Author's response to reviews

Title: CXCR7 is induced by hypoxia and mediates glioma cell migration towards SDF-1alpha

Authors:

Mine Esencay (mine.esencay@nyumc.org)
David Zagzag (david.zagzag@nyumc.org)

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Author's response to reviews: see over
May 19, 2013

Dafne Solera
Executive Editor, BioMed Central

Dear Dr. Solera,

We would like to thank you and the Executive Editorial Board of BMC Cancer for reviewing our manuscript (Manuscript ID: 1191713407874422) entitled, “CXCR7 is induced by hypoxia and mediates glioma cell migration towards SDF-1alpha”. We appreciate your consideration of our revised version. Our responses to the reviewers’ comments are discussed below, point by point. The revisions in the text have also been underlined to aid the Reviewers. We have added Yasmeen Sarfraz as a co-author to the manuscript.

**Responses to Reviewers:**

We appreciate that both reviewers found our manuscript to be “an important current research subject” and “is a very interesting paper that addresses an important topic of great clinical relevance”. In addition, they found the paper to be “well written” and the results “straight forward and of high quality”. They also mentioned that, “the conclusions are clearly supported by the results. The relevant literature is appropriately cited and discussed”.

**Reviewer 1**

**Comment 1:** Measurement of CXCR7 expression: The main problem is the quantitative determination of CXCR7, which was done by Western blots. Since the reference beta-actin is overexposed in some blots, the relatively small changes in CXCR7 are not always convincing. An accurate determination would involve at least 3 independent cell culture experiments with corresponding blots, not overexposed for the reference, and a densitometric analysis. Since in contrast to CXCR4 there is still some controversy about CXCR7 regulation by hypoxia (e.g. Schutyser et al. Eur Cytokine Netw 2007;18:59-70), it would be mandatory to measure also the transcription of CXCR7 mRNA e.g. by quantitative RT-PCR.

**Response:** As a first step to investigate the possible mechanisms of CXCR7 upregulation in hypoxic conditions, we performed qRT-PCR in U87MG glioma cells cultured under normoxic and hypoxic conditions. CXCR7 mRNA was collected after 6, 18, and 24 hours of exposure to hypoxia (1% O2). We observed that CXCR7 mRNA levels were significantly upregulated in hypoxic conditions. Please see results below:
Comment 2: *CXCR7 and CXCR4 expression on the glioma cells used:* Since some of the effects could also be mediated by CXCR4 (that is addressed by experiments with the specific inhibitor AM3100, but not by use of specific CXCR7 inhibitors), it is important to know if CXCR4 is expressed on the cell lines used, and if it is induced by hypoxia. I miss corresponding blots and qRT-PCR determinations!

Response: Previously, we showed that hypoxia upregulates CXCR4 protein expression in U87MG, LN229 and LN308 glioma cell lines (Zagzag et al., 2008). We also showed that hypoxia upregulates CXCR4 mRNA levels in U87MG and LN308 glioma cells (Zagzag et al., 2006). In this manuscript, we blocked CXCR4 signaling by AMD3100 (a CXCR4 inhibitor) and we inhibited CXCR7 signaling by shRNA sequences directed against CXCR7. Before commencing our experiments, we checked the specificity of the shCXCR7 sequences by studying the protein expression of CXCR4 in the glioma cells that were infected with shCXCR7. We did not observe any difference in CXCR4 protein levels under these conditions and thus confirmed that the shCXCR7 sequences were specific for CXCR7, with no effect on CXCR4 expression. AMD3100, at the concentration used in our manuscript, also had no effect on CXCR7 signaling. Therefore, the data we present here are attributed solely to the specific inhibition of either CXCR4 or CXCR7, where indicated.

Comment 3: *Chemotaxis assays:* The number of independent experiments is too low (n = 2). With some cell lines also the number of migrated cells (especially in controls) appears comparatively low.

Response: Each cell line has its own intrinsic capacity for migratory behavior. Therefore, while some cell lines will migrate more, others will migrate less under the same conditions. Even the intrinsic migratory capacity of glioma cell lines we used in these experiments are a little different among themselves, with LN229 glioma cells tending to migrate slightly more than U87MG and LN308. One possible explanation for this phenomenon is the grade of the tumor and the genomic mutations present in that cell line. The migration assays in this manuscript were carried out in the presence of low amounts of FBS (1%) and reduced incubation time (8 hours). These conditions
were established on purpose in order to better observe the significance of the events. Higher amounts of FBS and longer incubation times would result in a higher number of migrated cells in the controls. However, the presence of growth factors in the FBS, plus cell proliferation induced by longer hours of incubation would interfere with the results of the experiments, and make it impossible to accurately study the significance of events. Examples from literature support our experimental conditions and show that establishing a low number of migrated cells in controls excludes the outside factors contributing to cell migration, and promote better analysis of significance (Jarjour et al., 2011; Delamarre et al., 2009). Although the number of independent experiments is two, results are data that were pooled from both experiments and not the results of one experiment as representation. In addition, the error bars indicate standard deviation (not standard error of mean), and the small range shows that the values were similar between the two independent experiments.

**Comment 4:** Also use the alternative novel nomenclature, CXCL12, for SDF-1 in the introduction.

**Response:** As the reviewer requested, we have added “CXCL12” next to SDF-1 in the Abstract, Background and Methods.

**Comment 5:** Hypoxia, Figure legends: hypoxic condition in figure legends should be specified to 1% oxygen (as described in Methods).

**Response:** Besides Methods, now we also specify hypoxia as 1% O₂ in the Figure Legends.

**Comment 6:** Concentration, Figure legends: Give SDF-1-concentration in nM instead of ng/ml – the Molecular mass is known.

