

Galectin-8 Favors VEGF-Induced Angiogenesis: *In Vitro* Study in Human Umbilical Vein Endothelial Cells and *In Vivo* Study in Chick Chorioallantoic Membrane

LENKA VARINSKÁ^{1,2}, LENKA FÁBER¹, EVA PETROVOVÁ³, LUDMILA BALÁŽOVÁ⁴,
ELEONÓRA IVANČOVÁ⁵, MICHAL KOLÁŘ⁶ and PETER GÁL^{2,7,8}

¹Department of Pharmacology, Faculty of Medicine, Pavol Jozef Šafárik University, Košice, Slovak Republic;

²Department of Biomedical Research, East-Slovak Institute of Cardiovascular Diseases Inc., Košice, Slovak Republic;

³Department of Anatomy, University of Veterinary Medicine and Pharmacy, Košice, Slovak Republic;

⁴Department of Pharmacognosy and Botany, University of Veterinary Medicine and Pharmacy, Košice, Slovak Republic;

⁵Department of Stomatology and Maxillofacial Surgery,

Pavol Jozef Šafárik University and Louis Pasteur University Hospital, Košice, Slovak Republic;

⁶Laboratory of Genomics and Bioinformatics,

Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic;

⁷Laboratory of Cell Interactions, MediPark, Pavol Jozef Šafárik University, Košice, Slovak Republic;

⁸Prague Burn Center, Third Faculty of Medicine, Charles University, Prague, Czech Republic

Abstract. *Background/Aim:* Although it has been accepted that the tandem repeat galectin-8 (Gal-8) is linked to angiogenesis, the underlying mechanisms in endothelial cells has remained poorly understood. In this study we aimed to investigate the effect of Gal-8 on selected biological processes linked to angiogenesis in *in vitro* and *in vivo* models. *Materials and Methods:* In detail, we assessed how exogenously added human recombinant Gal-8 (with or without vascular endothelial growth factor – VEGF) affects selected steps involved in vessel formation in human umbilical vein endothelial cells (HUVECs) as well as using the chick chorioallantoic membrane (CAM) assay. *Gene expression profiling of HUVECs was performed to extend the scope of our investigation. Results:* Our findings demonstrate that Gal-8 in combination with VEGF enhanced cell proliferation and migration, two cellular events linked to angiogenesis. However, Gal-8 alone did not exhibit any significant effects on cell proliferation or on cell migration. The molecular analysis revealed that Gal-8 in the presence of VEGF influenced cytokine-cytokine receptor interactions,

HIF-1 and PI3K/AKT signaling pathways. Gal-8 alone also targeted cytokine-cytokine receptor interactions, but with a different expression profile as well as a modulated focal adhesion and TNF signaling. *Conclusion:* Gal-8 promotes a pro-angiogenic phenotype possibly in a synergistic manner with VEGF.

The process of angiogenesis has been the focus of extended research as an attempt to treat cancers with poor prognosis (1, 2), particularly targeting a key regulator of angiogenesis - the vascular endothelial growth factor (VEGF) (3). Although a single line anti-angiogenic therapy has not led to any success (4, 5), the anti-angiogenic therapy has been well established as an adjuvant treatment administered in combination with conventional chemotherapy in the management of many solid tumors (6-8). In addition to VEGF, the regulation of angiogenesis involves the interplay of many other regulatory molecules (9). Recent evidence has been provided on the tandem repeat β -galactoside binding proteins, galectins (Gal), which present a class of sugar-binding proteins and are capable of inducing cell-cell and/or cell matrix communication (10-12), also emerging in many cancers, such as in chromophobe and papillary carcinomas, squamous cell carcinoma, pediatric ependymoma, chronic lymphocytic leukemia and many others (13-23). Gal-1 and -3 have been the most intensively studied in different aspects of cell biology and immunology (24). Gal-1 positively contributes towards endothelial cell (EC) proliferation, migration, sprouting and tube-like structure formation (25-

Correspondence to: Peter Gál, Department of Biomedical Research, East-Slovak Institute of Cardiovascular Diseases, Inc., Ondavská 8, 040 11 Košice, Slovak Republic. Tel: +421 552343533, Fax: +421 557891613, e-mail: pgal@vusck.sk or galovci@yahoo.com

Key Words: Tumor growth, wound healing, vessel sprouting, glycobiology, sugar code.

28). Gal-1 binds to neuropilin-1 or to VEGF receptor 2 (VEGFR-2) followed by VEGFR-2 phosphorylation and signal transduction through the Raf/extracellular signal-regulated kinase and Akt (25). The interactions of Gal-1 with complex N-glycans on VEGFR-2 in ECs contribute towards preserved angiogenesis in anti-VEGF-resistant tumors (29). This leads to the notion that the over-expression of Gal-1 may be considered as a biomarker for the diagnosis, prognosis and treatment condition in many types of cancers. The mechanisms of Gal-3-mediated pro-angiogenic responses described so far include the binding to and activation of VEGFR-2 (30) in ECs and of neuron-glia antigen 2 (NG2) at the pericyte-EC interface (31). Moreover, Gal-3 also binds to $\alpha\beta3$ integrin and this in turn facilitates basic fibroblast growth factor (bFGF) and VEGF-induced angiogenic responses (32). Furthermore, under hypoxic conditions, Gal-3 directly binds to JAG1 and triggers vessel sprouting by JAG1/Notch-1 signaling activation in ECs (33).

A particular member of the galectin family, Gal-8, is heavily involved with different types of cancer (34) and can be found in many tissues, including lung, liver, kidney, spleen and others [reviewed in (35)]; however, its role in tumorigenesis is still not well understood. Gal-8 is expressed in the cytoplasm as well as nucleus of ECs in normal and tumor-associated blood vessels, but also in lymphatic ECs [reviewed in (36)]. This protein contributes to cell adhesion, either positively or negatively depending on its subcellular localization (37, 38), prevents tumor cell apoptosis (39), and may also control metastatic progression (40). Several studies have also shown that Gal-8 expression differs in various types of tumors and can serve as a new prognostic factor for the overall survival and disease-free survival of cancer patients (41-46). The role of Gal-8 *in vivo* also involves eNOS activation, S-nitrosylation of adherens junction components and induction of hyperpermeability, possibly by S-nitrosylation, which potentially contributes to the malignancy of tumors (47). Moreover, Gal-8, as an angiogenic modulator, recognizes different ligands in blood (36, 48) and lymphatic endothelia (49, 50). One such molecule, CD166/ALCAM (activated leukocyte cell adhesion molecule), has been identified as a major ligand for Gal-8 in bovine aortic ECs and at least partially regulates Gal-8-mediated angiogenesis and migration. In lymphatic ECs, a Gal-8-dependent cross-talk among VEGF-C, PDPN, and integrin pathways ($\alpha1\beta1$ or $\alpha5\beta1$ integrins) plays a critical role in lymphangiogenesis (51).

Although it is now accepted that Gal-8 is linked to angiogenesis, still little is known regarding its exact functions in ECs. For this reason, in the current study, we studied how exogenously added human recombinant Gal-8, alone and in combination with VEGF, affects endothelial cell biology. On the *in vitro* level we used primary cultures of human umbilical vein endothelial cells (HUVECs) whereas

the *in vivo* study was realized using as our model the chick chorioallantoic membrane (CAM). Furthermore, gene expression profiling of HUVECs was performed to extend the scope of our investigation.

Materials and Methods

Cell culture. In the present study we used primary cultures of HUVECs that were isolated and expanded following published protocol (52) and with respect to the Helsinki Declaration (53) with an informed consent from donors. The study was approved by the Ethical committee of the Faculty of Pharmacy, Comenius University in Bratislava (06/2019). All *in vitro* experiments were performed using HUVECs at passage 2-3 isolated from 3 donors. Cells were cultured on gelatin-coated dishes in M199 supplemented with 20% heat-inactivated new born calf serum (both from Cambrex, Verviers, Belgium), 150 $\mu\text{g/ml}$ crude endothelial cell growth factor (ECGF), 5 U/ml heparin, 100 IU/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin (all from Invitrogen, Carlsbad, CA, USA) at 37°C under 5% CO₂/95% air atmosphere. Twenty-four hours prior to the experiments the endothelial cell cultures were refreshed with Opti-MEM™ I Reduced Serum Medium (Thermo Fisher Scientific, Waltham, MA, USA). Cell viability, estimated by trypan blue exclusion, was higher than 95% before each experiment. As a positive control, 25 ng/ml of VEGF (R&D Systems, Minneapolis, MN, USA) (54), was used in all experiments. Recombinant human Gal-8 (R&D Systems) was tested at the following concentrations: i) 0.004, ii) 0.02, iii) 0.1, iv) 0.5, and v) 1.0 $\mu\text{g/ml}$.

