Author's response to reviews

Title: Presence of intratumoral platelets is associated with tumor vessel structure and metastasis

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Author's response to reviews: see over
Major comments

1. “To induce thrombocytopenia the authors used 2.5 ug/g anti-GPIb# antibody every 3 days, and state that this resulted in a 95% reduction in circulating platelets at 12 h post injection. It has previously been reported that using the same anti-platelet antibody at similar concentrations results in platelet depletion within minutes, and that platelet counts remain less than 2.5% for approx. 24 hrs (Goerge et al. 2008 Blood), while using lower concentrations of the same anti-platelet antibody results in platelet counts less than 50% for approx. 48 hrs (Stone et al. 2012 NEJM). To confirm the activity of the anti-platelet antibody in the current study, did the authors carry out experiments to determine the dose and time dependant effect of the antibody on mouse platelet counts in vivo? Also did the authors carry out any experiments to confirm the specificity of this anti-platelet antibody, in order to exclude the possibility that antibody recognition of cells other than platelets (e.g. pericytes, tumor, or endothelial cells), might account for observed anti-tumor and/or anti-angiogenic effects?”

Response. As reviewer pointed, we have noted the difference in the platelet counts with treatment of anti-platelet antibody. According to the instruction from Emfret Analytics, this preparation of antiplatelet antibody is optimized to reduce the platelet count by >95% within 60 minutes after IV injection of 2 µg/g. Based on previous publication (Demers, et al 2011. Cancer Research), we induced thrombocytopenia in mice by I.P. injection of an anti-GPIbα antibody (2.5ug/g), resulted in ≥95% reduction in circulating platelets at 12 h post-injection in all mice. We have also done platelet counts at the different day, and resulted in a 90% and 50% reduction at 48 h and 72 h, respectively. We have not done any experiments to confirm the specificity of this anti-platelet antibody, however, we found that platelet depletion inhibited the recruitment of pericyte in vivo.

2. “It has previously been reported in the literature that inducing thrombocytopenia in a mouse model significantly reduces tumor growth (Stone et al. 2012 NEJM, Demers et al 2011 Cancer Research, etc…). In this current study the authors observed no decrease in tumor growth despite inducing a 95% reduction in platelets. Can the authors offer an explanation for the observed differences? (Furthermore, the authors later state that co-implantation of B16/F10 with platelets led to increased tumor growth, which appears to contradict this earlier stated result.)”

Response. In response to this suggestion, we carefully review the two publications mentioned by reviewer. Firstly, compared with previous publication by Stone et al (Stone et al. 2012 NEJM), the injecting time of anti-platelet antibody is different. We injected platelet antibody started tumors grew to ~500 mm$^3$ in size in B16/F10 and ~250 mm$^3$ in size in 4T1, respectively, every 3 days until 24 days post-injection, in contrast, Stone et al started the injection of anti-platelet when tumor cell implantation; Secondly, like our finding, Demers et al. showed a no difference in tumor growth
between control an platelet depletion alone groups. Platelet depletion only increases paclitaxel delivery to mammary tumors and enhances its tumoricidal effects. Thirdly, we found that co-implantation of B16/F10 with platelets led to increased tumor growth. However, whether the presence of platelets will induce tumor cells proliferation, further studies are required to determine the precise mechanisms.

3. “Thrombocytopenia has previously been shown to cause severe bleeding that is specific to the tumor site and not seen elsewhere in the mouse model (e.g. Ho-Tin-Noe et al. 2008 Cancer Research). This result is confirmed in this present study where the authors demonstrate that platelet depletion induces vessel leakage (Figure 3). However, the VEGF data is not convincing. The results in figure 3B appear to be skewed by saline having an effect on Evans Blue detection. In the control sample, the OD EB is approx. 1 g/tissue when injected with saline, and this figure increases just over 2 fold to approx. 2.5 g/tissue when injected with VEGF. Similarly in the platelet depleted sample, the OD EB is approx. 2 g/tissue when injected with saline, and this figure increases just over 2 fold to approx. 4.5 g/tissue when injected with VEGF. If the saline effect is negated out of this set of results, is VEGF-mediated hyperpermeability still significantly increased in the platelet depleted sample? Is it a volume effect?”

