Author's response to reviews

Title: The endogenous soluble VEGF Receptor-2 isoform suppresses lymph node metastasis in a mouse immunocompetent mammary cancer model

Authors:

Masa-Aki MS Shibata (shibatam@art.osaka-med.ac.jp)
Jayakrishna JA Ambati (jamba2@email.uky.edu)
Eiko ES Shibata (belleiko@ri.ncvc.go.jp)
Romulo RA Albuquerque (rjca12@gmail.com)
Junji JM Morimoto (eac001@art.osaka-med.ac.jp)
Yuko YI Ito (an1006@art.osaka-med.ac.jp)
Yoshinori YO Otsuki (an1001@art.osaka-med.ac.jp)

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Title: The endogenous soluble VEGF Receptor-2 isoform suppresses lymph node metastasis in a mouse immunocompetent mammary cancer model

Dear Editor,

I would like to thank the reviewers for constructive criticism and for the opportunity to make improvements to our manuscript, which is now resubmitted for further consideration for publication in BMC Medicine or BMC Cancer. We would prefer to publish in BMC Medicine.

We have made a number of changes (indicating with red) in line with the reviewers’ suggestions, as detailed below.

Thank you very much for your efforts on our behalf.

Sincerely yours,

Dr. Masa-Aki Shibata
Division of Life Sciences
Department of Anatomy and Cell Biology
Osaka Medical College
e-mail: shibatam@art.osaka-med.ac.jp
Reviewer: Airo Tsubura
Thank you very much for excellent comments to improve our research.

General comments:
1. BJMC3879luc2 and the parent cells express VEGF-C (J. Gene Med., 8, 335-352, 2006). Additional description has been made in the “Cell line and animals” section (page 6).
2. Basically Lyve-1 and podoplanin show same reaction with lymphatic endothelial cells. Many investigators use them for demonstration of lymphatic vessels. However, podoplanin antibody additionally cross react with perineurium. Therefore, we paid a special attention to count lymphatic vessels in tumors.
3-4. Typing errors have been corrected. In Figures 1 and 3 and Table 1, “pesVEGFR-2” has been corrected.
5. Journal style of the references has been checked.

Reviewer: Norihiko Tsuchiya
Thank you very much for excellent comments to improve our research.

Major compulsory revisions:
1) Mouse mammary carcinoma BJMC3879luc2 cells used in the present study express VEGF-C, VEGFR-2 and VEGFR-3 (unpublished data). Also, in BJMC3879 cells (without incorporation of luciferase gene) that were parent cells of BJMC3879luc cells, expression of VEGF-C and VEGFR-3 were reported (J. Gene Med., 8, 335-352, 2006). Additional description has been made in the “Cell line and animals” section (line 5 from the bottom, page 6).
2) Unfortunately, we did not measure blood esVEGFR-2 levels in the pes VEGFR-2 group. Since pesVEGFR-2 expression vector contains secretion signal, the observed effects may be due to circulating esVEGFR-2.
3) Table 1 has been altered. The revised Table 1 has contained average number of each organ with metastasis. Although pesVEGFR-2 significantly decreased number of lymph node and lung metastatic nodules in Figure 3A and B, the revised Table 1 showed that pesVEGFR-2 was not able to inhibit metastasis to the lungs, ovaries, kidneys and adrenals as counted by unilateral or bilateral metastasis. Therefore, “lung metastasis” from the title has been deleted as follows: “The endogenous soluble VEGF Receptor-2 isoform suppresses lymph node metastasis in a mouse immunocompetent mammary cancer model”. Description regarding the revised
Table 1 has been added in “Overall metastasis” of the Results section (lines 16-23, page 13) and the Discussion section (lines 17~, page 16 to lines 1-4, page 17).

4) Descriptions concerning counting for blood vessels and lymphatic vessels has been made in the “Blood and lymphatic microvascular densities in mammary tumors” section (lines 7-21, page 9) and the “Dilated lymphatic vessels with cancer cell invasion” section (lines 1-10, page 10).

Minor essential revisions:
Typing errors have been corrected. In Figures 1 and 3 and Table 1, “pesVEGFR-2” has been corrected.

Reviewer: Bronislaw Pytowski
Thank you very much for excellent comments to improve our research.

Major revisions or comments from authors required:
1. The reason why chosen electrogene transfer is as follows: Although in vivo gene electrotransfer was previously believed to have very low transfection efficiency, it was recently reported that, under suitable conditions, transduction was found to be approximately equivalent to that using an adenoviral vector concentration of $10^6$ transduction units/ml. The technique offers some attractive advantages over the use of viral vectors. In the first place, since vector immunogenicity is not an issue, accumulation of gene transduction is possible through repeated transfections. In addition, the vector is much easier to manipulate than a viral vector, and the procedure is safe in vivo and does not depend on cell cycles within target tissues. Several studies have further reported the efficacy of using in vivo gene electrotransfer in animal cancer models (Proc. Natl. Acad. Sci. USA, 97, 354-359, 2000; Cancer Res., 61, 3281-3284, 2001; Cancer Gene Ther., 9, 16-27, 2002).

sVEGFR-3 decoy experiment is actually conducting in Shibata’s laboratory. We also believe to sVEGFR-3 therapeutic approach is an important issue in mammary cancer. However, in the present study, we wanted to investigate naturally occurring molecules, hence esVEGFR-2 and endostatin (not recombinant proteins) were used. The description has been made in the Background section (lines 22-24, page 5).

