Author's response to reviews

Title: Deletion of the thrombin cleavage domain of osteopontin mediates breast cancer cell adhesion, proteolytic activity, tumorigenicity, and metastasis

Authors:

Michel S Beausoleil (mbeausol@hotmail.com)
Erika B Schulze (eschulze@uwo.ca)
David Goodale (david.goodale@lhsc.on.ca)
Carl O Postenka (cpostenk@uwo.ca)
Alison L Allan (alison.allan@lhsc.on.ca)

Version: 2 Date: 23 September 2010

Author's response to reviews: see over
Response to Reviewer’s Comments

We thank the Editor and the Reviewers for their positive feedback and helpful comments. We have addressed specific Editor and Reviewers’ points as follows:

Editor:

1. Please can you state whether your research conformed to the Helsinki Declaration (http://www.wma.net/e/policy/b3.htm), and to local legislation. Please add a paragraph in your methods section stating this, and name the ethics committee which approved the research.

Our research did conform to the Helsinki Declaration and to local legislation. We have now added a paragraph in our Methods section stating this, and named the ethics committee which approved the research (Page 9-10 of the revised manuscript).

Reviewer 1:

1. “The manuscript is well written, clear and concise. The data are well presented and the experiments have been well performed. The findings are interesting and potentially reveal that inhibiting OPN cleavage leads to an increase in breast tumour growth and metastasis”.

We thank the reviewer for his positive feedback.

2. “As it stands it appears that the authors are suggesting that the lack of cleavage is the reason for the effects. It is clear that deleting the thrombin cleavage domain leads to an increase in tumour growth and metastasis. However, it is not clear whether this is because the deleted version is refractory to cleavage or whether the deletion has had some other effect such as altering the conformation of OPN or inhibiting an undefined interaction. As it stands I am not convinced that it’s the lack of cleavage that is responsible for the observed functional effects. The conclusions could be strengthened by generating point mutations to abrogate cleavage rather than deleting several amino acids”.

Based on the western blot analysis presented in Figure 1A, we are confident that the ∆TC-OPN is refractory to cleavage. Figure 1A shows that, relative to 468-CON cells, 468-OPN and 468-∆TC cells expressed high levels of OPN protein. However, only the 468-OPN cells expressed the N-terminal cleavage product of OPN, indicating that the thrombin expressed by the cells is capable of cleaving only the wildtype OPN produced by 468-OPN cells and not the thrombin uncleavable OPN produced by 468-∆TC cells.

However, the reviewer makes a good point from the perspective that the deletion may indeed be imparting additional effects such as altered conformation of OPN or inhibiting other protein
interactions that in turn influence the functional changes observed. We have added discussion of these possibilities to the Conclusions on Page 20 of the revised manuscript. The suggestion to generate a series of point mutations to further test the influence on thrombin cleavage of OPN and downstream functional behavior is an excellent idea; however it is beyond the scope of the present study.

3. “Related to point 1. In Fig. 1 there is an additional band of approximately 55kDa in the deltaTC lane of the OPN western. What is this band? Is it potentially related to the observed functional effects?”

We thank the reviewer for this important observation. Due to post-translational modifications such as glycosylation/sialation and phosphorylation, the molecular weight of OPN in monomeric form varies widely (41-75 kDa) (see reference 22). However, the numerous forms of post-translationally modified OPN are very poorly understood, particularly with regards to their differential functional effects on cell behavior. It is very possible that the additional ~55kDa band in the ∆TC-OPN lane represents a novel post-translational modification, and that this is related to the observed functional effects. We have now included this observation in the Results (Page 13) and Discussion (Page 17-18) of the revised manuscript.

4. “The title needs adjusting. The data do not show that the thrombin cleavage domain of osteopontin mediates breast cancer cell adhesion, proteolytic activity, tumorigenicity, and metastasis. They show the opposite, since deletion of this domain leads to an increase in these properties.”

