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Galectins: multitask signaling molecules linking fibroblast, endothelial and immune cell programs in the tumor microenvironment

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Running Title: Galectins in the tumor microenvironment.

Abstract

Tumor cells corrupt surrounding normal cells instructing them to support proliferative, pro-angiogenic and immunosuppressive networks that favor tumorigenesis and metastasis. This dynamic cross-talk is sustained by a range of intracellular signals and extracellular mediators produced by both tumoral and non-tumoral cells. Galectins - whether secreted or intracellularly expressed- play central roles in the tumorigenic process by delivering regulatory signals that contribute to reprogram fibroblasts, endothelial and immune cell programs. Through glycosylation-dependent or independent mechanisms, these endogenous lectins control a variety of cellular events leading to tumor cell proliferation, survival, migration, inflammation, angiogenesis and immune escape. Here we discuss the role of galectin-driven pathways, particularly those activated in non-tumoral stromal cells, in modulating tumor progression.

Key words: Galectins / Tumor microenvironment / Fibroblasts / Endothelial Cells/ Immune Cells

1. Introduction

The tumor microenvironment (TME) includes a diversity of cell types such as fibroblasts/myofibroblasts, vascular endothelial cells (ECs), lymphatic endothelial cells (LECs) and immune/inflammatory cells [1]. These cells induce tumorigenesis by controlling angiogenesis, inflammation and immune escape through the coordinated action of soluble mediators, including cytokines, chemokines and growth factors. Interestingly, multifunctional cytokines such as transforming growth factor-β_1 (TGF-β_1), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) may concurrently affect fibroblast, endothelial and immune cell programs that coordinately sustain tumor growth [2,3]. In this way, non-tumor cells can profoundly affect the tumorigenic process by conditioning the associated microenvironment through a limited number of 'multitask' signaling molecules that facilitate tumor survival, proliferation, and metastasis [3].

Galectins, a family of glycan-binding proteins with affinity for N-acetyllactosamine (LacNAc; Galβ1-4GlcNAc)-containing glycoconjugates, have broad influence on fibroblast, vascular and immune cell biology [4-7]. From a structural viewpoint, these endogenous lectins display at least one carbohydrate recognition domain (CRD) in a single polypeptide chain that recognizes specific glycans on a variety of cell surface receptors and the extracellular matrix (ECM). Whereas mono-CRD (proto-type) galectins, including galectin (Gal)-1, -2, -5, -7, -10, -11, -13, -14 and -15, occur as biologically active monomers or homodimers, bi-CRD (tandem-repeat) galectins (Gal-4, -6, -8, -9, 12) share two distinct CRDs connected by a linker peptide. On the other hand, Gal-3 -the only representative member of the chimera galectin-type subfamily- is composed of an N-terminal collagen-like domain and a C-terminal domain containing a single CRD. In most human cancers, altered (up- or down-regulated) expression of galectins correlates with poor clinical outcome and acquisition of metastatic phenotype [8]. Through glycosylation-dependent or -independent mechanisms these endogenous lectins have been shown to play key roles in tumor growth, homotypic and heterotypic cell aggregation, survival, migration, angiogenesis, lymphangiogenesis and immune escape [6,8,9]. Galectins in the TME have been shown to play key roles in tumor progression. In breast cancer, high levels of Gal-1 expression in stromal cells were observed in invasive carcinoma compared to carcinoma *in situ*, which showed positive correlation with TNM stage and axillary lymph node metastasis [10]. In this sense, Gal-1-positive stroma is preferentially found in triple negative and human epidermal growth factor receptor 2 (HER2)-positive breast tumors and correlates with epidermal growth factor receptor (EGFR)-positive, Ki67-positive cells and mutated p53 [11]. Here we discuss the role of galectins, particularly those synthesized and released by non-tumoral stromal cells such as fibroblasts, endothelial and immune cells in shaping the TME (Fig. 1).

2. Galectins from fibroblasts

In the TME, fibroblasts and myofibroblasts are collectively designated as carcinoma-associated fibroblasts (CAFs). CAFs promote tumor growth, invasion and angiogenesis and produce ECM, proteinases, cytokines, chemokines and growth factors [12-14]. Myofibroblasts are α-smooth muscle actin (α-SMA)-positive fibroblasts, a hallmark of activated fibroblasts [15,16]. Various cell types such as resident normal fibroblasts, ECs, pericytes, smooth muscle cells, pre-adipocytes and bone marrow-derived progenitors, such as fibrocytes and mesenchymal stem cells can generate tumor-

promoting myofibroblasts [17-19]. Moreover, soluble factors secreted by CAFs can promote tumor growth and metastasis through diverse mechanisms [20,21].

Recently, CAFs have been postulated to induce intratumoral fibrosis as a result of the deposition of a cross-linked collagen matrix, and such fibrotic process has been shown to exert mechanical forces creating a biochemical milieu that shapes intratumoral immunity and influences tumor cell metastatic behavior. Hence, metastasis results from a change in the type of stromal collagen cross-link, and fibrosis and inflammation perpetuate each other through proteolytic and chemotactic mediators released into the tumor stroma [22].

2.1 Galectin-1 in fibroblasts

Gal-1 was the first galectin cloned from fibroblasts [23], and its accumulation in the TME surrounding thyroid [24], head and neck [25], colon [26], and prostate carcinoma [27] has been well documented. In ovary carcinoma, Gal-1 preferentially expressed in contiguous peritumoral fibroblasts. Conditioned medium (CM) obtained from ovary carcinoma cells induced increased Gal-1 expression in fibroblasts [28]. Downregulation of Gal-1 secretion by fibroblasts decreased MMP-2 expression in ovarian cells, suggesting that this lectin might affect the invasive capacity of tumor cells [29]. In head and neck squamous carcinoma, upregulation of Gal-1 by α-SMA-positive CAFs correlated with known poor-prognosis factors [30]. In oral squamous cell carcinoma, Gal-1 overexpression has been associated with fibroblast activation: exogenously-added Gal-1 stimulated transdifferentiation of normal fibroblasts (NFs) to myofibroblasts, while Gal-1 knockdown normalized CAFs upon activation (Fig. 2). Conditioned medium from Gal-1 knockdown CAFs significantly inhibited oral squamous tumor cell migration and invasion, and down-regulated the expression of monocyte chemotactic protein-1 (MCP-1). Notably, Gal-1 knockdown in CAFs significantly inhibited CAF-induced tumor growth and intravasation *in vivo* when fibroblasts were co-inoculated with oral squamous cell carcinoma cells in nude mice [31] (Fig. 2). In co-cultures of hypopharyngeal carcinoma cells and fibroblasts, increased intracytoplasmic staining for Gal-1 and fibronectin (FN) was detected in fibroblasts, which secreted both proteins to the ECM [32] (Fig. 2). In gastric tissues, CAFs exhibited strong expression of Gal-1, and co-cultures of Gal-1 knockdown CAFs with gastric cancer cells showed inhibition of tumor cell migration and invasion as compared with co-incubation with Gal-1-sufficient control CAFs [33]. Expression of Gal-1 in cell lysates and CM was higher in gastric CAFs than in NFs, and exogenous Gal-1 stimulated NFs transformation into CAFs in a dose-dependent manner. Moreover, CM from Gal-1-positive CAFs promoted gastric cancer cell migration and invasion, an effect that was inhibited when CAFs were pretreated with Gal-1 siRNA [34].