5-bromo-2'-deoxyuridine (BrdU) cell proliferation assay. Cell proliferation was examined by quantification of BrdU incorporated into the genomic DNA during the cell cycle. DNA synthesis was assessed using the colorimetric cell proliferation ELISA assay (Roche Diagnostics GmbH, Mannheim, Germany) following the vendor's protocol. Briefly, 4×10^3 cells in 80 μl of medium per well were placed into a 96-well plate. The next day, cells were treated with tested concentrations of Gal-8 (0.004-1.0 $\mu\text{g/ml}$) in the presence or absence of 25 ng/ml of VEGF and further cultured. VEGF (25 ng/ml) without Gal-8 was used as a positive control. After 24 h of treatment, cells were incubated with BrdU labeling solution for another 24 h followed by fixation and incubation with anti-BrdU peroxidase conjugate for an additional 1.5 h at room temperature. Following substrate reaction, color intensity was measured using the Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek, Winooski, VT, USA) at 450 nm (reference wavelength: 690 nm).

Two-dimensional migration (wound healing) assay. Migration of HUVECs was evaluated using a scratch-assay (55). Briefly, a confluent layer of cells cultured on a 24-well plate in the cM199 medium was scratched using a pipette tip creating a "wound". Afterwards, the medium was replaced with Opti-MEM™ I Reduced Serum Medium containing Gal-8 at tested concentrations (0.004-1.0 $\mu\text{g/ml}$) in the presence or absence of 25 ng/ml VEGF. VEGF (25 ng/ml) without Gal-8 was used as positive control. The wounded area was photographed at the start (t=0 h) and at a specific time point t=16 h. The migration distance (gap size) was determined using the ImageJ image analysis software (Bethesda, MD, USA). The experiments were performed in duplicate wells and repeated three times with cells from different donors.

RNA isolation and cDNA Synthesis. Following HUVECs treatment with tested Gal-8 concentrations (0.004-1 µg/ml), with or without VEGF (25 ng/ml), the total RNA was extracted using the Qiagen RNeasy® Mini Kit (#74104) according to the manufacturer's recommendations. VEGF alone (25 ng/ml) was used as positive control. RNA samples were handled by DNase. RNA was spectrophotometrically quantified (ND-1000; NanoDrop Products, Thermo Fisher Scientific, Wilmington, Delaware, USA) and 250 ng of total RNA was analyzed by agarose gel electrophoresis to confirm its integrity. The residual RNA was stored at -80°C. Only samples pure enough (A260/A230 ratio>1.8, A260/A280 ratio=1.8-2.0), with a reasonably high concentration (>100 ng/µl), were used as templates for cDNA synthesis. First-strand complementary DNA was synthesized from total RNA (0.5 µg) using the RT² First-Strand Kit (#330401; QIAGEN Sciences, MD, USA). Briefly, 0.5 µg of total RNA was added to 2 µl of Buffer GE (5x gDNA Elimination Buffer) and filled with RNase-free water to the final volume of 10 µl. The mixture was denatured at 42°C for 5 min and then immediately cooled by placing on ice for 1 min. Reverse transcription was performed after adding 10 µl of the reverse transcription mix. The reaction mixture was incubated at 42°C for 15 min and the reaction was terminated by heating the mixture at 95°C for 5 min. The generated cDNA was diluted with 91 µl of RNase-free water and stored at -20°C until use.

Gene expression profiling. Quantitative PCR was performed on the Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fischer Scientific, MA, USA). Gene transcription was evaluated using the Human Angiogenesis RT² Profiler™ PCR Array (#330231; QIAGEN Sciences, MD, USA). The array contains built-in primers for 84 tested and 5 housekeeping genes and positive control elements to determine i) the efficiency of the reverse transcription reaction, ii) performance of the PCR reaction, and iii) detection of genomic DNA contamination. The PCR mixture includes 1350 µl of RT² SYBR Green ROX™ qPCR Mastermix (#330523, QIAGEN Sciences), 102 µl cDNA template, and 1248 µl RNase-free water. The PCR reaction mix was added to the wells of the PCR plate in equal amounts (25 µl), and then the real-time PCR cycling program was initiated. The thermal cycling program recommended by the plates' manufacturer (QIAGEN Sciences) was as follows: 10 min at 95°C followed by 40 cycles: denaturation at 95°C for 15 s, with 60 s annealing and elongation at 60°C, followed by melting curve analysis.

PCR data analysis and statistics. Data obtained from the quantitative PCR were analyzed using the SABiosciences data analysis software and the $\Delta\Delta C_t$ method (<https://www.qiagen.com/sk/shop/genes-and-pathways/data-analysis-center-overview-page/?akamai-feo=off>). In detail, fold-changes for each gene were calculated as a difference in gene expression between Gal-8 exposure in the presence or absence of VEGF and the untreated control or the VEGF-treated positive control. A positive value indicated gene up-regulation and a negative value indicated gene down-regulation. Each experiment was independently repeated twice (as recommended by the manufacturer). Genes with greater than 1.5-fold change in expression compared to the respective control were identified as statistically significant ($p<0.05$).

To visualize observed changes in context of signaling pathways, we used Pathview Web with default parameters (56). Log FC values

greater than 10 (respectively less than -10) were trimmed to 10 (respectively -10). The Venn diagram was used to show the number of unique and differentially regulated genes between the tested cell treatment conditions.

Chicken embryo chorioallantoic membrane (CAM) assay. The CAM assay was performed as described in our previous study (57). Briefly, fertilized chicken eggs (Parovske Haje, Slovak Republic) were cleaned with 70% ethanol and incubated with a storage blunt end up in a forced-draft incubator at 37.5°C, with approximately 60-65% humidity. On embryonic day (ED) 3 of incubation period, 2 ml of albumin was aspirated using a syringe needle (20 G) so as to detach the developing CAM from the top part of the shell. On ED 7, a window of around 1.5-2.0 cm² was gently opened on the blunt end of the egg without damaging the embryo. A sterilized silicone ring (inner diameter - 6 mm, Fisher Slovakia, Bratislava, Slovak Republic) was positioned on the CAM surface avoiding major blood vessels and 30 µl of the sample, Gal-8 (0.004-1.0 µg/ml) with or without VEGF (25 ng/ml), was placed within the ring. VEGF alone (25 ng/ml) was used as positive control.

After 72 h, the vascularization of CAM was evaluated. The angiogenesis index was measured as the difference between the number of vessels (in percentage) after 72 h and the number of vessels (in percentage) before each treatment. The photographs of CAM blood vessels forming inside the rings were obtained using a stereomicroscope Olympus SZ61 and a digital camera ARTCAM-300MI (both Tokyo, Japan). The experiments were repeated three times using 5 eggs per group.

Statistical analysis. Results are expressed as mean±SD (standard deviation). Statistical comparison of obtained data was performed using the one-way ANOVA followed by Dunnett's test. Difference was considered to be statistically significant when $p<0.05$.

Results

Effect of Gal-8 on BrdU incorporation. In order to evaluate the proliferating potential of HUVECs exposed to different concentrations of Gal-8 (0.004-1.0 µg/ml), the BrdU proliferation assay was employed. As shown in Figure 1, Gal-8 treatment had no effect on BrdU incorporation in the absence of VEGF.

In striking contrast, Gal-8 significantly facilitated the positive effect of VEGF on cell proliferation when compared to the control. Moreover, the absorption of BrdU incorporation in cells treated with Gal-8 at 0.004-0.5 µg/ml and VEGF was significantly increased ($p<0.001$) compared to the positive control (VEGF only).

Gal-8 promotes VEGF-stimulated migratory ability of HUVECs. It is a well-known fact that endothelial cell migration is essential to angiogenesis, thus we examined how different concentrations of Gal-8 (0.004-1.0 µg/ml) affects 2-D wound healing *in vitro*. As illustrated in Figure 2, the HUVECs migrated into the wounded area actively in the positive control (VEGF only) after 16 h of incubation. In contrast to cell proliferation, Gal-8 alone stimulated the

