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Open Wound Healing *In Vivo*: Monitoring Binding and Presence of Adhesion/Growth-Regulatory Galectins in Rat Skin during the Course of Complete Re-Epithelialization

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Galectins are a family of carbohydrate-binding proteins that modulate inflammation and immunity. This functional versatility prompted us to perform a histochemical study of their occurrence during wound healing using rat skin as an *in vivo* model. Wound healing is a dynamic process that exhibits three basic phases: inflammation, proliferation, and maturation. In this study antibodies against keratins-10 and -14, wide-spectrum cytokeratin, vimentin, and fibronectin, and non-cross-reactive antibodies to galectins-1, -2, and -3 were applied to frozen sections of skin specimens two days (inflammatory phase), seven days (proliferation phase), and twenty-one days (maturation phase) after wounding. The presence of binding sites for galectins-1, -2, -3, and -7 as a measure for assessing changes in reactivity was determined using labeled proteins as probes. Our study detected a series of alterations in galectin parameters during the different phases of wound healing. Presence of galectin-1, for example, increased during the early phase of healing, whereas galectin-3 rapidly decreased in newly formed granulation tissue. In addition, nuclear reactivity of epidermal cells for galectin-2 occurred seven days post-trauma. The dynamic regulation of galectins during re-epithelialization intimates a role of these proteins in skin wound healing, most notably for galectin-1 increasing during the early phases and galectin-3 then slightly increasing during later phases of healing. Such changes may identify a potential target for the development of novel drugs to aid in wound repair and patients' care.

Key words: differentiation, lectin, migration, proliferation, repair

I. Introduction

The integrity of skin is essential, because it forms a mechanically flexible barrier protecting higher organisms from infections. Replacement or repair of this barrier requires an intricate healing process that starts immediately after surgery and/or trauma. Successful wound healing involves an orchestration of several processes encompassing cell migration [47], proliferation [44], and differentiation [29]. Remodeling and formation of the extracellular matrix (ECM) [10] requires a sequence of molecular-, cellular-, and tissue-level events including cell-cell and cell-matrix interactions. On the molecular level, growth factors,

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chemo- and cytokines are known to play important roles in coordinating the events that lead to complete posttraumatic skin repair and finally to scar formation [3, 13, 49].

It is evident that the clinical presentation of non-healing wounds calls for better understanding of the basic biological mechanisms underlying the repair processes of higher organisms [41]. Hence, in addition to further exploring the regulatory pathways involved in wound healing, such studies can have clinical and socioeconomic implications. Toward this end, we focused on glycans as versatile biochemical signals and endogenous lectins as efficient signal-transduction elements, embodied by what is now called the sugar code: the transfer of information between cells via the shape and structure of glycan determinants [for recent reviews, see 16].

The recent application of array technology to study wound re-epithelialization has paved the way for discovering differential regulation of gene expression for enzymes involved in glycan remodeling and for the identification of distinct lectins such as galectins-1, -3, -4 and -7 [6, 7, 37]. In fact, members of this protein family are known to be potent regulators of cell adhesion, growth, and migration, via protein/glycan and protein/protein interactions [17, 40, 42, 45]. The marked effects of galectins on cell migration, observed in colon cancer cells and also in keratinocytes [20, 23], as well as the expression of galectins in malignancies of squamous epithelia, which in certain cases can correlate to tumor progression [4, 9, 28, 31, 38, 39], encouraged us to investigate galectin expression in skin during different phases of wound healing. Because galectin activity is also regulated on the level of ligand availability, e.g. by displaying distinct, highly reactive glycan epitopes on, to give examples, ganglioside GM1, the fibronectin receptor or CD7 in suited density through the action of a tumor suppressor or cell activation/differentiation [1, 14, 17, 26, 32, 35, 36, 46], parallel testing of accessibility of binding sites with galectins as tools provides insights into regulatory events on this level.

Wound healing is an intricately orchestrated cascade of events separated into the phases of inflammation, proliferation and maturation [2]. In our study, we systematically determined presence of adhesion/growth-regulatory galectins and the tissue reactivity to these proteins in Sprague-Dawley rats at three time points post-trauma, i.e. day two (inflammation), day seven (proliferation), and day twenty-one (maturation) after surgery. Tissue specimens were processed under identical conditions to exclude any factor other than the time-point that would affect signal occurrence and intensity.

In detail, we have monitored, the expression and reactivity of proto-type galectins-1 and -2 as well as the chimera-type galectin-3 using (immuno/galectin)histochemical techniques. This galectin-related work was flanked by examining keratin presence as marker to characterize the level of cell differentiation [15]. Among the keratins, keratin-10 is an indicator of early stages of keratinocyte differentiation [8, 19, 33], whereas keratin-14 is considered as a key feature of poorly differentiated epidermal cells located in the basal epidermal layer [30, 33]. In addition, to complete the study, wide-spectrum cytokeratin, vimentin and fibronectin were also localized.

II. Materials and Methods

Animal model

This study was approved by the State Veterinary and Food Administration of the Slovak Republic.

One-year-old male Sprague-Dawley rats (n=17) were included into the experiment. In 15 rats, surgery was performed under general anesthesia induced by administration of ketamine (40 mg/kg; Narkamon a.u.v., Spofa, Prague, Czech Republic), xylazine (15 mg/kg; Rometar a.u.v., Spofa) and tramadol 5 mg/kg (Tramadol-K; Krka, Novo Mesto, Slovenia). Under aseptic conditions one round full-thickness skin wound, 10 mm in diameter, was inflicted to the back of each rat. Five rats were sacrificed at each time point by ether inhalation, i.e. after two, seven, and twenty-one days, respectively. Two rats that remained uninjured were included as control.

Histology

Either uninjured skin or skin-wound specimens were removed from rats sacrificed by ether inhalation at each evaluated time point and routinely processed for classical histological staining (fixation in 4% buffered formaldehyde, dehydration using increasing concentration of ethanol, paraffin embedding, sectioning, and staining). Deparaffinized sections were stained with hematoxylin-eosin (HE–basic staining) and Van Gieson (VG–non-specific collagen staining).

Immunohistochemistry and lectin histochemistry

In parallel, skin-wound specimens were cryoprotected by Tissue-Tek (Sakura, Zoeterwoude, Netherlands) and deeply frozen in liquid nitrogen. Ten-μm-thick cryocut sections obtained by microtome use (Reichert-Jung, Vienna, Austria) were first mounted on the surface of poly-L-lysine-treated supporting glass slides (Sigma-Aldrich, St. Louis, MO, USA), and then fixed using 2% (w/v) paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) for 10 min. Non-specific binding of the applied secondary antibody was precluded by a pre-incubation step of sections with normal swine serum (DakoCytomation, Glostrup, Denmark) diluted with PBS (1:100) for 30 min.

Primary and secondary antibodies as well as the biotinylated galectins used in this study are described in Table 1. The commercially available antibodies were diluted as recommended by supplier and antibodies against galectins as well as the biotinylated galectins were used at the concentration of 20 μg/ml in reaction medium. DNA in cell nuclei was stained by 4,6-diamino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA). Controls for specificity of the immunohistochemical reaction included: 1) replacement of the specific by an irrelevant antibody (in the

Table 1. *Reagents for immunohistochemistry and lectin histochemistry*

primary antibody	abbrevation	host	produced by	secondary antibody	produced by	channel
vimentin	VIM	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	Sigma-Aldrich, St. Louis, MO, USA	TRITC-red
keratin-10	K10	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	Sigma-Aldrich, St. Louis, MO, USA	TRITC-red
keratin-14	K14	mouse monoclonal	Sigma-Aldrich, St. Louis, MO, USA	goat anti-mouse	Sigma-Aldrich, St. Louis, MO, USA	TRITC-red
fibronectin	FIBR	rabbit polyclonal	Dakopatts, Glostrup, Denmark	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA, USA)	FITC-green
wide spectrum cytokeratin	WSK	rabbit polyclonal	Abcam, Cambridge Science, Cambridge, UK	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA, USA)	FITC-green
Galectin-1	Gal-1	rabbit polyclonal	house-made, Gabius laboratory	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA, USA)	FITC-green
Galectin-2	Gal-2	rabbit polyclonal	house-made, Gabius laboratory	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA, USA)	FITC-green
Galectin-3	Gal-3	rabbit polyclonal	house-made, Gabius laboratory	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA, USA)	FITC-green
biotinylated lectin	abbrevation		produced by	second-step reagent	produced by	channel
Galectin-1-binding site	Gal-1-BS		house-made, Gabius laboratory	ExtrAvidin	Sigma-Aldrich, St. Louis, MO, USA	TRITC-red
Galectin-2-binding site	Gal-2-BS		house-made, Gabius laboratory	ExtrAvidin	Sigma-Aldrich, St. Louis, MO, USA	TRITC-red
Galectin-3-binding site	Gal-3-BS		house-made, Gabius laboratory	ExtrAvidin	Sigma-Aldrich, St. Louis, MO, USA	TRITC-red
Galectin-7-binding site	Gal-7-BS		house-made, Gabius laboratory	ExtrAvidin	Sigma-Aldrich, St. Louis, MO, USA	TRITC-red

Table 2. *Results of the semi-quantitative assessment of selected cellular processes/structures*

	re-epitheliliazation (WSK+)	PMNL	fibroblasts (Vim+)	new vessels	fibronectin
02d	+	+++	–	–	+
07d	+	++	++++	++	++++
21d	++++	–	++	++	++

case of monoclonals of the same isotype), and 2) omission of the incubation step with the primary antibody to exclude antigen-independent signal generation. Involvement of the carbohydrate recognition domain in the lectin histochemical reaction was ascertained by pre-incubation of biotin-labeled lectins with 5 mM lactose (Sigma-Aldrich, St. Louis, MO, USA) as previously described [31]. Specimens were mounted by Vectashield (Vector Laboratories, Burlingame, CA, USA). The analyses of the specimens and data acquisition/storage were performed using a Nikon Eclipse-90i fluorescence microscope equipped by specific filter-blocks for DAPI, FITC, and TRITC (Nikon, Prague, Czech Republic) as well as by a Cool-1300Q CCD camera (Vosskühler, Osnabrück, Germany) and a computer-assisted image analysis system LUCIA 5.1 (Laboratory Imaging, Prague, Czech Republic).

Histological assessment

The status of re-epithelialization, the presence of polymorphonuclear leukocytes (PMNL), fibroblasts, newly formed vessels, and collagen were assessed according to the semi-quantitative scale system: –, +, ++, +++, and ++++ [18]. The extent of the immuno- and galectin histochemical reaction in injured epidermis and dermis was assessed by ranking the signal intensity according to the scale: –, +, ++, +++ [5]. Data are presented as median.

III. Results

During the post-surgical period all animals remained healthy without clinical symptoms of infection. The data of the semi-quantitative analysis of the histological sections are summarized in Table 2 and Figure 3. The microphotographs presented are organized into two plates presenting hematoxilin-eosin stained sections (Fig. 1, top panel), and immunohistochemical localization profiles of marker proteins (Fig. 1 for fibronectin, vimentin and cytokeratin at all time points) as well as the galectin-related data (Fig. 2). In order to visually summarize the way galectin-related parameters are affected in the course of wound healing we

present our data graphically in Figure 3, separating epidermis from dermis/granulation tissue. A graphical survey of these results for galectin presence and reactivity is given in Figure 3. A detailed account of the results obtained at each time point is reported below.

Two days post-surgery

The analysis started with marker protein monitoring. Near the site of injury a slight increase for fibronectin deposition was seen (Fig. 1–2d Fibronectin). The epidermis was thickened at its cut edges (not shown). A demarcation line, rich in PMNL, was formed and separated the necrosis from vital tissue (Fig. 1–2d H+E insert). The number of fibroblasts was slightly increased in the dermis near the wounded area, as evidenced by vimentin-dependent staining (Fig. 1–2d K+Vim). Presence of both vimentin and cytokeratin was rarely observed in cells separated from the epithelial leading edge. Both keratin-10 and -14-positive cell populations were present in the epidermis and epidermal leading edge (data not shown).

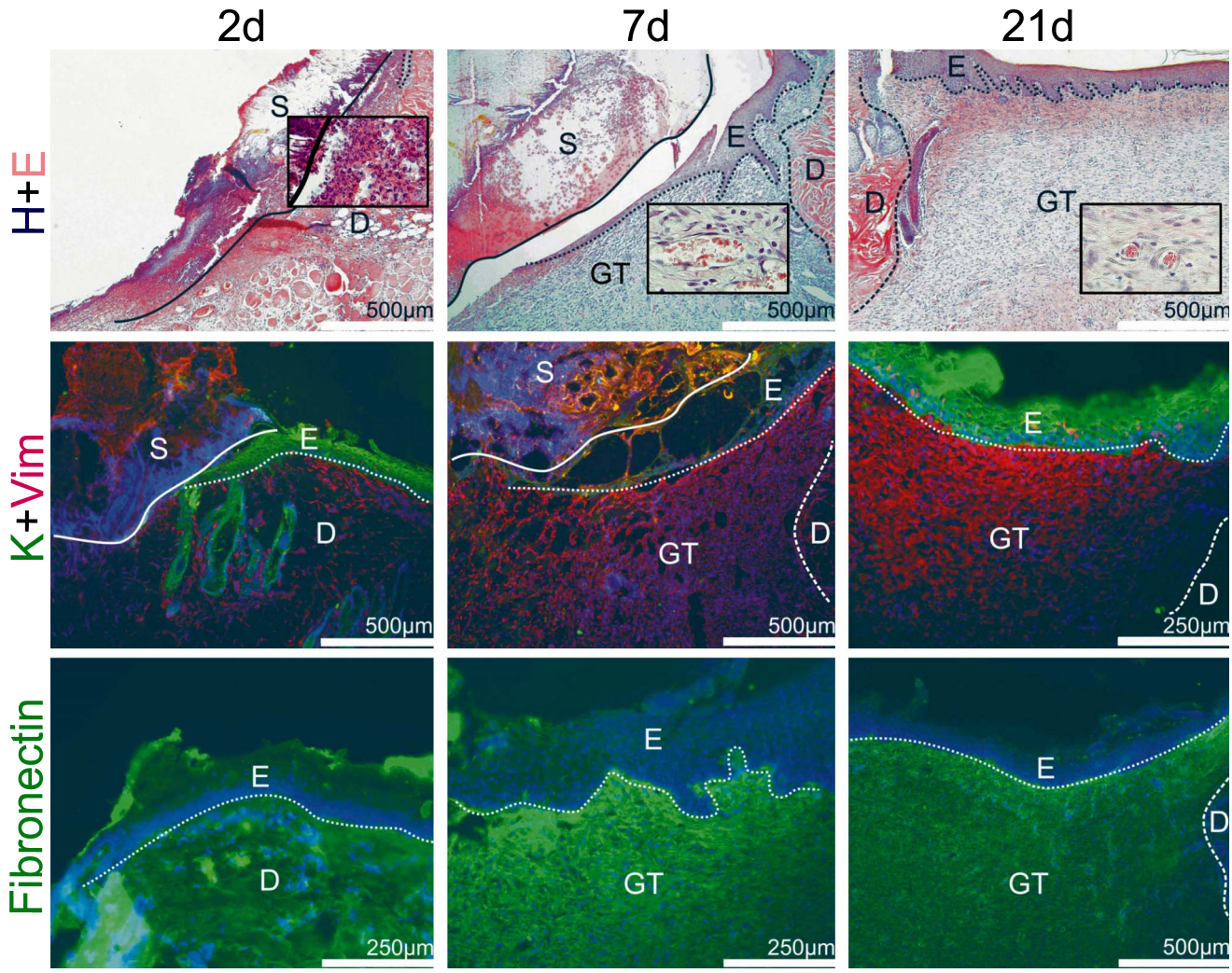

Fig. 1. H+E: Hematoxylin and eosin staining of skin wounds at three different stages of the healing process, starting at day 2: (2d): presence of tissue necrosis (S), formation of the demarcation line beneath the scab consisting mainly of polymorphonuclear leucocytes (see insert); seven-days healing wound (7d): migration of epidermal (E) cells over the wound, forming of the granulation tissue (GT) rich on fibroblasts and high-caliber vessels (see insert); 21-days healing wound (21d): completed epidermis regeneration, well-formed granulation tissue with decreased number of vessels and fibroblast (see insert) establishing into the scar. K+Vim: wide-spectrum cytokeratin+vimentin double-staining immunohistochemistry of healing skin wounds at the same time points: 2d: migration of epidermal cells beneath the scab; 7d: formation of the granulation tissue rich on vimentin-positive cells; 21d: completed epidermis regeneration, well-formed granulation tissue with decreased number of vimentin-positive cells. Fibronectin: simple staining immunohistochemical localization in the course of healing of skin wounds: 2d: wounds with low-level expression of fibronectin near the wound edge; 7d: granulation tissue rich on fibronectin; 21d: low-level presence of fibronectin in the developing scar. For orientation solid/dotted/broken lines are given separating distinct regions referred by the following abbreviations: E, epidermis; D, dermis; GT, granulation tissue; S, scab; in detail, a dotted line sets epidermis apart from dermis and/or granulation tissue; the broken line distinguishes dermis from granulation tissue and the solid line scab/necrosis from vital tissue.

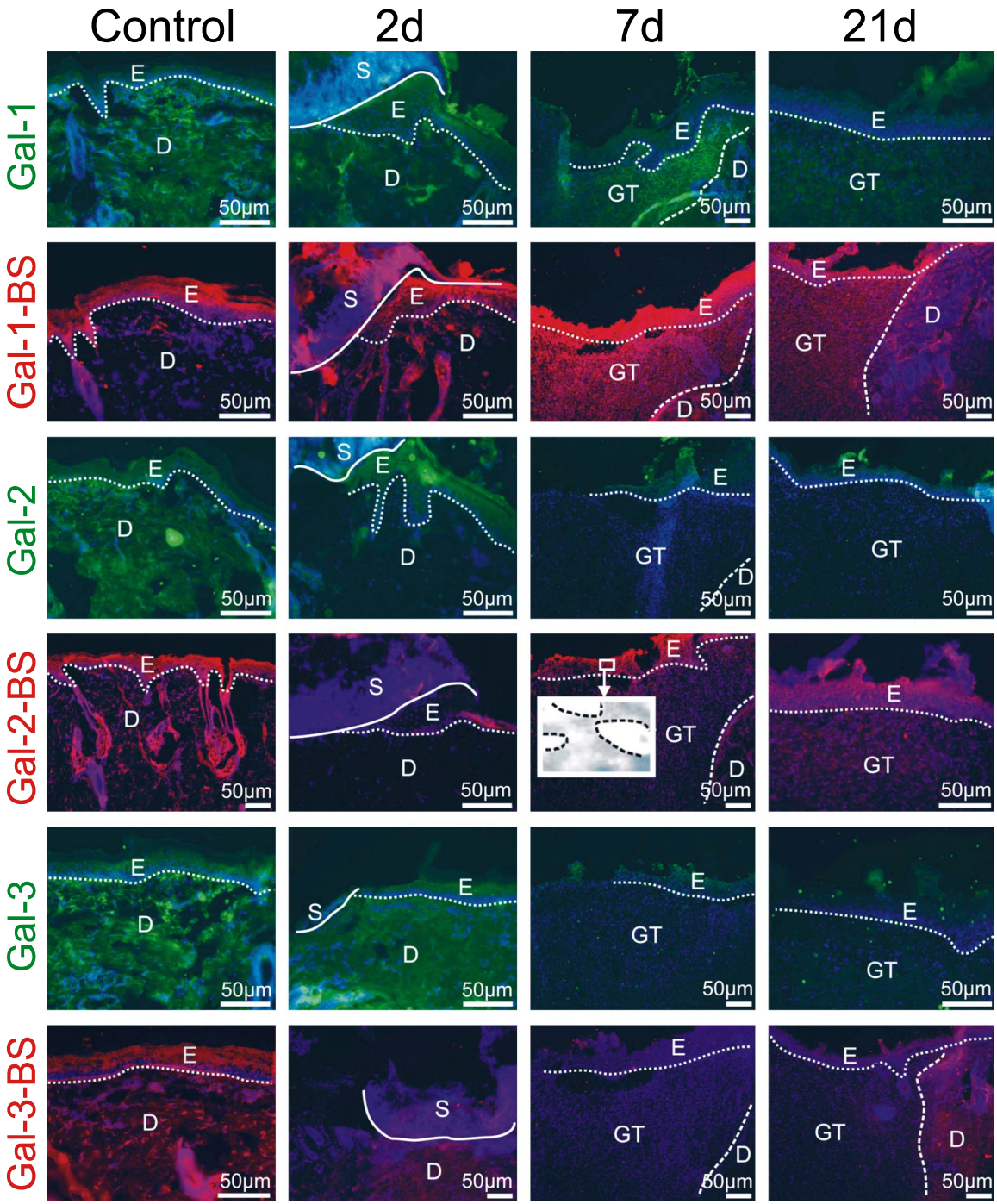

Fig. 2. Illustration of immunohistochemical galectin detection and localization of accessible binding sites (BS) for labeled galectins in the epidermis and in the dermis/granulation tissue during healing. Comparison between control data (first vertical panel) and specimens at each studied time point during healing (2d: second vertical panel; 7d: third vertical panel; 21d: fourth vertical panel) is thus made possible for each marker along each horizontal panel. In detail, the following assignments of type of probe and time point are given. First panel (Gal-1): strong signal intensity for galectin-1 two days after injury in both epidermis and dermis near the wound edge, decreasing over time to minimal presence in the dermis at day 21; second panel (Gal-1-BS): low level of galectin-1 reactivity in uninjured skin and wounds two and 21 days post wounding, increased reactivity to Gal-1-BS in the granulation tissue; third panel (Gal-2): galectin-2 detection in the epidermis, absence in granulation tissue; fourth panel (Gal-2-BS): galectin-2 reactivity in uninjured skin and wounds localized to epidermis, low-level presence of binding sites in the dermis, insert—galectin-2 nuclear reactivity in the epidermis (dashed line marks the nuclei of keratinocytes); fifth panel (Gal-3): presence in the suprabasal epidermal layer and dermis, low-level signal intensity in the granulation tissue; sixth panel Gal-3-BS: present in the suprabasal epidermal layer and in the surrounding dermis, low abundance presence in the scar forming. For orientation solid/dotted/broken lines are given separating distinct regions referred by the following abbreviations: E, epidermis; D, dermis; GT, granulation tissue; S, scab; in detail, a dotted line sets epidermis apart from dermis and/or granulation tissue; the broken line distinguishes dermis from granulation tissue and the solid line scab/necrosis from vital tissue.

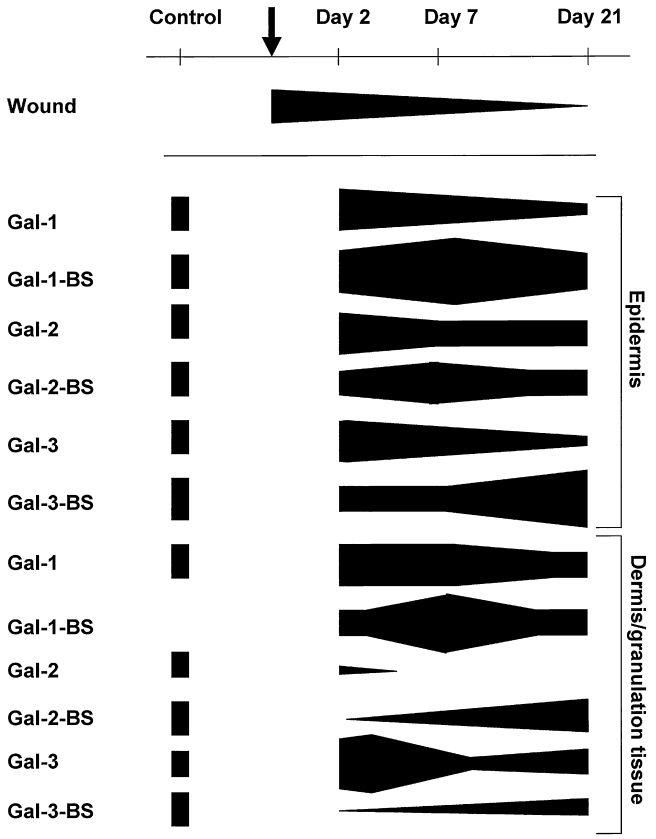

Fig. 3. Computation of the staining data on semi-quantitative scale for the tested galectins and respective binding sites in epidermis and in the dermis/granulation tissue at the three given time points during the healing process (top row) with respect to wound closure. Arrow indicates the moment of wounding.

The galectin-related data (presence of galectins-1, -2, and -3; reactivity to galectins-1, -2 and -3) are presented in pairs for each protein in horizontal panels in Figure 2. Each panel starts with the control to set the reference (first vertical panel in Fig. 2). Moving to the second vertical panel of Figure 2, the status after two days is exemplarily illustrated. Near the injury site a moderate level of galectin-1 expression and reactivity in both epidermis and dermis was observed. Galectin-2 was seen in all layers of the epidermis but not in the dermis. Reactivity to galectin-2 correlated with its expression; thus, it was confined to the epidermis. Galectin-3 positivity resembled the profile of galectin-1 in the dermis, but it was restricted to the suprabasal layer of epidermis. Rather weak signals were recorded for binding of labeled galectin-3 in the epidermis near the injury site.

