.05% Tween 20-TBS plus 5% skim milk and then incubated with primary antibodies against galectin-3 (Abcam), galectin-3 binding protein (Sigma), PTPκ (Abcam), TIMP-1 (Sigma), and PC5A (Abcam). After incubation with HRP-labeled secondary antibodies (Cell Signaling), membranes were allowed to react with ECLTM Western blotting detection reagents (Pharmacia) and exposed to X-ray film for 1–2 min. The band intensity was calculated from the digitalized, scanned files using ImageJ software (http://rsbweb.nih.gov/ij/).

### 2.5. Immunofluorescence

Mock and stably transfected cells were trypsinized and plated on cover slips in 6-well plates for 24 h. Cell mono-layers were washed with PBS, fixed with 4% paraformaldehyde, and completely washed with excess PBS-Tween 20 (0.02% v/v). After blocking with 1 % (w/v) BSA, cells were treated with rabbit polyclonal antibody raised against PTPκ overnight at 4 °C, as mentioned described previously [5]. Cells were washed with PBS buffer three times for 5 min, and treated with goat anti-rabbit immunoglobulin G antibody conjugated with TRITC fluorescent dye (Santa Cruz Biotechnology) in the blocking buffer at room temperature for 1 h. The cells were subsequently washed with excess volumes of PBS buffer four times and stained with DAPI (Sigma). Finally, each slide was examined by confocal fluorescence microscopy (LSM 510 Meta, Zeiss). Representative images were chosen and digitally recorded at the same sensitivity and magnification.

### 2.6. Cell migration assay

Cell migration assays were performed using 12-well Transwell chambers (Corning) with polycarbonate inserts containing 8-μm pores, as described elsewhere [10]. Briefly, $2 \times 10^5$ cells were placed inside the upper chamber and maintained in RPMI 1640 medium plus 1% BSA. Cells were incubated for 12 h to migrate to the lower chamber containing culture medium and fibronectin (100 ng/ml). Cells that had migrated across the porous membrane were fixed in methanol, stained with Toluidine blue, and counted with a microscope at 400×.

## 3. Results

### 3.1. PC5A cleaves the ectodomain of PTPκ in WiDr colon cancer cells

Protein tyrosine phosphatase kappa (PTPκ) has dual functions: one involves dephosphorylating intracellular target proteins and the other is in maintaining cell adhesions. We have previously reported that the increased PTPκ shedding results in the mitigated cell adhesions, promoting cancer cell migration in vitro [5]. However, how the shedding process of PTPκ is regulated during cancer progression has not been fully elucidated. Identification of the responsible protease(s) has been considered a key factor necessary to address this question.

The short form of proprotein convertase 5 (PC5A) is a secreted form of PC5 and is reportedly responsible for the cleavage of PTPμ [11]. To address the role of PC5 in PTPκ shedding, the in vitro cleavage of PTPκ by purified PC5A was investigated. PC5A tagged with 6xHis residues at the C-terminus was expressed in CHO cells and purified on a $Ni^{2+}$-column. Meanwhile, a fusion protein was produced in CHO cells by fusing the PTPκ ectodomain (PTPκECD) to the Fc gamma region of immunoglobulin G (IgGFc). The fusion protein was purified on a protein-G column and used as a substrate for PC5A. The limited proteolysis of PTPκECD-IgGFc fusion protein by PC5A revealed that extracellular domain of PTPκ was cleaved by PC5A in vitro (Fig. 1A). To confirm this, cell lines were generated in which the PC5A gene was silenced to different extents (Fig. 1B). The cleavage of PTPκ was also suppressed by the down-regulation of PC5A expression, which was dependent on the efficiency of PC5A silencing. As expected, the cleavage rate was increased in the PC5A-over-expressing WiDr cells compared to mock-transfected cells (Fig. 1C). Involvement of PC5A in the cleavage of PTPκ was further confirmed by a competition assay where a synthetic peptide, a part of the PTPκ ectodomain with the target RXXR sequence, was added into the culture media at the concentration of 5 or 20 μg/ml (Fig. 1D). The shedding of PTPκ was decreased by the addition of the synthetic peptide in a concentration-dependent manner.

