Title: Silencing Dkk1 Expression Rescues Dexamethasone-Induced Suppression of Primary Human Osteoblast Differentiation

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Version: 2 Date: 16 June 2010

Author’s response to reviews: see over
Dear Dr Nguyen,

Firstly we wish to express our gratitude for the constructive review of our manuscript entitled ‘Silencing Dkk1 Expression Rescues Dexamethasone-Induced Suppression of Primary Human Osteoblast Function’ (MS: 1236928709370297).

As advised in your email (7th May 2010) we have revised this manuscript accordingly (corrections in grey) and resubmitted it for review. We feel that the comments made by the reviewers and the subsequent corrections have significantly improved the strength and clarity of the manuscript.

We will now address each of the reviewers’ comments in a point-by-point fashion in order to satisfy the editorial board of this manuscript’s suitability for publication. I acknowledge that there has been some disagreement among the reviewers and I hope that the revised version of this manuscript addresses these issues adequately.

**Reviewer #1 Comments:** (Feng-Sheng Wang)

*The reviewer has concerns whether the protocols for harvest of primary human osteoblast cultures from normal hip are approved by the IRB.*

The primary human osteoblasts used in our experiments were purchased commercially from Promocell (Cat.No. C-12720). These cells were obtained from normal human hip samples and processed at Promocell’s cell culture facility (Heidelberg, Germany). In brief, shortly after isolation, osteoblasts were cryopreserved at passage 2 (P2) using the serum-free freezing medium, Cryo-SFM. Quality control tests performed on our batch of primary human osteoblasts included cell morphology, adherence rate, and cell viability, in addition to the histochemical tests of alkaline phosphatase activity and bone mineralization. A detailed certificate of analysis was also provided for our batch. At our research facility, cells were cultured in Promocell’s proprietary Osteoblast Growth Medium (containing 10% FCS and antibiotics (100 IU/ml penicillin and 100µg/ml streptomycin) at 37°C, 5% CO₂.

Institutional board review was received by the Mater Misericordiae University Hospital Research Ethics Committee for this work under the project title ‘The identification of key genes and gene clusters underpinning osteoporosis’. This has now been included in the manuscript (Methods Section, paragraph 1).

*The concentration of dexamethasone used in this study is controversial. 10⁻⁸ M glucocorticoid is a physiological level for osteoblast cultures.*

This in vitro model of dexamethasone-induced osteoporosis involved primary human osteoblasts being exposed to 10⁻⁸ M dexamethasone. This concentration was utilised as a result of microarray analysis data from our group previously published looking at gene expression in primary human osteoblasts exposed to dexamethasone of the same concentration.² Our group used this concentration to validate our previous findings at a gene expression level and correlate osteoblast gene expression with osteoblast phenotypic changes. This concentration was found to significantly suppress both osteoblast differentiation and
Wnt/β-catenin signaling (Figures 1a, 1b) in our experiments. This dose has also been previously shown to significantly effect Dkk1 expression in human osteoblasts.

Hurson CJ, Butler JS, Keating DT, Murray DW, Sadlier DM, O'Byrne JM, Doran PP. Gene expression analysis in human osteoblasts exposed to dexamethasone identifies altered developmental pathways as putative drivers of osteoporosis. BMC Musculoskelet Disord 2007 Feb 12;8:12.


To prove the efficiency of Dkk1 RNA interference, they may need to provide Dkk1 immunoblotting of cell cultures or soluble Dkk1 levels in culture medium.

Quantitative RT-PCR was used to confirm gene knockdown, however, the efficiency of Dkk1 RNA interference was assessed when the Dkk1 concentration of cell supernatant was analysed using ELISA. A 72% reduction in Dkk1 concentration was seen in the cell supernatant of siRNA transfected cells relative to untransfected control cells. This represented a significant reduction when compared to both scrambled control and transfection control cells (Results, paragraph 7; Figure 4b).

Authors may need to provide additional evidence of TCF reporter activity and β-catenin translocation in Dkk1 siRNA-transfected cell cultures.

In order to confirm that knockdown of Dkk1 was inhibiting Wnt/β-catenin signaling in primary human osteoblasts, we firstly examined β-catenin trafficking using immunofluorescence analysis. Primary human osteoblasts were grown to confluency and transfected with siRNA targeting Dkk1 expression or scrambled control RNA. Scrambled control primary human osteoblasts demonstrated a strong perinuclear and intranuclear staining of β-catenin, representing activated Wnt/β-catenin signalling. siRNA transfected primary human osteoblasts demonstrated a reduction in intracellular staining for β-catenin, representing a significant inhibition of Wnt/β-catenin signaling.