Response. We thank the reviewer for making this comment. As shown in Fig 3B, platelet depletion exhibited increased vascular leakage compared with baseline. Indeed, VEGF in either platelet depletion or control group markedly induced vascular leakage after 30 mins. As pointed by reviewer, the data is similar as the VEGF:Saline ratio between platelet depletion and control group, suggesting that platelet play a role for VEGF in regulating EC barrier function, further studies are required to determine the precise mechanisms.

4. “The authors state TGF-beta levels were significantly increased in mice co-implanted with cancer cells and platelets compared to those implanted with cancer cells alone or platelet depletion mice (Figure 6A-C). In order to make this statement the authors would have had to include another control set where the mice were injected with platelets only. Platelets are a major source of TGF-beta in the vasculature. Therefore, adding platelets to any one mice, would result in an increase in TGF-beta levels. In the discussion the authors state “most serum VEGF is derived from platelets, which are activated upon coagulation”, similar is true for TGF-beta and must be considered when interpreting these results. Similarly, platelets are also a source for MMP-2 and MMP-9 (Fernandez-Patron et al. 1999 Thrombosis and Haemostasis) and thus results presented in Figure 6D must be interpreted with caution when no platelet alone control sample is included.”

Response. We thank the reviewer for making this comment. With other reviewers’ suggestions and concerns that only deal with platelet depletion, we deleted the results of co-implantation experiments from the revised manuscript.
5. “The authors present an extensive and complex group of experiments looking at the effect of platelet depletion on the progression of melanoma. The final section of their results includes one set of experiments looking at the effect of platelet depletion on growth and metastasis of lung cancer. This experiment is not consistent with the rest the work presented in this manuscript.”

Response. We thank the reviewer for making this comment. The main objectives of our study were to examine the effect of platelet depletion on tumor growth and metastasis and to study the role of platelet-derived growth factors in regulating tumor vessel function. Key findings of our study were that platelet depletion showed no change in tumor growth and reduced lung metastasis in B16/F10 implanted mice and associated with changed tumor vessel functions. We agree that the rest experiments examining the effect of platelet depletion on 4T1 implanted mice would be of interest and relevant to our manuscript under consideration. However, we do not have remaining tissue from 4T1 implanted mice for further experiments.

Minor Revisions

1. Figure 1, graph A doesn’t appear to ‘match’ graph B. In graph A at 24 hours, the control appears to be higher than the platelet depletion samples, in graph B these results appear to be the opposite way around. Figure 1, there is some mis-labelling, Western Blot analyses is Figure 1D, not 1E as is referred to in the text.

Response. We thank the reviewer for making this comment. We have corrected the error accordingly. We have replaced it with new Figure 1.

2. Figure 2 is difficult to interpret. Figure 2A, could the authors please have 4 images for the control and platelet depleted, comprising of: (i) PECAM-1 staining (ii) alpha-SMA staining (iii) DAPI staining and (iv) merged image. Figure 2C Should the Y-axis read alpha-SMA and not alpha-SMA/PECAM-1? If it is indeed supposed to read alpha-SMA/PECAM-1, why have the authors merged these results when they have already shown the PECAM-1 result in Figure 2B?

Response. As suggested by the reviewer, we have replaced Figure 2A with a more representative image, including (i) PECAM-1 staining (ii) alpha-SMA staining (iii) merged image. We also deleted Figure 2B, and have modified the new Figure 2 accordingly.

3. Three-dimensional co-culture assay: The authors state that the cancer cells alone or cancer cells/platelet suspension were incubated in standard conditions for 72 hrs. Matrigel experiment should generally be preformed for no longer than 24 hours, after this time point the cancer cells that have migration/invasion can proliferate and thus skew the results. Could the authors justify why they picked this time point of 72 hrs, and
also if they took into account when calculated the number of invaded cells. They state the number of invaded cells was ‘manually counted’. Please expand on this calculation.