A high cleavage rate of PTPκ would mean a decreased pool of the intact molecules remaining on the cell surface, so the level of intact PTPκ on the cell surface was investigated by immunofluorescence using an antibody recognizing the ectodomain of PTPκ. The levels of intact PTPκ molecules were observed to be significantly reduced at the surface of the PC5A-over-expressing cells, whereas intact PTPκ was present at an even higher concentration on the surface of the PC5A-silenced cells (Fig. 1E–G). These results, obtained from the independent approaches, indicate that PTPκ cleavage is catalyzed by PC5A at the least, although other proteases may be partly involved in PTPκ shedding.

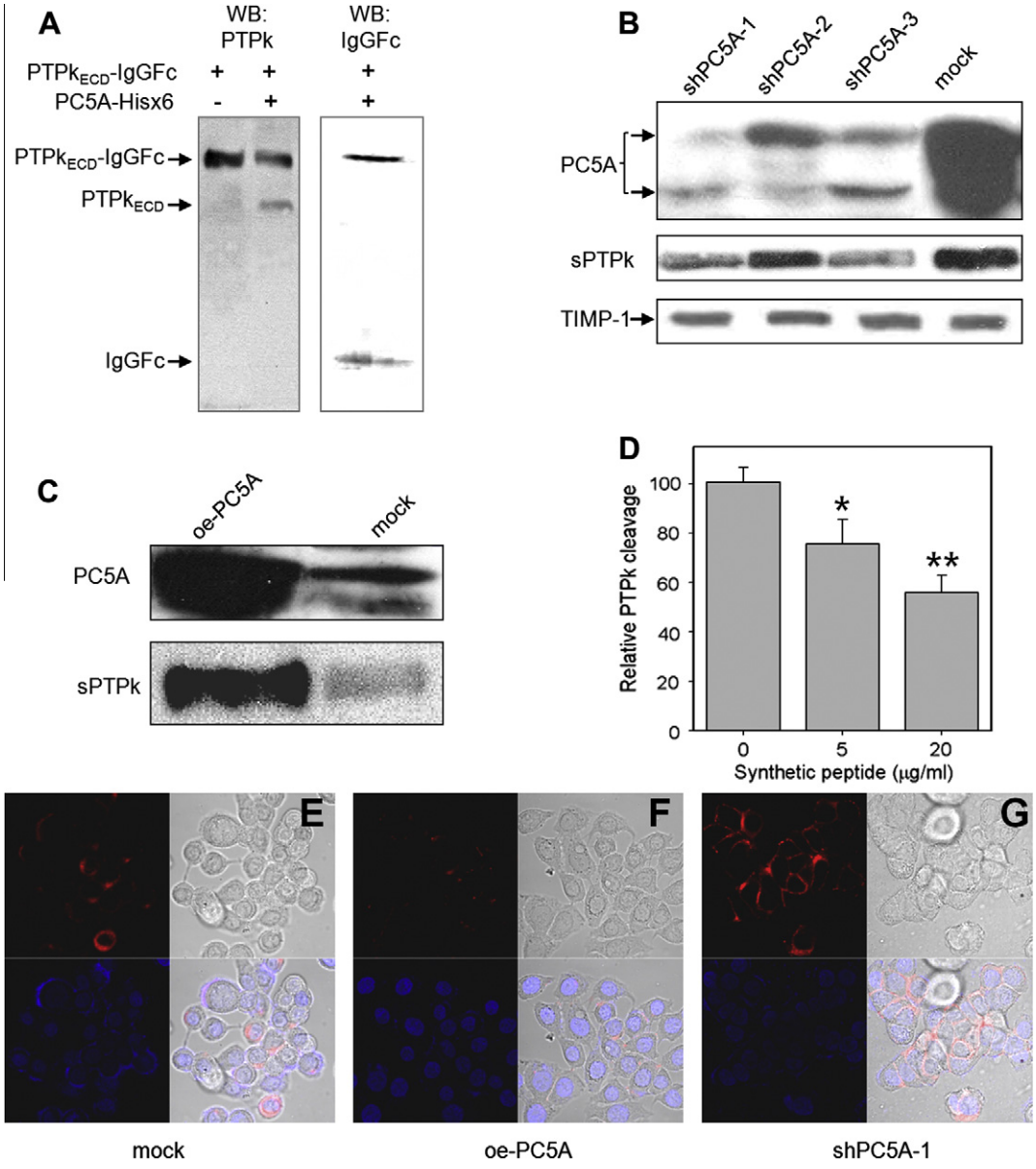


**Fig. 1.** Proprotein convertase 5A (PC5A) cleaves the extracellular domain of PTPκ. (A) Silencing of PC5A gene and the effect on PTPκ cleavage. Gene silencing allowed the establishment of PC5A-knockdown WiDr cells with various levels of gene suppression. The PC5A level was proportional to PTPκ cleavage products (sPTPκ). (B) Establishment of PC5A over-expressing WiDr cells and the effect on PTPκ cleavage. (C) Over-expression of PC5A (oe-PC5A) resulted in the higher PTPκ shedding compared to mock. (D) Competition study using a synthetic peptide for a fragment of PTPκ ectodomain. PTPκ cleavage by PC5A was competed with a synthetic peptide with sequence of ELHPHRTKREAGAME containing RXKR, a target sequence for PC5A. The values are averages with standard error obtained from the three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$. (E–G) Immunofluorescence of intact PTPκ on the surface of PC5A-manipulated cells. The manipulation of PC5A level in the extra-cellular compartment resulted in different levels of intact PTPκ remaining on the cell surface as assessed by anti-PTPκ antibody recognizing the PTPκ ectodomain.

### *3.2. The ratio of galectin-3 binding protein to galectin-3 affects PTPκ shedding*

Galectin-3 binding protein is one of the interaction partners for galectin-3 and is reportedly up-regulated in cancer cells [6]. To test whether galectin-3 binding protein is involved in the cleavage of PTPκ in colon cancer cells, alterations in PTPκ cleavage were monitored in several transfected cell variants in which the expression level of galectin-3 or galectin-3 binding protein had been manipulated. First, we established a galectin-3 binding protein knockdown cell line (Fig. 2A). The transfected cells exhibited diminished PTPκ cleavage. Conversely, when galectin-3 was silenced by stable expression of interference