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Secreted factors from brain endothelial cells maintain glioblastoma stem-like cell expansion through the mTOR pathway

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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 01 December 2010

Thank you for the submission of your manuscript to our editorial office. We have now received the enclosed reports on it.

I am sorry to say that the evaluation of your manuscript is not a positive one. I will not detail their reports here, but as you will see, while the referees agree that the study is potentially interesting, they also indicate that it is not novel that the PI3K-AKT-mTOR pathway is required for GSC growth and furthermore, that the novel part of the study is premature. All referees point out that the conclusion that endothelial cells (EC) promote GSC growth in culture via the mTOR pathway is not sufficiently supported by experimental data, as the effect of EC-CM on mTOR pathway activity in GSC may be indirect. Importantly, referees 1 and 2 stress that the culture medium for EC and 293T cells is different, which makes it impossible to distinguish between effects of the medium versus secreted factors from the EC on GSC. Referees 1 and 3 further add that it needs to be examined whether EC-CM promotes proliferation or survival of GSC.

Referees 2 and 3 also point out that the marker sox2 does not distinguish between GSC and EC and that it is therefore unclear whether EC-CM does indeed induce the proliferation of GSC or rather other cells in the culture. All three referees also mention that it should be investigated which factors are secreted by the EC that maintain GSC in culture. Since all referees raise this point, we strongly recommend that it should be addressed, however, we also feel that it would not be an absolute

requirement for publication in EMBO reports.

Considering the nature of these criticisms, the partial novelty and the premature state of the study, the amount of work required to make the study conclusive, and the fact that, due to pressure for space, EMBO reports can only invite revision of papers that receive enthusiastic support from a majority of referees, I am afraid that we do not feel it would be productive to call for a revised version of your manuscript at this stage and therefore we cannot offer to publish it.

Given the potential interest of the findings, we would, however, have no objection to consider a new manuscript on the same topic if at some time in the near future you obtained data that would considerably strengthen the message of the study that EC promote GSC growth in culture via the mTOR pathway, and that address the referees concerns in full (as mentioned above and in their reports). To be completely clear, however, I would like to stress that if you were to send a new manuscript this would be treated as a new submission rather than a revision and would be reviewed afresh, also with respect to the literature and the novelty of your findings at the time of resubmission.

At this stage of analysis though, I am sorry to have to disappoint you. I nevertheless hope, that the referee comments will be helpful in your continued work in this area and I thank you once more for the opportunity to consider your study.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The current manuscript seeks to integrate two paradigms in glioma stem cells: PI3K-mTOR signaling and the perivascular niche. Although the concepts are interesting and may be correct, the study is premature for publication.

Major concerns:

1. The cancer stem cell models are not well developed. Inherent in the claim of a cancer stem cell is the ability to demonstrate a cellular hierarchy in the ability to generate tumors. The putative glioma stem cells (GSC) in this study were derived from patient specimens that were permitted to grow up to 47 days before the initial formation of neurospheres. The reliance on induced differentiation as a comparison is improper as this culture condition is not able to recapitulate the expression profile of the original tumor (Lee et al. Cancer Cell 2006). A much better approach would be to compare the self renewal, tumor growth, etc. of prospectively segregated populations. The authors have previously dismissed some of the major stem cell markers (CD133, CD15/SSEA-1, and CD34) (Patru et al. BMC Cancer 2010) but these studies never used freshly derived tumors so conclusions cannot be made.
2. The comparison of GSC and differentiated cells is not designed well because the culture conditions are drastically different and may account for the observed differences in pathway activation. These issues should be addressed in the design of the studies and the assessment of PI3K-mTOR activation immediately after tumor removal without culture would be important.
3. The PI3K-AKT-mTOR studies are not novel. There have been several studies that address this pathway in GSC with different inhibitors, including:
a. Gallia GL, Tyler BM, Hann CL, Siu IM, Giranda VL, Vescovi AL, Brem H, Riggins GJ. Inhibition of Akt inhibits growth of glioblastoma and glioblastoma stem-like cells. Mol Cancer Ther. 2009 Feb;8(2):386-93.
b. Eyler CE, Foo WC, LaFiura KM, McLendon RE, Hjelmeland AB, Rich JN. Brain cancer stem cells display preferential sensitivity to Akt inhibition. Stem Cells. 2008 Dec;26(12):3027-36.

