THE
EMBO
JOURNAL

Infiltrative and drug-resistant slow-cycling cells support metabolic heterogeneity in glioblastoma

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Review timeline:

Submission date:	6th Dec 2017
Editorial Decision:	30th Jan 2018
Revision received:	16th Jul 2018
Editorial Decision:	7th Aug 2018
Revision received:	20th Aug 2018
Accepted:	24th Aug 2018

Editor: Daniel Klimmeck

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30th Jan 2018

Thank you for the submission of your manuscript (EMBOJ-2017-98772) to The EMBO Journal. My apologies for the extended duration of the review process of the manuscript at this time of the year. Three referees have originally been assigned to your manuscript, however one of them did not come back to us even after repeated messages. We have now received reports from two referees, which I enclose below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of major issues that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In more detail, referee #2 states concerns about the physiological relevance of your results and accordingly asks you to corroborate your claims on differential metabolic dependences of SCCs versus FCCs by additional in vivo experiments (complementary assays, loss-of function and inhibition approaches; Ref#1, pts. 5,6,8,10,11). Ref#3 agrees in that the claims on metabolic heterogeneity are not sufficiently well supported at this stage, and in addition is concerned about the robustness of the SCC specification and stemness features as well as relative contribution of SCCs versus bulk cells to the phenotypes observed (ref#3, pts. 1, 2, 4). In addition, the referees list a number of technical issues and controls related to statistics and missing controls that would need to be conclusively addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. Please note however, that we would need strong support from the referees on such a revised version of the manuscript to move towards publication. I agree that it would be essential to

consolidate the in vivo relevance of distinctive metabolic features of slow- versus fast-cycling glioblastoma cells.

REFEREE COMMENTS

Referee #2:

In their report, Hoang-Minh et al describe the metabolic heterogeneity present in glioblastoma cells. This manuscript will be of high interest to readers because of the extensive transcriptomic and metabolomic profiling of SCCs (slow cycling cells), which have been demonstrated to mediate invasiveness, chemoresistance, and recurrence. The combination of multiple in vitro and in vivo experiments from cells derived from multiple primary human tissues is another great strength. This report also highlights tumor heterogeneity and suggests that targeting of both glycolytic processes as well as oxidative phosphorylation will be most effective for tumor control. The manuscript could be greatly strengthened by the addition of in vivo experiments demonstrating the efficacy of FABP7 inhibitors and combinatorial studies with inhibitors of glycolysis. The novelty of the findings presented in this manuscript is somewhat limited by the publication of similar studies in other cancer types1-3 as well as in glioblastoma.4 Overall, the report is strong and I would recommend acceptance following adequate responses to the following points:

Major concerns

1. The title is not precisely indicative of the studies. The authors find that SCC are more invasive but are not testing the invasive cells directly. The fast cycling cells form tumors more quickly and may invade given time. It would be better to focus directly on the way that the cells are separated, i.e. the SCC. It would be interesting to select cells based on invasion and measure the SCC:FCC ratio and metabolic profile, but that was not directly tested.
2. Throughout the manuscript, the authors normalize the assays to each group. This is understandable, but the raw values should be presented, because it is not surprising that metabolism is different in cells with different proliferation.
3. The authors find that SCC are more resistant than FCC to TMZ, but it would be helpful to directly measure the ratios of SCC to FCC in TMZ resistant lines.
4. I am also somewhat confused by the overall premise. Tumors don't have exclusively SCC or FCC cells. What happens to the population over time? Is there an equilibrium? What are the responses to different stimuli when the cells are in combination? It would seem that the assays are missing controls of the combined or bulk cells. Are all the resistant and invasive cells from the SCC?
5. I would suggest that the authors use caution in interpreting the gene expression profiles and mitochondrial tracking dyes as evidence of metabolic states. How does the cell division process change the level of mitochondrial dyes if the mitochondria have to replicate themselves? Is this like label retention as well? It would be standard to use Seahorse measurements as more direct study of metabolism. Direct ox phos vs. glycolysis should be tested.
6. I was surprised by the relatively limited number of in vivo studies tested. There seem to be a single model with one replicate for each study done and no testing of metabolism in vivo, except for Figure 1I. The mitoctracker dye could be used in vivo, it seems.
7. Figure 3: How much faster to FCCs grow than SCCs? Do FCCs show decreased viability in low glucose conditions simply because they proliferate more rapidly and exhaust the supply of glucose within the media?
8. In figure 4, the authors claim that SCCs do not rely on glycolysis for energy production. To support this statement, the authors should treat cells with inhibitors of glycolysis (or perform knockdown of key glycolytic genes) and show a differential sensitivity between SCCs and FCCs (to parallel the OxPhos inhibition studies). This would be a more appropriate control than using only glucose restriction in vitro or low carbohydrate diet in vivo. This would be a very important experiment to support the authors main hypothesis of differential metabolic dependencies between SCCs and FCCs.
9. In Figure 4G/H, the effect of the combination of low glucose with rotenone/metformin appears to be additive and not synergistic (as might be expected from targeting both SCCs and FCCs). This combination of two cellular insults leading to impaired cell proliferation does not specifically support the authors conclusions. Additionally, the terms "HG" and "PG" are not clearly defined in the text or figure legends related to Figure 4.

