Extracellular Matrix of Galectin-1-exposed Dermal and Tumor-associated Fibroblasts Favors Growth of Human Umbilical Vein Endothelial Cells *In Vitro*: A Short Report

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Abstract. *Background/Aim: Stromal cells in the tumor microenvironment are primarily considered as sources of promalignant factors. The objective of our study was to define the effect of extracellular matrix (ECM) produced by normal dermal or cancer-associated fibroblasts exposed to adhesion/growth-regulatory lectin galectin-1 on human umbilical vein endothelial cells (HUVECs). Materials and Methods: Fibroblasts were cultured for 10 days with lectin, followed by removing cellular constituents after an osmotic shock. Freshly-isolated HUVECs were placed on the ECM. In parallel, HUVECs were seeded on untreated and gelatin-coated surfaces as controls. A positive control for growth of HUVECs culture using medium supplemented with vascular endothelial growth factor completed the test panel. Cells were kept in contact to the substratum for two days and then processed for immunocytochemistry. Results: HUVECs seeded on fibroblast-generated ECM presented a comparatively high degree of proliferation. Furthermore, contact to substratum produced by tumor-associated fibroblasts led to generation of a meshwork especially rich in fibronectin. Conclusion: Galectin-1 is apparently capable to trigger ECM production favorable for growth of HUVECs, prompting further work on characterizing structural features of the ECM and in situ correlation of lectin presence, ECM constitution and neoangiogenesis.*

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The tumor microenvironment is formed by both malignant and non-malignant cells as well as by extracellular matrix (ECM) glycoproteins and various types of soluble mediators (1, 2). While the study of its impact on tumor cells is an established focus of research, another cellular aspect deserves attention. Nutrient supply and spread *via* circulation critically depend on neoangiogenesis (3). Broadening the biomedical scope beyond malignancy, this process can drive granulation tissue formation during wound healing, adding incentive to examine the impact of microenvironment features on vessel growth (4). In this respect, constituents of the ECM have proven capable to stimulate angiogenesis, *e.g.* by stabilizing blood vessels (5). Hence, rational modulation of endothelial cell-ECM interactions may open a route contributing to advancing wound healing and interfering with cancer.

With focus on cancer, we have previously shown that fibroblasts obtained from tumor stroma can affect malignant cells *in vitro*. These purported cancer-associated fibroblasts

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(CAFs) apparently acquire a distinct phenotype different from normal fibroblasts and, by a re-programming within gene expression, provide pro-inflammatory/tumoral effectors to their vicinity (6-9). In addition, these cells also shape their vicinity by generating ECM, whose functional significance warrants investigation. Toward this end, we here address the issue of testing whether ECM affects vessel growth, considering the impact of a potent effector for conversion of fibroblasts to myofibroblasts and ECM production, *i.e.* the adhesion/growth-regulatory galectin-1 (Gal-1) (10-12). This endogenous lectin, known for triggering diverse cellular responses such as growth modulation, invasion or motility (12-14) and production of vascular endothelial growth factor-C (VEGF-C) (15), is herein studied for its impact on the qualities of ECM to sustain proliferation of endothelial cells.

Materials and Methods

Human umbilical vein endothelial cells (HUVECs). HUVECs were isolated, cultured, and characterized as described previously (16-17). Cells were cultured on gelatin-coated dishes in cM199 (M199 medium (Cambrex, Verviers, Belgium), supplemented with 10% heat-inactivated human serum (PAA, Pasching, Austria), 10% heat-inactivated newborn calf serum (Cambrex, Verviers, Belgium), 150 μg/ml crude endothelial cell growth factor (ECGF) (Cambrex, Verviers, Belgium), 5 U/ml heparin (Cambrex, Verviers, Belgium), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Biochrom, Berlin, Germany)) at 37˚C with a 5% CO_2/95% air atmosphere. Twenty-four hours prior to the experiments, medium of the endothelial cell cultures was exchanged with a solution lacking crude ECGF and human serum. Cell viability, estimated by trypan blue exclusion, was 95% before starting each experiment.

Human dermal fibroblasts (HDFs) and squamous cell carcinoma fibroblasts (SCCFs). HDFs were isolated from residual skin specimens obtained from healthy donors with the informed consent of the patients (in complete agreement with the Helsinki Declaration after approval by the Local Ethical Committee) at the Department of Aesthetic Surgery (Charles University, 3rd Faculty of Medicine, Prague, Czech Republic) and the cultures expanded in Dulbecco's medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin/streptomycin (ATB) (all from Biochrom, Berlin, Germany) at 37˚C and 5% CO_2/95% air atmosphere.