RNA, a higher cleavage rate of PTPκ cleavage was observed compared to the rate in mock-transfected cells. The manipulations to expressions of galectin-3 and galectin-3 binding protein had no influence on the expression of PC5A. These results indicate that galectin-3 binding protein, in contrast to galectin-3, positively regulates the cleavage of the PTPκ ectodomain, implying that PTPκ cleavage is affected by the ratio of galectin-3 binding protein to galectin-3.

Regulation of PTPκ cleavage by galectin-3 binding protein was further confirmed in cells that over-express galectin-3 binding protein. Because galectin-3 binding protein is already expressed at high level in the parental WiDr cells, we could not obtain transfected cells with a remarkable increase in expression compared to the parental cells. However, two transfectant cell-lines showed an increase in the ratio of galectin-3 binding protein to galectin-3 by 1.5- and 1.8-fold, respectively. Albeit not a marked increase,

the ratio was proportional to the extent of PTPκ cleavage in vitro (Fig. 2B).

The involvement of galectin-3 and galectin-3 binding protein in PTPκ cleavage was also confirmed through an immunofluorescence-based approach. PTPκ was more resistant to cleavage in cells where the galectin-3 binding protein had been silenced, and suppression of galectin-3 gene expression led to an increase in PTPκ shedding (Fig. 2C). These results indicate an involvement of galectin-3 binding protein in the cleavage of the cell-adhesion molecule, PTPκ.

### 3.3. Galectin-3 binding protein may facilitate PTPκ cleavage by reducing the cell surface-bound galectin-3

Galectin-3 binds preferentially to carbohydrates containing galactose, and N-acetyllactosamine (LacNAc) is one of the preferable natural ligands [12]. Based on the results depicted in Fig. 2, we hypothesized that binding of galectin-3 to the LacNAc residues of PTPκ interferes with the proteolytic action of PC5A, either by acting independently or by recruiting additional relevant interaction partners. To address this possibility, LacNAc was added to the serum-free media at 0.5 mM to compete with glycan substrates on glycoproteins, and PTPκ shedding was investigated. The addition of LacNAc produced more soluble PTPκ ectodomain when normalized to the amount of PC5A ($p < 0.01$), which was consistent with the result obtained when anti-galectin-3 polyclonal antibody was added to the culture media at 20 μg/ml. In addition, the simultaneous addition of the two components resulted in a cumulative effect on the cleavage (Fig. 3A).

