Glycobiology Advance Access published August 12, 2014

Regulatory role of glycans in the control of hypoxia-driven angiogenesis and sensitivity to anti-angiogenic treatment

Diego O. Croci[1], Juan P. Cerliani[1], Nicolas A. Pinto[1], Luciano G. Morosi[1] and Gabriel A. Rabinovich[1,2]

[1]Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental (IBYME), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), 1428 Buenos Aires, Argentina

[2]Laboratorio de Glicómica Funcional. Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina

To whom correspondence should be addressed: e-mail: gabyrabi@gmail.com (G.A.R.)

Abstract

Abnormal glycosylation is a typical hallmark of the transition from healthy to neoplastic tissues. Although the importance of glycans and glycan-binding proteins in cancer-related processes such as tumor cell adhesion, migration, metastasis and immune escape has been largely appreciated, our awareness of the impact of lectin-glycan recognition in tumor vascularization is relatively new. Regulated glycosylation can influence vascular biology by controlling trafficking, endocytosis and signaling of endothelial cell (EC) receptors including vascular endothelial growth factor receptors (VEGFRs), platelet endothelial cell adhesion molecule (PECAM), Notch and integrins. In addition, glycans may control angiogenesis by regulating migration of endothelial tip cells and influencing EC survival and vascular permeability. Recent evidence indicated that changes in the EC surface glycome may also serve 'on-and-off' switches that control galectin binding to signaling receptors by displaying or masking specific glycan epitopes. These glycosylation-depedent lectin-receptor interactions can link tumor hypoxia to EC signaling and control tumor sensitivity to anti-angiogenic treatment.

Keywords: Angiogenesis; Galectins; Galectin-1; Glycosylation; Hypoxia; Immunotherapy; Lectins; Vasculature

Glycobiology of the tumor microenvironment

Glycosylation, the dynamic process responsible for creating the complex cellular portfolio of glycan structures, involves the synchronized action of glycosyltransferases and glycosidases (Ohtsubo and Marth, 2006). This process is dynamically regulated during cellular activation and differentiation and changes dramatically in response to cellular stress and environmental signals (Rabinovich and Croci, 2012). At the cellular level, different glycan structures can selectively regulate trafficking, localization and turnover of glycoprotein receptors (Boscher et al., 2011) and play essential roles in cellular recognition, adhesion, communication and signaling (Ohtsubo and Marth, 2006). Deciphering the information encoded by the cellular glycome has proven to be challenging because of the non-template nature of carbohydrate synthesis and the macro- and micro-heterogeneity of glycosylation patterns (Mariño et al., 2010). However, it is now clear that endogenous glycan-binding proteins or lectins can decode and translate glycan-containing information into functional cellular responses (van Kooyk and Rabinovich, 2008).

Abnormal glycosylation has been largely appreciated as a hallmark of the transition from healthy to neoplastic tissue (Varki et al., 2009). In fact, glycans and glycan-binding proteins contribute to tumor progression by influencing homotypic and heterotypic cellular interactions, promoting tumor cell migration and metastasis and fostering immune escape strategies. Moreover, it has become increasingly evident that glycans also play important roles in tumor angiogenesis. Here we will review recent data on the role of lectin-glycan recognitions sytems in endothelial cell (EC) signaling and tumor vascularization and will discuss their contribution to angiogenic rescue programs developed in response to anti-angiogenic therapy.

Glycans in vascular signaling programs: regulation and function

Blood vessels deliver oxygen and nutrients, remove waste and represent the central highway through which immune cells migrate (Potente et al., 2011). Vessels are comprised of a monolayer of ECs that are covered by vascular smooth muscle cells (also called pericytes) that establish direct cell-cell interactions and offer mechanical and functional support (Kerbel, 2008). In response to environmental cues, ECs are capable of displaying a variety of metabolic and immunological functions (Potente et al., 2011). In adult healthy organs, vessels are quiescent and rarely form new branches. However, under pathological conditions such as cancer, ischemia, inflammation and infectious diseases, ECs restart growing programs and respond to angiogenic signals to form new blood vessels from existing ones; a process termed angiogenesis (Carmeliet and Jain, 2011). However, in spite of the formation of a highly dense vascular network, tumor-associated vessels are often abnormal, leaky and immature, leading to aggravation of tumor hypoxia, promotion of tumor metastasis and resistance to treatments. Abnormal angiogenesis, thus represents an important tumor Achilles' heel and an advantage for the development of novel anti-tumor treatments (Carmeliet and Jain, 2011).

Analysis of gene expression profiles revealed distinct sets of genes that are up- or down-regulated in healthy versus tumor-associated vessels. Of 170 transcripts predominantly expressed in the endothelium, 46 were specifically elevated in the endothelium associated to malignant colorectal tissues compared to normal blood vessels (St. Croix et al., 2000). Moreover, another study identified 17 genes (including vimentin, CD59, HMGB1 and IGFBP7) that were specifically overexpressed in tumor-associated vessels as compared to angiogenic endothelium of normal tissues (van Veijnum et al., 2006). Interestingly, glycan-related genes including glycosyltransferases and glycosidases can also be up- or down-regulated during the angiogenesis process (Garcia-Vallejo et al., 2006; Willhauck-Fleckenstein et al., 2010). Garcia-Vallejo and colleagues identified a set of glycosyltransferases, mannosidases and

sulfotransferases that are differentially expressed in activated versus resting human ECs (Garcia-Vallejo et al., 2006).

Remarkably, the EC glycome is highly sensitive to environmental signals, including cytokines and growth factors (Croci et al., 2014). In response to immunosuppressive (IL-10 or TGF-β_1) or pro-angiogenic (fibroblast growth factor-2; FGF2) cytokines, human ECs showed increased branching of β1,6 *N*-glycan structures and elongation of poly-LacNAc terminals, while displayed reduced expression of α2,6-linked sialic acid. These glycosylation changes facilitated binding of the endogenous lectin galectin-1 (Gal-1) to ECs and favored activation of pro-angiogenic signaling pathways (Croci et al., 2014). In contrast, ECs exposed to pro-inflammatory cytokines (IFN-γ, IL-17) showed reduced β1,6-N-glycan branching and increased α2,6-sialylation which prevented Gal-1 binding and angiogenesis. However, not only cytokines and growth factors altered the EC glycophenotype as hypoxia (a hallmark feature of the tumor microenvironment) induced pronounced up-regulation of neutral *N*-glycans and diminished expression of tri- and tetra-sialylated *N*-glycans on ECs, which enhanced Gal-1 binding, EC signaling and angiogenesis (Croci et al., 2014). Thus, hypoxic, immunosuppressive or pro-inflammatory stimuli may serve as 'on-and-off' switches that selectively unmask or mask Gal-1-specific glyco-epitopes and controls EC signaling and angiogenesis. This particular 'glycan switch' (characterized by low expression of α2,6 sialic acid) is not restricted to ECs, as it is also a hallmark of immunological processes such as differentiation of T helper (Th)1 and Th17 cells (Toscano et al., 2007), dendritic cell maturation (Bax et al., 2007) and conversion of microglial cells toward an M1 phenotype (Starossom et al., 2012). Whether a distinctive glycosylation signature could delineate the vasculature of tumor-associated versus inflammatory microenvironments or could serve to distinguish vessels at different stages of tumor progression still remains to be explored.