10. The authors posit an innovative hypothesis that targeting of both glycolytic and OxPhos pathways via FABP7 inhibition would be an ideal method to decrease in vivo tumor formation by targeting both SCCs and FCCs. In vivo experiments utilizing this FABP7 inhibitor in combination with a glycolytic inhibitor would greatly heighten the impact of the findings. There should be some in vivo studies, hopefully in more than one model.
11. I was surprised that the authors did not test FABP7 knockdown directly, but rather only an inhibitor, and there were few studies of proliferation, invasion, etc.
12. The authors discuss stem cells, but this is not developed. None of the studies seem to be directly related to stem cell pathways and stem cell assays are not tested.
13. Supplementary figure 6 is interesting, but I would suggest staining for FABP7 and Ki67 to see if there is overlap. The images shown have very high non-specific staining.

Minor concerns
1. The authors propose that ZEB1 contributes to invasiveness of slow cycling cells in vivo. Could the authors perform a knockdown of ZEB1 in SCCs and determine if this diminishes invasiveness in this model?
2. Doses for in vitro temozolomide treatment may not be physiologically relevant, as the authors suggest. According to one report5, the maximum TMZ concentration within the brain is around 3-5uM (0.6ug/mL +/- 0.3). Could the authors explain their use of 50-1000uM of temozolomide in this study?
3. Presentation of data using Enrichment maps (http://baderlab.org/Software/EnrichmentMap/UserManual) may allow for more customizable and aesthetically pleasing representations of data in Figure 2 and supplementary Figure 2.
4. The authors may also consider exploring differential endogenous lipid synthesis processes in SCCs as opposed to focusing only on lipid update from the microenvironment.
5. Further validation of the specificity of the FABP7 inhibitor (or a reference) would be helpful.

References
1 Lagadinou, E. D. et al. BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. Cell Stem Cell 12, 329-341, doi:10.1016/j.stem.2012.12.013 (2013).
2 Sancho, P. et al. MYC/PGC-1alpha Balance Determines the Metabolic Phenotype and Plasticity of Pancreatic Cancer Stem Cells. Cell metabolism 22, 590-605, doi:10.1016/j.cmet.2015.08.015 (2015).
3 Ye, X. Q. et al. Mitochondrial and energy metabolism-related properties as novel indicators of lung cancer stem cells. International journal of cancer. Journal international du cancer 129, 820-831, doi:10.1002/ijc.25944 (2011).
4 Janiszewska, M. et al. Imp2 controls oxidative phosphorylation and is crucial for preserving glioblastoma cancer stem cells. Genes Dev 26, 1926-1944, doi:10.1101/gad.188292.112 (2012).
5 Portnow, J. et al. The neuropharmacokinetics of temozolomide in patients with resectable brain tumors: potential implications for the current approach to chemoradiation. Clinical cancer research : an official journal of the American Association for Cancer Research 15, 7092-7098, doi:10.1158/1078-0432.CCR-09-1349 (2009).

Referee #3:

In this study, the authors provide a comprehensive characterization of glioblastoma cell subpopulations according to metabolic features. The main finding is that the subpopulation of slow-cycling glioblastoma cells depend more on mitochondrial oxidative phosphorylation, while fast-cycling tumor cells more on aerobic glycolysis. More differences are reported, like higher lipid droplet content and utilization as a rescue mechanism by slow -cycling cells; significant differences in lysosomes; and a molecular involvement of the FABP family of proteins.

The experiments are conducted in a careful and thoughtful manner, and the data included in this manuscript are rich, and support most conclusions well.