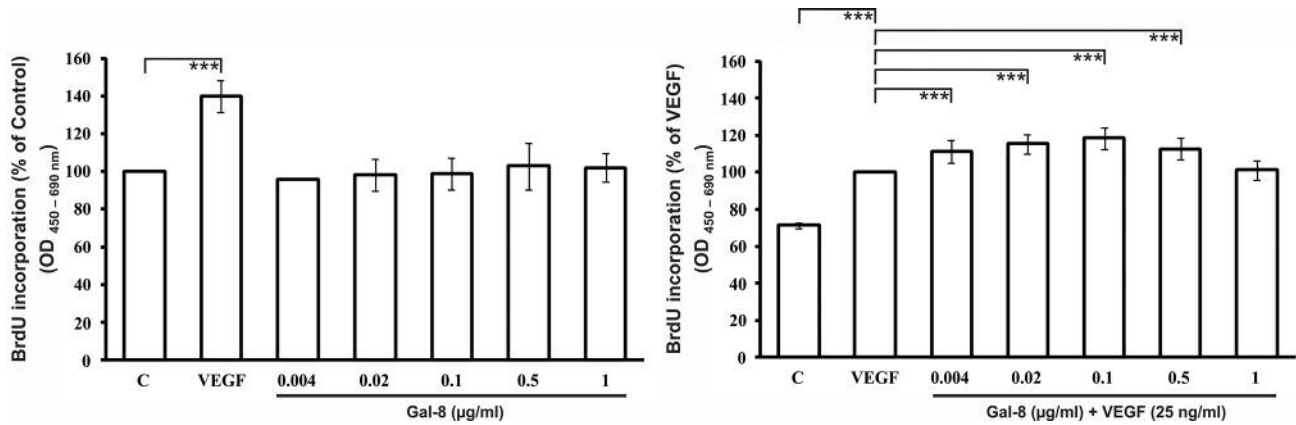


Figure 1. Proliferation 5-bromo-2'-deoxyuridine (BrdU) incorporation assay: positive effect of Gal-8 + VEGF (25 ng/ml) treatment on the proliferation of human umbilical vein endothelial cells (HUVECs). Data represent mean±standard deviation of three independent experiments. The left graph demonstrates the comparison of positive control HUVECs (+VEGF, 25 ng/ml) and HUVECs with tested concentrations of Gal-8 (0.004-1.0 µg/ml) to the untreated control, respectively, whereas the right graph shows the comparison of untreated control HUVECs and HUVECs with tested concentrations of Gal-8 (0.004-1.0 µg/ml) + VEGF (25 ng/ml) to the positive control (+VEGF 25 ng/ml), respectively. *** $p < 0.001$ C: Untreated control; OD: optical density.

migration ability of HUVECs, however, the difference was not significant when compared to the control.

Gal-8 co-incubation with VEGF facilitated HUVECs' migration to the wounded area at a concentration between 0.004-0.02 µg/ml. However, the difference was only significant at the concentration of 0.004 µg/ml ($p < 0.05$). Gal-8 at the highest tested concentration (1.0 µg/ml) in either the absence or presence of VEGF reduced cell migration as compared to the control and positive control (VEGF), respectively.

Pro-angiogenic effects of Gal-8 on HUVECs in the CAM model in vivo. We also evaluated the *in vivo* angiogenic activity of Gal-8 (0.004-1 µg/ml) by using the CAM assay. As shown in Figure 3, Gal-8 treatment in the absence of VEGF induced neovascularization in the concentration range of 0.004-0.5 µg/ml (145%-120%, respectively). However, the difference was only significant when Gal-8 was applied at 0.1 µg/ml ($p < 0.01$). Of note, Gal-8 in the absence of VEGF at the concentration of 1 µg/ml rather inhibited (72%) vessel formation.

In addition, treatment with Gal-8 in the presence of VEGF caused an increase in the branching of new capillaries from the exiting basal vessels compared to treatment with VEGF alone. Quantitative analysis revealed that Gal-8 at concentrations of 0.004, 0.02, and 0.1 µg/ml significantly increased the angiogenesis index in chicken embryos by 116% ($p < 0.01$), 122% ($p < 0.001$), and 115% ($p < 0.01$), respectively. The difference was significant at 0.5 µg/ml of Gal-8, however the angiogenesis index decreased to 86% ($p < 0.05$) compared to the VEGF control.

Gene profiling of HUVECs after Gal-8 treatment. The Human Angiogenesis RT² Profiler PCR Array evaluates the expression of 84 key angiogenesis-related genes. Quality control parameters included in the array showed good reproducibility and efficiency based on the web based software provided by the manufacturer.

Comparison of the various gene expressions of HUVECs after exposure to Gal-8 (0.004 µg/ml) vs. untreated control showed that 33 genes were up-regulated and 20 genes were down-regulated (Table I). After treatment with Gal-8 co-incubated with VEGF vs. VEGF only 17 genes were down-regulated while 53 genes were up-regulated (Table II).

Furthermore, the number of up- and down-regulated genes in the two main groups was counted and plotted as a Venn diagram (Figure 4). The comparative analysis revealed 20 commonly up-regulated genes (*ANGPT2*, *ANPEP*, *CDH5*, *CTGF*, *CXCL10*, *CXCL6*, *CXCL5*, *EFNA1*, *EFNB2*, *FGF2*, *HGF*, *IL6*, *JAG1*, *KDR*, *NRP2*, *PDGFA*, *PECAM1*, *PTGS1*, *TGFBR1*, *TIMP1*) when Gal-8 vs. C and Gal-8+VEGF vs. VEGF were compared. On the other hand, 6 genes (*COL18A1*, *HPSE*, *ID1*, *PF4*, *PGF*, *VEGFC*) were commonly down-regulated in the comparison of Gal-8 vs. C and Gal-8+VEGF vs. VEGF. The fold-change of all down-regulated genes was increased when cells were treated with the combination of Gal-8 and VEGF as compared to single Gal-8 treatment. The group of up-regulated genes revealed slightly different patterns. Whereas in the most of genes (*ANPEP*, *ANGPT2*, *CDH5*, *CTGF*, *CXCL6*, *CXCL10*, *EFNB2*, *FGF2*, *KDR*, *NRP2*, *PECAM1*, *PDGFA*, *PTGS1*, *TGFBR1*) the up-regulation was increased, in four genes (*CXCL5*, *EFNA1*, *HGF*, *JAG1*, *TIMP1*) the fold-change was decreased and one gene remained

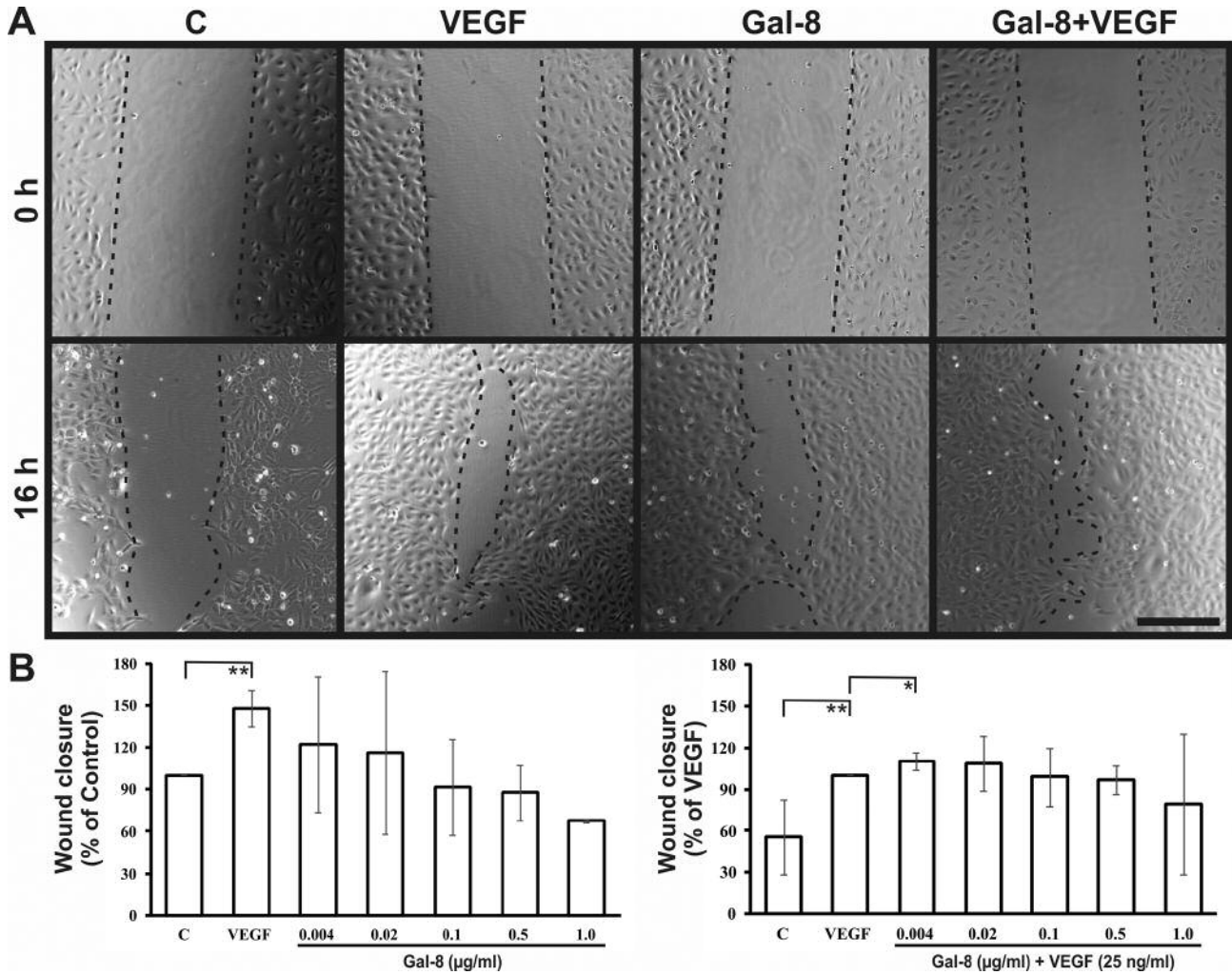


Figure 2. Migration/wound healing (WH) assay. (A) Representative photographs of the positive effect of Gal-8 (0.004 $\mu\text{g/ml}$) and Gal-8 (0.004 $\mu\text{g/ml}$) + VEGF (25 ng/ml) treatments on the migration of human umbilical vein endothelial cells (HUVECs) (scale=100 μm). (B) Both graphs present summarized data from the WH assays presented as mean \pm standard deviation of three independent experiments. The left graph demonstrates the comparison of positive control HUVECs (+VEGF, 25 ng/ml) and HUVECs with tested concentrations of Gal-8 (0.004-1.0 $\mu\text{g/ml}$) to the untreated control, respectively, whereas the right graph shows the comparison of untreated control HUVECs and HUVECs with tested concentrations of Gal-8 (0.004-1.0 $\mu\text{g/ml}$) + VEGF (25 ng/ml) to the positive control (+VEGF 25 ng/ml), respectively. * $p < 0.05$, ** $p < 0.01$ C: Untreated control; VEGF: Positive control.