Although less appreciated, compared to the well-established roles of glycans in the control of innate and adaptive immunity (Rabinovich and Croci, 2012), compeling evidence indicates that glycosylation is integral to different angiogenesis-related processes. An

example illustrating this concept is the dual regulation of angiogenesis by Notch receptor signaling depending on its glycosylation profile. Notch can be modulated by various posttranslational modifications of the receptors, such as the addition of fucose residues by protein O-fucosyltransferase 1 (POFUT1) to the extracellular epidermal growth factor (EGF)-like repeats, which can be further modified by the Fringe family of β-1,3-N-acetyl-glucosaminyltransferases. Fringe enhances the activation of Notch in response to Delta-like ligands, but has the opposite effect for Serrate/Jagged ligands (Stanley and Guidos, 2009). It has been demonstrated that, in cells expressing the Fringe glycosyltransferase, Jagged1 acts a potent proangiogenic regulator that antagonizes Dll4-Notch signaling and controls EC tip formation (Benedito et al., 2009). Thus, Notch glycosylation may serve to differentially control vascularization programs and sprouting angiogenesis. Interestingly, another example highlighting the influence of glycosylation in angioregulatory circuits was provided by Kitazume and colleagues (2010) who demonstrated a central role for α2,6-linked sialic acid in modulating homophilic interactions of PECAM and controlling EC survival and angiogenesis. Additionally, Xu et al. (2011) showed a pivotal role for heparan sulfate proteoglycans in limiting vascular endothelial growth factor (VEGF)-induced vascular hyperpermeability. In this regard, interruption of heparan sulfate biosynthesis using a peracetylated 4-deoxy analogue of the heparan sulfate constituent GlcNAc, which was activated intracellularly into UDP-4-deoxy-GlcNAc, attenuated angiogenic signaling and prevented neovessel formation (van Wijk et al., 2013). Thus, regulated glycosylation can control different events in the angiogenesis cascade including ligand-binding activity, receptor trafficking and signaling, EC tip formation, sprouting and vascular permeability.

Lectin-glycan recognition systems in vascular biology

As mentioned above, subtle variations in the cellular glycophenotype could alter vascular processes by displaying or masking ligands for endogenous lectins, which translate glycan-containing information into functional responses (Garner and Baum, 2008). Currently,

imited information is available regarding the contribution of C-type lectins or siglecs to the control of angiogenesis, with the exception of CLEC-14a, a C-type lectin involved in EC migration and filopodia formation (Ki et al., 2013). In contrast, an increasing number of studies support the central role of galectins, a family of β-galactoside-binding lectins, in the control of vascular signaling programs (Thijssen et al., 2013). Galectins (Gal-1, -3, -8 and -9) can differentially control angiogenesis programs by engaging a different set of EC surface receptors, activating distinct signaling pathways and/or regulating different events in the angiogenic cascade (Thijssen et al., 2013). In this regard, Gal-1 binds to neuropilin-1 (NRP-1) or to VEGFR2 where it modulates receptor segregation, internalization and trafficking through glycosylation-dependent mechanisms, leading to VEGFR2 phosphorylation and signaling via the Raf/extracellular signal-regulated kinase (ERK) and Akt (Hsieh et al, 2008; Thijssen et al., 2010; Croci et al., 2012; Mathieu et al., 2012; D'Haenne et al., 2013, Croci et al., 2014). More recently, Wu and colleagues showed that, in addition to its role in the regulation of EC proliferation, migration and morphogenesis, Gal-1 also plays a role in the control of vascular permeability through activation of NRP-1, VEGFR1 and Akt signaling (Wu et al., 2014).

On the other hand, Gal-3 controls EC biology through binding to *N*-glycans on $\alpha_v\beta_3$ integrin and modulating cell surface retention of VEGFR2 (Nangia-Makker et al., 2000; Markowska et al., 2010; Markowska et al., 2011), whereas Gal-8 triggers EC signaling through binding to the activated leukocyte cell adhesion molecule (ALCAM; CD166) (Cardenas-Delgado et al., 2011). Interestingly, recent evidence indicated a dose- and context-dependent effect of the Gal-9Δ5, a splice variant isoform of Gal-9, on EC proliferation, migration and morphogenesis (Heusschen et al., 2014). Moreover, induction of platelet-derived angiogenic molecules (including VEGF-A and endostatin) has been documented as an alternative regulatory pathway by which galectins can control angiogenesis (Etulain et al., 2014). As different galectins may be up- or down-regulated in different tumor microenvironments (Langbein et al., 2007; Laderach et al., 2013; Dalotto-

Moreno et al., 2013) a detailed 'galectin signature' of different tumor types will disclose the best targets for anti-angiogenic therapy.

Recent evidence showed that specific interactions between Gal-1 and complex *N*-glycans may serve to link tumor hypoxia to vascularization programs in models of Kaposi's sarcoma, melanoma, lung adenocarcinoma and T-cell lymphoma (Croci et al., 2012; 2014). Remarkably, hypoxia favored a Gal-1-specific glycophenotype in ECs, as it increased the amounts of β1-6GlcNAc-branched *N*-glycans and poly-LacNAc structures and reduced α2,6 sialylation. Furthermore, exposure to hypoxic conditions up-regulated Gal-1 expression in different tumor types through HIF-1-dependent (Le et al., 2005; Zhao et al., 2011) or ROS/NF-κB-dependent (Croci et al., 2012) mechanisms.

