Glioblastoma stem cells differentiation through epigenetic modulation is driven by miR-296-5p/HMGA1/Sox2 axis

Veronica Ferrucci[1,2,3], **Massimo Zollo**[1,2,3]

[1]Department of Molecular Medicine and Medical Biotechnology, 'Federico II' University of Naples, Via Pansini 5, Naples, Italy; [2]Ceinge Biotecnologie Avanzate, Naples, Italy; [3]European School of Molecular Medicine (SEMM), Milan, Italy

Correspondence to: Prof. Massimo Zollo. Department of Molecular Medicine and Medical Biotechnology, 'Federico II' University of Naples, Via Pansini 5, Naples, Italy; Ceinge Biotecnologie Avanzate, Via Gaetano Salvatore 486, Naples, Italy. Email: massimo.zollo@unina.it.

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Abstract: Glioma Stem Cells (GSCs) biology is finely regulated by a cross-talk of epigenetic mechanisms of regulation and the action of Oct4/Sox2 stemness gene drivers. Recently, a novel mechanism of regulation of miRNA-296-5p/Sox2 axis together with a new gene target (HMGA1) was described. The results raised questions of great relevance in glioma therapy, especially for the failure of the current radio/chemo-therapies. Can miR-296-5p/HMGA1/Sox2 axis trigger the differentiation of GSCs subpopulation? Can these findings be used in a short term to develop novel therapeutics to fight glioma? We thus believe that targeting HMGA1 in GSCs, via nano-liposomes miRNA delivery together with epigenetic drugs, could overcome the failure of the current therapies. Additionally, *in silico* analyses to identify other gene targets of miR-296-5p with data available in literature, found a remarkable association with new gene targets whose functions are related to cell differentiation processes. Taken altogether these findings make miR-296-5p an attractive target for human brain cancer treatment.

Keywords: Cancer-stem cells; epigenetics; glioma; miR-296-5p; Sox2/Oct4/HMGA1 axis

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Cancer stem cells (CSCs) are defined as "a small subset of cancerous population responsible for tumor initiation and growth, which also possess the characteristic properties of quiescence, indefinite self-renewal, intrinsic resistance to chemo- and radio-therapy and capability to give rise to differentiated progeny" (1). The biological function of normal stem cells (SCs) in their tissue counterparts is to provide a supply of terminally differentiated and functional cells in the tissues with higher cellular turnover (2). Similar to normal SCs, CSCs also contain the biological properties of unlimited proliferation, self-renewal and differentiation into specialized cell types. Nowadays, the deregulation of SCs function has been reported to have a role in the genesis of some tumors (2). Because of these common features, it was suggested that tumorigenic cells could undergo processes that are analogous to the self-renewal and differentiation of SCs (3). However, SCs and CSCs are wrongly considered more similar than they really are (2). Normal SCs are notable for their vigilance in proliferation control and for their care of genomic integrity maintaining. Instead, CSCs are distinguished by their lack of control of such processes.

Several oncogenic pathways, such as the Notch, Sonic hedgehog (Shh) and Wnt signalling cascades, have been reported to regulate self-renewal and normal development in SCs and to be dysregulated during malignant transformation (3). For the above reasons, the hypothesis that SCs themselves may represent the target of transformation in certain tumors generates new ideas for the development of novel therapeutic strategies for cancer treatment. However, there is much yet to be learned about how the mechanisms that regulate normal SCs may be used by cancer cells to propagate themselves.

An additional level of complexity is given by the epigenetic alterations, including DNA methylation and histone modifications that are the key factors in the differentiation of SCs into different tissue subtypes. During tumorigenesis, the generation of CSCs may involve a similar epigenetic reprogramming where, in contrast, it leads to the loss of expression of genes specific to the differentiated state and regaining of stem cell-specific characteristics (4). These events do not take in consideration the mutations-driven clonal evolution of tumorigenic cells that will be a topic for further discussions (5).

