Author’s response to reviews

Title: Vasculature analysis of patient derived tumor xenografts using species-specific PCR assays: evidence of tumor endothelial cells and atypical VEGFA-VEGFR1/2 signalings

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Author's response to reviews: see over
Dafne Solera
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Dear Dafne Solera,

Please find enclosed the revised manuscript MS: 979451641091120 ‘Vasculature analysis of patient derived tumor xenografts using species-specific PCR assays: evidence of tumor endothelial cells and atypical VEGFA-VEGFR1/2 signalings’ submitted for publication in BMC Cancer. We appreciated the constructive comments made by your referees. We have carefully addressed them and provide a point-by-point reply in the accompanying letter to the reviewers. Accordingly, we have modified the manuscript in response to the specific criticisms raised by the reviewers, changes are indicated in red.

We hope that these corrections will further clarify the work presented in this manuscript and that it will meet your favourable consideration for publication in BMC Cancer.

On behalf of all of the authors
Yours sincerely,

Virginie DANGLES-MARIE
DVM, PhD
Answers to Referees

Referee: Jean-Philippe Gratton

Point 1: ‘Since the supplementary Table 2 is referenced multiple times throughout the manuscript its description should be improved. In its present form, it is very difficult to understand and interpret the data.’

As requested by the referee, the supplementary Table 2 has been modified as follows to provide a better understanding:
- Figures in the different columns are right-aligned
- Target mRNA levels that were not detected or at very low level (Ct > 38) in tumor samples were scored ‘0’ for non-expressed.
- Target mRNA levels that were weak (35< Ct < 38) in tumor samples were scored ‘<1’
- In other cases, normalized values of gene expression are expressed as a whole number without decimal places

Besides, because of its central interest, this supplemental table has been moved to the main manuscript as Table 1. Tables have been accordingly renumbered.

Point 2: ‘Labels for Y-axes in figure 2 (a-d) are missing. Legend for Figure 2 could be more explanatory and the labeling of the bar graphs could be improved’

Labels for Y axes and the color code of the bar graphs were missing and have been added (revised Figure 2).
Referee: Andras Nagy

**Point 1:** ‘Multiple reference genes should be selected for Q-PCR data normalization (see e.g. PLoS One. 2013;8(3):e59180), unless the author could explain why TBP alone is better than a set of house keeping genes.’

We selected the TBP gene as an endogenous control because the prevalence of its transcripts is moderate (Ct values between 24 and 26 in the tissue samples) both in mouse and human tissues, and because there are no known TBP retropseudogenes (retropseudogenes lead to co-amplification of contaminating genomic DNA and thus interfere with RT-PCR, despite the use of primers in separate exons). We have added a sentence dealing with this matter in the ’Methods’ section (page 13, lines 25-27).

We therefore rejected the β-actin, GAPDH, HSPCB, PPIA and HPRT genes as endogenous controls because of the existence of corresponding retropseudogenes (Jacob et al, PLoS One 2013, 8:e59180 ; Dinhofer et al, Trends Genet 1995, 11(10):380-1; Sellner et al, Mol Cell Probes 1996, 10(6):481-3); we also rejected the RRN18S (human 18S rRNA) and the RPS13 genes, which are intronless and has very high abundance of transcripts.

**Point 2:** ‘To validate the estimate of mouse/human cell ratio in the xenografts, the authors should show the accuracy of the estimates by performing a standard curve obtained from a set of artificially created mouse human cell ratio (mouse/human: 0/100, 10/90, … , 90/10, 100/0).’

By performing a RT-PCR analysis of a standard curve, we demonstrate below the robustness of the quantitative assessment of mouse content in PDXs using TBP as a ubiquitously expressed housekeeping gene with similar expression in human and mouse tissues.

For that, 1 µg of RNAs from 2 sources of human cells (referred as to H1 and H2) and from 2 sources of mouse cells (M1 and M2) have been reverse transcribed into cDNAs. The four resulting cDNA solutions have been then diluted to perform standard curves by mixing {H1 cDNA with M1 cDNA} and {H2 cDNA with M2 cDNA}. We have obtained the following artificial human/mouse cell ratios (0/100, 5/95,10/90; 25/75; 50/50; 75/25; 90/10; 95/5 and 100:0), as suggested by the referee. As mentioned in the article, specific mouse Tbp gene expression and the expression of both the mouse and the human TBP genes have been studied by real-time qRT-PCR using the mouse Tbp as target gene and the ‘Total-TBP’ as endogenous RNA control. The final result gives an estimate of the proportion of mouse cell content within the 2 mixture sets (Figure a).