Targeting Gal-1 expression eliminated vascularization and suppressed growth in several tumor types including melanoma (Thijssen et al., 2006; 2010; Mathieu et al., 2012; Croci et al., 2014), Kaposi's sarcoma (Croci et al., 2012), prostate carcinoma (Laderach et al., 2013), lung adenocarcinoma (Croci et al., 2014), T-cell lymphoma (Croci et al., 2014), pancreatic adenocarcinoma (Martinez-Bosch et al., 2014) and glioblastoma (Verschuere et al., 2014). Furthermore, interfering with Gal-1-induced angiogenesis has demonstrated clinical benefits not only in cancer settings but also in pregnancy-associated pathologies including pre-eclampsia (Freitag et al., 2013) and endometriosis (Baston et al., 2014), thus emphasizing the key role of Gal-1 as a general target of anti-angiogenic therapies. Analysis of human tumor biopsies revealed that Gal-1 expression correlated with the number of blood vessels in prostate adenocarcinoma (Laderach et al., 2013), non-small cell lung adenocarcinoma (NSCLC) (Carlini et al., 2014) and Kaposi's sarcoma (Croci et al., 2012). Interestingly, Gal-1-induced angiogenesis appeared to be independent of canonical pro-angiogenic factors including VEGF, FGF2, oncostatin M, angiopoietin-like 4 (ANGPTL-4) and platelet-derived growth factor (PDGF)-α (Croci et al., 2012; 2014; Laderach et al., 2013).. In contrast, recent studies indicated that targeting Gal-3 in the stroma or parenchyma of melanoma cells impaired angiogenesis through modulation of VEGF- and TGF-β-dependent

pathways (Machado et al., 2014). These findings are consistent with the ability of Gal-3 to potentiate VEGF- and FGF2-mediated angiogenesis through mechanisms involving binding to complex *N*-glycans on integrin $\alpha_v\beta_3$ and cell surface retention of VEGFR2 (Markowska et al., 2010; 2012). Notably, Nangia-Makker and colleagues showed that cleavage of the N-terminus of Gal-3 by matrix metaloproteinases (MMPs) represents a critical step for stimulating breast cancer angiogenesis (Nangia-Makker et al., 2010). Furthermore, LGALS3BP, a protein known to specifically bind Gal-3, functions as a pro-angiogenic mediator through a dual mechanism involving induction of tumor VEGF or stimulation of EC function by Gal-3 (Piccolo et al., 2013).

Regarding other members of the galectin family, recent studies documented an indirect role of Gal-2, Gal-4 and Gal-8 in angiogenesis programs by inducing the secretion of EC-derived cytokines and chemokines (G-CSF, IL-6, MCP-1 and GROα), which in turn can stimulate EC signaling (Chen et al., 2014). Altogether, these studies indicate non-redundant roles of individual members of the galectin family in the control of EC biology, which may support angiogenesis through different mechanisms involving: a) engagement of distinct EC receptors; b) activation of divergent signaling pathways; and/or c) independence or interdependence of canonical angiogenic ligands.

Mechanisms of resistance to anti-angiogenic therapies: the glycan connection

The initial experiments of Judah Folkman were the inspiration for targeting angiogenesis as a mean of eradicating tumors (Folkman et al., 1971). Later, the identification of VEGF as a central mediator of angiogenesis and the elucidation of its specific receptors (VEGFRs) have enabled the design of selective inhibitors that block the vascularization process (Ferrara et al., 2004). The master pro-angiogenic factor VEGF acts through activation (dimerization, phosphorylation and signaling) of VEGFRs including VEGFR1, VEGFR2 and VEGFR3 on ECs (Ferrara et al., 2004).

Most anti-angiogenic therapies are designed to disrupt VEGF-VEGFR interactions through: a) sequestering soluble VEGF using an anti-VEGF blocking Ab (bevacizumab), b) inhibiting VEGFR tyrosine kinase activity using receptor tyrosine kinase (RTK) inhibitors such as sunitinib, sorafenib, pazopanib, vandetanib, cabozantinib, tivozatinib, linifanib and axitinib) that target VEGFRs through direct competition with ATP to the intracellular tyrosine kinase binding domain (Loges et al., 2009).

Although VEGF-targeted therapies have increased progression-free survival and in some cases overall survival in patients with colorectal cancer, NSCLC, metastatic breast cancer, renal cell carcinoma and advanced hepatocarcinoma, the clinical benefits conferred by these therapies are, at most temporary, and tumors eventually reinitiate growth, suggesting that alternative angiogenic pathways may be invoked in the absence of VEGF signaling to preserve tumor vascularization (Bergers and Hanahan, 2008; Ebos et al., 2009). In fact, tumors develop a number of strategies to circumvent antiangiogenic treatment. Whereas some tumors are intrinsically refractory to antiangiogenic therapies (intrinsic resistance), in most cases tumors develop adaptive resistance mechanisms to circumvent specific angiogenic blockade (evasive resistance) (Bergers and Hanahan, 2008). Pathways of evasive resistance involve the expression of alternative angiogenic factors including FGF2, placental growth factor (PIGF), PDGF-β, IL-6, IL-8, angiopoietins (Ang2) or hepatocyte growth factor (HGF), which stimulate angiogenic compensatory programs and limit the efficacy of anti-VEGF treatment (Berger and Hanahan, 2008, Shojaei et al., 2010). In addition anti-angiogenic treatments induce an initial 'vessel pruning' effect, which aggravates tumor hypoxia and favors revascularization and tumor metastasis (Paez-Ribes et al, 2009; Ebos et al., 2009). Indeed in Darwinian terms hypoxia acts as a pressure mechanism that selects tumor cell variants with increased aggressiveness and lower sensitivity to anti-angiogenic therapy. Finally, an additional mechanism involves mobilization of angio-competent myeloid cells, which in response to hypoxic conditions or to anti-angiogenic treatment, preserves vascularization programs. This includes the recruitment of $TIE2^{+}$

monocytes (De Palma et al., 2005), Bv8-expressing $CD11b^+$ $Gr1^+$ myeloid-derived suppressor cells (MDSCs) (Shojaei et al., 2007) and $VEGFR1^+$ macrophages (Hattori et al., 2002) which, upon reaching the tumor microenviornments secrete potent proangiogenic mediators such as VEGF, FGF2 and TGF-β (Murdoch et al., 2008). Interestingly, IL-17 (released by Th17 cells) induces the secretion of granulocyte colony-stimulating factor (G-CSF) by tumor-associated fibroblasts, which in turn promotes the mobilization of Bv8-expressing $CD11b^+Gr1^+$ MDSCs and stimulates tumor angiogenesis (Chung et al., 2013).

In recent studies, we identified a glycosylation-based mechanism mediated by Gal-1-*N*-glycan interactions that links tumor hypoxia to VEGFR2 signaling and preserves angiogenesis in the setting of VEGF blockade (Croci et al., 2014). We found that Gal-1 binds directly to non-sialylated *N*-glycans on VEGFR2 and promotes segregation and retention of this glycosylated receptor on the surface of ECs. This glycosylation-based mechanism leads to VEGFR2, Erk1/2 and Akt phosphorylation and mimics VEGF signaling. Although Gal-1 preferentially bound VEGFR2 (Croci et al., 2014), further studies should examine in detail the glycosylation status of other EC receptors including c-Met, FGFR and PDGFRs under different experimental conditions.