Laakkonen et al. reported that VEGFR-3 blocking antibody therapy significantly suppresses both angiogenesis and lymphangiogenesis (Cancer Res., 67, 593-599, 2007). In addition, Burton et al. reported that sVEGFR-3 significantly inhibits lymphangiogenesis but slight inhibition on tumor blood vasculature by sVEGFR-3 is also observed. They mentioned that it could likely be responsible for
the delay in tumor growth \textit{in vivo} (Cancer Res., 68, 7828-7837, 2008). Therefore, in the present study, since we wanted to study the effect of inhibiting lymphatic vessels alone, esVEGFR-2 was chosen. The description has been made in the Discussion section (lines 4-11, page 16).

2. Unfortunately, we did not measure blood esVEGFR-2 levels in the present study. Since pesVEGFR-2 expression vector contains secretion signal, the observed effects may be due to circulating esVEGFR-2.

3. Since endostatin exerts both inhibition of blood and lymphatic vessels and it is also naturally occurring molecules as in esVEGFR-2, endostatin is served as a positive control (lines 22-24, page 5 in the Background section). Thank you very much for informing the reference regarding anti-lymphagenic effects by endostatin (Cancer Res., 67, 11528-11535, 2007). The description has been made in the Discussion section (lines 9-20, page 18).

4. Data between the pEndo and pesVEGFR-2 groups were conducted by statistical analysis. Tumor volumes and metastases showed statistical significances between the pEndo and pesVEGFR-2 groups. Descriptions have been made in the “Survival rates, body weights and tumor growth ....” section (pages 11-12), the “Bioluminescent imaging” section (page 12), the “Metastasis to lymph node” section (pages 12-13), the “Metastasis to lungs” section (page 13), the “Metastasis to lungs” (page 13) and “Overall metastasis” (page 13), and also in the Discussion section (lines 9-20, page 18).

Minor essential revisions or comments from the authors:
1. Scale bars have been made in Figures 2, 4 and 5.
2. p53 immunohistochemistry section has been made and the method was described in lines 1-6, page 9. A p53 mouse monoclonal antibody (Clone Pab240, Santa Cruz Biotechnology) used in the present study reacts to the mutant protein in fixed specimens. This method is a standard technique for demonstrating p53 mutation. Sequencing is another way which gives us more detail information.
3. As suggested by the reviewer, Figure 1G regarding levels of the luciferase activity (photon counts, cpm) has been made. The levels were significantly decreased in the pesVEGFR-2 and pEndo groups as compared to the control group. The description has been made in the “Survival rates, body weights and tumor growth ....” section (lines 23~, page 11 to lines 1-2, page 12), the “Bioluminescent imaging” section (lines 7-11, page 12), the “Metastasis to lymph nodes” section (line 25~, page 12 to lines 1-2, page 13) and the “Metastasis to lungs” section (lines 8-9, page 13).
4. Since arrows indicating vessels are contained endothelial cells, they are vessels. However, as the reviewer pointed, since it is not uncertain whether blood vessels or lymphatic vessels. Description has been altered in Figure 5F legend. Arrows indicating vessels are brownish, suggesting necrotic vessels histopathologically. Since blood vessel density was significantly decreased in the pEndo, it is considered that apoptosis of tumor cells may be due to injury of the blood vessels (blockage of oxygen supply and nutrition to tumor cells). Therefore, it is possibly that apoptosis of tumor cells were observed along damaged (brownish) blood vessels.

5. Table 1 has been altered. The revised Table 1 has contained average number of each organ with metastasis. Although pesVEGFR-2 significantly decreased number of lymph node and lung metastatic nodules in Figure 3A and B, the revised Table 1 showed that pesVEGFR-2 was not able to inhibit metastasis to the lungs, ovaries, kidneys and adrenals as counted by unilateral or bilateral metastasis. Therefore, “lung metastasis” from the title has been deleted as follows: “The endogenous soluble VEGF Receptor-2 isoform suppresses lymph node metastasis in a mouse immunocompetent mammary cancer model”. Description regarding the revised Table 1 has been added in “Overall metastasis” of the Results section (lines 16-23, page 13) and the Discussion section (lines 17~, page 16 to lines 1-4, page 17).

6. Descriptions regarding blocking VEGFR-3 with antagonist antibody or soluble VEGFR-3 with corresponding references have been made in the Background (lines 17-20, page 4) and in the Discussion (lines 3-10, page 6). Thank you very much for informing the references.

7. Descriptions regarding possibility of the reduction of metastasis due to smaller tumors have been made in the Discussion section (lines 8-11, page 15).

Minor comments:
1. Descriptions regarding origin of BJMC3879 cells have been made in the “Cell line and animals” section (page 6).
2. The sentence in the “Blood and lymphatic microvascular densities in mammary tumors” section in the Methods has been revised (page 9).
3. Description regarding tumor volumes has been changed in Figure 1C legend.
4. As suggested by the reviewer, the description has been deleted in Figure 2 legend.
5. If all Figure 3 (A-F) is moved to Figure 5 (the end), it will not good position for Figures 3A and B (metastasis data). We would like to keep Figure 3 in the present position.
6. In the Introduction section, “therapeutic target” has been changed to “therapeutic
tool” (line 9 from bottom, page 5).