c. Sunayama J, Sato A, Matsuda KI, Tachibana K, Suzuki K, Narita Y, Shibui S, Sakurada K, Kayama T, Tomiyama A, Kitanaka C. Dual blocking of mTor and PI3K elicits a prodifferentiation effect on glioblastoma stem-like cells. Neuro Oncol. 2010 Sep 22. [Epub ahead of print]
d. Sunayama J, Matsuda KI, Sato A, Tachibana K, Suzuki K, Narita Y, Shibui S, Sakurada K, Kayama T, Tomiyama A, Kitanaka C. Crosstalk Between the PI3K/mTOR and MEK/ERK Pathways Involved in the Maintenance of Self-Renewal and Tumorigenicity of Glioblastoma Stem-Like Cells. Stem Cells. 2010 Sep 20. [Epub ahead of print]
4. The lack of in vivo studies to develop the idea of a vascular niche is a major deficit and precludes interpretation.
5. The claim as to a secrete factor from endothelial cells is premature until molecules are identified. The concept that endothelial cells augment GSC growth is repeatedly described (e.g. Calabrese et al Cancer Cell 2007 and Borovski T, Verhoeff JJ, ten Cate R, Cameron K, de Vries NA, van Tellingen O, Richel DJ, van Furth WR, Medema JP, Sprick MR. Tumor microvasculature supports proliferation and expansion of glioma-propagating cells. Int J Cancer. 2009 Sep 1;125(5):1222-30.) This needs greater development.
6. Figure 4 is the most interesting part of the manuscript but is not developed fully and lacks mechanism.
7. There are no data to show that treatment of rapamycin, PP242, or PI103 is specifically inhibiting the effects of EC-CM. Because each drug is active on its own, it is hard to appreciate how these studies create a mechanism.
8. There are no studies to show that responses to rapamycin, PP242, or PI103 [or siRNA for mTOR1, Raptor1, Rictor1] are different between GSC and differentiated cells.
9. The cellular mechanism (self renewal, proliferation, survival) with 293T-CM vs. EC-CM is not developed.

Minor points:
1. The phosphorylated Westerns lack total protein bands for each protein (Akt, S6).
2. Statistical testing is not completed on any study.
3. Supplemental Figure 1 is premature.
4. There are typographical errors to correct.

Referee #2 (Remarks to the Author):

This manuscript by Gavard's group showed that mTOR pathway is important for GSC expansion in vitro. This is an interesting study. However, the evidence to support that brain endothelial cells maintain GSC expansion through the mTOR pathway is very weak. Thus, the major conclusion of this manuscript is not well supported by their data.

Major concerns:

1. Fig. 4. The culture medium used for 293T (DMEM) and endothelial cells (EBM2) were totally different, and the EBM2 medium (Lonza) for endothelial cells already contains several growth factors, it is really hard to tell whether the effect of endothelial cell conditioned media on GSCs is due to the growth factors already present in EBM2 culture medium or the factors secreted by endothelial cells. Authors should include the un-conditioned media for controls in order to make a right conclusion.
2. If only the EC conditioned medium (but not the un-conditioned medium) can activate p-Akt and p-S6 in GSCs, then it is true that some factors secreted by endothelial cells affect the GSCs. However, endothelial cells may produce many factors to activate a number of pathways on GSCs. To demonstrate that endothelial cells affect GSC expansion through mTOR, authors should examine whether mTOR knockdown can abolish the effect induced by EC conditioned medium.
3. What is the potential factor(s) secreted by endothelial cells to activate mTOR in GSCs? As the factors that can activate mTOR pathway have been identified in other cells, authors should be able to identify the key factor(s) to activate mTOR in GSCs. The molecular link between endothelial cells and mTOR activation in GSCs is not well addressed.