Remarkably, tumor refractory to VEGF blocakde (Lewis lung carcinoma; LLC1 and R1.1 T cell lymphoma) produced high amounts of Gal-1 in response to hypoxia or anti-VEGF treatment and their associated vasculature displayed glycosylation patterns that facilitated Gal-1-EC interactions, including increased β1-6GlcNAc branching, diminished display of α2-6-linked sialic acid and greater exposure of poly-LacNAc-extended glycans. In contrast, vessels associated to anti-VEGF sensitive tumors (B16 melanoma and CT26 colon carcinoma) displayed high amounts of α2,6-linked sialic acid in response to VEGF blockade, which prevented Gal-1 binding and angiogenesis. Accordingly, loss of α2-6-sialylation in tumor-associated vessels conferred reduced sensitivity to anti-VEGF treatment and favored compensatory angiogenesis mediated by Gal-1-receptor interactions. In contrast, lack of β1-6 GlcNAc-branched *N*-glycans in ECs or silencing of tumor-derived Gal-1 converted refractory

into anti-VEGF-sensitive tumors (Croci et al., 2014). Although host cells including ECs and stromal cells also express substantial amounts of Gal-1 (Thijssen et al., 2013), no considerable differences in microvessel density were observed when tumor cells from Kaposi's sarcoma or LLC1 were implanted into Gal-1-deficient (*Lgals1*$^{-/-}$) or wild-type mice (Croci et al., 2012; 2014). These findings highlight the relevance of EC surface glycosylation and tumor-derived Gal-1 as potential therapeutic targets to surmount anti-VEGF compensatory programs. Interestingly, recent studies disclosed a higher frequency of anti-Gal-1 antibodies in melanoma patients treated with a combination of anti-VEGF (bevacizumab) and anti-CTLA-4 (ipilumimab) antibodies (Hodi et al., 2014). Whether these antibodies are the result of increased amounts of circulating Gal-1 in treated patients remains to be explored.

Targeting Gal1-*N*-glycans interactions, using an anti-Gal1 monoclonal antibody, eliminated resistance to anti-VEGF treatment, suppressed the formation of aberrant tumor vascular networks and enhanced anti-tumor immune responses in several tumor models (Croci et al., 2012; 2014). Interestingly, antibody-mediated Gal-1 blockade promoted transient normalization of tumor-associated vasculature early after treatment, as shown by reduced vessel diameter, increased pericyte coverage and maturation and attenuation of tumor hypoxia. These effects, which favored influx of anti-tumor immune cells to the tumor parenchyma, were also verified in *Mgat5*$^{-/-}$ mice, thus emphasizing the critical role of complex *N*-glycans in the control of tumor vascularization and immunity (Croci et al., 2014). In addition, these findings underscore the dual effects of blocking Gal-1-*N*-glycan interactions, which influence tumor growth by attenuating aberrant angiogenesis and potentiating anti-tumor responses. Supporting these findings, treatment of tumors with both bevacizumab and anginex, an anti-angiogenic peptide known to bind Gal-1, normalized tumor vessels, increased oxygenation and improved responses to radiation therapy (Dings et al., 2007). Moreover, administration of OTX008, a synthetic compound that targets Gal-1, potentiated the activity of the tyrosine kinase inhibitor sunitinib in nude mice inoculated with

tumor xenografts (Zucchetti et al., 2013). These results support the use of combination therapies containing Gal-1-blocking agents to maximize the efficacy of anti-cancer treatments.

Conclusions and future challenges

In the present review we summarize the emerging roles of glycans and glycan-binding proteins (particularly galectins) in angiogenesis-related processes with particular emphasis in tumor vascularization and resistance to anti-angiogenic therapies. First, we discuss the relevance of glycosylation in regulating angiogenesis by controlling Notch signaling, EC migration and branching, EC survival and vascular permeability. Next, we highlight the role of lectin-glycan recognition systems, particularly those involving galectins, in regulating receptor segregation, endocytosis and signaling. Finally, we discuss the implications of a glycosylation-based mechanism mediated by direct Gal-1-receptor interactions that links tumor hypoxia to VEGFR2 signaling and preserves angiogenesis in the setting of VEGF blockade.

Challenges for the future will embrace: a) a systematic study of the EC glycome in tumor-associated vessels compared to those irrigating inflamed and healthy tissues in pre-clinical and clinical settings; b) a comprehensive analysis of different lectin-glycan systems (including those involving C-type lectins, siglecs and other galectin family members) in vascular signaling programs; and c) the integration of the Gal-1-*N*-glycan axis to other angiogenic rescue programs with the ultimate goal of maximizing the efficacy of anti-angiogenic treatments.

Future anti-cancer therapies will require the rational combination of tumor-targeted therapies (i.e. those aimed at disrupting biochemical and metabolic pathways in tumors; e.g. EGFR inhibitors); immunotherapeutic approaches (i.e. those targeting negative regulatory checkpoints, such as CTLA-4 or PD-1/PD-L1) and anti-angiogenic agents (i.e. those promoting vessel pruning or normalization). Given its dual immunostimulatory and anti-

angiogenic effects, targeting Gal-1 (and probably other galectins in the tumor microenvironment) might serve to potentiate current anti-cancer strategies and maximize their therapeutic efficacy. Future pre-clinical studies should be aimed at exploring these combination strategies, studying their pharmacokinetics and distribution and analyzing their toxicity and potential side effects.

Funding

Work in G.A.R.'s lab is supported by grants from the Argentinean Agency for Promotion of Science and Technology (Ministry of Science and Technology), Argentinean National Council Research (CONICET), University of Buenos Aires and Sales Foundation.

Abbreviations

ALCAM, activated leukocyte cell adhesion molecule; ANGPTL-4, angiopoietin-like 4; CLEC14a, C-type lectin domain family 14 member A; CTLA-4, cytotoxic T-lymphocyte antigen 4; ECs, endothelial cells; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; Gal, galectin; G-CSF, granulocyte colony-stimulating factor; HUVEC, human umbilical vein endothelial cells; IFN-γ, interferon-γ; MDSCs, myeloid-derived suppressor cells; Mgat5, *N*-acetylglucosaminyltransferase 5; MMP, matrix metaloproteinases; NRP-1, neuropilin-1; PD-1, programmed cell death-1; PDGF, platelet-derived growth factor; PECAM, platelet endothelial cell adhesion molecule; ST6Gal1, α2,6-sialyltransferase 1; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

REFERENCES

Bastón JI, Barañao RI, Ricci AG, Bilotas MA, Olivares CN, Singla JJ, Gonzalez AM, Stupirski JC, Croci DO, Rabinovich GA, Meresman GF. 2014. Targeting galectin-1-induced angiogenesis mitigates the severity of endometriosis. *J Pathol.* Jun 30 [Epub ahead of print]

Bax M, García-Vallejo JJ, Jang-Lee J, North SJ, Gilmartin TJ, Hernández G, Crocker PR, Leffler H, Head SR, Haslam SM, Dell A, van Kooyk Y. 2007. Dendritic cell maturation results in pronounced changes in glycan expression affecting recognition by siglecs and galectins. *J Immunol.* 179:8216-8224.