Response. We thank the reviewer for making this comment. Given reviewers’ suggestions and concerns, we deleted the results of co-implantation experiments from the revised manuscript.

Comments of Reviewer 2 and Response

In this manuscript, the authors reported that platelets play a crucial role in vascular permeability and tumor metastasis. To clarify the role of platelets in tumor development and metastasis, the authors used rat anti-GPIbα antibody to deplete platelets in vivo. The platelet depletion resulted in the reduction of blood vessel density, pericyte coverage of tumor vessels, and Met phosphorylation in B16/F10 tumor. Moreover, co-implantation of tumor cells with platelets increased primary tumor volume, the number of metastatic lung foci, and TGF-β levels in mice. Although the authors showed the expression of some molecules, such as Met, Angiopoietin-1, Angiopoietin-2, TGF-β, MMP-9, MMP-2, and PAI-1, were affected by platelet depletion or co-implantation of tumor cells with platelets, no definitive data is provided to certify the critical roles of these molecules in the tumor vascular permeability and the cancer metastasis.

1) The authors induced thrombocytopenia by intraperitoneal injections of 2.5 microgram/g of mouse platelet-depleted antibody every 3 days. This method is not clear whether platelets are sufficiently depleted at 72 h of post-injection in mice. The authors should perform time course analysis of the number of platelets.

Response. In response to this suggestion, and based on previous publication (Demers, et al 2011. Cancer Research), we induced thrombocytopenia in mice by I.P. injection of an anti-GPIbα antibody (2.5ug/g), resulted in ≥95% reduction in circulating platelets at 12 h post-injection in all mice. We have also done platelet counts at the different day, and resulted in a 90% and 50% reduction at 48 h and 72 h, respectively.

2) Although the authors stated “a finding that may help in the development of effective anti-metastasis therapies” in Conclusion, it is not investigated whether the molecules that were affected by platelet-depletion are really associated with cancer metastasis. The authors should perform some additional experiments by using small molecular inhibitors, siRNAs, or shRNAs targeting these molecules.

Response. We thank the reviewer for making this comment. The main objectives of our study were to examine the effect of platelet depletion on tumor growth and metastasis and to study whether platelet depletion affect tumor vessel function. We agree that the rest experiments examining the effect of platelet depletion-related molecules on tumor growth and metastasis would be of interest and relevant to our manuscript under consideration. We appreciate the significance of these comments, however performing
the suggested experiments are beyond the scope of the current study. The main conclusions of our manuscript are not affected by this.

3) The authors stated “Expression of Met was significantly increased in the tumor of co-implanted mice compared to B16/F10 alone mice” in Result (page 8, line 4 from the bottom). However, there is no evidence that platelets do not express Met on their surface. The authors should confirm the expression of c-Met and check the phosphorylated-Met level in the platelet by Western blotting.

Response. We thank the reviewer for making this comment. However, performing the suggested experiments are beyond the scope of the current study. The main conclusions of our manuscript are not affected by this.

4) Since the authors showed the decreases of vessel density and pericyte coverage in the tumors of platelet depleted mice, it would be better to investigate whether co-implantation of tumor cells with platelets increases tumor vessel density and pericyte coverage by IHC staining.

Response. We thank the reviewer for making this comment. Based on reviewers’ suggestions and concerns, we deleted the results of co-implantation experiments from the revised manuscript.

5) Although platelet depletion did not affect the tumor growth, tumor volume was increased by co-implantation of tumor with platelets (Figure 1A and 4A). The authors should comment on the apparent inconsistency of these results.

Response. In response to this suggestion, we carefully review the two publications mentioned by reviewer. Firstly, compared with previous publication by Stone et al (Stone et al. 2012 NEJM), the injecting time of anti-platelet antibody is different. We injected platelet antibody started tumors grew to ~500 mm$^3$ in size in B16/F10 and ~250 mm$^3$ in size in 4T1, respectively, every 3 days until 24 days post-injection, in contrast, Stone et al started the injection of anti-platelet when tumor cell implantation; Secondly, like our finding, Demers et al. showed a no difference in tumor growth between control and platelet depletion alone groups. Platelet depletion only increases paclitaxel delivery to mammary tumors and enhances its tumoricidal effects. Thirdly, we found that co-implantation of B16/F10 with platelets led to increased tumor growth. However, whether the presence of platelets will induce tumor cells proliferation, further studies are required to determine the precise mechanisms.

