Author's response to reviews

Title: Cross-linking of CD24 inhibits growth of MCF-7 breast cancer cells

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Author's response to reviews: see over
Letter to Editor

Dear Dr. Abigail Brown

Thank you so much for giving us the opportunity to revise this manuscript. Please find enclosed our revised manuscript entitled

“CD24 cross-linking induces apoptosis in, and inhibits migration of, MCF-7 breast cancer cells”.

The reviewers’ very helpful comments were addressed as follows:

Reviewer-1: Glen Kristiansen
Major points.
The work is basically of great interest, since the effects of CD24 crosslinking on breast cancer cell lines are only insufficiently characterized. However, the impact of the presented results would be enhanced, if more than one CD24-positive cell line was analysed. I suggest to analyse at least another CD24 positive breast cancer cell line with the assays described to confound the findings. Also, the choice of the blocking antibody (rabbit polyclonal) should be explained, given the wide range of available monoclonal CD24 antibodies (which might be more suitable for a therapeutic in vivo application).

→ Instead of breast cancer cell line, we provide inhibition of growth in the MCF-10A expressing CD24 as an alternative cell line in supplementary figure.
   We selected rabbit-polyclonal anti-CD24 (FL-80) antibody for this studying with paper reported. Chen et al showed that P-selectin ligand CD24 mediates the binding of platelets to prostate cancer line DU145 cells in vitro using Polyclonal anti-CD24 antibody (FL-80) obtained from Santa Cruz Biotechnology in the Proceedings of the Western Pharmacology Society 2004, 47:28-29.

Please describe antibody specificity and the epitope detected by the antibody. This is important, since CD24 is heavily glycosylated.

→ We inserted and provide the sentence about antibody specificity, the epitope, and about CD24 (FL-80) antibody used in this studying in Page 5 line 24-25.

Please explain the choice of cell lines. MCF-7 is generally considered a model for a less aggressive breast cancer, whereas MDA-MB231 is considered highly invasive. Would it be possible that the suggested anti-CD24 therapy would in vivo target only less aggressive tumour cells but spare more aggressive populations?

→ We inserted about characteristics of MCF-7 in Page (3) line 24-25 and Page (4) line
The manuscript has a few references that have been cited either inappropriately or in a biased fashion. These phrases should be carefully re-written: Background, second paragraph, last sentence. Please check the validity of reference 2, because it does not deal with functional properties of rat carcinoma cell lines.

→ We omitted the sentence and reference indicated.

Discussion, line 1. “CD24 plays important roles in progression, migration, and metastasis of human breast cancer”. For this matter of factly sounding statement, references should be cited. However, the role of CD24 in breast cancer is quite unclear. Apart from merely correlative studies that demonstrated a prognostic value of CD24 in primary breast cancer and functional studies, that demonstrated CD24 as an alternative ligand to P-selectin in breast cancer cells, functional data on the role of CD24 in “progression, migration and metastasis” of breast cancer are scarce. Conversely, different groups have suggested that aggressive breast cancer cells/cases are CD24 negative/low (please see e.g. Breast Cancer Res. 2006;8(5):R59, Proc Natl Acad Sci U S A. 2003 Apr 1;100(7):3983-8., N Engl J Med. 2007 Jan 18;356(3):217-26.), which could be added to the discussion.

→ We inserted the reference and sentences in Page (12) line 10-20.

Discussion, second sentence. The statement that adhesion was inhibited by CD24 crosslinking is not supported by the authors data (“adhesion (…) was reduced a little, but not significantly”, results, last paragraph) and should be omitted.

→ We omitted the sentence indicated

Discussion, line 19 – “consistent with previous studies showing that polyclonal immunoglobulin inhibits growth of cancer cells” should be further specified since the polyclonal immunoglobulins in the cited study were directed against tumor vasculature.

→ We showed the sentence indicated in Page (10) line 16-20 with reference 19, 20.