Seven days post-surgery

By seven days after surgery, the skin edges separated by the open wound *in vivo* were not yet completely bridged by a new layer of epithelium (Fig. 1–7d H+E). The wounds were positive for keratins-10 and -14 and were only lightly infiltrated with PMNL (data not shown). The newly formed granulation tissue was rich on fibronectin (Fig. 1–7d Fibronectin), fibroblasts (Fig. 1–7d K+Vim), and high-caliber vessels (Fig. 1–7d H+E insert).

The galectin-related parameters presented similarities and conspicuous changes that are clearly illustrated in the third vertical panel of Figure 2. Increased expression of galectin-1 was maintained in the epidermis and granulation tissue, and the reactivity for this galectin was particularly strong during this time period of healing. Of note in view of the close homology between the two prototype galectins, the galectin-2 parameters were relatively unchanged, with evidence for nuclear reactivity in the epidermis (Fig. 2–insert, nuclei surrounded with dashed lines). There was no signal for galectin-3 presence and reactivity in the granulation tissue, but the expression of galectin-3 remained present suprabasally in the epidermis, excluding a false-negative result.

Twenty-one days post-surgery

At this stage, the presence of keratin layer in wounds demonstrated a normal course of keratinocyte differentiation and completed process of epidermis regeneration (Fig. 1–21d H+E). The number of luminized vessels in the granulation tissue decreased (Fig. 1–21d H+E insert). Equally typically, the level of presence of fibronectin in the granulation tissue had leveled off (Fig. 1–21d Fibronectin), while the content of collagen had increased (data not shown).

The galectin-related parameters are documented in the fourth vertical panel of Figure 2. At this stage, galectin-1 presence decreased to a minimum, signal intensity for binding sites of this lectin was also slightly reduced in both epidermis and granulation tissue and moderately in the surrounding dermis. The galectin-2-related parameters remained at relatively low levels, notably without nuclear reactivity for galectin-2 in the epidermis over the developing scar. Galectin-3 parameters also appeared to re-normalize, with slight increase observed in expression and reactivity. As a further specificity control for galectin binding we added analysis with biotinylated prototype galectin-7. As a result, no staining was detectable with this homodimeric protein, an indication for the specificity of the interaction among prototype galectins.

IV. Discussion

The proteins studied are versatile effectors in cell adhesion, growth regulation and other cellular processes, by virtue of binding distinct epitopes [17, 45, 48]. Of note, even the closely related prototype galectins-1 and -2 are known to have their characteristic activity profile, and functional competition between galectins-1 and -3 has also been documented [25, 35, 43]. By using non-cross-reactive antibodies and biotinylated galectins as probes we compared the expression and reactivity profiles for two prototype (Gal-1, -2) and the chimera-type (Gal-3) galectins. Our study resolved distinct aspects of the issue on galectin presence in wound healing: localization profiles and signal intensity were clearly different. Despite pronounced sequence homol-

ogy each family member tested had its characteristic pattern during the course of wound healing.

Indications for a co-regulation of lectin expression/ reactivity were discerned for galectin-1, first increasing, then leveling-off during scar formation. Of note, when compared to porcine skin such changes were less marked in rats [24], revealing interspecies differences. In functional terms, galectin-1 is known as a potent inductor of ECM formation and TGF-β-independent conversion of fibroblasts into myofibroblasts [12]. In addition, wound treatment with recombinant human galectin-1 resulted in significantly increased wound contraction in rats [12]. In contrast, galectins-3 and -7, but not galectin-1, have been shown to play a role in reepithelialization of murine corneal wounds [7]. From this point of view, the galectin-dependent regulation of wound healing might be different for epidermis and dermis.

Monitored in parallel in this model, the close relative of galectin-1, i.e. galectin-2, appeared to follow its own independent course. The proliferation phase was associated with nuclear reactivity to this galectin in the epidermis, adding to our previous observations of the nuclear galectin-2 presence following physical, chemical, and/or biological treatment modalities [11, 34]. The question whether galectin-2 joins the category of nuclear lectins has herewith been answered on the *in vivo* level as well.

In contrast to galectin-1, the expression pattern of the chimera-type galectin-3 was clearly different, with only a slight increase observed during the maturation phase. In semiquantitative terms, extent of galectin-3 signal intensity was lower than for galectin-1, and the presence in the dermis was confined to galectin-1, comparable to the situation in porcine skin [24]. The reported obvious differences in galectin regulation give this research a clear future direction. Because the rat epidermis is also known to express the tandem-repeat-type galectin-9 [27], galectin fingerprinting in skin wound healing, initiated here, can thus be extended to the members of this galectin group.

In summary, our study initiated characterization of galectin presence and reactivity in the course of healing of skin wounds in the rat model. Galectins-1 and -3 are differentially regulated during skin wound healing. Whereas galectin-1 seems to play a role in the early phases of healing and wound contraction, observations on the role of galectin-3 in hepatic [21] and renal [22] fibrosis combined with the presented evidence intimate that this lectin might be able to modulate scaring. An extrapolation from this experimental to the clinical situation is, however, not possible due to interspecies variability, but the general molecular regulation of wound healing should be similar. Respective investigations are thus encouraged by this study in the rat model.

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Original Article

Early Stages of Trachea Healing Process: (Immuno/Lectin) Histochemical Monitoring of Selected Markers and Adhesion/ Growth-Regulatory Endogenous Lectins

(wound healing / tissue repair / regeneration / galectin / glycophenotype / tracheotomy)

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Abstract. Tracheotomy may be associated with numerous acute and chronic complications including extensive formation of granulation tissue. The emerging functional versatility of the adhesion/growth-regulatory galectins prompted us to perform a histochemical study of wound healing using rat trachea as model. By using non-cross-reactive antibodies and the labelled tissue lectins we addressed the issue of the presence and regulation of galectin reactivity during trachea wound healing. Beside localization of high-molecular-weight keratin, wide-spectrum cytokeratin, keratins 10 and 14, α-smooth muscle actin, vimentin, fibronectin, and Sox-2, galectins -1, -2, and -3 and their reactivity profiles were measured in frozen sections of wounded and control trachea specimens 7, 14, and 28 days after trauma. A clear trend for decreased galectin-1 presence and increased reactivity for galectin-1 was revealed from day 7 to day 28. Sox-2-positive cells were present after seven days and found in the wound bed. Interestingly, several similarities were observed in comparison to skin wound healing including regulation of galectin-1 parameters.

Introduction

Tracheotomy may be followed by numerous acute and chronic complications. The most frequent reaction manifested late as response to the surgical procedure is the development of excess granulation tissue. Its presence may lead to airway occlusion. Such a reaction may occur in up to 65 % of the patients (Wood and Mathiesen, 1991; Sue and Susanto, 2003), requiring prompt treatment. Several studies have been published pointing to an impact of various factors within the management of tracheal stenosis (Talas et al., 2002; Liman et al., 2005; Sarper et al., 2005; Herrington et al., 2006).

It is generally accepted that the key problems of trachea healing are associated with extensive formation of granulation tissue. In detail, this process leads to hypertrophic scar formation and may finally result in tracheal

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Abbreviations: Gal – galectin, HE – haematoxylin-eosin, M/TM – monocytes/tissue macrophages, PBS – phosphate-buffered saline, PMNL – polymorphonuclear leukocytes, VG – Van Gieson.

stenosis (Talas et al., 2002). Different techniques have been developed and successfully used to treat tracheal stenosis, such as implementation of stents (Fernando et al., 2010). However, these techniques are rather time- and money-consuming and they cause additional stress for the patient because further surgical interventions are often inevitable. Hence, intervention by medications favouring trachea wound healing and preventing stenosis formation would reduce costs and be beneficial for patients.

At the molecular level, growth factors as well as chemo- and cytokines are known to play significant roles in the coordinated events leading to complete post-traumatic tissue repair and regeneration (Gomperts et al., 2007; Sandulache et al., 2009). In addition, increasing attention is given to the role of glycosylation of proteins/lipids, the sugar-encoded information being translated into cellular activities by endogenous lectins (Gabius et al., 2004, 2011). Of note, the presence of distinct endogenous lectins and the presentation of appropriate glycan epitopes on counter-receptors such as the fibronectin receptor are orchestrated in a finely tuned manner to generate potent signals regulating e.g. cell growth (for concept and overview see Gabius, 2009; for model studies on control exerted by a tumour suppressor or within T-cell communication see André et al., 2007; Wang et al., 2009; Sanchez-Ruderisch et al., 2010; Wu et al., 2011). In the mentioned cases, members of the galectin family serve as the lectin part capable to effectively regulate cell adhesion, growth and migration, via protein/glycan and protein/protein interactions (Gabius, 2006; Smetana Jr. et al., 2006; Schwartz-Albiez, 2009; Kaltner and Gabius, 2012). This emerging activity profile prompted us to study the presence of adhesion/growth-regulatory galectins focusing on the homodimeric proto-type galectins -1 and -2 (Gal-1, Gal-2) as well as the chimera-type galectin-3 (Gal-3). Special care was taken to preclude any cross-reactivity in immunodetection, and purified galectins were in parallel applied as sensors for changes in cellular reactivity. Beside application of these tools, formation of the extracellular matrix and the signature of keratin expression were assessed.

Hence, in this investigation, distribution of endogenous lectins and their binding sites were set in relation to keratin expression and extracellular matrix formation. Since the status of sialylation is known to regulate galectin reactivity markedly, with α-2,6-sialylation acting as stop signal except for the presence on termini in repeats (Ahmed et al., 2002), this parameter was additionally monitored by two plant lectins. In view of recent evidence that adult stem cells play a remarkable role in wound healing, the presence of Sox-2, nucleostemin, and vimentin was evaluated as well. Tissue specimens were processed under identical conditions to rigorously exclude any factor other than the time point to affect signal occurrence and intensity.

Material and Methods

Animal model

The study was approved by the local ethical committee and by the State Veterinary and Food Administration of the Slovak Republic.

One-year-old male Sprague-Dawley rats (N = 20) were included into the experiment. In all rats, surgery was performed under general anaesthesia induced by administration of ketamine (40 mg/kg; Narkamon a.u.v.; Spofa, Prague, Czech Republic), xylazine (15 mg/kg; Rometar a.u.v.; Spofa) and tramadol (5 mg/kg; Tramadol-K; Krka, Novo Mesto, Slovenia). Prior to tracheotomy each rat was intubated. Under aseptic conditions, skin and subcutaneous tissue were incised, then strap muscles were separated and retracted laterally to enable access to the front tracheal wall. Next, median incision was performed from the second to the fifth tracheal cartilage ring. Subsequently, the incision was closed using two simple interrupted sutures (Chiraflon 6/0; Chirmax, Prague, Czech Republic), as was done for the wound in anatomical layers (Chiraflon 5/0). Overall, six rats were killed by ether inhalation 7, 14, and 28 days after surgery, respectively. Intact trachea was removed from two control rats, which did not undergo surgery.

Human trachea

A sample of human trachea was obtained from a non-smoking volunteer suffering from tracheal stenosis after punction tracheostomy. The sample was obtained after informed consent of the patient with agreement of the local ethical committee.

Basic histology

Tracheae were removed from one intact rat and four wounded rats killed at each evaluated time point and routinely processed for light microscopy (fixation in 4% buffered formaldehyde, dehydration, paraffin embedding, sectioning, and staining). Paraffin sections were stained with haematoxylin-eosin (HE – basic staining) and Van Gieson (VG – non-specific collagen staining).

Immunohistochemistry and lectin histochemistry

Tracheae were cryoprotected by Tissue-Tek from one intact rat and two wounded rats killed at each given time point (Sakura, Zoeterwoude, The Netherlands) and stored in liquid nitrogen. Frozen sections were fixed in 2% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) (pH = 7.2). Non-specific binding of secondary antibody was blocked by preincubation with normal swine serum (DAKO, Glostrup, Denmark) diluted in PBS for 30 min. The origin of the reagents for immunohistochemistry and lectin histochemistry used in this study is listed in Table 1. Purification of galectins, rigorous controls for purity, their labelling under activity-preserving conditions and activity controls as well as preparation of the antibodies, testing for and removal of

Table 1. Reagents used for immunohistochemistry and lectin histochemistry

primary antibody	abbreviation	host	produced by	secondary antibody	produced by	channel
high-molecular-weight cytokeratin	HMWK	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	(Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
vimentin	Vim	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	(both Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
cytokeratin 10	K10	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	(both Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
cytokeratin 14	K14	mouse monoclonal	Sigma, Saint Louis, Missouri	goat anti-mouse	(both Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
wide-spectrum cytokeratin	WSK	rabbit polyclonal	Abcam, Cambridge Science, Cambridge UK	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA)	FITC-green
fibronectin	Fibr	rabbit polyclonal	Dakopatts, Glostrup, Denmark	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA)	FITC-green
Sox-2	Sox2	rabbit polyclonal	Abcam, Cambridge Science, Cambridge UK	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA)	FITC-green
galectin 1	Gal-1	rabbit polyclonal	Gabius laboratory	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA)	FITC-green
galectin 2	Gal-2	rabbit polyclonal	Gabius laboratory	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA)	FITC-green
galectin 3	Gal-3	rabbit polyclonal	Gabius laboratory	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA)	FITC-green
biotinylated lectin	**abbreviation**		**produced by**	**second step reagent**	**produced by**	**channel**
galectin 1 binding site	Gal-1BS		Gabius laboratory	ExtrAvidin	(Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
galectin 2 binding site	Gal-2BS		Gabius laboratory	ExtrAvidin	(Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
galectin 3 binding site	Gal-3BS		Gabius laboratory	ExtrAvidin	(Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
Maackia amurensis (α2,3-linked NeuNAc)	MAA		Vector Laboratories, Burlingame, CA	ExtrAvidin	(Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
Sambucus nigra (α2,6-linked NeuNAc)	SNA		Vector Laboratories, Burlingame, CA	ExtrAvidin	(Sigma-Aldrich, Prague, Czech Republic)	TRITC-red

any cross-reactivity among galectins and quality controls by Western blotting/ELISA were performed as described previously (Gabius et al., 1991; Purkrábková et al., 2003). All antibodies were routinely tested against galectins -1, -2, -3, -4, -5, -7, -8, and -9 (galectin-6 is restricted in occurrence to several mouse strains). Nuclei were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO). Specimens were mounted to Vectashield (Vector Laboratories, Burlingame, CA). The analysis of specimens and data storage were performed using an Optiphot-2 fluorescence microscope equipped with filter blocks specific for DAPI, FITC, and TRITC (Nikon, Tokyo, Japan), a CCD camera (COHU) and computer-assisted image analysis system LUCIA 3.2 (Laboratory Imaging, Prague, Czech Republic), as described for nucleostemin monitoring previously (Čada et al., 2007).

Morphometric and semi-quantitative evaluation of histological sections

The area covered by granulation tissue was determined in a section obtained from each individual animal, based on VG staining. The specimen analysis was performed using an Olympus BX51 microscope (Olympus, Tokyo, Japan) with an Olympus DP71 CCD camera and QuickPHOTO MICRO 2.2 (Promicra, Prague, Czech Republic) software. Data are expressed as mean ± standard deviation. A semi-quantitative method (Grendel et al., 2011) was used to evaluate re-epithelization, the presence of inflammatory cells (polymorphonuclear leukocytes [PMNL]), the presence of chondroblasts and of newly formed vessels. Sections were examined as coded slides by two well-trained experts according to the scale ranging from 0 to 3 (Table 2). Similarly, signal intensi-

Table 2. Scale for the semi-quantitative evaluation of histological parameters in healing trachea

Scale	re-epithelization	PMNL	new vessels	new collagen	new cartilage	fluorescence signal
–	absent	absent	absent	absent	absent	absent
+	migration of cells	mild	mild	mild	minimal	mild
++	bridging incision	moderate	moderate	moderate	moderate	moderate
+++	complete regeneration	marked	marked	marked	marked	marked

Table 3. Results from the semi-quantitative evaluation of galectin expression/reactivity

	Gal-1	Gal-1BS	Gal-2	Gal-2BS	Gal-3	Gal-3BS	MAA	SNA
HT	+	+	++	+	++	+	–	+
RT	+	+	++	+	++	+	–	++
7d	++	+	++	++	+	+	–	++
14d	+	+	+++	++	+	++	–	++
28d	+	++	++	++	+	+	–	++

(HT – human trachea, RT – rat trachea)

Table 4. Results from the semi-quantitative evaluation of histological structures/changes during trachea wound healing

	re-epithelization	PMNL	fibronectin	collagen	new cartilage
7d	++	+	+++	–	–
14d	+++	–	++	–	+
28d	+++	–	+	+	++

ties of the galectin presence and reactivity were assessed by a semi-quantitative method according to this scale (Table 2) as previously described in detail (Čada et al., 2009).

Results

During the post-surgical period all animals remained healthy, without any clinical symptoms of infection. The data of the semi-quantitative analysis of histological sections are summarized in Table 3 and Table 4. A detailed account of the obtained results is given for each time point as follows.

Intact trachea

Similar glyco- and immunophenotypes were observed for both human and rat tracheae (Fig. 1, Fig. 2). In contrast to the wide-spectrum cytokeratin antibody, which stained the entire epithelial population, the expression of high-molecular-weight keratin was detectable in a rather limited population of cells (Fig. 2). Similarly, keratin 14 positivity was restricted to the non-ciliated cell population (Fig. 2), whereas keratin 10-positive cells were not found in the tracheal epithelium (not shown). Control trachea was positive for the plant lectin reactive with α-2,6-sialylation, whereas no α-2,3-sialylation on type II termini (i.e. Gal β1, 4GlcNAc) was

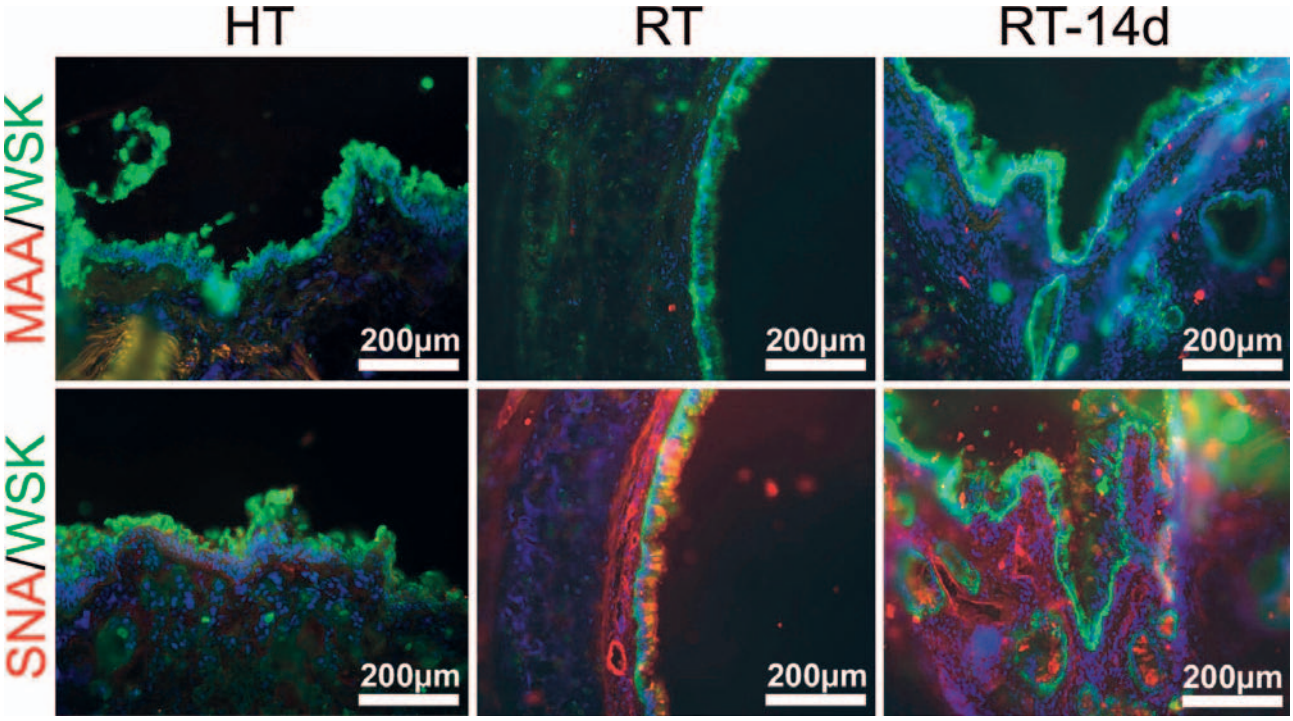

Fig. 1. Binding patterns of the two plant lectins selective for α-2,3- and α-2,6-sialylation in control and injured trachea: a – control trachea/b – wounded trachea 14d post surgery – lack of MAA reactivity under experimental conditions; c – control trachea/d – wounded trachea – SNA reactivity; 200×.

a	b	c
d	e	f

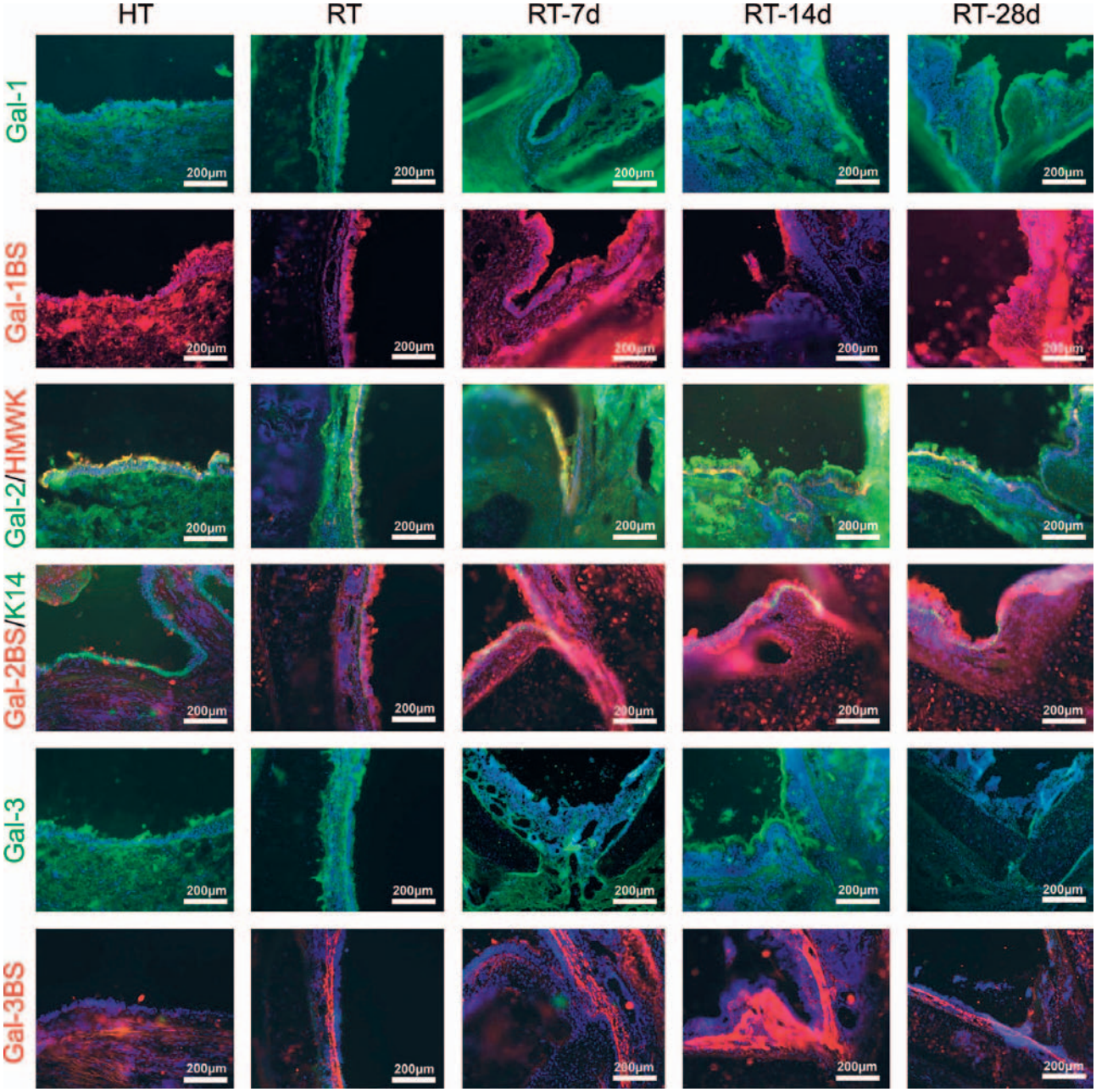

Fig. 2. Galectin (immuno)histochemistry of control and injured tracheae: control (intact) human trachea – a, f, k, p, u, and z; control (intact) rat trachea – b, g, l, q, v, and a1; trachea 7d post surgery – c, h, m, r, w, and b1; trachea 14d post surgery – d, i, n, s, x, and c1; trachea 28d post surgery – e, j, o, t, y, and d1; 200×.

a	b	c	d	e
f	g	h	i	j
k	l	m	n	o
p	q	r	s	t
u	v	w	x	y
z	a1	b1	c1	d1

detectable (Fig. 1). The presence of galectins and their binding sites was as follows (Fig. 2): moderate staining intensity for galectins was seen in the epithelium and in subepithelial tissue. The cartilage and the chondrocytes did not reveal any signal for any of the tested galectins. The binding patterns for Gal-1 and -2 were similar and were located both in the epithelium and in the subepithelial tissue, with moderate up-regulation of Gal-1 on day 7 following injury. In contrast, binding sites for Gal-3 were strictly confined to a thin layer of the subepithelial tissue.

