Human galectin-3: Molecular switch of gene expression in dermal fibroblasts *in vitro* and of skin collagen organization in open wounds and tensile strength in incisions *in vivo*

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Abstract. Understanding the molecular and cellular processes in skin wound healing can pave the way for devising innovative concepts by turning the identified natural effectors into therapeutic tools. Based on the concept of broad-scale engagement of members of the family of galactoside-binding lectins (galectins) in pathophysiological processes, such as cancer or tissue repair/regeneration, the present study investigated the potential of galectins-1 (Gal-1) and -3 (Gal-3) in wound healing. Human dermal fibroblasts, which are key cells involved in skin wound healing, responded to galectin exposure (Gal-1 at 300 or Gal-3 at 600 ng/ml) with selective changes in gene expression among a panel of 84 wound-healing-related genes, as well as remodeling of the extracellular matrix. In the case of Gal-3, positive expression of Ki67 and cell number increased when using a decellularized matrix produced by Gal-3-treated fibroblasts as substrate for culture of interfollicular keratinocytes. *In vivo* wounds were topically treated with 20 μg/ml Gal-1 or -3, and collagen score was found to be elevated in excisional wound repair in rats treated with Gal-3. The tensile strength measured in incisions was significantly increased from 79.5±17.5 g/mm^2 in controls to 103.1±21.4 g/mm^2 after 21 days of healing. These data warrant further testing mixtures of galectins and other types of compounds, for example a combination of galectins and TGF-β1.

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Introduction

It is commonly known that acute and chronic wounds pose serious and unresolved treatment issues that increase morbidity and mortality, thus leading to increased healthcare costs. Bioinspired approaches derived from insights into mechanisms of healing can help to find solutions that improve skin repair. Initial steps in the direction of finding practical possibilities in this respect have been taken by exposing organ

cultured human skin and wounded adult newts to plant agglutinins, such as phytohemagglutinin or concanavalin A (1,2). Endogenous lectins in the skin, which are involved in healing pathways, would be superior candidates over exogenous reagents. Indeed, the skin is home to a variety of glycan receptors, including C-type lectins and galactoside-binding lectins, or galectins.

Galectins are a family of β-sheet proteins synthesized on free ribosomes that exert various context-specific activities both intracellularly and following non-conventional secretion that do not involve the endo reticulum-Golgi route (3-12). First detected (as Mac-2 antigen) in the keratinocytes of murine skin (13) and also human skin (SL66) fibroblasts (14,15), galectin-3 (Gal-3) has become a focus of research in this type of lectin in dermatology (16-23). In addition to its ability to bridge cell surface counterreceptors, thus reducing the rate of their dynamic endocytosis, Gal-3 is able to trigger changes in gene expression profiles by outside-in signaling, not only in human skin fibroblasts, but also in other types of cells (24-26). This evidence has prompted further exploration beyond Gal-3 to obtain a full view on galectin representation in skin. Since the presence of galectins has been described in human skin as a network (27), our previous studies examined their expression profiles in pig and rat skin during wound healing (28,29). Based on the presence of several galectins in the skin and emerging insights into their regulation during wound healing, together with their known multifunctionality, it may be hypothesized that galectins may mechanistically be involved in this regenerative process.

In our previous study, this hypothesis was tested for six galectins *in vitro*, demonstrating their capacity to convert dermal fibroblasts into myofibroblasts and to remodel the extracellular matrix (ECM) (30). Moreover, these observations were dependent on galectin type and provided evidence that Gal-1 could reduce the area of an excisional wound *in vivo* in rats (30). In the present study, expression profiling of wound-healing-related genes was carried out in human dermal fibroblasts *in vitro* following exposure to Gal-1 and -3. Moreover, immunocytochemical analysis of keratinocytes cultured on an ECM substratum derived from the galectin-treated fibroblasts, histology of rat skin wounds, including collagen staining, and measurements of tensile strength of an incisional wound were also conducted *in vivo*. The present findings pointed to a potential beneficial role for human Gal-3 in the healing of skin incisions.

Materials and methods

Galectins. Human wild-type (WT) Gal-1 and -3 were obtained by recombinant production and purified by affinity chromatography on lactosylated Sepharose 4B prepared by ligand conjugation after activation of resin by divinyl sulfone. In the case His-tagged Gal-1 (E71Q) mutant (mutant that lost lectin activity by the site-directed mutagenesis), Ni-CAMTM HC (Sigma-Aldrich; Merck KGaA) was used. This was followed by the removal of any lipopolysaccharide contamination and desorption of bound (His-tagged) proteins by histidine (31,32). Product analysis was performed using one- and two-dimensional gel electrophoresis, gel filtration and mass spectrometry, as well as carbohydrate-inhibitable hemagglutination and solid-phase assays in order to ascertain β-galactoside binding (or its loss) (33,34).

Human dermal fibroblast (HDF) and human interfollicular keratinocyte (HIK) primary culture. HDFs and HIKs were isolated from the skin of healthy donors who underwent routine aesthetic surgery at the Department of Aesthetic Surgery, Third Faculty of Medicine, Charles University (Prague, Czech Republic). Written informed consent was obtained from all donors, in agreement with the Declaration of Helsinki and with approval from The Ethics Committee of University Hospital Kralovske Vinohrady and Third Faculty of Medicine, Charles University (approval no. 100/1947/2005). HDF cultures were expanded in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS (both from Biochrom, Ltd.) and penicillin (100 U/ml)/streptomycin (100 μg/ml; both from Biochrom, Ltd.), while HIKs were cultured in a mixture of DMEM and F12 (BioConcept AG) medium (3:1 vol:vol) containing 10% FBS that was further enriched with insulin (0.12 U/ml; Novo Nordisk A/S), cholera toxin (1 nM; Sigma-Aldrich; Merck KGaA), hydrocortisone (0.4 μg/ml; Sigma-Aldrich; Merck KGaA) and epidermal growth factor (10 ng/ml; Sigma-Aldrich; Merck KGaA) at 37˚C with 5% CO_2, as described previously (35).

