Aberrant cancer glycosylation: a potent regulator of tumor growth and anti-tumor immunity

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2021

document version
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Thesis Summary

Cancer represents a large family of diseases, which are characterized by abnormal cell growth and the potential to invade or disseminate to other parts of the body. Just like every single cell in our organism (e.g endothelial cells) (**Figure 1**) [1], cancer cells are also covered with a dense layer of carbohydrates (sugar molecules decorating cell-surface glycoproteins or glycolipids). This layer is called the glycocalyx. However, malignant cells are marked by glycosylation signatures that are significantly different compared to the ones found on their adjacent non-malignant counterparts, with representative examples being the enhanced levels of sialylation, the truncated or shorter *O*-glycans and the increased expression of terminally fucosylated (Lewis) antigens [2]. Today, we know that aberrant cancer glycosylation is implicated in several aspects of tumor progression and metastasis, including cell growth, angiogenesis, immune modulation and cancer stemness [3, 4].

Figure 1. Microscopic image of the cell-surface glycocalyx of an endothelial cell. Adapted from Rutledge et al. [1]

Our immune system has evolved in order to protect us against different diseases and it is divided into two major arms: the innate and the adaptive immune system. Particularly, the innate immune system responds quickly to pathogen-associated molecular patterns (PAMPs) via the expression of pattern recognition receptors (PRRs) and at the same time it trains the adaptive immune system in order to respond to specific antigens, eliminate danger and provide long-term protection to the host [5]. In the case of cancer, aberrant glycosylation of tumor cells can be efficiently scanned by the immune system through glycan-binding proteins, such as the sialic acid-binding immunoglobulin-type lectins (Siglecs) [6] and the C-type lectin receptors (CLRs) [7]. Characteristic examples of well-studied CLRs are the dendritic cell-specific intercellular adhesion molecule-3-grabbing non integrin (DC-SIGN) that preferentially binds to high-mannose and fucosylated antigens [8] and the macrophage galactose-type lectin (MGL) that binds to terminal GalNAc residues, such as the Tn antigen [9]. However, triggering of these receptors by tumor-associated glycans has been shown to often induce a strong tolerogenic phenotype on the immune cells expressing them, which might exert a negative impact on anti-tumor immunity [10]. For this reason, the tumor glyco-code has been proposed as a novel immune checkpoint that can be targeted during personalized cancer immunotherapy in the future.

As highlighted in the beginning of the current thesis, colorectal cancer (CRC) is a very common type of malignancy and analysis of primary CRC cells, tissues and sera has

revealed significant changes in terms of glycosylation compared to the samples obtained from healthy individuals [11]. Moreover, detailed *N*-glycan and *O*-glycan analysis of human CRC cell lines has indicated an interesting association between glycosylation and cellular differentiation [12, 13]. Given the importance of cell lines as reliable research models to study tumor glycosylation, we initially examined different methodologies (in-solution PNGase F, PVDF membrane-based PNGase F and *N*-mode hydrazinolysis) that are usually followed for *N*-glycan release and subsequent glycoprofiling (**Chapter 2**) [14]. Our goal was to establish and standardize glycoproteomic technologies for quantitative glycan analysis from cultured (CRC) cell lines. Interestingly, we found that in-solution PNGase F is the most robust method regarding processing time, sensitivity and reproducibility and it can be applied as a standard approach for *N*-glycoprofiling on a broad range of samples.

Next, we focused on the role of sialylation in the context of CRC. According to the current dogma, sialic acids have an immune-suppressive function, because they are bound by Siglec receptors, the majority of which contain an intracellular immunoreceptor tyrosine-based inhibitory (ITIM) motif [6]. Since tumor cells present high levels of sialylation on their surface (hypersialylation) as a mechanism to escape from immune destruction, current therapeutic approaches aim at dismantling the sialoglycan coat of cancer cells. However, the effect of complete desialylation on the tumor growth and anti-tumor immunity has not been fully examined yet. To address this, we used as our main research model MC38 cells, a well-known mouse CRC cell line [15]. Using CRISPR-Cas9, we knocked out the *Cmas* gene in MC38 cells, a key gene involved in the sialylation pathway (**Chapter 3**) [16]. Unexpectedly, complete desialylation of MC38 cells (confirmed with liquid-chromatography mass-spectrometry following the in-solution PNGase F *N*-glycan release method tested in **Chapter 2**), led to increased tumor growth *in vivo* compared to control cells. In addition, we identified a reduced frequency of $CD8^+$ T cells at the tumor site, while culture supernatants of the de-sialylated MC38 cells could induce $CD8^+$ T cell apoptosis *in vitro*, providing a possible explanation for the obtained *in vivo* phenotype. In conclusion, we have observed a detrimental effect of complete tumor desialylation on colorectal cancer tumor growth and anti-tumor immune response, which can markedly impact the design of novel cancer therapeutics aimed at altering the tumor glycosylation profile (**Figure 2A**).