7d post surgery

At this time point, the epithelial sheet completely bridged the incision (Fig. 3). Well-formed granulation tissue was present in wounds removed on day 7 (granulation tissue area = 87.925 ± 21.547 μm^2). Near the site of injury, a slight increase of fibronectin deposition was seen (Fig. 3). No α-smooth muscle actin was detectable (not shown). Keratin 14-positive cell populations were present in the tracheal epithelium (Fig. 2). No keratin 10-positive cells were seen in the epithelium (not

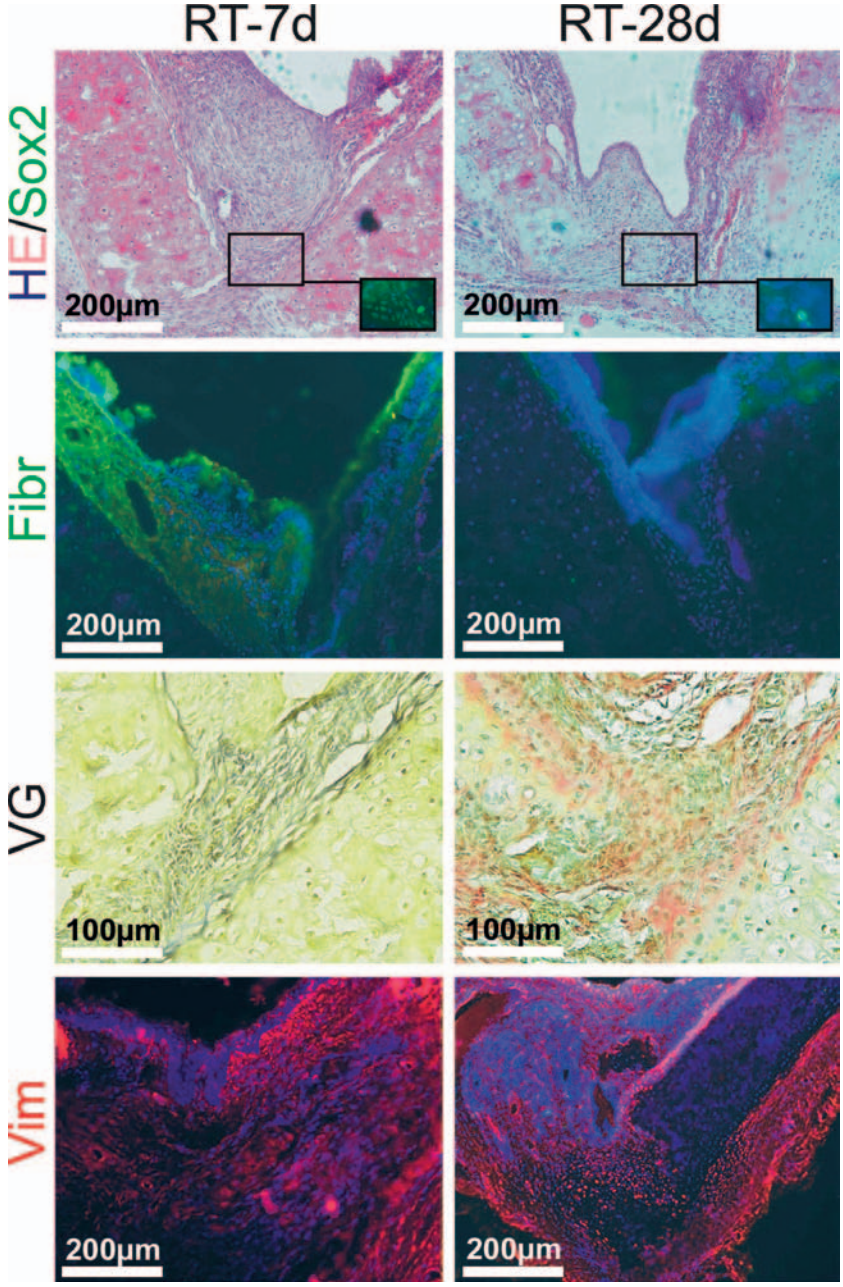

Fig. 3. Wounded trachea: a – 7d post surgery (HE (200×) – staining, well-formed granulation tissue, insert – Sox-2-positive cells in the wound bed); b – 28d post surgery (HE (200×) – staining, reduced granulation tissue, insert – reduced number of Sox-2-positive cells in the wound bed); c – 7d post surgery (fibronectin staining (200×), presence of fibronectin at the injury site); d – 28d post surgery (fibronectin staining (200×), absence of fibronectin at the injury site); e – 7d (VG (400×) – staining, absence of collagen at the injury site); f – 28d post surgery (VG (400×) – staining, presence of collagen at the injury site); g – 7d post surgery (vimentin-positive cells in the granulation tissue (200×)); h – 28d post surgery (presence of vimentin-positive cells (200×), formation of new cartilage).

a	**b**
c	**d**
e	**f**
g	**h**

shown). Moreover, several Sox-2-positive cells were found in the wound bed (Fig. 1 – insert). Monitoring galectin presence revealed a marked level of Gal-1 expression at the site of injury; Gal-2 was seen in the epithelium and subepithelial tissue of the trachea (Fig. 2). Gal-3 staining was weak, present in the subepithelial tissue and epithelium (Fig. 2). Moderate signals were obtained for binding of labelled Gal-1 and -2 in the epithelium and subepithelial tissue, Gal-3 reactivity was maintained in a thin layer of the subepithelial tissue (Fig. 2). As seen in control trachea, evidence for α-2,6--sialylation was obtained at the site of injury (not shown).

14d post surgery

The number of PMNL remained unchanged, while the number of monocytes/macrophages increased when compared to sections removed on day 7 (not shown). The largest number of new vessels in the granulation

tissue was observed at this time point (not shown). Its area decreased to 75.591 ± 8.878 μm^2. The content of fibronectin also decreased in the granulation tissue, whereas the collagen content increased (not shown). Furthermore, the number of Sox-2-positive cells decreased (not shown). Positivity for Gal-1 and -2 was maintained in the epithelium and subepithelial tissue (Fig. 2). There was no signal for the Gal presence in the cartilage. Mild reactivity for Gal-1 was determined, Gal-3 binding slightly increased (Fig. 2). The profile for α-2,6-sialylation remained unchanged 14 days post-wounding (Fig. 1).

28d post surgery

A month after surgery, the remodelling/maturation phase of healing was still in progress (Fig. 3). The number of newly formed vessels decreased, together with the overall area of granulation tissue (67.612 ± 10.612 μm^2). The number of PMNL reached its minimum, whereas the number of monocytes/macrophages remained rather moderate (not shown). Moreover, the density of fibronectin in the granulation tissue also decreased to its minimum, while the content of collagen continued to increase (Fig. 3). On the other hand, the number of Sox-2-positive cells was diminished (Fig. 1 – insert). As already noted above, a concomitant reduction in Gal-1 was seen (Fig. 2). Conversely, the signal intensity for binding sites of this lectin reached its highest level (Fig. 2). Binding of Gal-2 and -3 followed a constant pattern. In addition, reactivity for Gal-2 was also observed in the chondroblasts of the cartilage (Fig. 2). No change in the α-2,6-sialylation was seen (not shown).

Discussion

The range of physiological activities of endogenous lectins prompted us to systematically determine the presence of three adhesion/growth-regulatory galectins at three time points post-trauma and in uninjured control tissue, along with several known marker proteins. According to our observations complete wound covering with a layer of epithelial cells was already seen on day 7. Most epithelial cells were positive for keratin 14, but all cells were negative for keratin 10. The epithelial cells expressed Gal-1, -2 and -3. Interestingly, high-molecular-weight keratin antibody did not stain any cell; its presence was rather seen in the basal layer of the cylindrical epithelium and not in ciliated cells, similar to the positivity profile for keratin 14.

Our experiments with non-cross-reactive antibodies and biotinylated galectins answered the questions on the level of presence, regulation in the course of healing and inter-galectin differences. Galectin presence could be detected in all cases. By testing two plant lectins no evidence for a change in the sialylation status, a decisive parameter in T-cell activation and tumour suppressor activity controlling galectin reactivity (André et al., 2007; Bi and Baum, 2009; Sanchez-Ruderisch et al., 2011), was detected. However, applying the tissue lectins as tools, different profiles among the tested galectins were observed during the course of the repair processes. Both localization patterns and signal intensity were clearly disparate. Despite the overall sequence homology, each protein tested followed its characteristic course and, of note, regulation within the healing process. Most prominently, Gal-1 appeared to be up-regulated in the early phase of tissue repair and regeneration, then leveled off, with matching changes in tissue reactivity for Gal-1. Co-regulation has been observed to direct e.g. anoikis induction by a tumour suppressor as master regulator (André et al., 2007). The changes in Gal-1 and -3 were similar when compared to rat skin wound healing (Gál et al., 2011). Obvious differences among galectins give research a clear direction into deciphering mechanisms underlying gene regulation, based on the results of mining proximal promoter regions for putative sites binding transcription factors (Sturm et al., 2004; Lohr et al., 2007, 2008). This conclusion is supported by the finding of disparate profiles also in wound healing of pig skin (Klíma et al., 2009).

A peak in the proliferation phase among the evaluated time intervals of trachea healing was observed on day 7. At this time point, an increased number of vessels and vimentin-positive cells was observed, suggesting an involvement in such parameter changes. When compared to skin wound healing in rats, there is a slight delay for these alterations to occur (Gál et al., 2008). Together with the increase in the number of cells, the collagen content in the trachea wound was elevated. Of note, the contents of fibronectin and collagen had an inverse relationship. This relationship is necessary for the sufficient biomechanical stiffness of the injured tissue to be accomplished. On the other hand, hyperproliferation of granulation tissue might result in collagen overproduction. Later on, a hypertrophic scar can be formed, a manifestation often underlying airway occlusion (Liman et al., 2005).

Sox-2 is an evolutionarily conserved transcription factor that plays an important role in oesophagus and trachea development (Que et al., 2009). In that study, it was shown that Sox-2 impairs proliferation and differentiation of adult tracheal epithelium during regenerative processes. In our histological study, we demonstrated that Sox-2 was expressed in the cells that form an endoluminal wound bed of healing trachea, and its expression decreased with healing time. From this point of view, it may be suggested that – in addition to epithelial stem cells – a limited population of adult stem cells is located in the perichondrium and wound bed and that these cells might contribute to the formation of granulation tissue during trachea wound healing.

In summary, our study characterized the galectin presence and reactivity in the course of healing of tracheal incisional wounds in the rat model. The detected differences argue in favour of functional individuality and also intracellular activities of the tested proteins. Since the sialophenotypes of intact human and rat tra-

chea were similar, it may be suggested that the rat model might have relevance for the clinical situation. Consequently, analysis of clinically relevant specimens following this concept is now warranted.

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Human Galectins Induce Conversion of Dermal Fibroblasts into Myofibroblasts and Production of Extracellular Matrix: Potential Application in Tissue Engineering and Wound Repair

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Key Words
Extracellular matrix · Fibronectin · Keratinocyte · Lectin · Tissue engineering

Abstract
Members of the galectin family of endogenous lectins are potent adhesion/growth-regulatory effectors. Their multifunctionality opens possibilities for their use in bioapplications. We studied whether human galectins induce the conversion of human dermal fibroblasts into myofibroblasts (MFBs) and the production of a bioactive extracellular matrix scaffold is suitable for cell culture. Testing a panel of galectins of all three subgroups, including natural and engineered variants, we detected activity for the proto-type galectin-1 and galectin-7, the chimera-type galectin-3 and the tandem-repeat-type galectin-4. The activity of galectin-1 required the integrity of the carbohydrate recognition domain. It was independent of the presence of TGF-β1, but it yielded an additive effect. The resulting MFBs, relevant, for example, for tumor progression, generated a matrix scaffold rich in fibronectin and galectin-1 that supported keratinocyte culture without feeder cells. Of note, keratinocytes cultured on this substratum presented a stem-like cell phenotype with small size and keratin-19 expression. In vivo in rats, galectin-1 had a positive effect on skin wound closure 21 days after surgery. In conclusion, we describe the differential potential of certain human galectins to induce the conversion of dermal fibroblasts into MFBs and the generation of a bioactive cell culture substratum.

Abbreviations used in this paper

ECM	extracellular matrix
MFBs	myofibroblasts

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Introduction

Stem cells hold an enormous therapeutic potential, but obstacles remain to be overcome for their successful clinical application, for example, concerning the generation of a suitable microenvironment to maintain stemness and to ensure anchorage [Voog and Jones, 2010]. In this context, the components of the extracellular matrix (ECM) play pivotal roles in docking cells and engaging them in the complex molecular interplay essential for the cells' functionality. As a consequence, aberrations in this complex network, either engineered in mice or detected in patients, can lead to diseases [Hennet, 2009; Honke and Taniguchi, 2009]. Such observations guide interest especially to the glycan part of the ECM, a highly versatile platform for coding biochemical information, as embodied in the concept of the sugar code [Gabius, 2009]. Indeed, simple monosaccharides used as a matrix, inspired by this bioactivity of glycans, can already serve as a bioactive substratum, even substituting for feeder cells in the case of epidermal keratinocytes [Labský et al., 2003]. In order to achieve the natural complexity, it would be ideal to direct cells to produce suitable ECM using endogenous effectors.

Toward this end, observations that an endogenous lectin, such as galectin-1, has proven capable of promoting the attachment of diverse types of cells, also in concert with matrix glycoproteins or chitosan, have given our research a clear direction [André et al., 1999; Chang et al., 2004; Kadri et al., 2005]. This type of galectin binds the $\alpha_5\beta_1$-integrin, as well as the associated ganglioside GM1, that influences cell adhesion and growth [André et al., 2007; Wang et al., 2009]. It can redirect gene expression, for example of α2(I) collagen via TGF-β/Smad3 signaling, and the exposure of fetal skin or mesenchymal stem cells leads to conversion to the myogenic lineage and even mature myotubes in vitro [Goldring et al., 2002a, b; Fischer et al., 2005; Chan et al., 2006; Okano et al., 2008]. In addition to potential medical relevance, we have detected a strong galectin-1 presence in the stroma of squamous cell carcinomas and in healing wounds [Lacina et al., 2007; Klíma et al., 2009]. Here, the presence of myofibroblasts (MFBs), a distinct type of fibroblast positive for α-smooth muscle actin, is frequent [Hinz 2007; De Wever et al., 2008]. Their generation by conversion in situ is assumed to be induced by TGF-β [Brenmoehl et al., 2009], their presence then contributing to promote wound healing/contraction and tumor progression of squamous carcinoma cells. Obviously, the ECM of MFBs can be conducive in programming cell activities. This concept led us to test the hypothesis whether galectin-1 is involved in MFB and ECM generation and then to investigate its bioactivity as a culture substratum for human keratinocytes. In other words, galectin-1 was studied as a potential inducer of the conversion of adult dermal fibroblasts into MFBs, using TGF-β1 and TGF-β3 as positive controls. Following these experiments, we addressed the issue of measuring the profile of this activity within the family of human galectins. This family consists of three groups, which differ in the spatial presentation of the lectin domain, with characteristic expression in human skin [Villalobo et al., 2006; Čada et al., 2009]. As a consequence, homodimeric galectin-1, galectin-2 and galectin-7, the chimeric galectin-3 and the tandem-repeat-type galectin-4 and galectin-8 were comparatively studied under identical conditions. Natural variants, that is, truncated galectin-3 after proteolytic removal of the stalk region and the two galectin-8 forms differing in linker length, as well as an engineered form of galectin-4 without linker and a galectin-1 mutant without lectin activity, completed the test panel used in the present study. In addition to testing the ability of the lectins in the panel to induce MFBs and the capacity of their ECM to support keratinocyte culture and for wound healing in vivo, we also contribute to defining the extent of functional divergence among these homologous endogenous lectins.

Materials and Methods

Preparation of Galectins

Human galectins (table 1), including the linkerless variant of galectin-4 and the E71Q mutant of galectin-1, were obtained by recombinant production and purified by affinity chromatography on lactosylated Sepharose 4B (or Ni-CAM™ HC resin from Sigma, Munich, Germany, in the case of the His-tagged galectin-1 mutant without lectin activity) as a crucial step [Gabius et al., 1985], routinely followed by the removal of any lipopolysaccharide contamination [Sarter et al., 2009]. Product analysis was performed using one- and two-dimensional gel electrophoresis, gel filtration and mass spectrometry as well as hemagglutination, solid-phase and cell assays to ascertain activity [André et al., 2006, 2007, 2008; Beer et al., 2008]. Proteolytic truncation of galectin-3 with cleavage at the Tyr106/Gly107 and Glu229/Ile230 peptide bonds was performed using collagenase digestion [Kopitz et al., 2001; Kübler et al., 2008].

The Influence of Galectins on Human Dermal Fibroblasts

Dermal fibroblasts were isolated from skin specimens obtained from healthy donors with their informed consent (in agreement with the Helsinki Declaration after approval by the Local Ethical Committee) in the Department of Aesthetic Surgery (Charles University, 3rd Faculty of Medicine). Sterile solutions of human galectins were prepared in Dulbecco's culture medium

Table 1. Galectins used in experiments

Type of galectin	Galectin	Comment
Homodimeric (proto-type)	galectin-1	
	galectin-1-E71Q	strongly reduced affinity for carbohydrates
	galectin-2	
	galectin-7	
Chimera	galectin-3	
	galectin-3-truncated	proteolytically processed form lacks the collagenase-sensitive N-terminal section, reducing ability for cross-linking
Tandem-repeat	galectin-4-linkerless	prototype-like galectin-4 with covalent domain linkage after engineered removal of the linker peptide
	galectin-8-short	different lengths of the linker
	galectin-8-long	

(DMEM) containing 10% fetal calf serum and antibiotics (all from Biochrom, Berlin, Germany). Human dermal fibroblasts were seeded at a density of 2,000 cells/cm^2 and cultured for 3–4 weeks. The culture medium was changed three times per week. The sensitivity of fibroblasts to TGF-β1/-β3 was tested at 10 ng/ml, as described elsewhere [Brenmoehl et al., 2009]. The contribution of galectins to MFB formation was assessed by using galectins (table 1) in concentrations of 200, 300, 400 and 600 ng/ml. The additive effect of both galectin-1 and TGF-β1 (10 μg/ml) was tested by incubating cells with both substances. The role of galectin was, moreover, verified by application of 20 μg/ml of a polyclonal anti-galectin-1 antibody, developed in our laboratory and carefully tested for lack of cross-reactivity with other types of galectins (please see below). To exclude the nonspecific effect of immunoglobulins, the commercial anti-thyroglobulin rabbit polyclonal antibody (Dako, Glostrup, Denmark) was also tested.

Using a commercial ELISA kit (R&D Systems, Minneapolis, Minn., USA), the concentration of TGF-β1, the best-known inducer of MFB formation, was measured in conditioned media from cultures of human dermal fibroblasts in the absence or presence of galectins in a Multiskan RC reader (Labsystem, Helsinki, Finland) at 450 nm.

Human Keratinocytes and Their Culture on Matrices Induced by Galectins

The ECM scaffolds produced by cells exposed to galectins on the surface of coverslips were tested as substrates for the culture of human keratinocytes obtained as described above. The coverslips with the three-dimensional scaffolds were incubated with sterile distilled water for 60 min to devitalize MFBs by osmotic shock (as ascertained by subsequent culture), then the supports were incubated with culture medium for 24 h to prevent osmotic stress during the subsequent culturing of keratinocytes. These cells were obtained from the same clinical source and under identical ethical conditions as the fibroblasts. They were seeded at a density of 40 × 10^3 cells/cm^2 and cultured in keratinocyte medium [Strnad et al., 2009] at 5% CO_2 and 37°C for 1 week. Part of these experimental acellular supports with three-dimensional ECM lattices was fixed with 0.25% glutaraldehyde (Sigma-Aldrich, Prague, Czech Republic) for 30 min at laboratory temperature. After extensive washing they were used as described above. Cells cultured on untreated coverslips were used as a control.

Immunocytochemical Analysis of Cultured Cells

The staining procedure, including fluorescence profiling, had been described recently in detail [Szabo et al., 2009]. Briefly, the tested specimens were fixed with 2% buffered paraformaldehyde (pH 7.2–7.4) for 5 min and washed with PBS. The cells were permeabilized by exposure to Triton X-100 (Sigma-Aldrich), and sites for the antigen-independent adsorption of antibodies were blocked by bovine serum albumin and/or nonimmune porcine serum, serum application preventing the binding of antibodies via the interaction of the Fc fragments of immunoglobulins with cellular Fc receptors. Commercial antibodies were diluted as recommended by the suppliers. The primary and secondary antibodies used in this study are summarized in table 2. The lack of cross-reactivity of our homemade anti-galectin antibodies was ascertained, if required, after affinity depletion on resin with covalenty conjugated galectins [Kaltner et al., 2002; Saal et al., 2005]. The specificity of the immunohistochemical reaction was verified by the replacement of specific antibodies by an irrelevant antibody recognizing thyroglobulin instead of the first-step antibody and/or by the omission of the first-step antibody as well as by processing positive control specimens. Cell nuclei were counterstained by 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The specimens were mounted in Vectashield (Vector Laboratories, Burlingame, Calif., USA) and inspected by an Eclipse 90i fluorescence microscope (Nikon, Prague, Czech Republic) equipped with filter blocks for FITC, 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).

Experimental Animals and in vivo Study

The study design with 1-year-old male Sprague-Dawley rats (n = 48) was approved by the Ethical Committee of the Faculty of Medicine of Šafárik University and by the State Veterinary Administration of the Slovak Republic. Surgery was performed un-

Table 2. Primary and secondary antibodies

Antigen	Type of antibody	Source
Gal-1, Gal-2, Gal-3, Gal-4, Gal-7	rabbit polyclonal	Faculty of Veterinary Medicine, Ludwig-Maximilians-University, Munich, Germany
Gal-1	mouse monoclonal	Vector Laboratories, Burlingame, Calif., USA
Fibronectin	rabbit polyclonal	Dako
Fibronectin	mouse monoclonal	Abcam, Cambridge, UK
Collagen-1 Collagen-3	mouse monoclonal mouse monoclonal	Sigma-Aldrich, Prague, Czech Republic
Smooth muscle actin	mouse monoclonal	Dako
Keratin (wide spectrum)	rabbit polyclonal	Abcam
Keratin-19 Thyroglobulin Thyroglobulin	mouse monoclonal mouse monoclonal rabbit polyclonal	Dako
Rabbit immunoglobulins Mouse immunoglobulins	FITC-labeled swine serum FITC-labeled swine serum	Alseva, Prague, Czech Republic
Mouse immunoglobulins	TRITC-labeled goat serum	Sigma-Aldrich

der general anesthesia induced by the administration of ketamine (40 mg/kg; Narkamon a.u.v.; Spofa, Prague, Czech Republic), xylazine (15 mg/kg; Rometar a.u.v.; Spofa) and tramadol (5 mg/kg; Tramadol-K; Krka, Novo Mesto, Slovenia). One full-thickness incision, 4 cm long, was made on the back of each rat. Cranially and caudally from the incision, round full-thickness skin excisions, 1 cm in diameter, were performed. The incision was then closed using an intradermal running suture (Chiraflon 5/0; Chirmax, Prague, Czech Republic). Subsequently, the rats were randomly assigned to four groups: (1) testing wild-type galectin-1, (2) testing the E71Q mutant, (3) testing full-length galectin-3 and (4) mock treatment. Wounds of rats from groups 1–3 were treated daily with the respective galectin starting from the day of surgery until the third postoperative day. All rats, including the control group, were kept in cages with an oval opening over the wound to ensure similar levels of stress for each animal during the experiment.

The healing of wounds was measured from standardized photographs as follows. Wounds were photographed with a scale immediately after surgery and at days 7 and 21 using an Olympus E330 digital camera equipped with a digital ED 50 mm f 2.0 macro objective and a ring set flash SRF-11 (Olympus, Tokyo, Japan). The wound area was then measured on the images using Quick Photo Micro 2.2 software (Premiere, Prague, Czech Republic) and expressed as a percentage of the original wound area generated on the day of surgery to reflect the level of wound healing. Statistical processing of the measured data was done using one-way ANOVA followed by the Tuckey-Kramer post hoc test. The quality of wound healing was evaluated in paraffin-embedded sections stained with hematoxylin-eosin.