Isolation of RNA and reverse transcription (RT). Following 48-h treatment with Gal-1 (300 ng/ml) and Gal-3 (600 ng/ml), total cellular RNA was isolated from HDFs using an RNeasy® Mini kit (Qiagen GmbH) according to the manufacturer's instructions. RNA quantification was performed with a NanoDrop™ 1000 Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.), after which 250 ng total RNA was subjected to 1% agarose gel electrophoresis to confirm its integrity. The RNA samples were stored at -80˚C. For analytical profiling, RNA was reverse transcribed using the RT^2 First Strand kit (Qiagen GmbH) following the manufacturer's instructions.

RT-quantitative (RT-q)PCR. RT^2 Profiler PCR Array for Human Wound Healing (Qiagen GmbH) was carried out using SYBR-Green as the reporter dye, according to the manufacturer's instructions. The RT array included reference genes, a control for excluding the presence of genomic DNA, three reverse-transcription controls and set of 84 wound-repair-related genes. Three positive RNA controls were also present. The sequences of forward and reverse primers were designed and supplied by Qiagen GmbH. Two biological replicates were used for each sample group in two independent experimental batches and mRNA profiling was performed at two time points (24 and 48 h of culture). PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Inc.). The thermocycling conditions were set as follows: Initial denaturation step (10 min, 95˚C) was followed by 40 cycles each consisting of a denaturation step for 15 sec at 95˚C and annealing for 1 min at 60˚C. The obtained data were analyzed using the $2^{-\Delta\Delta Cq}$ method (36), with the average expression of four housekeeping genes used as a reference (β-actin, β2-microglobulin, GAPDH and hypoxanthine phosphoribosyltransferase 1) to obtain relative expression values for each gene. The analysis was carried

out using limma (version 3.42.2) (37). To detect differentially transcribed genes following treatment with Gal-1, -3 and TGF-β1 (30 ng/ml; PeproTech EC Ltd.), groups were individually compared with the untreated control using a linear model and a moderated t-test. Genes with >2-fold up- or down-regulation relative to the untreated control were considered statistically significant. Data and overlaps of differentially transcribed genes were visualized using gplots (version 3.0.3; https://github.com/talgalili/gplots) and Vennerable (version 3.1.0.9000; https://github.com/js229/Vennerable) packages in R version 3.6.3 (https://www.r-project.org).

Culture of HIKs on ECM produced by galectin-treated HDFs. HDFs were seeded at a density of 2,000 cells/cm^2 and cultured for 10 days in the absence or presence of Gal-1 (WT or E71Q mutant) or Gal-3. Sterile solutions containing either no additive or Gal-1 (WT or E71Q mutant) at 300 ng/ml and Gal-3 at 600 ng/ml, which were determined to be the most effective concentrations based on a previous experiment (30), were prepared in DMEM containing 10% FBS and antibiotics. ECM scaffolds produced by the cell preparations cultured in parallel on the surface of glass microscope slide cover slips were tested as substrate for the HIKs. Cells were removed from cover slips by osmotic shock (exposure to sterile distilled water for 60 min), then surfaces were covered with keratinocyte medium (mixture of DMEM and F12; 3:1) and cover slips were incubated for 24 h. HIKs were seeded at a density of 10,000 cells/cm^2 and kept for 7 days at 5% CO_2 and 37˚C prior to immunocytochemistry.

Immunocytochemistry of cultured cells. The tested specimens were fixed with 2% buffered paraformaldehyde (pH 7.2) for 5 min at room temperature and washed with phosphate-buffered saline (PBS; pH 7.2). Cells were permeabilized by exposure to Triton X-100 (Sigma-Aldrich; Merck KGaA), and sites for antigen-independent binding of antibodies were blocked by incubation with porcine serum albumin (diluted in PBS, 1:30; Dako; Agilent Technologies, Inc.) for 30 min at room temperature. Commercial antibodies were used at concentrations recommended by the suppliers (Table I). Incubations with primary and secondary antibodies were performed for 90 and 45 min at room temperature, respectively. Nuclear DNA was stained using DAPI for 1 min at room temperature. All specimens were mounted to Vectashield (Vector Laboratories, Inc.) and examined using an Eclipse 90i microscope equipped with filter blocks for the three types of dyes (Nikon Corporation), as well as a Cool-1300Q CCD camera (Vosskühler) and a computer-assisted image analysis system LUCIA 5.1 (Laboratory Imaging).

Cell counting. *In vitro* experiments were repeated twice to assess the expression of keratin-19 (K-19; marker of low-degree keratinocyte differentiation) and Ki67 (marker of proliferation). Staining (1 min at room temperature) of nuclear DNA with DAPI (Sigma-Aldrich; Merck KGaA) was performed so that the total numbers of HIKs per three visualization fields (magnification, x200) of each biological replicate could be counted, then positive expression of Ki67 and K-19 in cell populations was quantitated. The data are given as a percentage of the total number of counted cells. The aforementioned imaging system described for immunocytochemistry was also utilized for this assay.

Animal model. This study was approved by the Ethical Committee of the Faculty of Medicine of the Pavol Jozef Šafárik University (Košice, Slovak Republic) and by the State Veterinary Administration of the Slovak Republic. It was performed as described previously (30). A total of 96 male Sprague-Dawley rats (age, 1 years old; weight, 507±48 g) were included in the study. Animals were housed in plexiglass cages (22-24˚C, 50-70% relative humidity, 12/12 h light/dark cycles) with free access to food and water. Surgery was performed under general anesthesia induced by intramuscular administration of 40 mg/kg ketamine, 15 mg/kg xylazine and 5 mg/kg tramadol (38,39). Under strict aseptic conditions, two 1-cm, round, full-thickness, excisional skin wounds and one 4-cm, full-thickness skin incision were inflicted to the back of each rat at the position depicted in Fig. 1. The incision was subsequently sutured using intradermal running suture. In each group, 6 rats were sacrificed on day 7 and 21 post-surgery, respectively. Allocation of rats to treatment groups is shown in Table II.