Benedito R, Roca C, Sorensen I, Adams S, Gossler A, Fruttiger M, Adams R H. 2009. The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell.* 137:1124-1135.

Bergers G and Hanahan D. 2008. Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer.* 8:592-603.

Boscher C, Dennis JW, Nabi IR. 2011. Glycosylation, galectins and cellular signaling. *Curr Opin Cell Biol* 23:383-392.

Cardenas-Delgado VM, Nugnes LG, Colombo LL, Troncoso MF, Fernandez MM, Malchiodi EL Malchiodi, EL, Frahm I, Croci DO, Compagno D, Rabinovich GA, Wolfenstein-Todel C and Elola MT. 2011. Modulation of endothelial cell migration and angiogenesis: a novel function for the tandem-repeat lectin galectin-8. *FASEB J.* 25:242–254.

Carlini MJ, Roitman P, Nuñez M, Pallotta MG, Boggio G, Smith D, Salatino M, Joffé ED, Rabinovich GA, Puricelli LI. 2014. Clinical relevance of galectin-1 expression in non-small cell lung cancer patients. *Lung Cancer*. 84:73-78.

Carmeliet P, and Jain RK. 2011. Molecular mechanisms and clinical applications of angiogenesis. *Nature.* 473:298-307.

Chen C, Duckworth CA, Fu B, Pritchard DM, Rhodes JM, Yu LG. 2014. Circulating galectins -2, -4 and -8 in cancer patients make important contributions to the increased circulation of several cytokines and chemokines that promote angiogenesis and metastasis. *Br J Cancer*. 110:741-752.

Chung AS, Wu X, Zhuang G, Ngu H, Kasman I, Zhang J, Vernes JM, Jiang Z, Meng YG, Peale FV. 2013. An IL-17-mediated paracrine network promotes tumor resistance to anti-angiogenic therapy. *Nat Med. 19*:1114-1123

Croci DO, Salatino M, Rubinstein N, Cerliani JP, Cavallin LE, Leung HJ, Ouyang J, Ilarregui JM, Toscano MA, Domaica CI, Croci MC, Shipp MA, Mesri EA, Albini A, Rabinovich GA. 2012. Disrupting galectin-1 interactions with N-glycans suppresses hypoxia-driven angiogenesis and tumorigenesis in Kaposi's sarcoma. *J Exp Med*. 209:1985-2000.

Croci DO, Cerliani JP, Dalotto-Moreno T, Méndez-Huergo SP, Mascanfroni DI. Dergan-Dylon LS, Toscano MA, Caramelo JJ, García-Vallejo JJ. Ouyang J, Mesri EA. Junttila MR, Bais C, Shipp MA, Salatino M and Rabinovich GA. 2014. Glycosylation-dependent lectin-receptor interactions preserve angiogenesis in anti-VEGF refractory tumors. *Cell*. 156:744-758.

Dalotto-Moreno T, Croci DO, Cerliani JP, Martinez-Allo VC, Dergan-Dylon S, Méndez-Huergo SP, Stupirski JC, Mazal D, Osinaga E, Toscano MA, Sundblad V, Rabinovich GA, Salatino M. 2013. Targeting galectin-1 overcomes breast cancer-associated immunosuppression and prevents metastatic disease. *Cancer Res*. 73:1107-1117.

De Palma M, Venneri MA, Galli R, Sergi Sergi L, Politi LS, Sampaolesi M, Naldini L. 2005. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell.* 8:211-226

D'Haene N, Sauvage S, Maris C, Adanja I, Le Mercier M, Decaestecker C, Baum L, and Salmon I. 2013. VEGFR1 and VEGFR2 involvement in extracellular galectin-1- and galectin-3-induced angiogenesis. *PLoS One.* 8, e67029.

Dings RP, Loren M, Heun H, McNiel E, Griffioen AW, Mayo KH, Griffin RJ. 2007. Scheduling of radiation with angiogenesis inhibitors anginex and Avastin improves therapeutic outcome via vessel normalization. *Clin Cancer Res*. 13:3395-3402.

Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. 2009. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell.* 15:232-239.

Etulain J, Negrotto S, Tribulatti MV, Croci DO, Carabelli J, Campetella O, Rabinovich GA, Schattner M. 2014. Control of angiogenesis by galectins involves the release of platelet-derived proangiogenic factors. *PLoS One*. 4:e96402.

Ferrara N, Hillan KJ, Gerber HP, Novotny W. 2004. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov*. 5:391-400.

Freitag N, Tirado-González I, Barrientos G, Herse F, Thijssen VL, Weedon-Fekjær SM, Schulz H, Wallukat G, Klapp BF, Nevers T, Sharma S, Staff AC, Dechend R, Blois SM. 2013. Interfering with Gal-1-mediated angiogenesis contributes to the pathogenesis of preeclampsia. *Proc Natl Acad Sci U S A*. 110:11451-11456.

Folkman J. Tumor angiogenesis: therapeutic implications. 1971. *N Engl J Med*. 285:1182-1186

Garcia-Vallejo JJ, Van Dijk W, Van Het Hof, Van Die I, Engelse MA, Van Hinsbergh VW, and Gringhuis SI. 2006. Activation of human endothelial cells by tumor necrosis factor-alpha results in profound changes in the expression of glycosylation-related genes. *J Cell Physiol*. 206:203–210.

Garner OB, Baum LG. 2008. Galectin-glycan lattices regulate cell-surface glycoprotein organization and signalling. *Biochem Soc Trans*. 6:1472-1477.

Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, Hicklin DJ, Zhu Z, Bohlen P, Witte L . 2002. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat Med*. 8:841–849.

Heusschen R, Schulkens IA, van Beijnum J, Griffioen AW, Thijssen VL. 2014. Endothelial LGALS9 splice variant expression in endothelial cell biology and angiogenesis. *Biochim Biophys Acta*. 18422:284-292.

