Differential Stimulation of VEGF-C Production by Adhesion/Growth-regulatory Galectins and Plant Lectins in Human Breast Cancer Cells

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Abstract. *The present study tested the hypothesis that the production of vascular endothelial growth factor C (VEGF-C), a key lymphangiogenic factor, by human breast cancer cells can be stimulated by human lectins, using plant lectins as controls. Materials and Methods: The effects of human galectins and five plant lectins reacting with distinct determinants of N- and O-glycans on the accumulation of VEGF-C in serum-free cell culture media of human breast cancer cells endowed with high (MDA-MB-231) and low (MCF7, T-47D, and SK-BR-3) VEGF-C-producing abilities were examined. Results: All tested lectins stimulated VEGF-C production by MDA-MB-231 cells, albeit with different potency. Concanavalin A, but not galectins, was also able to stimulate VEGF-C production by low VEGF-C-producing cell lines MCF7 and T-47D. Both VEGF-C mRNA and protein were strongly up-regulated in SK-BR-3 cells by concanavalin A and wheat germ agglutinin, but not jacalin. Conclusion: The differential response of breast cancer cell lines separated by the endogenous level of VEGF-C production suggests that galectins may contribute to tumor-associated lymphangiogenesis in a cell-specific manner.*

Vascular endothelial growth factor-C (VEGF-C) is a key lymphangiogenic factor, which can be produced by both normal and cancer cells for stimulation of lymphangiogenesis (1, 2). Exogenous VEGF-C has been employed to treat experimental lymphedema resulting from a deficiency of lymphatic vessels in animal models (3, 4). Transgenic induction of the *VEGF-C* gene leads to persistent lymphatic hyperplasia in murine adult tissues (5), and overproduction of VEGF-C *in situ* promotes tumor-associated lymphangiogenesis and lymphatic metastasis of cancer (6, 7). VEGF-C synthesis by cells can be stimulated by a variety of factors in the cellular micro environment and several transcription factors such as NF-κB, Sp-1, and AP-2 can be involved in the combinatorial regulation of *VEGF-C* gene expression (8).

Recently, it was found that concanavalin A (Con A), a mitogenic mannose-specific plant lectin that up-regulates COX-2, as well as an *N*-acetyl-D-glucosamine-specific wheat germ agglutinin (WGA), stimulated VEGF-C synthesis by MDA-MB-231 breast cancer cells (2, 9). These data indicate that glycan–protein interactions might be a part of molecular mechanisms regulating VEGF-C production *in vitro*. Indeed, cell surface glycans are increasingly considered as bioactive docking sites, initiating contacts with human endogenous lectins as a step toward triggering biosignaling (10). Growth regulation and mediator release are examples of clinically relevant responses to lectin binding (11). Furthermore, the cellular glycomic profile and/or expression of glycogenes such as those for glycosyltransferases and glycosidases can be under the control of master regulators such as tumor suppressors, tested for $p16^{INK4a}$, activin type 2 receptor, absent in melanoma 2, and transforming growth factor β-type 2 receptor, to orchestrate the interplay with endogenous lectins in cancer cells (12, 13). In this context, members of the galectin family of endogenous lectins are potent effectors translating the sugar-encoded signals into effects, with potential for antagonistic activities of closely related proteins such as galectin-1 and -3 (12, 14).

In this study it was hypothesized that this glycobiological route of biosignaling can affect VEGF-C synthesis in cancer cells, thus promoting tumor-associated lymphangiogenesis. As the first step in testing this hypothesis, the present study was designed to probe the potential of distinct glycan

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epitopes by (i) five plant lectins and (ii) five human galectins to regulate VEGF-C production by four human breast cancer cell lines (MDA-MB-231, MCF7, T-47D, and SK-BR-3) with varying degrees of endogenous VEGF-C production.

