Author's response to reviews

Title: Stimulation of ST3 expression in mouse fibroblasts by cytokines, collagen and co-culture with human breast cancer cell lines

Authors:

Saxon Selvey (l.griffiths@griffith.edu.au)
Larisa M Haupt (lhaaupt@imcb.a-star.edu.sg)
Erik W Thnompson (rik@foo.medstv.unimelb.edu.au)
Klaus I Matthaei (Klaus.Matthaei@anu.edu.au)
Michael G Irving (michael_irving@bond.edu.au)
Lyn R Griffiths (l.griffiths@griffith.edu.au)

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Author's response to reviews: see over
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THE EDITOR
BMC Cancer

Dear Editor,

Please find enclosed the manuscript entitled “Stimulation of MMP-11 (stromelysin-3) expression in mouse fibroblasts by cytokines, collagen and co-culture with human breast cancer cell lines” by S. Selvey, L.M. Haupt, E. W. Thompson, K. I. Matthaei, M. G. Irving and L. R. Griffiths. The manuscript has been revised following comments by the reviewer discussing the importance of MMP expression studies in relation to cancer. The reviewer makes a number of specific suggestions for the revision of the manuscript and our detailed responses to these criticisms are outlined in the accompanying material.

Correspondance concerning this publication should be directed to:
Professor Lyn Griffiths
School of Health Science
Griffith University, Gold Coast 4217
QLD, Australia
Fax No: +61-7-555-28908
Tel No: +61-7-555-28664
Email: l.griffiths@griffith.edu.au

We thankyou in advance for your consideration and await your response.

Regards,

Professor Lyn Griffiths
School of Health Science
Griffith University
The reviewer discusses the importance of MMP expression studies in relation to cancer and makes a number of specific suggestions for the revision of the manuscript. Our responses to these detailed criticisms are:

1. The authors should fix the track changes before upload and submission of manuscript.

The authors apologise for the confusion, the track changes has been disabled in the resubmitted manuscript.

2. Title: Spell out ST3

As suggested by the reviewer, ST3 has been replaced in the title and throughout the manuscript by the MMP family name of MMP-11 (stromelysin-3).

3. Pages 4 and 5: This content would fit better in the discussion section, with some modifications.

As suggested by the reviewer, content on pages 4 and 5 has been modified. Some of this content is now in the discussion section with minor modifications.

4. Page 6, Methods, Cell lines: 1) The primary cultures were established allowing the cells after homogenization to attach for a period between 6 and 12 hours prior to use. Was there any assess to determine the contamination with other cell types? 2) How was the invasive and metastatic potential of the breast cancer cell lines listed in Table 1 determined? Are those based on Thompson et al. 1992? 3) What are NCL-adr and MCF-7stv cell lines?

1) The procedure used to obtain MEFs is one we have routinely used. The procedure yields a predominant fibroblast population (approx 90%), as assessed by microscopy, which becomes less heterogeneous in culture.

2) Yes, the invasive and metastatic potential of the HBCs used in the study are based upon Thompson et al, 1992. This is indicated in the title of Table 1 and referenced appropriately. The authors accept the influence of long term in vitro propagation on cell line stability. As such, effort was made to source cells as close as possible in terms of age and population doubling to those characterised in the Thompson et al study.

3) We apologise for the confusion, the cells in question were part of a larger study that is not relevant to the current manuscript. All references to these cell lines in the manuscript have been removed.

5. Page 6, Methods, Collagen coats: 1) Inform the source of the collagens. 2) Were the cultures treated for 24 hours (last line of this section) or a range from 3 to 48 hours as shown in Figures-1 and -2?

1) The collagens were all sourced from Sigma Aldrich. This is now indicated in the Methods, Collagen Coats section on Page 5.

2) We apologise for the confusion. After allowing the collagen coats to set for 1 hour, cells were seeded at the stated density and incubated for two timepoints, 24 hours and 48 hours. Following these two time-point incubations, RNA was extracted as detailed. The manuscript has been modified to reflect this clarification in the Methods, Collagen Coats section on Page 6. Figures 1 and 2 (now Tables 3, 4, 5 and 6) have now been tabulated to alleviate this confusion.

6. Page 7, Methods, Con A and Fibronectin Treatment: 1) Why was the fibronectin treatment different from the collagen treatment? 2) Inform source of reagents. 3) Were treated cultures incubated for 24 hours before RNA isolation or incubated at different timepoints?

1) The authors apologise for the confusion raised in this excellent question. Indeed the fibronectin treatment was similar to that performed with the collagen coats. The fibronectin was allowed to coat the tissue culture plastic for 1 hour, followed by seeding of the cells. Treatments were performed for two timepoints, 24 hours and 48 hours followed by RNA extraction. The Methods, Collagen Coats and Methods, Con A and Fibronectin Treatments sections on Pages 5 and 6 have been modified accordingly.

2) Fibronectin was obtained from Promega as indicated.
3) The fibronectin treatments were performed at two timepoints, 24 hours and 48 hours followed by RNA extraction. The Methods, Collagen Coats and Fibronectin Treatments section on Pages 5 and 6 has been modified to reflect this clarification.