These experiments, done in duplicate, clearly show that this approach allows an accurate estimation of the mouse cell proportion within xenograft samples. This validation point has been added in the ‘Results and discussion’ section (page 6, lines 7-8).

**Figure a.** Standard curve of mouse/human cell ratio.

![Standard curve of mouse/human cell ratio](image-url)
Point 3: ‘The current analysis is overtly simple. There is much more the authors could do with the data. They should perform, for example, cluster analysis and principle component analysis on the 150 xenografts (having ten Q-RT-PCR values each) to show variance within and between tumour types and the order of genes regarding their discrimination value’

As suggested by the referee, cluster analysis and PCA have been performed in order to get potential new information. The 10 studied genes were used for clustering analyses but led to mixed groups of tumor types. By contrast, the hierarchical clustering of the 10 genes showed that mCd105, mVegfr1, mVegfr2 cluster together, closely to mCd31, and to hVEGFA (Figure b), as found by the correlation analysis in the revised Table 2 (ex Table 1) ‘Relationships between mouse (m) and human (h) mRNA levels in the 150 human tumor xenografts’. Likewise, PCA was also informative. As shown in Figure c, the majority of the xenograft samples forms a relatively compact group, while the renal cell carcinoma (sky blue, 12 out of 15 models) and less massively, cutaneous melanoma (bright blue) clearly stand out from the global panel. This visualization could then underline both that the GBM are highly vascularized and the CM cells are able to express hCD105. These clustering approaches have not been added to the revised manuscript as they rather confirm results than demonstrate new data.

Figure b. Hierarchical clustering of the 10 genes in the 150 patient tumor derived xenografts and magnification of the dendogram.

Figure c. Principle Component Analysis of the 5 mouse and 5 human genes within the 150 samples.


**Point 4:** ‘The authors should point out better that the human TEC could come from two sources: 1) Primary tumour resident “normal” endothelial cells and 2) Endothelial like cells converted from tumour cells. They should also emphasize that this study was not able to discriminate between these two sources.’

It is commonly admitted that the human stroma in patient tumor-derived xenografts is eventually replaced by stroma of mouse origin, as illustrated by histological analyses (immunostaining against mouse specific stromal markers or ALU probe in situ hybridization) and by gene expression analysis (recurrent variations of gene expression between patient tumors and xenografts correspond to disappearance of human stromal compartment genes) (Julien et al, Clin Cancer Research 2012, 18:5314 ; Reyal et al, Breast Cancer Res 2012;14(1):R11 ; Mattie et al, Neoplasia 2013;15(10):1124). Nevertheless, components of human stroma could be maintained in early passages and the timing of this switch human/mouse stroma remains to be clarified.

Thus, all the xenografts used here correspond to a passage between P5 and P12 in mice (as added in the ‘Results and discussion’ and ‘Methods’ sections, page 6, lines 14-15 and page 13, line 7, respectively), reducing the chance of maintaining residual human stroma cells. Nevertheless, as real-time qRT-PCR is a most sensitive technique than histological analyses, it can not be formally excluded that the signal of human endothelial markers are due to remaining human stroma cells. This aspect has been discussed in the revised manuscript (page 8, lines 24-27).

**Point 5:** ‘The authors should provide immunohistochemistry analyses on tumor samples. For example, co-staining of TEC markers (CD31+/CD105+) with human mitochondria marker will show the human TEC component). The quantitation of immunohistochemistry sections should agree with their estimates based on Q-RT-PCR data.’

Identification of tumor endothelial cells by immunohistochemistry analyses have been already reported and could confirm the qRT-PCR findings. To our knowledge, these positive analyses, demonstrating the presence of human TDEC, have never been done directly in patient-derived xenografts but in conditions facilitating TDEC discrimination:

- GFP expression by tumor cells. All tumor cells, including TDECs, are then GFP+, avoiding specific staining for cancer cell identification (Mouse glioblastoma models co-expressing activated oncogenes and GFP or lenti-GFP-transduced human glioblastoma spheres, Soda et al, Proc Natl Acad Sci U S A 2011,108:4274).