Minor points:

1. Fig 3A, why Sox2 was positive in ECs only? How can authors distinguish GSCs and ECs in the co-culture?
2. In the text and figure legend, the authors described differentiated cell derived from GSC as "differentiated GSC". This caused confusion. If the cell is differentiated, it lost stem cell properties and is no longer GSC..
3. Fig. 1B, please show total Akt and S6. Are total Akt and S6 different between # and NS?
4. Fig 1B, 1C: why PTEN expression pattern is not consistent with mTOR activation in 4 GSC lines? PTEN is upstream of mTOR, why PTEN is unlikely to impact on mTOR activation?
5. Fig 1A, the authors used Olig2 as a marker for Oligodentrocytes. But Olig2 is a well-defined marker for GSCs. Most GSCs express Olig2, but differentiated cells lost Olig2 expression. Galc is a good marker for oligodentrocytes.

Referee #3 (Remarks to the Author):

This manuscript by the laboratory of Dr. Gavad addresses the mechanism that endothelial cells enhance the stem cell characteristics of tumor cells adjacent to them. Overall this is a nice, focused paper that shows:
1) p-S6 and p-Akt are active in GSC .
2) blocking MTOR signaling decreases GSC number.
3) Endothelial cells secrete factors that can promote GSC number (or rather, block decreases in GSC number).

The authors have established four patient derived neurosphere lines that express Sox2 and Nestin and differentiate into GFAP+ and Tuj1+ cells (1A). These neurosphere lines express p-AKT and PS6 under neurosphere but not under differentiating conditions (1B). Treatment of these lines with MTOR inhibitors (rapamycin, PP242, PI103) blocks downstream signaling (2A), decreases Sox2 expression (2B), decreases the number of neurospheres/FOV (2C) and decreases the total amount of cells as assayed via MTT (2D). The authors show the same results when they use siRNA to MTOR, Raptor and Rictor (figures 2E-H). The authors conclude from this that MTOR signaling might function to maintain GSC integrity. The authors also show that their neurosphere lines maintain Sox2 expression when grown in the presence of endothelial cells or endothelial cell-conditioned media (EC-CM). As a control for this they use 293T-CM. Again they use nsph/FOV and MTT to quantify effects of endothelial vs. epithelial cells and conclude that nsphs fare better under endothelial cell CM than epithelial cell CM. Last, the authors show that EC-CM maintains p-Akt and p-S6 at higher levels that epithelial cell CM and try to show that the positive effects of EC-CM on the GSC is mediated via MTOR as inhibition of MTOR signaling blocks EC-CM mediated growth of nsphs.

The topic is timely as the mechanism for this effect is likely complicated and under investigation in several laboratories. There are a few issues that could be addressed to strengthen the paper:

1. The MTT assay shows that with MTOR inhibition there are less cells- thus there seems likely to be less spheres/ FOV even if MTOR has no effect on GSC integrity- a better measure of "stemness" or self renewal is secondary neurosphere formation. All the authors have shown is that MTOR is responsible for the survival and/or proliferation of some cells within the spheres - not necessarily the GSC

2. The authors use sox2 expression as proof that GSC have retained stem character and not differentiated in the presence of endothelial cells. This is not completely convincing. Again, differentiation into different cell types and/or secondary sphere formation are functional measures of 'stemness'.

3. It is unclear that the Sox2-expressing cells grown in the presence of endothelial cells are the GSC. In fact, seems that the EC alone cultures have some Sox2 expressing cells -it seems hard to be sure that the Sox2+ cells are the GSC. This is an important fact because the effects on proliferation in response to EC factors could be on more differentiated cells and not on GSC. Additionally, here is another place where secondary sphere formation would clarify things.

4. Although it may be beyond the scope of this focused manuscript, it would be interesting if the authors investigated what factors from the endothelial secretome contributed to the growth/survival of the GSC. It would also be interesting for the authors to determine whether the EC-CM promotes proliferation or survival of the GSC. All MTT really does is determine relative cell number.

5. The authors show that MTOR signaling plays a role in GSC survival and/or proliferation. They also show that endothelial cells promote growth of their GSC when compared to epithelial cells. The data demonstrating that EC promote GSC growth via MTOR is less convincing. Is it possible that the EC-CM effects on MTOR signaling may be an indirect effect of EC-CM promoting general survival/proliferation of the GSC? The same question holds for the blockade of MTOR signaling in the presence of EC-CM - this does not prove that EC-CM mediates its effects via MTOR, but rather, that MTOR signaling is required for GSC survival, which they already showed in figure 1.