The level of galectin-3 bound to the cell surface was traced in several transfected WiDr cell variants (Fig. 3B). WiDr cells were detached with EDTA and briefly washed in PBS buffer with gentle pipetting to avoid cell rupture by mechanical force. The cells were treated with 10 mM LacNAc to detach bound galectin-3 from the cell surfaces, and the levels of released galectin-3 were quantified by immune-blot analysis. Reduced expression of galectin-3 binding protein resulted in more galectin-3 remaining bound to cell surfaces. Manipulations to galectin-3 expression led to changes in the level of galectin-3 at the cell surface. Galectin-3 was not observed in the surface of cells that had been maintained in the presence of 10 mM LacNAc. Importantly, the amount of cell surface-bound galectin-3 was inversely proportional to the level of PTPκ cleavage. Based on these results, it is tempting to speculate that the cell surface-bound, or more specifically PTPκ-bound, galectin-3 is a negative regulator of PTPκ cleavage by PC5A. Conversely, galectin-3 binding protein appears to facilitate proteolysis by PC5A, possibly by directing galectin-3 from the cell surface to an unbound state from the cell surfaces. However, how cell surface-bound galectin-3 exerts the negative effect on the PTPκ cleavage remains elusive.

### 3.4. Galectin-3 binding protein is associated with cancer cell growth and migration

PTPκ has a dual function, one of which is related to cell–cell adhesion. PTPκ is a member of PTPμ subfamily that has a MEM domain at the N-terminal region, which participates in the

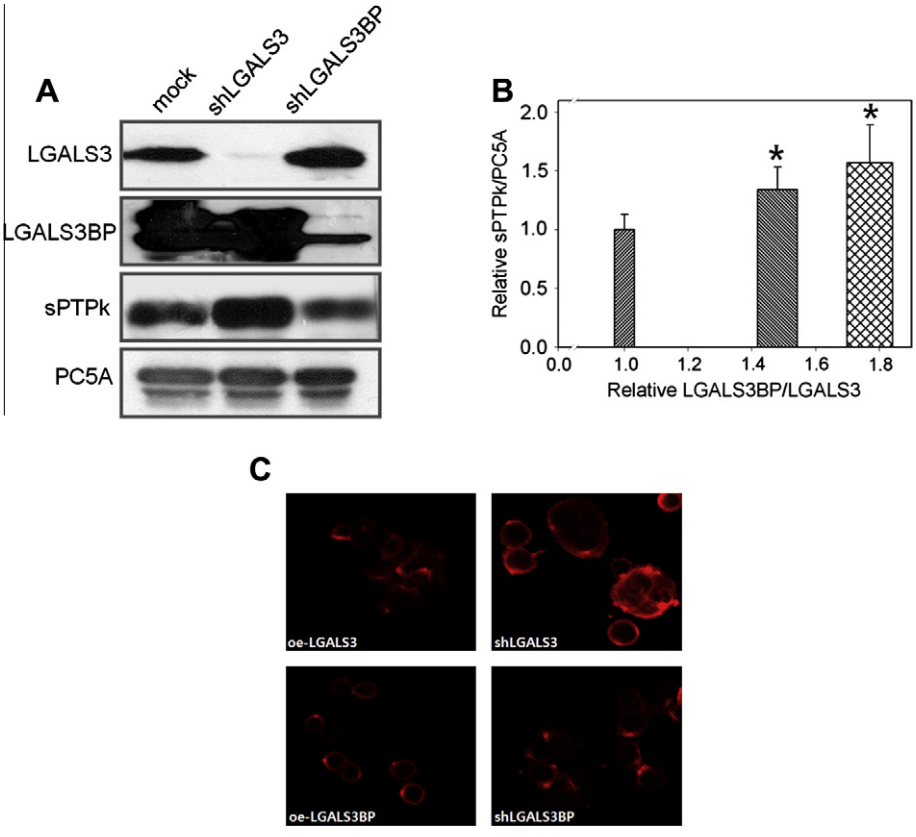


**Fig. 2.** High ratio of galectin-3 binding protein to galectin-3 results in elevated PTPκ shedding. (A) Suppression of galectin-3 and galectin-3 binding protein and the effect on PTPκ cleavage. Suppressed expression of galectin-3 resulted in an elevation of PTPκ cleavage, while that of galectin-3 binding protein was responsible for poor cleavage of PTPκ compared to mock. (B) Relationship between the ratio of galectin-3 binding protein to galectin-3 and PC5A-normalized PTPκ cleavage rate. The ratio is proportional to the PC5A cleavage rate in WiDr cells. The width of bars indicates the standard errors of each LGALS3BP/LGALS3 ratio ($N = 3$). *$p < 0.05$. (C) Immunofluorescence of intact PTPκ on the cell surfaces of various transfectant cell-lines. PTPκ cleavage is sensitized by the suppression of galectin-3, while over-expression of galectin-3 binding protein leads to stimulated PTPκ cleavages.