In pancreatic carcinoma, strong Gal-1 immunostaining was observed in CAFs of the tumor ECM in comparison to the normal pancreatic stroma [35]. In fact, Gal-1 has been proposed to be one of the main factors that modulate the pancreatic TME, characterized by abundant stromal desmoplasia, a fibroinflammatory reaction composed of a dense ECM, and immune, endothelial and stellate (resident fibroblasts) cells. Normal pancreatic stellate cells (PSCs) are star-shaped fibroblasts, usually activated from their quiescent phenotype into myofibroblast-like cells in response to pancreatic injury. Gal-1 is strongly expressed in activated PSCs, and recombinant Gal-1 increased their proliferation favoring the activation of relevant signaling pathways including activating protein-1 (AP-1), extracellular-regulated kinase-1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and nuclear factor-kappa B (NF-κB) as well as the production of MCP-1, cytokine-induced neutrophil chemoattractant-1 (CINC-1) [36] and collagen

[37]. Gal-1 is expressed and released by activated PSCs, enhancing pancreatic cancer cell proliferation and infiltration in co-cultured invasion assays. Interestingly, both platelet-derived growth factor (PDGF) and transforming-growth factor-β_1 (TGF-β_1) promoted Gal-1 expression in cultured PSCs [38]. Endogenous Gal-1 in PSCs was found to be upregulated by TGF-β_1, inducing MMP2/ MMP9 and invasion of tumor pancreatic cells. In fact, in mouse xenografts, a strong signal for Gal-1 was detected in the TME when malignant PSCs were implanted in the presence of tumor cells as compared to tumors derived from injection of normal PSCs and tumor cells [39]. Genetic ablation of Gal-1 in a mouse model of pancreatic cancer suppressed tumor progression by reducing fibroblast activation, and Gal-1 down-regulation in CAFs altered the Hedgehog signaling pathway [40]. In liver tissues, proteomics of cellular and secreted proteins of normal (quiescent) and activated rat hepatic stellate cells (HSCs) showed up-regulation of Gal-1 upon cellular activation [41]. Expression of this lectin was upregulated in activated rat HSCs and in liver fibrosis and promoted higher proliferation and migration of activated HSCs through an ERK1/2 signaling pathway [42].

In human breast cancer, positive Gal-1 expression in CAFs correlated with enhanced regional lymph node metastasis [43]. Silencing Gal-1 in human breast CAFs markedly reduced the expression of α-SMA, fibroblast activation protein (FAP), and FN, while transfection with Gal-1 in NFs dramatically increased the expression of α-SMA, FAP, and FN. Moreover, CM from CAFs induced the migration and invasion of human breast cancer cells and TGF-β_1 secreted by breast cancer cells induced Gal-1 expression, transdifferentiation and MMP-9 secretion in NFs [44].

Fibroblasts differentiation into myofibroblasts driven by TGF-β_1 was accompanied by up-regulation of Gal-1 via phosphatidylinositide 3-kinase (PI3K)/AKT and p38 MAPK activation; Gal-1 knockdown inhibited TGF-β_1-induced fibroblast differentiation (Fig. 2). Moreover, Gal-1 up-regulation -in the absence of TGF-β_1 treatment- triggered FN and α-SMA expression [45]. In addition, in gastric tumors, Gal-1 knockdown in NFs abolished TGF-β-induced α-SMA expression. Accordingly, Gal-1 promoted TGF-β-driven NF transformation into CAF by enhancing Smad2 activation [34]. Thus, galectins shape the TME, at least in part, by modulating fibroblast signaling programs, through autocrine or paracrine mechanisms.

2.2 Galectin-3 in fibroblasts

Expression and secretion of Gal-3 has been well documented in NFs [46-50] and its silencing at the genetic level led to inhibition of fibroblast proliferation [51]. Similarly, Gal-3 knockout in mouse embryonic fibroblasts (MEFs) or Gal-3-depletion in human skin NFs decreased cell proliferation and increased premature senescence, highlighting a role for Gal-3 in senescence regulation [52]. As a result of their cross-talk with tumor cells, NFs inhibit early proliferation and motility of malignant cells. In this sense, co-cultures of NFs and prostatic PC3 tumor cells revealed that Gal-3 was one of the eight highly secreted proteins to the CM, contributing to inhibition of tumor cell proliferation and motility [50] (Fig. 2).

Interestingly, Gal-3 expressed by myofibroblasts also contributed to inflammatory responses. In fact, in fibrotic liver, Gal-3 exerts growth-promoting effects on HSCs through ERK1/2 activation [42]. Disruption of Gal-3 gene suppressed myofibroblast activation and pro-collagen expression *in vitro* and *in vivo*, an effect that was prevented by adding exogenous Gal-3. Similarly, Gal-3 expression was upregulated in myofibroblasts during *in vivo* liver injury, and Gal-3 knockdown inhibited this effect. Moreover, TGF-β failed to transactivate Gal-3-deficient HSCs [53,54]. Likewise, TGF-

β- and bleomycin-induced lung fibrosis was dramatically reduced in mice deficient in Gal-3, as revealed by reduced TGF-β–induced epithelial to mesenchymal transition (EMT), myofibroblast activation, and collagen production [55]. In intestinal tissues, Gal-3 from epithelial cells was identified as a strong activator of human colon NFs, inducing NF-κB activation and interleukin-8 (IL8) secretion in primary intestinal fibroblasts and stimulating their migration. Likewise; in human intestinal fibroblasts, exogenous Gal-3 also induced IL-8 secretion, promoting inflammation [56,57]. Accordingly, this lectin also induced secretion of IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF), CXCL8, and MMP-3 in fibroblasts, producing high secretion of tumor necrosis factor (TNF) and CCL2, CCL3, and CCL5 chemokines [58]. In rat cardiac NFs, exogenous Gal-3 induced cell proliferation, collagen production, and cyclin D1 expression [59], while in human corneal NFs this lectin induced up-regulated expression of α-SMA and connective tissue growth factor (CTGF), a fibrosis-related growth factor [60] (Fig. 2).

Pioneering experiments demonstrated that Gal-3 overexpression in NFs caused actin microfilament reorganization, anchorage-independent growth and morphological transformation *in vitro*, but not tumorigenicity *in vivo*, while Gal-3 transfection in fibrosarcoma cells resulted in increased lung metastases in mice [61] (Fig. 2). Thus, Gal-3 is a central player in fibroblast differentiation, inducing the conversion of NFs to myofibroblasts with up-regulation of α-SMA [62], displaying multiple roles in different stromal cells [63,64]. In breast carcinoma, CAFs in the tumor stroma revealed positive staining for Gal-3, while Gal-3 expression in tumor stroma negatively correlated with patient prognosis [65,66].

In uterine carcinomas, Gal-3 was detected in single-cell suspensions of stromal CAFs from fresh tumor biopsies [67]. In thyroid tissues, Gal-3 mRNA was detected in stromal NFs with similar levels to those observed in normal thyroid tissues, follicular adenomas and carcinomas [68]. In gastric tissues, Gal-3 has been found in NFs of the submucosal layer [69], mediating cell activation and migration in the gastrointestinal tract [57]. Telomerase reverse transcriptase (TERT) mRNA was shown to be decreased in Gal-3 knockout MEFs, while Gal-3 mRNA was also reduced in TERT knockout MEFs, suggesting that Gal-3 and TERT regulate each other. Overexpression of TERT in human NFs increased cell proliferation, reducing $p27^{kip1}$ (a senescence inducer) protein levels and senescence, while Gal-3 knockdown prevented these effects. Thus, Gal-3 from fibroblasts regulates TERT expression and function in cellular senescence, which was also observed in gastric cancer cells [70].