Discussion, second paragraph – “Using in vitro migrations assays(matrigel) and in vivo immunohistochemical staining, Senner et al. (6) showed that CD24 induces migration of glioblastomas, (…).”, is a simplification. Correct is, that Senner et al. have found in a rat model CD24-positive gliomas more aggressive than CD24 negative implants, but did not observe a greater migration rate of CD24-positive cells in matrigel assays.

→ We rewrite the sentence as indicated

Discussion, 4th paragraph – “Furthermore, CD24 is expressed in most neuroendocrine carcinomas of the skin and can thus be applied as a diagnostic marker as well (17). Since CD24 is widely expressed in human neoplasms, it cannot be recommended as an entity-specific diagnostic marker!
We omitted the sentence indicated
The rate of CD24 positivity in the abstract (2% and 65%) is different to the results shown later (2% and 61%), please check.

We corrected the percentages to 66% replacing 65% and 61%

Reviewer-2: Periasamy Selvaraj

Major Compulsory Revisions
1. In figure 1A, the results show that control rabbit IgG (this is referred as ‘anti rabbit IgG’ in many places) at 1 and 2 microgram/ml concentration dramatically inhibited proliferation of MCF-7 cells. It is not clear why a control rabbit IgG as low as 2 microgram/ml can inhibit 96% of MCF-7 cell growth? Is the rabbit IgG stock had preservatives such as sodium azide? No explanation was provided. Why use distilled water instead of medium or saline as a control? Addition of water to culture medium will change the osmolarity of the medium and may kill the cells. Therefore the results presented in the manuscript may not be not reliable.

We added a comment in Page (8) line 23-25 and Page (9) line 1-2
We examined cell growth in five different culture media. However, we did not find difference in cell growth in each culture condition

2. Reference 15 is wrongly interpreted and quoted. The results described in manuscript 15 show that addition of anti-CD24 mAb blocks the T cell costimulation dependent T cell proliferation and do not show anything about induction of apoptosis in T cells due to CD24 cross-linking. But authors quote ‘cross-linking of CD24 induced apoptosis in several cells, including human T cells [15]’

We omitted the reference

3. The authors use terms such as ‘antibody binding’, ‘cross-linking’, and ‘antibody blockage’ to refer the same thing. These words do not refer to same thing; they mean different events. Example: ‘functional blocking of CD24 through cross-linking in MCF-7’

We decided to use the term ‘cross-linking’ replacing ‘antibody binding’, or ‘antibody blockage’

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct) The manuscript is poorly written. The authors did not even bother to proof read the manuscript. Apart from many grammatical mistakes, many typos were not corrected. Some examples: microgram is referred as ‘ug’ and micrometer is referred as ‘um’; CD24 is referred throughout the reference as ‘cd24’; and there are square symbols to refer centigrade!

We corrected typos as indicated such as µg, µm, and CD24 in all part of manuscript
Reviewer-3: Nobutaka Kiyokawa

Discretionary Revisions (which are recommendations for improvement but which the author can choose to ignore)

1) Is inhibition of cell growth induced by CD24 cross-linking general phenomenon for CD24 expressing breast cancer cells? Similar experiment using CD24-expressing breast cancer cell lines other than MCF-7 should strengthen the claim of the author.

→ Instead of breast cancer cell line, we provide inhibition of growth in the MCF-10A expressing CD24 as an alternative cell line in supplementary figure.

2) If authors consider application of their observation to clinical therapy in the future, employment of monoclonal antibody instead of polyclonal antibody should be convenient. Do monoclonal anti-CD24-antibody have same effect as that of polyclonal antibody?

→ We inserted and provide the sentence about antibody specificity, the epitope, and about CD24 (FL-80) antibody used in this studying in Page 5 line 24-25.