rather unchanged (*IL6*) when the profile was compared between Gal-8 vs. C and Gal-8+VEGF vs. VEGF.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) graph-visualized changes (Supplementary Figures 1 and 2) in the context of potentially modulated signaling pathways revealed that Gal-8 in the presence of VEGF influenced cytokine-cytokine receptor interaction, HIF-1 and PI3K/AKT signaling pathways (Supplementary Figure 1). Gal-8 alone also targeted the cytokine-cytokine receptor interaction, but with a different expression profile as well as modulated focal adhesion and TNF signaling pathway (Supplementary Figure 2).

Discussion

It was previously shown that very low doses (5-20 nM) of Gal-8 can activate ECs to form an extensive capillary network (48). On the other hand, an over 10-fold higher concentration (250 nM) of immobilized Gal-8 inhibited tube formation of lymphatic endothelial cells (LECs) in a 3D tube formation assay (49). However, under these experimental conditions, Gal-8 stimulated LECs adhesion and migration, but prevented LEC tubulogenesis. Of note, in the study performed by Cunei and Detmar (49) the formation of tube-like structures by HUVECs was not affected. Similarly, our current experiment

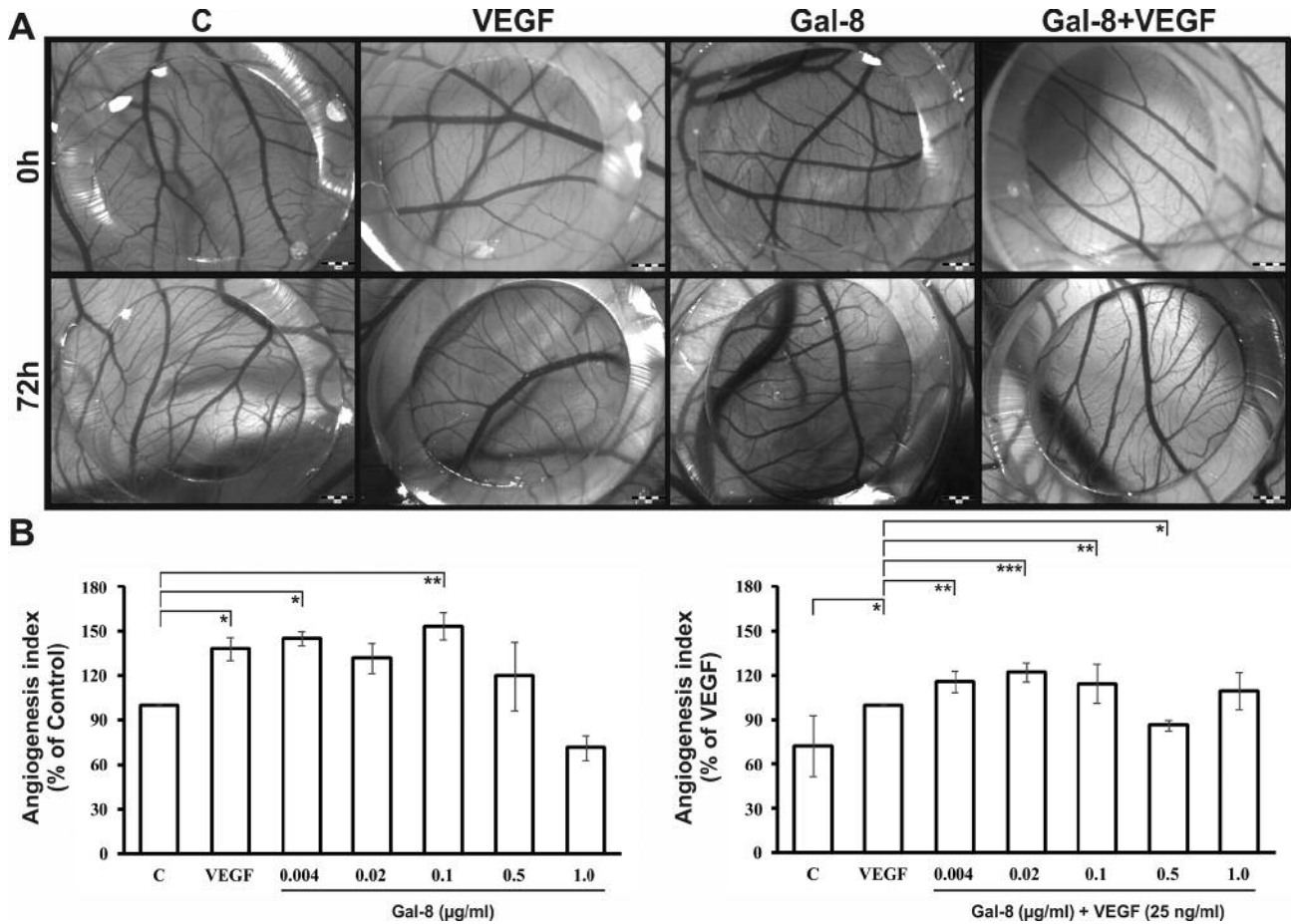


Figure 3. Chick chorioallantoic membrane (CAM) assay. (A) Representative photographs of the positive effect of Gal-8 (0.004 µg/ml) and Gal-8 (0.004 µg/ml) + VEGF (25 ng/ml) treatments on vessel sprouting and branching (scale=1 mm). (B) The graphs show summarized data from the chick CAM assay presented as mean±standard deviation (SD) of three independent experiments. The results are summarized in the graph as the angiogenesis index (the mean±SD of new vessels per field) for each experimental variable. The left graph demonstrates the comparison of positive control (+VEGF, 25 ng/ml) and tested concentrations of Gal-8 (0.004-1.0 µg/ml) to the untreated control, respectively, whereas the right graph shows the comparison of untreated control and tested concentrations of Gal-8 (0.004-1.0 µg/ml) + VEGF (25 ng/ml) to the positive control (+VEGF 25 ng/ml), respectively. *p<0.05, **p<0.01, ***p<0.001 C: Untreated control; VEGF: Positive control.

revealed that Gal-8 as a single soluble molecule did not significantly trigger migration of HUVECs and had no effect on cell proliferation. In contrast to single molecule treatment, both the proliferation and migration tests revealed that this member of the galectin family rather acted in a synergistic manner with VEGF. Furthermore, application of Gal-8 increased the number of vessel endpoints in both experimental conditions, i) Gal-8 alone and ii) Gal-8 co-incubated with VEGF, but the combination treatment elicited a higher efficiency. Of note, the use of a single efficient concentration of VEGF presents the first limitation of the current study and suggests a direction to forthcoming experiments. Although all performed *in vitro* experiments reflected a dose-dependent response with an inhibitory effect of Gal-8 at the highest tested

concentration, the CAM assay did not confirm this trend. Galectins are well known for their biphasic modulation of cell growth. For example, while high doses (~1 µM) of the recombinant prototype Gal-1 inhibit cell proliferation independently of Gal-1 sugar-binding activity, low doses (~1 nM) of Gal-1 are mitogenic (58, 59). Although the concentrations differ for the tandem repeat Gal-8, we may suggest that a similar biphasic effect may also contribute towards the observed differences between the *in vitro* and *in vivo* responses.

In detail, the molecular analysis showed that Gal-8 alone and Gal-8 with VEGF regulate the expression profile of HUVECs differently. Although the cytokine-cytokine receptor interactions were affected in both treatments, the gene

Table I. Log₂-fold change of gene expression in HUVECs exposed to Gal-8 (0.004 µg/ml) compared to Opti-MEMTM 1 reduced serum medium-treated control*.