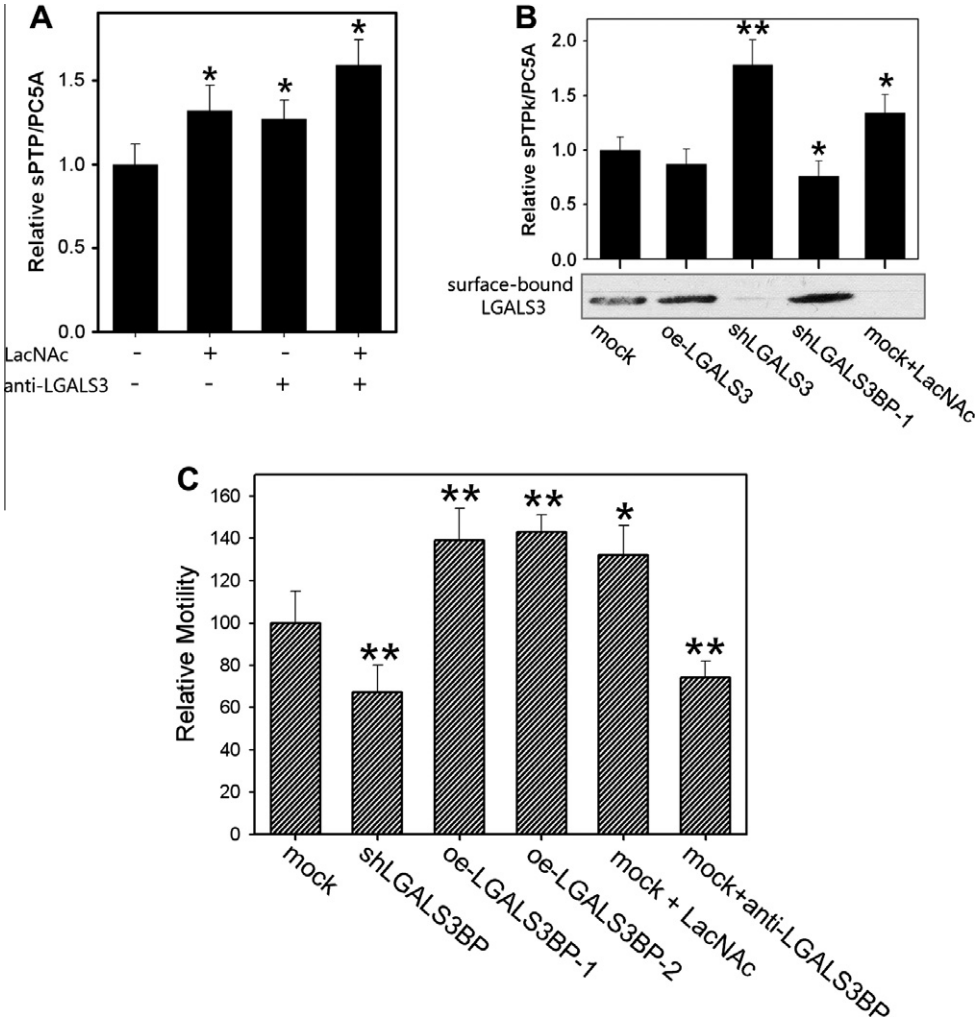


**Fig. 3.** Galectin-3 binding protein promotes cancer cell motility by acting as a negative regulator of cell-surface galectin-3 in PTPκ shedding. (A) Enhanced PTPκ cleavage by inhibition of cell surface binding of galectin-3. WiDr cells were treated with either N-acetyllactosamine (LacNAc) at 0.5 mM, anti-galectin-3 polyclonal antibody (10 μg/ml), or both for 2 days. The cleaved PTPκ product was normalized to the amount of PC5A, as assessed by immunoblot analysis. $N = 3$, $^{*}p < 0.05$; $^{**}p < 0.01$. (B) Effect of the regulated galectin-3 cell surface levels on PC5A-normalized PTPκ cleavage. Various transfected cells were incubated in serum-free RPMI 1640 media for 2 days, in the presence of 0.5 mM LacNAc when necessary, and the conditioned media was used for analysis of PTPκ cleavage products and PC5A. Cell mono-layers were detached from the plate with 5 mM EDTA and briefly washed with phosphate-buffered saline (PBS). The cell surface-bound galectin-3 was allowed to elute from the cells following incubation with 10 mM LacNAc in PBS for 1 h at room temperature. The eluate was used for immune-blot analysis of galectin-3. $N = 3$, $^{*}p < 0.05$; $^{**}p < 0.01$. (C) In vitro migration assay of various transfected and mock-transfected cells treated with LacNAc at 0.5 mM or anti-galectin-3 binding protein polyclonal antibody at 10 μg/ml, when necessary. High levels of effective galectin-3 binding protein relative to galectin-3 are related to cancer cell motility in vitro. $N = 3$, $^{*}p < 0.05$; $^{**}p < 0.01$.