Appeal Letter 22 December 2010

As a courtesy, we would like to notify you that a revised version of our manuscript entitled "Brain endothelial cells maintain glioblastoma stem-like cell expansion through the mTOR pathway" (EMBOR-2010-34674V1 to substitute for EMBOR2010-34545V1) by E.M. Galan-Moya, A. Le Guelte, E. Lima Fernandes, C. Thirant, J. Dwyer, N. Bidere, P.O. Couraud, M.G.H. Scott, M.P. Junier, H. Chneiweiss and J. Gavard has been resubmitted online today.

We have tried our best to clarify some misinterpretations and confusions. For example, we now explain in detail the experimental design and the condition medium preparation (Fig S1). In addition, GSC integrity is now monitored through a multi-parametric based approach: nestin and Sox2 expression, secondary neurosphere formation, proliferation and cell survival (MTT assays and flow cytometry analysis), and morphological signs of differentiation (adhesion, neurite elongation and Tubulin bIII staining). We further provide western-blot analysis that firmly establishes Sox2 as a marker for GSC and not EC in co-culture experiments (Fig S3).

Following the reviewers' suggestions and comments, we performed substantial new experimentation that confirms and strengthens our conclusions. All western-blot experiments have been complemented with appropriate loading controls (Fig 1 & 4) and quantifications are now enriched with statistical analysis (Fig 2, 3 & 4). Secondary neurosphere formation assays have also been performed and show the importance of the mTOR pathway in GSC self-renewal in response to mitogens and EC-CM (Fig 2, 3 & 4). As suggested, we also explored more deeply how blocking the mTOR pathway impacts on differentiation and survival (Fig 2 & S2). Keeping with this, we provide evidences that EC-CM favours GSC survival rather than proliferation (Fig 3). Furthermore, we address the environmental activation of mTOR by the endothelial secretome and its involvement in GSC expansion using both pharmacological drugs and siRNA (Fig 4). Finally, we show that a myriad of soluble factors are released by endothelial cells to maintain GSC in culture (Fig S4), and provides a molecular framework for the newly described concept of "angiogrine factors". This is now discussed throughout the paper.

We believe the new information discussed above, both strengthens and clarifies our initial manuscript. We appreciate your hard work on our manuscript and hope that it is now suitable for the EMBO Reports.

Authors' Response to Referees 22 December 2010

Please let me thank you and the Reviewers for these comments that had helped us to improve our manuscript. We have now carried out additional experimental work to address the queries of the reviewers (Fig 1B, 2B, 2C, 2G, 2H, 3C, 3E, 3F, 4B, 4D, 4E, S1, S2, S3 and S4).

Please find below our point-by-point discussion in which we reiterate the reviewers' points and provide an answer.

Point-by-point answer to referees:

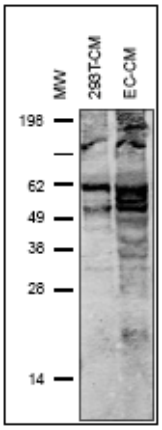
Referee #1:

1. The patient derived glioblastoma stem-like cells used in our study have been extensively studied and characterised previously (Patru et al., 2010). In fact, this specific subpopulation of glioma cells represents a a powerful and well-accepted model to generate long-term human adult tumour stable cell lines that might phenocopy parental tumour behaviour with the unique ability to self-renew and initiate tumours (Galli et al. Cancer Res 2004; Singh et al., Nature 2004; De Witt Hamer et al, 2008; Lee et al, 2006; Patru et al, 2010). However, the concerns raised by Reviewer 1 are valid. We therefore discuss more deeply this model in the new version of the manuscript and substitute the general term of glioma stem cell by a more appropriate one (long-term glioblastoma stem-like cell).
2. We agree that this set of experiments might sound confusing at first, and we clarify the manuscript accordingly. Nonetheless, this approach intended to compare the influence of the environment on mTOR activation, including culture conditions (Fig 1). Because floating growing neurospheres and attached differentiated cells exhibit different levels of Akt and S6 phosphorylation, we favour a model in which extrinsic activation is active. Indeed, we further address the environmental activation of mTOR in GSC by either the mitogens in their regular medium or the endothelial secretome (Fig 4).

