Author's response to reviews

Title: Gene expression analysis in human osteoblasts exposed to dexamethasone identifies altered developmental pathways as putative drivers of osteoporosis

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Author's response to reviews:

Reviewer 1

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

1. The title of the manuscript is not appropriately chosen. Although putative dexamethasone responsive genes are found, the manuscript does not provide any functional data. Therefore, it can not be stated that de genes (or developmental pathways) are indeed drivers of osteoporosis. In the abstract the authors mention that they have used "an integrated genomics and computational biology to identify key genes and genes clusters whose differential expression drives osteoporosis". However, what they provide in this paper is a list of genes and gene clusters that might be involved in the regulation of dexamethasone-induced osteoporosis and again functional data are lacking to support this conclusion. Furthermore, since the dexamethasone treatment was only applied to osteoblasts it can not be excluded that at least part of the regulated genes, are involved in a general cellular response to dexamethasone treatment and do not represent an osteoblast-specific effect. The conclusions drawn in this paper should much more carefully be formulated.

Response
We thank the reviewer for this comment. We have modified the title of the manuscript as requested. However we would draw the reviewers attention to the last paragraph of the discussion "In aggregate the data presented herein lend further weight to the hypothesis that osteoporosis arises, at least in part, from alterations in the developmental control pathways in human osteoblasts. Further studies will aim to articulate the exact mechanism of dysregulation of these developmental genes, characterise the effect of altered expression on osteoblast biology with a view to further characterising these mediators as indices of disease activity, diagnostic markers and therapeutic targets in osteoporosis" which articulates that the date presented herein provide support to a hypothesis and that further experimentation, which is currently ongoing, is required to further describe the role of developmental processes in osteoporosis. Furthermore we have revised the statement in the abstract to read, "In this study we have used an integrated genomics profiling and computational biology based strategy to identify the key osteoblast genes and gene clusters whose expression is altered in response to dexamethasone exposure".

2. The exact experimental design is not clear. Do the cells receive fresh medium after o/n incubation on serum-free medium or is dexamethasone added to the cultures? If fresh medium is added to the cells appropriate controls are required in the microarray experiment (non-dexamethasone-treated samples of each time point).

Response
We thank the reviewer for this comment and are able to confirm that dexamethasone is added directly to the medium following overnight incubation in serum free medium. We have amended the appropriate methods section to clarify this.

3. A gene profiling study on dexamethasone-treated osteoblasts as a model for glucocorticoid-induced osteoporosis is not new. A similar study has been published by Leclerc et.al., 2004, J Mol Endocrinol, who studied dexamethasone-induced gene expression in the murine osteoblast cell line MC3T3. The authors...
should at least review their data with respect to the already published data.
Response
This point from the reviewer is duly noted. We have now included a discussion of this manuscript and included the reference. We would also like to point out that the study presented herein complements the existing work of Leclerc et al. We are of the opinion that the data presented in our manuscript may be of more utility as a primary human osteoblast model system is used, as opposed to transformed mouse osteoblast-like cells.

4. The mRNA expression levels in figure 4 are shown relative to control. However, the authors do not show the gene expression levels on similar time points in the absence of dexamethasone. For this reason, it can not be concluded that the responses are specifically triggered by dexamethasone. To strengthen the hypothesis that dexamethasone-induced expression modulation of development related genes may be associated with osteoporosis, the authors should include data showing that dexamethasone treatment indeed affects osteoblast differentiation in their experimental setup.
Response
We note the reviewers opinion on this point. We recognize that in long term exposures non dexamethasone associated changes may cloud the results of the expression studies. However we believe that in this case a single control as reference is appropriate, due to the short nature of the time exposure (maximum 4 hours). We would not expect significant baseline changes in gene expression over this short time period.

5. On page 8, it is mentioned that 106 genes were development associated genes. In table 1, a number of 39 developmental genes are shown as being up-regulated. So apparently, 67 developmental genes are down-regulated. On page 7, however, the authors state that activation of developmental pathways may underlie osteoblast activity in osteoporosis. Since more developmental genes are down-regulated, it may thus also be reasonable to conclude that inactivation of developmental pathways is at the basis of dexamethasone-induced osteoporosis. It would be of interest to include a table of down-regulated genes as well. Furthermore, based on KEGG pathway analysis, a distinction could be made between pathways that may be either activated, or inactivated by dexamethasone.
Response
We thank the reviewer for this point. We have now also included a table of development associated genes whose expression is decreased in response to dexamethasone exposure, and referred to same in the text (Table 2). Regarding actication or inactivation we would suggest that downregulation of genes, is not in itself, indicative of pathway inactivation. For example, downregulation of negative regulators of signaling pathways may actually enhance pathway activation.

6. The results of figure 4 should be compared with the microarray data. A comparison between table 1 and figure 4 suggests that a discrepancy between the results obtained by the two assays is present. Furthermore, the authors should indicate in the text that frizzled 2 and 7 are not present in table 1 since these genes are identified as down-regulated genes (see also note 5).
Response
We acknowledge this important point. Firstly we again note the inclusion of new table 2 showing downregulated genes in response to dexamethasone exposure. Secondly we accept the variation in quantification between Microarray and real Time PCR expression indices. This is a common issue in microarray studies and further supports the need for careful validation of microarray data. In this study we have validated four individual transcripts to ensure the reliability of the array predictions and to demonstrate the reproducibility of the findings.