The potential translational relevance is apparent, and should be more explicitly discussed with specific suggestions for diets and trial options in the discussion section. Other parts of the discussion section appear somewhat lengthy and can be shortened.

I have several major, principle issues, however, that limit my enthusiasm for the current version of this manuscript significantly, and that need to be addressed before publication can be recommended.

1.) On the glioblastoma cell line level, the authors compare slow vs. fast cycling cells, thus on heterogeneity of two tumor cell populations, while the multiple human data (in silico analyses of existing databases) is purely performed with bulk sequencing/RNA expression datasets. This makes it very difficult to draw conclusions from the one and transfer it to the other. It would make a lot of sense to rather use RNA expression data of single cell analyses of the different glioma types, which are publicly available today, too, and investigate whether two principle "metabolic subtypes" can be detected here, too.

2. It is of crucial importance to better characterize the SCC vs. FCC subpopulations, which are identified by a dye retention method (CellTrace dyes). Using other methods to identify fast- vs. slow-cycling cancer cells (genetic models, cell cycle reporters etc.), it needs to be checked whether dye retention is indeed a marker for slower tumor cell division, or rather for another cellular feature. Particularly in light of the gross metabolic differences (including lipid), a dye retention system where the dye binds to lipids in the cell can be relevantly influenced by features other than mitosis frequency; particularly when considering that the more dye-retaining ("slow cycling") cells were found to contain more lipid droplets...in other words, in a worst case scenario, the findings are somewhat a self-fulfilling phenomenon, and have nothing to do with cell cycling tumor cell subpopulations. In this context, a cell cycle FACS or similar (cell cyle state according to gene expression classification) should complement the data here.

3. Furthmore, at multiple sites of the manuscript, the authors link slow-cycling to stemness (or at least progenitor-ness), which is clearly something frequently done in the field. However, it is extremely important for general conclusions of the study, and the impact of the results of the experiments reported here, to understand whether SCC are indeed more tumorigenic in vivo (dilution assays with decreasing amount of cells implanted), and also whether they show typical stem-like features in ex vivo assays (clonogeneity etc.).

4.) A similar and also very important point is that the authors need to provide much more data on the general glioblastoma cell population (not sorted according to dye retention), both in vivo and in vitro. This would also allow to get some idea about the relevance of the SCC vs. FCC subpopulations for tumor generation, tumor progression, and treatment resistance, in line with my comments above (3.)). Specifically, this information would be important for the data shown in Figs. 1B,C,D; Fig. 4E,G,H. Without that data, it is not possible to clearly assess the overall effect of metabolic interventions for tumor growth in malignant gliomas, and thus very difficult to draw translational conclusions. Next to the fact that different cell populations in glioblastoma will most likely interact with each other in a very complex way in vivo (as shown for the EGFRvIII-pos. vs. -neg. cells), the particular relevance of SCC vs. FCC GB cells for glioma progression and resistance can be clarified by these additional experiments in a coherent way.

Minor points:
Fig. 1B: the differences in invasion in vivo can be just due to the smaller size (?) of SCC - derived tumors. Please quantify the full region that is occupied by tumor cell (volume/diameter).
Fig. 1D,E: please provide proper Kaplan-Maier curves for survival, and show the statistics for them.
Fig. 3A: please provide images of the mitochondria of SCCs vs. FCCs, and please try to quantify the size, shape, number per cell etc. - are there differences beyond the relative mean of intensity provided that could give a clue about different mitochondrial state and function?

1st Revision - authors' response 16th Jul 2018

Point-by-point response

Referee #2:

Major concerns:

1. ***The title is not precisely indicative of the studies. The authors find that SCC are more invasive but are not testing the invasive cells directly. The fast cycling cells form tumors more quickly and may invade given time. It would be better to focus directly on the way that the cells are separated, i.e. the SCC. It would be interesting to select cells based on invasion and measure the SCC:FCC ratio and metabolic profile, but that was not directly tested.***

The title of our study, "*Infiltrative and drug-resistant slow-cycling cells support metabolic heterogeneity in glioblastoma*" reflects that the goal of our studies is to characterize the properties of SCCs, particularly as they relate to metabolism. We have also considered the possibility that FCCs might exhibit slow cycling cell features as the tumor grows and start invading given time. However, our *in vivo* analysis of FCC invasion was done ten weeks after implantation, the approximate survival endpoint for mice implanted with non-SCCs (including FCCs), and no tumor cell invasion was detected at that endpoint stage. The reviewer makes another great point, and previous studies have indeed shown that invading cells can be mostly non-dividing/slowly cycling. Our goal in the present study is to assess the invasive potential of SCC-derived cells and compare it with FCCs', and we have found that SCC-derived cells are more invasive than FCC-derived cells, whether SCC and FCC populations are implanted alone or together in a 1:1 ratio (**Fig 1C**).