Characterizing the epigenetic marks of CSCs and their signalling cascades might help to develop therapeutic strategies against the chemo-resistant tumors (4). CSCs-targeted therapeutic approaches would improve patient survival by reducing tumor relapse. The differentiation of CSCs is of importance for cancer therapy. The differentiation therapy is an emerging therapeutic approach in which CSCs are induced to differentiate toward a mature differentiated form, through the activation of differentiation-related signalling pathways, miRNA-mediated alterations and epigenetic changes. Thus, understanding the origin of CSCs and their epigenetic regulation is crucial to develop a treatment strategy against not only to the heterogeneous population of cancer cells but also to CSCs.

To date, CSCs have been isolated from several human tumors, including brain cancer (6-9).

Diffuse gliomas are the most common primary tumors of the central nervous system occurring in both adults and children with an incidence of ~5 cases per 100,000 (10,11). The mutational spectrum, hystopathology and methylation status defined the current classification for gliomas approved by the World Health Organization (WHO) (10). To date, adult primary Glioblastoma (GBM) and pediatric Hemispheric high-grade glioma (HGG) are often fatal due to the resistance to the current standard of care therapies (surgery, radiation and chemotherapy) (10) in almost 90% (12) of the recurrent gliomas).

The presence of a cancer-stem-like cells subpopulation in glioma, also termed glioma stem cells (GSCs), has been reported as a leading factor contributing to drug-resistance and relapse following the conventional radio and chemo-therapy for glioma treatment (10,13). GSCs are a highly tumorigenic subpopulation of tumor cells with the potential to initiate and maintain growth of glioma thanks to their properties in terms of self-renewal capacity, multilineage differentiation potential and expression of stemness markers (14,15). The phenotypic plasticity of GSCs, including their differentiation, is believed to be also regulated by epigenetic mechanisms because their genetic alterations are the same of those in the more differentiated cancer cells to which they give rise (10,15). Because of the potential reversibility of the epigenetic alterations, the use of epigenetic drugs would be an attractive approach to trigger GSCs differentiation abolishing their tumor-propagating properties and suppressing tumorigenesis (10,13). Nowdays, a great impact is given by the functions of non-coding RNAs, including microRNAs (miRNAs), which are acting as epigenetic regulators of GSCs differentiation (15). Moreover, recent findings have suggested the potential role of miRNAs in the regulation of gene expression networks in GSCs responsible for their differentiation programme (15,16).

A disregulation of miRNAs profile in glioma has been reported in both tumor tissues and serum and a small set of differentially expressed circulating miRNAs has been described as potential novel diagnostic biomarker (17,18).

In this issue, we discuss the findings of microRNA-296-5p (miR-296-5p) by Lopez-Bertoni *et al.* (19). MiR-296-5p is involved in tumorigenic processes acting as both cancer-promoting (*oncomiR*) or tumor-suppressor miRNA in several tumors. Its oncogenic role has been described in gastric cancer, where it promoted tumor progression through the repression of its target *Caudal*-related homeobox 1 (CDX1) (20). Furthermore, miR-296-5p has been demonstrated to be positively correlated with radioresistance and relapse in early-stage of laryngeal carcinoma (21). Nonetheless, miR-296-5p was found to act as a tumor-suppressor miRNA in prostate (22), breast (23) and non-small cell lung cancer (24) through the repression of different target mRNAs. MiR-296-5p has been also found as a "risk" miRNA within a miRNAs signature that was developed as a prognostic marker for patients risk stratification in anaplastic gliomas, Secondary and Proneural GBMs to identify those with a high risk of unfavorable outcome (18). Therefore, miR-296-5p has been also described as an "angiomiRs" with a role in the regulation of neovascularization in glioma (25). Indeed, miR-296-5p was found up-regulated in tumor blood vessels isolated from human GMB tumors where it functionally promoted the angiogenic phenotype by directly inhibiting the expression of hepatocyte growth factor-regulated tyrosine kinase substrate (HGS) (25).