A critical TME factor is oxygen, and Gal-3 was one of the up-regulated genes in murine NFs under hypoxic conditions. A comparative analysis between wild-type and hypoxia-inducible factor $1\alpha^{-/-}$ fibroblasts demonstrated that Gal-3 up-regulation under hypoxia was HIF-1α -dependent [71]. A HIF-1α-binding site in Gal-3 promoter was found in MEFs and in HeLa cervical cancer cells, and hypoxia induced Gal-3 mRNA, protein, and promoter activity in MEFs derived from wild-type mice but not in those from HIF-$1\alpha^{-/-}$ mice [72] (Fig. 2). Moreover, Gal-3 was found to be a key binding partner of K-Ras GTP protein. In Gal-$3^{-/-}$ MEFs, marked reduction of K-Ras was found, and Gal-3 transfection increased K-Ras GTP activity. In fact, this lectin negatively regulated Let-7 miRNA expression in MEFs, leading to increased K-Ras expression [73], fuelling NF transformation. Thus, a coordinated network of galectins and their glycosylated ligands control fibroblast signaling in the TME.

3. Galectins in vascular and lymphatic endothelial cell compartments

3.1 Galectin-1 in endothelial cells

The role of galectins in vascular endothelium and tumorigenesis has been widely demonstrated [74]. Activated ECs synthesize high amounts of Gal-1 [75,76] and this lectin has been implicated in tumor angiogenesis [77]. Incubation of ECs with CM from prostate carcinoma cells augmented Gal-1 expression, an effect that was critical for tumor-EC adhesion [78]. Modulation of EC programs including EC proliferation, migration, and capillary-tube formation has been well documented *in vitro* and *in vivo* [77,79-81]. Noteworthy, Gal-1 may be synthesized or taken up by ECs to promote angiogenesis in an autocrine or paracrine manner [82]. Whereas Gal-1-*N*-glycan interactions in Kaposi´s sarcoma can link tumor hypoxia to vascularization through HIF-1α-independent mechanisms [80], expression of this lectin involves HIF-1α-dependent pathways in the microenvironment of other tumor types including human renal carcinoma and acute myeloid leukemia [83,84]. Interestingly, in prostate cancer patients Gal-1 directly correlated with tumor vascularization and poor clinical outcome, and promoted vascularization independently of other pro-angiogenic factors [85]. This pro-angiogenic effect was mediated by direct interactions with neuropilin-1 (NRP-1) and/or VEGFR2 and involved signaling via ERK1/2 and AKT [79,86]. Remarkably, Gal-1-driven VEGFR2 phosphorylation was sustained even in the absence of the canonical ligand VEGF, suggesting the potential role of this lectin as a mechanism of resistance to anti-VEGF therapies. Blockade of Gal-1 using neutralizing mAb or disruption of specific ligands (complex *N*-glycan branching) by genetic ablation of the *N*-acetylglucosaminyltransferase V (MGAT5) converted refractory into anti-VEGF sensitive tumors [81]. In contrast, removal of α2,6-linked sialic acid from ECs by disruption of α2,6-sialyltransferase 1 (ST6GAL1) eliminated sensitivity to anti-VEGF mAb and favored tumor angiogenesis by unmasking Gal-1-specific glyco-epitopes [81] (Fig. 3). Notably, Gal-1 blockade also induced vessel normalization as revealed by pericyte coverage of ECs, reduction of tumor hypoxia and augmented influx of immune cells into the tumor parenchyma [81], suggesting a major role for this lectin in connecting ECs, pericytes and immune cell networks. Interestingly, genome-wide functional analysis recently identified Gal-1 as a pivotal regulator of lymphatic vascular growth and maintenance of the lymphatic endothelial phenotype via lectin interaction with VEGFR2 [87].

3.2 Galectin-3 and -8 in endothelial cells

Early studies demonstrated that Gal-3 induces EC tube formation and chemotaxis *in vitro* and *in vivo* in models of breast cancer [88]. In fact, Gal-3 has been proposed as a binding partner for aminopeptidase N/CD13 (APN), a Zn^{2+}-dependent metalloexopeptidase in ECs [89]. Silencing Gal-3 markedly reduced VEGF-A and bFGF-mediated cell migration and angiogenesis *in vitro* and *in vivo*, suggesting that, in contrast to Gal-1, this lectin functions by regulating activity of pro-angiogenic ligands. Disruption of complex branched *N*-glycans in ECs lacking MGAT5 reduced Gal-3-induced angiogenesis. Moreover, function-blocking mAbs against $\alpha_v\beta_3$ integrins markedly reduced Gal-3-induced angiogenesis [90]. Like Gal-1, Gal-3 also binds and activates VEGFR2 in ECs, inducing VEGFR2 phosphorylation. Knocking down Gal-3 or MGAT5 reduced VEGF-A-mediated angiogenesis. Therefore, Gal-3 modulates EC biology by directly interacting with VEGFR2 *N*-glycans and increasing the angiogenic response to its canonical ligand [91] (Fig. 3).

Like Gal-1 and Gal-3, Gal-8 is one of the most prominent galectins found in ECs [76]. Either exogenously added or endogenously regulated, this two-CRD galectin

promotes EC migration and capillary tube formation, and facilitates angiogenesis *in vivo*. Remarkably, activated leukocyte cell adhesion molecule (ALCAM/CD166) was identified as a specific Gal-8-binding partner in vascular ECs (Fig. 3), and anti-ALCAM blocking antibodies diminished Gal-8-induced angiogenesis [92]. Furthermore, Gal-8 also bound to LECs and promoted lymphangiogenesis [93]. A mechanistic analysis revealed that Gal-8 induces lymphangiogenesis by interacting with podoplanin (PDPN) and $\alpha_1\beta_1$ or $\alpha_5\beta_1$ integrins in a VEGFR3-independent fashion. However, Gal-8 also potentiates VEGF-C-induced lymphangiogenesis *in vitro* and *in vivo*, through a mechanism involving PDPN/Gal-8/integrin interactions and potentiation of VEGFR3 signaling [94] (Fig. 3). Thus, galectins, particularly Gal-1, -3 and -8, promote angiogenesis and lymphangiogenesis via interaction with a selected repertoire of glycosylated receptors on ECs and LECs.

4. Galectins in immune cell compartments

Galectins favor tumor growth by inhibiting immune surveillance through several mechanisms, including promotion of T-cell apoptosis, suppression of T-cell activation, shift toward an anti-inflammatory Th2 profile, expansion of $Foxp3^+$ T regulatory cells (Tregs), differentiation of IL-27^+ IL-10^+ tolerogenic dendritic cells (DCs), inhibition of NK cell cytotoxicity, and polarization of macrophages toward an M2 phenotype [95,96]. These immunoregulatory pathways are summarized in Fig. 1.

4.1 Galectin-1 induces T-cell apoptosis and shifts the balance toward a Th2 cytokine profile

In different cancer settings, Gal-1 has been proposed to induce T-cell death. Expression of this lectin correlated with the extent of tumor-induced T-cell death in both murine and human melanoma cells. In a melanoma model, Gal-1 blockade markedly enhanced tumor rejection by enhancing Th1 responses, suggesting that Gal-1 contributes to tumor immune privilege by modulating T-cell survival and cytokine production [97]. In head and neck squamous cell carcinomas, Gal-1 overexpression inversely correlated with the number of infiltrating T cells and was an independent prognostic factor for shorter overall survival [98].

In neuroblastoma cells, Gal-1 acted as a pro-apoptotic soluble factor that compromised T-cell function. Inhibition of Gal-1 gene expression reduced primary tumor growth and prevented metastasis in a murine model. Splenocytes from mice inoculated with Gal-1-silenced neuroblastoma cells showed higher cytotoxic activity when compared with splenocytes from mice inoculated with Gal-1-overexpressing tumor cells. At the cellular level, tumor-derived Gal-1 impaired T-cell effector functions by promoting T-cell apoptosis, reducing IFN-γ secretion and decreasing T-cell recruitment to tumor parenchyma [99].