Results

In vitro Experiments

Galectins Induce MFB Formation

Cultures of normal dermal fibroblasts contained none or only a very limited number of MFBs (fig. 1a, b). To test the effector activity of the galectins, we systematically used galectin-1 and detected a significant increase in the number of MFBs in the third week of culture (fig. 1c). Next, we tested all the selected galectins (galectin-1, galectin-1-E71Q mutant, galectin-2, galectin-3, truncated version of galectin-3, galectin-4, linkerless variant of galectin-4 and both, that is, short and long, versions of galectin-8) in the concentration range of 200–800 ng/ml over the course of 3 weeks. The experiments revealed that the activity depends on the type of galectin and its concentration (fig. 2a–f). The strongest induction of the conversion of fibroblasts to MFBs was achieved using homodimeric galectin-1 (fig. 2a). Impairing its carbohydrate-binding activity by introducing the E71Q mutation led to a drastic reduction of its stimulatory ability (fig. 2b). Activity was also found for galectin-3, depending on the presence of the N-terminal stalk, because the truncated version of this galectin was inactive (fig. 2c, d). The natural version of tandem-repeat galectin-4 (fig. 2e) was also active concerning MFB formation. Parallel experiments

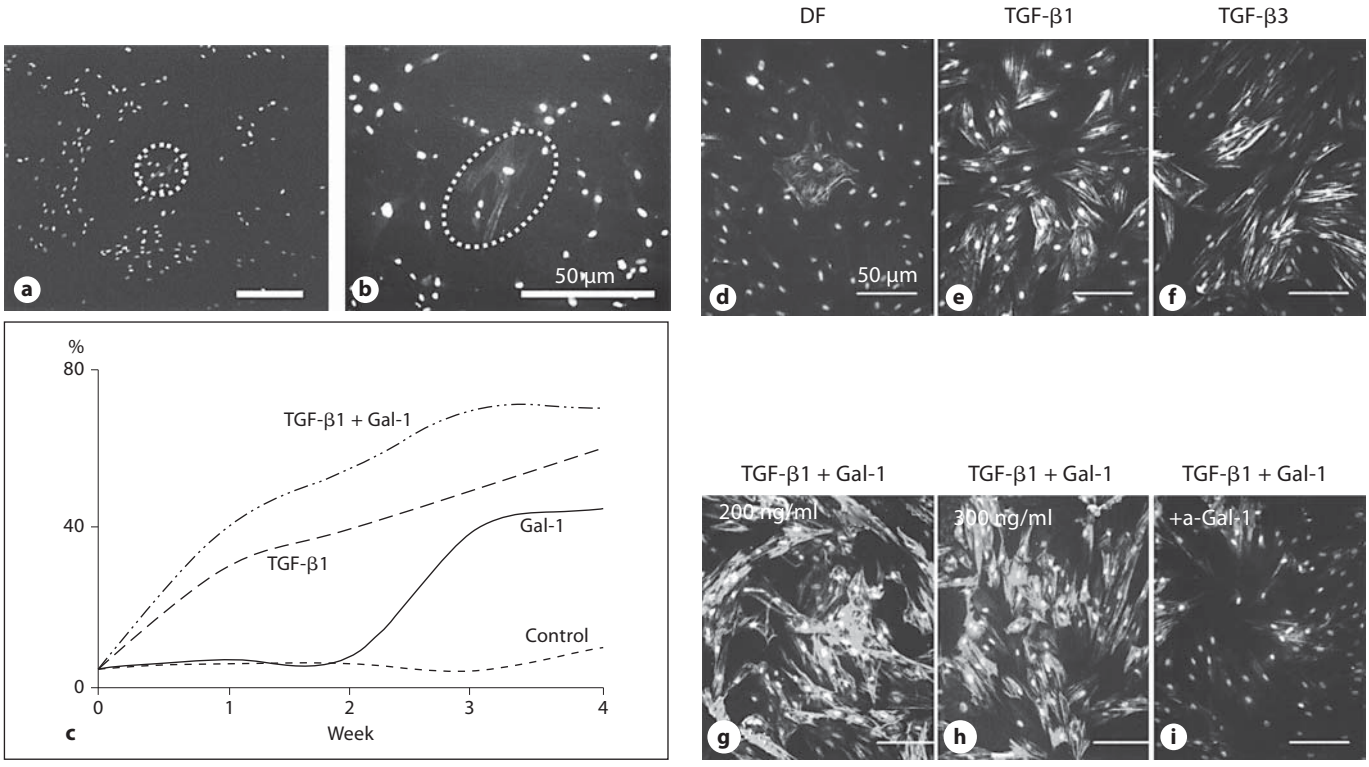

Fig. 1. Detection of smooth muscle actin in control cultures of dermal fibroblasts (**a**, **b**) and illustration of the influence of TGF-β1 and galectin-1 (200 ng/ml) on the formation of smooth muscle actin-positive myofibroblasts. The number of myofibroblasts was estimated as percentage of the number of cell nuclei occurring in smooth muscle actin-positive myofibroblast clusters related to the total number of nuclei in the evaluated area in three independent experiments. The representative figures demonstrating at the same level of magnification the control culture (**d**), the effect of TGF-β1 (**e**), TGF-β3 (**f**), combination of TGF-β1 with two concentrations of galectin-1 (**c**, **g**, **h**) and with antibody neutralizing galectin-1 are also included (**i**). The results show that myofibroblasts with low extent of smooth muscle actin expression are very rarely present in control (untreated) cultures of dermal fibroblasts (**a**, **b**, encircled). While the number of myofibroblasts is very low and stable in control cultures (**a–d**) during the testing period, TGF-β1 (**c**, **e**), TGF-β3 (**f**) and galectin-1 (**c**) have stimulatory effect on myfibroblast formation, with additive effect when both substances were applied simultaneously (**c**, **g**, **h**), the TGF being added to the system at the beginning of cell culture. The introduction of anti-galectin-1 antibody to the system, in which both the TGF-β1 and galectin-1 were applied simultaneously, strongly reduced the number of myofibroblasts (**i**).

with its linkerless variant delineated the importance of this part of the protein for bioactivity, because this version of galectin-4 was inactive (not shown). Galectin-7, as the second representative of homodimeric galectins, was also able to stimulate MFB formation (fig. 2f). Galectin-2, as the closest relative of galectin-1, was not a significant activator of MFB formation, similarly as both natural forms of tandem-repeat-type galectin-8 (not shown).

Obviously, certain galectins can serve as effectors for the conversion of dermal fibroblasts to MFBs, an activity known for TGF-β. Thus, we proceeded to determine whether these two types of inductors may act additively. Since galectins may act via inducing the secretion of TGF-β1 by cultured fibroblasts, we first determined the concentration of TGF-β1 in conditioned media, that is, controls and samples from cultures in the presence of galectin-1, galectin-3 and galectin-7. Except for two values (fig. 3), detectable concentrations of TGF-β1 were evidently not reached. Comparing these data with the physiological levels of TGF-β1 in serum [Lin et al., 2009] and with the concentration necessary for conversion (10 ng/ml), the measured data provided no evidence for TGF-β1 secretion upon galectin presence (fig. 3). As expected, both TGF-β1 and TGF-β3 induced dermal fibroblasts to generate MFBs (fig. 1c–f). When galectin-1 was added to medium containing TGF-β1, an additive effect was observed (fig. 1c, g, h). An antibody against the lectin strongly reduced the formation of MFBs (fig. 1i).

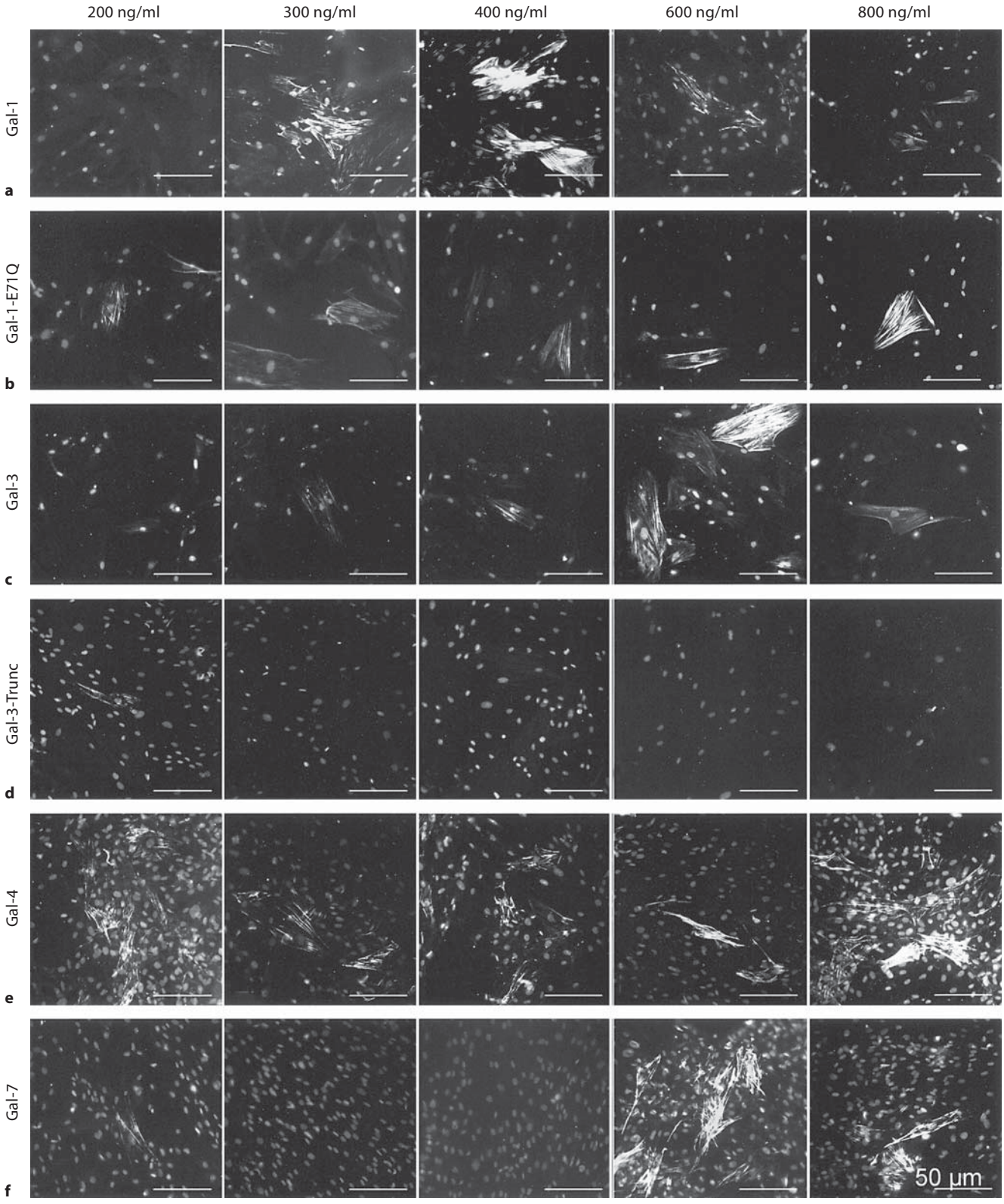

Fig. 2. Influence of galectins (Gal), Gal-1, Gal-1-E71Q mutant, Gal-3, the proteolytically truncated Galectin-3 (Gal-3-Trunc), Gal-4 and Gal-7 on the formation of myfibroblasts detected by monitoring smooth muscle actin. Nuclei are counterstained by DAPI.

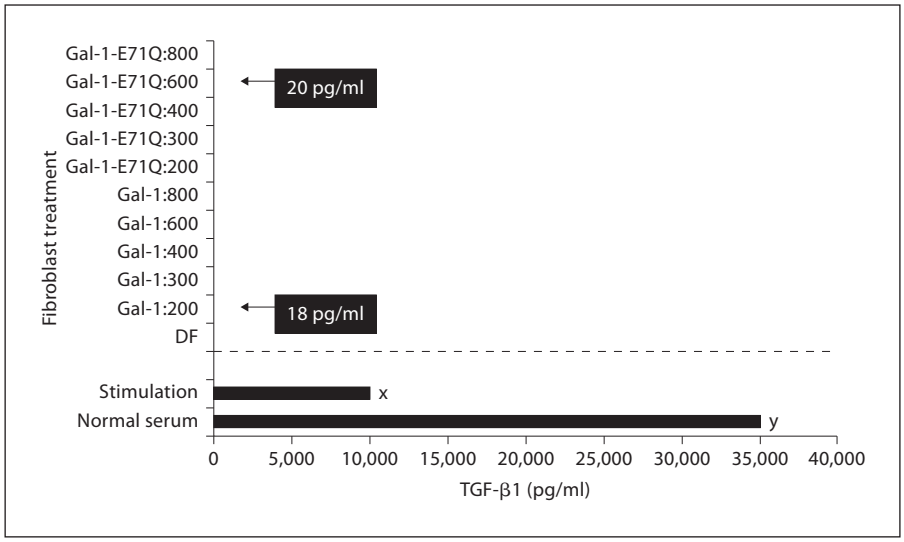

Fig. 3. Influence of wild-type galectin-1 and its E71Q mutant on the concentration of TGF-β1 in cultures of normal dermal fibroblasts (DF). Two measured concentrations (arrows) are very low in comparison to the TGF-β1 concentration used for stimulating myofibroblast formation (x) and that found in normal plasma in humans (y).

Galectins Stimulate ECM Formation

We found that the presence of certain galectins led to the appearance of three-dimensional ECM scaffold (fig. 4a–h). Namely, galectin-1, galectin-4 and galectin-7 (fig. 4b, c, d, g, h) were responsible for the marked production of a complex three-dimensional network of fibers of 140 ± 60 nm in diameter. The galectin-1-E71Q mutant was not able to stimulate ECM production (fig. 4b–d). Other galectins, including galectin-3, were inactive (fig. 4e, f). ECM fibers were composed from fibronectin (fig. 4a–h, 5a, b) and galectin-1 (fig. 5b, e) with colocalization of these two proteins. When probing for collagen-1 and collagen-3, only a very limited amount of collagen-1 was detected in cultures of the stimulated cells (fig. 5c, d). Interestingly, using galectin-4 and galectin-7 as inductors, the cultured cells were able to produce an ECM enriched in galectin-1 but not in galectin-4 or galectin-7 (fig. 5e, f).

ECM Scaffolds Produced by Galectin-Stimulated Fibroblasts Influence Phenotype of Cultured Keratinocytes

To determine whether such an ECM scaffold produced by fibroblasts/MFBs can find applications in cell culture, we next tested the panel of galectins given in the introduction in culture of keratinocytes without feeder cells. Keratinocytes cultured on these coverslips with a three-dimensional ECM network composed from fibronectin and galectin-1 appeared to resorb this matrix (fig. 5g). In comparison to control experiments (fig. 5h, i), the cells' size was significantly smaller, and they expressed keratin-19 as one of the putative markers of poorly differentiated keratinocytes. When the coverslips with the substratum were pretreated with glutaraldehyde to crosslink its components, the ECM lattices became resistant to resorption, but the cells remained negative for keratin-19 (not shown).

In vivo Experiments

Galectins Influence Wound Healing

To take the observed in vitro activity to the in vivo level, we finally addressed the issue of rat skin-wound healing in the presence of galectins (galectin-1, galectin-1-E71Q mutant, galectin-3). No remarkable effect on wound re-epithelialization was observed when the animals were treated with any of the tested galectins as the extent of re-epithelialization was identical with that seen with galectin-free controls and the wounds were completely re-epithelialized 3 weeks after the surgery (fig. 6a–d). Measuring the extent of wound contraction also revealed no effect of galectins at day 7, when wound re-epithelialization was only partial. Continued observation revealed an effect for galectin-1. Compared to its control (E71Q), galectin-1 gave a positive result after 21 days, not seen for galectin-3 (fig. 6e–i).

Discussion

Our study addresses four issues pertinent to evaluating the potential of galectins for application in cell-based and topical therapy modalities in a long-term perspective. Building on histopathological observations of the

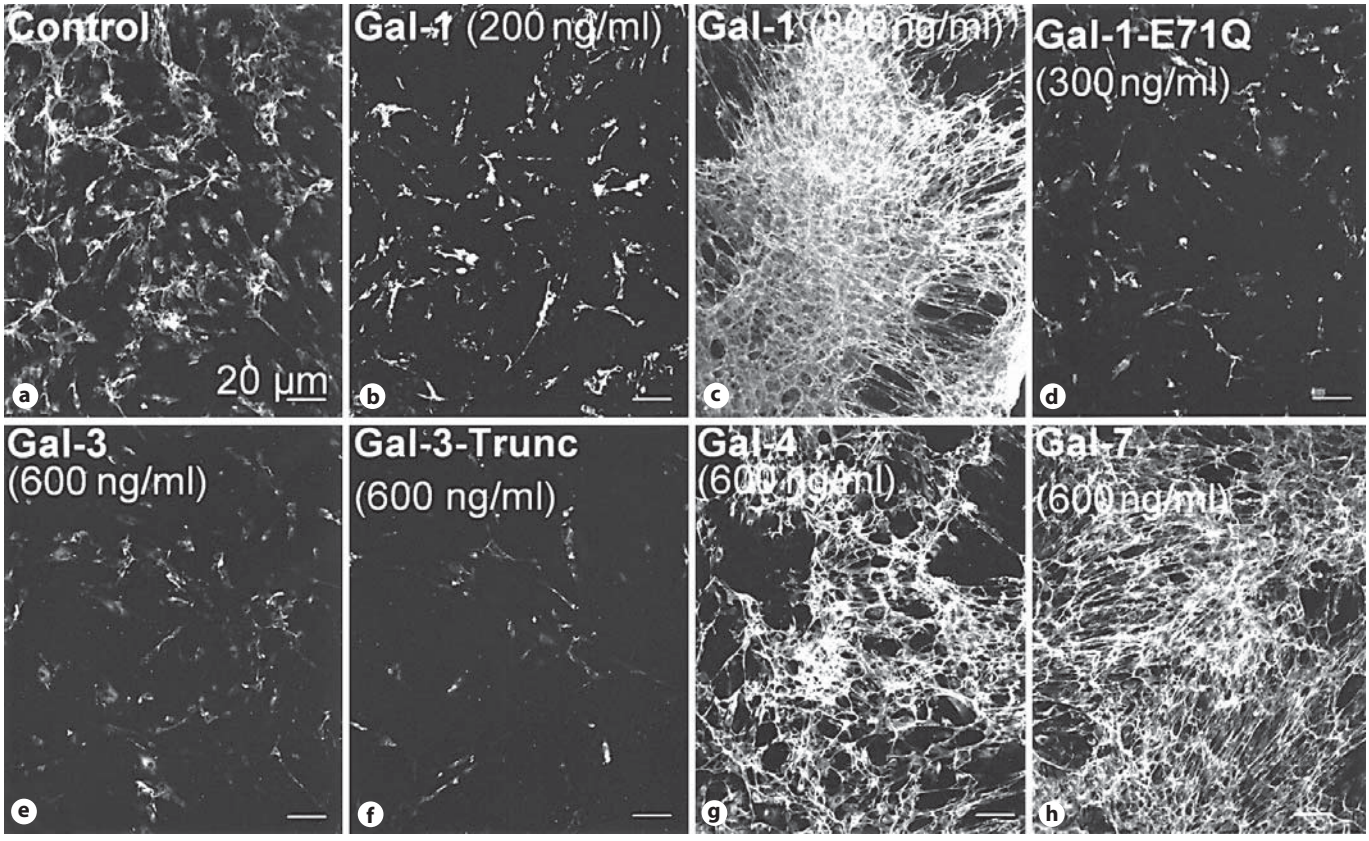

Fig. 4. Immunocytochemical detection of fibronectin in control culture of dermal fibroblasts (**a**) and fibroblasts treated with galectin-1 in two concentrations (**b**, **c**), with mutated galectin-1 (**d**), galectin-3 (**e**), truncated version of galectin-3 (**f**), galectin-4 (**g**) and galectin-7 (**h**) simultaneously. The extensive production of a three-dimensional fibronectin network was observed in cultures treated with galectin-1 (300 ng/ml, **c**), galectin-4 (**g**) or galectin-7 (**h**).

presence of stromal galectin and the capacity of galectin-1 to direct mesenchymal stem cells and fetal fibroblasts towards the myogenic lineage as described in the introduction, we tested this protein on adult dermal fibroblasts and found that it was able to convert them to MFBs at submicrogram/milliliter concentrations. Of note, this activity was abolished when the lectin site's functionality was impaired by the E71Q mutation. The previously reported TGF-like activity of rat galectin-1 on DNA synthesis and anchorage-independent growth of 3T3 cells, in contrast, was not sensitive to haptenic sugar inhibition [Yamaoka et al., 1993]. The induction of TGF-β secretion as a downstream effector could be excluded, while an additive effect of galectin-1 and the growth factor could be documented.

This ability of galectin-1 led us to monitor other human galectins, that is, to explore the functionality profile among these homologous proteins. We detected a conspicuous functional divergence within the class of prototype and tandem-repeat-type galectins. Also, a requirement for the presence of the stalk region in galectin-3, which is responsible for cross-linking activity, was seen. Evidently, even structurally closely related family members can differ markedly in terms of functionality. Such divergence has previously been seen for the growth regulation of human neuroblastoma and pancreatic carcinoma cells as well as activated T cells [Kopitz et al., 2001, 2010; Sturm et al., 2004; Sanchez-Ruderisch et al., 2010]. Since galectin secretion can make these proteins available in the stroma, this described ability to increase the presence of MFBs may eventually contribute to tumor progression, analogously to TGF-β, for example in colon cancer where galectin-1 and galectin-4 in combination have a strongly negative impact on prognosis [Nagy et al.,

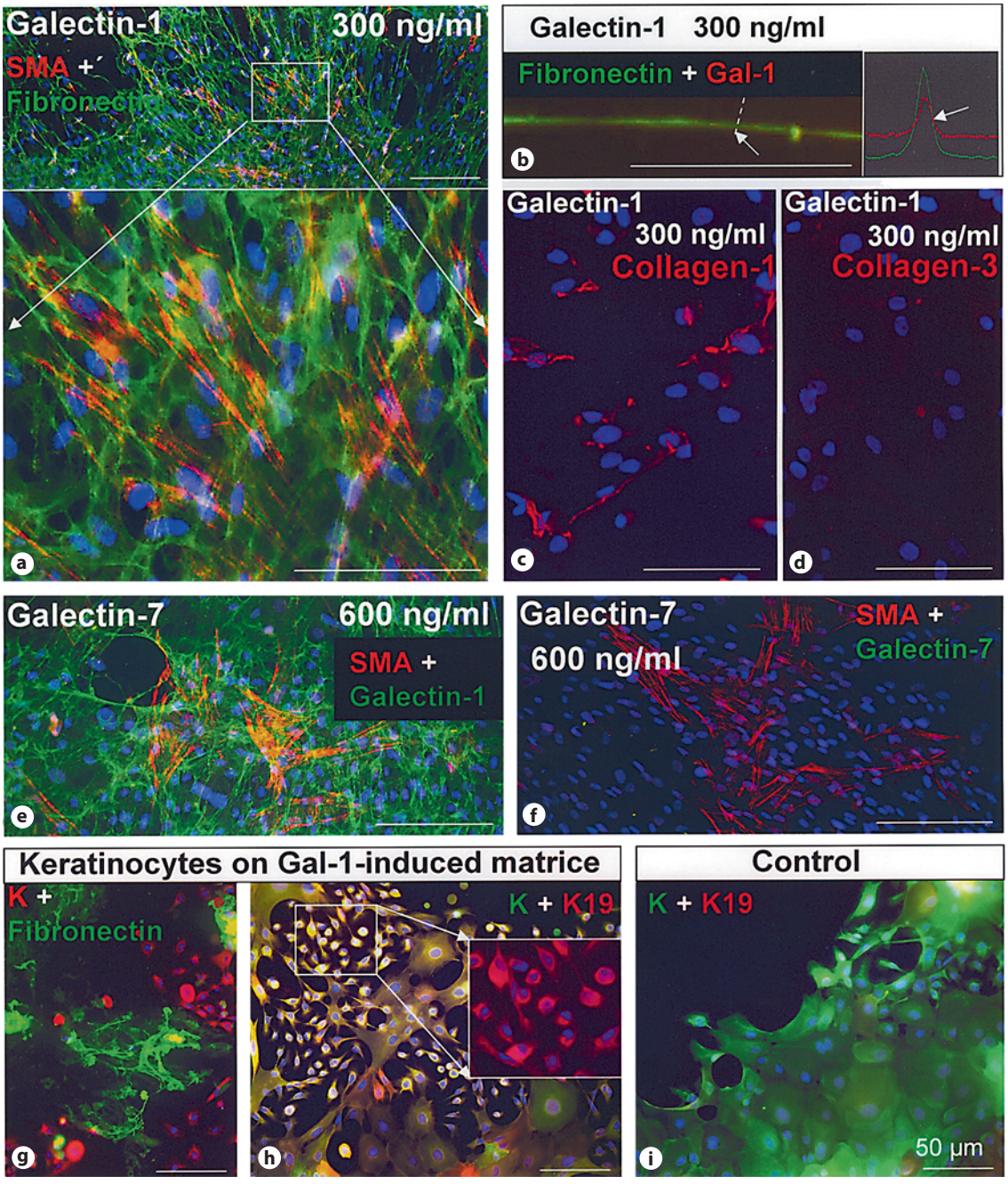

Fig. 5. Influence of galectin-1 on the production of fibronectin (green signal, **a**, **b**) and a galectin-1 (red signal, **b**)-containing three-dimensional network, compared to a weak effect on collagen-1 synthesis (red signal, **c**) and a lack of effect on collagen-3 synthesis (red signal, **d**). Analyzing the presence of fibronectin and galectin-1 (**b**), both proteins were detected and the distribution measured by fluorescence profiles. Other types of galectins such as galectin-7 also induced the production of galectin-1 (green signal, **e**) but not of galectin-7 (green signal, **f**). Visualization of smooth muscle actin (red signal, **a**, **e**, **f**) indicated that myofibroblasts are located in the extracellular matrix network. When keratinocytes (red signal-keratin, **g**) were seeded on an ECM lattice formed by galectin-1-induced fibroblasts/myofibroblasts, resorption of ECM lattice was apparent (green signal, fibronectin, **g**). These keratinocytes were very small and expressed keratin-19 (red signal, **h**) in addition to pan-kreatin (green, **h**). Keratinocytes cultured on coverslips are shown for comparison (**i**). Nuclei are counterstained by DAPI.