No.	Gene symbol	Log fold change	Gene symbol	Log fold change
1	TNF ^t	11.03	PF4 ^p	-4.01
2	LEP ^t	6.50	TGFA ^t	-3.95
3	TIMP1 ^p	5.72	FN1 ^t	-3.37
4	MMP9 ^t	5.28	EDN1 ^t	-2.39
5	PROK2 ^t	4.72	ADGRB1 ^t	-2.16
6	JAG1 ^p	3.95	ID1 ^p	-2.13
7	CXCL5 ^p	3.91	TYMP ^t	-2.09
8	EFNA1 ^p	3.01	TIE1 ^t	-1.26
9	ANPEP ^p	2.43	ANGPTL4 ^t	-1.14
10	IL6 ^p	2.23	ANG ^t	-1.13
11	FGF2 ^p	1.97	ERBB2 ^t	-1.02
12	NRP2 ^p	1.91	COL18A1 ^p	-0.89
13	EFNB2 ^p	1.83	CCL11 ^t	-0.87
14	LECT1 ^t	1.83	IL1B ^t	-0.68
15	KDR ^p	1.82	TIMP2 ^t	-0.68
16	IFNG ^t	1.54	VEGFC ^p	-0.65
17	SERPINE1 ^t	1.49	PGFP	-0.64
18	PECAM1 ^p	1.46	ACTB ^t	-0.63
19	TGFB2 ^t	1.48	HPSE ^p	-0.61
20	NOTCH4 ^t	1.43	HPRT1 ^t	-0.61
21	PTGS1 ^p	1.25		
22	IFNA1 ^t	1.23		
23	PDGFA ^p	1.22		
24	EGF ^t	1.19		
25	ANGPT2 ^p	1.12		
26	TGFBR1 ^p	1.15		
27	CXCL10 ^p	1.07		
28	HGF ^p	1.08		
29	CTGFP	0.95		
30	EPHB4 ^t	0.89		
31	ITGAV ^t	0.77		
32	CDH5 ^p	0.75		
33	CXCL6 ^p	0.64		

*Genes distribution based on the Venn diagram (Figure 4) is marked with upper index for turquoise (t) and purple (p).

expression profile differed. Our results not only confirm the pro-inflammatory role of Gal-8, but, together with published data (60), also suggest that this galectin is orchestrating the interaction between endothelial cells and other cell types, such as leukocytes and platelets. Furthermore, Gal-8 secreted by osteoarthritic chondrocytes induces a pro-degradative/inflammatory gene signature, largely under the control of NF-κB signaling (61). In particular, the observed TNF over-expression following Gal-8 treatment supports its pro-inflammatory role, an effect that was rather attenuated in the presence of VEGF. The comparative analysis using the Venn diagram revealed 20 genes (*ANGPT2*, *ANPEP*, *CDH5*, *CTGF*, *CXCL10*, *CXCL6*, *CXCL5*, *EFNA1*, *EFNB2*, *FGF2*, *HGF*, *IL6*, *JAG1*, *KDR*, *NRP2*, *PDGFA*, *PECAM1*, *PTGS1*, *TGFBR1*,

Table II. Log₂-fold change of gene expression in HUVECs exposed to Gal-8 (0.004 µg/ml) in the presence of VEGF compared to VEGF-treated positive control*.

No.	Gene symbol	Log fold change	Gene symbol	Log fold change
1	KDR ^p	21	ENG ^o	-21.13
2	FGF1 ^o	8.36	PGFP	-20.63
3	TYMP ^o	6.78	ID1 ^p	-19.56
4	ANGPT1 ^o	5.90	TGFB1 ^o	-17.78
5	S1PR1 ^o	5.72	VEGFB ^o	-17.72
6	ANGPTL4 ^o	5.56	COL18A1 ^p	-17.49
7	ANG ^o	5.03	EPHB4 ^o	-16.84
8	TIE1 ^o	4.36	TGFB2 ^o	-15.66
9	ERBB2 ^o	4.32	VEGFC ^p	-15.21
10	ADGRB1 ^o	4.01	HPSE ^p	-12.55
11	IL1B ^o	3.77	PF4 ^p	-6.08
12	FGFR3 ^o	3.76	EGF ^o	-5.34
13	SERPINF1 ^o	3.65	LECT1 ^o	-5.14
14	FGF2 ^p	3.61	MMP9 ^o	-3.16
15	ANPEP ^p	3.57	PROK2 ^o	-1.89
16	CDH5 ^p	3.24	LEP ^o	-1.77
17	FIGF ^o	3.05	ITGAV ^o	-0.63
18	F3 ^o	2.83		
19	THBS1 ^o	2.80		
20	NOS3 ^o	2.74		
21	CXCL5 ^p	2.74		
22	ITGB3 ^o	2.70		
23	NRP2 ^p	2.56		
24	EFNA1 ^p	2.54		
25	ANGPT2 ^p	2.49		
26	PECAM1 ^p	2.47		
27	CXCL10 ^p	2.28		
28	CCL2 ^o	2.28		
29	PDGFA ^p	2.27		
30	IL6 ^p	2.26		
31	TIMP3 ^o	2.22		
32	CXCL8 ^o	2.19		
33	NRP1 ^o	2.17		
34	EFNB2 ^p	2.14		
35	CXCL1 ^o	2.07		
36	TIMP1 ^p	2.03		
37	FN1 ^o	2.03		
38	PTGS1 ^p	1.92		
39	MMP14 ^o	1.89		
40	FLT1 ^o	1.75		
41	JAG1 ^p	1.53		
42	CTGFP	1.50		
43	MDK ^o	1.50		
44	TEK ^o	1.46		
45	TGFBR1 ^p	1.43		
46	HIF1A ^o	1.25		
47	MMP2 ^o	1.21		
48	CXCL6 ^p	1.19		
49	EDN1 ^o	1.14		
50	CCL11 ^o	1.04		
51	TGFA ^o	1		
52	SPHK1 ^o	0.80		
53	HGF ^p	0.68		

*Genes distribution based on the Venn diagram (Figure 4) is marked with upper index for orange (o) and purple (p).

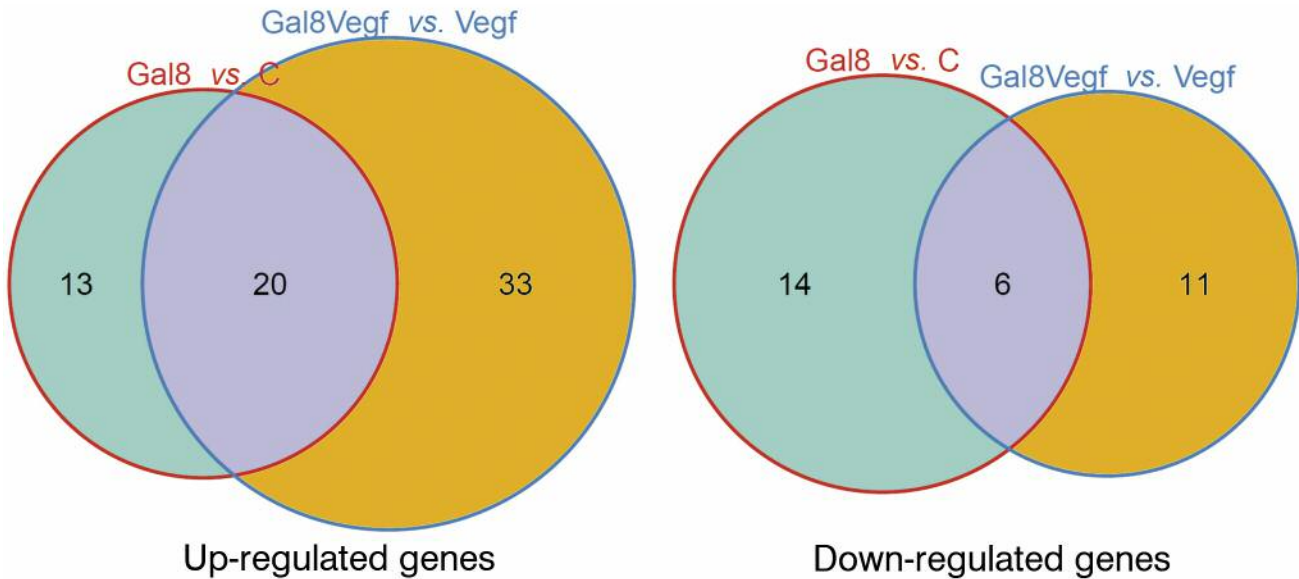


Figure 4. The Venn diagram of up- and down-regulated genes of two comparisons, i.e. Gal-8 (0.004 µg/ml) vs. untreated control (C) and Gal-8 (0.004 µg/ml) + VEGF (25 ng/ml) vs. VEGF (25 ng/ml) positive control. The fold-changes of genes are listed in Table 1 (Gal-8 vs. C) and 2 (Gal-8+VEGF vs. VEGF).