In Lewis lung carcinoma (LLC), tumoral Gal-1 was found to be more important than host Gal-1 in enhancing tumor growth and metastasis, through immune-mediated mechanisms. When wild-type or Gal-1 null mice were inoculated subcutaneously with control or Gal-1-silenced LLC cells, expression of tumor Gal-1, rather than that of the host, was essential for tumor growth and spontaneous metastasis. Lung metastasis was only found in mice bearing Gal-1-expressing tumors and not in mice bearing Gal-1-silenced tumors. Increased apoptosis of $CD8^+$- and $CD4^+$-T cells was observed in the microenvironment of control versus Gal-1 knockdown tumors in both wild-type and Gal-1 null mutant mice. The effects of Gal-1 on tumor growth and metastasis were abrogated in immunocompromised mouse models, highlighting the critical role of immune cells as targets of the pro-tumorigenic effects of this lectin. In addition, more

viable $CD4^+$ and $CD8^+$ T-cells were detected in the hypoxic areas of Gal-1 knockdown compared to control Gal-1-sufficient tumors. In fact, intratumoral hypoxia augmented Gal-1 secretion and diffusion, which promoted T-cell apoptosis [100].

Interestingly, in co-cultures of primary human PSCs and T cells, overexpression of Gal-1 in PSCs induced T cell apoptosis while knockdown of Gal-1 in PSCs increased $CD4^+$ T cell and $CD8^+$ T cell viability. Supernatants from T cells co-cultured with Gal-1-overexpressing PSCs increased the levels of Th2 cytokines (IL-4 and IL-5) and decreased the amounts of Th1 cytokines (IL-2 and IFN-γ). Thus, PSC-derived Gal-1 induces T-cell apoptosis and contributes to Th2 cytokine shift, regulating PSC-dependent immune privilege in the pancreatic TME [101]. A mechanistic analysis of Gal-1 effects revealed the ability of this lectin to selectively delete Th1 and Th17 lymphocytes through differential glycosylation of these cells. In brief, Th1 and Th17 cells share the repertoire of glycans that are important for Gal-1 binding and T-cell death. In contrast, Th2 cells are protected from Gal-1 through α2,6 sialylation of these cells [102]. Thus, Gal-1 impairs T-cell function by promoting T-cell apoptosis and modulating T helper cytokine balance.

4.2 Galectin-1 promotes expansion of $Foxp3^+$ and $Foxp3^-$ regulatory T cells

Inducible $CD4^+$ regulatory T cells (iTregs) are generated outside the thymic compartment to regulate peripheral immune tolerance, whereas thymus-derived naturally occurring $CD4^+$ regulatory T cells (nTregs) are generated within the thymus. Notably, iTregs may be divided into two subsets: the classical TGF-β_1-induced $CD4^+$ $Foxp3^+$ Tregs and the $CD4^+$ $Foxp3^-$ type 1 regulatory T (Tr1) cells [103]. Upregulation of Gal-1 was evident in $Foxp3^+$ Tregs compared to activated effector T cells at the mRNA level [104]. Garin and colleagues confirmed the abundance of Gal-1 protein in $Foxp3^+$ Tregs and provided evidence of its contribution to the suppressive activity of these cells. Disruption of Gal-1 attenuated the inhibitory effects of human and mouse $Foxp3^+$ Tregs, suggesting the involvement of this lectin in Treg-mediated immunosuppression [105]. In a breast cancer model, silencing tumor-derived Gal-1 reduced the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs within tumor, draining lymph nodes, spleen and lung metastases. Tregs isolated from mice bearing Gal-1-depleted tumors exerted poor immunosuppressive activity when co-cultured with conventional T cells. Considerable downregulation of linker of activated T cells (LAT) was detected in Tregs isolated from the metastatic lungs or from the tumor-draining lymph nodes of mice bearing Gal-1-deficient tumors, suggesting an association between Gal-1, LAT and the immunosuppressive activity of these cells [106].

Reed-Sternberg cells from classical Hodgkin lymphoma overexpressed Gal-1 through an AP-1-dependent enhancer. In co-cultures of activated T cells and Hodgkin tumor cell lines, blockade of tumoral Gal-1 enhanced T-cell viability and restored the Th1/Th2 cytokine balance. In contrast, Gal-1 treatment of activated T cells favored secretion of Th2 cytokines and facilitated expansion of $CD4^+$ $CD25^{high}$ $Foxp3^+$ Tregs, protecting Reed-Sternberg cells from immune attack [107]. Of note, expression of Gal-1 together with c-Jun contributed to delineate Hodgkin lymphoma from other lymphoma types [108]. On the other hand, Gal-1 binding to unpolarized activated T cells induced an immunoregulatory signature defined by IL-10, suggesting that this lectin also induces differentiation of $Foxp3^-$ Tr1 cells. In fact, Gal-1 association with cell surface glycoproteins on CD4 T cells induced polarization toward an immunoregulatory Tr1 phenotype characterized by IL-10 production through mechanisms involving IL-21 and the c-Maf and aryl hydrocarbon receptor (AHR) pathways [109]. Thus, Gal-1-glycan interactions foster differentiation of both $Foxp3^+$ Tregs and $Foxp3^-$ Tr1 cells.

4.3 Galectin-1 induces tolerogenic dendritic cells

Soluble factors secreted by tumor cells or upregulated in the TME may instruct dendritic cells (DCs) to become tolerogenic and suppress T-cell responses. We demonstrated that DCs differentiated or matured in a Gal-1-enriched microenvironment acquired regulatory or tolerogenic signatures characterized by low expression of CD11c (mouse DCs) or CD1a (human DCs), high expression of CD45RB, phosphorylation of signal transducer and activator of transcription-3 (STAT-3) and secretion of IL-27 and IL-10. When transferred *in vivo*, these DCs promoted T-cell tolerance in antigen-specific and cancer settings (particularly melanoma), generating an immunoregulatory circuit linking Gal-1, IL-27 and IL-10 [110]. Moreover, when mouse bone marrow-derived DCs (BMDCs) were cultured in the presence of CM collected from Gal-1-expressing neuroblastoma cells, lower expression of co-stimulatory molecules was detected on $CD11c^+$ BMDCs. Thus, neuroblastoma-derived Gal-1 controls effector T cell responses indirectly via promotion of immature or tolerogenic DCs [99].

In lung cancer settings, Gal-1 also promoted tolerogenic DCs, which in turn favored the induction of $Foxp3^+$ Tregs. Further examination of the mechanisms underlying these effects revealed the ability of this lectin to alter the function of DCs through an IL-10-dependent autocrine effect, regulated by the inhibitor of DNA binding 3 (Id3) transcription factor [111]. Interestingly, recent studies showed that expression of the special AT-rich sequence-binding protein-1 (Satb1) chromatin organizer on tumor-associated DCs controls their immunosuppressive activity through mechanisms involving Gal-1. Although Satb1 expression on wild-type DCs was sufficient to accelerate tumor growth when admixed *in vivo* with ovary cancer cells, this effect was abrogated when DCs lacked Gal-1 expression [112]. Moreover, Hsu and colleagues found that, in response to Gal-1, CAFs up-regulated expression of tryptophan 2,3-dioxygenase (TDO2) and produced the tryptophan metabolite kynurenine that inhibited DC differentiation and induced lung cancer growth. These results demonstrate a critical role for Gal-1 in creating immunosuppressive microenvironments by linking lung cancer cells, CAFs and tolerogenic DCs [113].