2. ***Throughout the manuscript, the authors normalize the assays to each group. This is understandable, but the raw values should be presented, because it is not surprising that metabolism is different in cells with different proliferation.***

We understand, and the raw data has been included in the revised manuscript (**Fig EV3** and **Fig EV6**).

3. ***The authors find that SCC are more resistant than FCC to TMZ, but it would be helpful to directly measure the ratios of SCC to FCC in TMZ resistant lines.***

We agree with the reviewer that it would be interesting to compare the ratio of SCCs to FCCs within the different experimental populations, including among TMZ-resistant cells. However, the label-retention method employed in our study does not allow for the comparative analysis of SCC and FCC numbers/ratios between different cell populations. As described in our methods section, the gating for the selection of the SCC and FCC populations was placed at the upper and lower 10% extremities of the label intensity histogram. Therefore, using our labeling paradigm, the SCC to FCC ratio remains constant (= 1), regardless of the cell population. However, **Figure EV1F** shows that the TMZr population is mostly composed of slowly dividing cells with expansion rates that are similar to those of sorted SCCs. This result is now described in more detail in the revised version of the manuscript (**page 5**).

4. ***I am also somewhat confused by the overall premise. Tumors don't have exclusively SCC or FCC cells. What happens to the population over time? Is there an equilibrium? What are the responses to different stimuli when the cells are in combination? It would seem that the assays are missing controls of the combined or bulk cells. Are all the resistant and invasive cells from the SCC?***

As mentioned in the response to comment #3 above, the FCC and SCC populations are selected as the 10% most and least dye intense (most and least dividing, respectively) cell populations based on our label retention assays, so the rest of the tumors would contain a mixture of more or less rapidly cycling cells. In this manuscript, we focused on the extremes in order to obtain less heterogenous populations and address more accurately their relative contribution to heterogeneity, specifically at the metabolic level. Regardless of the dynamic properties and hierarchical relationship between the SCC and FCC subpopulations, our previous studies show that label retention is a powerful method to select subpopulations with stem cell characteristics (Deleyrolle *et. al.* 2011) that have been linked to treatment resistance and ability to drive tumor recurrence . To address the reviewer's comment about the responses of combined/bulk cells, unsorted total population groups have been added to the experiments investigating the invasion and TMZ resistance properties of the cells, *in vitro* and *in vivo* (**Fig 1B, E, Fig EV1C**). Of note, the responses of the unsorted total cell populations to TMZ (*in vitro* and *in vivo*) have previously been reported (Siebzehnrubl et al. EMBO Mol Med 2013). Additionally, we have conducted studies assessing the effects of glucose restriction, 2-deoxyglucose (2-DG)-mediated glycolytic inhibition, rotenone or metformin-mediated mitochondrial inhibition, and FABP7 inhibition of

fatty acid transport on the bulk cell populations from multiple patient-derived cell lines, *in vitro* and *in vivo*. The results are presented in **Figures 4G-I, 7O-Q** and **Figures EV4D-E, EV8A, and EV8C-D**.

5. I would suggest that the authors use caution in interpreting the gene expression profiles and mitochondrial tracking dyes as evidence of metabolic states. How does the cell division process change the level of mitochondrial dyes if the mitochondria have to replicate themselves? Is this like label retention as well? It would be standard to use Seahorse measurements as more direct study of metabolism. Direct ox phos vs. glycolysis should be tested.

We understand the reviewer's concerns. For the mitochondrial tracking dye experiments, the signal was analyzed within minutes of staining. For this reason, cell proliferation would not affect the results. Additionally, we used other methods to show mitochondria-related differences between SCCs and FCCs, such as the staining and quantification of VDAC1 and complexes I/V, which complement the gene expression profile and tracking dye data. However, as suggested, Seahorse experiments have been conducted to directly compare metabolic activities and complement our findings. These new data are presented in **Figure 3K-N** and **Figure EV3E-F**.

6. I was surprised by the relatively limited number of in vivo studies tested. There seem to be a single model with one replicate for each study done and no testing of metabolism in vivo, except for Figure 1I. The mitoctracker dye could be used in vivo, it seems.