Hodi FS, Lawrence D, Lezcano C, Wu X, Zhou J, Sasada T, Zeng W, Giobbie-Hurder A, Atkins MB, Ibrahim N, Friedlander P, Flaherty KT, Murphy GF, Rodig S, Velazquez EF, Mihm MC, Russell S, DiPiro PJ, Yap JT, Ramaiya N, Van den Abbeele AD, Gargano M, McDermott D. 2014. Bevacizumab plus Ipilimumab in Patients with Metastatic Melanoma. *Cancer Immunol Res*. Apr. [Epub ahead of print]

Hsieh SH, Ying NW, Wu MH, Chiang WF, Hsu CL, Wong TY. 2008. Galectin-1, a novel ligand of neuropilin-1, activates VEGFR-2 signaling and modulates the migration of vascular endothelial cells. *Oncogene.* 26:3746–3753.

Kerbel RS. 2008. Tumor angiogenesis. *N Engl J Med*. 358:2039-2049.

Ki MK, Jeoung MH, Choi JR, Rho SS, Kwon YG, Shim H, Chung J, Hong HJ, Song BD, Lee S. 2013. Human antibodies targeting the C-type lectin-like domain of the tumor endothelial cell marker clec14a regulate angiogenic properties in vitro. *Oncogene.* 48:5449-5457

Kitazume S, Imamaki R, Ogawa K, Komi Y, Futakawa S, Kojima S, Hashimoto Y, Marth JD, Paulson JC, Taniguchi N. 2010. α2,6-sialic acid on platelet endothelial cell adhesion molecule (PECAM) regulates its homophilic interactions and downstream antiapoptotic signaling. *J Biol Chem.* 285:6515-6521.

Laderach DJ, Gentilini LD, Giribaldi L, Delgado VC, Nugnes L, Croci DO, Al Nakouzi N, Sacca P, Casas G, Mazza O, Shipp MA, Vazquez E, Chauchereau A, Kutok JL, Rodig SJ, Elola MT, Compagno D, Rabinovich GA. 2013. A unique galectin signature in human prostate cancer progression suggests galectin-1 as a key target for treatment of advanced disease. *Cancer Res*. 73:86-96.

Langbein S, Brade J, Badawi JK, Hatzinger M, Kaltner H, Lensch M, Specht K, André S, Brinck U, Alken P, Gabius HJ. 2007. Gene-expression signature of adhesion/growth-regulatory tissue lectins (galectins) in transitional cell cancer and its prognostic relevance. *Histopathology*. 51:681-690.

Le QT, Shi G, Cao H, Nelson DW, Wang Y, Chen EY, Zhao S, Kong C, Richardson D, O'Byrne KJ. 2005. Galectin-1: a link between tumor hypoxia and tumor immune privilege. *J Clin Oncol*. 23:8932-8941.

Loges S, Mazzone M, Hohensinner P, Carmeliet P. 2009. Silencing or fueling metastasis with VEGF inhibitors: antiangiogenesis revisited. *Cancer cell.* 15:167-70.

Machado CM, Andrade LN, Teixeira VR, Costa FF, Melo CM, dos Santos SN, Nonogaki S, Liu FT, Bernardes ES, Camargo AA, Chammas R. 2014. Galectin-3 disruption impaired tumoral angiogenesis by reducing VEGF secretion from TGFβ1-induced macrophages. *Cancer Med.* 3:201-214.

Mariño K, Bones J, Kattla JJ, Rudd PM. 2010. A systematic approach to protein glycosylation analysis: a path through the maze. *Nat Chem Biol*. 6:713-23.

Markowska AI, Jefferies KC, Panjwani N. 2011. Galectin-3 protein modulates cell surface expression and activation of vascular endothelial growth factor receptor 2 in human endothelial cells. *J Biol Chem*. 286:29913–29921.

Markowska AI, Liu FT, Panjwani N. 2010. Galectin-3 is an important mediator of VEGF- and bFGF-mediated angiogenic response. *J Exp Med.* 207:1981-1993.

Martínez-Bosch N, Fernández-Barrena MG, Moreno M, Ortiz-Zapater E, Munné-Collado J, Iglesias M, André S, Gabius HJ, Hwang RF, Poirier F, Navas C, Guerra C, Fernández-Zapico ME, Navarro P. 2014. Galectin-1 Drives Pancreatic Carcinogenesis through Stroma Remodeling and Hedgehog Signaling Activation. *Cancer Res*. May 8. [Epub ahead of print].

Mathieu V, de Lassalle EM, Toelen J, Mohr T, Bellahcene A, Van Goietsenoven G, Verschuere T, Bouzin C, Debyser Z, De Vleeschouwer S. 2012. Galectin-1 in melanoma biology and related neo-angiogenesis processes. *J Invest Dermatol*. 132, 2245-2254.

Murdoch C, Muthana M, Coffelt SB, Lewis CE. 2008. The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer* 8:618-631.

Nangia-Makker P, Honjo Y, Sarvis R, Akahani S, Hogan V, Pienta KJ, Raz A. 2000. Galectin-3 induces endothelial cell morphogenesis and angiogenesis. *Am J Pathol.* 156:899-909.

Nangia-Makker P, Wang Y, Raz T, Tait L, Balan V, Hogan V, Raz A. 2010. Cleavage of galectin-3 by matrix metalloproteases induces angiogenesis in breast cancer. *Int J Cancer*. 127:2530-2541.

Ohtsubo K, MarthJD. 2006. Glycosylation in cellular mechanisms of health and disease. *Cell*. 126: 855-6.

Pàez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Viñals F, Inoue M, Bergers G, Hanahan D, Casanovas O. 2009. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell*. 15:220-231

Piccolo E, Tinari N, Semeraro D, Traini S, Fichera I, Cumashi A, La Sorda R, Spinella F, Bagnato A, Lattanzio R, D'Egidio M, Di Risio A, Stampolidis P, Piantelli M, Natoli C, Ullrich A, Iacobelli S. 2013. LGALS3BP, lectin galactoside-binding soluble 3 binding protein, induces vascular endothelial growth factor in human breast cancer cells and promotes angiogenesis. *J Mol Med (Berl).* 91:83-94.

Potente M, Gerhardt H, and Carmeliet P. 2011. Basic and therapeutic aspects of angiogenesis. *Cell* 146:873-887.

Rabinovich GA and Croci DO. 2012. Regulatory circuits mediated by lectin-glycan Interactions in autoimmunity and cancer. *Immunity*. 36:322–335.

Shojaei F, Lee JH, Simmons BH, Wong A, Esparza CO, Plumlee PA, Feng, Stewart AE, Hu-Lowe DD, and James G. Christensen. 2010. HGF/c-Met Acts as an Alternative Angiogenic Pathway in Sunitinib-Resistant Tumors. *Cancer Res.* 70:10090-10100

Shojaei F, Wu X, Zhong C, Yu L, Liang XH, Yao J, Blanchard D, Bais C, Peale FV, van Bruggen N, Ho C, Ross J, Tan M, Carano RD, Meng YG, Ferrara N. 2007. Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature*. 450:825-31

St. Croix BS, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A,Riggins GJ, Lengauer C, Vogelstein B, Kinzler KW. 2000. Genes expressed in human tumor endothelium. *Science*. 289:1197-1202.