SCCFs were prepared from tumor specimen of a case of squamous cell carcinoma located in the root of tongue with the informed consent of the patient (in complete agreement with the Helsinki Declaration after approval by the Local Ethical Committee) according to a basic protocol (18) modified as described in detail (6) at the Department of Stomatology (Charles University, 1st Faculty of Medicine). SCCFs migrating from small pieces of tumor biopsy were collected and cultures then expanded in DMEM with 10% FBS and ATB (all from Biochrom) at 37˚C and 5% CO_2/95% air atmosphere.

HUVECs and their culture on matrices induced by Gal-1. Sterile solutions containing human Gal-1, produced, stabilized by iodoacetamide treatment, controlled for purity and tested for bioactivity as described previously (10) at the concentration of 300 ng/ml, were prepared in DMEM containing 10% FBS and ATB. HDFs or SCCFs were seeded at a density of 5,000 cells/cm^2 and cultured for 10 days. ECM scaffolds (Figure 1) produced by cells exposed to Gal-1 on the surface of coverslips were tested as substratum for the culture of HUVECs obtained as described above. To remove the cells, the coverslips were incubated with sterile distilled water for 60 minutes exerting an osmotic shock to cells (as ascertained by subsequent culture), then the supports were incubated with culture medium for 24 hours to prevent osmotic stress during subsequent culturing of HUVECs (Figure 2A). HUVECs were seeded at a density of 10,000 cells/cm^2 and kept for 48 h at 5% CO_2/95% air atmosphere and 37˚C. Cells brought into contact with untreated and/or gelatin-coated coverslips (in the presence or absence of 25 ng/ml of recombinant VEGF) were used as controls.

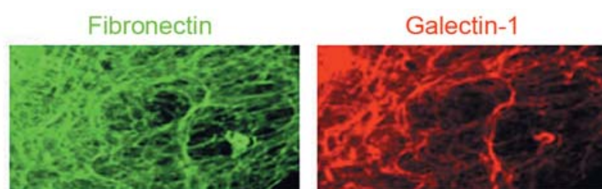

Figure 1. *Representative figure of decellularized ECM produced by fibroblasts following 10-days lasting galectin-1 stimulation and after an osmotic shock.*

Immunocytochemical analysis of cultured cells. The tested specimens were fixed with 2% buffered paraformaldehyde (pH 7.2) for 5 min and washed with phosphate-buffered saline. Cells were permeabilized (except for HUVEC cells processed for CD31 staining (endothelium marker)) by exposure to Triton X-100 (Sigma-Aldrich, Prague, Czech Republic) and sites for the antigen-independent binding of antibodies were blocked by porcine serum albumin. Commercial antibodies were diluted as recommended by the suppliers. The set of primary (CD31, galectin-1, Ki67, vimentin, fibronectin) and secondary antibodies used in this study is summarized in Table I. The specificity of the immunocytochemical reaction was ascertained by replacing the specific first-step antibodies by an irrelevant antibody, by omission of the first-step antibody from processing and by processing positive control specimens. Cell nuclei were counterstained by 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). All specimens were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and inspected by an Eclipse 90i fluorescence microscope (Nikon, Tokyo, Japan) equipped with filterblocks for fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) and DAPI, and a Cool-1300Q CCD camera (Vosskühler, Osnabrück, Germany); data were analyzed by a LUCIA 5.1 computer-assisted image analysis system (Laboratory Imaging, Prague, Czech Republic).

Cell counting and statistical analysis. Cell nuclei (based on DAPI staining) were counted in three randomly selected visualization fields in each case. Furthermore, Ki67 positivity was assessed in three fields per specimen. The proliferation activity was then expressed as mean number of cells per visualization field and also as percentage of Ki67-positive cells. All experiments were performed in triplicates.