4.4 Galectin-1 impairs NK cell function

NK cells protect against early tumor formation through mechanisms involving cytotoxic activity against transformed cells and secretion of pro-inflammatory cytokines. In Kaposi´s sarcoma murine models, we demonstrated therapeutic benefits of a Gal-1–specific mAb through mechanisms involving inhibition of aberrant tumor angiogenesis and increased recruitment of NK cells [80]. Whereas no differences were found in the frequency of infiltrating $B220^+$ B cells and $F4/80^+$ macrophages between xenografts treated with a Gal-1–specific mAb [114] or isotype control, Gal-1 blockade resulted in an increased percentage of tumor-infiltrating NK cells [80].

Furthermore, Baker and coworkers showed that glioblastoma cells suppress NK cell-mediated immune surveillance by overexpressing Gal-1. Conversely, Gal-1-deficient glioma cells could be eradicated by host NK cells before the initiation of an antitumor T-cell response. Knocking down Gal-1 in glioma cells led to rapid accumulation of cytotoxic NK cells within the brain TME allowing complete tumor eradication in the absence of an ongoing anti-tumor T-cell response. *In vitro*, Gal-1-deficient glioma cells were 3-fold more sensitive to NK cell-mediated tumor lysis than Gal-1 overexpressing tumor cells [115]. In fact, knocking down Gal-1 in glioma cells improved their inflammatory status leading to rapid recruitment of $Gr\text{-}1^+$ $CD11b^+$

myeloid cells and NK cells, inducing tumor clearance in mice lacking mature T- and B-cells. Immunodepletion of Gr-1^+ myeloid cells allowed growth of Gal-1-deficient glioma cells despite the presence of NK cells, thus demonstrating an essential role for myeloid cells in the clearance of Gal-1-deficient glioma [116]. Thus, NK cells as well as myeloid cells are essential targets of the immune inhibitory activity of Gal-1 in the TME.

4.5 Galectin-1 contributes to the immunoregulatory and pro-tumoral activities of macrophages and myeloid-derived suppressor cells

In addition to DCs, the TME is often infiltrated by other myeloid cells such as macrophages and myeloid-derived suppressor cells (MDSCs). Within the monocyte/macrophage compartment Gal-1 contributes to de-activation of these cells and polarization from an M1 pro-inflammatory toward an M2 anti-inflammatory phenotype [117-120]. This effect has been confirmed in microglia cells during central nervous system inflammation [121]. Recently, Van Woensel and coworkers showed that intranasal delivery of Gal-1 siRNA induced a remarkable switch in the TME composition, reducing macrophage polarization from M1 to M2 and inhibiting recruitment of monocytic MDSCs during glioblastoma multiforme progression [122]. In this sense, Gal-1 levels positively correlated with soluble CD163, a biomarker of M2 macrophage activation in sera from multiple myeloma patients [123].

MDSCs are a heterogeneous population of immature cells shown to accumulate in the blood and TME of human and murine tumors, which can influence anticancer immunity by altering macrophage, NK cell and T-cell effector functions [124]. Two major MDSC subsets are recognized: granulocytic (Ly-$6G^{high}$ $CD11b^+$ Ly-$6C^{low}$) and monocytic (Ly-$6G^{low}$ $CD11b^+$ Ly-$6C^{high}$) MDSCs [125]. In ovarian cancer models, MDSCs and γδ-T cells are major Gal-1 producers, contributing to systemic immunosuppression. This effect is tightly controlled by commensal microbiota, TLR5 signaling and pro-inflammatory cytokines particularly IL-6 [126]. Briefly, TLR5-dependent IL-6 production triggered by commensal bacteria favored malignant progression at extramucosal locations through mobilization of MDSCs and further expansion of γδ-T cells producing high amounts of Gal-1 [126]. On the other hand, in glioblastoma models, Gal-1 silencing reduced recruitment of MDSCs to the TME highlighting a role for this lectin in modulating the function of this heterogeneous immunosuppressive cell population [120].

Finally, in patients with chronic lymphocytic leukemia (CLL), Gal-1 was found to be upregulated in nurse cells, a subset of non-tumoral $CD68^+$ myeloid cells responsible of sustaining proliferation and survival of leukemic B cells. In co-cultures of nurse cells and CLL cells, this lectin augmented B-cell receptor signaling, providing an appropriate niche for tumor progression [127].

4.6 Immunoregulatory effects mediated by Gal-3, -8 and -9

Gal-3 has been shown to play key immunomodulatory roles promoting T-cell apoptosis and modulating T-cell receptor (TCR) signaling [128,129]. Extracellular Gal-3 promotes apoptosis in both human and murine T cells upon binding to T-cell surface glycoproteins CD7 and CD29, providing an alternative mechanism by which cancer cells may evade immune responses [128]. Similarly, colorectal tumor-reactive T cells become apoptotic in response to Gal-3 signaling leading to enhanced tumor growth *in vitro* and *in vivo* [130]. Accordingly, a glycopeptide capable of binding Gal-3 inhibited Gal-3-mediated T-cell apoptosis and suppressed prostate cancer metastasis [131].

Remarkably, human $CD8^+$ tumor-infiltrating lymphocytes (TILs) from different solid tumors and ascites, but not CD8+ circulating T cells, showed impaired IFN-γ secretion following *ex vivo* restimulation. However, these cells evidenced enhanced IFN-γ production upon treatment with the saccharide *N*-acetyllactosamine (a galectin-specific inhibitor) [132]. Furthermore, GCS-100, a modified polysaccharide pectin that interrupts Gal-3 signaling, as well as *N*-acetyllactosamine treatment promoted colocalization of the TCR with coreceptors CD8 or CD4 on the surface of TILs, by detaching Gal-3 from the cell surface and increasing TCR mobility [133]. More recently, Gal-3 has been shown modulate the availability of glycosylated soluble factors including IFN-γ, thus limiting T-cell infiltration and antitumor immunity [134]. Hence, administration of Gal-3 antagonists results in higher activation of the tumor infiltrating T cells with critical implications in tumor immunity.

In pancreatic ductal carcinoma models, tumor-derived Gal-3 limited the expansion of plasmacytoid DCs. Interestingly, this lectin bound to antigen-experienced $CD8^+$ T cells only in the TME, and Gal-3-deficient mice exhibited improved $CD8^+$ T-cell effector function and increased expression of several inflammatory genes. At the molecular level, Gal-3 bound to lymphocyte activation gene-3 (LAG-3), an inhibitory checkpoint molecule engaged by tumors to suppress $CD8^+$ T-cell responses [135]. Thus, blocking Gal-3 may contribute to checkpoint blockade immunotherapies by interrupting LAG-3 function. On the other hand, Gal-9 has been shown to bind the T-cell immunoglobulin and mucin domain-3 (TIM-3) [136], another important checkpoint molecule, suggesting essential roles of individual galectins in engaging immune inhibitory receptors.

In addition to its role in T-cell biology, Gal-3 expression has also been associated to macrophage polarization towards an M2 phenotype [137], as well as to inhibition of NK cell activity [138-140]. This lectin impairs NK cell functions through binding to the NKp30 receptor, thus allowing tumor cell evasion of NK cell attack [140]. Additionally, Gal-3 can impair NK cell activity by inhibiting interactions between NKG2D, an activating NK cell receptor and major histocompatibility complex class I-related chain A (MICA) [139], a ligand highly expressed on the surface of tumors, endothelial and lymphoid cells [139,141].