homophilic interaction with the same domain in neighboring cells [13]. The involvement of PTPκ in cell adhesion was confirmed in a cancer cell-line, where shedding of PTPκ was responsible for higher cancer cell motility during cancer progression [5]. This prompted us to investigate the effect of expression of galectin-3 binding protein on in vitro cell migration.

As is shown in Fig. 3C, in vitro cell migration of WiDr cells was dependent on the expression level of galectin-3 binding protein. Suppressed galectin-3 binding protein decreased the cell motility compared to the parental cells, whereas high levels of galectin-3 binding protein led to increased migration. Furthermore, treatment with LacNAc to remove galectin-3 from the cell surface led to the increased cell migration. Neutralization of galectin-3 binding protein with an anti-galectin-3 binding protein polyclonal antibody also resulted in diminished motility. These results suggest that in colon cancer cells, over-expressed galectin-3 binding protein plays a role in cancer cell motility by weakening cell adhesion by facilitating PTPκ shedding.

### *3.5. The ratio of galectin-3 binding protein to galectin-3 can be a molecular indicator for cancer progression*

Galectin-3 binding protein is known to be up-regulated in several malignant cells and tissues. Therefore, we investigated the expression of the protein in colon cancer cells and clinical tissues. As is seen in Fig. 4A, galectin-3 binding protein was expressed and secreted in the five colon cancer cell lines, although there were differences in the levels among the cell lines. Galectin-3 and galectin-3 binding protein were also monitored in cancer tissues and their normal counterparts (Fig. 4B). Galectin-3 was expressed even in normal tissues, but it was galectin-3 binding protein that showed a cancer tissue-specific expression. These results imply that tumor cells originating from at least colonic tissues promote cancer progression via cleavage of PTPκ by regulating the expression of galectin-3 binding protein.

Cancer cells successfully metastasize by implementing various molecular machineries that favor cell growth, movement, nutrient