Lopez-Bertoni *et al.* (19) identified the high mobility group AT-hook 1 (HMGA1) protein as a novel target of miR-296-5p in GBM. Importantly, Lopez-Bertoni *et al.* (19)

provided clarity to how the cross-talk between epigenetic modifications, miR-296-5p repression and transcription factors inducing stemness, can regulate GSCs phenotype. Lopez-Bertoni *et al.* (26) had previously identified some potential miRNAs with a role in GBM stem-like phenotype induced by the transcriptional factors Oct4 and Sox2 (26). Briefly, they found a positive correlation between the expression of Oct4/Sox2 and the stemness properties in GBM-neurospheres mediated by DNA methyltransferase 1 (DNMT1) (26). In their proposed model, the acquisition of stemness properties in GSCs cells was mediated by Oct4/Sox2-induced DNMT1 that in turn switched-off the expression of different miRNAs through DNA methylation (26). They used a Human Brain Cancer miRNA PCR array (SABioscience) to look for changes in miRNAs profile after Oct4/Sox2-induced stem like cell phenotype in GSCs (26). However, this approach may not allow the identification of the yet-undiscovered miRNAs or those recently identified having a role in brain tumors. One example is taken by microRNA-199b-5p (miR-199b-5p) that had been reported to impair CSCs in medulloblastoma (MB) (27) and in another study in glioma cells (28). A list of other putative miRNAs influencing MB Cancer Stem Cells function and relation to tumor progression are presented (29). Using this approach based on miRNA PCR array, the authors found twenty-three downregulated and ten upregulated miRNAs with different fold-change (≥2) following Oct4/Sox2 overexpression (26). Then, the authors chose a subset of those potential regulated miRNAs to further validate the array and to evaluate their promoter CpG island methylation status after Oct4/Sox2 overexpression. In their analyses, unfortunately, they did not take into account the upregulated miRNAs. These results might be of importance to discuss other signalling networks involved in this axis of regulation. Hoverer, among those downregulated, they focused on miR-296-5p (19) whose promoter DNA methylation increased from 67 to 100% in GSCs expressing Oct4/Sox2 (26).

In addition, they found the tumor-suppressor microRNA-34a (miR-34a) among the downregulated miRNAs. The anti-tumorigenic properties of miR-34a were deeply studied in brain tumors, including neuroblastoma (30), medulloblastoma (31,32) and glioma in which it was previously reported to inhibit tumor growth of glioma stem cell (33). The link between miR-34a and *Oct4/Sox2* gene drivers regulation and how it controls the epigenetic actions, will be of great importance to study GSCs biology.

The authors further investigated the mechanisms beyond the decrease of miR-296-5p expression levels in GSCs (19). They suggested that endogenous miR-296-5p can act as an inhibitor of the stem-like cell phenotype in GBM neurospheres with decreased pre-miR-296-5p levels (from 50% to 90% reduction) in those overexpressing Oct4/Sox2. They showed that the inhibition of miR-296-5p, via an antisense miRNA (or miR-296-5p *sponge*), increased self-renewal property and stem cell driver factors (including Sox2) in GSCs-neurospheres (19). The authors also investigated the effects of miRNA precursor (pre-miR-296-5p) overexpression in GSCs using a Doxycycline-inducible system. As expected, they found a decrease in sphere-forming ability and proliferation with the downregulation of both stemness markers and drivers.

The anti-tumorigenic effects of miR-296-5p have been shown also *in vivo* (19). For this purpose, pre-miR-296-5p-inducible GSCs were subcutaneously injected into the flanks of immuno-compromised mice (19). The miRNA inhibited the tumor-growth and reduced tumor burdens *in vivo* (19). One important limitation of this study is related to the unexpected use of immuno-compromised mice, instead of using syngenic animal models of glioma, to study the effects of the miR-296-5p on glioma growth *in vivo*. Growing evidence indicates that cancer cells can grow by creating a favorable environment that includes vascular endothelial cells, stromal cells and different elements of the immune system which are responsible for the malignant progression (34). The brain tumor microenvironment is infiltrated by several populations of immune cells: myeloid-derived suppressor cells (MDSCs), T-lymphocytes, dendritic cells and tumor-associated macrophages (TAMs) (35). Therefore, immuno-deficient mice xenografts of human tumor cell lines have poor predictive power in the translation of cancer therapeutics into clinical settings failing to reproduce the tumor microenvironment (36). Taking into account also the brain tumor microenvironment, it will be also important to clarify the previously reported pro-angiogenic role of miR-296-5p in glioma endothelial cells (25).