7. Page 7, Methods, Cytokine Treatments: 1) How was the specific concentration for each cytokine treatment media? 2) What does TGF-β mean? Pan or specific b1, b2 or b3? 3) Was there serum in the treatment media? 4) How was the effect of residual serum avoided?

1) The concentration of each of the cytokine treatments used was based on the cited publications 16-23.

2) A pan TGF-β obtained from Sigma was used for these studies. The Methods, Cytokine Treatments section on Page 6 has been altered to clarify this.

3) As indicated in the Methods section throughout, all cultures were maintained in 10% FCS during treatments.

4) As the serum level was maintained, residual serum was not relevant.

8. Page 10, Methods, RT-PCR: Were the RNA samples treated with RNase enzyme before RT reaction? How was DNA contamination controlled?

RNA samples were certainly not treated with RNase prior to reverse transcription. However, as part of the RNA extraction protocol referred to in the manuscript, a DNase digestion step was routinely performed to make sure there was no DNA contamination.

9. Page 12, Results: The authors start to abbreviate the primary fibroblast cultures as PMF (primary mouse fibroblasts). The English literature frequently uses MEF, as mouse embryonic fibroblasts. Furthermore, the use of the abbreviation is not consistent throughout the manuscript.

We thank the reviewer for noticing this oversight. As he correctly points out, the standard abbreviation is MEF. This abbreviation is now consistent throughout the manuscript with all references to these cells changed appropriately.

10. Results, Figures: 1) It is not clear in the text and in the legend figures if Figures 1-4 represent MEF or NIH3T3? 2) In Figures 1-4, it is very difficult to see the expression levels, since the lines frequently overlap. Furthermore, it is not clear if all genes are expressed in all timepoint or not. One way to improve that would be to convert the graphics into tables.

1) We thank the reviewer for this observation. Figures 1-6 have now been clarified and presented in tabulated form.

2) Again, we concur with the reviewer. The graphed data from Figures 1-6 is now tabulated and presented in Tables 2-8 clarify the data.

11. Page 12, Results: 1) Which are the basal levels of MMP-11 and MMP-14 expression in the fibroblast cell lines, if there are? 2) Since all experiments were made in triplicate, why don’t show average ± SEM? Furthermore, why don’t apply statistical analyses? 3) Although the authors do a great job using competitive RT-PCR for amplification control, no controls for the treatment effects are included. For example, no timepoint 0 (no treatment) is included and used as reference to comparative effect.

1) As the levels for MT1-MMP and MMP-11 were identical for timepoint 0 and the 3 Hour timepoint, the data is presented beginning with the 3 Hour timepoint.

2) As stated above, the Figures have now been tabulated to help clarify this data, included in the Tables are the SEM values.

3) As clarified above, the data obtained at timepoint 0 and the 3 hour timepoint was identical, in an effort to conserve space, only the 3 Hour timepoint has been presented.

12. Pages 12 and 13, Results, Induction Treatments: 1) The authors stated that MMP-14 expression was not affected by any cytokine. For example, comparing the timepoints 3 and 12 hours in Figure 1, an
increase higher than 3-fold is observed for several cytokines. Minor variations? 2) Did other housekeeping genes give the same results as 18s rRNA? 3) The authors state: “In our opinion, MT1-MMP expression should be considered unaffected by IL-1β, IL-2 and fibronectin treatments during this time schedule”. This conclusion can no be raised without additional controls and experiments. Furthermore, the results disagree to some extent with previously published data. In association with the expression studies, specific assays to measure amount of protein and/or activity are necessary.

1) Although there was some initial increase in MMP-14 gene expression at earlier timepoints, the overall target expression pattern, when normalised to the control demonstrated no significant affect by the cytokine treatments used. The Results, Induction Treatments, MT1-MMP: 3, 6, 12 and 24 Hour Treatments section on Page 11 has been altered to reflect this.

2) We have previously demonstrated the unsuitability of β-actin for use as a positive control and “housekeeping gene” for these type of assays (Selvey et al 192001). That study also highlighted the suitability of 18S rRNA. As a result 18S rRNA was used in all subsequent experiments.

3) The advantage of the competitive quantitative RT-PCR procedure within this study is that it provides several layers of internal controls to quantify gene expression levels. Specifically, primer design of both target and control primers along with generation of cDNA competitive templates to eliminate spurious and fluctuating data, and the capillary electrophoresis and Genescan analysis. As such, the data presented represent quantitive expression data only as influenced by the treatments performed. The authors agree that further data examining MMP protein levels and activity are warranted but are clearly another extensive further study.

13. Page 16, Discussion. If it is true that MMP-14 can be down regulated for some cytokines, how may this affect tumour progression?

As suggested earlier, the Discussion section has been modified slightly. Included in this the possible consequences of cytokines on MMP induction has been addressed. Specifically, MT1-MMP may represent a more important target for the cytokines in terms of tumour progression because of its interaction with MMP-2. This has now been postulated in the Discussion section on Page 16.