Given reviewers’ suggestions and concerns, we deleted the results of co-implantation experiments from the revised manuscript.
6) Although the authors stated about HIF-1# and hypoxia in Discussion (page 12, line 12 from bottom), no data about HIF-1# and hypoxia was provided in this manuscript. The authors need to show these data or correct the sentences.

Response. We have previously done this experiment, and we included the data in Results (pp. 8) and Figure 4A,B.

7) In Figure 2D and 3A, vascular leakage was examined by FITC-dextran and Evans Blue respectively. Although Figure 2D indicated that control mice was more leaky than platelet-depleted mice, Figure 3A indicated the opposite result. The authors would be better to comment on these differences in Discussion.

Response. We thank the reviewer for making this comment. As we described in Methods and Results, FITC-dextran was used to evaluate tumor perfusion, and the ratio of tumor fluorescence/plasma fluorescence reflects the extent of tumor blood vessel perfusion. In contrast, evans blue was used in Miles vascular permeability assay for in vivo permeability. The results are discussed on page 11-12.

Minor Revision

1) Almost all Western blot images are saturated. The authors should substitute more clear images and quantify the band intensity again.

Response. In response to reviewer, we have replaced it with a more representative image and quantified the band intensity again.

2) In Figure 1D, the c-Met band intensity seemed to be equal. The authors should quantify the band intensity again.

Response. In response to reviewer, in revised Figure 4C, we have replaced it with a more representative image and quantified the band intensity again.

3) In Figure 1C, images are longitudinally extending and these background colors are different from each other. The authors would be better to substitute more clear ones.

Response. In response to reviewer, we have replaced it with a more representative image.

4) The authors stated “Zymographic analysis of microdialysates revealed that the intensity of MMP-9 and MMP-2 bands in PLT-depletion were lower than in control groups.” (page 9, line 3 from the bottom). However, down regulations of MMP-9 and -2 is not clear in Figure 6D. The authors should quantify the relative band density.

Response. In response to reviewer, we included quantification of relative band density in revised Figure 6D.
5) Although the authors examined the expression level of MMP-2 by gelatin zymography as well as MMP-9, there is no comment on MMP-2 change. The authors need to comment the MMP-2 change.

Response. In response to reviewer, we included MMP-2 change in Results and Discussion.

6) In Figure 1D, figure numbers does not match with the text. (Western blotting analysis of Met is “Figure 1E” in the text (page 7, line 16 from the top), while that is shown as “Figure 1D” in the Figure.). The authors should change E to D in the text.

Response. In response to reviewer, and we included the data in Results (pp. 8) and Figure 4C.

7) In Figure 1D and 3A, horizontal axis labels of graphs could not be found. The authors should show labels.

Response. In response to reviewer, we have corrected it accordingly.

8) Although the authors stated “Proteolytic enzymes, such as MMP and PAI-1” in Discussion (page12, line 4 from the top), PAI-1 is not enzyme. The authors should correct the sentence.

Response. In response to reviewer, we have corrected the sentence accordingly.

Comments of Reviewer 3 and Response

Overall comments: To some extent, many of the findings in this manuscript lack novelty because it is known that platelets protect vasculature, and that increases in vascular permeability allow platelets to escape from the vasculature and be activated by collagen, etc. However, I found the results provided to be quite informative and I think the paper would be quoted by others. The results presented on changes to the vasculature induced by platelet depletion were useful. I was less convinced by the experiments in which platelets were added to tumour cells and shown to increase tumour growth and metastasis, because the chances of inducing artefacts as a consequence of platelet aggregation and clotting would be higher. On the whole I would have been happier if the manuscript just dealt with platelet depletion.