Furthermore, the future use of miR-296-5p as an anti-cancer therapeutic in glioma treatment would be also investigated using these approaches. However, one of the major issue to be addressed is its delivery *in vivo*. An attractive strategy would be to obtain a targeted delivery of miR-296-5p using a lipid-based nano-carrier coupled to a specific marker for GBM cells. For this purpose, (CTX), a scorpion-derived peptide, was recently reported as a specific marker for glioma and CTX-coupled Stable Nucleic Acid

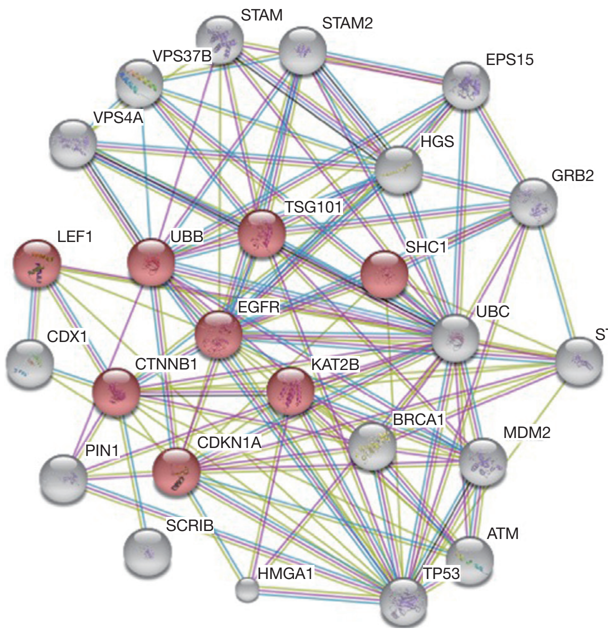

Figure 1 The protein-protein interaction network using the proteins identified by literature search being targets of miR-296-5p (CDX1, Pin1, HGS, SCRIB, HMGA1) was analyzed with the STRING software (Search Tool for the Retrieval of Interacting Genes/Proteins) available at http://string-db.org. Within this network, the proteins identified are represented as nodes with the colors of the lines connecting the nodes that are representing different evidence types for protein linkage. Then, the proteins labeled in red here listed (LEF1, lymphoid enhancer binding factor 1; KAT2B, lysine acetyltransferase 2B; EGFR, epidermal growth factor receptor; CTNNB1, catenin beta 1; TSG101, tumor susceptibility 101; CDKN1A, cyclin dependent kinase inhibitor 1a; SHC1, SHC adaptor protein 1; UBB, ubiquitin B) are known to be involved in cell differentiation processes with a significant statistical p-value as described by their False Discovery Rate (fdr).

Lipid Particles (SNALPs) were found to be effective in mediating the nucleic acid delivery to GBM cells also *in vivo* (37). Altogether, these strategies would be of importance to bring the findings of Lopez-Bertoni *et al.* (19) in translational studies investigating the ability of miR-296-5p to induce the differentiation of GSCs *in vivo*. Because of the clear positive correlation between GSCs and radio/chemo-resistance, targeting stem-like propagating cells in glioma would be of impact to develop novel anti-cancer therapeutics based on miRNAs. Nonetheless, more research is needed to optimize the strategies for miRNAs delivery and to assess the immunogenicity of the administrated nanoparticles to determine a safety profile of SNALP-containing miRNA by evaluating the activation of microglia and the pro-inflammatory citokines in the treated immuno-competent mice (38).