Wound treatment. *In vivo* experiments were performed in parallel under identical conditions, first on an exploratory level (n=48; data not shown) with galectin concentrations of 10 μg/ml (lyophilized protein containing K/Na-phosphate salts as buffer substances dissolved in physiological saline solution), then systematically (n=48) with galectin concentrations of 20 μg/ml applied topically on the wound surface (using an eye dropper) during the first 3 post-operative days (three times a day).

Histology. Specimens of wounds were removed from rats sacrificed by cervical dislocation following ether anesthesia (using a vaporizer) at the two given time points and routinely processed for classical histological staining performed at room temperature (fixation in 4% buffered formaldehyde for 10 min, dehydration using a series of solutions with increasing concentration of ethanol, paraffin embedding, sectioning). Deparaffinized sections (5 μm thick) were stained with Van Gieson's solution (non-specific collagen staining) and also with hematoxylin for 10 min and eosin at 4 min, according to a previous study (30). Sections were examined under an Olympus BX51 microscope equipped with an Olympus DP73 CCD camera (Olympus Corporation).

Immunohistochemistry. A second set of specimens of wounds was cryoprotected using Tissue-Tek (Sakura Finetek Europe B.V.) and frozen in liquid nitrogen. Tissue sections (~10 μm thickness) were first mounted on the surface of poly-l-lysine-treated glass slides (Sigma-Aldrich; Merck KGaA), then fixed at room temperature using 2% (w/v) paraformaldehyde in PBS for 10 min. Non-specific binding of the secondary antibody was blocked at room temperature with normal swine serum (DakoCytomation; Agilent Technologies, Inc.) diluted with PBS (1:30) for 30 min.

Solutions of commercially available primary and secondary antibodies are shown in Table I. Incubation with primary and

Table I. Antibodies used for immunocytochemistry and lectin histochemistry.

A, Primary antibodies

Name	Host	Supplier	Cat. no.	Dilution
α-smooth muscle actin	Mouse monoclonal	Dako (Agilent Technologies, Inc.)	M0851	1:50
Vimentin	Mouse monoclonal	Dako (Agilent Technologies, Inc.)	M0725	1:200
Tenascin	Mouse monoclonal	Sigma-Aldrich (Merck KGaA)	T3413	1:200
Fibronectin	Rabbit polyclonal	Dako (Agilent Technologies, Inc.)	A0245	1:1,000
Ki67	Rabbit polyclonal	EMD Millipore	AB9260	1:200

B, Secondary antibodies

Name	Host	Supplier	Cat. no.	Dilution
Anti-mouse; TRITC	Goat	Sigma-Aldrich (Merck KGaA)	T5393	1:30
Anti-rabbit; FITC	Swine	Dako (Agilent Technologies, Inc.)	F0205	1:100

TRITC, tetramethyl rhodamine isothiocyanate; FITC, fluorescein isothiocyanate.

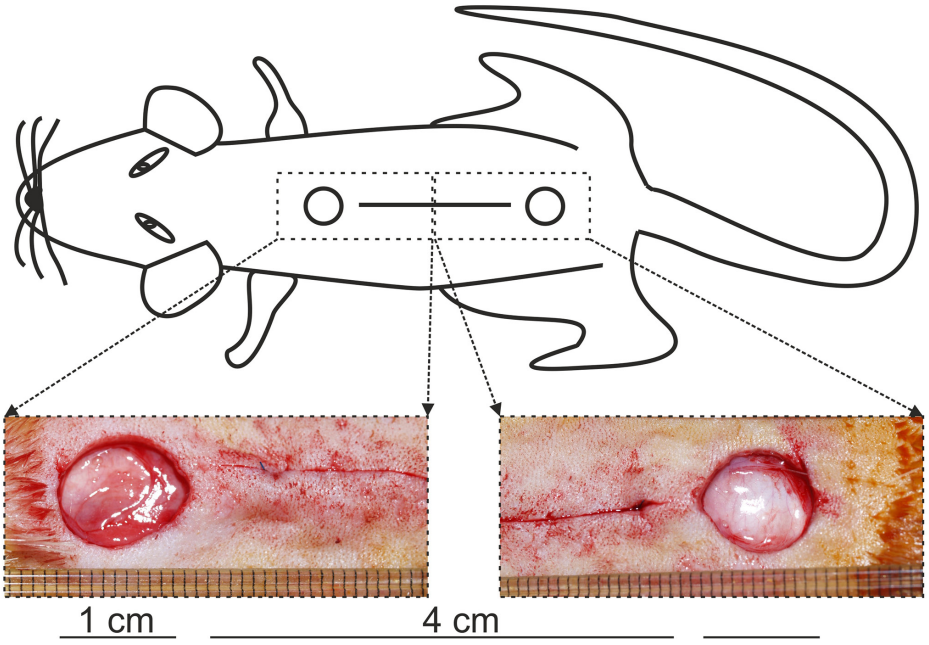

Figure 1. Schematic illustration and photograph showing the position and shape of wounds inflicted on the back of each rat. Two open wounds 1 cm in diameter each and one sutured incision 4 cm in length.

secondary antibodies was performed for 90 and 45 min (at room temperature), respectively. Nuclear DNA was stained using DAPI for 1 min at room temperature. Controls for specificity of the immunohistochemical detection were as follows: i) Replacement of the target-specific antibody by an irrelevant antibody (in the case of monoclonal antibodies of the same isotype); and ii) omission of the incubation step with the primary antibody to exclude antigen-independent signal generation. Specimens were mounted using Vectashield (Vector Laboratories, Inc.) and examined under an Eclipse 90i microscope (Nikon Corporation), as aforementioned.

Semi-quantitative scoring of histological sections. The status of re-epithelialization and extent of the presence of polymorphonuclear leukocytes (PMNL), fibroblasts, newly formed vessels and collagen were assessed according to a semi-quantitative scale system, as defined in Table III (40).

Wound tensile strength (TS). Measurement of force to rupture an incisional wound was facilitated by equipment assembled in our laboratory using commercial devices (41). Briefly, an adequately shaped horizontal arm was used to pull at one side of a specimen, while the opposite side was fixed to a sensor tip of a force meter

Table II. Allocation of rats in treatment groups.