Remarkably, Gal-8 promotes the differentiation of $Foxp3^+$ Tregs through modulation of TGF-β_1 and IL-2 signaling [142], in addition to its documented roles in tumor angiogenesis [92] and migration [143], emphasizing the potential role of this lectin in tumor immunosuppression. Moreover, Dardalhon and colleagues found that activation of the Gal-9/Tim-3 pathway increased the number of $CD11b^+$ $Ly\text{-}6G^+$ granulocytic MDSCs, which negatively controlled T-cell responses *in vivo* [144]. Furthermore, Gal-9 promoted Treg differentiation and stability through binding to CD44 and modulation of TGF-β_1-driven SMAD signaling [145]. More recently, in the context of pancreatic adenocarcinoma, Gal-9 has been shown to interact with Dectin-1 on the surface of tumor-associated macrophages reprogramming these cells toward an immunosuppressive phenotype. Blockade of the Dectin 1/Gal-9 axis reinvigorated $CD4^+$ and $CD8^+$ T cell responses and contributed to eradicate pancreatic tumors in different models [146]. Strikingly, granulocytic MDSCs infiltrating pancreatic ductal adenocarcinoma were found to be the main source of Gal-9, thus emphasizing the relevance of non-tumoral galectins in tumor progression [146]. Thus, different members of the galectin family may influence tumor immunity by modulating lymphoid and myeloid programs.

Conclusions

In this review we focus on the pro-tumorigenic roles of galectins in the TME. First, we discuss the role of galectins within the fibroblast compartment. An emerging hypothesis proposes fibroblasts as initiators of cancer pathogenesis, due to their inherent heterogeneity, plasticity and function, which enable them to modulate normal and tumor epithelia. While NFs from disease-free tissues inhibit tumor growth, CAFs favor tumor progression as they secrete soluble factors that enhance tumor cell proliferation, angiogenesis and immunosuppression. CAFs can also suffer vasculogenic mimicry, as tumor cells do, and may promote angiogenesis and tumor-immune escape by releasing a variety of pro-angiogenic and immunosuppressive mediators including galectins. Second, we discuss the role of galectins, particularly Gal-1, Gal-3 and Gal-8 in modulating angiogenesis and lymphangiogenesis, effects that ultimately lead to tumor progression. Finally, we analyze the role of galectins in modulating immune signaling programs influencing T cells, NK cells, DCs, macrophages and MDSCs. Particularly we highlight the role of immune cells, both of lymphoid and myeloid origin, as major galectin sources and regulators of local and distant tumor growth. Hence, galectin-dependent circuits involving CAFs, ECs, LECs and immune cells, cooperate with tumor cells to create hostile microenvironments that sustain tumor growth and metastasis. Thus, targeting selected members of the galectin family in both tumoral and non-tumor cells, may contribute to extinguish tumor progression by controlling abnormal vascularization, thwarting immunosuppression and preventing metastasis.

Noteworthy, most experimental models used to study galectins in cancer show several limitations precluding clear discrimination of the role of tumor-derived galectins versus those expressed in non-tumoral cells within the TME. Most in vivo studies have been accomplished in galectin-deficient mice or wild-type mice [80-82], which have been inoculated with syngeneic tumor cells expressing normal, upregulated or downregulated galectin levels. However, the contribution of each cellular compartment to the overall galectin effects has not yet been elucidated. In this regard, some *in vivo* models including selective immunodepletion of myeloid cell populations in immunodeficient mice (with impaired T- and B-cells) inoculated with Gal-1-deficient tumor cells were used to dissect the role of individual immune cell types as selective targets of this protein [116]. Further studies are awaited to dissect the role of galectins in the TME using conditional knockout selectively overexpressing or eliminating galectins in different TME compartments [147], including CAFs, pericytes, ECs, innate immune cells (macrophages, neutrophils, DCs, MDSCs) as well as adaptive immune cells (T cells, B cells). Furthermore, the use of clinically-relevant, genetically engineered mouse models crossed with galectin-deficient strains will be important to faithfully recapitulate clinical features of the human neoplastic disease.

Different galectin blocking compounds have been tested *in vitro* and/or *in vivo*, including specific anti-galectin neutralizing antibodies [80,81]. Moreover, glycan-based galectin inhibitors have also been investigated and proved to be promising low-nanomolar galectin antagonists [148-153]. Furthermore, plant pectins and galactomannans have also shown antitumoral activities by virtue of their ability to bind galectins [154,155]; yet recent studies demonstrated that these naturally-occurring saccharides exhibit low inhibitory potency towards galectins CRDs [156]. Peptide-based galectin inhibitors such as anginex [77,157] or its derivatives 6DBF7 [158] or OTX008 [159,160] have also been shown to inhibit galectin functions including tumor angiogenesis and cell migration. Further studies should be aimed at evaluating this

portfolio of anti-galectin compounds in pre-clinical settings and clinically-relevant tumor models.

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Disclosure of Potential Conflicts of Interest

The authors declare no competing financial interests.

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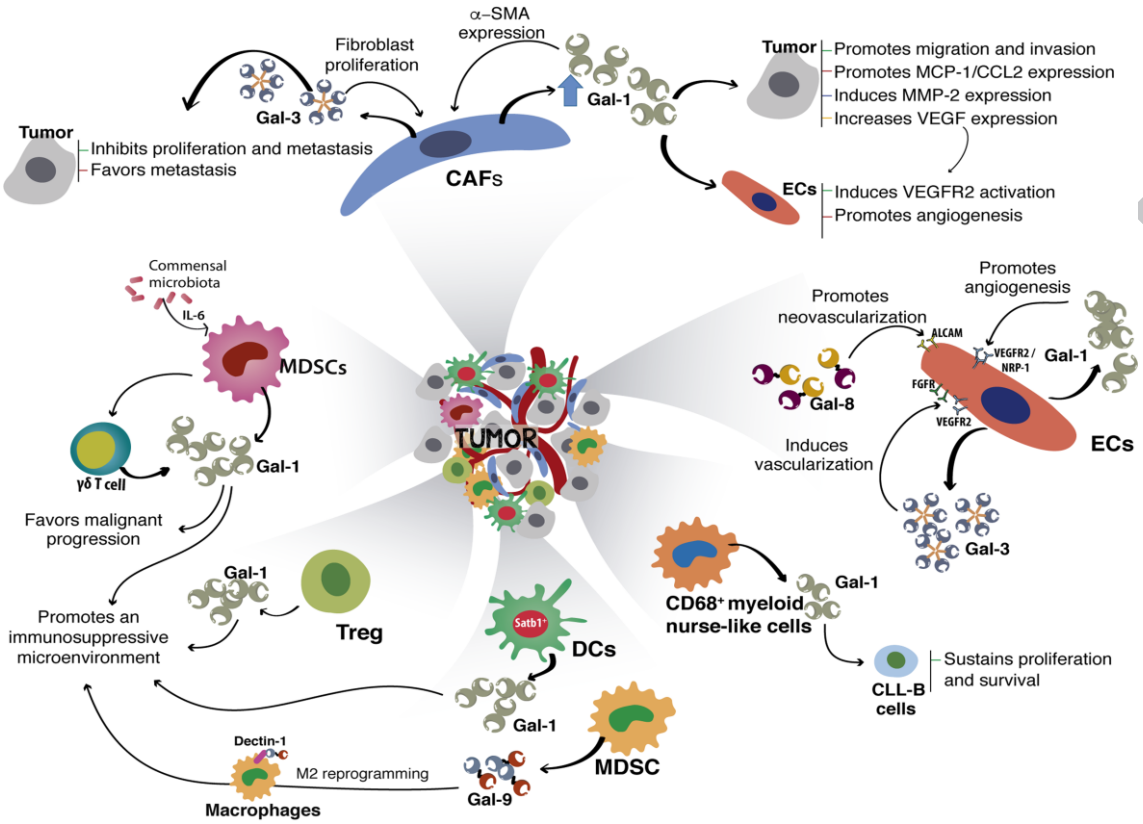
Figure Legends