Materials and Methods

Reagents. DMEM, McCoy's 5A medium (modified), RPMI-1640, DPBS and all cell culture reagents were obtained from Invitrogen (Invitrogen/GIBCO, Burlington, ON, USA). Con A, bovine serum albumin (BSA), wheat germ agglutinin (WGA), crystal violet and glutaraldehyde were from Sigma-Aldrich (Oakville, ON, USA). Jacalin was from Calbiochem (San Diego, CA, USA). *Sambucus nigra* agglutinin (SNA) was from Lektinotest (Lviv, Ukraine). Peanut agglutinin (PNA) was isolated from peanut seeds by affinity chromatography on lactosylated Sepharose 4B as described elsewhere and tested for purity by one- and two-dimensional gel electrophoresis (15).

Preparation of galectins. Extracts containing the human galectins, *i.e.* proto-type galectins-1, -2, and -7, the chimera-type galectin-3 and the tandem-repeat-type galectin-4, were obtained after recombinant production and were processed for galectin purification ensuring absence of any contamination by lipopolysaccharide as previously described (12, 15, 16).

Cell culture. The four breast cancer cell lines (MDA-MB-231, MCF7, T-47D, and SK-BR-3) were obtained from American Type Culture Collection (Manassas, VA, USA). MDA-MB-231 cells were cultured in DMEM, SK-BR-3 cells in McCoy's 5A medium (modified), and MCF7 and T-47D in RPMI-1640 medium, all supplemented with 9% FBS, L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 25 mM HEPES in a humidified 5% (v/v) atmosphere at 37˚C.

Cell-substratum (lectin) adhesion. Adhesion assays were performed according to the standard protocol using Costar 3590 polystyrene 96-well plates from Corning (Corning, NY, USA) with surface-adsorbed lectins and the crystal violet quantification method (17). Briefly, the 96-well plates were coated with lectins (20 μg/ml, 100 μl per well) overnight at 4˚C, blocked with 200 μl of heat-denatured BSA solution (10 mg/ml) for 30 min at room temperature, and rinsed with DPBS immediately before adding 50 μl of the working suspension of cells (5×10^5 cells/ml in DMEM) to each well containing 50 μl DPBS. The cells were allowed to adhere to the bottom of the wells for 60 min at 37˚C in a 5% (v/v) CO_2 incubator with the microtiter plate lid off. The non-adherent cells were removed by gently washing the wells three times with 100 μl DPBS and then the attached cells were fixed at 4˚C overnight by adding 100 μl of 5% glutaraldehyde, rinsed three times with 100 μl water and stained by adding 100 μl of 0.1% crystal violet solution in 0.2 M 2-(*N*-morpholino)ethanesulfonic acid, pH 6.0 for 60 min at room temperature. To quantify cell adhesion, the stained cells in each well were rinsed three times with 200 μl water, the dye was extracted in 100 μl of 10% acetic acid on an orbital shaker (150 rpm, 5 min) at room temperature, and the absorbance was measured at 570 nm using a plate reader.

Preparation of cell culture samples and human VEGF-A and VEGF-C assays. Cells were grown in 6- or 12-well tissue culture plates up to 80-90% confluency, rinsed with serum- and antibiotics-free medium, and treated with sterile lectin solutions in DMEM for 24 h in a 5% (v/v) CO_2 incubator. The cell culture supernatants were collected as 0.5 ml aliquots, and kept frozen for several days at –20˚C prior to VEGF measurements. Quantitative determination of VEGF in the cell culture media was performed by a solid sandwich ELISA using Human VEGF-A and VEGF-C Assay Kits from IBL (Takasaki, Japan).

Real time RT-PCR. Primers for human VEGF-C (forward, 5'-CGGGAGGTGTGTATAGATGTG-3', reverse, 5'-ATTGGCTGGGGA AGAGTTTG-3'), and GAPDH (forward, 5'-ACCACAGTCCAT GCCATCAC-3'; reverse, 5'-TCCACCACCCTGTTGCTGTA-3') were synthesized locally at the UWO Oligo Factory (London, Canada) and their quality was proved by a conventional RT-PCR using GeneAmp PCR System from Perkin Elmer (Norwalk, CT, USA). Real-time quantitative PCR (qPCR) was performed in single microcapillary tubes using the LightCycler™ (Roche Diagnostic, Laval, Canada) and SYBR® Green Tag ReadyMix™ (Sigma, St. Louis, USA) as previously described (2). All data were normalized to the expression of GAPDH mRNA in respective samples.

