



The CXCL12/CXCR4 pathway or the autocrine proliferative loop of the glioblastoma stem cells

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Comment on: Calinescu AA, Yadav VN, Carballo E, *et al.* Survival and Proliferation of Neural Progenitor-Derived Glioblastomas Under Hypoxic Stress is Controlled by a CXCL12/CXCR4 Autocrine-Positive Feedback Mechanism. *Clin Cancer Res* 2017;23:1250-62.

Submitted Feb 01, 2017. Accepted for publication Feb 08, 2017.

doi: 10.21037/tcr.2017.03.47

View this article at: <http://dx.doi.org/10.21037/tcr.2017.03.47>

Despite multimodal therapy (the classical Stupp's protocol) including surgical resection (as large as possible), radiotherapy and chemotherapy, glioblastoma (GBM) remain a burden as the global survival rate at two years reaches barely 9% of patients (1). This situation is mainly a consequence of a systematic recurrence and this recurrence is itself a consequence of various causes, acting alone or synergistically: heterogeneous nature of the disease (2), the presence of the brain-blood barrier which impedes the potentially active drugs to get into the brain (3), the lack of targeted chemotherapeutic molecules and the persistence of GBM-initiating or stem cells (GSC) (4).

The recent study of Calinescu *et al.* (5) specifically address the question of GSC as those cells have been demonstrated to be resistant to chemo- and radiotherapy (6,7) and responsible for promoting neo-angiogenesis in tumors (8). Indeed, the authors induced brain tumors in a model recently described and based on a plasmid which allow a knock-in of immature brain cells using a *Sleeping Beauty* transposase (9). The inserted genes are the *simian virus 40 large T antigen (SV40-LgT)* and a constitutively active human NRAS oncogene (*NRAS*). The plasmid construct was injected in the right lateral ventricle of post-natal day 1 mice expressing the dsRed fluorescent protein under the control of the CXCL12 promoter. Ten days after the plasmid injection, one can observe clusters of cells in the sub-ventricular zone (SVZ) expressing the SV40-LgT, but also Nestin and Olig2, two markers of cell immaturity in the brain. These cell clusters are near capillary where endothelial cells express the chemokine CXCL12. Nineteen days after the plasmid injection, the tumors can be

observed, some present in the SVZ and some in the brain parenchyma. Those tumors are surrounded by reactive astrocytes expressing high levels of GFAP. Those tumors express Nestin and Olig2 but also exhibit several GBM hallmarks (multinucleated cells, numerous mitosis, vascular proliferation, pseudopalisading necrosis). Moreover, there is a lack of an efficient brain-blood barrier as intravascular dextrans are able to diffuse into the tumor.

Those tumors have been put in cultures and Calinescu *et al.* demonstrated that some tumoral cells grown as spheres, express CD133 and are able to generate tumors when transplanted back in naïve mice. All these features are specificities of GSC. Therefore, the authors compare the transcriptome of those cells (named M7) to two other GBM established mouse cell lines (GL26 and GL26A1, the latter is overexpressing NRAS). They found a differential expression for 5866 probe sets, in which several chemokines: CCL2, CXCL1, CCL7 and CXCL12. They focused to CXCL12 for two reasons: (I) they wanted to characterize the molecular environment of GSC that is responsible for the set-up of a niche for these tumor stem cells; and (II) CXCL12 has been previously shown to regulate neural and hematopoietic niches, acting through its CXCR4 receptor (10). Indeed, it appears that, at the phylogenetical point of view, the CXCL12/CXCR4 axis is very old, existing before the appearance of an adaptive immune response, with ancestral role in the nervous system (11).

Calinescu *et al.* quantified and characterized the expression of both CXCL12 and CXCR4 in their GBM model. They showed that the cytokine is expressed at high levels in the GBM induced by the transforming plasmid

but also in the surrounding brain tissue. CXCR4, the CXCL12 receptor, is expressed at high level by tumoral cells, by immune infiltrating cells but not by normal brain cells. Finally, CXCR7, the other and less known CXCL12 receptor, is barely detectable in the system. CXCL12 and CXCR4 are also expressed by cultivated tumoral cells and AMD3100 or Plerixafor, the classical CXCR4-inhibitor, stimulates *in vitro* the early apoptosis (modestly) and inhibits (more strongly) the cell proliferation. Indeed, cell cycle analysis revealed that AMD3100 maintains cells in G2/M phases and decreases the number of cells in S-phase. This could be a consequence of a decrease of expression of early G1 cyclins and CDKs like cyclin D1, CDK4 and CDK6.