TIMP1) that were up-regulated in both comparisons, i.e. Gal-8 vs. C and Gal-8+VEGF vs. VEGF. In contrast, only 6 genes (*COL18A1*, *HPSE*, *ID1*, *PF4*, *PGF*, *VEGFC*) were down-regulated in these comparisons. For example, the basic fibroblast growth factor, product of the *FGF2* gene and a potent inducer/regulator of angiogenesis (62), was up-regulated with almost double-fold increase when cells were co-treated with Gal-8 and VEGF compared to VEGF positive control. Furthermore, the *KDR* gene coding the VEGFR2, was up-regulated over ten times when Gal-8 was combined with VEGF and data were compared to VEGF positive control. Following VEGF blockade Gal-1 may preserve angiogenesis via the VEGFR2 signaling (63). From this point of view, Gal-8-induced VEGFR2 up-regulation might be considered clinically relevant. The down-regulation of the *COL18A1* gene may also indirectly contribute to the pro-angiogenic effect of Gal-8, since the anti-angiogenic molecule endostatin is a fragment of the collagen XVIII that it encodes (64). In human platelets, Gal-1, -3, and -8 trigger VEGF release from α-granules (cellular component of platelets containing several growth factors, clotting proteins and active molecules), but only Gal-8 also induces the secretion of endostatin (65). On the one hand it supports the paradigm that we have different types of α-granules (storing different cargo proteins with opposite effects) that may selectively be activated, leading to the release of either pro or anti-angiogenic factors. More importantly, it also points to a competitive role of Gal-8 in biological processes associated with angiogenesis. However, missing data regarding the protein expression levels of the

identified angiogenesis-related key genes present the second limitation of our study and warrant further research.

While several Gal-8-binding proteins have been identified that might underlie its angiogenic activity, e.g., CD166, integrins, adhesion molecules (e.g. activated leukocyte cell adhesion molecule) and others (36, 48, 66), a direct interaction with VEGFR has not yet been reported, as has been seen, for example, in the case of Gal-1 and -3 (28). Nevertheless, the molecular data obtained in the present study, in particular regarding the *KDR* gene, suggest that VEGF signaling might be affected by Gal-8. The Venn diagram revealed that approximately in a half of deregulated genes the fold-change differs when Gal-8 vs. C and Gal-8+VEGF vs. VEGF were compared. However, among the commonly regulated genes Gal-8 and VEGF acted rather in a synergistic manner. Therefore, the question whether Gal-8 is able to directly interact with VEGF or its receptors remains to be answered in future experimental studies.

In conclusion, we present for the first time the gene expression profiling of HUVECs following Gal-8 treatment alone and when co-incubated with VEGF. Our findings demonstrate that Gal-8 with VEGF enhanced cell proliferation, migration and vessel sprouting compared to VEGF alone. However, Gal-8 alone does not exhibit any significant effects on cell proliferation and migration. These data indicate that Gal-8 promotes the pro-angiogenic phenotype rather in a synergistic manner with VEGF. Although, several clinical trials are being conducted focusing on the use of galectins for the treatment of neoplasms, they

only investigate inhibitors of Gal-1 and -3 (67). Therefore, further research is warranted to assess the role of Gal-8 in cancer growth, angiogenesis and occurrence of metastasis.

Supplementary Material

The Supplementary Figures are available at: <https://www.upjs.sk/en/faculty-of-medicine/center-of-research/laboratory-of-cell-interactions/varinska-supplementary-figure/>.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Authors' Contributions

Conceptualization: Lenka Varinská and Peter Gál; Methodology: Lenka Varinská, Lenka Faber, Eva Petrovová, Ludmila Balážová, Eleonóra Ivančová and Michal Kolář; Supervision: Peter Gál; Writing – original draft: Lenka Varinská and Peter Gál.

Acknowledgements

This work was supported by the Slovak Research and Development Agency (Nos. APVV-0408-12, APVV-16-0207 and APVV-14-0731), the Slovak Grant Agency (VEGA-1/0561/18), the Charles University, Prague, Czech Republic (PROGRES Q37), by the Operational Programme Research, Development and Education ("Center for Tumor Ecology - Research of the Cancer Microenvironment Supporting Cancer Growth and Spread", reg. No. CZ.02.1.01/0.0/0.0/16_019/0000785), and project implementation: Medical University Science Park in Košice (MediPark, Košice - Phase II) ITMS2014+ 313011D103 supported by the Operational Programme Research & Innovation, funded by the ERDF.

References

- Folkman J: The role of angiogenesis in tumor growth. *Semin Cancer Biol* 3(2): 65-71, 1992. PMID: 1378311.
- Kim HS, Jang JM, Yun SY, Zhou D, Piao Y, Ha HC, Back MJ, Shin IC and Kim DK: Effect of robinia pseudoacacia leaf extract on interleukin-1beta-mediated tumor angiogenesis. *In Vivo* 33(6): 1901-1910, 2019. PMID: 31662518. DOI: 10.21873/invivo.11684
- Ferrara N: Vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol* 29(6): 789-791, 2009. PMID: 19164810. DOI: 10.1161/ATVBAHA.108.179663
- Dvorak HF: Tumor stroma, tumor blood vessels, and antiangiogenesis therapy. *Cancer J* 21(4): 237-243, 2015. PMID: 26222073. DOI: 10.1097/PPO.000000000000124
- Itatani Y, Kawada K, Yamamoto T and Sakai Y: Resistance to anti-angiogenic therapy in cancer-alterations to anti-vegfr pathway. *Int J Mol Sci* 19(4), 2018. PMID: 29670046. DOI: 10.3390/ijms19041232
- Vasudev NS and Reynolds AR: Anti-angiogenic therapy for cancer: Current progress, unresolved questions and future directions. *Angiogenesis* 17(3): 471-494, 2014. PMID: 24482243. DOI: 10.1007/s10456-014-9420-y
- Tampellini M, Sonetto C and Scagliotti GV: Novel anti-angiogenic therapeutic strategies in colorectal cancer. *Expert Opin Investig Drugs* 25(5): 507-520, 2016. PMID: 26938715. DOI: 10.1517/13543784.2016.1161754
- Zhang W, Shen Z, Luo H, Hu X, Zheng L and Zhu X: The benefits and side effects of bevacizumab for the treatment of recurrent ovarian cancer. *Curr Drug Targets* 18(10): 1125-1131, 2017. PMID: 27138763. DOI: 10.2174/138945011766160502150237
- Rajabi M and Mousa SA: The role of angiogenesis in cancer treatment. *Biomedicines* 5(2): pii: E34, 2017. PMID: 28635679. DOI: 10.3390/biomedicines5020034
- Elola MT, Ferragut F, Mendez-Huergo SP, Croci DO, Bracalente C and Rabinovich GA: Galectins: Multitask signaling molecules linking fibroblast, endothelial and immune cell programs in the tumor microenvironment. *Cell Immunol* 333: 34-45, 2018. PMID: 29602445. DOI: 10.1016/j.cellimm.2018.03.008
- Kaltner H and Gabius HJ: Sensing glycans as biochemical messages by tissue lectins: The sugar code at work in vascular biology. *Thromb Haemost* 119(4): 517-533, 2019. PMID: 30620995. DOI: 10.1055/s-0038-1676968
- Rabinovich GA and Toscano MA: Turning 'sweet' on immunity: Galectin-glycan interactions in immune tolerance and inflammation. *Nat Rev Immunol* 9(5): 338-352, 2009. PMID: 19365409. DOI: 10.1038/nri2536
- Nabi IR, Shankar J and Dennis JW: The galectin lattice at a glance. *J Cell Sci* 128(13): 2213-2219, 2015. PMID: 26092931. DOI: 10.1242/jcs.151159
- Kaltner H and Gabius HJ: A toolbox of lectins for translating the sugar code: The galectin network in phylogenesis and tumors. *Histol Histopathol* 27(4): 397-416, 2012. PMID: 22374719. DOI: 10.14670/HH-27.397
- Rabinovich GA and Croci DO: Regulatory circuits mediated by lectin-glycan interactions in autoimmunity and cancer. *Immunity* 36(3): 322-335, 2012. PMID: 22444630. DOI: 10.1016/j.immuni.2012.03.004
- Jiang ZJ, Shen QH, Chen HY, Yang Z, Shuai MQ and Zheng SS: Galectin-1 gene silencing inhibits the activation and proliferation but induces the apoptosis of hepatic stellate cells from mice with liver fibrosis. *Int J Mol Med* 43(1): 103-116, 2019. PMID: 30365068. DOI: 10.3892/ijmm.2018.3950
- Dong R, Zhang M, Hu QY, Zheng S, Soh A, Zheng YJ and Yuan H: Galectin-3 as a novel biomarker for disease diagnosis and a target for therapy. *Int J Mol Med* 41(2): 599-614, 2018. PMID: 29207027. DOI: 10.3892/ijmm.2017.3311
- Cioca A, Muntean D, Bungardean C, Raica M and Cimpean AM: Expression and distribution of galectin-3 in chromophobe and papillary carcinomas. *Anticancer Res* 38(1): 259-263, 2018. PMID: 29277781. DOI: 10.21873/anticancer.12216
- Zivicova V, Gal P, Mifkova A, Novak S, Kaltner H, Kolar M, Strnad H, Sachova J, Hradilova M, Chovanec M, Gabius HJ, Smetana K, Jr. and Fik Z: Detection of distinct changes in gene-expression profiles in specimens of tumors and transition zones of tenascin-positive/-negative head and neck squamous cell carcinoma. *Anticancer Res* 38(3): 1279-1290, 2018. PMID: 29491051. DOI: 10.21873/anticancer.12350
- Michalova Z, Coma M, Kicova M, Gabzdilova J, Dedinska K, Guman T, Hajikova M, Veselinyova D, Giertlova M, Gal P and Sarisky M: Overexpression of galectin-3 in chronic lymphocytic leukemia is associated with 17p deletion: A short