1. Is the question posed by the authors well defined? The end of the introduction contains the sentence “The current study was designed to test the hypothesis that platelets influence metastasis by mediating the local vascular activity as well as tumor
cell invasiveness.” To me, most of the manuscript addresses the question of how platelet depletion affects tumour structure and dynamics.

**Responses.** We thank the reviewer for making this comment. Based on reviewers’ suggestions and concerns, we deleted the results of co-implantation experiments from the revised manuscript.

2. Are the methods appropriate and well described? Yes, I had no trouble in following them but was concerned about the experiments with mixed tumour cells and platelets.

**Responses.** Based on reviewers’ suggestions and concerns, we deleted the results of co-implantation experiments from the revised manuscript.

3. Are the data sound? The histology in Fig. 1 relies a lot on the interpretation of the pathologist, because it is not possible to conclude this from the photograph supplied. I would just like assurance that the images have been checked properly. Also, as stated above I might question the conclusion about metastasis. If tumours grow faster they may metastasise earlier. Should the frequency of metastases be compared at the same tumour volume?

**Responses.** We thank the reviewer for making this comment. Metastases were identified via histopathological analysis and metastatic area was quantified by NIH ImageJ software, which was widely used to evaluate the tumor distant metastasis in studying of nude tumor implantation model. The frequency of metastases was compared at the same tumour volume. We injected platelet antibody started tumors grew to ~500 mm$^3$ in size in B16/F10 and ~250 mm$^3$ in size in 4T1, respectively. We included this change in Methods.

4. Does the manuscript adhere to the relevant standards for reporting and data deposition? Yes, with the reservations stated above

5. Are the discussion and conclusions well balanced and adequately supported by the data? See above. Most of the data are on platelet depletion and I would have liked to see more discussion on this as opposed to the platelet addition experiments.

**Responses.** Based on reviewers’ suggestions and concerns, we deleted the results of co-implantation experiments from the revised manuscript and focused on the studying of platelet depletion. We added new data for elucidating the effect of platelet depletion on tumor hypoxia in Results (pp. 8) and Figure 4A,B.

6. Are limitations of the work clearly stated? Not for the platelet mixing experiments

7. Do the authors clearly acknowledge any work upon which they are building, both published and unpublished? Yes
8. Do the title and abstract accurately convey what has been found? See comments above; there is very little about platelet depletion.

9. Is the writing acceptable? There are minor stylistic errors in English, but these could easily be adjusted editorially.

**Comments of Reviewer 4 and Response**

**RE.** Presence of intratumoral platelets promotes tumor vascular permeability and metastasis

In this paper the authors investigated whether a platelet-tumor cell interaction outside of the bloodstream plays a role in regulating primary tumor growth, vascular permeability and metastasis initiation. Their findings demonstrated that platelets within the primary tumor microenvironment play a critical role in the induction of vascular permeability and initiation of tumor metastasis. All performed experiments are well designed and the data obtained has been analyzed by the appropriate molecular and statistical methods.

1. General comment:
   **Major compulsory:** In Background section the assertion “Previous studies demonstrated that the depletion or reduction of circulating platelets…….” need a reference.

   **Response.** In response to reviewer, we have corrected it accordingly in Reference 10, 11.

2. **Major compulsory:** In Results section was mentioned the figure 1E concerning Met and pMet but in the panel 1 there is not figure E, also it is not described in the figure legends.

   **Response.** In response to reviewer, we have corrected it accordingly.

3. **Major compulsory:** In Figure Legend section, there is no description of fig. 2C what is present in the panel 2.

   **Response.** In response to reviewer, we have corrected it accordingly.

4. **Major compulsory:** In the Reference section, ref 1 (pag 3427-3236, uncorrected). Reference 15 was absent in the Text.

   **Response.** In response to reviewer, we have corrected it accordingly.

5. **Minor compulsory:** References: 2 that should be included to support their ideas—

   1. Hwang SJ, Luo JC, Li CP et al. Thrombocytosis: a paraneoplastic syndrome in
patients with hepatocellular carcinoma. World J Gastroenterol. 2004 Sep 1;10(17):2472-7

Response. In response to reviewer, we have corrected it accordingly.