The reviewer makes a good point. We immunostained i*n vivo* tumors derived from SCC or FCC xenografts with the mitochondrial marker MTCO2 and found a higher number of mitochondria in SCC-derived tumors. This finding was confirmed using electron microscopy analysis, which showed more mitochondria per cell in SCCs than in FCCs. These results are presented in **Figure 3A-C** and **Figure EV3A**.

7. Figure 3: How much faster to FCCs grow than SCCs? Do FCCs show decreased viability in low glucose conditions simply because they proliferate more rapidly and exhaust the supply of glucose within the media?

We understand the reviewer's concerns. Media glucose concentrations were monitored daily and maintained constant throughout the experiments by adding glucose to the cell cultures as needed, which prevented the glucose supply exhaustion that might have occurred due to FCCs' higher division rate. This point has now been clearly stated in the revised manuscript (**page 17**).

8. In figure 4, the authors claim that SCCs do not rely on glycolysis for energy production. To support this statement, the authors should treat cells with inhibitors of glycolysis (or perform knockdown of key glycolytic genes) and show a differential sensitivity between SCCs and FCCs (to parallel the OxPhos inhibition studies). This would be a more appropriate control than using only glucose restriction in vitro or low carbohydrate diet in vivo. This would be a very important experiment to support the authors main hypothesis of differential metabolic dependencies between SCCs and FCCs.

We would like to thank the reviewer for this great suggestion. We treated the cells with 2-deoxyglucose (2-DG), a pharmacological inhibitor of glycolysis, and found that SCCs are less sensitive to this pharmacological inhibition than FCCs, thus complementing the results we found using glucose restriction. These data are presented in **Figure 4B and Figure EV4B**.

9. In Figure 4G/H, the effect of the combination of low glucose with rotenone/metformin appears to be additive and not synergistic (as might be expected from targeting both SCCs and FCCs). This combination of two cellular insults leading to impaired cell proliferation does not specifically support the authors conclusions. Additionally, the terms "HG" and "PG" are not clearly defined in the text or figure legends related to Figure 4.

The effects of the combined treatments have been verified as being synergistic (see manuscript **page 9,** statistical method section and **Fig 4H-I & EV4DE**), and the terms "HG" and "PG" have been defined in the "Methods" section of the revised manuscript (**page 17**).

10. The authors posit an innovative hypothesis that targeting of both glycolytic and OxPhos pathways via FABP7 inhibition would be an ideal method to decrease in vivo tumor formation by targeting both SCCs and FCCs. In vivo experiments utilizing this FABP7 inhibitor in combination

with a glycolytic inhibitor would greatly heighten the impact of the findings. There should be some in vivo studies, hopefully in more than one model.

We thank the reviewer for this suggestion. *In vivo* experiments assessing the effects of FABP7 inhibition (FABP7i), alone or in combination with pharmacological targeting of glycolysis with 2-DG, have been conducted on xenograft-derived tumors using three different GBM patient-derived cells lines, and the results of these experiments are presented in **Figure 7O-Q** and **Figure EV8C-D** and described in the last section of the results (**page 12**). For one cell line-derived tumor, we observed a synergistic effect between 2-DG and FABP7i (**Fig. 7O).** The effect of the treatments was less robust for the other line-derived tumors, emphasizing inter-tumor heterogeneity. However, when modeling the results obtained across all three cell lines using a Cox - Frailty model, we found that survival is significantly improved when tumors are treated with FABP7i, alone or in combination with 2-DG, with the largest significant effect occurring after the combined treatment (**Fig. 7P-Q)**. Together, these results demonstrate the importance of targeting SCCs via FABP7 inhibition to achieve significant therapeutic benefit and also confirm an evident degree of metabolic heterogeneity in GBM.

11. I was surprised that the authors did not test FABP7 knockdown directly, but rather only an inhibitor, and there were few studies of proliferation, invasion, etc.

Additional *in vitro* migration experiments were conducted in the presence of different concentrations of FABP7i, and the results show decreased migration of SCCs in response to the inhibitor for all three patient-derived GBM cell lines (**Fig EV8A**). FABP7 inhibition was also assessed *in vivo*. Tumors from animals treated with FABP7i showed reduced invasion (**Fig EV8B**1**.** Knockout of FABP7 was performed using CRISPR/Cas9 genome editing (**Fig EV1E**), and its effects on sensitivity to glycolysis inhibition and/or lipid uptake have been included in the present manuscript (**Fig 7L-N**).