Another established alteration on the surface of cancer cells is the presence of abnormally truncated or shorter *O*-glycans, such as the Tn antigen. In particular, the Tn antigen is recognized by tolerogenic dendritic cells and tumor-associated macrophages (TAMs) via MGL [17, 18] and it has already served as a target for CAR-T cell therapy in leukemia and in pancreatic cancer [18]. Yet, its potential involvement in the induction of an immune suppressive tumor microenvironment in CRC remains unknown. For this reason, we have generated MC38-Tn^{High} cells by knocking out the *Cosmc* gene with the use of the CRISPR-Cas9 system (**Chapter 4**). COSMC is a chaperone that plays a pivotal role in *O*-glycan elongation and its loss results in enhanced synthesis of the Tn glycan structure. Interestingly, RNA-sequencing analysis revealed major differences in genes related to the anti-tumor immune response between MC38-Tn^{High} and control cells. Furthermore, MC38-Tn^{High} tumors exhibited increased growth *in vivo*, with a higher percentage of myeloid-derived suppressor cells (MDSCs) and a reduced frequency of cytotoxic $CD8^+$ T cells at the tumor site relative to control tumors (**Figure 2B**). Taken together, our findings demonstrate for the first time that Tn antigen expression affects the tumor-associated immune cell repertoire in the context of CRC.

Fucosylation is a specific type of glycosylation and it represents the transfer of a fucose residue to oligosaccharides carried by cell-surface glycoproteins (*N*-glycans, *O*-glycans) or glycosphingolipids. Increased levels of fucosylation on cancer cells, including type I and type II Lewis antigen overexpression, is linked to tumor progression and metastasis (**Chapter 5**) [4]. Of note, aberrant fucosylation is the result of the deregulated expression of pertinent fucosyltransferases (FUTs) in malignant cells due to genetic or epigenetic alterations. Hence, in **Chapter 6** we tried to mimic and further assess tumor-specific fucosylation by utilizing one of the latest applications of the CRISPR-Cas9 system, the CRISPR-dCas9-VPR technology. We followed this method in order to transcriptionally activate CRC-associated *Fut* genes that are not normally expressed in wild-type MC38 cells, such as the *Fut9* gene. Induction of expression of these FUTs resulted in potent Lewis^{x}-specific *N*-glycome alterations (analyzed following the in-solution PNGase F *N*-glycan release method optimized in **Chapter 2**) (**Figure 2C**). Since FUT9 has been proposed as a metabolic driver of CRC [19], in **Chapter 7**, we performed RNA sequencing-based regulon analysis of our MC38 glyco-engineered cells and we identified key gene regulatory networks linking FUT9 expression to cancer stemness. Also, MC38-FUT9-expressing cells displayed several phenotypic and functional characteristics of cancer stem-like cells. Likewise, both in human CRC cell lines and in primary cells, FUT9 expression was associated with the acquisition of stemness traits. Our study revealed that FUT9 programs a stem cell-like fate in colon cancer cells and thus, its expression is of utmost importance during malignant transformation and tumor fueling. Cancer stem cells are characterized by immune-suppressive functions [20, 21] and they have the ability to escape immune recognition and subsequent elimination [22]. Therefore, we next wondered whether FUT9 expression in MC38 cells could have a negative effect on the anti-tumor immune response. Particularly, FUT9 neo-expression in MC38 cells resulted in increased tumor growth *in vivo* (immune-competent mice) and this phenotype was accompanied by increased infiltration of PD-L1-expressing tumor-associated macrophages and decreased expression of MHC-I on the surface of tumor cells (**Chapter 8**). Therefore, our preliminary findings underscore the role of FUT9 as a candidate regulator of immune resistance in CRC.

In summary, this thesis is a paradigm of how the CRISPR-Cas9 gene editing technology can be implemented in glycobiology research and utilized for cell line glyco-engineering and assessment of the biological roles of aberrant cancer glycosylation. We hereby provide evidence that the altered glycosylation profile of CRC tumors is not only a bystander effect during disease progression, but instead, it can deeply affect tumor growth and the corresponding immune cell repertoire. Therefore, future research should be oriented towards the complete functional characterization of the tumor cell glycome in a pan-cancer level and the development of novel glycan-based therapeutic approaches that can be combined with the already existing regimes for a better outcome.