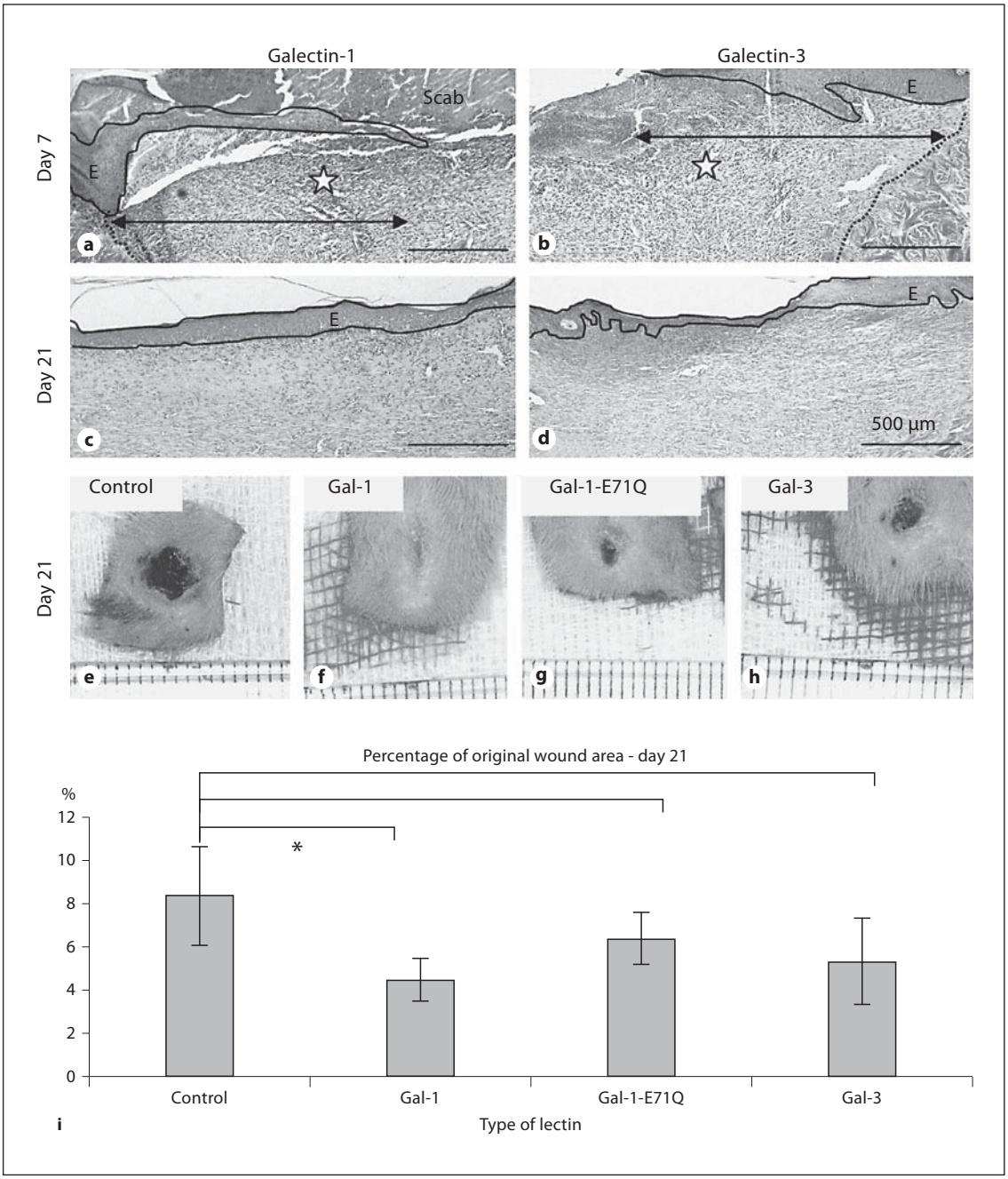

Fig. 6. Effect of galectins on the re-epithelialization and contraction of skin wounds. Partial re-epithelialization as reached one week after the administration of galectin-1 (**a**) or galectin-3 (**b**). The double-headed arrow marks the distance between the wound margin (dotted line) and the edge of regenerating epithelium. The complete reaction is shown 3 weeks after the surgery (**c**, **d**). Galectins (**e–i**) stimulated wound contraction in comparison to physiological saline as a control (**e**, **i**) with the highest efficiency seen for wild-type galectin-1 (**f**, **i**), which surpassed the threshold for statistical significance after 3 weeks ($p < 0.05$).

2003] or in breast cancer, where stromal galectin-3 is a negative prognostic factor [Moisa et al., 2007]. This study thus broadens the experimental evidence for galectins as effectors for MFB generation and delineates marked activity differences.

In addition to cellular conversion, the formation of a complex scaffold rich in fibronectin and galectin-1 by MFBs was detected immunocytochemically. Its assumed bioactivity was ascertained by culturing human keratinocytes on this substratum without feeder cells. Strikingly, their phenotype, with a small diameter and keratin-19 expression, is evocative of a low differentiation status [Dvořánková et al., 2005], implying a beneficial role also in wound healing in vivo together with MFB generation [Kwon et al., 2006]. In the last part of our study, we report a positive effect of galectin-1 application on skin wound healing in rats at day 21 after surgery.

In summary, this study describes a TGF-β-like activity of galectin-1 on MFB generation from adult dermal fibroblasts and detects functional divergence in this respect among this family of human lectins. The matrix scaffold produced in vitro supports keratinocyte culture without feeder cells. In vivo, the application of galectin-1 appears favorable for skin-wound healing after 3 weeks. Beyond wound healing, the described addition to the activity profile of galectins, considered as biomaterials, can also have relevance for explaining the physiological significance of the presence of stromal galectin in tumors.

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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.*

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.

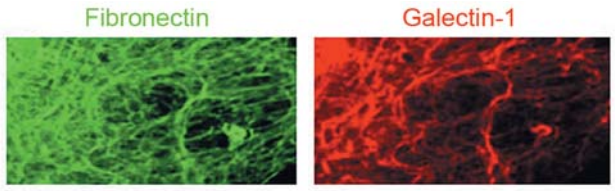

Figure 1. *Representative figure of decellularized ECM produced by fibroblasts following 10-days lasting galectin-1 stimulation and after an osmotic shock.*

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.

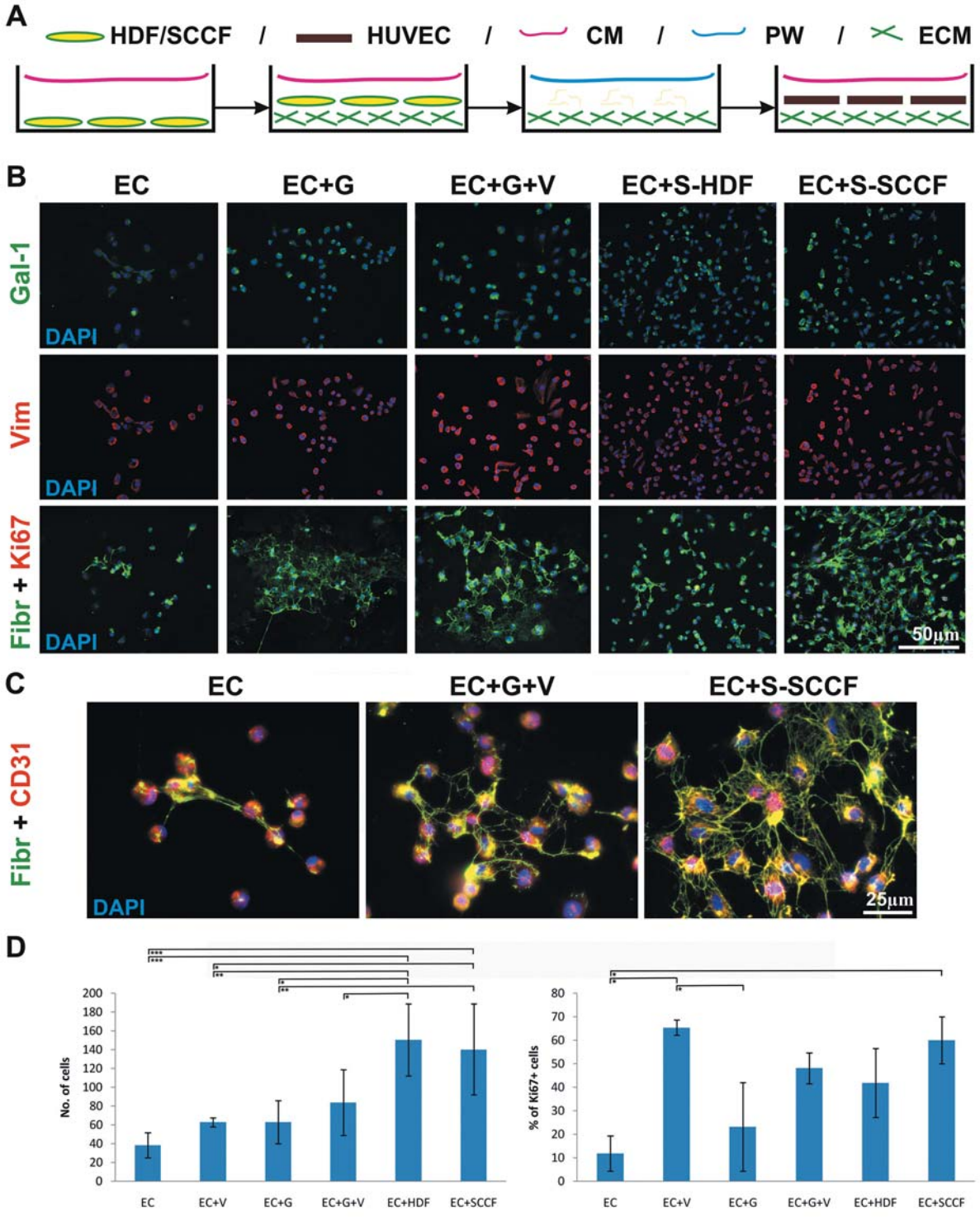

Figure 2. *A: Schematic presentation of experimental design for building the bioactive scaffold produced by fibroblasts. B: Cultures of human umbilical vein endothelial cells (EC) seeded on various types of surfaces: EC – cells seeded on the untreated surface in standard medium, EC+G – cells seeded on 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.

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Smooth muscle actin-expressing stromal fibroblasts in head and neck squamous cell carcinoma: Increased expression of galectin-1 and induction of poor prognosis factors

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Tumor stroma is an active part influencing the biological properties of malignancies *via* molecular cross-talk. Cancer-associated fibroblasts play a significant role in this interaction. These cells frequently express smooth muscle actin and can be classified as myofibroblasts. The adhesion/growth-regulatory lectin galectin-1 is an effector for their generation. In our study, we set the presence of smooth muscle actin-positive cancer-associated fibroblasts in relation to this endogenous lectin and an *in vivo* competitor (galectin-3). In squamous cell carcinomas of head and neck, upregulation of galectin-1 presence was highly significantly correlated to presence of smooth muscle actin-positive cancer-associated fibroblasts in the tumor ($p = 4 \times 10^{-8}$). To pinpoint further correlations on the molecular level, we applied microarray analyses to the transcription profiles of the corresponding tumors. Significant correlations of several transcripts were detected with the protein level of galectin-1 in the cancer-associated fibroblasts. These activated genes (*MAP3K2*, *TRIM23*, *PTPLAD1*, *FUSIP1*, *SLC25A40* and *SPIN1*) are related to known squamous-cell-carcinoma poor-prognosis factors, NF-κB upregulation and splicing downregulation. These results provide new insights into the significance of presence of myofibroblasts in squamous cell carcinoma.

Key words: carcinoma, stroma, myofibroblast, actin, lectin
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Increasing attention is being turned to the stroma part in carcinomas. It is formed by fibroblasts producing the extracellular matrix, macrophages and other inflammatory cells as well as blood/lymphatic capillaries.[1] Recent progress in cancer and skin cell biology has markedly changed our view on the functional significance of the stroma. Classically, the tumor stroma has been considered as a milieu suitable for growth of capillaries that guarantee the supply with oxygen and nutrients for the cancer epithelium. With the new data being accrued, the stroma can be described as an active microenvironment, which modulates the biology of the tumor including cancer stem (initiating) cells by mechanisms similar to the function of the niche in the case of tissue stem cells.[2] As a consequence, a focus of this research is given to the study of mutual epithelial-mesenchymal interactions. This interplay is known to be crucial for embryonic development, and a major role in the route of tumor progression is likely.[2,3]

The fibroblasts of the stroma, that is, cancer-associated fibroblasts (CAF), frequently contain fibers of smooth muscle α-actin (SMA and ACTA2), showing notable similarities to myofibroblasts responsible for the contraction of a wound in the course of a healing process.[4] The origin of CAF is not

well established: they can derive from the local mesenchyme by influence of the paracrine activity of cancer cells, by transition from other cell types present in the tumor site such as the tumor epithelium, pericytes, macrophages, endothelial cells[5] or from mesenchymal stem cells, which have entered the tumor site.[6] CAF are known to exert strong biological activities: fibroblasts isolated from tumors of squamous cell epithelia such as basal cell carcinoma of the skin or squamous cell carcinoma of head and neck are able to alter characteristics of the phenotype of normal epithelial cells and change their differentiation status into the direction of epidermal stem cells or cancer cells.[7,8] For example, CAF differ from normal fibroblasts in the gene expression profiles, with deviations detected for 560 genes, and in the secretion of cytokines strongly acting on epithelial cells.[9] In addition to these well-known effectors, CAF also produce and secrete other classes of potent modulators of cells.

An emerging class of proteins relevant in this context is endogenous lectins from the galectin family. They share β-sandwich folding and affinity to a galactoside core and are capable to elicit regulation of adhesion and growth *via* carbohydrate/protein-protein interactions.[10] The immunohistochemical monitoring of galectin presence in tumors has indicated cell type/differentiation-dependent profiles, which have given respective fingerprinting diagnostic and prognostic value.[11–13]

Previous studies have indicated that tumor stroma and also granulation tissue of wounds are frequently very rich for the homodimeric proto-type galectin-1 (Gal-1 and LGALS1).[14,15] At both sites, presence of myofibroblasts is documented, and SMA-positive CAF express Gal-1.[16] Functionally important, an epithelial-stromal cross-talk has been inferred in pancreatic carcinoma involving a dual autocrine-paracrine signaling loop with Gal-1 and tissue plasminogen activator in the transition zone, contribution to cell invasion, migration and proliferation.[17] Thus, Gal-1 secreted from CAF and tumor cells can be a mechanistically versatile constituent of this microenvironment. Secretion of Gal-1 and active cross-talk is also known from immune regulation exerted by regulatory T cells, with relevance for autoimmune diseases.[18,19] An influence on angiogenesis has been described, as also a pivotal role in the activity spectrum of the tumor suppressor $p16^{INK4a}$.[20,21] In laryngeal tumors, it is a negative prognostic indicator.[22] Sodium butyrate treatment, which induces cells to acquire the squamous phenotype, results in increased Gal-1 expression in the tumor, while retinoic acid treatment, which inhibits establishment of this phenotype, decreases Gal-1 presence.[23]

Galectin-3 (Gal-3 and LGALS3) is of interest in this context, because it can block Gal-1 activity on tumor cells.[24,25] Gal-3 presence is documented in squamous epithelia under physiological conditions and in cancer tissue.[26,27] The level of its expression increases with the differentiation status of head and neck squamous cell carcinomas (HNSCC) and correlates with keratinization.[23,28] These data on presence and functional divergence from Gal-1 prompted us to add monitoring for Gal-3 to our study.

Based on this body of knowledge, we analyzed occurrence of SMA-positive CAF (myofibroblasts) set in relation to both mentioned galectins in HNSCC, in normal tissue and in surgical margins (SM) using immunofluorescence. Because a relationship between biological properties of HNSCC and human papilloma virus (HPV) positivity has been established,[29] occurrence of correlation between Gal-1, SMA-positive CAF and HPV was also tested. A strong association between SMA and Gal-1 but not Gal-3 was revealed. This result led us to define expression differences caused by the presence of Gal-1-positive stromal myofibroblasts on the transcription profile of the tumor.

Material and Methods

Sample collection

The specimens were obtained from patients suffering from HNSCC after their informed consent in full agreement with the local ethical committee based on the Declaration of Helsinki. Samples from squamous cell carcinoma (SCC), SM of the resecate and normal epithelium (non-neoplastic epithelium) (NE) were collected from 31 patients. The tissue was protected by RNA-Later (Ambion, Austin, TX), deeply frozen in liquid nitrogen and stored at −85°C to prevent activity of endogenous RNases. Frozen sections (Cryocut-E, Reichert-Jung, Vienna, Austria) were used for RNA isolation and for immunocytochemical detection of SMA, Gal-1 and Gal-3.

Immunohistochemical detection of SMA, Gal-1, Gal-3, CD31 and MAP3K2

Cryocut sections were washed and rehydrated by phosphate-buffered saline (PBS, pH 7.2), fixed by paraformaldehyde in PBS for 5 min and, after extensive washing, subjected to processing to detect the proteins listed above. The mouse monoclonal antibody for the detection of SMA was purchased from DAKO (Glostrup, Denmark), the rabbit polyclonal antibody against MAP3K2 from LifeSpan BioSciences (Seattle, WA) and anti-CD31 from (Abcam, Cambridge, UK). Primary antibodies were diluted according to the recommendation of the supplier. Homemade rabbit antibodies against Gal-1 and Gal-3 were used in dilution of 1:50. These antibody fractions had been systematically tested for specificity and lack of cross-reactivity among the galectin family, with affinity depletion being performed by affinity chromatography in each positive case followed by another round of controls by ELISA.[11,30,31] Fluorescein isothiocyanate-labeled swine anti-rabbit antibody (DAKO, Glostrup, Denmark) and tetramethylrhodamine isothiocyanate-labeled goat antimouse antibody (Sigma, Prague, Czech Republic), both diluted as recommended by supplier, were used as the second-step reagents. Nuclear DNA was visualized by 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Prague, Czech Republic). Controls of specificity had been performed by omission/replacement of specific antibodies by irrelevant isotypic antibodies of preimmune serum. Specimens were mounted to Vectashield (Vector Laboratories, Burlingame, CA), then

inspected by an Eclipse 90i fluorescence microscope (Nikon, Prague, Czech Republic) equipped with filterblocks for FITC, TRITC and DAPI and a Cool-1300Q CCD camera (Vosskühler, Osnabrück, Germany); images were analyzed by a LUCIA 5.1 computer-assisted image analysis system (Laboratory Imaging, Prague, Czech Republic) to evaluate the levels of fluorescence intensity in defined areas of specimens (epithelium, mesenchymal component) in arbitrary units [FI (AU)]. Background levels were estimated from a negative control and galectin-negative areas of experimental specimens.

Detection of HPV

Detection of HPV with high oncogenic potential (high-risk HPV group: HPV16, 19, 31, 33 and 45) was performed by OneStep reverse transcription RT-PCR kit (Qiagen, Hilden, Germany) on cRNA templates. The cRNA was prepared by linear amplification of mRNA and thus only transcriptionally active HPVs were detected. Primers specific for the selected HPV subtypes were designed to target the viral genome in the area encoding the *E6* and *E7* oncogenes and used as mixture in multiplexed PCR (see Supporting Information Table 1 for primer sequences and product lengths). The sensitivity of the procedure was tested on cRNA from HeLa cells (HPV-positive). Input amount of 50 ng of cRNA was estimated to be sufficient for the analysis. One-step RT-PCR was performed according to manufacturer's instructions using the following program: 50°C/30 min, 95°C/15 min, 40 cycles of (94°C/1 min, 50°C/1 min and 72°C/1 min) and 72°C/10 min. Possibility for cross-hybridization of primers was tested and was not observed under the given conditions.

Microarray analysis

The microarray analyses were performed on material from a subset of 21 patients (Table 1). Total RNA was isolated from the cryostat sections using RNeasy Micro Kit (Qiagen), checked for integrity, amplified and hybridized on an Illumina HumanWG-6 v3 Expression BeadChip (Illumina). The raw data were analyzed and processed using the beadarray package of the Bioconductor, as described previously.[32] In short, the transcription profiles were background corrected using the normal-exponential model, quantile normalized and variance stabilized using base-2 logarithmic transformation. Transcriptionally active genes correlated to Gal-1 presence (detected by immunohistochemistry, IHC) were identified using the standard function (cor.test) of the R statistical environment (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/). As we could not expect a linear correlation *a priori*, we used Spearman rank correlation as correlation method within the test. To remove spuriously correlated transcripts, an at least two-fold change difference in the gene's transcriptional activity over the range of Gal-1 presence was required. Storey's q-value[33] was used to adjust p-values on multiple testing. Transcripts were considered correlated when $q < 0.1$. Detected transcripts were annotated using the beadchip manifest (HumanWG-6_V3_0_R2_11282955_A.bgx, Illumina). The transcription data are MIAME-compliant and deposited in the ArrayExpress database (accession #: E-MTAB-850).

Statistical analysis

We have used Fisher's exact test for the univariate association studies between clinical markers and presence of SMA-positive CAF. Disease-free survival curves have been compared using Mantel-Cox log-rank test. Statistical significance was considered at the level of $p = 0.05$. Statistical analysis of the microarray data was performed as described above.

Results

Clinical characterization of the samples

The characteristics of the tumor samples used for immunohistochemical detection are listed in Table 1. It demonstrates that, among the 31 tumors, the majority was of the clinical stage T2 (45%) and histological grade G2 (48%). The lymph node stage was distributed approximately evenly among N0 (42%), N1 (23%) and N2 (35%). All patients were of the stage M0, that is, without distant organ metastases. Concerning the localization, tonsillar carcinomas prevailed (32%).

SMA-positive CAF were present in 61% of the SCC samples

When we evaluated NE and the SM of the resecate, we observed no SMA-positive myofibroblasts (Figs. 1*a*, 1*b*, 1*e* and 1*f*). The apparent signal came from SMA-positive smooth muscle cells in the wall of vessels, which can be considered as an internal control for the specificity of the histochemical reaction. Within the tumors, SMA-positive CAF were present in >60% of the tested SCC (19 patients of the total of 31; Table 1, Figs. 1*d* and 1*g*). The remaining tumors apparently did not contain these cells (12 patients of 31, Fig. 2*c*). These specimens exhibited a strong signal of SMA in the wall of vessels, only, with an exception of a few round cells per view field (*e.g.*, three positive cells in Fig. 1*b*). The colocalization experiments, in which SMA and CD31 endothelial marker were detected simultaneously, verified that the SMA signal was in the stroma attributed dominantly to myofibroblasts and not to myoblasts of the wall of the small vessels (Supporting Information Fig. 1).

Association of the clinical data with SMA positivity of CAF

We observed no association between the clinical stage of the tumors or their histological grade with SMA positivity (Figs. 2*a*–2*c*). We, however, observed indications, yet not of a statistically significant level, for an association of disease-free two-year survival among patients with SMA-positive CAF, in contrast to published data[34] (Fig. 2*d*), possibly attributed to the low coverage of the survival data (Table 1). No statistically significant association was detected between HPV and SMA positivity of CAF (Fig. 2*e*). A statistically significant ($p = 0.04$, Fisher's exact test) majority of the tumors with SMA-positive CAF was present in SCC of palatine tonsils (Fig. 2*f*), while tumors with SMA-negative CAF tended to localize in the oral cavity, pharyngeal wall and soft palate.