12. The authors discuss stem cells, but this is not developed. None of the studies seem to be directly related to stem cell pathways and stem cell assays are not tested.

The stemness characteristics of SCCs have been reported in our previous publication (Deleyrolle *et.al*., 2011). This previous report indicates a phenotypic and functional enrichment of SCCs in stem-like features, including greater tumorigenicity determined by *in vivo* limiting dilution transplantation assays. We have added in the revised manuscript (**page 4**) a statement summarizing these studies and emphasizing the link between SCCs and cancer stem cells. Additionally, we have included the results of a gene set enrichment analysis (**Fig EV1A**) that was performed with the RNA sequencing data presented in this paper and demonstrates that SCCs overexpress a gene module defined as a stem cell signature and described by Wong *et. al.* 2008.

13. Supplementary figure 6 is interesting, but I would suggest staining for FABP7 and Ki67 to see if there is overlap. The images shown have very high non-specific staining.

Higher quality and magnification images showing specific FABP7 staining in tissue sections have been provided in **Figure EV7A-B**. Bioinformatics analysis showed no correlation between FABP7 and Ki67 ($p = 0.76$). However, to address this particular point and correlate the expression of FABP7 with the SCC phenotype, we identified all the genes that are positively correlated with FABP7 using the GlioVis data portal. We then performed a gene set enrichment analysis to compare the levels of expression of these FABP7-correlated genes between the SCCs and FCCs isolated from the 3 different patients included in our study (**Fig EV7C**, **Supp Table 10**). The results of this analysis support that FABP7 and the FABP7-positively correlated gene signature are overexpressed in SCCs. Additionally, single cell-RNA sequencing analyses from available databases (Venteicher et. al. 2017) also confirmed the over-expression of FABP7 in the slow-cycling cell lineage defined based on the expression of cell cycle genes as described in Tirosh *et. al.* 2016 (**Supp Table 11**).

Minor concerns

1. The authors propose that ZEB1 contributes to invasiveness of slow cycling cells in vivo. Could the authors perform a knockdown of ZEB1 in SCCs and determine if this diminishes invasiveness in this model?

We purified SCCs from ZEB1 shRNA knockdown and control cells from two patient-derived GBM cell lines and investigated their invasion potential after intracranial xenotransplantation (n = 5 mice/line). For both cell lines, SCCs derived from ZEB1 knockdown cells were significantly less invasive than SCCs from control cells. These results are shown in **Figure 1D** and **Figure EV1E** and to the manuscript on page 5.

2. Doses for in vitro temozolomide treatment may not be physiologically relevant, as the authors suggest. According to one report5, the maximum TMZ concentration within the brain is around 3-5uM (0.6ug/mL +/- 0.3). Could the authors explain their use of 50-1000uM of temozolomide in this study?

We applied a range of *in vitro* TMZ concentrations that go above physiological levels in order to comprehensively capture the difference in drug sensitivity between the cell populations. Divergent IC50 values between *in vitro* and *in vivo* systems are fairly common and usually linked to different pharmacokinetics and -dynamics between cell-based assays and the organism. The *in vitro* concentrations we applied are in a similar range as those used in many other studies. Furthermore, we would like to point out that our *in* vitro findings are supported by *in vivo* experiments where the temozolomide concentration reflects human equivalent doses [50-150 mg/m^2] converted for mice [20-50 mg/kg], as described in Zhou et. al., 2007 and Reagan-Shaw et. al., 2008.

3. Presentation of data using Enrichment maps (http://baderlab.org/Software/EnrichmentMap/UserManual) may allow for more customizable and aesthetically pleasing representations of data in Figure 2 and supplementary Figure 2.

Clearer gene networks are now presented in **Figure EV2**.

4. The authors may also consider exploring differential endogenous lipid synthesis processes in SCCs as opposed to focusing only on lipid update from the microenvironment.

We agree with the reviewer that other mechanisms of lipid metabolism may be involved, and our gene expression profiling supports this statement. However, the functional investigation of additional lipid pathways might be beyond the scope of this manuscript and will be performed in future studies, as mentioned in the discussion.

5. Further validation of the specificity of the FABP7 inhibitor (or a reference) would be helpful.

A reference providing specific information about the FABP7 inhibitor we used in our studies has been added to the revised manuscript (i.e., Kaczocha et. al., 2014).