3) In the page 9, lines 15-17, the authors described that “blocking CD24 through cross-linking is … to inhibit tumor growth, adhesion and invasion in MDA-MB-231 and MCF-7”. Is, however, the phenomenon that the authors observed really mediated by “blocking CD24”? The cross-linking of CD24 induce cell signals by it self. Why can the authors declare it is “blocking CD24” but not “activating CD24”? It should be explain or discus on this point.

→ We put a sentence indicating as title of manuscript

- Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

1) Few type miss were found. In the page 6, line 1 and page 8 line7, “MDA-MD-231“ should be ”MDA-MB-231”.

→ We corrected as indicated

2) In the page 9, lines 15-17, the authors stated that “blocking CD24 through cross-linking is … to inhibit tumor growth, adhesion and invasion in MDA-MB-231 and MCF-7”, whereas this reviewer think that the data and description in the manuscript (Figs. 1 & 2, etc.) indicate that MDA-MB-231 is a negative control and CD24-crosslinking only affect MCF-7.

→ We rewritten the sentence indicated and correct the wrong statement.

3) The authors stated that “Annexin V-unstained survived cell population decreased by 10% and 26%…” in the page 9, line. However, this reviewer think that the data of Figure 5 are revealing that the “PI-unstained survived cell population decreased by 10%
We deleted and correct the sentence.

4) The quality of Fig. 3A is low and nothing can see for this reviewer as judged by PDF file. Thus this picture should be replaced with better one.

5) In the page 10, line 33; what is “ER”? This reviewer could not find the abbreviation of ER in other part of this manuscript.

- Major Compulsory Revisions (which the author must respond to before a decision on publication can be reached)

For this reviewer, the description of Fig. 2 is not sufficient and how they drew the graphs from their experiments is not clear. However, considering the description of the Methods, Figure 2 exhibits the results from cell counting using trypan blue staining as the ratio of viable cells against total cells. From this data, they concluded that “cross-linking of CD24 inhibit cell growth” (Abstract, page4, lines 18-19), “whereas they also indicate that CD24 cross-linking increased apoptosis” (Abstract, page4, lines 14-15) based on the data presented in Fig.5. This reviewer think that the “Inhibition of cell growth” and “decrease in viable cell number induced by cell death” are totally distinct events. If the decrease in viable cell number is due to increase in apoptosis, the title “Cross-linking of CD24 inhibits growth of MCF-7 breast cancer cells” may need to be change. Therefore, the authors should make the point clear whether the decrease in the ratio of viable cell population presented in Fig. 2C is mediated by ether cell growth inhibition or increase in cell death. For this purpose, it should be helpful to also indicate the data of actual number of living and dead cells in the Fig. 2 and discuss on them.

We showed the results from cell counting using trypan blue staining as growth curve of viable cells against total cells in figure 2C.

Since the Discussion is redundant and obscure, it should be reorganized and revised. For example, the descriptions in page 9, lines 14-32 and page 10, lines 11-14 are almost same and should be get together. On the other hand, the description in page 9, lines 32-37 is same as that described in results and it may be unnecessary to described here again. In addition, the point of discussion that described in page 9, line 27 to page 10, line 3 is not clear for this reviewer, and thus it should be better to make clear the point of argument in this part.

We reorganized and revised in discussion.

- Have you in the past five years received reimbursements, fees, funding, or salary from
an organisation that may in any way gain or lose financially from the publication of this paper, either now or in the future?
- No
- Do you hold any stocks or shares in an organisation that may in any way gain or lose financially from the publication of this paper, either now or in the future?
  - No
Do you hold or are you currently applying for any patents relating to the content of the manuscript?
  - No
Have you received reimbursements, fees, funding, or salary from an organization that holds or has applied for patents relating to the content of the manuscript?
  - No
- Do you have any other financial competing interests?
  - No
- Do you have any non-financial competing interests in relation to this paper?
  - No

We thank all the valuable comments from the reviewers. We hope that after these corrections our manuscript will be acceptable for publication in *BMC Cancer*.

Sincerely yours,

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