Stanley P, and Guidos CJ. 2009. Regulation of Notch signaling during T- and B-cell development by O-fucose glycans. *Immunol Rev*. 230:201-215.

Starossom SC, Mascanfroni ID, Imitola J, Cao L, Raddassi K, Hernandez SF, Bassil R, Croci DO, Cerliani JP, Delacour D, Wang Y, Elyaman W, Khoury SJ, Rabinovich GA. 2012. Galectin-1 deactivates classically activated microglia and protects from inflammation-induced neurodegeneration. *Immunity*. 37:249-263.

Thijssen VL, Barkan B, Shoji H, Aries IM, Mathieu V, Deltour L, Hackeng TM, Kiss R, Kloog Y, Poirier F, Griffioen AW. 2010. Tumor cells secrete galectin-1 to enhance endothelial cell activity. *Cancer Res*. 70:6216-6224.

Thijssen VL, Postel R, Brandwijk RJ, Dings RP, Nesmelova I, Satijn S, Verhofstad N, Nakabeppu Y, Baum LG, Bakkers J. 2006. Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy. *Proc Natl Acad Sci U S A.* 103:15975-15980.

Thijssen VL, Rabinovich GA, Griffioen AW. 2013. Vascular galectins: Regulators of tumor progression and targets for cancer therapy. *Cytokine Growth Factor Rev*. 10:1359-6101.

Toscano MA, Bianco GA, Ilarregui JM, Croci DO, Correale J, Hernandez JD, Zwirner NW, Poirier F, Riley EM, Baum LG, Rabinovich GA. 2007. Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death. *Nat Immunol* 8:825-834.

van Beijnum JR, Dings RP, van der Linden E, Zwaans BM, Ramaekers FC, Mayo KH, Griffioen AW. 2006. Gene expression of tumor angiogenesis dissected: specific targeting of colon cancer angiogenic vasculature. *Blood* 108:2339-2348.

van Kooyk Y, and Rabinovich GA. 2008. Protein-glycan interactions in the control of innate and adaptive immune responses. *Nat Immunol.* 9:593–601.

van Wijk XM, Thijssen VL, Lawrence R, van den Broek SA, Dona M, Naidu N, Oosterhof A, van de Westerlo EM, Kusters LJ, Khaled Y, Jokela TA, Nowak-Sliwinska P, Kremer H, Stringer SE, Griffioen AW, van Wijk E, van Delft FL, van Kuppevelt TH. 2013. Interfering with UDP-GlcNAc metabolism and heparan sulfate expression using a sugar analogue reduces angiogenesis. *ACS Chem Biol*. 8:2331-2338.

Varki A, Kannagi R, Toole BP. 2009. Glycosylation changes in cancer. In: Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME, editors. *Essentials of Glycobiology* (Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009. Chapter 44.

Verschuere T, Toelen J, Maes W, Poirier F, Boon L, Tousseyn T, Mathivet T, Gerhardt H, Mathieu V, Kiss R, Lefranc F, Van Gool SW, De Vleeschouwer S. 2014. Glioma-derived galectin-1 regulates innate and adaptive antitumor immunity*. Int J Cancer*. 134:873-884.

Willhauck-Fleckenstein M, Moehler TM, Merling A, Pusunc S, Goldschmidt H, and Schwartz-Albiez R. 2010. Transcriptional regulation of the vascular endothelial glycome by angiogenic and inflammatory signalling. *Angiogenesis.* 13:25–42.

Wu MH1, Ying NW, Hong TM, Chiang WF, Lin YT, Chen YL. 2014. Galectin-1 induces vascular permeability through the neuropilin-1/vascular endothelial growth factor receptor-1 complex. *Angiogenesis*. Apr 10. [Epub ahead of print]

Xu D, Fuster MM, Lawrence R, Esko JD. 2011. Heparan sulfate regulates VEGF165- and VEGF121-mediated vascular hyperpermeability. *J Biol Chem*. 286:737-745.

Zhao XY, Zhao KW, Jiang Y, Zhao M, Chen GQ. 2011. Synergistic induction of galectin-1 by CCAAT/enhancer binding protein alpha and hypoxia-inducible factor 1alpha and its role in differentiation of acute myeloid leukemic cells. *J Biol Chem.* 286:36808-36819.

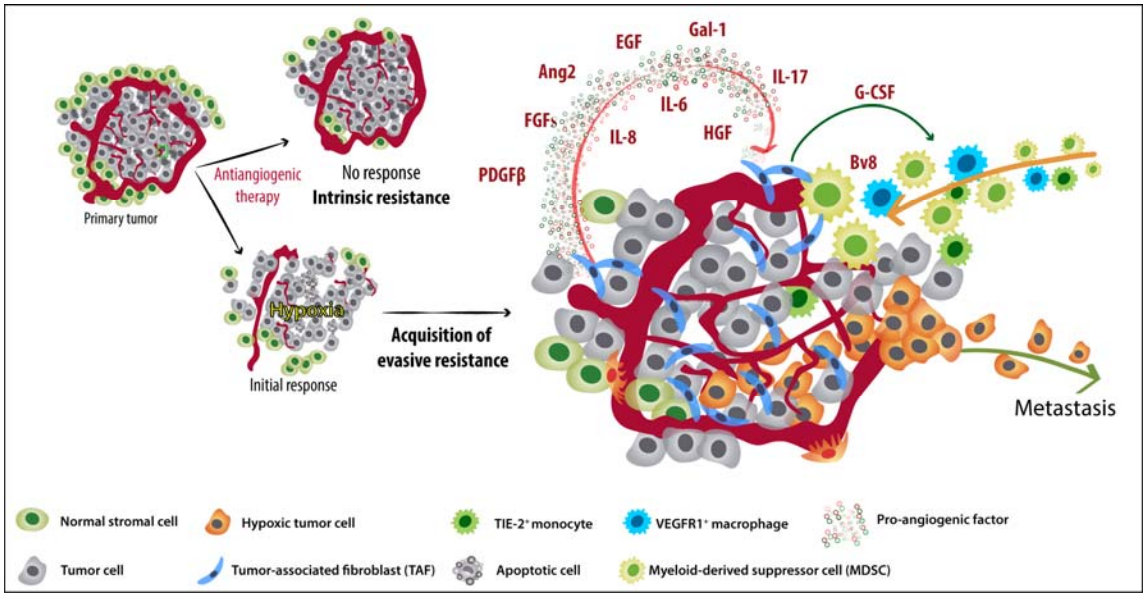
Zucchetti M, Bonezzi K, Frapolli R, Sala F, Borsotti P, Zangarini M, Cvitkovic E, Noel K, Ubezio P, Giavazzi R, D'Incalci M, Taraboletti G. 2013. Pharmacokinetics and antineoplastic activity of galectin-1-targeting OTX008 in combination with sunitinib. *Cancer Chemother Pharmacol*. 72:879-887.