Statistical analysis. All data are shown as mean with standard deviation of at least four independent assays. Two-tailed Student's *t*-test was used to analyze the data and *p*-values less than 0.05 were considered significant.

Results

The test panel comprised five plant lectins (WGA and ConA to test reactivity of *N*-acetylglucosamine or mannose as controls, PNA and jacalin as probes for reactivity of the mucin-type core 1 O-glycan disaccharide and sialylated derivatives, respectively, and SNA of α2,6-sialylated N-glycans) and members of each galectin subgroup with galectin-1, -2 and -7 (proto-type), galectin-3 (chimera-type) and galectin-4 (tandem-repeat type). Cell-to-substratum adhesion assay was used to examine the ability of MDA-MB-231 cells to attach to immobilized lectins. Positive responses signified bioactivity of the tested ligands. Figure 1A demonstrates that MDA-MB-231 cells adhered most strongly to galectin-2, -4, and -7, and Con A, moderately to galectin-1 and -3, PNA, and SNA, and to jacalin and WGA at the same level as compared with untreated plastic wells.

Given that MDA-MB-231 cells reacted efficiently with the majority of the tested lectins, the accumulation of VEGF-A and VEGF-C in cell culture media following a 24 h treatment with lectins was investigated next. Con A and also WGA were used as positive controls as reported earlier (2, 9). As shown in Figure 1B, the production of VEGF-A was increased in response to all galectins, Con A, and WGA, whereas no change was observed in response to jacalin and PNA, which bind to mucin-type core 1 *O*-glycans. In the case of VEGF-C, a significant increase in production was measurable in the presence of all tested plant lectins (Con A, jacalin, PNA, SNA, and WGA), Con A and WGA being most efficient (Figure 1C). This also happened in the presence of