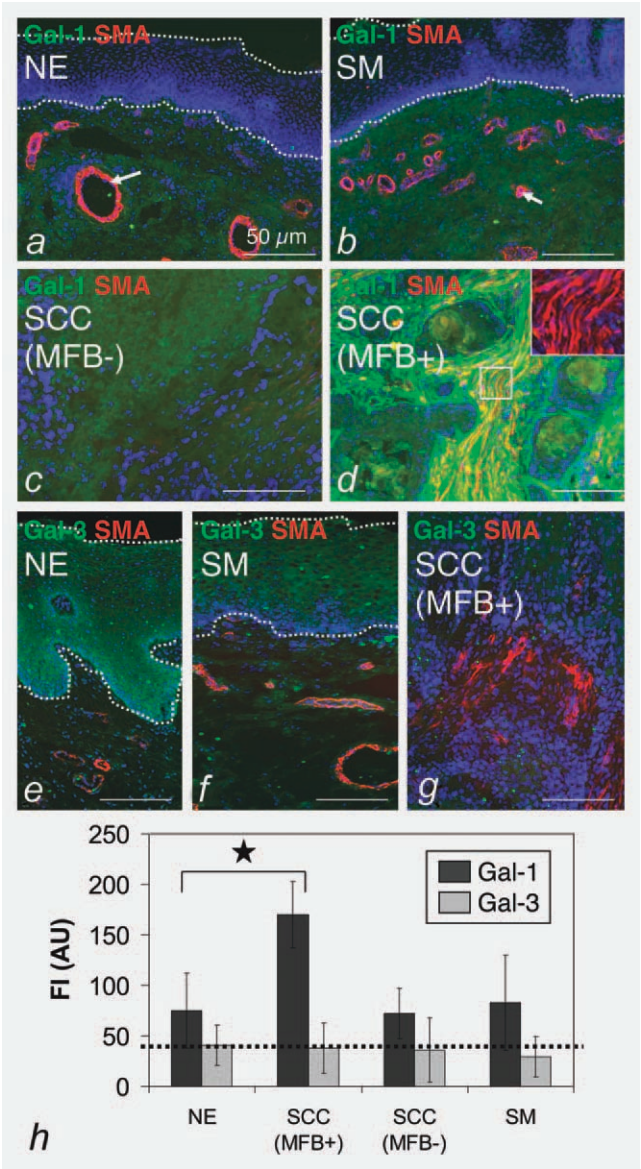
Table 1. Characterization of the patient cohort

Sample Name	SMA-positive CAF	Gal-1 (IHC)	Gal-3 (IHC)	Sex	TNM classification	Grade	Stage	Keratinization	Localization	HPV positivity	Disease-free survival		Microarray
											Status	Day	
P011C1S1	+	179	NA	Male	T4 N0 M0	G2	IV	N	Base of tongue	N	Withdrawn	73	Y
P029C1S1	+	220	20	Female	T2 N0 M0	G2	II	N	Oropharynx part	N	Disease free	1421	Y
P041C1S1	+	148	53	Female	T2 N1 M0	G2	III	Y	Tonsils	NA	Disease free	840	N
P048C1S1	+	209	36	Male	T2 N1 M0	G2	III	N	Tonsils	Y	Disease free	1165	Y
P078C1S1	+	123	42	Male	T4 N0 M0	G1	IV	Y	Larynx	N	Disease free	735	Y
P087C1S1	+	181	NA	Male	T2 N2 M0	G3	IV	N	Tonsils	Y	Disease free	1105	Y
P090C1S1	+	207	NA	Male	T3 N2 M0	G3	IV	Y	Larynx	N	Disease free	1079	Y
P103C1S1	+	182	NA	Female	T2 N2 M0	G2	IV	N	Tonsils	NA	Disease free	266	N
P111C1S1	+	NA	NA	Male	T3 N0 M0	G3	III	N	Larynx	N	EUR	351	Y
P118C1S1	+	NA	NA	Male	T3 N1 M0	G2	III	Y	Tonsils	NA	Withdrawn	0	N
P119C1S1	+	161	9	Male	T2 N2 M0	G2	IV	Y	Tonsils	Y	Disease free	736	Y
P121C1S1	+	166	NA	Male	T2 N2 M0	G3	IV	N	Tonsils	Y	Disease free	122	Y
P123C1S1	+	213	NA	Male	T2 N0 M0	G2	II	N	Base of tongue	NA	Disease free	710	N
P124C1S1	+	NA	23	Male	T3 N2 M0	G2	IV	Y	Larynx	N	Recidive	330	Y
P126C1S1	+	NA	NA	Female	T1 N0 M0	G3	I	N	Tonsils	N	Recidive	138	Y
P127C1S1	+	129	25	Male	T3 N0 M0	G1	III	Y	Base of tongue	N	EUR	142	Y
P131C1S1	+	132	NA	Male	T3 N0 M0	G2	III	Y	Larynx	NA	Recidive	352	N
P132C1S1	+	206	98	Male	T2 N2 M0	G3	IV	N	Oral cavity	NA	Disease free	881	N
P146C1S1	+	148	NA	Male	T3 N0 M0	G1	III	Y	Tonsils	NA	Disease free	597	N
P005C1S1	–	68	NA	Male	T1 N0 M0	G3	I	N	Base of tongue	NA	Disease free	1536	N
P074C1S1	–	NA	NA	Male	T2 N1 M0	G2	III	Y	Oropharynx part	Y	EAR	5	Y
P080C1S1	–	NA	79	Female	T4 N0 M0	G3	IV	N	Larynx	N	EUR	495	Y
P088C1S1	–	79	NA	Male	T1 N2 M0	G2	IV	Y	Oropharynx part	N	EUR	885	Y
P104C1S1	–	102	NA	Male	T2 N2 M0	G3	IV	Y	Oral cavity	N	EUR	374	Y
P120C1S1	–	93	45	Female	T2 N1 M0	G2	III	NA	Tonsils	NA	Disease free	774	N
P125C1S1	–	43	15	Male	T1 N0 M0	G3	I	Y	Oropharynx part	N	EUR	264	Y
P128C1S1	–	47	NA	Male	T2 N1 M0	G1	III	NA	Oral cavity	N	ED	177	Y
P129C1S1	–	99	NA	Male	T4 N2 M0	G3	IV	N	Larynx	N	Recidive	394	Y
P134C1S1	–	85	NA	Male	T4 N1 M0	G2	IV	N	Base of tongue	N	Disease free	821	Y
P135C1S1	–	34	NA	Male	T3 N2 M0	G2	IV	Y	Oral cavity	N	Recidive	137	Y
P160C1S1	–	NA	8	Female	T2 N0 M0	G3	II	Y	Oropharynx part	NA	Disease free	940	N

The tumor loci are listed in the following groups: Tonsils (tonsillar carcinoma), larynx (supraglottical carcinoma, glottical carcinoma and transglottical carcinoma), oral cavity (carcinoma of floor of mouth, lip carcinoma and tongue carcinoma), base of tongue (carcinoma of the base of tongue) and oropharynx part (lateral pharyngeal wall carcinoma and soft palate carcinoma). Disease-free survival is specified by the outcome status (status) and the day at which the status was reported relative to the day of operation (day). Microarray: availability of mRNA microarray data for the sample. Abbreviations: ED, exitus of the disease; EAR, exitus of another reason; EUR, exitus of unknown reason; Y, yes; N, not; NA, data not available; IHC, intensity of immunohistochemical staining in myofibroblasts.

Presence of Gal-1 correlated with presence of SMA-positive CAF and absence of Gal-3

Gal-1 was not detectable in normal and malignant epithelium (Figs. 1*a*–1*c*). In contrast, a much stronger signal was seen in the stroma of all tumors with presence of SMA-positive CAF (Figs. 1*d* and 1*h*). The stroma of the tumors lacking SMA-positive CAF exhibited a very weak signal for Gal-1 (Figs. 1*c* and 1*h*) with fluorescence intensity levels comparable to the signal detected in normal tissues (Figs. 1*a*, 1*b* and 1*h*). When probing the Gal-3 presence, epithelial cells of NE and SM exhibited a distinct signal (Figs. 1*e* and 1*f*), while cancer cells were negative or they exhibited distinct, but a very weak signal just above the background level (Fig. 1*g*). No signal of Gal-3 was obtained in connective tissue of all three types of studied specimens including the tumors with SMA-positive CAF in their stroma (Figs. 1*e*–1*h*).



Transcription data of the bulk tumor samples differed from the immunohistochemical signal intensities in a predicted way

The results of immunohistochemical inspection were compared to mRNA microarray analysis on a subset of 21 selected patients (Table 1). The transcription profiles were obtained from partially homogenized bulk samples, which included epithelial parts and other types of tissue. Overall, there are two sites, where *SMA* and *Gal-1* genes are transcribed: smooth muscle cells of vessels[20,35] and stromal myofibroblasts.[4,16,36] When evaluating correlations of the two factors, normal tissue with no stromal fibroblasts and hence only vascular production was expected to present such a correlation of transcriptional intensity for these two genes. Indeed, transcriptional activity of *Gal-1* and *SMA* genes correlated very well in NE (p-value = 0.0002, Fig. 3*a*). On the contrary, in tumors, both vessels and stromal myofibroblasts were present, thus the correlation was lost ($p = 0.4$; Fig. 3*a*). The same held also true for other markers of smooth muscle cells and myofibroblasts (*MYH11* and *CDH11*; Supporting Information Figs. 2*a* and 2*b*). Angiogenesis, as represented by *VEGF* and *EDN1*, was highly correlated ($p = 0.001$, 0.01, respectively) with level of *Gal-1* transcription in tumors with SMA-negative CAF, but not in tumors with SMA-positive CAF ($p = 0.9$, 0.2, respectively, Figs. 3*b* and 3*c*). That implies that while in a tumor with SMA-negative CAF *Gal-1* was produced only or predominantly in vessels, in tumors with SMA-positive CAF, another source of *Gal-1* production was present, that is, the myofibroblasts.

Figure 1. Normal epithelium (NE) (*a*, *e*), the tissue area of the surgical margin of tumors (SM) (*b*, *f*) and minority of tumor tissues (*c*) contained no smooth muscle actin (SMA) (red signal)-positive myofibroblasts (MFB). The positive signal was exclusively observed in smooth muscle cells of vessels (white arrow). Majority (61%) of tumors contained CAF positive for SMA (*d*, *g*). No presence of Gal-1 (Gal-1, green signal) was observed in normal and marginal epithelial cells (*a*–*d*). The connective tissue component of normal epithelium (*a*), the SM (*b*) and stroma with SMA-negative CAF (*c*) presented a very low but specific signal intensity for Gal-1 in contrast to tumors with SMA-positive CAF in the stroma (*d*). Weak but specific signal for presence of Gal-3, with intensity well above the background level, was detected in normal epithelial cells (*e*) and cells from the epithelium of the SM (*f*). Malignant epithelial cells were negative (*g*). No reactivity was observed in connective tissue of normal epithelium (*e*), the SM (*f*) and the tumor stroma (*g*). When the extent of presence of Gal-1 (black column) and Gal-3 (gray column) was compared in connective tissue of epithelium and tumor stroma (positive or negative for MFB) by measurements of fluorescence intensity (*h*), the stroma of tumors with SMA-positive CAF was stained significantly stronger than normal tissue or the SM (asterisk; paired *t*-test). The stromal signal for Gal-3 presence was very close to the background level (dashed line). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

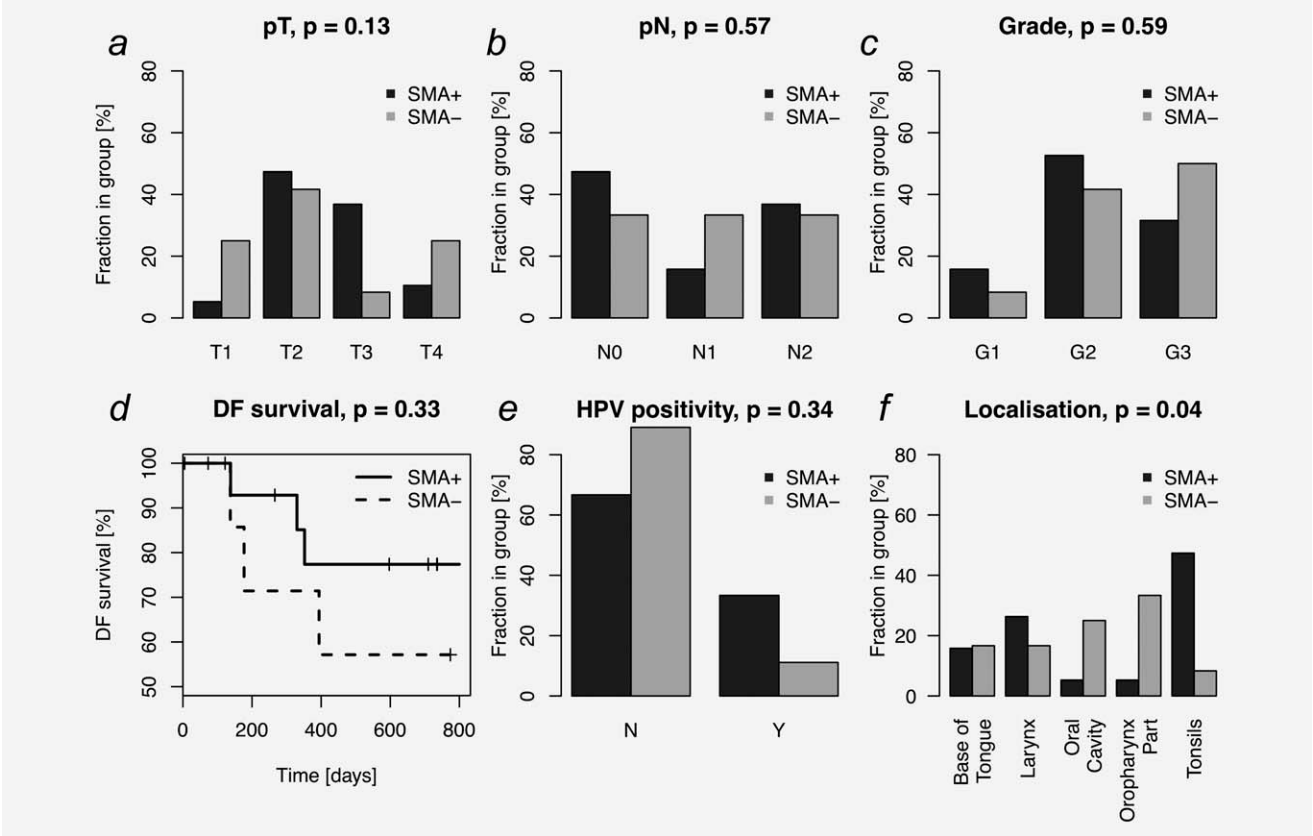


Figure 2. Lack of a statistically significant association between presence of SMA-positive CAF and TNM classification (*a*, *b*), histological grade (*c*), disease-free survival (*d*) and transcriptional activity of HPV in the tumor (*e*). The tumors with SMA-positive CAF tended to localize in palatile tonsils, while tumors with SMA-negative CAF localized to the oral cavity, pharyngeal wall and soft palate (*f*). *p*-Values of the Fisher's exact test (*a*–*c*, *e* and *f*) and Mantel-Cox log-rank test (*d*) are given.

Transcription profiles of the SMA-positive CAF and their influence on the transcription profile of the tumors were apparently masked by the transcription profile of smooth muscle cells of vessels and could not be assessed directly. This apparent complexity could, however, be dissected by correlation analyses.

Transcription of several genes in tumors was highly correlated to the presence of Gal-1 in myofibroblasts

To delineate changes of the tumor transcription profile caused by myofibroblasts only, we identified transcripts associated with the presence of SMA-positive CAF by correlation analysis of the microarray data with the detection of CAF-related Gal-1 at the protein level, as estimated by semi-quantitative immunohistochemical staining. Thus, only transcripts present in or induced by SMA-positive CAF were detected. Toward this end, we used data on 16 patients, for whom both microarray and immunohistochemical data (IHC) were available. We identified six transcripts significantly correlated with Gal-1 presence (as estimated by IHC). They were significantly upregulated in high-Gal-1 tissue samples with transcription varying at least two-fold over the range of Gal-1 intensities (see Table 2 for the list of the detected transcripts as well as Supporting Information Figs. 3 and 4 for the correlation plots). The measured upregulation of the production of the *MAP3K2* transcript was ascertained immunohistochemically. A marked difference between tumors with SMA-positive and SMA-negative CAF was observed (Fig. 4).

Discussion

The presence of myofibroblasts, SMA-producing CAF, in the stroma of SCC has been associated with poor prognosis.[5] In view of the emerging role of Gal-1 as effector in autocrine and paracrine communication between stroma and tumor parts in the local microenvironment we have focused our study on Gal-1. Here, we have detected several transcripts associated with Gal-1 production by the stromal myofibroblasts in head and neck SCC (Table 2). The majority of these detected transcripts directs synthesis of proteins involved in processes that have been associated with poor prognosis. Thus, this regulation within SMA-positive CAF is suggested to contribute to their activity at the clinical level.

Presence of myofibroblasts is associated with tumor localization

We have observed that the SMA-positive CAF-containing tumors are more often localized to palatine tonsils, while the SMA-negative CAF containing tumors localize more likely to

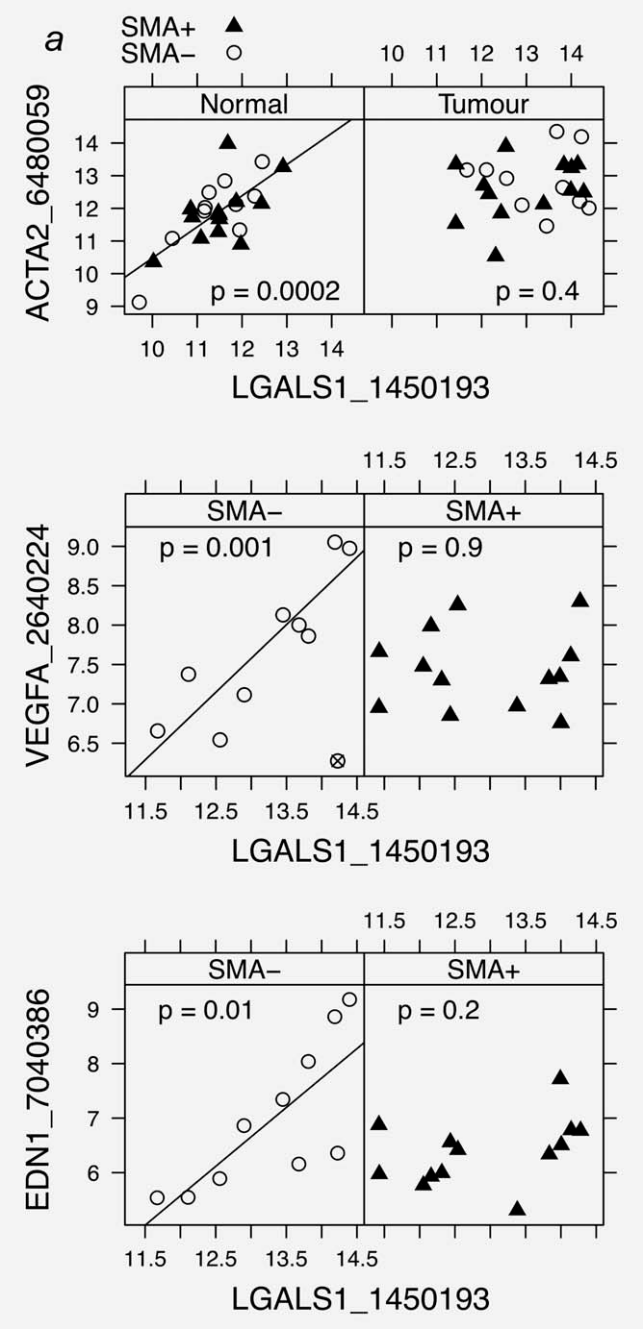


Figure 3. Two sites of *Gal-1* production in SMA-positive tumors. Correlation of the transcriptional activity for *Gal-1* (*LGALS1*) and *SMA* (*ACTA2*) revealed differences between normal and tumor tissues. While the Pearson correlation of the log-intensities of the two transcripts in the normal tissue was highly significant ($p = 0.0002$), the correlation was lost in tumors ($p = 0.4$) (*a*). Transcriptional activity of vascular smooth muscle markers *VEGFA* (*b*) and *EDN1* (*c*) correlated well with that of *Gal-1* (*LGALS1*) in tumors with SMA-negative CAF but not in tumors with SMA-positive CAF. Statistical significance of the Pearson correlation of the log-intensities was evaluated, and the *p*-values are given. In the case of *VEGFA*, an outlier case (marked) had been removed from the statistical analysis. Considering this case, the *p*-value was 0.09.

the oral cavity, pharyngeal wall and soft palate ($p = 0.04$). No significant differences in TMN parameters, grading or HPV classification were seen.

Presence of Gal-1 correlates within the presence of myofibroblasts

The results demonstrate a distinct relationship between Gal-1 presence in the tumor stroma and presence of SMA-expressing CAF in tumors. Of note, we previously reported that exogenous Gal-1 has a stimulatory effect on the transition of fibroblasts to myofibroblasts under *in vitro* condition. The extent of this effect of Gal-1 is lower than the activity of both TGF-β1 and TGF-β3, but it has a strong additive effect when combined with these proteins.[37] We have not observed any association of Gal-3 presence with the SMA-positive CAF.

Several cellular processes worsening disease prognosis are correlated with Gal-1 production by myofibroblasts

A small number of transcripts was detected, which significantly correlated with the presence of Gal-1 in myofibroblasts. These genes cover aspects of several important cancer-related processes, that is, downregulation of splicing (*FUSIP1*), NF-κB activation (*TRIM23*, *PTPLAD1* and *MAP3K2*), ubiquitinylation (*TRIM23*), cell cycle control (*SPIN1*), autophagy (*PTPLAD1*), protection against mitochondrial oxidative stress (*SLC25A40*) and MAPK signaling (*MAP3K2*).

Downregulation of splicing

Chung *et al.*[38] observed that splicing is negatively affected in poor-prognosis SCC of head and neck. We have detected upregulation of the gene for FUS interacting protein (FUSIP1) with Gal-1 presence. FUSIP1 is, despite its typical SR protein structure, a splicing repressor activated by dephosphorylation during the M phase and after a heat shock.[39] Its principal function is to repress, rather than activate, splicing. FUSIP1 contains seven putative high-stringency 14-3-3 interacting domains, and it binds to ectopic 14-3-3σ (stratifin SFN).[40] It then protects its binding partner from dephosphorylation and activation.[41] Stratifin has been shown to be a poor-prognosis predictor in SCC, the mechanism of its action being yet unknown.[38]

NF-κB activation

Activation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) has recently been linked to poor prognosis in head and neck SCC.[38] We have detected three transcripts (*TRIM23*, *PTPLAD1* and *MAP3K2*) correlated with Gal-1 presence by SMA-positive CAF and related to NF-κB activation. As these transcripts are all produced in cardiac myocytes and smooth muscle cells,[35,42] they may be generated also directly by SMA-positive myofibroblasts and can cause the difference between the tumors with SMA-positive CAF and SMA-negative CAF by activation of NF-κB. That would link poor prognosis of tumors with activated NF-κB

Table 2. Transcripts significantly correlated with expression of Gal-1 in CAF

RefSeq ID	Entrez Gene ID	Symbol	Definition	Spearman rank correlation	*q*-Value	Mean log2-intensity	Minimal log2-intensity	Maximal log2-intensity
NM_006717.2	10,927	SPIN1	Spindlin 1	0.879	$<10^{-6}$	6.56	5.59	7.71
NM_054016.1	10,772	FUSIP1	FUS interacting protein (serine/arginine-rich) 1 and transcript variant 2	0.856	$<10^{-6}$	5.99	5.42	7.10
NM_001656.3	373	TRIM23	Tripartite motif-containing 23 and transcript variant alpha	0.853	$<10^{-6}$	5.97	5.35	6.89
NM_018843.2	55,972	SLC25A40	Solute carrier family 25, member 40 and nuclear gene encoding mitochondrial protein	0.850	0.0092	6.52	6.06	7.63
NM_016395.2	51,495	PTPLAD1	Protein tyrosine phosphatase-like A domain containing	0.847	0.039	7.69	7.35	10.20
XM_001128799.1	10,746	MAP3K2	Mitogen-activated protein kinase kinase kinase 2	0.841	0.096	6.74	6.13	8.09
Detection threshold 5.35								

Statistical significance of the Spearman rank correlation was estimated by correlation test with *p*-values adjusted on multiple testing by the Storey's *q*-value. The range of transcript intensities in the tumor samples is also given. For respective correlation plots, see Supporting Information Figures 3 and 4.