		Gal-1 treatment group, n		Gal-1(E71Q) treatment group, n		Gal-3 treatment group	
Time/group	Untreated control, n	10 μg/ml	20 μg/ml	10 μg/ml	20 μg/ml	10 μg/ml	20 μg/ml
7 days	12	6	6	6	6	6	6
21 days	12	6	6	6	6	6	6

Gal, galectin.

Table III. Scale used for the semi-quantitative evaluation of histological sections.

Scale	Epithelialization	PMNL	Fibroblasts	Luminized vessels	Collagen
0	Thickness of cut edges	Absent	Absent	Absent	Absent
1	Migration of cells (<50%)	Mild-ST	Mild-ST	Mild-SCT	Mild-GT
2	Migration of cells (≥50%)	Mild-DL/GT	Mild-GT	Mild-GT	Moderate-GT
3	Bridging the excision	Moderate-DL/GT	Moderate-GT	Moderate-GT	Marked-GT
4	Complete keratinization	Marked-DL/GT	Marked-GT	Marked-GT	Organized-GT

DL, demarcation line; GT, granulation tissue; PMNL, polymorphonuclear leucocyte; SCT, subcutaneous tissue; ST, surrounding tissue.

unit (Omega Engineering, Inc.). The moving arm was driven by a high-precision stepper motor MDI-17 (Intelligent Motion Systems Inc.) through a linear slider. The technique for determining the TS had been described in detail previously (42). Briefly, following euthanasia, two 1-cm-wide skin strips were removed from each incisional wound and placed lengthwise between the clamps of the TS testing device. Pulling was performed perpendicularly to the original direction of the incision. The maximal strength of rupture was measured for each specimen. TS (g/mm^2) was calculated as TS=MRS/A, where MRS is the maximal rupture strength (g) and A is the wound area (mm^2).

Statistical analysis. Continuous data are presented as the mean ± SD. Categorical data are presented as the median. Data obtained from cell counting were compared using one-way ANOVA followed by the Tukey-Kramer post hoc test. Two-way ANOVA followed by Tukey's post hoc test was used to compare the effects of treatment modalities and time on wound TS. The Kruskal-Wallis test with multiple comparison (Dunn's test) was applied to compare non-parametric semi-quantitative data. The aforementioned statistical analyses were performed in SPSS v22 software (IBM Corp.). RT-qPCR data were analyzed using a moderated t-test in limma (version 3.42.2) (37) R version 3.6.3 (https://www.r-project.org) and Bioconductor version 3.1.0 software packages (http://www.bioconductor.org). P<0.05 was considered to indicate a statistically significant difference.

Results

In vitro study. The influence of administration of the two galectins on biochemical and cellular features was tested *in vitro* in two experimental settings.

HDF gene expression profiling. In order to determine whether Gal-1 and -3 could affect HDF gene expression *in vitro,* the effective concentration required to induce conversion to myofibroblasts or ECM redesign (30), the Qiagen RT^2 Profiler PCR Array for Human Wound Healing system was used to monitor expression levels of 84 selected genes relevant for wound healing. Technically, the sets of experiments recommended for stringent quality control completely satisfied the standards of this commercial system (data not shown). Indeed, Gal-1 led to significant changes of signal intensity relative to the control ($\log_2$ fold change, 1.62-6.00; Fig. 2A). Genes with increased expression are presented in Table SI. Gal-3 treatment also caused deviations from the respective control from 1.14 to 6.28 $\log_2$ fold change upregulation relative to the control (Fig. 2A). There was considerable overlap between the lists of upregulated genes for Gal-1 and -3, including *ANGPT1*, *IGF1*, *FGF10*, *F13A1* and *ACTC1* (Fig. 2B). In order to ascertain common responsiveness of cell populations to a known effector of fibroblast activation, TGF-β1 was tested in parallel as a positive control (Table SI). Of note, a full overlap of profiles between TGF-β1 and a Gal-1 was observed, whereas Gal-3 treatment resulted in uniquely regulated genes overlapping neither Gal-1 nor TGF-β1 profiles, such as *IL6*, *CDH1* and *F3* (Fig. 2B).

HIK immunocytochemistry. The low-level fibronectin staining at day 10 following HIK seeding demonstrated that the original three-dimensional structure of the ECM was completely replaced by a confluent layer of epithelial cells (Fig. 3A). The presence of fibronectin was restricted to small intracellular granules. K-19 was present in typically small cells, and a relative increase in the red signal was seen in the Gal-1-treated group, compared with the control, Gal-1(E71Q) mutant and Gal-3 conditions (Fig. 3A and B). Of note, the loss-of-glycan-binding

