Reviewer's report

Title: Spatial morphological and molecular differences within solid tumors may contribute to the failure of vascular disruptive agent treatments.

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Reviewer: Gillian Tozer

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Nguyen et al 2012

Please note: my original comments are in brackets.

(This manuscript addresses a very important issue regarding the differences within the microenvironment at the tumour periphery versus the tumour centre, which could explain the resistance of the former to vascular disrupting agents such as the combretastatins. The aims of the study are straightforward and the manuscript is clearly written. Some further staining and quantification of results are needed to justify the conclusions).

The authors have addressed most of my previous comments. There are just a few remaining issues to sort out.

Major Compulsory Revisions

1) (On pages 6 & 7, the authors state the numbers of tumours used in the analysis but information should be given about numbers and sizes of tumours in each liver and whether the whole of the liver tissue was used. Statistical analysis needs to take into account inter-animal variation as well as inter-tumour variation and error bars should reflect this. It is not clear whether this has been done and statistical advice should be sought).

The additional information in supplementary data is useful here but the authors need to ensure that inter-animal variation as well as inter-tumour variation is reflected in the plot and analysis – authors do not say whether this has been addressed. Statistical advice should be sought on how to present these data.

2) (The methods for quantifying the staining data are unclear and need to be described in more detail. It is unclear whether counts were made by a fully automated method or whether there was manual input. In either case, the criteria for assessing ‘positively stained vessels’, for instance, should be given. Similar details need to be given for CD3, FOXP3 and F4/80. Quality control methods and whether analysts were blinded need to be stated. The axis labeling needs some clarification. For instance, in Fig 1, CD34 positive MVD is not very informative and should be replaced by ‘number of vessels per mm^2’, if this is what the graph shows. Similarly, ‘pericyte coverage’ needs defining clearly).

These points have been clarified.
3) (Quantified results should be provided for hypoxia, VEGF, FGF, HIF-1alpha, AT1R, TGFbeta).

Point taken re. quantifying hypoxia. A semi-quantitative analysis of the markers shown in Fig 3 seems reasonable. However, it is incorrect to provide means and standard errors of these scores. Statistical advice should be sought on how to present these data.

4) Results – Fig 1.

(The antibody for CD34 is clearly only staining a small fraction of vessels, which appears to be the larger ones. The authors’ justification for using CD34 as their endothelial cell marker is that it is expressed on vessels undergoing neangiogenesis and cite unpublished work to support this statement. However, there is no direct evidence for this. The images for #-SMA staining for pericytes are rather poor quality. Better images are required to demonstrate that there is more #-SMA staining on vessels in the periphery than in the centre. Some lower power images should be shown, as has been done for CD34. CD31 staining should also be carried out, as this should highlight many more vessels and aid in identifying #-SMA co-localised to vessels. It is not at all clear how the pericyte coverage data was calculated, considering the high level of #-SMA staining shown in C in non-vascular structures, presumably myofibroblasts. Authors need to explain how this was done and define ‘pericyte coverage’ (see above)).

Most of these issues have been addressed. However, it is clear from the new Suppl Fig 2 that staining for CD34 is highlighting many more tumour vessels than is apparent in the main figure 1. Better images of CD34 staining should be shown in Fig 1. Also, no new high power images of a-SMA staining have been provided, rather the original high power images are no longer in the main fig 1 but appear in Suppl fig 3 (note that the title of this fig is incorrect). The results for a-SMA staining shown in Fig 1B are clear but it is still not clear how these were obtained. There is a little more information given on page 5-6 but this does not explain how vessels were identified at the periphery, where there appears to be so much staining in myofibroblasts. This needs inclusion. Was it just the very large co-opted vessels that were counted? If so, this should be made clear. Also, the number of vessels analysed for a-SMA should be given. If no better images of a-SMA can be provided, I think that the Discussion should at least outline limitations with this analysis and be somewhat more circumspect in the conclusions regarding vessel maturity in the periphery.

5) (The Ang1 data is insufficient to back-up the conclusion that there are more mature vessels in the periphery. Evidence from #-SMA staining should be strengthened, as above, or a second pericyte marker should be included (see below)).

The image for Ang 1 is an improvement. Data are still limited but as long as the a-SMA data is strengthened – see comments above – this is OK.

6) Fig 3
(Results for these markers should be quantified to support the statements made in Results. In particular, distribution of HIF1alpha did not obviously mirror pimonidazole staining, as stated in the text. These do not appear to be serial sections and so it is difficult to see the evidence for this statement. Although there is very little pimonidazole staining at the periphery of the tumour shown, the HIF1alpha staining appears to extend to the periphery. B-FGF staining is unclear – does the expanded area include normal liver as well as tumour tissue? There is a lot of positive staining for b-FGF and TGF-beta in normal liver – how do controls compare with these? Is this specific staining? These issues need to be clarified).

These issues have been addressed.

7) Fig 6
(Extensive necrosis is seen at 1h after treatment in the figure. Is this typical across the tumours? The necrosis data should be quantified for all the tumours over the full time-course to show overall response to treatment and a comment should be made on how this compares with other tumour models in the literature).

This issue has been addressed.

Minor Essential Revisions

1) Fig 1
(The arrows in A appear to be indicating vessels outside the tumour mass. Since the paper is concerned with vessels in the tumour periphery, this should be changed).
Done

2) Fig 5
(What does the asterix in Fig5C (24h) signify?
What evidence is there for tumour vessels being leaky at 1h after treatment? It is not possible to tell this from the figure and the statement should be deleted. The statement in the text about patent vessels in the periphery at 24h should refer to the 24h image, not the 1h image, I think).
Done

3) Discussion
(Repetition on page 14, in paragraph 2).
Done

Additional Essential Revisions

1) All images need to be higher resolution.
2) Supplementary figures – title of Suppl Fig3 needs correcting. mm should be µm.

3) Page 7 – need to give the suppliers for all the antibodies.

4) Page 8 – Authors say that only viable tumour areas were analysed but in Fig 5, CD34 staining in A is clearly shown in necrotic regions and presumably included in the analysis. This needs clarifying in the text.

5) In Fig 2, the asterices showing significance on the graphs need replacing.

6) Page 17 – please use an original reference rather than ref 3, which is a review, for the proposal that tumor cells in the periphery survive due to close proximity to host vessels. Ref 3 is however suitable for the following statement about retained perfusion, as currently quoted.

Discretionary Revisions

1) (A second pericycle marker would help to clarify the issue of whether the vessels at the periphery are more mature than in the centre, since the Ang1 result is unconvincing. NG2 would be a possibility).

2) (It would be helpful, in Methods, to group the antigens into different categories e.g. FOXP3 and CD3 are for T-cells etc).

3) (Were any other markers investigated that did not have higher expression at the periphery? – this would be useful information too).

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**

I declare that I have no competing interests