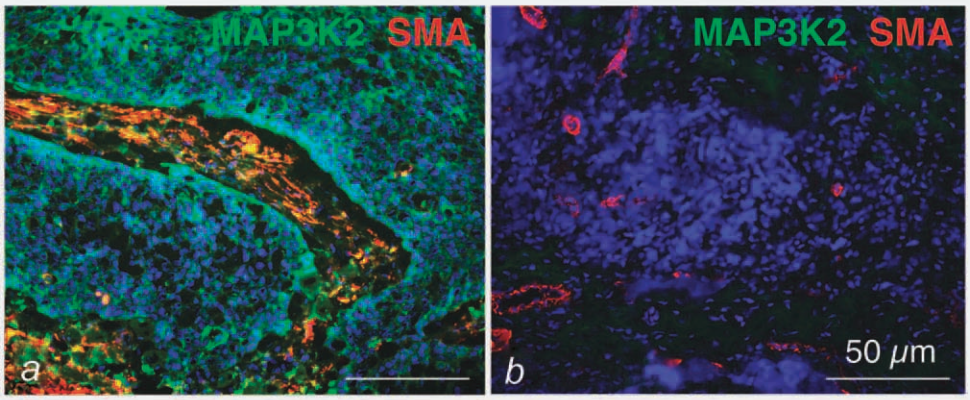


Figure 4. Detection of MAP3K2 (green signal) in tumors containing SMA (red signal) in CAF of the tumor stroma (*a*) and in tumors containing SMA exclusively in smooth muscle cells of vascular wall (*b*). A signal for MAP3K2 was confined to cancer cells of tumors characterized by the presence of SMA-expressing CAF. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and the observation of association of poor-prognosis and SMA-positive CAF in SCC.[5]

Tripartite motif-containing 23 (TRIM23) is an E3 ubiquitin ligase, which adds ubiquitin to NF-κB essential modulator (NEMO). Consistently with this NEMO modification, TRIM23 expression considerably and dose-dependently regulates the NF-κB promoter activity.[43] Mitogen-activated protein kinase kinase kinase 2 (MAP3K2 and MEKK2) is a MEK kinase for the ERK5 and JNK pathways,[44] which is activated by EGF and stress stimuli. It regulates delayed activation of NF-κB in response to cytokine stimulation (TNF-α and IL-1α), being a candidate NEMO interactor as identified by protein microarray screening.[45] MAP3K2 is also critical for both B-cell and T-cell development and interacts with FUS, an association partner of FUSIP1.[46] A large-scale RNAi screen revealed that MAP3K2 regulates mitochondrial abundance and function.[47] NF-κB-related factor is the protein tyrosine phosphatase-like A domain-containing protein (PTPLAD1). It has been described to belong to the set of butyrate-induced genes and is also known as B-ind1. Transfection experiments have shown that PTPLAD1 acts downstream of the small GTPase Rac1 in the pathway leading to NF-κB activation and potentiates JNK activation[48] and is a member of the autophagy network.[49]

Other deregulated transcripts

Solute carrier family 25 member 40 (SLC25A40) is a putative manganese-containing superoxide dismutase, playing a critical role in protection against mitochondria oxidative stress. Thus, it is essential for cell survival[50] and may play a role in tumor proliferation. Spindlin 1 (SPIN1) is an important mitotic spindle component. Its overexpression may disrupt cell cycle progression and can lead to tumorigenesis.[51]

Conclusions

Distinct galectins and myofibroblasts appear to play pivotal roles in three distinct pathological processes: wound healing, fibrosis and carcinomas.[34] On the cellular level, these processes share the stages of tissue damage, repair, remodeling and cell proliferation. It is reasonable to assume that tumors misuse pathways of tissue healing for their proliferation as predicted by Dvorak.[52] To extend our knowledge on the potential of Gal-1 in this respect, we describe its expression profile and identify several transcripts that are associated with Gal-1 production by SMA-positive CAF. Of note, they are associated with biological processes proven to worsen disease prognosis. As Gal-1, directly, these gene products may qualify as targets to interfere with disease progression.

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Review

Emerging role of tissue lectins as microenvironmental effectors in tumors and wounds

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Summary. Detailed comparative analysis of at first sight not related process cascades is a means toward this aim: to trace common effector mechanisms and hereby eventually inspire innovative routes for therapeutic management. Following this concept, promotion of tumor progression by stroma, especially cancer-associated fibroblasts and smooth muscle actin-positive myofibroblasts, and beneficial activity of respective cells in wound healing have helped to delineate the involvement of endogenous lectins of the family of galectins. In addition to initiating conversion of fibroblasts to myofibroblasts, galectin-1 instructs the cells to produce a structurally complex extracellular matrix. This bioscaffold is useful for keratinocyte culture, also apparently operative in ameliorating wound healing. These functional aspects encourage to study in detail how lectin-(glycan) counterreceptor display is orchestrated. Such insights are assumed to have potential to contribute to rationally manipulate stem/precursor cells as resource in regenerative medicine.

Key words: Chemokine, Fibroblast, Glycosylation, Lectin, Myofibroblast, Stem cell

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Introduction

Operating in different physiological contexts, a bioeffector can underlie diverse outcomes. The detection of this versatility is the starting point for research work with therapeutical perspective. To draw analogies between separate bioprocesses and to trace recurring molecular themes within them are prerequisites to identify routes for application. In this sense, Harold Dvorak's article "Tumors: wounds that do not heal" published nearly 30 years ago was instructive to turn attention to remarkable similarities between the connective tissue reaction in wounds and in cancer. Ensuing work comparing regeneration/wound healing with aspects of malignancy revealed that these two process cascades have even more in common (Smetana et al., 2013a; Rybinski et al., 2014). Owing to the emerging physiological significance of the microenvironment, our review focuses on cells and mediators from this region. They can program cell fate and thus become of interest for controlled manipulations with therapeutic intentions.

Stem cells under physiological conditions and in cancer

A central role in growth/regeneration is played by stem cells. They were first described in the process of hematopoiesis (Loutit, 1968). The following broad-scale research, which even led to founding journals exclusively devoted to this topic, has described their

occurrence, route of differentiation and potential for applications. As to totipotent stem cells, they can be prepared from the early embryo at the stage of several blastomers. Each cell has, as the term 'totipotency' implies, unrestricted capacity to form cell lineages. Pluripotent (embryonic) stem cells are isolated from the embryoblast of a blastocyst, and their daughter cells can practically be differentiated into most types of cells. In contrast to stem cells of prenatal origin, both multipotent stem and progenitor cells are present in the body throughout all periods of life of an organism, and almost all types of tissues harbor their own stem cell pool (Hansis, 2006; Mimeault and Batra, 2006; Yamanaka et al., 2008).

These tissue/adult stem cells are usually located in distinct regions. For example, epidermal and neural crest-originated stem cells reside in the bulge region of the outer root sheath of hair follicles (Sieber-Blum et al., 2004; Blanpain and Fuchs, 2006). They have a very slow rate of proliferation. As a consequence, when labeled by a pulse of radioactive nucleotides, the stem cell pool maintains positivity for a very long period of time (label-retaining cells). When proliferating, their division is asymmetric; this means that the first daughter cell keeps its stem cell properties. In contrast, the second one, the so-called transit-amplifying cell, is the source feeding the differentiation cascade. The transit-amplifying cell rapidly goes through the cell cycle stages to mitosis. The overall number of possible mitotic rounds yet is restricted. Characteristically, these adult tissue stem cells are equipped with protein pumps in their membrane. They efficiently export toxic agents such as xenobiotics from the cytoplasm (Challen and Little, 2006; Mimeaut and Batra, 2006; Inaba and Yamashita, 2012). Hereby, stem cells minimize the risk of damage to their genome. While work with stem cells *in vitro* has been accomplished, it is being noticed that adult tissue stem cells *in vivo* thrive in a special microenvironment. This is called the niche (Watt and Hogan, 2000; Das and Zouani, 2014). A current challenge to further applicability of stem cells is to define the niche's properties in detail.

Malignancies of blood cells are assumed to arise due to aberrations from the regular course of differentiation of bone marrow stem cells. These molecular deviations and their consequences then account for the production of abnormal cells, which are released into circulation. In view of the success rate to graft solid tumor cells to a genetically non-identical donor of the same species, the existence of cells with properties of stem cells had also been proposed for solid tumors (Glinsky et al., 2008; Sell, 2010). Work on teratoma cells supported the concept for tumor stem cells. In fact, when introduced into the cavity of a blastocyst, such cells even took part in forming the embryo and adult animals, with phenotypic properties dependent on the teratoma cell donor (Mintz and Illmensee, 1975; Solter, 2006). These data harmonize well with observations on the fate of embryonic stem cells, which are the source of a teratoma/teratocarcinoma when grafted to the adult host. These findings point to two important conclusions: i) stem cells have potential to become malignant, and ii) the microenvironment has a respective bearing on these rather undifferentiated but genetically normal cells. Further work on different tumor types showed that cancer stem cells can play salient roles in the majority of the tested carcinomas, such as those developing in breast (Owens and Naylor, 2013), prostate (Chen et al., 2013), colon/rectum (Fanali et al., 2014), lung (Singh and Chellapan, 2014), skin (Shakhova, 2014), in the head and neck region (squamous cell carcinomas) (Chovanec et al., 2005; Zhang et al., 2012) and/or in brain (Pointer et al., 2014). It is quite likely that cancer stem cells underlie complications in cancer therapy, especially with respect to minimal residual disease. Here, the cells, which survive tumor therapy, are at the heart of initiating tumor relapse. As a down-side for the success of chemotherapy, these cells can remove cytostatic drugs from their cytoplasm by the efficient transport mechanism mentioned above (Motlík et al., 2007). Having herewith emphasized the relevance of stem cells for onset and propagation of malignancy, it is instructive to next deal with the potential of host factors to affect disease progression.

In this context, the paradigm in tumor biology has shifted from rather exclusively focusing on tumor cells to the microenvironment, with its immune and stromal cells as well as mediator proteins produced by these cell types (de Visser et al., 2006; Le Bitoux and Stamenkovic, 2008; Mbeunkui and Johanen, 2009; Grivennikov et al., 2010; Galdiero et al., 2013; McAllister and Weinberg, 2014; Marcucci et al., 2014). In addition to cancer-associated fibroblasts (CAFs), which are frequently positive for α-smooth muscle actin (SMA), and infiltrating leukocytes such as cancer-associated macrophages (CAMs), several biochemical components of the extracellular matrix (ECM) play a role to endow the microenvironment with pro-tumoral properties (Fig. 1) (Plzák et al., 2010; Gatazzo et al., 2014).

Cancer-associated fibroblasts

The origin of CAFs is not yet fully clear. Its ancestry is traced to different sources, one of them epithelial-mesenchymal transition (Petersen et al., 2003; De Wever et al., 2008; Haviv et al., 2009). Another route to CAFs can start from bone marrow-derived mesenchymal stem cells (Mishra et al., 2008; Nishimura et al., 2012). Acting on malignant cells, such stromal cells can significantly stimulate both tumor growth and metastatic behavior (Karnoub et al., 2007) as well as suppress immune recognition of cancer cells (Ling et al., 2014). They are thus considered as "culprits in tumor growth, immunosuppression and invasion" (Stromnes et al., 2014).

Bone and/or cartilaginous metaplasia are also present in malignant tumors such as squamous cell

(Katase et al., 2008) and breast carcinomas (Downs-Kelly et al., 2009). Occurrence of bone or cartilage in tumor stroma is an indicator for the presence of mesenchymal stem cells at this site and reflects their inherent plasticity for differentiation. Interestingly, CAFs isolated from basal cell carcinoma induced expression of transcription factors Oct-4 and Nanog, markers of embryonic stem cells, in co-cultured mouse 3T3 fibroblasts. Moreover, the capacity for differentiation of these 3T3 cells exposed to CAFs then comes close to the plasticity of mesenchymal stem cells (Szabo et al., 2011). These data add support to the growing notion that the stromal part is an active player for tumor biology. Of note, recent work on autochthonous mouse models of pancreatic cancer presenting intraepithelial neoplasia, acinar-to-ductal metaplasia and progression to ductal adenocarcinoma highlighted the possibility for a favorable aspect, i. e. host protection by precluding to let more aggressive tumor cells arise (Oezdemir et al., 2014; Rhim et al., 2014). This evident ambivalence justifies respective research efforts. In their vicinity, CAFs are apparently capable to reprogram cells to let them gain a stem cell-like character. As the test case of pancreatic cancer exemplifies, tumor cells may alternatively acquire a moderate or advanced status of differentiation (Gore and Korc, 2014).

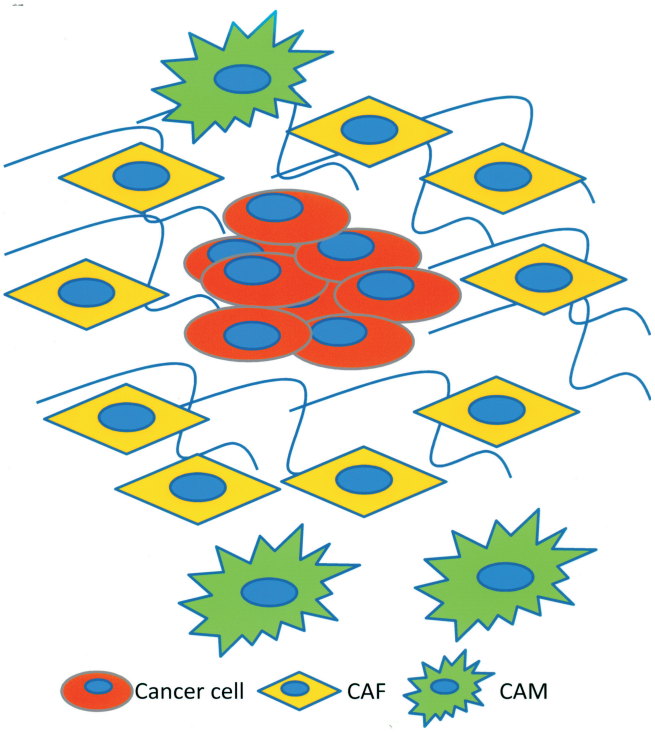

Fig. 1. Cancer-associated fibroblasts (CAFs) and distinct types of leukocytes such as cancer-associated macrophages (CAMs) contribute to establish a micromilieu that supports tumor cell growth and spreading.

In addition to the noted sources, CAFs can develop from fibroblasts of the local mesenchyme (Mueller et al., 2007), *in situ* harboring SMA (Cirri and Chiarugi, 2011). Bioactive fibroblasts, having properties similar to CAFs but without SMA, can also be generated under *in vitro* conditions by co-culture either with carcinoma cells or with normal keratinocytes (Kolář et al., 2012). As will further be discussed below, the pattern of expression of mediator proteins by fibroblasts is drastically altered, in turn changing the micro-environment (Fig. 2). By comparing the activity levels of normal fibroblasts and CAFs isolated from squamous cell carcinoma, one crucial difference was observed: whereas the activation of normal fibroblasts was time restricted, that of CAFs was prolonged to more than four weeks in culture (Szabo et al., 2013).

A key effector for the conversion of local fibroblasts to CAFs is the transforming growth factor-β1 (TGF-β1) (Casey et al., 2008; Brenmoehl et al., 2009). To pinpoint any effect of cancer cells on normal fibroblasts *in vitro*, both cell types were co-cultured. Although cancer cells alone were not able to induce production of SMA in normal dermal fibroblasts, proteomic analysis demonstrated a marked impact of the co-cultured epithelial cells on presence of proteins operative in the cytoskeleton, especially in actin functionality, such as caldesmon-1, cofilin and calponin-2 (Jarkovská et al., 2014). In addition, significant changes in serum levels of mRNA coding for apoptosis/growth-regulatory proteins of the p53 pathway such as p53 itself, p21, cyclin D, MDM2, CASP3, and MAX as well as Bcl-2 family proteins (Bcl-2, Bcl-XL, Bcl2L1, Mcl1, and BclAF1) were observed in patients with head and neck squamous cell cancer (Čapková et al., 2014). Evidently, intercellular communication in this system markedly influenced gene expression poised to reprogram motility and cell growth properties.

Turning back to TGF-β1 and its ability to alter cellular aspects within the microenvironment, a pertinent question was whether other proteins have similar capability. We have recently identified a new class of endogenous factors for CAF generation, i. e. adhesion/growth-regulatory lectins of the galectin family (for review, please see Cooper, 2002; Gabius et al., 2011; Kaltner and Gabius, 2012; Smetana et al., 2013b). Galectins share the β-sandwich fold and a sequence signature with a central Trp residue in the contact site for sugars, preferentially β-galactosides as reflected in the name (Barondes, 1997; Gabius, 1997; Kasai, 1997; Ahmad et al., 2002; Hirabayashi et al., 2002). Like other classes of lectins active extracellularly in cell adhesion and ordered cell migration (Gabius et al., 1985a; Gready and Zelensky, 2009; Schwartz-Albiez, 2009), galectins can serve as bridge between cells or cells and the ECM (Brewer, 1997). Equally important, bi- and oligovalency of galectins is instrumental for cargo selection and transport as well as cluster formation on membranes. For example, N-glycans with N-acetyllactosamine termini guide galectin-4-dependent apical or axonal glycoprotein

routing and status of microdomain integrity is a switch for galectin affinity (Stechly et al., 2009; Kopitz et al., 2010; Velasco et al., 2013), The capacity to read distinct glycan signatures on cellular structures (in terms of structure and topology of presentation) is readily revealed by applying human galectins as tool in cyto- and histochemistry (Gabius et al., 1991; Holíková et al., 2002; Habermann et al., 2011; Kopitz et al., 2013). The target-specific binding, e.g. to glycans of integrins, will induce outside-in signaling. Hereby, galectins elicit diverse cellular responses when binding cell surface glycans, for example mediator release or cell cycle arrest and anoikis/apoptosis (Villalobo et al., 2006; André et al., 2007; Wang et al., 2009). Following their secretion from a cell via a non-classical pathway, they thus become intimately involved in intercellular cross-talk, as the case study on communication between activated regulatory/effector T cells exemplifies with clinical relevance (Wang et al., 2009; Wu et al., 2011a).

Building on its capacity to direct human dermal fibroblasts to the myogenic lineage (Goldring et al., 2002) and also giving heed to its role in tumor promotion by mesenchymal stromal cells (Szebeni et al., 2012), we tested galectin-1. It is a homodimeric protein with contact sites for glycans at opposing sides ideal for cross-linking (López-Lucendo et al., 2004). These assays revealed activity (Dvořánková et al., 2011). It was additive to and independent from that of TGF-β1 (Fig. 3). Thus, this human lectin is a potent elicitor of CAF generation. Because tumors can express a network of galectins, as demonstrated exemplarily for brain, breast, colon, salivary gland, skin and testicular tumors (Gabius et al., 1986; Camby et al., 2001; Kayser et al., 2003; Čada et al., 2009; Saussez et al., 2010; Remmelink et al., 2011; Dawson et al., 2013; for a recent review, please see Gabius and Kayser, 2014), we proceeded to test three further members of this family. Activity was revealed also for galectins-3 (the full-length protein but not its proteolytically truncated form), -4, and 7 (Dvořánková et al., 2011). These proteins belong to the three different subgroups of the galectin family, the non-covalently associated homodimers (galectins-1 and -7), the tandem-repeat-type proteins with two different lectin domains connected by a linker peptide (galectin-4) and the chimera-type galectin-3 with its tail of collagen-like repeats and an N-terminal peptide attached to the lectin domain (Kasai and Hirabayashi, 1996). Together with galectin-1, they often are present in tumors and their stroma, thus likely operative accordingly *in situ*. As consequence, endogenous lectins secreted from tumor cells or produced by stromal cells obviously deserve the same attention as put on growth factors.

Besides the effect on fibroblasts, galectin-1 also stimulates the production of a network of ECM fibers. This is rich in fibronectin, tenascin and galectin-1 itself (Dvořánková et al., 2011; Mifková et al., 2014). For endothelial (HUVEC) cells, the matrix is suited to stimulate proliferation (Perželóva et al., 2014). To address the issue on validity of extrapolation from *in vitro* to *in vivo* squamous cell carcinomas of the head and neck were analyzed. This work led to a significant correlation between presence of galectin-1 in tumor stroma and presence of SMA-positive CAFs. Further examining gene expression profiles by microarrays, cancer cells isolated from tumors rich in stromal CAFs and galectin-1 had higher signal intensities for genes implicated in cancer progression such as MAP3K2, TRIM23, PTPLAD1, FUSIP1, SLC25A40 and SPIN1

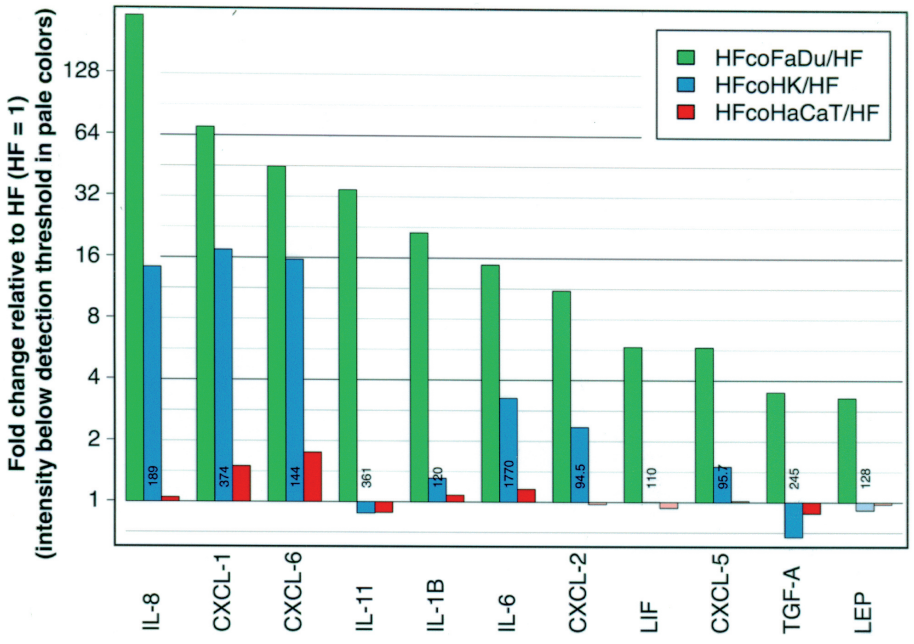

Fig. 2. Markedly elevated expression of genes for chemo- and cytokines as well as growth factors in normal human fibroblasts (HF; set to 1) by co-culture with cells of a squamous cell carcinoma (FaDu) or keratinocytes (K). The same procedure with non-tumorigenic immortalized cells (HaCaT) triggered comparatively minor effects; (kindly provided by Dr. Michal Kolář and Dr. Hynek Strnad from the Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic v.v.i. in Prague).

than preparations from cells isolated from tumors with low levels of the lectin and SMA positivity (Valach et al., 2012). That stromal presence of galectins can be associated with an unfavorable prognosis, as indicated for breast cancer and galectins-1 and -3, respectively (Jung et al., 2007; Moisa et al., 2007), fits into this concept. A rather general role of galectin-1 is indicated when further noting its respective activity in other types of carcinoma, e. g. oral squamous cell carcinoma with impact on SMA positivity, fibronectin/collagen I production and CCL2 presence (Wu et al., 2011b) or pancreatic ductal adenocarcinoma with enhanced Hedgehog pathway signaling in desmoplasia associated to tumor progression (Martínez-Bosch et al., 2014). Concerning the aspect of the age of normal fibroblasts, it is noteworthy that adult cells were found to produce more galectin-1 than foetal fibroblasts (Ho et al., 2014).

Will CAFs affect cell types other than malignant cells? CAFs are also able to even influence normal keratinocytes to acquire a poorly differentiated (tumor-like) phenotype, as we observed in basal/squamous cell carcinomas (Lacina et al., 2007a, b) and in benign tumors, here dermatofibroma (Kideryová et al., 2009). Of note, this phenotype is rather similar to that of epidermal stem or prenatal cells. An effect of stromal fibroblasts had also been noticed in other types of tumors such as malignancies of breast (Casey et al., 2009), pancreas (Hwang et al., 2008) and prostate (Hayward et al., 2001). On the cellular level, marked effects of CAFs on proliferation, epithelial-mesenchymal transition and migration had been reported (Orimo et al., 2005; Fujita et al., 2009; Martin et al., 2010). To contribute to resolve the arising issue on the relationship between the response and the origin of CAFs a comparative analysis was performed in homo- and heterologous systems. Fibroblasts isolated from basal/squamous cell carcinoma and melanoma affected breast cancer cells in a manner similar to that observed by co-culture with fibroblasts isolated from a skin metastasis of breast cancer (Dvořánková et al., 2012). These results indicated that the activity of CAFs will not be strictly tumor-type specific.