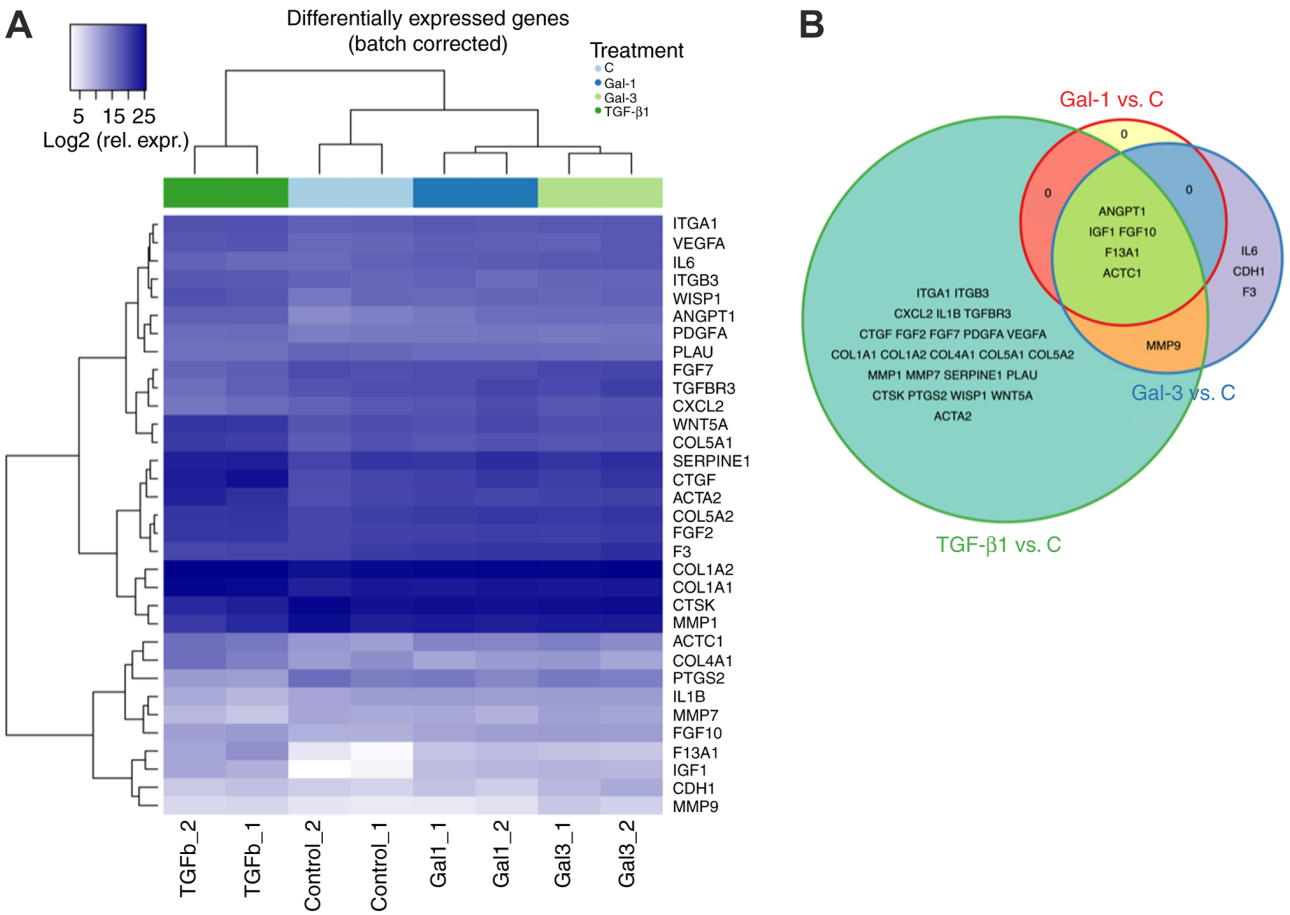

Figure 2. Expression profile of human dermal fibroblasts following treatment with Gal or TGF-β1. (A) Heat map and (B) Venn diagram of genes separately/commonly regulated in human dermal fibroblasts following treatment with Gal-1, Gal-3 or TGF-β1 compared to untreated control. Gal, galectin; C, control.

Gal-1 (Gal-1E71Q) single-site mutant did not trigger the pronounced effect of WT Gal-1 (Fig. 3A and B). As a measure of the growth fraction of cell populations, the Ki67 antigen status was assessed. Ki67-Positive HIKs were generally seen, most frequently for Gal-3 (Fig. 3B). Double staining suggested that K-19-positive cells did not present the Ki67 antigen (see insert in Fig. 3B), an observation made independent of the experimental condition.

Quantification of the observed parameters is presented in Fig. 3C-E. The increase in the positive expression of K-19 in Gal-1-treated cells was significant when compared with the control group, the Gal-1(E71Q) mutant- and Gal-3-treated cell populations (Fig. 3C). Positive expression of Ki67 was increased significantly in the Gal-1- and Gal-3-treated groups compared with the control and Gal-1(E71Q) mutant-treated cells, but not relative to each other (Fig. 3D). The total number of cells counted for each condition reached the most statistically significant level (among test groups) for Gal-3-exposed cells (Fig. 3E).

In vivo study. The effect of the two galectins on wound healing was tested on rats using two basic models of skin repair (open excision and sutured incision). During the post-surgical period, all animals remained healthy and did not show symptoms of infection. Initial experiments using concentrations of 10 μg/ml galectins showed no activity (data not shown). Thus, subsequent experiments were performed with 20 μg/ml concentrations (equal molar concentration due to similar molecular weights of Gal-1 homodimer and monomeric chimera-type Gal-3).

Histology of open excision wounds. The results of the extended histological analysis using immunohistochemistry and Van Gieson staining (top and middle panel) and of the semi-quantitative evaluation of distinct parameters of the wounds at the two time points (bottom panel) are shown in Fig. 4. At day 7, the newly formed granulation tissue (GT) was rich in fibronectin (Fig. 4A), populated by fibroblasts, and also notably vascularized. The inset in the photomicrograph of a section of a specimen of the Gal-1-treated group illustrates the already known capacity of this protein to generate SMA-positive myofibroblasts (Fig. 4A). When quantitated, the numbers of fibroblasts and luminized vessels were increased in both lectin-treated groups (Fig. 4C). An increase in collagen score was seen at this time point for Gal-3 (Fig. 4C).

At day 21, the level of fibronectin in the granulation tissue had leveled off, and no myofibroblasts were present in the granulation tissue of any group (data not shown). In contrast, the contents of collagen had further increased, as the Van Gieson staining in granulation tissue revealed (Fig. 4B and D). Inspection of such stained specimens under polarized light showed a marked effect of Gal-3 on collagen type-1 formation (Fig. 4B). Median values for scores of the measured characteristics reflect these microscopical observations, as they also indicate a tendency for an increase by presence of