3. The reviewer raises a good point. Although drugs blocking the mTOR pathway have been used previously (suggested references are now inserted), they might also have unrelated target effects. To circumvent this potential pitfall, we used siRNA against mTOR, Raptor and Rictor (Fig 2E-H & 4D-E). This powerful tool unambiguously demonstrates the key role of the mTOR components for GSC maintenance.

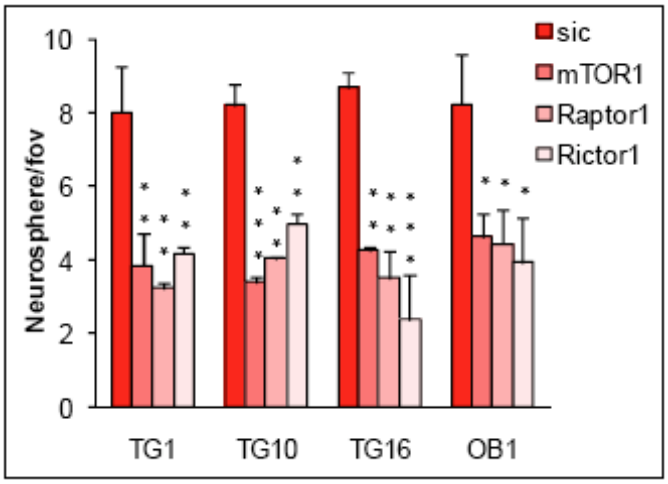
4. We agree with Referee 1 that "*in vivo studies to develop the idea of a vascular niche*" would be an exciting challenge, but we feel that this is beyond the scope of the present manuscript. Herein, we focused our research on an *in vitro* mimicking niche composed by cancer stem cells, endothelial cells, and their secreted factors.

5. As pointed out by Reviewer 1, identifying molecules secreted by endothelial cells would undoubtedly open new perspectives in the GSC field. However, our analysis of the endothelial secretome shows that a myriad of factors are released (protein concentration, Coomassie gel, Fig S4 and gel presented here), and suggests that a complex scenario likely takes place. This is in agreement with two recent published studies (Kobayashi et al., Nature Cell Biology 2010; Ding et al., Nature 2010). These unidentified factors that could act as environmental modifiers were termed angiocrines by Rafii's group.



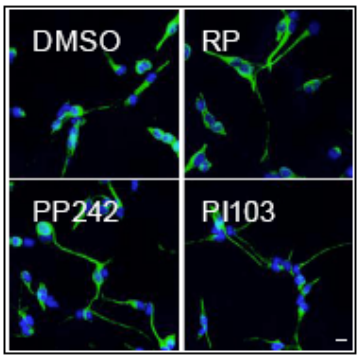
6. We show in our paper that endothelial, but not epithelial soluble factors can recapitulate the effects of regular medium on mTOR activation and GSC integrity. As suggested by the reviewer, we performed additional experiments to examine whether EC-CM acts on cell proliferation or survival.

Although no major changes in CFSE pattern were observed, less viable cells remained in 293T-CM, as more PIpositive cells were detected by flow cytometry (Fig 3E-F). Collectively, these data support a role for EC-CM on GSC survival rather than proliferation. This interesting aspect is now added in the new version of our manuscript. Finally, this study is also complemented with siRNA experiment, depicting no effects of the EC-CM on in secondary neurosphere formation (Fig 4, graph on the left hand side) and MTT values in the absence of mTOR, Raptor and Rictor (Fig 4E).



7. We apologize for not explaining enough our data. Experiments on Fig 2 show that these drugs hamper GSC growth in mitogen-containing medium, while independently blocking EC-CM-mediated recovery of GSC expansion (Fig 4). We rephrased this specific point for improved clarity. In addition, we performed additional experiments that show the effects of siRNA against mTOR, Raptor and Rictor (Fig 4D-E). See also point above.

8. This is a very insightful remark. We have performed Tubulin bIII staining to assess the effects of mTOR inhibitors on differentiated cells (Fig 2B, pictures on the left hand side). In addition, flow cytometry analysis demonstrates that mTOR inhibitors severely dampen cell viability (Fig S2). Altogether, our data converge to a pro-survival role for the mTOR signalling pathway in GSC.