In culture and *in situ*, CAFs can act via contacts and also via the production of cytokines/growth factors, proteolytic enzymes and ECM. As noted above, effectors such as lectins are known to act directly on cells or to act

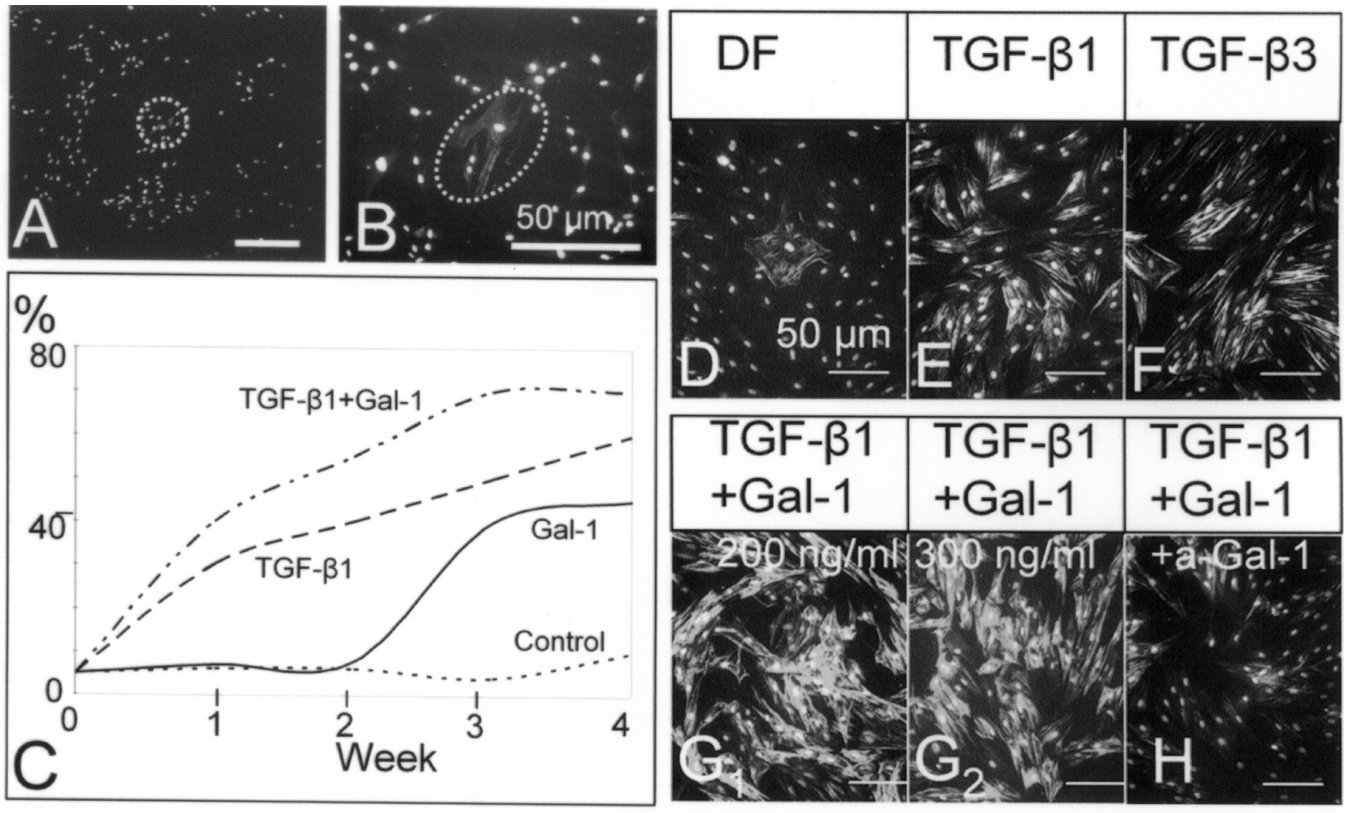

Fig. 3. Extent of occurrence of smooth muscle actin-positive myofibroblasts in control culture of normal human fibroblasts is very low **(A-D)**. Exposure of cells to galectin-1 **(C)**, TGF-β1 **(C, E)** and TGF-β3 **(F)** stimulates generation of these myofibroblasts from normal dermal fibroblasts. Galectin-1 exerts an additive effect to TGF-β1 **(G1, G2)**. Blocking of galectin-1 binding expectably reduces extent of myofibroblast generation. Figure is adopted from Dvořánková et al. (2011), with kind permission of S. Karger AG, Basel.

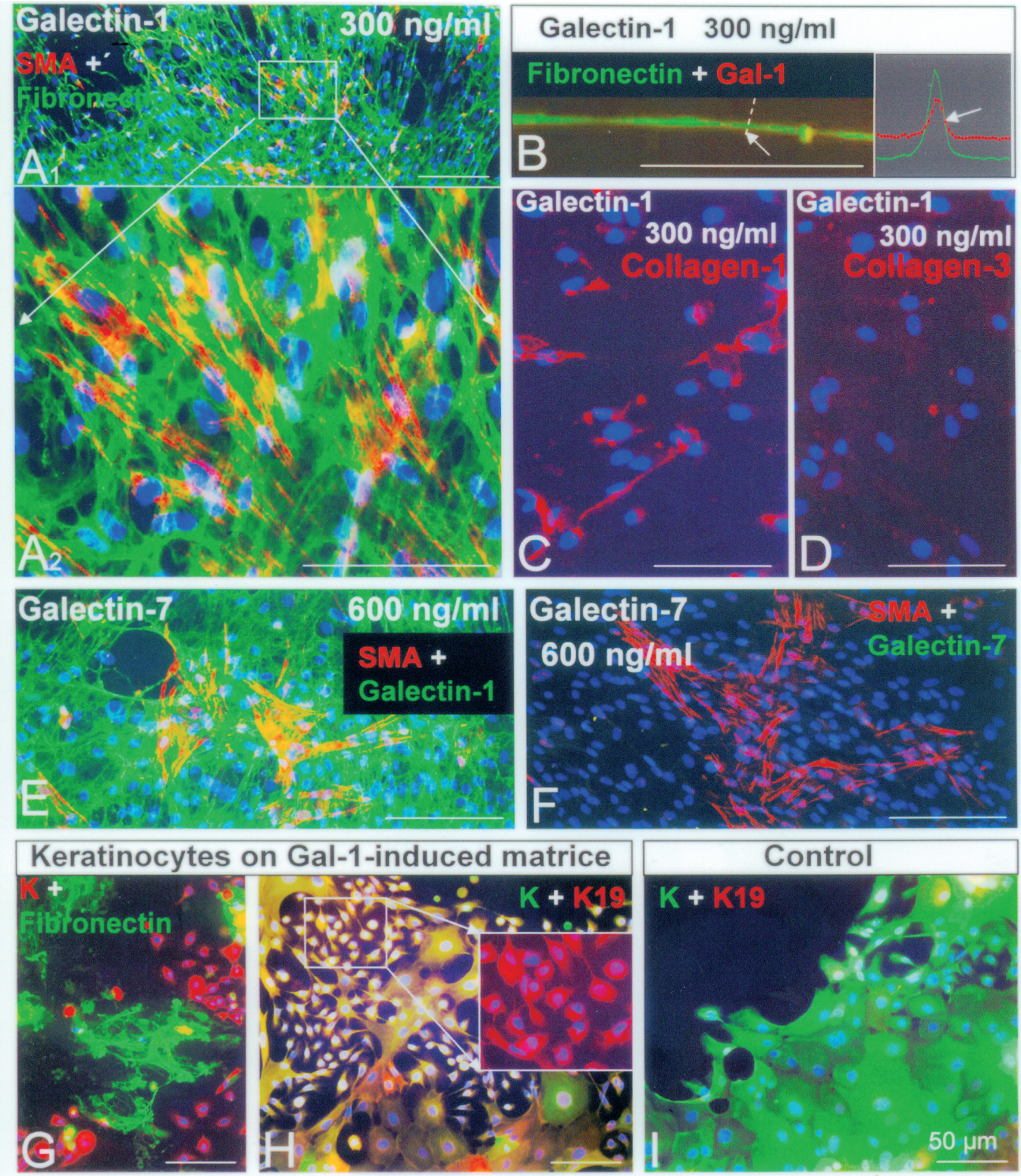

Fig. 4. Galectin-1 stimulates conversion of fibroblasts to smooth muscle actin-positive myofibroblasts **(A1, A2)**. These cells produce a complex network of fibers in the extracellular matrix rich in fibronectin and galectin-1, as demonstrated by the measurement of fluorescence profiles of these two components **(A1, A2, B)**. Production of collagen types 1 and 3 was negligible after the treatment **(C, D).** Besides galectin-1, the proto-type galectin-7 also turns fibroblasts into myofibroblasts **(E)**. Extracellular matrix produced by these cells contained galectin-1 **(E)** but not galectin-7 **(F)**. When this network of extracellular matrix was colonized by human keratinocytes *in vitro*, they actively resorbed this network **(G)**. Of note, these keratinocytes were very small and expressed a marker for low-degree of differentiation status, i. e. keratin 19 **(H)**. Such rather small keratinocytes including keratin 19 positive cells were not observed in classical culture on plastic **(I)**. Figure is adopted from Dvořánková et al. (2011), with kind permission of S. Karger AG, Basel.

as elicitors by induction of cytokines/growth factors (Gabius, 2001; Timoshenko et al., 2010; Ledeen et al., 2012). For example, galectin-3 augments transcription of genes for the chemokines CCL2, 5, 8 and 20 as well as CXCL8 in the range of 3.4-27fold in macrophages (Papaspyridonos et al., 2008) and stimulates production of CCL2, 3 and 5 in synovial fibroblasts suited to recruit mononuclear cells (Filer et al., 2009). These mediators will be discussed further below. That this lectin is a mitogen for fibroblasts and an inducer of collagen I gave reason to link its early on-set expression to failure of hypertrophied hearts (Sharma et al., 2004), broadening clinical correlation.

At this stage, it is also informative to more closely define differences between fibroblast preparations. When comparing gene expression profiles of normal fibroblasts and CAFs isolated from head and neck squamous cell carcinoma by microarrays, differences in nearly 600 genes were observed, among them *IGF2* and *BMP4* appearing as most noteworthy (Strnad et al., 2010). Important growth factors/cytokines produced by CAFs and acting on cancer cells are compiled in Table 1. These factors promote cancer cell proliferation and migration as well as the epithelial-mesenchymal transition, all relevant for progression and spreading of tumor cells from the primary site. To the same outcome, proteolytic enzymes produced by CAFs can likewise be important for epithelial-mesenchymal transitions and tumor progression with spread to distant organs (Stuelten et al., 2005; Orlichenko and Radisky, 2008; Saussez et al., 2009; Taddei et al., 2013). That matrix metalloproteinases (MMPs) -2 and -9 (together with increased filopodia occurrence) in oral squamous cell carcinoma cells and MMP-9 in murine lymphoma and HeLa cells are targets for upregulation by galectins-1 and -7 (Demers et al., 2005; Park et al., 2009; Wu et al., 2009) adds further evidence to the concept of galectin relevance for different effector routes. Matrix degradation by ADAM-15, in contrast, is negatively regulated with galectin-1 presence (Camby et al., 2005).

Turning to the ECM, it represents more than just an inert protection/stabilization scaffold for cells. It is organized either into a complex meshwork of connective tissue or it forms the basement membrane. The structure of the ECM and its composition dynamically reflect functional requirements of tissues, with an intricate balance between matrix production and breakdown by lytic enzymes. Because components of the ECM have been referred to as "Janus-faced" (Tímár et al., 2002), the actual context is a salient factor to foresee functional implications. As proof-of-principle representatives of the ECM in tumors, tenascins-C and W, modular proteins equipped to engage in multiple contacts, were proven to play a major role in the course of tumor growth (Brellier and Chiquet-Ehrismann, 2012), frequently in concert with laminins (Franz et al., 2006). Other ECM constituents that participate in tumor formation are periostin (Tilman et al., 2007) and heparan sulfate proteoglycans (Gomes et al., 2013). The glycosaminoglycan chains of the proteoglycans can serve as a storage place for chemo- and cytokines and growth factors (Buddecke, 2009). Fibronectin in the ECM of malignant tissue, a counterreceptor for galectins via its glycans (André et al., 1999), is able to influence vascularization of tumor stroma (van Obberghen-Schilling et al., 2011). As with the glycans, the three-dimensional architecture of the ECM will likely be pivotal, besides the composition. This topological aspect also works in the interplay of lectins an ECM. Because a commercial matrix (Matrigel) loaded with galectin-1 was highly efficient to present the lectin for inducing apoptosis of activated T cells (He and Baum, 2004), matrix properties can definitely modulate a lectin's *in situ* activity status. Moving from this (glycobiological) secreted effector to cells, the inflammatory cells infiltrating the tumor also deserve proper emphasis.

Inflammatory cells: a double-edged sword

Stimulation of the immune defence, with local infiltration by inflammatory cells, had been faithfully interpreted as favorable indicator, of benefit for patients. By uncovering unsuspected mechanisms, this view has been subject to a paradigmatic change. From the side of the stem cells, their own immunomodulatory properties minimize the risk of their recognition and destruction by defence mechanisms (Maccalli et al., 2014). In addition to such attenuation regulatory T cells, myeloid-derived suppressor cell and CAMs are able to downregulate cancer surveillance and increase the tolerance of the immune system to cancer cells. Toward the same outcome, cells such as CAMs have a strong tumor-supporting effect by locally enhancing the availability of pro-inflammatory (and tumor-stimulatory) cytokines such as interleukin-6, teaming up with CAFs (please see Fig. 2). Of note, TGF-β1, a member of the cytokine

Table 1. Examples of growth factors/cytokines/chemokines produced by CAFs in different types of cancer.

Type of cancer	Growth factor/cytokine/chemokine	Reference
Basal cell cancer	IGF-2, FGF-7, Lep, TGF-β3, GREMLIN	Sneddon et al., 2006; Szabo et al., 2011
Breast	CCL-5, IL-6, IL-8, CXCL-7, CXCL-12, SDF-1	Orimo et al., 2005; Karnoub et al., 2007; Korkaya et al., 2011
Pancreas	TGF-β1–3, BMP-4, FG2-1, FGF-2, FGF-7, FGF-10, HGF, CXCL-12, IL-6, LIF, NGF	Hua et al., 2006; Mahadevan and von Hoff, 2007
Prostate	FGF-2, TNF-α	Kaminski et al., 2006
Squamous cell cancer	IGF-2, BMP-4, IL-6, IL-8, CXCL-1	Strnad et al., 2010; Kolář et al., 2012

family, exerts anti-immune activities (Jackaman and Nelson, 2014; Sideras et al., 2014). Galectin-1 in the tumor stroma, as noted above, may augment immunosuppression by eliciting apoptosis in activated T cells (Pace and Baum, 1997; Smetana et al., 2013b). However, it should be added that suited glycan display can also make tumor cells susceptible to galectin-1-dependent anoikis/apoptosis induction, rendering the activity profile of this multifunctional lectin dependent on the context (Sanchez-Ruderisch et al., 2011; Smetana et al., 2013b). In conclusion, presence of inflammatory cells (and their secreted proteins) has inherent ambivalence precluding immediate and reliable predictions, a challenge for future research. The required monitoring will extend the data basis for allowing to draw analogies to other process cascades.

Wound/tissue healing

As previously highlighted in the seminal paper by Dvorak (1986), numerous cellular events appear to be shared by tumors and wounds, with a successful outcome in wound healing. Looking more closely at skin wound healing, the entire process can be divided into three phases. They cannot strictly be separated from each other (Barbul and Regan, 1993; Reinke and Sorg, 2012): i) inflammatory phase, ii) proliferation phase and iii) maturation/remodeling phase. Broadening its implications, it is justified to apply these three categories to other repair processes, too, for example in striated muscle (Bentzinger et al., 2013). Starting wound healing, clotting of blood and migration of inflammatory cells to the injury site occur. In the acute phase, polymorphonuclear leukocytes (PMNL) establish the demarcation line. It delimits necrotic/damaged tissue from vital parts. PMNL are replaced by tissue macrophages during the chronic phase of inflammation. Approximately two days following the injury, fibroblasts begin to populate the wound, proliferate and produce constituents of the ECM. They also contribute to the microenvironment in terms of its profile of chemo- and cytokines and growth factors (Table 2). Immune cells, predominantly micro- and macrophages, are responsible for removal of tissue debris, and they also protect the wound against infections, mainly by bacteria and fungi. In this defense line, lectins such as galectin-3 (MAC-2 antigen) or the tandem-repeat-type mannose receptor are engaged (Gabius, 2006; Quattroni et al., 2012). Obviously, the term "double-edged sword" fits well to describe the spectrum from beneficial to harmful activities of the local effector panel (Behm et al., 2012). In wound healing, lack of injury-site infiltration by inflammatory cells markedly retards the process (Grim et al., 1988). Fittingly, a poor inflammatory response resulting in a low level of scar formation is observed in neonates and newborns (Bermudez et al., 2011; Borský et al., 2012).

Having described the relevance of SMA-positive CAFs and aspects of galectin functionality, the question arises as to whether equivalent cells and any galectin are an active players of wound healing. Indeed, cellular accumulation in granulation tissue takes place, and galectin-1 reactivity, a prerequisite for activity, has been detected using the human lectin as histochemical tool (Klíma et al., 2009; Gál et al., 2011; Grendel et al., 2012). Using re-epithelialization of rat cornea as model, galectin-3 (and galectin-7 but not galectin-1) was active (Cao et al., 2002; Yabuta et al., 2014). Interestingly, galectin-7 is also implicated in repair following menstruation. Wound cell layers exposed to the lectin (at 2.5 μg/ml) showed transcriptional upregulation of ECM constituents including fibronectin and TGF-β1 (Evans et al., 2014). As then expectable, myofibroblasts positive for SMA are common in skin-wound granulation tissue, TGF-β also belonging to the local inductors secreted from fibroblasts as described for cancer. Due to these cells' contractility they are responsible for wound contraction that effectively reduces the area necessary for re-epithelialization (Werner et al., 2007; Kapoor et al., 2008). An insufficient level of presence of myofibroblasts and/or prolonged inflammation at the wound site can account for extensive scar formation, prompting to consider treatment of wounds with focus on proper functions of fibroblast/myofibroblasts as an attempt to minimize its extent in patients (van Beurden et al., 2005). Interestingly, when compared to skin healing, scarification is significantly reduced in adult oral mucosa, owing to similarities in the healing process seen in neonates (Mak et al., 2009). Combination of all factors mentioned above influences the rate of re-epithelialization in the case of skin wound repair, as it does for proliferation and ensuing differentiation of satellite cells to myoblasts and fusion to muscle fiber in striated muscle repair (Reinke and Sorg, 2012; Bentzinger et al., 2013). Stem or precursor cells, which receive signals for their proper functions from inflammatory cells and fibroblasts, serve as pool and source for the cell material in repair.

Mutatis mutandis, cell generation proceeds similarly in tumors, but terminal differentiation and "wound closure" are not attained (Smetana et al., 2013a). The

Table 2. Examples of main growth factors/cytokines/chemokines involved in wound healing.

Mediator	Producer	Target cell	Reference
VEGF	K, F, MF, E	E, MF	Behm et al., 2012
IGF-2	M, Ch, O	M, Ch, O	Koh et al., 2011
FGF-2	F	K	Peplow and Chatterjee, 2013
TGF-β1–3	K, F, MF, platelets	F, K, MF, E	Behm et al., 2012
IL-1	MF, K, F	E, MF, K, F	Behm et al., 2012
IL-6	F, E, MF, K	E, MF, K	Behm et al., 2012
IL-8	F, K	K, F, E	Gillitzer and Goebeler, 2001
CXCL-1	F, K	K	Gillitzer and Goebeler, 2001

K: keratinocytes, F: fibroblasts, E: endothelial cells, MF: macrophages, M: mesenchymal cells, Ch: chondrocytes, O: osteoblasts

last step of wound healing is represented by the remodeling of connective tissue, the basis of any scar formation. Due to the implications on elasticity its occurrence is physiologically undesirable. Proteolytic degradation and ECM remodeling underlie the reconstitution of the normal status. Again, such processes re-appear in cancer, with different consequences (Behm et al., 2012). If the inflicted damage by wounding is too serious, fibrosis can result. Here, functional cells are replaced by scar-like connective tissue. Fibrosis usually represents the final stage of organ damage with none or only very limited therapeutic perspectives for reversal, myofibroblasts a prominent cell type on the route to its establishment, evocative of their role in cancer (Lopéz-Novoa and Nieta, 2009; LeBleu et al., 2013). With respect to effectors, a galectin (i. e. galectin-3), again, has been delineated to be critically involved in fibrosis, as observed in model studies especially using knock-out mice and looking at heart, kidney, lung and pancreas (Wang et al., 2000; Henderson et al., 2006, 2008; Nishi et al., 2007; Liu et al., 2009; Cullinare et al., 2014). Potentially counterbalancing this profibrotic activity, galectin-9 (at 1-3 μg/ml) significantly increased the percentage of annexin V-positive activated human fibroblasts and was less expressed in patients with idiopathic pulmonary fibrosis (Matsumoto et al., 2013). These observations are indicative for a protective role.

From delineating analogies to envisioning perspectives

The aim of regenerative medicine is to rationally take advantage of the potential of stem cells in therapeutic protocols (Mironov et al., 2004). For example, mesenchymal stem cells can be a resource for correcting defects of the locomotory system (Kuhn and Tuan, 2010). Gaining detailed insights into the way growth factors help to shape a microenvironment suited for stem cell propagation can establish protocols for successful *in vitro* manipulation (Das and Zouani, 2014). Toward this end, ECM properties also come into play, e. g. by favoring growth of human umbilical vein endothelial cells (Perželóvá et al., 2014) or human keratinocytes. These cells acquired a low level of differentiation as reflected by positivity for keratin 19 (Fig. 4). In this respect, our work on galectins adds protein-carbohydrate recognition to the modes of molecular interactions, whose manipulation can have a therapeutic perspective.

Having been initially detected in malignant cells by haemagglutination in extracts of murine N-18 neuroblastoma cells (Teichberg et al., 1975), then purified by affinity chromatography from murine and human tumors (Gabius et al., 1984, 1985b) and localized in human (breast) tumors immuno-histochemically (Gabius et al., 1986), galectin-1 has become a role model for functional analysis in cancer biology and wound healing. Its presence directs production of a bioactive ECM and myofibroblast generation (Dvořánková et al., 2011), thus inspiring to target this process in tumors by unspecific means (Mifková et al., 2014) or by inhibitors blocking its binding to glycans (Murphy et al., 2013). Synthetic tailoring of the sugar headgroup and of the scaffold for topologically optimal modes of glycocluster preparation up to presentation on glycodendrimersomes are being merged to explore possibilities for selective galectin blocking at high inhibitory potency (André et al., 2003, 2010, 2011, 2012; Percec et al., 2013; Zhang et al., 2014). The controlled (beneficial) activity in wound healing, on the other hand, gives direction to consider protein engineering. Respective ideas for design can either be derived from the study of natural single nucleotide polymorphisms (Ruiz et al., 2014) or from performing systematic mutational re-designing of the lectin site or other regions (Imamura et al., 2011; Kopitz et al., 2014).

Alternatively, learning from physiological regulation of lectin presence, e. g. by metabolites such as butyrate (Katzenmaier et al., 2014), makes molecular switches available. Taking one step further, orchestration of expression of lectins, with intra-network coordination not only seen in tumor but also diseases such as osteoarthritis (Toegel et al., 2014), and of glycans acting as counterreceptors in growth control, e. g. on pancreatic carcinoma cells (Capan-1) *in vitro* by the tumor suppressor $p16^{INK4a}$ which downregulates α2,6-sialylation of the fibronectin receptor to make these cells susceptible to anoikis induction (Sanchez-Ruderisch et al., 2010; Amano et al., 2012), can inspire an innovative approach to make headway with tailoring stem cells to become tools for regenerative medicine (Mironov et al., 2004). Interestingly, the healing process in corneal wounds has a bearing on expression of glycosyltransferases implicated in the synthesis of galectin ligands. Remarkably, enzymes for T antigen synthesis, a ligand for galectin-3 (Krzeminski et al., 2011), are upregulated, that for α2,6-sialylation downregulated (Saravanan et al., 2010). Explicitly, the reprogramming of cell surface glycosylation by altering distinct expression properties of cell surface determinants such as a TGF-β1 receptor (Patsos et al., 2009), and of intracellular proteins such as the Rho GTPase Rac1 also involved in wound healing (André et al., 2014) or by changing a microenvironmental factor of inflammation (NO) (van de Wouwer et al., 2011) can be viewed as means toward regulating susceptibility to tissue lectins. Moreover, at the same time, manipulations of glycosylation can modulate availability of growth factor receptors. Such changes make their presence felt already at the folding stage and/or impair protein stability (Patsos and Corfield, 2009; Zuber and Roth, 2009). In fact, glycosylation then has a bearing on the extent of cell surface presence of glycoproteins, as recently observed for the epidermal growth factor receptor expressed in cell lines deficient in distinct aspects of galactosylation (Gabius et al., 2012).

By letting deciphering the cross-talk between tissue

lectins and their counterreceptors in tumor biology/would healing become a topic of research activity, using techniques from biophysical chemistry to cell biology for analysis (Solís et al., 2014), contributions to advance applicability of the potential of stem/precursor cells can be expected. Also considering tissue lectins as elicitors, e. g. by affecting production and secretion of chemo- and cytokines and growth factors and generating a particular composition of the ECM, shaping of microenvironmental properties can be envisioned. In this sense, monitoring glycan and lectin presence *in situ* has merits beyond a mere status description (Danguy et al., 1994). In view of the unsurpassed capacity of glycans for storing biological information and their emerging significance as versatile signals for diverse bioprocesses (Gabius et al., 2011), exploring this new ground can most likely be very fruitful.

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