Table I. *Reagents used for immunocytochemistry.*

Primary antibody	Abbreviation	Specificity	Host	Produced by	Secondary antibody	Produced by	Channel
CD31	CD31	Endothelial cell	Mouse monoclonal	Abcam, Cambridge Science, Cambridge, UK	Goat anti-mouse	Sigma-Aldrich, St. Louis, MO, USA	TRITC-red
Galectin-1	Gal-1	ECM/cell	Mouse monoclonal	Vector Laboratories, Burlingame, CA, USA	Goat anti-mouse	Sigma-Aldrich, St. Louis, MO, USA	TRITC-red
Ki67	Ki67	Proliferation marker	Mouse monoclonal	DakoCytomation, Glostrup, Denmark	Goat anti-mouse	Sigma-Aldrich, St. Louis, MO, USA	TRITC-red
Vimentin	Vim	Mesenchymal cell	Mouse monoclonal	DakoCytomation, Glostrup, Denmark	Goat anti-mouse	Sigma-Aldrich, St. Louis, MO, USA	TRITC-red
Fibronectin	Fibr	ECM	Rabbit polyclonal	DakoCytomation, Glostrup, Denmark	Swine anti-rabbit	Dako Cytomation, Glostrup, Denmark	FITC-green
Galectin-1	Gal-1	ECM/cell	Rabbit polyclonal	Gabius laboratory, LMU, Munich, Germany	Swine anti-rabbit	Dako Cytomation, Glostrup, Denmark	FITC-green

All data are expressed as mean±standard deviation (SD). Analysis of variance (ANOVA) followed by processing data by the Tukey-Kramer multiple comparison test were applied for statistically evaluating differences in total cell numbers and percentages of Ki67-positive cells. p Values less than 0.05, were considered statistically significant.

Results

Cell morphology. The mesenchymal origin of the cells was confirmed by vimentin staining, and cells were also positive for CD31, which is a commonly accepted endothelium marker (Figure 2B and C). Intracellular galectin-1 presence was moderate in all culture systems except for the control culture (seeded on the untreated surface without VEGF supplementation), where only a very weak signal was recorded (Figure 2B). Control cultures of HUVECs, seeded on the untreated surface without VEGF supplementation of the medium, expressed a comparatively small amount of fibronectin (Figure 2B). HUVECs seeded on a gelatin-coated surface and cultured in a VEGF-free medium were able to produce a fine-structured fibronectin-positive ECM. In contrast, cells in VEGF-stimulated cultures synthesized rather rough bundles of fibronectin in a comparatively reduced amount. Similarly, cells seeded on a HDF-generated bio-scaffold produced only a limited amount of fibronectin. In contrast, HUVECs in contact with a SCCFs-derived matrix produced the most prominent fibronectin network (Figure 2C).

Cell proliferation. In the next step, the number of cells per visualization field and the expression of the human nuclear antigen Ki67 were used to grade the effect of cell-surface/matrix interaction on HUVECs proliferation. Evidently, the lowest number of cells was present in the control culture, the highest in the culture where cells were seeded on the scaffold produced either by HDFs or SCCFs (Figure 2D). The percentages of Ki67-positive cells did not consistently correlate with the total number of cells (Figure 2D). With respect to this parameter, the highest level of Ki67 presence was observed in HUVECs seeded either on untreated glass and stimulated with VEGF or on a SCCF-produced matrix.

Discussion

Our previous experience with human keratinocytes, which could be expanded on a matrix produced by fibroblasts (rich in fibronectin and Gal-1) without feeder cells and having a phenotype characterized by a small diameter and keratin-19 positivity which reflects a low differentiation status (10, 19), prompted the current investigation. Accordingly, endothelial cells, routinely grown on gelatin-coated surfaces, were tested in this system as well. Herein we showed that ECM generated by HDFs and SCCFs in response to Gal-1 favors HUVECs growth in the culture.

The reported data make a strong case for directing further efforts to analyzing the properties of the ECM produced by fibroblasts beyond the attention given to the secretion of chemokines, cytokines and growth factors. Use of the described system provides availability of factors such as chemokine CXCL-1 and interleukins-6/-8, which are up-regulated (20, 22). With Gal-1 belonging to the set of proteins with increased expression documented in head and neck cancer stroma (22, 23), an effect on endothelial cells and so vascularization can be expected. Galectins have been proven to be potent mediators in different contexts, *e.g.* immunomodulation exerted by such parasite-derived effectors on immune cell activities (24-25). Consideration of this aspect can add to the currently defined multifunctionality of these lectins, a prerequisite to turn emerging insights into testable approaches to interfere with tumor growth or to promote wound healing.