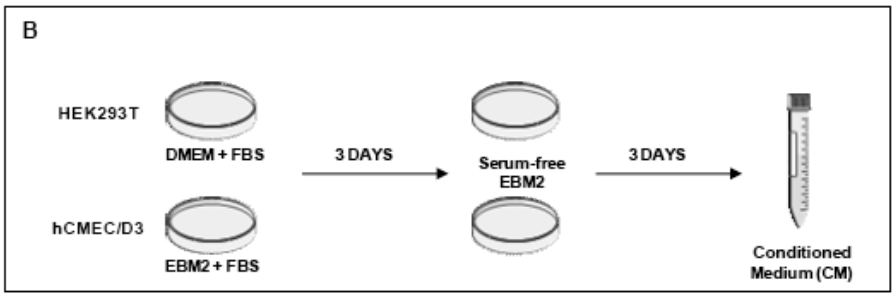


9. We have observed that protein concentration and contents are quite different between the two CM (Fig S4, see point 5). In addition, we have performed FACs analysis that demonstrates that the EC-CM promotes cell survival as compared to 293T-CM, while cell cycling rates remain unaffected (Fig 3E-F). Finally, we provide evidences that 293T-CM cannot promote mTOR-dependent GSC expansion (Fig 4A). Please see also point 6.

Minor points:
- Appropriate loading controls are now shown.
- Statistical analysis are now provided.

Referee #2:

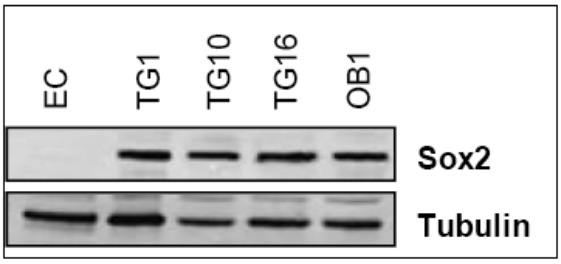
1. From the comments of Reviewer 2, it appears clear that we did not provide enough detail regarding experimental design. It is important to note that conditioned media were prepared with same serum-free and mitogen-free medium (EBM) applied to extensively PBS washed and quiescent 293T and endothelial hCMEC cultures in order to clearly distinguish between effects of their regular growing medium versus their secreted factors on GSC. We amend the methods section to avoid any confusion. We also provide a figure depicting the experimental design (Fig S1 and below).



2. This is a good point. Following reviewer 2 suggsetions, we have now performed siRNA experiments in GSC exposed to EC-CM (Fig 4D-E). Our data show that in the absence of functional mTOR pathway, endothelial factors are not sufficient to allow GSC expansion as monitored by MTT and secondary neurosphere assay formation (see point 6 in response to referee 1). These data are now included in the revised manuscript and improve our initial conclusions.

3. Our analysis of the endothelial secretome suggests that multiple factors are indeed released (protein concentration, Coomassie gel, Fig S4). Please see point 5 in response to reviewer 1.

Minor points:
- Sox2 essentially distributes in the nucleus of GSC, while a diffuse non-specific staining is detected in few endothelial cells (Fig 3A, picture quality has been improved). To support our claims, we performed the requested western-blot. This new data clearly establishes that Sox2 is specifically expressed in GSC and not in endothelial cells (Fig S3, and western-blot on the right hand side).



- We change the text accordingly.
- Appropriate loading controls are now shown.
- We rephrase the text to avoid any confusion. Whatever the status of PTEN expression, the Akt/mTOR pathway is found active in neurospheres.
- We remove this specific staining for clarity purpose.

Referee #3:

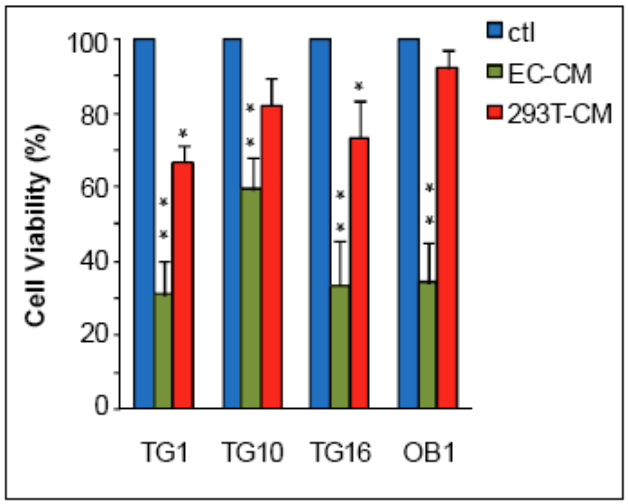
1-3. We would like to thank the reviewer for this cleaver remark. Following the referee suggestion, we have now assessed secondary neurosphere formation for all experiments in order better estimate

