Author's response to reviews

Title: Myoblast sensitivity and fibroblast insensitivity to osteogenic conversion by BMP-2 correlates with the expression of Bmpr-1a

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Author's response to reviews: see over
The Editor,
BMC Musculoskeletal Disorders,

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To the Editor:

Thank you for the positive response to our original paper, “Myoblast sensitivity and fibroblast insensitivity to osteogenic conversion by BMP-2 correlates with the expression of Bmpr-1a.”

We have endeavoured to address all of the comments and questions from the three reviewers and are pleased to submit an amended manuscript. This contains a number of minor revisions requested by the reviewers, including re-formatting of graphs (Figs 1-2), increased methodological detail and justification, and additional relevant discussion. These are summarised in detail in a Schedule of Changes.

We hope that editorial review will find our corrections satisfactory and that we can look forward to a rapid response.

Yours sincerely,

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Schedule of Changes

We would like to again thank the reviewers for their constructive comments. We have endeavoured to satisfy the majority of these revisions as described below:

**Reviewer 1:**

**Major Revisions:** None

**Minor Revisions:**

1. Typo in the first sentence of the Background.
   Fixed.

2. Wording of the last sentence of the 5th paragraph of the Background
   Reworded to improve clarity.

3. Clarify the decision to normalise QPCR data to GAPDH expression.

   We are not aware of any single “gold-standard” for normalisation that is completely static between all cell types and totally unresponsive to treatment with mitogenic compounds. The normalisation to GAPDH (or alternatively beta-actin) is an extremely common practice. In our procedure, we synthesize cDNA from total RNA using equivalent amounts of total RNA from spectroscopic measurement, and we have clarified this in the text.

   In our experience, the measurement of total RNA is less precise than normalising to a control gene and does not reflect differences in cDNA synthesis rates between samples.

4. Clarify the decision to normalise AP activity to cell viability data.

   The system we have used is published in the literature and is generally considered acceptable. Examples of peer-review papers that use this method include our own published results (Schindeler et al. *Biochem Biophys Res Commun.* 338(2):710-6, 2005) and other high-impact publications (Yu et al. *Proc Natl Acad Sci USA.* 101(31):11203-8, 2004; Mauney et al. *Tissue Eng.* 10(1-2):81-92, 2004)

   Furthermore, in additional samples we have directly stained for AP expression (data not shown), we have obtained a “total AP” value that is not normalised to cell number. These results broadly reflect our AP activity data, suggesting that our normalisation has not created spurious findings.

**Discretionary Revisions:**

1. Discuss differential mechanisms for regulating Bmpr-1a expression and BMP-2 activity in non-myogenic cell types.

   This is now more fully covered in the Discussion.

2. The text of the manuscript indicates that experiments were performed that show inhibition of myogenesis by BMP-2, but the data was not included.

   We have clarified that these findings belong to a previously published study, rather than our own work.

3. Inclusion of more detailed statistical information
As also desired by Reviewer 2, a detailed statistics section has been added to the Methods.

4. Alizarin Red Quantification
Alizarin Red staining was intended to qualitatively show the presence of mineralized matrix. Late stage osteogenic gene expression by QPCR (e.g. OCN) was intended as a quantitative measure.

5. Figure 3 is unnecessary as the result of the bioinformatics analysis is covered in the text. Also there seems to be a difference between the text and the figure.

We believe that Figure 3 schematically clarifies the findings and contains valuable positional data. Reviewer 1 is correct that there is an error in the text, and we have amended this.

6. The line graphs of PCR data suggest trends in the interpolated regions that are not supported by actual data. Clustered bar graphs or straight line graphs are suggested.

As recommended, we have changed the graphs to straight line graphs to rather than interpolated curves (bar graphs are far more challenging to digest).

7. Work by AJ Garcia et al. suggests that cell type dependent effects of Runx2/Cbfa1 in inducing osteogenesis may suggest a more complex mechanism, and could be cited.

We have made reference to this work in the Discussion.

Reviewer 2:
Major Revisions:

1. Explain the decision to use bone marrow cells rather than calvaria-derived cells?
The choice of using bone marrow osteoprogenitors rather than calvarial derived cells was a deliberate one. Calvarial cultures reflect mature cells taken from bone, whereas the cells isolated from the marrow (often termed mesenchymal stem cells) are immature cells with recognised osteogenic potential.

The goal of this paper was to compare myogenic progenitors with other immature cell types that may be present in an orthopaedic context (e.g. marrow osteoprogenitors or fibroblasts). Mature osteoblasts such as those present in calvarial cultures are not present in significant numbers at the onset of an orthopaedic injury. We have attempted to clarify this further in the Background and Methods sections.

2. Cells were infected with a MyoD-expressing lentiviral vector and treated with BMP. Why was a BMP-expressing or BMPR1A-expressing lentiviral vector used?
BMPs are secreted proteins that act upon cell surface receptors (BMPRs) that can robustly induce osteogenic differentiation in vitro and in vivo. We applied recombinant BMP protein directly to cells as a clinically relevant model of BMP-induced differentiation.

In contrast, MyoD is an intracellular (nuclear) transcription factor that would not induce myogenic differentiation as an exogenously added protein. Initially, we attempted to use a MyoD expression plasmid similar to that used by Komaki et al. (Journal of Cell Science, 117:1457-68, 2004), but transfection rates were sub-optimal even with a range of transfection reagents (data not shown). Thus we adopted a lentiviral system that was able to transduce cells at high efficiency.
At no stage were cells treated with BMPR-1A (protein or virus), as this was an outcome measure rather than an intervention. We plan to undertake future experiments aimed at reducing BMPR-1A expression similar to that done by Lavery et al. (*Journal of Biological Chemistry*, 283:20948-58, 2008), but these are not within the scope of this study.

3. The authors described only in vitro study; it is necessary to describe the results of an ex vivo study using these treated cells.

We are a bit confused by this comment – an *in vitro* study and an *ex vivo* study are generally considered equivalent (“in glass” vs “out of the body”). We tend to use the *ex vivo* phrase to describe primary cell cultures, which were performed as part of this study in addition to classical *in vitro* cell lines. We welcome any editorial clarification as to this statement.

4. The details of statistical analysis were not described.

Statistical methods were present in the figure legends, but to improve clarity we have added a detailed statistics section to the Methods.

**Minor Revisions:**

1. Replot figs 1E and 2E as bar graphs because they are not time course studies.

These figures have been replotted as bar graphs.

**Reviewer 3:**

**General Comments:**

Dr Goumans positive remarks are welcome, and the general comments section contained several key issues we are able to clarify:

The NIH3T3 cell line was selected to represent a fibroblastic cell type rather than the alternative C3H10T1/2 line. Although C3H10T1/2 cells are often referred to as fibroblasts, they behave more like multipotent mesenchymal cells and can readily assume a variety of cell fates. In contrast, the NIH3T3 line behaves more like cells found within the fibrous tissue of orthopaedic injuries that exhibit fibrosis and poor bone repair.

The decision to analyse the BMPRs for myogenic binding sites was due to the observed correlation between myogenic gene expression and BMP sensitivity. We have amended this section to better define our reasoning as well as the importance of other receptors based on *in silico* analysis. Some of the final questions raised by Dr Groumans, such as the functional role of ALK3 in mediating the MyoD-induced sensitivity of fibroblasts are intriguing and represent issues that are the subject of further study.

**Minor Revisions:**

1. Please check the text carefully for mistakes

A number of typographical errors including those identified specifically by the reviewer have been changed.