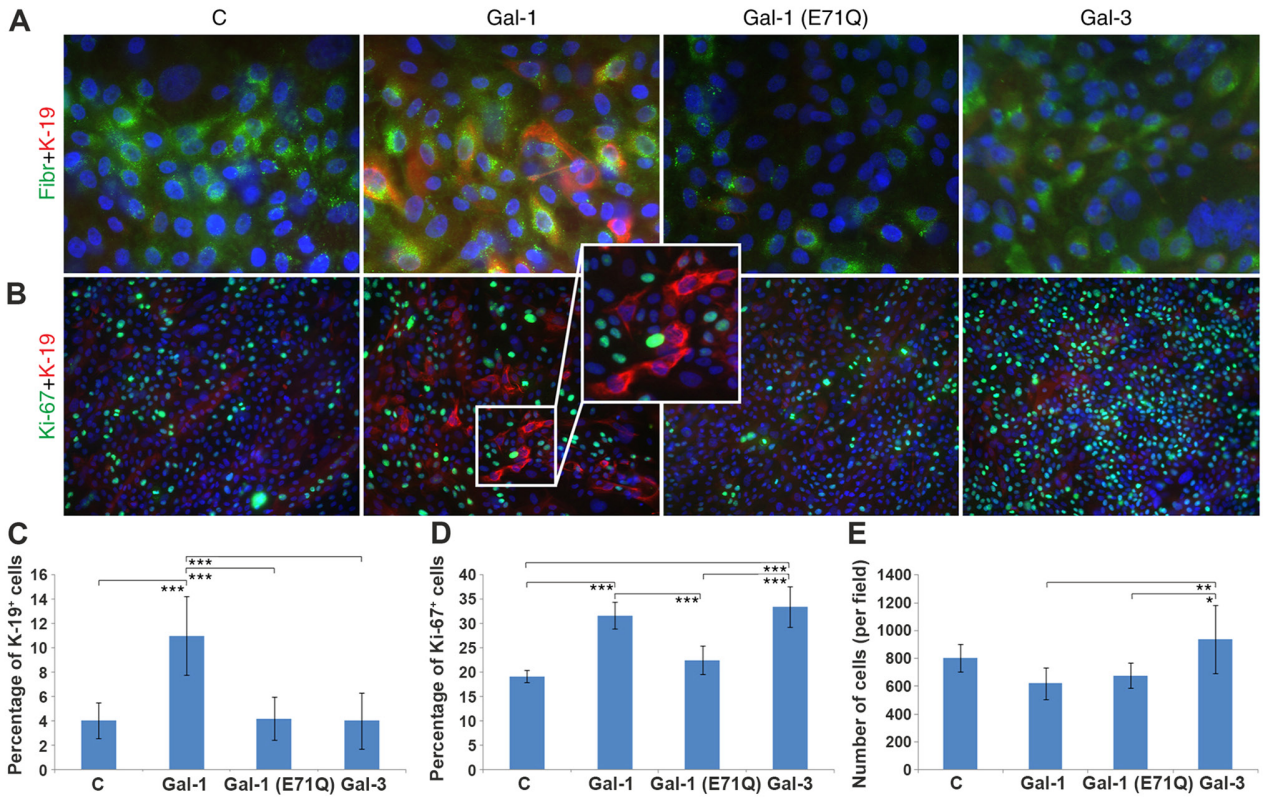

Figure 3. Characteristics of human interfollicular keratinocytes grown on an ECM produced by untreated or galectin-treated human dermal fibroblast cultures. Nuclei are counterstained with DAPI. (A) Cells were immunostained for the presence of Fibr (green signal) and K-19 (red signal). Magnification, x600. (B) Cells positive for Ki67 (green signal) and K-19 (red signal). Magnification, x200. Insert magnification, x400. (see insert, magnification 400x). Quantification of the studied parameters. (C) Frequency of K-19$^+$ keratinocytes. (D) Frequency of Ki67$^+$ cells. (E) Total number of cells in cultures using ECM from each group. *P<0.05, **P<0.01, ***P<0.001. Gal, galectin; C, control; Fibr, fibronectin; ECM, extracellular matrix.

Gal-1 and -3 of the scores on re-epithelialization and number of fibroblasts (Fig. 4D).

TS of sutured incision wounds. Following the systematic determination of TS values of incisional wounds (Fig. 5), the results of the statistical analysis of these experimental series using the two-way ANOVA are summarized in Table IV. Data analysis using one-way ANOVA is presented in Fig. 5A and B.

As expected, a significant increase of wound TS was observed between day 7 and 21. The data from the Gal-3-treated group significantly differed from those of all other groups. At day 7, TS in the Gal-3 group reached 29.6±8.2 g/mm^2, compared with 14.3±9.6 g/mm^2 for the control, 20.2±7.6 g/mm^2 for Gal-1 and 17.4±2.8 g/mm^2 for Gal-1(E71Q) (Fig. 5A).

At day 21 after surgery, the Gal-3 group continued to exhibit a significantly increased TS when compared with the untreated control (Gal-3, 103.1±21.4 g/mm^2 vs. control, 79.5±17.5 g/mm^2), the Gal-1 group (86.7±19.4 g/mm^2) and the Gal-1(E71Q) mutant (86.5±18.8 g/mm^2) (Fig. 5B). Thus, TS is a parameter separating galectin activity in incisional skin wound healing.

Discussion

The data of the present study provided further evidence of the favorable effect of Gal-1 and -3 on excisional/open and incisional/sutured skin wound healing. Similar to TGF-β1, the positive control, both galectins acted as molecular switches for the expression of genes related to the repair of skin wounds. Genes for *IGF1*, *FGF10* and *ANGPT1* were among the most upregulated genes, all of which have previously been implicated in healing processes (43-45). Multiplex cytokine assays of human skin fibroblasts after Gal-3 exposure have demonstrated the increased concentrations of interleukin-6, granulocyte-macrophage colony-stimulating factor, C-X-C motif chemokine ligand 8 and matrix metalloproteinase (MMP)-3, an overall pro-inflammatory/degradative signature (24), in osteoarthritic chondrocytes (25,46). On the other hand, the increase in the expression of MMP-9, cleaving the Ala62-Tyr63 bond in Gal-3's N-terminal stalk (47), can indicate a regulatory loop to put restrictions to prolonged Gal-3 activity for Gal-3-induced neutrophil activation by neutralizing contacts between lectin domains (48).