- report. *Anticancer Res* 39(6): 2805-2810, 2019. PMID: 31177117. DOI: 10.21873/anticancer.13408
- 21 Sherazi AA, Jariwala KA, Cybulski AN, Lewis JW, Karagiannis J, Cumming RC and Timoshenko AV: Effects of global o-glcNAcylation on galectin gene-expression profiles in human cancer cell lines. *Anticancer Res* 38(12): 6691-6697, 2018. PMID: 30504378. DOI: 10.21873/anticancer.13037
 - 22 Tsangaris GT, Papatouasiou C, Adamopoulos PG, Scorilas A, Vorgias CE, Prodromou N, Stathopoulou FT, Stravopodis DJ and Anagnostopoulos AK: Pediatric ependymoma: A proteomics perspective. *Cancer Genomics Proteomics* 14(2): 127-136, 2017. PMID: 28387652. DOI: 10.21873/cgp.20025
 - 23 Zivicova V, Broz P, Fik Z, Mifkova A, Plzak J, Cada Z, Kaltner H, Kucerova JF, Gabius HJ and Smetana K, Jr.: Genome-wide expression profiling (with focus on the galectin network) in tumor, transition zone and normal tissue of head and neck cancer: Marked differences between individual patients and the site of specimen origin. *Anticancer Res* 37(5): 2275-2288, 2017. PMID: 28476793. DOI: 10.21873/anticancer.11565
 - 24 Chou FC, Chen HY, Kuo CC and Sytwu HK: Role of galectins in tumors and in clinical immunotherapy. *Int J Mol Sci* 19(2), 2018. PMID: 29389859. DOI: 10.3390/ijms19020430
 - 25 Hsieh SH, Ying NW, Wu MH, Chiang WF, Hsu CL, Wong TY, Jin YT, Hong TM and Chen YL: Galectin-1, a novel ligand of neuropilin-1, activates vegfr-2 signaling and modulates the migration of vascular endothelial cells. *Oncogene* 27(26): 3746-3753, 2008. PMID: 18223683. DOI: 10.1038/sj.onc.1211029
 - 26 Thijssen VL, Barkan B, Shoji H, Aries IM, Mathieu V, Deltour L, Hackeng TM, Kiss R, Kloog Y, Poirier F and Griffioen AW: Tumor cells secrete galectin-1 to enhance endothelial cell activity. *Cancer Res* 70(15): 6216-6224, 2010. PMID: 20647324. DOI: 10.1158/0008-5472
 - 27 Ito K, Scott SA, Cutler S, Dong LF, Neuzil J, Blanchard H and Ralph SJ: Thiodigalactoside inhibits murine cancers by concurrently blocking effects of galectin-1 on immune dysregulation, angiogenesis and protection against oxidative stress. *Angiogenesis* 14(3): 293-307, 2011. PMID: 21523436. DOI: 10.1007/s10456-011-9213-5
 - 28 D'Haene N, Sauvage S, Maris C, Adanja I, Le Mercier M, Decaestecker C, Baum L and Salmon I: Vegfr1 and vegfr2 involvement in extracellular galectin-1-and galectin-3-induced angiogenesis. *Plos One* 8(6): e67029, 2013. PMID: 23799140. DOI: 10.1371/journal.pone.0067029
 - 29 Croci DO, Cerliani JP, Dalotto-Moreno T, Mendez-Huergo SP, Mascanfroni ID, Dergan-Dylon S, Toscano MA, Caramelo JJ, Garcia-Vallejo JJ, Ouyang J, Mesri EA, Junttila MR, Bais C, Shipp MA, Salatino M and Rabinovich GA: Glycosylation-dependent lectin-receptor interactions preserve angiogenesis in anti-vegfr refractory tumors. *Cell* 156(4): 744-758, 2014. PMID: 24529377. DOI: 10.1016/j.cell.2014.01.043
 - 30 Markowska AI, Jefferies KC and Panjwani N: Galectin-3 protein modulates cell surface expression and activation of vascular endothelial growth factor receptor 2 in human endothelial cells. *J Biol Chem* 286(34): 29913-29921, 2011. PMID: 21715322. DOI: 10.1074/jbc.M111.226423
 - 31 Fukushi J, Makagiarsar IT and Stallcup WB: Ng2 proteoglycan promotes endothelial cell motility and angiogenesis via engagement of galectin-3 and alpha3beta1 integrin. *Mol Biol Cell* 15(8): 3580-3590, 2004. PMID: 15181153. DOI: 10.1091/mbc.e04-03-0236.
 - 32 Markowska AI, Liu FT and Panjwani N: Galectin-3 is an important mediator of vegf- and bfgf-mediated angiogenic response. *J Exp Med* 207(9): 1981-1993, 2010. PMID: 20713592. DOI: 10.1084/jem.20090121
 - 33 dos Santos SN, Sheldon H, Pereira JX, Paluch C, Bridges EM, El-Cheikh MC, Harris AL and Bernardes ES: Galectin-3 acts as an angiogenic switch to induce tumor angiogenesis via jagged-1/notch activation. *Oncotarget* 8(30): 49484-49501, 2017. PMID: 28533486. DOI: 10.18632/oncotarget.17718
 - 34 Thijssen VL, Heusschen R, Caers J and Griffioen AW: Galectin expression in cancer diagnosis and prognosis: A systematic review. *Biochim Biophys Acta* 1855(2): 235-247, 2015. PMID: 25819524. DOI: 10.1016/j.bbcan.2015.03.003
 - 35 Bidon N, Brichory F, Bourguet P, Le Pennec JP and Dazord L: Galectin-8: A complex sub-family of galectins (review). *Int J Mol Med* 8(3): 245-250, 2001. PMID: 11494049. DOI: 10.3892/ijmm.8.3.245
 - 36 Troncoso MF, Ferragut F, Bacigalupo ML, Cardenas Delgado VM, Nugnes LG, Gentilini L, Laderach D, Wolfenstein-Todel C, Compagno D, Rabinovich GA and Elola MT: Galectin-8: A matricellular lectin with key roles in angiogenesis. *Glycobiology* 24(10): 907-914, 2014. PMID: 24939370. DOI: 10.1093/glycob/cwu054
 - 37 Levy Y, Arbel-Goren R, Hadari YR, Eshhar S, Ronen D, Elhanany E, Geiger B and Zick Y: Galectin-8 functions as a matricellular modulator of cell adhesion. *J Biol Chem* 276(33): 31285-31295, 2001. PMID: 11371555. DOI: 10.1074/jbc.M100340200
 - 38 Zick Y, Eisenstein M, Goren RA, Hadari YR, Levy Y and Ronen D: Role of galectin-8 as a modulator of cell adhesion and cell growth. *Glycoconj J* 19(7-9): 517-526, 2002. PMID: 14758075. DOI: 10.1023/B:GLYC.0000014081.55445.af
 - 39 Metz C, Doger R, Riquelme E, Cortes P, Holmes C, Shaughnessy R, Oyanadel C, Grabowski C, Gonzalez A and Soza A: Galectin-8 promotes migration and proliferation and prevents apoptosis in u87 glioblastoma cells. *Biol Res* 49(1): 33, 2016. PMID: 27459991. DOI: 10.1186/s40659-016-0091-6
 - 40 Gentilini LD, Jaworski FM, Tiraboschi C, Perez IG, Kotler ML, Chauchereau A, Laderach DJ and Compagno D: Stable and high expression of galectin-8 tightly controls metastatic progression of prostate cancer. *Oncotarget* 8(27): 44654-44668, 2017. PMID: 28591719. DOI: 10.18632/oncotarget.17963
 - 41 Danguy A, Rorive S, Decaestecker C, Bronckart Y, Kaltner H, Hadari YR, Goren R, Zich Y, Petein M, Salmon I, Gabius HJ and Kiss R: Immunohistochemical profile of galectin-8 expression in benign and malignant tumors of epithelial, mesenchymatous and adipous origins, and of the nervous system. *Histol Histopathol* 16(3): 861-868, 2001. PMID: 11510978. DOI: 10.14670/HH-16.861
 - 42 Dong GW, Kim J, Park JH, Choi JY, Cho SI and Lim SC: Galectin-8 expression in laryngeal squamous cell carcinoma. *Clin Exp Otorhinolaryngol* 2(1): 13-19, 2009. PMID: 19434286. DOI: 10.3342/ceo.2009.2.1.13
 - 43 Wollina U, Graefe T, Feldrappe S, Andre S, Wasano K, Kaltner H, Zick Y and Gabius HJ: Galectin fingerprinting by immuno- and lectin histochemistry in cutaneous lymphoma. *J Cancer Res Clin Oncol* 128(2): 103-110, 2002. PMID: 11862481. DOI: 10.1007/s00432-001-0304-3
 - 44 Savin S, Cvejic D, Jankovic M, Isic T, Paunovic I and Tatic S: Evaluation of galectin-8 expression in thyroid tumors. *Med Oncol* 26(3): 314-318, 2009. PMID: 19009371. DOI: 10.1007/s12032-008-9122-7