In the current study, deletion of the thrombin cleavage domain lead to a decrease in cell adhesion, as well as an increase in proteolytic activity, tumorigenicity, and metastasis relative to wildtype OPN. Therefore, we believe that “mediate” is the correct word to collectively and succinctly describe the results of the study in the title. However, we have adjusted the title to: “Deletion of the thrombin cleavage domain of osteopontin mediates breast cancer cell adhesion, proteolytic activity, tumorigenicity, and metastasis” in order to help address the reviewer’s concerns (Page 1 of the revised manuscript).

Reviewer 2:
1. “In the adhesion studies (Fig 2) the authors need to also use antibodies against CD44”.

The integrin binding sites of OPN (RGD: [arginine\textsuperscript{159}- aspartic acid\textsuperscript{161}] and SVVYLR [serine\textsuperscript{162}-arginine\textsuperscript{168}]) are located on the N-terminal in very close proximity to the thrombin cleavage domain (RSK [arginine\textsuperscript{168}-lysine\textsuperscript{170}]), and it has been hypothesized that cleavage of OPN by thrombin influences cell behavior, potentially by increasing access to these integrin binding domains. In contrast, the CD44 binding domain is believed to be present on the C-terminal cleavage product of OPN. Therefore, for the adhesion studies we were primarily interested in how deletion of the thrombin cleavage region would influence integrin-mediated adhesion, and designed the experiments in this manner.

Although we agree with the reviewer that it would be interesting see if CD44 is also differentially involved in cell adhesion mediated by wildtype OPN versus ∆TC-OPN, this
experiment would be fairly difficult to do and still compare the functional effects of integrin versus CD44 blocking antibodies. For example, the plates would need to coated with a CD44 ligand (i.e. hyaluronic acid or OPN) instead of the αvβ1 and αvβ5 integrin ligand vitronectin that was used. If OPN were used to coat the plates, this would further complicate analysis due to the presence of both exogenous recombinant OPN and cell-produced OPN. We believe that these experiments are beyond the scope of the current study.

2. “In Figure 1A that shows secreted OPN in the three cell lines, it is clear that there is an extra band above 50 kDa that is secreted by the deleted thrombin cleavage site cells. The authors did not comment on this band and whether it could be responsible for the changed behavior in these cells”.

Please see response to Reviewer 1, Comment 3.

3. “The authors also did not provide clear explanation or at least proposal for the reason why deletion of thrombin cleavage site of OPN would increase tumor size and metastasis. Is this due to the mere deletion or is it due to actual change in the cell proliferation and migration machinery and for that, in vitro studies and cell proliferation analysis are mandatory to elaborate on this point”.

The in vitro and expression studies presented in Figures 2, 4, and 5 suggest that deletion of the thrombin cleavage domain of OPN increases tumor size and metastasis by several potential mechanisms: (1) Decreased adhesion/increased detachment to allow cell dissemination/metastasis (loss of expression of the MCAM adhesion molecule also supports this); (2) Increased proteolytic activity and expression of uPA (and decreased expression of the uPA inhibitor Maspin) may increase invasion/metastasis; and (3) Decreased expression of the pro-apoptotic protein TRAIL, which is consistent with the decreased tumor latency and increased tumor size observed in the in vivo studies. This is clearly summarized in the Results section (Page 16), throughout the Discussion (Pages 16-20), and in the Conclusions (Page 20) of the revised manuscript.

We also investigated whether differences in cell proliferation and cell migration may be contributing to the observed in vivo differences between 468-OPN and 468-∆TC cells, and we have now included this data as Supplementary Figure 2. We observed that there was no difference between 468-CON, 468-OPN, and 468-∆TC cell lines with regards to cell proliferation in vitro (Supplementary Figure 2A). Although we did observe that both 468-OPN and 468-∆TC cells showed a significant increase in migratory ability compared to 468-CON cells, there was no significant difference in migration between 468-OPN and 468-∆TC cells (Supplementary Figure 2B). Both the proliferation and the migration data are consistent with our previous studies using the 468-CON and 468-OPN cell lines (see references 21, 24). Thus, it is unlikely that the in vivo differences in tumorigenicity and metastasis seen between 468-OPN and 468-∆TC cells in this study are due to change in the cell proliferation and migration machinery. To support the inclusion of Supplemental Figure 2, we have made additions to the Materials and Methods (Pages 8-9), the Results (Page 14), and the Figure Legends (Page 33) in the revised manuscript.
4. “Finally, the authors conclude that translating their results into the clinic could provide new therapeutic opportunities. My question is this: is deletion of thrombin cleavage site of OPN a frequently encountered phenomenon in breast cancer patients? And if so, how often and does this correlate with more invasive behavior and less survival.”