GSC integrity (Fig 2C, 2G, 3C, 4B and 4D). This assay clearly refines our previous findings.

3. Please see also response to referee 2, minor point 1. Our western blot analysis clearly demonstrates that Sox2 is specifically expressed in GSC and not in endothelial cells (Fig S3).

4. Analysis of the endothelial secretome shows that a myriad of factors are released (protein concentration, Coomassie gel, Fig S4), and suggests that a complex scenario likely takes place. Please see also point 5 in response to reviewer 1.

5. As pointed out in our response to reviewer 1, point 6, additional flow cytometry-based experiments were performed. Our data favour a role for EC-CM on GSC survival rather than proliferation (Fig 3E-F and graph on the right hand side). Similar experiments have been conducted in the presence of mTOR inhibitors (Fig S2), suggesting a pro-survival action of the mTOR pathway.



We have incorporated this new information into a revised version of the paper, which we believe is both strengthened and clarified.

We hope that the manuscript is now suitable for publication in Embo Reports.

2nd Editorial Decision 01 February 2011

Thank you for the submission of your revised manuscript to EMBO reports. I am sorry for the delay in getting back to you, which is due to the fact that referees 1 and 2 are not in agreement and that referee 3 is not available to review the revised manuscript. As you will see, while referee 1 does not support publication of the revised manuscript, referee 2 has no further concerns. I have discussed your manuscript with our senior editor Prof. Jacobs and we agree that the concerns raised by referee 1 do not prevent publication of the study in EMBO reports and that you have successfully addressed referee 3's concerns. The study could therefore in principle be accepted.

Going through the manuscript, I however feel that the data describing the effect of EC-CM on GSC-like cell survival versus proliferation need to be explained better. It is not clear what the graphs in figure 3E depict exactly but for TG1 and TG10 there are certainly differences in the number of cycling cells between EC-CM and 293T-CM. If these differences indicate an effect of the conditioned media on cell proliferation, this does not support your conclusion that EC-CM promotes survival rather than proliferation. Please explain the flow cytometry panels in figure 3E in more detail. I also noticed that panel B and C of figure 3 are not correctly referred to in the figure legend.

Another explanation I could not find in the manuscript (but I may have missed it) is whether you have corrected for the different protein concentrations in the EC-CM and 293T-CM when adding them to the GSC-like cells. Figure S4 is further missing the explanation of the error bars in the figure legend.

These remaining concerns require clarification before we can proceed with the acceptance of your manuscript. Please resubmit a revised manuscript addressing the remaining issues via the EMBO reports website. Do not hesitate to contact me if you have any further questions.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS

Referee #1 (Revision Comments):

The current manuscript asks two important questions: 1) What role does mTOR signaling play in glioblastoma stem-like cells? 2) How do endothelial cells increase glioblastoma stem-like cell survival? These are important questions that have been previously addressed by multiple prior groups but unresolved. Unfortunately, this manuscript does relatively little to advance the field. It certainly does not reach the threshold of publication for a high impact journal like EMBO Journal. It is disappointing that the authors did not make a great effort in the response to critiques.