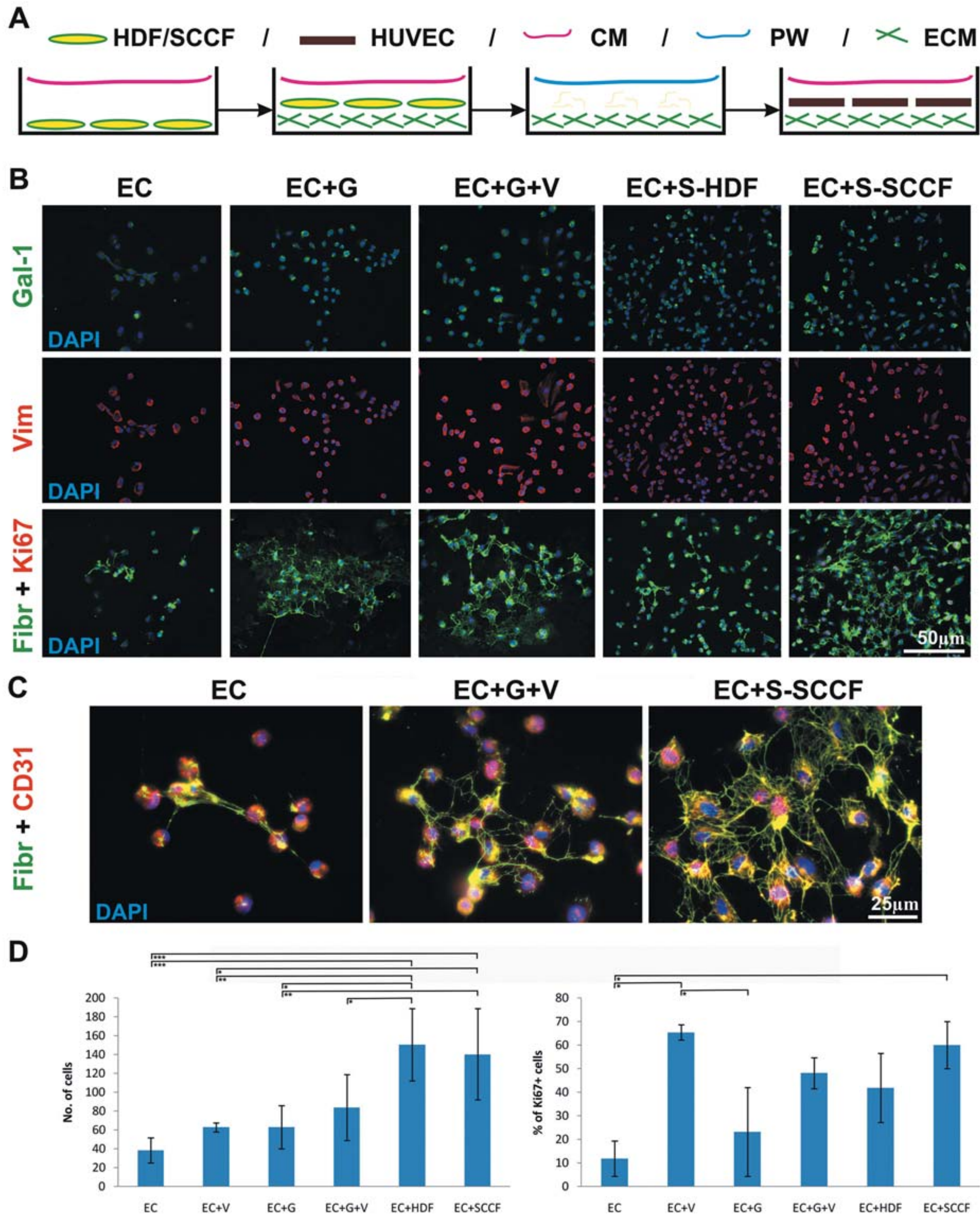

Figure 2. *A: Schematic presentation of experimental design for building the bioactive scaffold produced by fibroblasts. B: Cultures of human umbilical vein endothelial cells (EC) seeded on various types of surfaces: EC – cells seeded on the untreated surface in standard medium, EC+G – cells seeded on a gelatin-coated surface in standard medium, EC+G+V – cells seeded on a gelatin-coated surface in medium supplemented with VEGF (V), EC+S+HDF – cells seeded on the matrix produced by human dermal fibroblasts (HDF), EC+S+SCCF – cells seeded on the matrix produced by fibroblasts prepared from a human squamous cell carcinoma (SCCF). Figure B also shows the effect of cultivation condition on expression of galectin, vimentin and proliferation marker Ki67 as well as production of fibronectin. C: The effect on extracellular matrix rich for fibronectin production is shown with simultaneous detection of endothelial marker CD31. D: Graph shows cell numbers (left) and percentages of Ki67-positive cells (right) in the tested culture systems (*p<0.05; **p<0.01; ***p<0.001).*