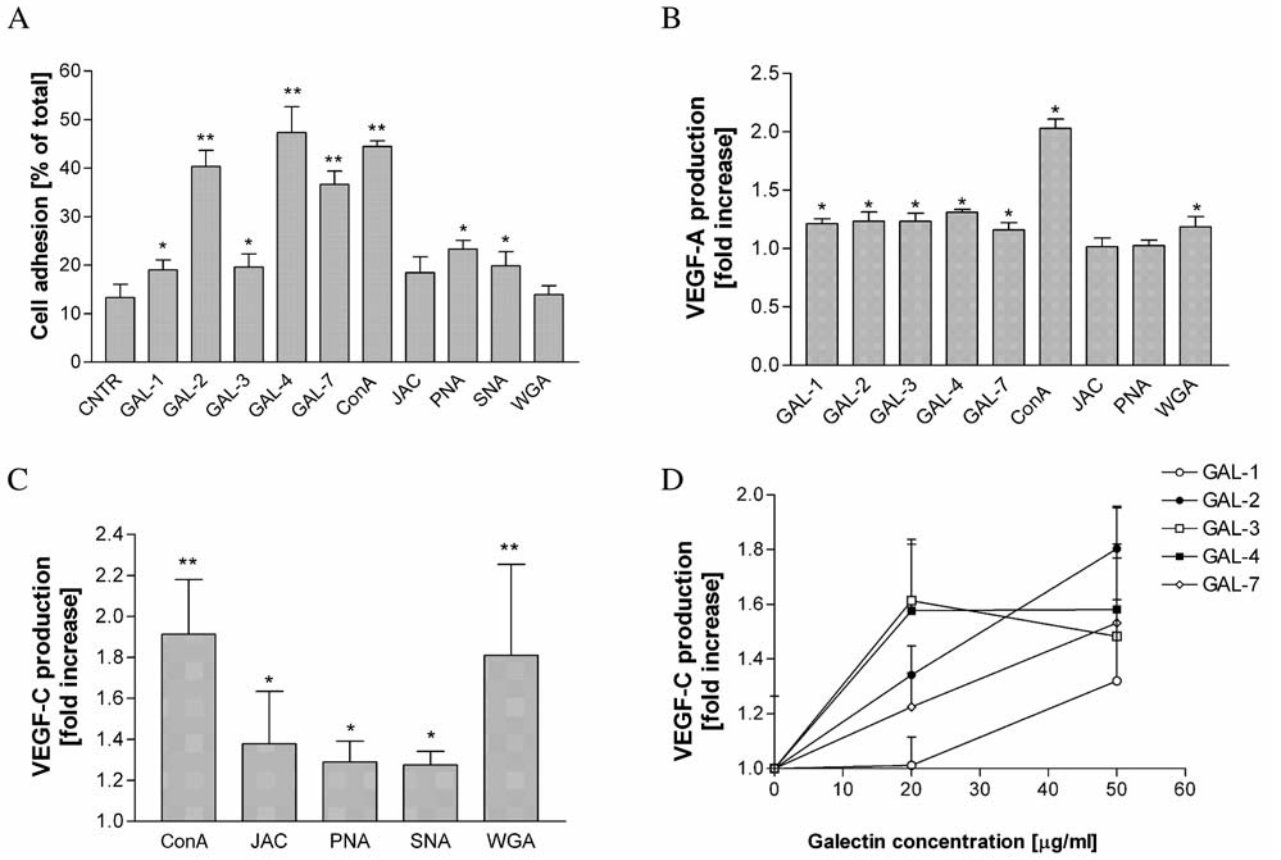

Figure 1. *Effect of plant lectins and human galectins on adhesion and VEGF-A or VEGF-C production by MDA-MB-231 cells. (A) adhesion of cells to immobilized lectins. (B) Production of VEGF-A by the cells in response to galectins (20 μg/ml) and plant lectins (5 μg/ml) at 24 h. (C) Production of VEGF-C by the cells in response to plant lectins (5 μg/ml) at 24 h. (D) Concentration-dependent production of VEGF-C by the cells in response to human galectins at 24 h. The basal production of VEGF-C by untreated cells was 1127±343 pg/ml (n=4). *p<0.05, **p<0.001.*

all tested galectins, demonstrating a dose-dependent trend (Figure 1D). Importantly, in the presence of 25 mM lactose, the haptenic inhibitor, all galectin effects at a concentration of 50 μg/ml were impaired, attesting to the carbohydrate-dependent nature of the effects (data not shown).

Given that Con A and galectins served as effective stimuli for MDA-MB-231 cells high in VEGF-C production, it was tested whether these lectins were able to trigger VEGF-C synthesis by low VEGF-C-producing MCF7 and T-47D breast cancer cell lines. As shown in Figure 2, only treatment with 10 μg/ml Con A resulted in evident stimulation of VEGF-C accumulation in cell culture media at 24 h, whereas no galectins tested at a concentration of 25 μg/ml affected the basal low level of VEGF-C production by these cells. Given that plant lectins were more efficient in stimulating VEGF-C production by breast cancer cells in comparison with galectins, finally it was explored whether plant lectins were able to up-regulate *VEGF-C* gene expression. To investigate this, low VEGF-C-producing SK-BR-3 cells were treated with 5 μg/ml of Con A, WGA, or jacalin for 24 h followed by collection of cell culture medium and total RNA extraction from cell monolayers. As shown in Figure 3, both Con A and WGA induced a consistent increase in VEGF-C mRNA and protein levels indicative of up-regulation of VEGF-C gene expression by these lectins. Jacalin failed to affect *VEGF-C* gene/protein expression underscoring the dependence on the type of reactive glycan epitope.

Discussion

This study demonstrated that both plant and human lectins are able to stimulate the production of VEGF-C by breast cancer cells. This effect depended on the type of lectin and the level of VEGF-C-producing capacity of cells. Of clinical relevance, human galectins stimulated VEGF-C production only in strongly VEGF-C-producing MDA-MB-231 cells. In contrast, plant lectins, *e.g.* Con A, up-regulated VEGF-C production by this cell line as well as by weakly VEGF-C producing MCF7, T-47D, and SK-BR-3 cells. In the latter case, this study showed that this was associated with an up-