The ECM of the HDFs is then subject to a remodeling by Gal-3 that in turn induces proliferation of keratinocytes, extending our previous data on responses to Gal-1 presence (30). In detail, we observed significantly upregulated *CDH1* (49) and *MMP9* (50) transcripts in Gal-3 treated fibroblasts, which supports the role of lectins in the re-epithelialization of wounds. Reduced Gal-3 expression has been implicated in defective skin wound repair in patients with diabetes by two mechanisms: i) Inverse correlation between Gal-3 and advanced glycation end products; and ii) decreased Gal-3 in chronic human wounds leading to delayed re-epithelialization as seen also in Gal-3 knockout mice (20,21). Of note, Gal-1 has been shown to accelerate

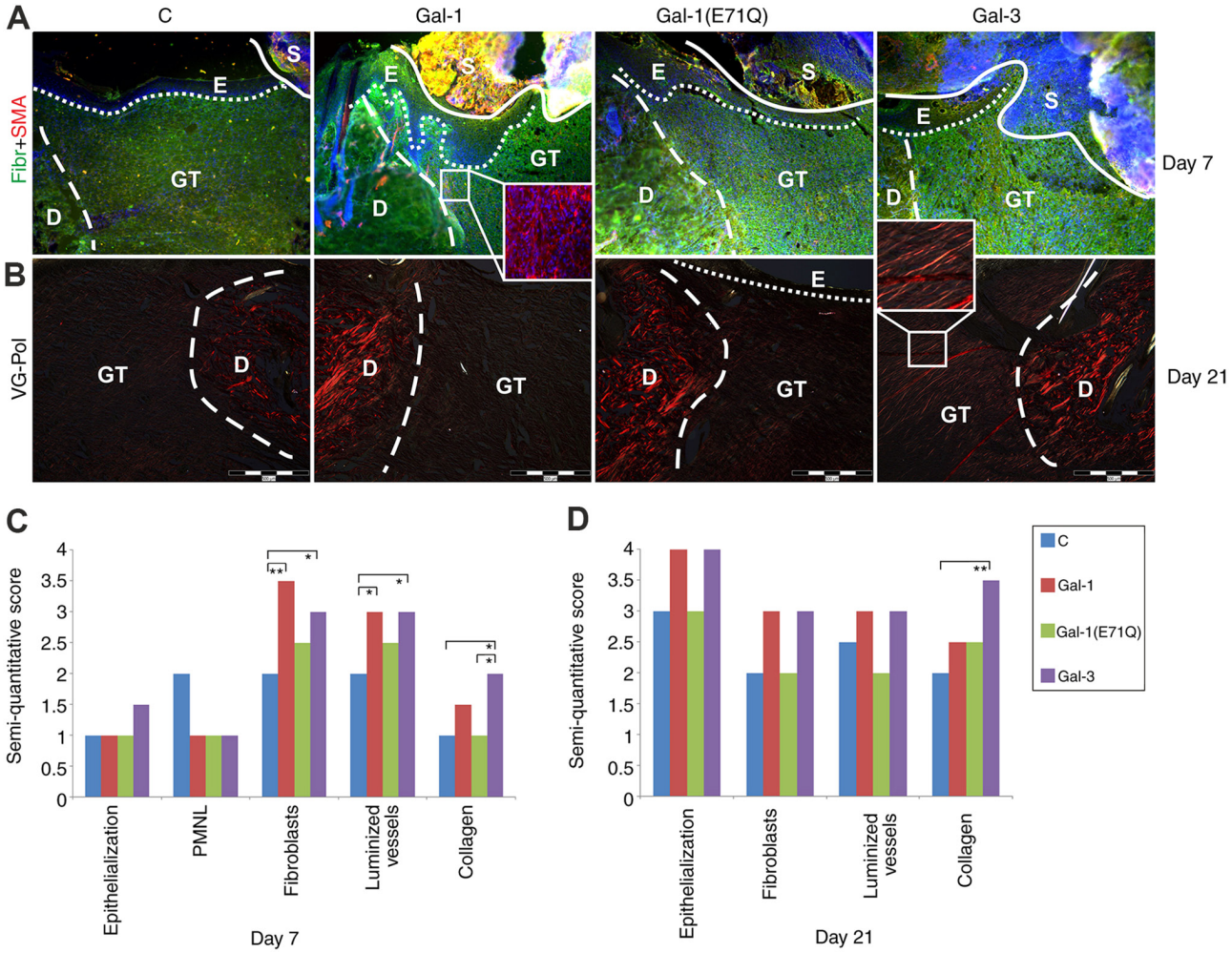

Figure 4. Characteristics of open wounds following treatment with tested galectins. Untreated group was used as C. Nuclei are counterstained with DAPI. (A) Sections of excisional wounds immunostained for Fibr (green signal) and SMA (red signal) seven days after surgery, presenting well-formed fibronectin-rich GT with SMA^+ vessels and re-epithelialization beneath scab can be seen in all groups, only Gal-1-treated wounds are characterized by increased number of SMA-positive myofibroblast-like cells. Magnification, x100. Insert magnification, x200. (B) VG-stained wounds monitored under polarized light 21 days after surgery, wounds treated with Gal-3 exhibited collagen organized into polarized light-reflecting fibers. Magnification, x100. Insert magnification, x200. Semi-quantitative analysis of histological parameters/changes. Data are presented as the median in two separate graphs for (C) day 7 and (D) day 21. PMNL was not evaluated at day 21. $^*P<0.05$, $^{**}P<0.01$. Gal, galectin; C, control; SMA, α-smooth muscle actin; Fibr, fibronectin; VG-Pol, Van Gieson-polarized light; D, dermis; E, epidermis; GT, granulation tissue; S, scab.