Referee #3:

Major concerns:

1. On the glioblastoma cell line level, the authors compare slow vs. fast cycling cells, thus on heterogeneity of two tumor cell populations, while the multiple human data (in silico analyses of existing databases) is purely performed with bulk sequencing/RNA expression datasets. This makes it very difficult to draw conclusions from the one and transfer it to the other. It would make a lot of sense to rather use RNA expression data of single cell analyses of the different glioma types, which are publicly available today, too, and investigate whether two principle "metabolic subtypes" can be detected here, too.

Single-cell RNA sequencing data from existing databases were analyzed and included in the revised paper. Specifically, we investigated, in high-grade glioma cells associated with lower cell cycling frequency, the lipid metabolism phenotype identified both in recurrent GBMs from the TCGA database and the SCC population from our 3 patient-derived GBM cell lines. The results of this analysis, presented in **Figure 2B** and **Figure EV2D-E**, support a model of metabolic heterogeneity in GBM, with a specific lipid metabolic signature observed in SCCs (defined by label retention as well as cell cycle gene expression) and recurrent GBMs.

2. It is of crucial importance to better characterize the SCC vs. FCC subpopulations, which are identified by a dye retention method (CellTrace dyes). Using other methods to identify fast- vs. slow-cycling cancer cells (genetic models, cell cycle reporters etc.), it needs to be checked whether dye retention is indeed a marker for slower tumor cell division, or rather for another cellular feature. Particularly in light of the gross metabolic differences (including lipid), a dye retention system where the dye binds to lipids in the cell can be relevantly influenced by features other than mitosis frequency; particularly when considering that the more dye-retaining ("slow cycling") cells were found to contain more lipid droplets...in other words, in a worst case scenario, the findings are

somewhat a self-fulfilling phenomenon, and have nothing to do with cell cycling tumor cell subpopulations. In this context, a cell cycle FACS or similar (cell cyle state according to gene expression classification) should complement the data here.

Most of these concerns have been addressed in our previous reports (Deleyrolle *et. al*., 2011, Deleyrolle et. al., 2012 and Azari, 2018). When labeled, all the cells exhibit similar levels of dye uptake and exhibit a narrow range of fluorescence on the day of staining, when the fluorescence spectrum width is less than one order of magnitude, and not more than three, as when we separate SCCs and FCCs on day 6-8. This would suggest that dye incorporation/uptake is relatively homogeneous and independent of lipid droplet content or cell division frequency. The SCCs are not defined by the amount of dye that is incorporated at day 0, but rather by their ability to retain the stain overtime. We have previously demonstrated that SCCs show the ability to retain labeling by using multiple assays, such as CellTrace-CFSE, which binds covalently to protein amine groups upon cleavage by endogenous esterases and is considered a protein-esterified dye (Wallace et. al. 2007, Lyons et. al. 2000 and Jensen et. al.2012). Furthermore, SCCs have been defined by their ability to also retain BrdU (Deleyrolle *et. al.*, 2011), as well as lipophilic dyes such as PHK26 (Roesch et. al 2010, Campos *et. al.* 2014, Richichi *et. al.* 2013) or DiI (Deminski *et. al.* 2009).

3. Furthermore, at multiple sites of the manuscript, the authors link slow-cycling to stemness (or at least progenitor-ness), which is clearly something frequently done in the field. However, it is extremely important for general conclusions of the study, and the impact of the results of the experiments reported here, to understand whether SCC are indeed more tumorigenic in vivo (dilution assays with decreasing amount of cells implanted), and also whether they show typical stem- like features in ex vivo assays (clonogeneity etc.).

Similar to our response to the reviewer #2 point 12, the stemness characteristics of SCCs have been reported in our previous publications. Those previous reports indicate a phenotypic and functional enrichment of SCCs in stem-like features, including greater tumorigenicity determined by *in vivo* limiting dilution transplantation assays. We have added in the revised manuscript (page 4) a statement summarizing these studies and emphasizing the link between SCCs and cancer stem cells. Additionally, we have included the results of a gene set enrichment analysis (**Fig EV1A**) that was performed with the RNA sequencing data presented in this paper and demonstrates that SCCs overexpress a gene module defined as a stem cell signature described by Wong *et. al.* 2008.