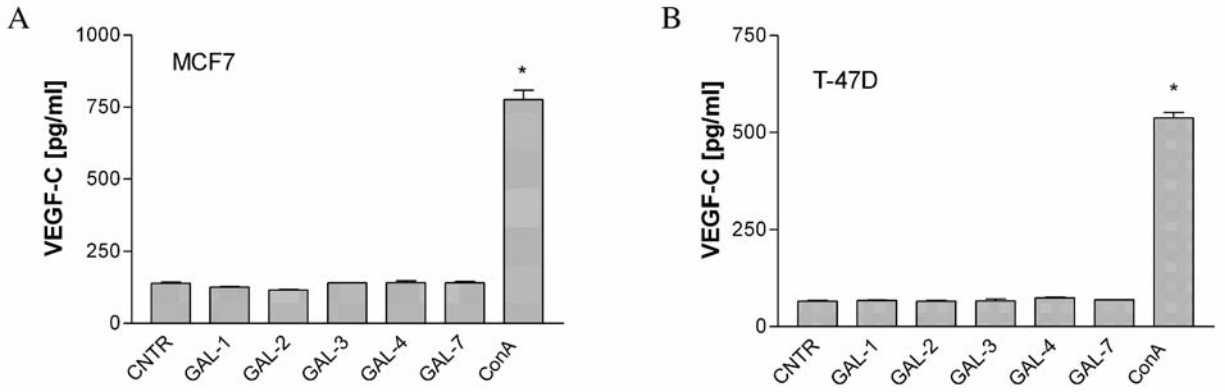

Figure 2. *Effects of human galectins and Con A on VEGF-C production by poorly metastatic breast cancer cells. Subconfluent monolayers of the cells in 6-well plates were treated with human galectins (GAL; 25 μg/ml) or Con A (10 μg/ml) for 24 h, 37˚C and the cell supernatants were assayed for VEGF-C concentration. A: MCF7 breast cancer cell line. B: T-47D breast cancer cell line. *p<0.001 in comparison with the basal production of VEGF-C by untreated cells.*

regulation of *VEGF-C* gene expression. Whether galectin stimulation of VEGF-C production in strongly VEGF-C-generating cells occurs by a further transcriptional up-regulation of the *VEGF-C* gene or an increase in mRNA stability and/or its translation and/or stability at the protein level remains to be investigated.

It is interesting to note that the adhesion of MDA-MB-231 cells to immobilized lectins used as substratum did not correlate with the ability of respective lectins to induce VEGF-C production by the cells. While there was no increased adhesion of cells to immobilized jacalin and WGA, these lectins were capable of stimulating VEGF-C production by the cells. Evidently, these two processes should not be considered to be functionally associated with mutual predictive power. In other words, different glycan epitopes may well be responsible for adhesion and VEGF-C modulation.

A salient aspect of this study is the discovery of the capacity of galectins to enhance production of VEGF-A/-C in tumor cells with high endogenous level of protein generation. While the influence was similar for VEGF-A, it was differential for VEGF-C, with up to 1.8-fold enhancement at a statistically significant level. Endogenous production of galectins may thus be relevant in this context. Of note, human breast cancer cells are characterized by remarkable variations in galectin-specific mRNA or galectin protein profiles holding galectin-1 as a common positive marker in majority of cell lines (18-20). Since galectin-1 is known to regulate Sp1 phosphorylation in carcinoma cells (21), a transcriptional activation by the similar route may be suggested. In breast cancer, the negative prognostic impact of stromal presence of galectin-3 (22) may be established by such activation processes. However, the presence of galectins is not necessarily linked to angiogenesis, as seen in laryngeal carcinomas (23). Apart from the delineated production capacity of breast cancer cell lines further studies

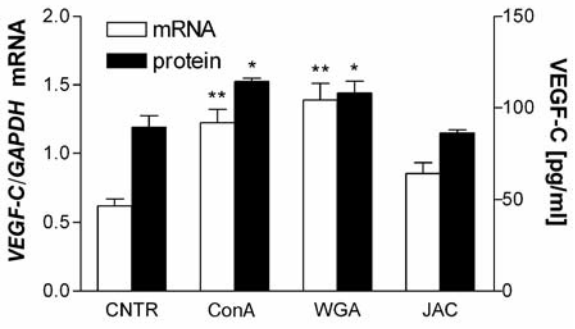

Figure 3. *Effect of lectins on VEGF-C gene expression and VEGF-C protein secretion by SK-BR-3 cells. Cell monolayers were treated with 5 μg/ml of lectins in serum-free DMEM medium for 24 h. Significant up-regulation of VEGF-C was induced at both gene and protein levels by Con A and WGA, while jacalin failed to stimulate VEGF-C. *p<0.05, **p<0.01 in comparison with control values for untreated cells.*

can now be performed on different tumor types to unravel the ultimate contribution of galectins in stimulating tumor-associated lymph- and angiogenesis, based on histopathological galectin fingerprinting (24).