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. The graphs in Figure 1 are not labeled, abbreviations are not explained. Figure 1 and Figure 2 do not contribute to a biological understanding of the effect of dexamethasone on osteoblasts.
Response
We acknowledge this point and have revised the figures in the paper.

2. In the Results section, some known effects of the Wnt pathway on bone are mentioned, but referencing is very poor or completely lacking.
Response
We acknowledge this point and have substantially added to the discussion and referencing.

3. The authors mention in the abstract that they identified 500 osteoblast genes. In the Results section it is mentioned that 31, 83, 130, and 300 genes are modulated at the different time points measured. It is not clear whether these are distinct genes, or if there is any overlap.
The abstract states approximately 500 genes were found to be significantly dysregulated. There is a degree of overlap between the gene lists found to be altered at the different time points.

4. The manuscript contains very many typographic errors. The usage of capital or lower case letters for gene names is not consistent and many incomplete or incorrect sentences are present in the manuscript.

Response
We acknowledge this point and have corrected these errors

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

1. In microarray data analysis, authors take the log ratio 0.6 of signal and control as a criterion to identify the differentially expressed (DE) genes. This criterion may be arbitrary for determination of DE genes. Authors maybe can explain it in detail or list the corresponding references.

Response
We thank the reviewer for this comment and are happy to address it. As can be readily appreciated the assigning of cut off points for which differential expression is significant or not is fraught with difficulties. We have now included a more complete description in the materials and methods section and added an additional reference to justify the use of SLR of 0.6. The included paragraph is "As each in vivo time-point was microarrayed in duplicate an average RMA value was computed. To ensure the average was statistically representative a t-test and p-value were generated. Only those genes with a p-value of d 0.01 were included in subsequent bioinformatic analysis. Thereafter, expression data for each time point was compared to control and a signal log ratio of 0.6 or greater (equivalent to a fold change in expression of 1.5 or greater) was taken to identify significant differential regulation [20]"

2. In this study, authors use the real time RT-PCR to confirm some DE genes. It is better to show and explain the results in the results section.

Response
We apologise that we do not completely understand the reviewer's point. All differential expression data is reported in the results section.

3. In results section, authors found a lot of apoptosis associated genes were relative to this study. However according to Panel A in Figure 3, more Oxidative stress associated genes are identified. The functions of these genes may be also important and authors can discuss them in this paper.

4. In Table 1, according to the criterion (0.6), some genes seems not to be DE genes, for example, CYR61, HLX1, SLIT3, EPAS1, BST2. Authors may elucidate the reason to select these genes.

Response
We acknowledge this point and thank the reviewer. Having identified development associated genes as being of importance using functional classification strategies we expanded these investigations to include all development associated genes. As this analysis was focused on development associated genes, the gene selection stringency parameters were relaxed, (SLI of 35 and a SLR GBP -0.4 and +0.4 in one or more timepoints compared to control (T1)). We have discussed this in the results section of the paper.

5. In discussion section, authors should discuss more details about the relationship between steroid and osteoporosis. How dexamethasone pulse treatment can stimulate osteoblast activation?

Response
The role of dexamethasone in osteoporosis has been discussed in more detail in the discussion section as per the reviewers request.

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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 authors should pay more attention to the grammatical errors, and some sentences are hard to read, such as:
Page 3, line 3
After aging and sex steroid deficiency, the therapeutic use of glucocorticoids is the most common cause.
Page3, line 4
Osteoporotic fractures are an important cause of morbidity and mortality
However increased, long term use of steroid is in no small way contributing to increased burden of osteoporosis globally.

Response
These sentences have been reformulated where appropriate.

Reviewer 3
This is a limited study of one human osteoblast cell line and the effects of dexamethasone on gene expression at 4 time points. There are duplicate Affymetrix microarray data for control and 4 time points, with the major conclusion that apoptotic pathways are increased and wnt pathways are selectively altered by glucocorticoids.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)
Need to include 24hr time point to see long term effects on expression and test at least a 1hr, 4hr and 24hr dataset treating the cells in the presence of serum with same concentration of dexamethasone. Redo the functional classification using the more up to date David program and present in a format with the Ease Score and genes that are in each significant class. You could make a Web link to the data analysis using the new David-Ease program. Run data using a statistical model such as limma or SAM with some values for modified t statistic or Baysian statistic or FDR.

Response
We thank the reviewer for these useful suggestions. Regarding the inclusion of cells treated in the presence of serum, we find this suggestion very interesting. Whilst our goal in this study has been to attempt to identify genes that are strictly dexamethasone responsive the impact of serum should also be considered. Due to the restricted time window available for completion of this review, we are unable to complete further microarray classification. Development associated genes have been classified using DAVID. Further details of statistical approach have been included in the 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)
Figure 1 is really unnecessary if correct normalization and limma analysis are carried out with list of statistically significant expression changes.

Figure 2 Panel A is not clear and not sure why showing clustering of the duplicate array and average data. Panel B can be presented in a form that shows the data output with the up and downregulate genes.

Figure 3 can be eliminated if David-Ease data shown in the correct format as suggested above. Why all the missing data in Panel B of Figure 3?

Response
The revised figures presented address these issues.

What is the role of long term elevated Dkk1 and Frz7. Is Wnt signaling suppressed by dexamethasone in this model? Please address.

Response
This has been discussed in more detail in the revised version of the manuscript.