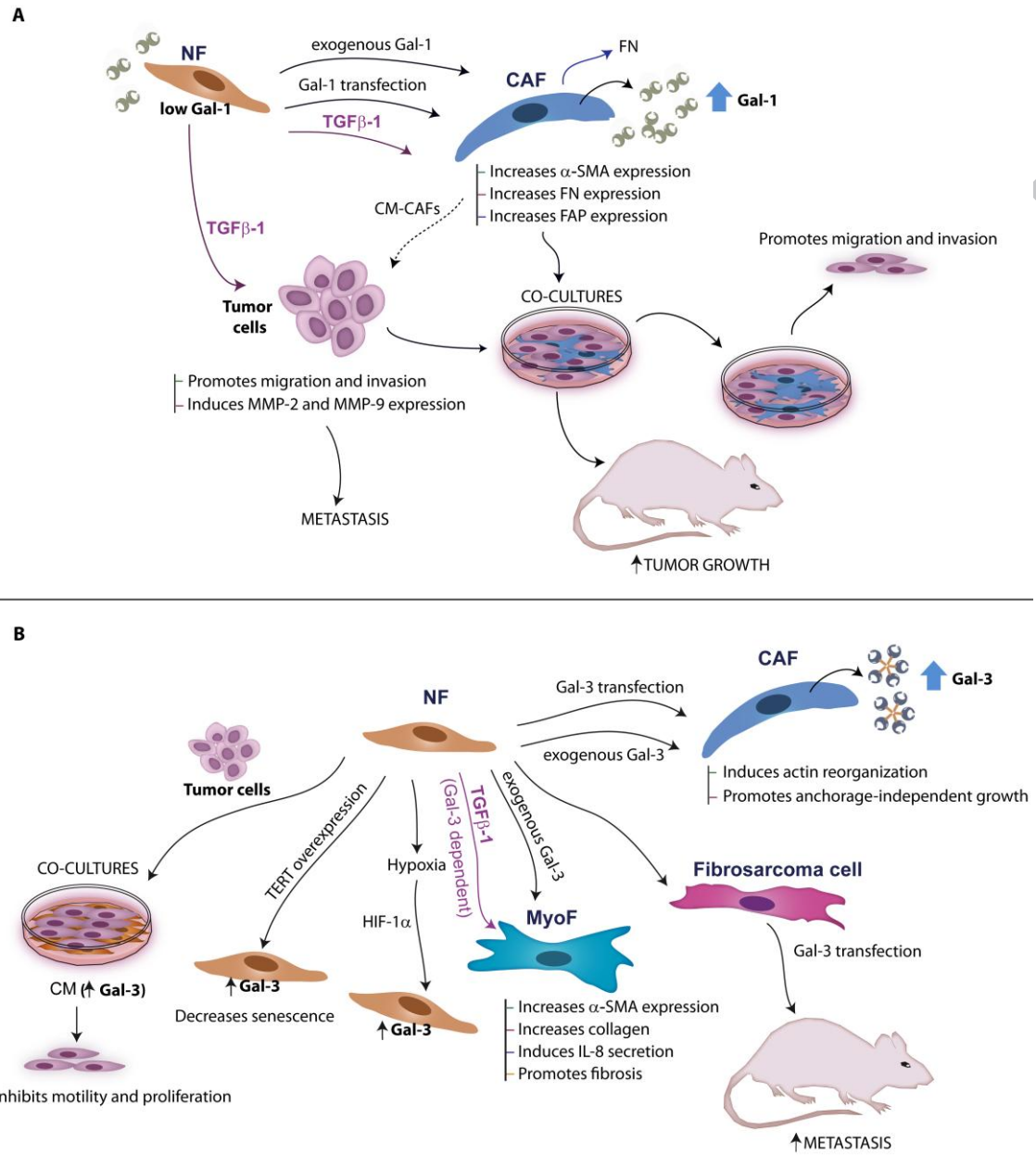
Figure 1: Functions of galectins in the tumor microenvironment. Different effects of galectins, particularly those produced by non-tumoral cells are illustrated. Cancer-associated fibroblasts (CAFs) express and secrete galectins (e.g. Gal-1 and Gal-3) which control tumorigenic events including tumor cell migration, proliferation, angiogenesis, immunosuppression and metastasis, and promote expression of metalloproteinases (e.g. MMP-2), chemokines (e.g. MCP-1/CCL-2) and pro-angiogenic factors (e.g. VEGF). Endothelial cells (ECs) also synthesize galectins which contribute to tumor progression through different mechanisms. Gal-1, -3- and -8 control both vascular EC and lymphatic EC (LEC) programs, and modulate angiogenesis and lymphangiogenesis by co-opting different signaling pathways and glycosylated receptors, including VEGFR2, VEGFR3, neuropilin-1 (NRP-1) and podoplanin. Innate and adaptive immune cells, including myeloid-derived suppresssor cells (MDSCs), γδ-T cells, regulatory T cells (Tregs), tolerogenic dendritic cells (DCs), macrophages and myeloid-derived nurse-like cells can influence tumorigenic programs via galectin-dependent circuits that foster immunosuppressive TME. The collective action of galectins and their specific glycans, modulating fibroblast, vascular EC, LEC and immune cell compartments, contribute to reprogram the TME by controlling angiogenesis, immunosuppression and metastasis. Abbreviations: α-SMA: α-smooth muscle actin; MCP-1/CCL2: monocyte chemotactic protein-1; MMP-2: matrix metalloproteinase-2; NRP-1: neuropilin-1; VEGF: vascular endothelial growth factor; VEGFR2: vascular endothelial growth factor receptor-2; FGFR: fibroblast growth factor receptor; ALCAM (CD166): activated leukocyte cell adhesion molecule; IL-6: interleukin 6.

Figure 2: Galectin functions in tumor-associated fibroblasts. A, Gal-1 in tumorigenesis: Exogenous Gal-1 induces normal fibroblasts (NFs) transdifferentiation to cancer-associated fibroblasts (CAFs). CAFs overexpress and secrete Gal-1, which remains associated to the extracellular matrix (ECM). Transfection of Gal-1 gene (*Lgals1*) in NFs increases the expression of α-smooth muscle actin (α-SMA), fibroblast activation protein (FAP) and fibronectin (FN) inducing a CAF phenotype. Conditioned medium (CM) obtained from CAFs promotes tumor cell migration and invasion, and *in vivo* metastasis. CM obtained from Gal-1-transfected NFs induces tumor cell migration and invasion. Co-cultures of tumor cells and CAFs stimulated *in vitro* tumor cell migration and invasion, and *in vivo* metastasis. TGF-β_1 induces Gal-1 expression in NFs and fibroblast transdifferentiation. **B, Gal-3 in tumorigenesis:** Co-cultures of tumor cells and NFs show increased Gal-3 secreted to the CM, which inhibits tumor cell proliferation and motility. Exogenous Gal-3 induces transdifferentiation of NFs to CAFs, which in turn secrete high levels of this lectin. Gal-3 transfection in NFs promotes actin microfilament reorganization and anchorage-independent growth, while Gal-3 transfection in fibrosarcoma cells promotes *in vivo* metastasis. NFs are differentiated into myofibroblasts (MyoF) upon treatment with exogenous Gal-3 or with TGF-β_1 (in a Gal-3-dependent manner), promoting fibrosis. Under hypoxia, NFs up-regulate Gal-3 in a HIF-1α–dependent fashion. Moreover, telomerase reverse transcriptase (TERT) transfection in NFs also up-regulates Gal-3, delaying senescence.