Recently, the authors identified additionally HMGA1 as the main direct functional downstream target of miR-296-5p involved in differentiating signals and GSCs phenotype (19). Of note, HMGA1 expression was found to be upregulated and inversely correlated with miR-296-5p in primary GBM neurospheres isolated from patients (19). HMGA1 was responsible for maintaining the expression of the transcription factors involved in reprogramming. Among these, Sox2 was shown to be the most activated by HMGA1 (19). Moreover, HMGA1 has been shown to bind Sox2 promoter through its two binding sites and to displace histone H1 from this location in response to Oct4/Sox2 overexpression (19). Their findings also suggest that miR-296-5p resides within a positive feedback loop responsible for the increase of Sox2 levels, one of the drivers of stem cell phenotype (19). Interestingly, Sox2 has been recently identified as the most enriched gene among the stemness signature in $CD133^+$ GBM stem-like cells (39). Targeting Sox2 expression in GSCs to inhibit tumor initiation as well as drug resistance would be an attractive therapeutic approach (40). Epigenetic drugs would be another interesting strategy to modulate the therapy sensitivity by targeting of GSCs. Briefly, epigenetic drugs, acting through the modulation of DNA methylation pattern, could rescue miR-296-5p expression levels and subsequently induce the differentiation of GSCs. This will be aimed to sensitize the glioma stem-cells subpopulation to the action of the chemotherapeutics currently used for glioma treatment (e.g., Temozolomide). To date, both short term and long-term Valproic Acid treatments have not been reported to increase Temozolomide sensivity *in vitro* (13). This failure was probably due to the mechanisms driving the clonal evolution of glioma tumorigenic cells under therapy with Temozolomide (5).

According to the authors, this study provides a "novel epigenetically circuit integrating miR296-5p, chromatin remodelling and Sox2 transcription factor to regulate GMB propagating stem cells". However, looking at the targets of miR296-5p, a notable evidence is the remarkable association between the microRNA and the cell differentiation process, including stem cell differentiation.

These findings are underpinning a picture that the validated targets of miR296-5p [CDX1 (22), Peptidyl-

Table 1 The list of proteins targets of miR-296-5p involved in "cell differentiation" (Gene Ontology term) together with their biological functions are shown below

GO_id	Term and biological function	Genes Names	P value_fdr
GO:0030182	Neuron differentiation	CTNNB1; STAT3; LEF1; SHC1; SCRIB; GRB2; UBB; EGFR	4.77E-4
GO:0030154	Cell differentiation	LEF1; KAT2B; EGFR; CTNNB1; TSG101; CDKN1A; SHC1; UBB	2.33E-1
GO:0030855	Epithelial cell differentiation	CTNNB1; TSG101; CDKN1A; SCRIB; LEF1	7.59E-3
GO:0045595	Regulation of cell differentiation	TP53; CTNNB1; PIN1; LEF1; KAT2B	3.19E-1
GO:0000904	Cell morphogenesis involved in differentiation	SCRIB; GRB2; LEF1; EGFR	1.1E-1
GO:0002065	Columnar/cuboidal epithelial cell differentiation	CDKN1A; SCRIB; LEF1	9.31E-3
GO:0010001	Glial cell differentiation	STAT3; CTNNB1; LEF1	1.4E-2
GO:0009913	Epidermal cell differentiation	CTNNB1; SCRIB; TSG101	1.7E-2
GO:0030098	Lymphocyte differentiation	ATM; TP53; CTNNB1	3.61E-2
GO:0048863	Stem cell differentiation	STAT3; CTNNB1; LEF1	8.88E-2
GO:0048667	Cell morphogenesis involved in neuron differentiation	SCRIB; GRB2; EGFR	3.28E-1

The proteins were taken from the predicted protein-protein interaction network (CDX1, PIN1, HGS, SCRIB, HMGA1) using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) software. Only those proteins showing a relevant function in cell differentiation processes, with P values >0.0001 (as corrected by "false discovery rates", fdr), were taken into account.

prolyl isomerase PIN1 (22), scribbled planar cell polarity protein SCRIB (23), HGS (25) and HMGA1 (19)] take part altogether to a predicted protein–protein interaction network involved in cell differentiation processes (see *Figure 1* and *Table 1*). This scenario opens new research hypotheses to find novel druggable targets belonging to the regulation of cell differentiation process driven by miR-296-5p/Sox2 axis through epigenetic mechanisms.

Studies in the near future should address these hypotheses and in particular if targeting HMGA1 in GSCs, by forcing the expression of miR-296-5p, via nano-liposomes (37), or using epigenetic drugs, or their combination, will improve the therapy of glioma.

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