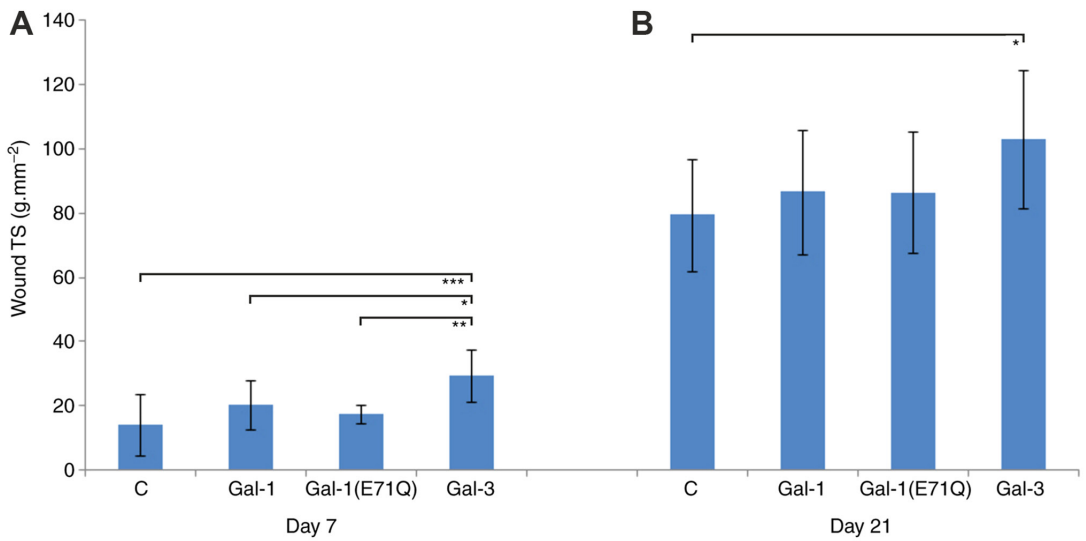

Figure 5. TS measurement of sutured wounds following treatment with Gal. TS of incisional wounds following treatment with Gal at (A) day 7 and (B) day 21. Untreated group was used as C. One-way ANOVA followed by the Tukey-Kramer post hoc test. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. TS, tensile strength; Gal, galectin; C, control.

Table IV. Results from the statistical comparison of wound tensile strength using two-way ANOVA.

A, Overall results

Group	P-value	95% CI
Time (7 days vs. 21 days)	<0.001	N/A
Group (C vs. Gal-1 vs. Gal-1(E71Q) vs. Gal-3)	<0.001	N/A
Time vs. Group	0.797	N/A

B, Time comparison

Group	P-value	95% CI
7 days vs. 21 days	0.0001	62.3995 to 74.7000

C, Treatment comparison

Group	P-value	95% CI
C vs. Gal-1	0.8815	-18.5813 to 5.5063
C vs. Gal-1(E71Q)	10.000	-17.1219 to 6.9657
C vs. Gal-3	0.0003	-31.8079 to -7.1180
Gal-1 vs. Gal-1(E71Q)	10.000	-9.8777 to 12.7964
Gal-1 vs. Gal-3	0.0214	-24.5819 to -1.2690
Gal-3 vs. Gal-1(E71Q)	0.0075	2.7284 to 26.0412

C, control; Gal, galectin.

cellular migration and proliferation (51), and augment skin wound repair in normal and diabetic mice (deficient for this galectin) (52). In parallel, these processes may also contribute to the stability of wound closure, which prompted us to proceed with the animal study. In the present study, the pro-fibrotic effect of Gal-3 was demonstrated, resulting in wound tensile strength increase and improved collagen organization (characteristic reflective appearance of collagen fibers under the polarized light) of healing skin incisions and excisions, respectively. Molecular analysis further revealed significantly increased transcripts of *IL6* and *F3* genes in Gal-3-treated cells, more so than Gal-1-treated cells. In particular, IL-6 and TGF-β1 play important roles in liver fibrosis and/or structural changes in human tendon, a wound healing-like processes characterized by the accumulation and turnover of ECM (53-55). On the other hand, the *F3* gene product (tissue factor), exerts potent pro-angiogenic activity (56), which may also contribute to wound healing improvement in Gal-3-treated rats.

When envisioning to further pursue this route of experimental testing, two aspects should be noted. Firstly, galectins are expressed as a network beyond Gal-1 and -3 with possibility for context-dependent functional antagonism and cooperation (26,57-60). For example, our previous study reported an additive effect of TGF-β1 (10 μg/ml) with Gal-1 (200 or 300 ng/ml) on inducing expression of SMA in HDF cultures (30). Further studies should investigate Gal-mixtures (also including other types of proteins such as TGF-β1), as they likely occur *in situ*, to attain the optimal efficacy, and the present study provides a solid foundation for this. Secondly, galectin function arises from their modular structure. Therefore, their protein architecture has become the subject of redesign using engineering, for example by creating homo-oligomers from natural dimers (61-64), in order to optimize a favorable activity. Of note, whether and how a Gal-1-like (homodimeric) Gal-3 or a Gal-3-like (monomeric, in solution) Gal-1 will influence *in vitro* and *in vivo* aspects of wound healing remains to be determined.

In summary, the present findings provided further evidence that galectins should be listed within the group of efficient wound healing modulators at several key steps of skin repair, including ECM formation/reorganization and re-epithelialization (65,66). This conclusion is incentive for further work. From a clinical perspective, the present data makes a strong case for directing further efforts to treat incisional and excisional wounds differently. In fact, Gal-1 seems to play a role in wound contraction, whereas Gal-3 seems to be a skin scar inductor. However, understanding the activity profile of each galectin *in vitro* and in acute and chronic wound repair, along with the potential of administration of mixtures, including custom-made variants in experimental *in vitro* and *in vivo* testing (67,68), will be important when investigating the potential applications of these endogenous effectors in the treatment of skin lesions.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PG, HJG, FS, JL and KS acquired funding and made substantial contributions to conception and design of the study. PG, TV, IK, MČ, JJ, MJ, VP, LU, MK, MN and JM performed the experiments. FS and JL supervised the animal study. HJG and KS validated the obtained data. PG and HJG wrote the original draft. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of clinical samples was approved by The Ethics Committee of University Hospital Kralovske Vinohrady and Third Faculty of Medicine, Charles University (approval no. 100/1947/2005). Human cells were isolated and cultured with informed consent from the donors. The animal study was approved by the Ethics Committee of the Faculty of Medicine of the P. J. Šafárik University and by the State Veterinary Administration of the Slovak Republic (approval nos. Ro-580/10-221a and Ro-2617/15-221a).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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