Then, the authors looked for a possible action of AMD3100 on several pathways previously demonstrated to be important in GBM cells. They thus observed a decrease (I) of expression and of phosphorylation of the tumor suppressor retinoblastoma (Rb) which is over-activated by mutation or various alterations in 70% of GBM; (II) of phospho-Akt which is also over-activated in GBM cells both by various tyrosine-kinase receptors (EGFR, PDGFRA, VEGFR2, ...) and by the inactivation of the phosphatase PTEN; (III) of the anti-apoptotic protein Bcl-XL previously shown to be over-expressed in GBM. It is important to note that the effects of AMD3100 on the apoptosis of GBM cells, on their proliferation and on the down-regulation of all these proteins required a long (at least 72 hours) stimulation.

CXCL12 is known to be overexpressed in hypoxic brain or by TGF β (12). Calinescu *et al.* showed that *in vitro*, *cxcl12* is 6-fold overexpressed with an increase of *bif1-a* and *tgf- β* expression, at 96 hours of culture, while the *cxr4* expression remains stable. AMD3100 blocks the increase of expression of *tgf- β* and *cxcl12* without any effect on the expression of *bif1-a*. These data suggest that the CXCL12/CXCR4 pathway operated in GSC cultures as an autocrine positive feedback loop, promoting the survival and the proliferation of these cells.

As their GBM model is artificial (overexpression of SV40lgT and the mutated NRAS in post-natal mouse neural precursors), Calinescu *et al.* compared at the molecular level the spheres of GBM cells cultivated from their induced GBM and various GBM cells established in cell lines (U251 and U87) or derived from a gliosarcoma (which is a glioma with a high proportion of cells harboring mesenchymal features) and known to be enriched in GSC. Their comparisons were obtained by western blot and concerned some classical GBM markers. The authors conclude that there is a similar molecular profile between

their induced-GBM cells and the human cells. However, a deeper molecular analysis, at the transcriptomic or at the proteomic level, would be useful here to assess the model used in the Calinescu's paper.

This deeper molecular analysis between the induced-GBM cells by the SV4LgT and NRAS and the other GBM cells, established as cell lines or derived from tumoral resection, is indeed mandatory as the expression of CXCL12 and CXCR4 but also the effect of a CXCL12- or an AMD3100-stimulation are various regarding other GBM cells tested by the authors.

Finally, Calinescu *et al.* treated by AMD3100 mice injected at post-natal day 1 with the transforming plasmid. The drug has been delivered by osmotic pumps that have been implanted at day 21 (when tumors develop macroscopically) and left in place for 5 days. The authors observe a non-significant tumor volume decrease when animals receive AMD3100 and a significant decrease of BrdU labeling index in the tumors. However, the median survival of AMD3100-treated animals was significantly higher (53 *vs.* 30 days) than animals that received saline. These results obtained with a pharmacological approach targeting the CXCR4 signalization were phenocopied using genetic approaches targeting the *cxr4* expression.

The merit of this study is to demonstrate very conclusively the role of the CXCL12/CXCR4 pathway in a model of GBM growth and invasion. However, the first (but also the main) criticism that one can raise is in this interesting paper is about the model. Indeed, the model used by Calinescu *et al.* has the main advantages to be highly reproducible, to generate operationally-defined GSC and to produce tumors with all the histological characteristics described for GBM. The disadvantages of this model are the cell transformation of postnatal neural precursor, the relationship of the tumor with an immature brain parenchyma and, at least so far, a clear description of the molecular profiling of the tumors both, between themselves and with the human GBM. Indeed, it shouldn't be now a big work to perform RNA sequencing assays of three to six of these induced-GBM and to compare with themselves and with data available at the TCGA. This analysis could also be very instructive about the GBM subtype [with or without IDH mutations (13), the classification of those GBM according to Verhaak (14), ...]. Concerning the age of the brain in which the GBM is induced, it could be important as in human patients, GBM in children and GBM in adult are different in their prognosis but also at a molecular level (15).

Finally, one can also have a regret. Indeed, in their model (with all the odds that we have explained), Calinescu

et al. clearly demonstrated that the CXCL12/CXCR4 pathway does play a role in tumor growth *in vivo* as the AMD3100 doubles the mice survival. It would have been interesting to look for a possible role of this signaling pathway in tumor arousal. One has to admit that implanting mini-osmotic pumps in newborn animals is technically difficult. However a targeted gene invalidation approach should be here very informative.

Acknowledgments

Funding: B Rogister's work is supported by grants from the National Fund for Scientific Research (FNRS/TELEVIE, PDR.15.3621) by the Special Funds of the University of Liège (CFRA.2394) and by a Léon Frédéricq grant.

Footnote

Provenance and Peer Review: This article was commissioned and reviewed by the Section Editor Ning Huang (Department of Neurosurgery, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China).

Conflicts of Interest: The author has completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2017.03.47>). The author has no conflicts of interest to declare.

Ethical Statement: The author is accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Cite this article as: Rogister B. The CXCL12/CXCR4 pathway or the autocrine proliferative loop of the glioblastoma stem cells. *Transl Cancer Res* 2017;6(Suppl 2):S388-S390. doi: 10.21037/tcr.2017.03.47