In summary, the findings of the present study of the ability of human lectins to stimulate VEGF-C synthesis by human breast cancer cells are novel. Although the detailed molecular mechanisms underlying this ability need to be investigated, these findings suggest that distinct endogenous lectins are capable to act by autocrine and paracrine pathways that may contribute to tumor-associated lymphangiogenesis. This may represent one of the multiple mechanisms in tumor progression, including the previously reported interplay of galectin-1 with tissue plasminogen activator (25). Taken together, these findings add to the functional interpretation of histopathological data based on galectin profiling.

Acknowledgements

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References

1 Joory KD, Levick JR, Mortimer PS and Bates DO: Vascular endothelial growth factor-C (VEGF-C) expression in normal human tissues. Lymphat Res Biol *4*: 73-82, 2006.
2 Timoshenko AV, Chakraborty C, Wagner GF and Lala PK: COX-2-mediated stimulation of the lymphangiogenic factor VEGF-C in human breast cancer. Br J Cancer *94*: 1154-1163, 2006.
3 Szuba A, Skobe M, Karkkainen MJ, Shin WS, Beynet DP, Rockson NB, Dakhil N, Spilman S, Goris ML, Strauss HW, Quertermous T, Alitalo K and Rockson SG: Therapeutic lymphangiogenesis with human recombinant VEGF-C. FASEB J *16*: 1985-1987, 2002.
4 Jin DP, An A, Liu J, Nakamura K and Rockson SG: Therapeutic responses to exogenous VEGF-C administration in experimental lymphedema: immunohistochemical and molecular characterization. Lymphat Res Biol *7*: 47-57, 2009.
5 Lohela M, Heloterä H, Haiko P, Dumont DJ and Alitalo K: Transgenic induction of vascular endothelial growth factor-C is strongly angiogenic in mouse embryos but leads to persistent lymphatic hyperplasia in adult tissues. Am J Pathol *173*: 1891-1901, 2008.
6 Pepper MS, Tille JC, Nisato R and Skobe M: Lymphangiogenesis and tumor metastasis. Cell Tissue Res *314*: 167-177, 2003.
7 He Y, Karpanen T and Alitalo K: Role of lymphangiogenic factors in tumor metastasis. Biochim Biophys Acta *1654*: 3-12, 2004.
8 Chilov D, Kukk E, Taira S, Jeltsch M, Kaukonen J, Palotie A, Joukov V and Alitalo K: Genomic organization of human and mouse genes for vascular endothelial growth factor C. J Biol Chem *272*: 25176-25183, 1997.
9 Timoshenko AV: Chitin hydrolysate stimulates VEGF-C synthesis by MDA-MB-231 breast cancer cells. Cell Biol Int, 2010.
10 Gabius HJ (ed.): The Sugar Code. Fundamentals of Glycosciences. Wiley-VCH, Weinheim, 2009.
11 Villalobo A, Nogales-Gonzalés A and Gabius HJ: A guide to signaling pathways connecting protein–glycan interaction with the emerging versatile effector functionality of mammalian lectins. Trends Glycosci Glycotechnol *18*: 1-37, 2006.
12 André S, Sanchez-Ruderisch H, Nakagawa H, Buchholz M, Kopitz J, Forberich P, Kemmner W, Böck C, Deguchi K, Detjen KM, Wiedenmann B, von Knebel-Döberitz M, Gress TM, Nishimura SI, Rosewicz S and Gabius HJ: Tumor suppressor $p16^{INK4a}$: modulator of glycomic profile and galectin-1 expression to increase susceptibility to carbohydrate-dependent induction of anoikis in pancreatic carcinoma cells. FEBS J *274*: 3233-3256, 2007.
