Reviewer's report

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

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Reviewer: Gillian Tozer

Reviewer's report:

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.

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.

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.

3) Quantified results should be provided for hypoxia, VEGF, FGF, HIF-1alpha, AT1R, TGFbeta.

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for using CD34 as their endothelial cell marker is that it is expressed on vessels undergoing neoangiogenesis and cite unpublished work to support this statement.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).

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).

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.

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.

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.

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.

3) Discussion
Repetition on page 14, in paragraph 2.
Discretionary Revisions

1) A second pericyte 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, and I have assessed the statistics in my report.

**Declaration of competing interests:**

I declare that I have no competing interests