**Response:** 100 ng/ml is a well-established concentration for SDF-1 that is known to have biological activity, used in many publications. However, now, we have also added the concentration in nM in Figure Legends.

**Comment 7:** Give molecular masses in the Western blots.

**Response:** We have indicated the molecular masses for the proteins analyzed by Western blotting in Methods to better guide the readers.

**Reviewer 2**

**Comment 1:** The methods should provide a bit more information on how the Western blot data was quantified and how the fold changes were calculated. Specifically, for the data shown in
In Figure 1, measurements were normalized to loading control, and in Figures 4 and 5, measurements were normalized to total ERK 1/2, AKT, and FAK. We have added this information to the Methods section.

Comment 2: For the shRNA knockdown studies, the authors state the following: “The efficiency of knockdown was confirmed by Western blot analysis.” They should either present this data or state “data not shown”.

Response: We have added “(data not shown)” after “The efficiency of knockdown was confirmed by Western blot analysis” in the Results section for shRNA knockdown studies.

Comment 3: In figure 5, the effects of AMD3100 without CXCR7 knockdown are not shown. It would be helpful if the authors discussed if what is known about CXCR4 signaling. Is it known if CXCR4 induces phosphorylation of ERK or AKT? If so, does AMD3100 inhibit this?

Response: CXCR4-mediated signaling is known to play an important role in angiogenesis, survival, migration, invasion, and proliferation of glioblastoma cell lines (discussed in detail in Zagzag et al., 2006 and Zagzag et al., 2008). Previously, we showed that SDF-1α induces the phosphorylation of FAK, AKT and ERK1/2 in LN308 glioma cells, a cell line that expresses high levels of CXCR4. AMD3100 inhibited the SDF-1α-induced phosphorylation of FAK, AKT and ERK1/2 in this cell line (Zagzag et al., 2008).

Comment 4: It is a very interesting finding that both CXCR4 and CXCR7 are required for SDF-1 induced migration of hypoxic glioma cells, but blocking both CXCR4 and CXCR7 does not provide an additive effect, either with regards to transfilter migration or phosphorylation of ERK and AKT. It is also very interesting that CXCR7 can be co-immunoprecipitated with CXCR4-HA. It would be nice if the paper included a little more discussion on how the authors might interpret these findings. For example, does SDF-1-induced migration require cross-talk between CXCR4 and CXCR7? Or do they have another potential mechanism in mind?

Response: The Reviewer raised an interesting point. We have added a discussion for a potential mechanism that we have in mind under Discussion.

Comment 5: It would be interesting if the authors expanded the discussion to include some mention of SDF-1, CXCR4 and CXCR7 expression in the different subtypes of glioblastoma. For example, they could query the TCGA database to see if there is any subtype specific differences in the expression. Do the levels of these signaling molecules tend to correlate with each other, as one might expect since they are all upregulated by hypoxia.
Response: Upon the suggestion of the Reviewer, we have queried the TCGA database for subtype specific differences in the expression of SDF-1, CXCR4 and CXCR7. Below we show the mRNA expression data, z-Score threshold +/-2 for the glioma subtypes:

All TCGA GBMs with mRNA data

Gene Set / Pathway is altered in 11.2% of all cases.
Glioblastoma (TCGA, Nature 2008)/Tumors with mRNA data: (206)/User-defined List/3 genes

Modify Query

<table>
<thead>
<tr>
<th>OncoPrint</th>
<th>Mutual Exclusivity</th>
<th>Plots</th>
<th>Survival</th>
<th>Network</th>
<th>Download</th>
<th>Bookmark</th>
</tr>
</thead>
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**Customize**

*Remove Unaltered Cases*  *Restore Case Order*  *Remove Whitespace*

Case Set: Tumors with mRNA data, All samples with mRNA expression data (206 samples)

Altered in 23 (11%) of cases

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<tr>
<td>CXCR4</td>
<td>3%</td>
</tr>
<tr>
<td>CXCR7</td>
<td>4%</td>
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</tbody>
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mRNA Upregulation  mRNA Downregulation

Classical

Gene Set / Pathway is altered in 1.9% of all cases.
Glioblastoma (TCGA, Nature 2008)/Expression Cluster Classical: (54)/User-defined List/3 genes

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<th>Mutual Exclusivity</th>
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</table>

**Customize**

*Remove Unaltered Cases*  *Restore Case Order*  *Remove Whitespace*

Case Set: Expression Cluster Classical: Tumors assigned to the "Classical" expression cluster (Verhaak et al., 2010, 54 samples).

Altered in 1 (2%) of cases

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<tr>
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<tr>
<td>CXCR7</td>
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Mesenchymal
Neural

Gene Set / Pathway is altered in 0% of all cases.
Glioblastoma (TCGA, Nature 2008)/Expression Cluster Neural: (29)/User-defined List/3 genes

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<td>CXCR4</td>
<td>0%</td>
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<tr>
<td>CXCR7</td>
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Proneural

Gene Set / Pathway is altered in 10.7% of all cases.
C自律性 (TCGA, Nature 2008)/Expression Cluster Proneural: (56)/User-defined List/3 genes

Modify Query

In summary, we found the Reviewers' comments very helpful in improving our manuscript. We trust these revisions will meet with your approval and hope our manuscript is now suitable for publication in BMC Cancer.

Sincerely,

David Zagzag M.D., Ph.D.
Associate Professor of Pathology and Neurosurgery
Chief, Division of Neuropathology
Director, Human Brain Tumor Bank
Director, Microvascular and Molecular Neuro-Oncology Laboratory
New York University School of Medicine