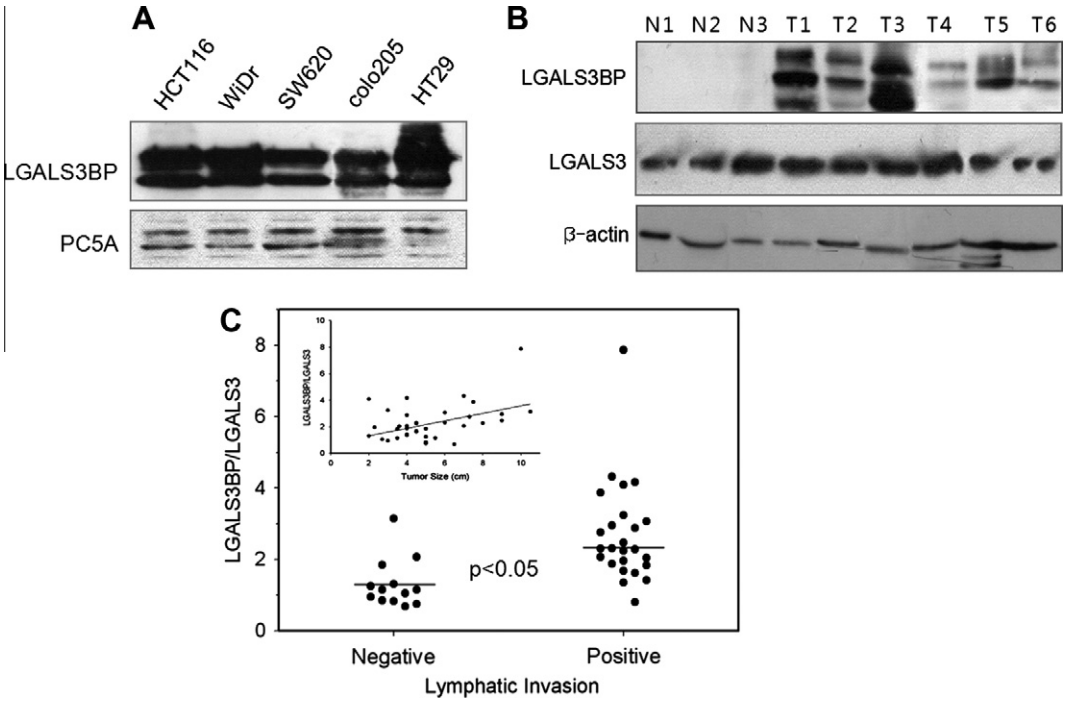


**Fig. 4.** High expression of galectin-3 binding protein in colon cancer cells and tissues, and the correlation with lymphatic invasion in clinical samples. (A) High expression of galectin-3 binding protein in several colon cancer cell-lines. Relatively high expression, as compared to PC5A, was observed in different types of colon cancer cell-lines, as assessed by immune-blot analysis. (B) Representative results of galectin-3 and galectin-3 binding protein in the cancer and normal tissues. Galectin-3 binding protein was found to be over-produced exclusively in colon cancer tissues, opposed to galectin-3, which was also observed in the normal tissues. (C) Correlation of the galectin-3 binding protein ratio to galectin-3 with lymphatic invasion. The LGALS3BP/LGALS3 ratio was analyzed in 38 colon cancer tissues and a high ratio was found to correlate with lymphatic invasion ($p < 0.01$). The inset shows a weak correlation of the ratio with tumor size (coefficient $r = 0.44$).

uptake, and cellular interactions. Cancer cell invasion is a decisive feature for malignant cells, and cell motility governs a large part of the invasiveness of cancer cells. Based on the results in Figs. 2 and 3, the correlation of galectin-3 binding protein/galectin-3 ratio to clinic-pathological variants was investigated in 38 colon cancer tissues. This analysis demonstrated that the ratio can be a discriminating molecular signal for lymphatic invasion: 25 tissues showing positive lymphatic invasion showed an average ratio of 2.13, which was significantly higher than that of 13 tissues (1.73) with negative lymphatic invasion (Fig. 4C). The ratio was not correlated with other factors, such as age, sex, stage, and type of colon cancer (data not shown). It is noteworthy to mention that the ratio was somewhat correlated to tumor size, although the degree of correlation was not very high, with the correlation coefficient ($r$) being 0.44. The clinical data suggest that the ratio of galectin-3 binding protein to galectin-3 confers advantages to cancer progression.

## 4. Discussions

Cleavage of membrane-bound proteins, termed shedding, is a biological process that is frequently observed in cancer cells and tissues. During the shedding process, growth-stimulating factors are generated that give cancer cells a proliferation advantage. However, the molecules involved, how they are regulated, and how shedding ultimately affects cancer progression are not understood for every shedding event. Herein, we report that galectin-3 binding protein is over-expressed in cancer cells and tissues, and that the surplus of this protein reversed the inhibitory effect of cell-surface galectin-3 on PTPκ shedding in colon cancer cells. Galectin-3 has various, often contradictory, effects on cancer progression, and it has never previously been reported that this protein is be involved in membrane protein shedding via binding to glycan residues. We report for the first time that the extracellular ratio of galectin-3 binding protein to galectin-3 is a critical factor for PTPκ shedding and cancer cell motility.

Galectin-3 binding protein is over-expressed in various cancer cell types including pancreatic, lung, and gastric cancers [14–16]. However, the role of galectin-3 binding protein is largely elusive, although it has been reported that this protein is involved in tumor cell adhesion to the extracellular matrix [17] and can enhance extracellular levels of several proteases in HT-29 cells [8]. Although it is suggested that galectin-3 binding protein acts as a positive regulator of PTPκ shedding, and thus cancer cell migration, the possibility cannot be ruled out that the high galectin-3 binding protein/galectin-3 ratio may result in the production of increased levels of growth stimulating proteins like EGF-family growth factors, thereby stimulating cancer cell proliferation via autocrine signaling. Given the role of galectin-3 binding protein in cancer progression, the idea of using galectin-3 binding protein as a target for colon cancer treatment is highly plausible.

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