4.) A similar and also very important point is that the authors need to provide much more data on the general glioblastoma cell population (not sorted according to dye retention), both in vivo and in vitro. This would also allow to get some idea about the relevance of the SCC vs. FCC subpopulations for tumor generation, tumor progression, and treatment resistance, in line with my comments above (3.)). Specifically, this information would be important for the data shown in Figs. 1B,C,D; Fig. 4E,G,H. Without that data, it is not possible to clearly assess the overall effect of metabolic interventions for tumor growth in malignant gliomas, and thus very difficult to draw translational conclusions. Next to the fact that different cell populations in glioblastoma will most likely interact with each other in a very complex way in vivo (as shown for the EGFRvIII-pos. vs. -neg. cells), the particular relance of SCC vs. FCC GB cells for glioma progression and resistance can be clarified by these additional experiments in a coherent way.

This comment is similar to referee #2's comment #4. In response to this concern, we have included an "unsorted group" condition to the experiments highlighted in **Figures 4G-I, 7O-Q** and **Figures EV4D-E, EV8A, and EV8C-D**.

Minor points:

Fig. 1B: the differences in invasion in vivo can be just due to the smaller size (?) of SCC - derived tumors. Please quantify the full region that is occupied by tumor cell (volume/diameter).

We agree that the SCC-derived tumors are smaller in overall size compared to the FCC-derived tumors at the same time points due to their slower growth rate. We define tumor invasion as the ability of cancer cells to infiltrate into the surrounding parenchyma, which is significantly reduced for FCC-derived tumors. Our method for quantification of invasion is independent of the tumor size (described in Siebzehnrubl *et. al.* EMBO Mol Med, 2013 and Silver *et. al.* J Neurosci, 2013). To directly test for endogenous differences in the invasion

potentials of SCC and FCC, and to exclude that lack of FCC invasion is influenced by the absence of SCCs in their environment, we chose to co-transplant GFP-labeled SCCs and RFP-labeled FCCs into the same animals. Moreover, we sacrificed these animals at early stages after implantation to preclude any effects of their different growth rates. This experiment validated that SCCs are more invasive than FCCs, even within the same tumor, and has been added to **Figure 1C** and **page 4** of the revised manuscript.

Fig. 1D,E: please provide proper Kaplan-Maier curves for survival, and show the statistics for them.

Survival curves and statistics are now presented (**Fig 1F-G, 4E-G, 7O-Q, EV8C-D**).

Fig. 3A: please provide images of the mitochondria of SCCs vs. FCCs, and please try to quantify the size, shape, number per cell etc. - are there differences beyond the relative mean of intensity provided that could give a clue about different mitochondrial state and function?

Mitochondria were visualized by electron microscopy and their numbers compared between SCCs and FCCs, as requested. The results, showing a higher number of mitochondria in SCCs, are presented in **Figure 3B-C**. We used multiple methods to show mitochondria-related differences between SCCs and FCCs, including staining for VDAC1 and electron transport chain complexes I/V, adding to the strength of the gene expression profile and tracking dye data. We have also added Seahorse experiment results (**Fig. 3K-N**), which further support greater mitochondrial function in SCCs, to this revised manuscript.

2nd Editorial Decision 7th Aug 2018

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Your revised study was sent back to the referees #2 and #3 for re-evaluation, and we have received comments from both of them, which I enclose below. As you will see the referees find that their concerns have been sufficiently addressed and they are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues regarding material and methods and formatting as outlined below, which need to be adjusted at re-submission.

REFEREE COMMENTS

Referee #2:

The authors are to be commended in attempting to respond to the prior review. Although there are some minor points that could be improved, I recommend publication of the manuscript. Of minor note, I would still advocate the removal of the word "infiltrative" in the title as this was really a secondary finding and not used for selection. Dying cells don't invade.

Referee #3:

The authors have responded well to all of my relevant concerns. I recommend acceptance of this manuscript.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Loic Deleyrolle
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2017-98772R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Methods (statistics setion)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Methods (statistics setion)
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Methods
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	For animal studies, randomization to cages was conducted after tumor implantation
For animal studies, include a statement about randomization even if no randomization was used.	For animal studies, randomization to cages was conducted after tumor implantation
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was conducted
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5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Methods (statistics setion)

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Is there an estimate of variation within each group of data?	Methods (statistics setion)
Is the variance similar between the groups that are being statistically compared?	Methods (statistics setion)

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Methods
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Methods

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Methods
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10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes

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12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Methods
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	A "Data Availability" section has been added to the Materials & Methods. RNA Sequencing data will be available on dbGAP and the metabolomics data will be deposited on Metabolomics Workbench. Accession numbers will be provided as soon as available.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Several datasets are provided as tables in the Expanded View materials.
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