Figure 3: Modulation of vascular and lymphatic endothelial cell signaling by galectins. A, Gal-1: Gal-1 directly binds to complex branched *N*-glycans on VEGFR2, and promotes VEGF-A-independent signaling. In tumors resistant to anti-VEGF treatment, vascular ECs display reduced α2,6 sialylation and increased *N*-glycan

branching, thus facilitating Gal-1 binding and angiogenesis. In contrast, in tumors sensitive to VEGF blockade, ECs show increased α2,6 sialylation, an effect that interrupts Gal-1 binding and inhibits vascularization. Blockade of Gal-1 or interruption of MGAT5-dependent *N*-glycan branching converted anti-VEGF resistant into anti-VEGF sensitive tumors by attenuating compensatory angiogenesis. Moreover, Gal-1 contributes to lymphangiogenesis by modulating LEC growth via specific interaction with VEGFR2. **B, Gal-3:** In vascular ECs, Gal-3 directly interacts with VEGFR2, inducing its phosphorylation and amplifying the angiogenic response to VEGF-A. Gal-3 promotes clustering of VEGFR2 through binding to complex branched *N*-glycans generated by MGAT5. In the presence of Gal-3, VEGFR2 is retained on the EC surface and angiogenesis proceeds. **C, Gal-8:** Gal-8 controls LEC programs through binding to podoplanin (PDPN), β_1 integrin and VEGFR3. In LECs, Gal-8 mediates lymphangiogenesis by interacting with PDPN and $\alpha_1\beta_1$ or $\alpha_5\beta_1$ integrins in a VEGFR3-independent pathway. Additionally, Gal-8 promotes VEGF-C-induced lymphangiogenesis *in vitro* and *in vivo* through a VEGFR3-dependent pathway, in which PDPN/Gal-8/integrin interactions potentiate VEGF-C/VEGFR3 signaling. In vascular ECs, Gal-8 promotes angiogenesis *in vitro* and *in vivo* through binding to ALCAM (CD166). Abbrevaitions: α2,6 SA: α2,6-linked sialic acid; α2,3 SA: α2,3-linked sialic acid.

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Increases α-SMA expression
Increases FN expression
Increases FAP expression
CM-CAFs
TGFβ-1
Tumor cells
Promotes migration and invasion
Induces MMP-2 and MMP-9 expression
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TUMOR GROWTH
B
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Gal-3 transfection
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Induces actin reorganization
Promotes anchorage-independent growth
CO-CULTURES
CM (↑ Gal-3)
Inhibits motility and proliferation
TERT overexpression
↑Gal-3
Decreases senescence
Hypoxia
HIF-1α
↑Gal-3
TGFβ-1
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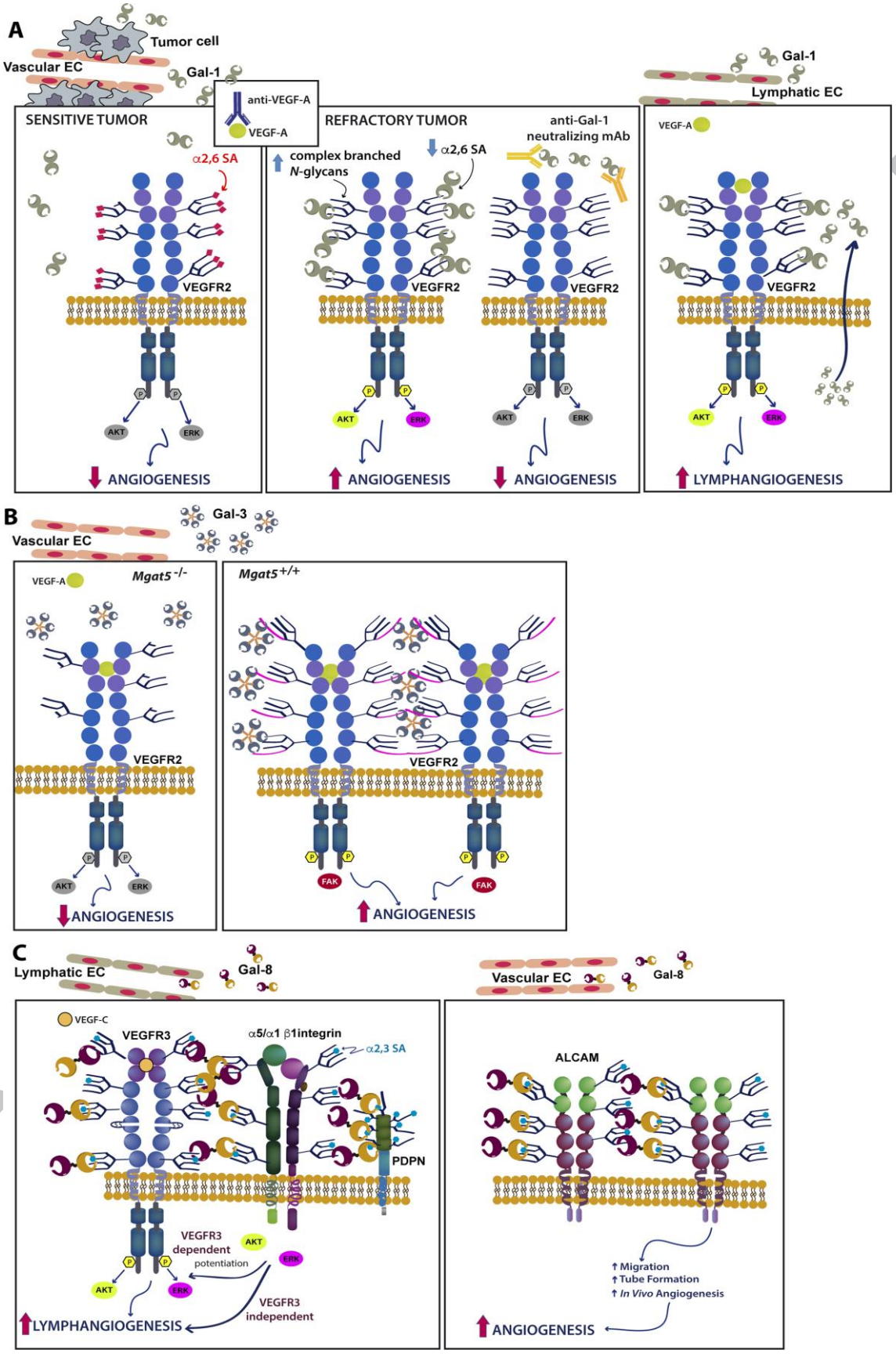
Increases collagen
Induces IL-8 secretion
Promotes fibrosis
Fibrosarcoma cell
Gal-3 transfection
↑METASTASIS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VEGF-A
α2,6 SA
complex branched N-glycans
α2,6 SA
VEGFR2
AKT
ERK
ANGIOGENESIS
LYMPHANGIOGENESIS
B
Gal-3
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VEGF-A
Mgat5 -/-
Mgat5 +/+
FAK
C
Lymphatic EC
Gal-8
Vascular EC
VEGF-C
VEGFR3
α5/α1 β1integrin
α2,3 SA
PDPN
ALCAM
VEGFR3 dependent potentiation
VEGFR3 independent
↑ Migration
↑ Tube Formation
↑ In Vivo Angiogenesis

Highlights

-Galectins influence tumor progression by reprogramming the tumor microenvironment
-Galectins are expressed and released by both tumoral and non-tumoral cells
-Galectins and glycans modulate fibroblast, endothelial and immune cell programs
-Galectins control tumor immunity by targeting lymphoid and myeloid cell function