The reviewer asks an interesting question. This has not yet been investigated in breast cancer patients, although we are interested in doing such a study in the future.

5. “It is not clear whether thrombin cleavage site deletion could have secondary effects on the different molecules that can affect the cells’ adhesion and metastatic behavior. For example, what is the status of secreted cathepsin D (CD) levels and activity? In the recent studies by the Sørensen group in JBC 2010, they show that CD cleavage of OPN supported minimal adhesion. It may be important to explore this possibility”.

The reviewer makes a good point that the deletion may indeed be imparting additional secondary effects on several different molecules (including but not limited to cathepsin D) that in turn influence the functional changes observed (see response to Reviewer 1, Comment 2). This is something that we are in the process of investigating further in detail, but is beyond the scope of the present study.

Reviewer 3:

1. “This is an unprecedented study to assess the functional role of the thrombin cleavage site of OPN in mediating effects on malignant properties of cells”.

We thank the reviewer for her positive feedback.

2. “In Figure 1A, the authors refer to the smaller band in the 468-OPN lane as that of N-terminal cleaved OPN. How do we know this? Is the antibody specific for recognition of the cleaved form”?

The antibody used is polyclonal anti-human antibody against OPN (hOPN1), a kind gift from Dr. Toshi Uede, Hokkaido University, Sapporo, Japan. This antibody binds to an epitope very close to the N-terminal end of the OPN protein, and thus recognizes both full-length OPN and the smaller N-terminal fragment generated upon thrombin cleavage. It does not detect the C-terminal cleavage product because of the epitope location (see reference 34). Therefore, we are confident that the smaller band observed in the 468-OPN lane (but not in the 468-ΔTC lane) is N-terminal cleaved OPN.

3. “In the same Figure, the OPN band in the deltaTC lane is identified as a ‘faster running’ band. There are two distinct bands in that lane. Could the authors comment on whether the band with the greater mass is due to post translational modifications of the delta TC band”?

Please see response to Reviewer 1, Comment 3.
4. “In the same Figure, it does appear that the band intensity for Thrombin is distinctly less than that seen in the CON and deltaTC lanes. Can the authors speculate on why this is the case? If there any mechanistic insight they can add to the discussion for this observation’’

Respectfully, we don’t believe that the band intensity for thrombin is significantly different between any of the cell lines. All three cell lines express both thrombin and TF at approximately equivalent levels, indicating that they would be present in the *in vitro* tissue culture environment during experimental assays.

5. “It will be helpful to show the entire dataset for Figure 1B. It could also be added as Supplemental Data...but the authors must consider giving the data for all the groups tested’’.

We thank the reviewer for this suggestion, and have now included the additional flow cytometry data as Supplemental Figure 1. To support the inclusion of Supplemental Figure 1, we have made additions to the Results (Page 13), and the Figure Legends (Page 33) in the revised manuscript.

6. “Figure 3B: Can the authors provide some insight, based on published literature, as to why they see an increase in uPA activity in the delta TC relative to OPN and CON cells? Also, based on literature, one would expect that the OPN cells will show an increase in uPA relative to vector-only cells. This is in fact not observed in these cells. Please comment’’.

Since this is the first study to report the functional consequences of deleting the thrombin cleavage region of OPN, we were not sure what to expect with regards to uPA activity and expression. The somewhat unexpected results may be explained to a certain degree by the fact that the uPA inhibitor Maspin is also downregulated in the 468-ΔTC cells, which may be a consequence of the deletion and may influence expression of uPA. We were also surprised that there was no increase in uPA in the 468-OPN versus 468-CON cells, since other studies in the literature have shown a relationship between OPN and uPA expression. However, we are not sure why this was not seen in our study, although it may be related to the genetic background of the MDA-MB-468 cell line versus the other breast cancer cell lines (MCF-7 and MDA-MB-231) used in previous studies.