- Pre-amplification of cancer stem cells before engraftment into mice. As TDECs arise from cancer stem cells, increase of TDEC frequency can be expected after engraftment of CSC-enriched population. This CSC enrichment, prior to implantation into mice, is reported by cancer cell culturing as tumor spheres (Soda et al, Proc Natl Acad Sci U S A 2011,108:4274; Ricci-Vitiani L et al, Nature. 2010, 468:824), by cell sorting for putative CSC markers (Wang et al, Nature 2010,468:829 ; Alvero et al, Stem Cells 2009, 27:2405) or by induction of in vivo chemoresistance which leads to both increase of cancer stem cells and emergence of hCD31 positive cancer cells (Marfels et al, BMC Cancer 2013,13:176).

- Selection of putative TDECs before engraftment into mice. Tumor tissues are dissociated for cell sorting and CD105+ cancer cells are injected into mice (Bussolati et al, Faseb J 2008, 22:3696).

Only one recent publication attempted to immunostain directly human CD31 in 3 human tumor xenografts, with no preliminary step of TDEC or CSC enrichment (Ghanekar et al, BMC Cancer 2013, 13:485). This study did not detect hCD31 and led the authors to conclude that endothelial cells in human hepatocellular carcinoma xenografts are of mouse rather than human origin, but did not allow them to absolutely exclude this possibility. Indeed, hCD31 is much less expressed than mCd31 in most cases (we found that 131 xenografts out of 150 display a ratio of hCD31/mCd31 inferior to 1%, including 56 xenografts with no expression). Unfortunately, we have currently no cryosections of the models expressing the highest levels of hCD31 for confocal immunofluorescence.

These elements have been added in the ‘Results and discussion’ section because of their high significance (page 8, lines 11-22).
**Point 6:** ‘The data described in the last paragraph of the result section is surprising. The author should discuss this in more depth. From this data, it looks that the mouse blood vessels respond to AVASTIN better than the human. According to the literature however, AVASTIN inhibits hVEGF better than mVEGF.’

The referee rightly notices that bevacizumab (by contrast to other anti-angiogenic agents like aflibercept) is unable to efficiently bind and neutralize mouse VEGFA because its efficacy is specifically directed against human VEGFA. Moreover, VEGFA and its receptors are known to display cross-species functionality in humans and mice (ie recombinant mouse VEGFA is active on Human Umbilical Vein Endothelial Cells and recombinant human VEGFA on mouse blood vessels).

As the majority of the studied tumor xenografts, NSCLC#3 and NSCLC#5 produce low level of mVEGFA compared to hVEGFA transcripts, showing that the major source of VEGFA is produced by cancer cells. These 2 models are also characterized by their high expression level of hVEGFR1 and hVEGFR2 (Table 1).

As mentioned in the article, the 2 NSCLC models, stabilized by the anti-VEGFA therapy, were highly responsive to bevacizumab, with no development of resistance to the anti-angiogenic drug. Levels of mCd31, mCd105, mVegfr1 and mVegfr2 transcripts were significantly reduced by bevacizumab treatment in these 2 NSCLC xenografts while the anti-VEGFA therapy did not impact the hCD105, hVEGFR1 and hVEGFR2 expression level or, on the contrary, increased hCD31.

Taken together, these data suggest that the mouse endothelial cells are more sensitive to anti-VEGFA therapy than human cells. In addition to a basic higher dependency of endothelial cell growth to VEGFA compared to that of cancer cells, several other reasons could be given: ‘classical’ endothelial cells are strictly dependent to free VEGFA while cancer cells are able to take advantage of autocrine intracellular VEGFA/VEGFR signalling (Lichtenberger et al, Cell 2010;140:268-279). Furthermore, transdifferentiation of tumor cells has been reported to be VEGF-independent but induced by HIF-1α (Soda et al, Proc Natl Acad Sci U S A 2011,108:4274). Hypoxia due to mouse endothelial cells destruction by VEGFA blockade may then in turn increase TDEC.

These distinct responses from human and mouse cells have been discussed in the revised manuscript (page 11, lines 3-16).

**Point 7:** The authors should give a more detailed description of the PDX. For example, what passage(s) were used for their analysis, what was the stage/or grade of the primary cancer?

For all xenografts, passages between 5 and 12 have been used for these experiments (see point 4). Depending upon the type of cancers, the collecting of patient tumor samples has been done through several collaborations, with different hospitals. Clinical data from each patient tumor varied with the various collaborations. We have consequently added for all xenografts models, in the Table 1 (previously referred as to Supplemental Table 2) the origin of the patient tumor as primary tumor or metastasis since if it is the only data we have for all xenografts.