- 45 Elola MT, Ferragut F, Delgado VMC, Nugnes LG, Gentilini L, Laderach D, Troncoso MF, Compagno D, Wolfenstein-Todel C and Rabinovich GA: Expression, localization and function of galectin-8, a tandem-repeat lectin, in human tumors. *Histol Histopathol* 29(9): 1093-1105, 2014. PMID: 24696431. DOI: 10.14670/HH-29.1093
- 46 Schulz H, Kuhn C, Hofmann S, Mayr D, Mahner S, Jeschke U and Schmoeckel E: Overall survival of ovarian cancer patients is determined by expression of galectins-8 and-9. *Int J Mol Sci* 19(1): pii: E323., 2018. PMID: 29361803. DOI: 10.3390/ijms19010323
- 47 Zamorano P, Koning T, Oyanadel C, Mardones GA, Ehrenfeld P, Boric MP, Gonzalez A, Soza A and Sanchez FA: Galectin-8 induces endothelial hyperpermeability through the enos pathway involving s-nitrosylation-mediated adherens junction disassembly. *Carcinogenesis* 40(2): 313-323, 2019. PMID: 30624618. DOI: 10.1093/carcin/bgz002
- 48 Delgado VMC, Nugnes LG, Colombo LL, Troncoso MF, Fernandez MM, Malchiodi EL, Frahm I, Croci DO, Compagno D, Rabinovich GA, Wolfenstein-Todel C and Elola MT: Modulation of endothelial cell migration and angiogenesis: A novel function for the "tandem-repeat" lectin galectin-8. *Faseb J* 25(1): 242-254, 2011. PMID: 20876211. DOI: 10.1096/fj.09-144907
- 49 Cueni LN and Detmar M: Galectin-8 interacts with podoplanin and modulates lymphatic endothelial cell functions. *Exp Cell Res* 315(10): 1715-1723, 2009. PMID: 19268462. DOI: 10.1016/j.yexcr.2009.02.021
- 50 Chen WS, Cao ZY, Sugaya S, Lopez MJ, Sendra VG, Laver N, Leffler H, Nilsson UJ, Fu JX, Song JH, Xia LJ, Hamrah P and Panjwani N: Pathological lymphangiogenesis is modulated by galectin-8-dependent crosstalk between podoplanin and integrin-associated vegfr-3. *Nature Communications* 7: 11302, 2016. PMID: 27066737. DOI: 10.1038/ncomms11302
51. Chen WS, Cao ZY, Leffler H, Nilsson U, Xia LJ and Panjwani N: Pathological lymphangiogenesis is regulated by galectin-8-dependent crosstalk among vegf-c, podoplanin and integrin pathways. *Invest Ophthalmol Vis Sci* 57, 2016.
- 52 Defilippi P, van Hinsbergh V, Bertolotto A, Rossino P, Silengo L and Tarone G: Differential distribution and modulation of expression of alpha 1/beta 1 integrin on human endothelial cells. *J Cell Biol* 114(4): 855-863, 1991. PMID: 1869591. DOI: 10.1083/jcb.114.4.855
- 53 World Medical Association: World medical association declaration of helsinki: Ethical principles for medical research involving human subjects. *JAMA* 310(20): 2191-2194, 2013. PMID: 24141714. DOI: 10.1001/jama.2013.281053
- 54 Poldervaart MT, Gremmels H, van Deventer K, Fledderus JO, Oner FC, Verhaar MC, Dhert WJ and Alblas J: Prolonged presence of vegf promotes vascularization in 3d bioprinted scaffolds with defined architecture. *J Control Release* 184: 58-66, 2014. PMID: 24727077. DOI: 10.1016/j.jconrel.2014.04.007
- 55 Martinez-Poveda B, Quesada AR and Medina MA: Hypericin in the dark inhibits key steps of angiogenesis *in vitro*. *Eur J Pharmacol* 516(2): 97-103, 2005. PMID: 15921677. DOI: 10.1016/j.ejphar.2005.03.047
- 56 Luo W, Pant G, Bhavnasi YK, Blanchard SG Jr. and Brouwer C: Pathview web: User friendly pathway visualization and data integration. *Nucleic Acids Res* 45(W1): W501-W508, 2017. PMID: 28482075. DOI: 10.1093/nar/gkx372
- 57 Varinska L, Faber L, Kello M, Petrovova E, Balazova L, Solar P, Coma M, Urdzik P, Mojzis J, Svajdlenka E, Mucaji P and Gal P: Beta-escin effectively modulates huvecs proliferation and tube formation. *Molecules* 23(1): pii: E197, 2018. PMID: 29342121. DOI: 10.3390/molecules23010197
- 58 Adams L, Scott GK and Weinberg CS: Biphasic modulation of cell growth by recombinant human galectin-1. *Biochim Biophys Acta* 1312(2): 137-144, 1996. PMID: 8672536. DOI: 10.1016/0167-4889(96)00031-6
- 59 Vas V, Fajka-Boja R, Ion G, Dudics V, Monostori E and Uher F: Biphasic effect of recombinant galectin-1 on the growth and death of early hematopoietic cells. *Stem Cells* 23(2): 279-287, 2005. PMID: 15671150. DOI: 10.1634/stemcells.2004-0084
- 60 Cattaneo V, Tribulatti MV, Carabelli J, Carestia A, Schattner M and Campetella O: Galectin-8 elicits pro-inflammatory activities in the endothelium. *Glycobiology* 24(10): 966-973, 2014. PMID: 24957054. DOI: 10.1093/glycob/cwu060
- 61 Weinmann D, Kenn M, Schmidt S, Schmidt K, Walzer SM, Kubista B, Windhager R, Schreiner W, Toegel S and Gabius HJ: Galectin-8 induces functional disease markers in human osteoarthritis and cooperates with galectins-1 and-3. *Cell Mol Life Sci* 75(22): 4187-4205, 2018. PMID: 29934665. DOI: 10.1007/s00018-018-2856-2
- 62 Murakami M and Simons M: Fibroblast growth factor regulation of neovascularization. *Curr Opin Hematol* 15(3): 215-220, 2008. PMID: 18391788. DOI: 10.1097/MOH.0b013e3282f97d98
- 63 Croci DO and Rabinovich GA: Linking tumor hypoxia with vegfr2 signaling and compensatory angiogenesis: Glycans make the difference. *Oncoimmunology* 3: e29380, 2014. PMID: 25114834. DOI: 10.4161/onci.29380
- 64 O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR and Folkman J: Endostatin: An endogenous inhibitor of angiogenesis and tumor growth. *Cell* 88(2): 277-285, 1997. PMID: 9008168. DOI: 10.1016/s0092-8674(00)81848-6
- 65 Etulain J, Negrotto S, Tribulatti MV, Croci DO, Carabelli J, Campetella O, Rabinovich GA and Schattner M: Control of angiogenesis by galectins involves the release of platelet-derived proangiogenic factors. *PLoS One* 9(4): e96402, 2014. PMID: 24788652. DOI: 10.1371/journal.pone.0096402
- 66 Hadari YR, Arbel-Goren R, Levy Y, Amsterdam A, Alon R, Zakut R and Zick Y: Galectin-8 binding to integrins inhibits cell adhesion and induces apoptosis. *J Cell Sci* 113(13): 2385-2397, 2000. PMID: 10852818.
- 67 Wdowiak K, Francuz T, Gallego-Colon E, Ruiz-Agamez N, Kubeczko M, Grochola I and Wojnar J: Galectin targeted therapy in oncology: Current knowledge and perspectives. *Int J Mol Sci* 19(1): pii: E210, 2018. PMID: 29320431. DOI: 10.3390/ijms19010210

Received April 6, 2020
 Revised April 20, 2020
 Accepted April 23, 2020