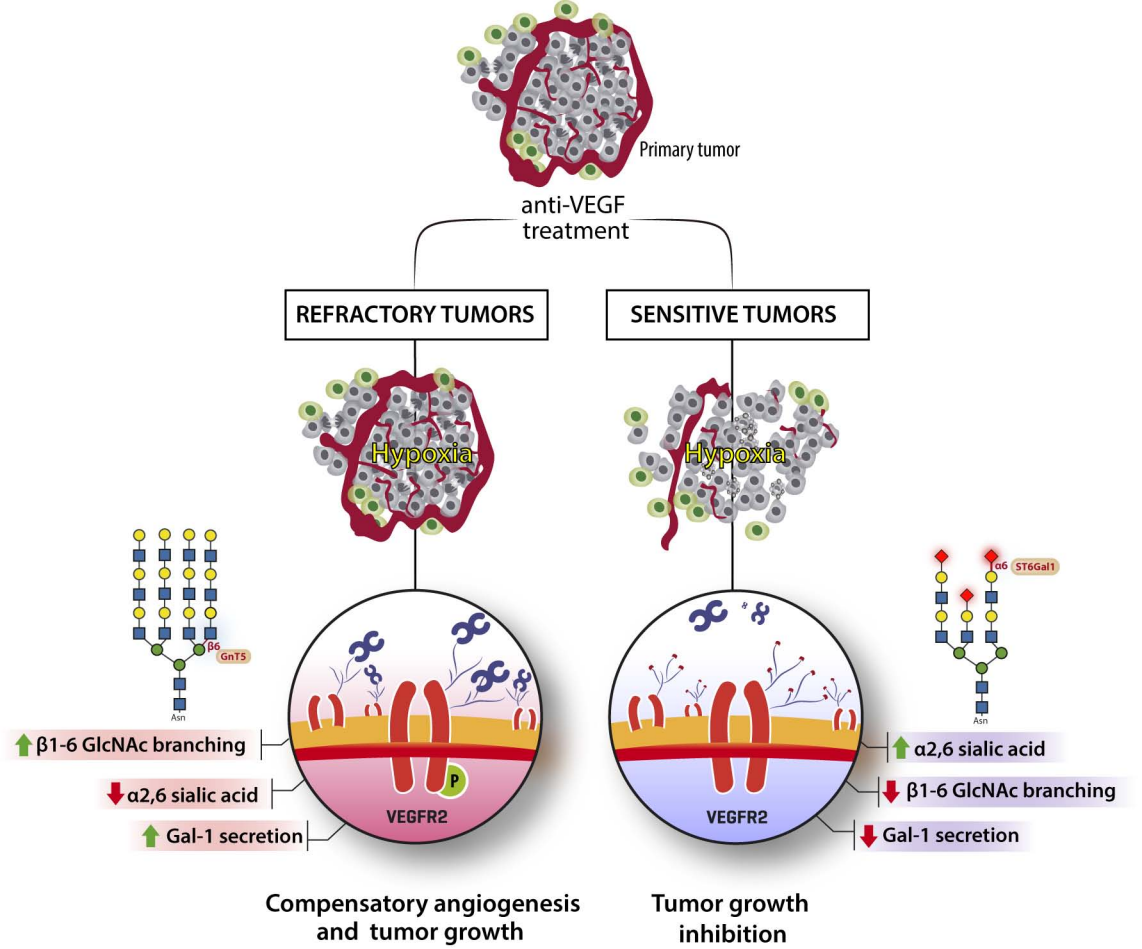
Legend to figures

Figure 1. **Mechanisms of resistance to anti-angiogenic therapies**. Tumors develop several strategies to evade anti-angiogenic treatment. While some tumors are intrinsically refractory and do not response to anti-angiogenic therapies even at early stages of treatment, others acquire evasive resistance mechanisms to circumvent angiogenic blockade (evasive resistance). Mechanisms of evasive resistance involve the secretion of alternative pro-angiogenic mediators including fibroblast growth factor-2 (FGF2), platelet-derived growth factor (PDGF)-β, IL-17, IL-6, IL-8, angiopoietin-2 (Ang-2) and hepatocyte growth factor (HGF), which may fuel revascularization programs and limit the efficacy of anti-VEGF treatment. Anti-angiogenic therapies may also lead to severe hypoxia as a result of vessel pruning which could act as a major driving force for the generation of angiogenic rescue programs and tumor metastasis. Moreover, mobilization of angio-competent myeloid regulatory cells ($TIE2^+$ monocytes, Bv8-expressing $CD11b^+$ $Gr1^+$ myeloid-derived suppressor cells (MDSCs) and $VEGFR1^+$ macrophages) may also preserve angiogenesis in anti-VEGF treated tumors through secretion of key proangiogenic factors. Emerging evidence indicates that Gal-1 interactions with complex *N*-glycans on ECs contribute to preserve angiogenesis in anti-VEGF refractory tumors.

Figure 2. Glycosylation-dependent Gal1-VEGFR2 interactions maintain angiogenesis in anti-VEGF refractory tumors. Hypoxic microenvironments generated in response to VEGF blockade instruct anti-VEGF refractory tumors (left pannel) to secrete higher amounts of Gal-1 and their associated vasculature displays Gal-1-specific glycans (increased β1-6GlcNAc branching and poly-LacNAc-extended glycans and diminished display of α2,6-linked sialic acid). This inducible EC glycophenotype facilitates Gal1 binding, compensatory angiogenesis and tumor growth. In contrast, vessels associated with anti-VEGF sensitive tumors (right panel) display higher amounts of α2,6-linked sialic acid, which

prevent Gal-1-VEGFR2 interactions. Gal-1 is depicted in blue in its prototypic dimeric form. For mechanistic details please see the text.




Primary tumor
anti-VEGF treatment
REFRACTORY TUMORS
SENSITIVE TUMORS
Hypoxia
Hypoxia
β6 GnT5
Asn
α6 ST6Gal1
Asn
β1-6 GlcNAc branching
α2,6 sialic acid
Gal-1 secretion
P
VEGFR2
α2,6 sialic acid
β1-6 GlcNAc branching
Gal-1 secretion
VEGFR2
Compensatory angiogenesis and tumor growth
Tumor growth inhibition