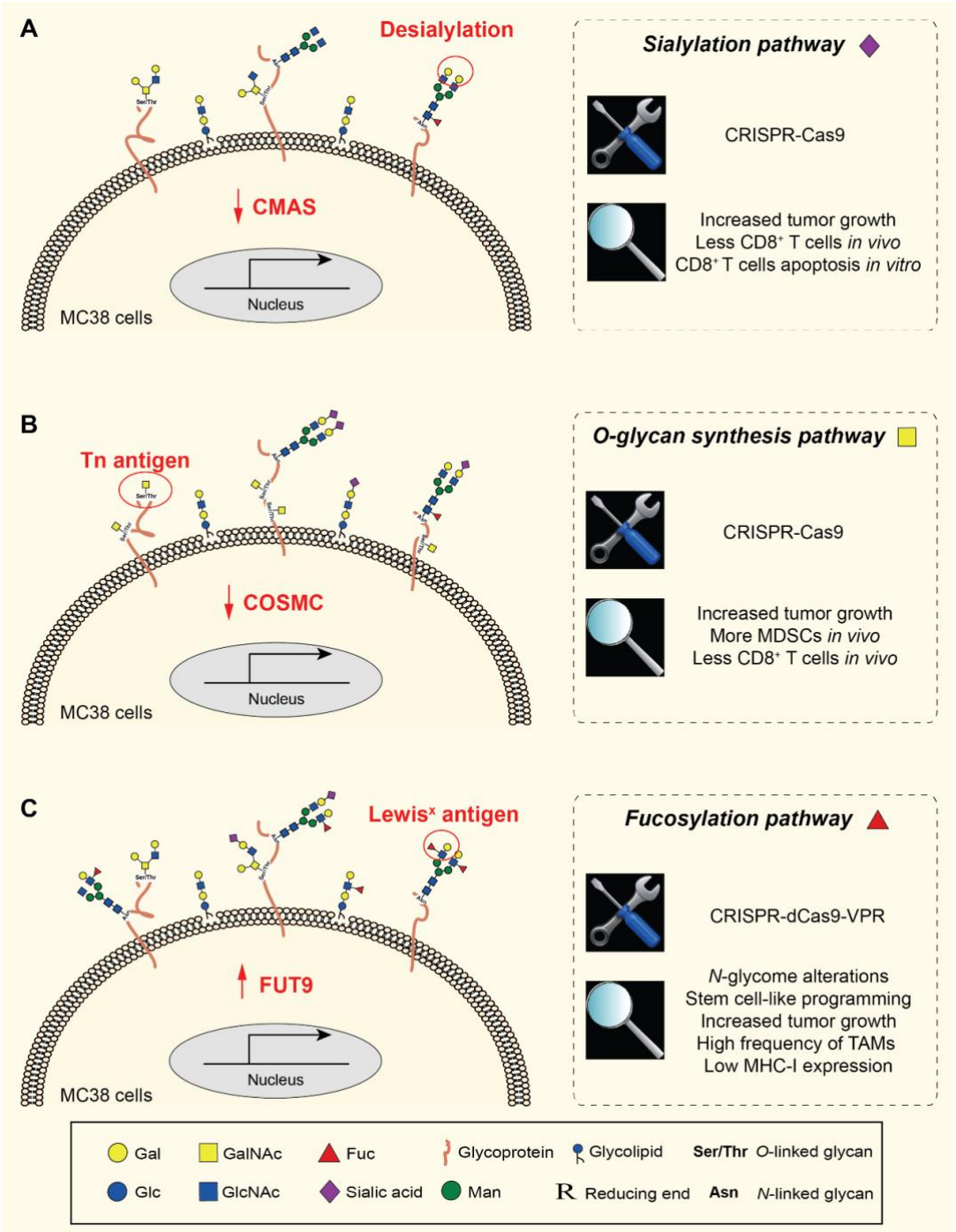

Figure 2. Summary of the main findings presented in this thesis, highlighting the role of aberrant cancer glycosylation as a potent regulator of tumor growth and anti-tumor immunity. A) Knock out of the *Cmas* gene (involved in the sialylation pathway) in MC38 cells using the CRISPR-Cas9 system leads to complete tumor desialylation (lack of sialic acid residues on cell-surface glycans) and is accompanied by increased tumor growth (relative to control tumors) and less $CD8^+$ T cells *in vivo* along with $CD8^+$ T cell apoptosis *in vitro*. B) Knock out of the *Cosmc* gene (*O*-glycan biosynthesis pathway) in MC38 cells using the CRISPR-Cas9 system results in MC38-Tn^{high} cells characterized by increased tumor growth as well as higher frequency of myeloid-derived suppressor cells (MDSCs) and $CD8^+$ T cells *in vivo.* C) Transcriptional activation of the *Fut9* gene (fucosylation pathway) in MC38 cells using the CRISPR-dCas9-VPR technology induces $Lewis^x$-specific *N*-glycome alterations, stem cell-like programming, increased tumor growth, higher frequency of tumor-associated macrophages (TAMs) and reduced MHC-I expression by cancer cells.