In conclusion, both dermal fibroblasts and cancer-associated fibroblasts respond to Gal-1 exposure *in vitro* by producing an ECM, which is favorable for sustained HUVECs culture. In addition to the aspect of ECM remodeling by matrix metalloproteinases (27), work on matrix constitution, produced by fibroblasts as integral part of tumor microenvironment, is thus warranted.

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Conflict of Interest

The Authors have no conflict of interest to declare.

References

1 Catalano V, Turdo A, Di Franco S, Dieli F, Todaro M and Stassi G: Tumor and its microenvironment: a synergistic interplay. Semin Cancer Biol *23*: 522-532, 2013.

2 Smetana K Jr., Dvořánková B and Lacina L: Phylogeny, regeneration, ageing and cancer: role of microenvironment and possibility of its therapeutic manipulation. Folia Biol (Praha) *59*: 207-216, 2013.

3 Bruno A, Pagani A, Magnani E, Rossi T, Noonan DM, Cantelmo AR and Albini A: Inflammatory angiogenesis and the tumor microenvironment as targets for cancer therapy and prevention. Cancer Treat Res *159*: 401-426, 2014.

4 Greaves NS, Ashcroft KJ, Baguneid M and Bayat A: Current understanding of molecular and cellular mechanisms in fibroplasia and angiogenesis during acute wound healing. J Dermatol Sci *72*: 206-217, 2013.

5 Nyberg P, Salo T and Kalluri R: Tumor microenvironment and angiogenesis. Front Biosci *13*: 6537-6553, 2008.

6 Lacina L, Smetana K Jr., Dvořánková B, Pytlík R, Kideryová L, Kucerová L, Plzáková Z, Štork J, Gabius H-J and André S: Stromal fibroblasts from basal cell carcinoma affect phenotype of normal keratinocytes. Br J Dermatol *156*: 819-829, 2007.

7 Kideryová L, Lacina L, Dvořánková B, Štork J, Čada Z, Szabo P, André S, Kaltner H, Gabius H-J and Smetana K Jr.: Phenotypic characterization of human keratinocytes in coculture reveals differential effects of fibroblasts from benign fibrous histiocytoma (dermatofibroma) as compared to cells from its malignant form and to normal fibroblasts. J Dermatol Sci *55*: 18-26, 2009.

8 Strnad H, Lacina L, Kolář M, Čada Z, Vlček Č, Dvořánková B, Betka J, Plzák J, Chovanec M, Sáchová J, Valach J, Urbanová M and Smetana K Jr.: Head and neck squamous cancer stromal fibroblasts produce growth factors influencing phenotype of normal human keratinocytes. Histochem Cell Biol *133*: 201-211, 2010.

9 Kim SH, Choe C, Shin YS, Jeon MJ, Choi SJ, Lee J, Bae GY, Cha HJ and Kim J: Human lung cancer-associated fibroblasts enhance motility of non-small cell lung cancer cells in co-culture. Anticancer Res *33*: 2001-2009, 2013.

10 Dvořánková B, Szabo P, Lacina L, Gál P, Uhrova J, Zima T, Kaltner H, André S, Gabius H-J, Sykova E and Smetana K Jr: Human galectins induce conversion of dermal fibroblasts into myofibroblasts and production of extracellular matrix: potential application in tissue engineering and wound repair. Cells Tissues Organs *194*: 469-480, 2011.

11 Kaltner H and Gabius H-J: A toolbox of lectins for translating the sugar code: the galectin network in phylogenesis and tumors. Histol Histopathol *27*: 397-416, 2012.

12 Smetana K Jr., André S, Kaltner H, Kopitz J and Gabius H-J: Context-dependent multifunctionality of galectin-1: a challenge for defining the lectin as therapeutic target. Expert Opin Ther Targets *17*: 379-392, 2013.