Major issues:
1. Model: While it is true that many reports have characterized glioblastoma stem-like cells, Patru et al. does a poor job in the "extensive characterization" as the in vivo studies that are critical are not well performed. The lack of markers in these cells to separate tumourigenic and non-tumourigenic cells means that the authors have made the poor choice of using serum to induce differentiation. This means that they cannot make good comparisons in most of their studies.
2. Culture: The use of different culture conditions for the comparisons for the more stem-like cells and differentiated cells has direct impact on TOR activation. This has not been resolved.
3. Mechanism: This is perhaps the greatest deficit in the manuscript. The proteomic studies are a poorly developed discovery set. It is not necessary to identify all of the secreted factors but there is not a single factor studied at all. The authors referenced recent studies with angiocrine factors. Why couldn't the authors have studied even one factor? Without these studies, there is little novelty.
4. Animal studies: It is strange to write of the perivascular niche without studying a single blood vessel. Why couldn't the authors have conceived of a single animal study to explore at least how the perivascular niche supports glioblastoma stem-like cells through mTOR? This is important and a major deficit.
5. The authors have reference the prior literature with the issue of therapy targeting the endothelial cells but this is also not addressed.

The current studies are useful but lack novelty and should have been further developed with a mechanism and in vivo confirmation. The tumour formation assay is the most important assay but this is lacking in these studies. I wish that the authors have put forth a greater effort in response.

Referee #2 (Remarks to the Author):

I think that the manuscript has been significantly improved. I don't have further concern.

2nd Revision - authors' response 03 February 2011

We first apologize that our description of flow cytometry experiments appeared rather confusing. In sake of clarity, we have now carefully detailed this set of data. Indeed, the overall ratio between proliferating and quiescent cells was similar although number of cells appears lower when cultured in 293T-CM (Fig 3E). Because cycling rates were monitored exclusively on viable cells, based on their size and granulosity (FSC/SSC dot plots), we inferred that endothelial cells might exert a pro-survival effect. This was further confirmed with propidium iodide incorporation experiments (Fig 3F). As for CM, they were not corrected by protein concentrations. Instead, reproducible CM samples were prepared by using same starting number of cells and densities on quiescent monolayers. This piece of information is now added in Fig S1 legend. We also would like to thank you for catching the mislabelling on Figure 3. This is now corrected. Last, we now indicated in the Fig S4 legend that error bars correspond to standard error from 3 independent experiments.

We have tried to clarify the remaining concerns in this revised manuscript, and hope that it is now suitable for publication.

3rd Editorial Decision 04 February 2011

Thank you for the submission of your revised manuscript to our journal. I appreciate that you have addressed the remaining concerns. However, the fact that the total protein concentration is significantly higher in EC-CM compared to 293T-CM (as shown in Figure S4) is not optimal. I have discussed this point also with our head of scientific publications, Dr. Pulverer, and we agree that the same total protein concentration should be used when comparing the effects of the two CM on GSC-like cells.

I would like to give you the opportunity to address this issue by performing one more experiment in which GSC-like cells are cultured in the different CM containing the same amount of total protein. It needs to be shown that under these conditions, neurosphere formation and sox2 and nestin expression is exclusively maintained in the case of EC-CM. It will not be necessary to repeat the analyses on cell viability and proliferation and the revised manuscript will also not be sent back to the referees if the results are clear. I am sorry for this additional request but we do feel that this is an important point as the different effects of the media may be simply due to the lower total protein levels in 293T-CM.

I hope you find our suggestion reasonable and I am looking forward to seeing a new revised version of your manuscript as soon as possible.

Yours sincerely

Editor
EMBO Reports

3rd Revision - authors' response 11 February 2011

We understand your concern regarding the differences in total protein concentrations between HEK293T- and endothelial cell (EC)-conditioned media (CM). Because we too felt that it was an important control, additional experiments were carried out. EC-CM and 293T-CM were therefore titrated from concentrations ranging between 0.2 and 1 mg/ml, and their effects on neurosphere

formation and stemness marker expression were assessed (see new Fig S4). We now report that regardless of the protein concentration, neurosphere formation was significantly higher in the presence of EC-CM, in comparison to 293T-CM when tested at the same dose (Fig S4C). Notably, the effects of EC-CM reach a plateau at 0.4 mg/ml. Stemness was further confirmed by immunostaining of Sox2 and Nestin (Fig S4D). Altogether, this additional information reinforces our model in which the nature of EC secreted proteins, more than the global protein concentration, is involved in the functional effects observed.
We hope that our manuscript is now suitable for publication in EMBO Reports.

4th Editorial Decision 15 February 2011

We are pleased to inform you that your manuscript has been accepted for publication and will be included in the next available issue of EMBO reports.

Yours sincerely

Editor
EMBO Reports