13 Patsos G, André S, Roeckel N, Gromes R, Gebert J, Kopitz J and Gabius HJ: Compensation of loss of protein function in microsatellite-unstable colon cancer cells (HCT116): a gene-dependent effect on the cell surface glycan profile. Glycobiology *19*: 726-734, 2009.
14 Sanchez-Ruderisch H, Fischer C, Detjen KM, Welzel M, Wimmel A, Manning JC, André S and Gabius HJ: Tumor suppressor $p16^{INK4a}$: downregulation of galectin-3, an endogenous competitor of the pro-anoikis effector galectin-1, in a pancreatic carcinoma model. FEBS J *277*: 3552-3563, 2010.
15 Gabius HJ: Influence of type of linkage and spacer on the interaction of β-galactoside-binding proteins with immobilized affinity ligands. Anal Biochem *189*: 91-94, 1990.
16 Sarter K, André S, Kaltner H, Lensch M, Schulze C, Urbonaviciute V, Schett G, Herrmann M and Gabius HJ: Detection and chromatographic removal of lipopolysaccharide in preparations of multifunctional galectins. Biochem Biophys Res Commun *379*: 155-159, 2009.
17 Humphries MJ: Cell-substrate adhesion assays. *In*: Curr Protoc Cell Biol. Bonifacio JS *et al*. (eds.). John Wiley & Sons, New York, sections 9.1.1-9.1.11, 1998.
18 Lahm H, André S, Hoeflich A, Fischer JR, Sordat B, Kaltner H, Wolf E and Gabius HJ: Comprehensive galectin fingerprinting in a panel of 61 human tumor cell lines by RT-PCR and its implications for diagnostic and therapeutic procedures. J Cancer Res Clin Oncol *127*: 375-386, 2001.
19 Satelli A, Rao PS, Gupta PK, Lockman PR, Srivenugopal KS and Rao US: Varied expression and localization of multiple galectins in different cancer cell lines. Oncol Rep *19*: 587-594, 2008.
20 Imai K, Ichibangase T, Saitoh R and Hoshikawa Y: A proteomics study on human breast cancer cell lines by fluorogenic derivatization-liquid chromatography/tandem mass spectrometry. Biomed Chromatogr *22*: 1304-1314, 2008.
21 Fischer C, Sanchez-Ruderisch H, Welzel M, Wiedenmann B, Sakai T, André S, Gabius HJ, Khachigian L, Detjen K and Rosewicz S: Galectin-1 interacts with the α5β1 fibronectin receptor to restrict carcinoma cell growth *via* induction of *p21* and *p27*. J Biol Chem *280*: 37266-37277, 2005.
22 Moisa A, Fritz P, Eck A, Wehner HD, Mürdter T, Simon W and Gabius HJ: Growth/adhesion-regulatory tissue lectin galectin-3: stromal presence but not cytoplasmic/nuclear expression of tumor cells as negative prognostic factor in breast cancer. Anticancer Res *27*: 2131-2140, 2007.
23 Saussez S, Decaestecker C, Cludts S, Ernoux P, Chevalier D, Smetana K, André S, Leroy X and Gabius HJ: Adhesion/growth-regulatory tissue lectin galectin-1 in relation to angiogenesis/lymphocyte infiltration and prognostic relevance of stromal up-regulation in laryngeal carcinomas. Anticancer Res *29*: 59-65, 2009.
24 Cludts S, Decaestecker C, Mahillon V, Chevalier D, Kaltner H, André S, Remmelink M, Leroy X, Gabius HJ, Saussez S: Galectin-8 up-regulation during hypopharyngeal and laryngeal tumor progression and comparison with galectin-1, -3 and -7. Anticancer Res *29*: 4933-4940, 2009.
25 Roda O, Ortiz-Zapater E, Martínez-Bosch N, Gutiérrez-Gallego R, Vila-Perelló M, Ampurdanés C, Gabius HJ, André S, Andreu D, Real FX and Navarro P: Galectin-1 is a novel functional receptor for tissue plasminogen activator in pancreatic cancer. Gastroenterology *136*: 1379-1390, 2009.

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