13 Wiest I, Alexiou C, Kuhn C, Schulze S, Kunze S, Mayr D, Betz P, Jeschke U and Dian D: Expression of different carbohydrate tumour markers and galectins 1 and 3 in normal squamous and malignant epithelia of the upper aaerodigestive tract. Anticancer Res *32*: 2023-2029, 2012.

14 Amano M, Eriksson H, Manning JC, Detjen KM, André S, Nishimura S-I, Lehtiö J and Gabius H-J: Tumour suppressor $p16^{INK4a}$: anoikis-favouring decrease in N/O-glycan/cell surface sialylation by down-regulation of enzymes in sialic acid biosynthesis in tandem in a pancreatic carcinoma model. FEBS J *279*: 4062-4080, 2012.

15 Timoshenko AV, Kaltner H, André S, Gabius H-J and Lala PK: Differential stimulation of VEGF-C production by adhesion/growth-regulatory galectins and plant lectins in human breast cancer cells. Anticancer Res *30*: 4829-4833, 2010.

16 van Hinsbergh VW, Sprengers ED and Kooistra EA:Effect of thrombin on the production of plasminogen activators and PA inhibitor-1 by human foreskin microvascular endothelial cells. Thromb Haemost *57*: 148-153, 1987.

17 Defilippi P, van Hinsbergh VWM, Bertolotto A, Rossino P, Silengo L and Tarone G: Differential distribution and modulation of expression of alpha1/beta1 integrin on human endothelial cells. J Cell Biol *114*: 855-863, 1991.

18 Grando SA, Schofield O, Skubitz APN, Kist DA, Zelickson BD and Zachary CB: Nodular basal cell carcinomain vivo vs *in vitro*. Arch Dermatol *132*: 1185-1193,1996.

19 Dvořánková B, Smetana K Jr, Chovanec M, Lacina L, Štork J, Plzáková Z, Galovičová M and Gabius H-J:Transient expression of keratin 19 is induced in originally negative interfollicular epidermal cells by adhesion of suspended cells. Int J Mol Med *16*: 525-531, 2005.

20 Kolář M, Szabo P, Dvořánková B, Lacina L, Gabius H-J, Strnad H, Sáchová J, Vlček C, Plzák J, Chovanec M, Čada Z, Betka J, Fík Z, Pačes J, Kovářová H, Motlík J, Jarkovská K and Smetana K Jr.: Up-regulation of IL-6, IL-8 and CXCL-1 production in dermal fibroblasts by normal/malignant epithelial cells *in vitro*: Immunohistochemical and transcriptomic analyses. Biol Cell *104*: 738-751, 2012.

21 Szabo P, Valach J, Smetana K Jr. and Dvořánková B. Comparative analysis of IL-8 and CXCL-1 production by normal and cancer stromal fibroblasts. Folia Biol (Praha) *59*: 134-137, 2013.

22 Valach J, Fík Z, Strnad H, Chovanec M, Plzák J, Čada Z, Szabo P, Sáchová J, Hroudová M, Urbanová M, Šteffl M, Pačes J, Mazánek J, Vlček Č, Betka J, Kaltner H, André S, Gabius H-J, Kodet R, Smetana K Jr, Gál P and Kolář M: Smooth muscle actin-expressing stromal fibroblasts in head and neck squamous cell carcinoma: increased expression of galectin-1 and induction of poor prognosis factors. Int J Cancer *131*: 2499-2508, 2012.
23 Saussez S, Decaestecker C, Cludts S, Ernoux P, Chevalier D, Smetana K Jr., André S, Leroy X and Gabius H-J: 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 Kim JY, Cho MK, Choi SH, Lee KH, Ahn SC, Kim DH and Yu HS: Inhibition of dextran sulfate sodium (DSS)-induced intestinal inflammation via enhanced IL-10 and TGF-beta production by galectin-9 homologues isolated from intestinal parasites. Mol Biochem Parasitol *174*: 53-61, 2010.
25 Wang W, Yuan C, Wang S, Song X, Xu L, Yan R, Hasson IA and Li X: Transcriptional and proteomic analysis reveal recombinant galectins of Haemonchus contortus down-regulated functions of goat PBMC and modulation of several signaling cascades *in vitro*. J Proteomics *98C*: 123-137, 2014.
26 Goruppi S and Dotto GP: Mesenchymal stroma: primary determinant and therapeutictarget for epithelial cancer. Trends Cell Biol *23*: 593-602, 2013.

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