These changes in intracellular β-catenin trafficking were accompanied by changes in Wnt/β-catenin signaling at a transcriptional level. Primary human osteoblasts transfected with Wnt-luciferase reporter construct pBAR and the control reporter pfuBAR, were subsequently treated with siRNA targeting Dkk1 expression or scrambled control RNA. A significant reduction in luciferase activity was observed in the siRNA transfected cells when compared to scrambled control cells (p<0.001; Student’s t-test) in the pBAR reporter cells, whilst the luciferase activity in the pfuBAR reporter cells remained unchanged during treatment. (Results, paragraph 8,9, Figures 5a, 5b,5c).

Many terms, including pro-osteoporotic agent, markers of osteoblast function and markers of bone turnover, in the subtitle and text are inappropriate. It will be more pertinent to re-term ‘osteoblast function’ and ‘bone turnover’ as ‘osteoblast differentiation’.

These terms have been corrected throughout the revised manuscript, including a title change.
In the Discussion, most part of text mentions the previous experimental findings of other groups. Authors need to pertinently re-write the text and discuss the originality of their experimental findings in this study.

Primary human osteoblasts exposed to dexamethasone in vitro display a reduction in alkaline phosphatase activity over a 72hr time course when compared to control osteoblasts. These phenotypic changes are driven by a Wnt/β-catenin-dependent mechanism as clearly demonstrated by both alterations in β-catenin trafficking and changes in TCF/LEF-mediated transcription. Silencing Dkk1 expression rescues dexamethasone-induced suppression of primary human osteoblast function. Increased alkaline phosphatase activity was displayed by primary human osteoblasts treated with siRNA targeting Dkk1 expression when compared to scrambled control cells over a 72h time course of dexamethasone exposure. (Discussion, paragraphs 4,5)

**Reviewer #2 Comments:** (Ya-Wei Qiang)

There are concerns about the experimental design for determining the effect of Dex on Osteoblast differentiation and the role of silencing Dkk1 expression in this process. The time for inducing osteoblast differentiation and then measured by ALP and Alizarin Red-S (ARS) analysis to determine the role of knockdown Dkk1 expression in Dex-impaired to osteogenic differentiation in this study is much shorter compared with that of well-established ALP and ARS, which has been utilized by many studies to determine osteoblast differentiation in both mouse and human in vitro cells model. Based on these studies and our experience to determine the osteogenesis differentiation, it takes 12 to 21 days for the positive in ARS and at least 72 hours for ALP activity be detected when human mesenchymal stem cells are cultured in medium containing osteoblastic differentiation media. In this study, the authors measured the ALP activity and calcium deposit using ARS analysis starting from 4 hours and the long time was at 48 hours after treatment of the cell with Dex.

As also highlighted by Reviewer 3, we have now re-examined the effects of dexamethasone treatment on ALP activity in primary human osteoblasts over a new prolonged time course of extended to 72h (the 4h time point has been removed). We have also examined the effects of silencing Dkk1 on ALP activity in osteoblasts exposed to dexamethasone over this new prolonged time course of 72h. The original figures dealing with Alizarin Red staining have been removed from the revised manuscript eliminating the source of conflict which the reviewers have expressed (Results, paragraphs 2,10, Figures 1, 6).

It is not clear if silencing Dkk1 expression had functional suppression in Wnt/beta-catenin function in the studies. The β-catenin measured by immunofluorescent staining analysis for β-catenin and TCF transcriptional activity by luciferase report assays should be done after silencing Dkk1.

In order to confirm that knockdown of Dkk1 was inhibiting Wnt/β-catenin signaling in primary human osteoblasts, we firstly examined β-catenin trafficking using immunofluorescence analysis. Primary human osteoblasts were grown to confluency and transfected with siRNA targeting Dkk1 expression or scrambled control RNA. Scrambled control primary human osteoblasts demonstrated a strong perinuclear and intranuclear staining of β-catenin, representing activated Wnt/β-catenin signalling. siRNA transfected primary human osteoblasts demonstrated a reduction in intracellular staining for β-catenin, representing a significant inhibition of Wnt/β-catenin signaling.
These changes in intracellular β-catenin trafficking were accompanied by changes in Wnt/β-catenin signaling at a transcriptional level. Primary human osteoblasts transfected with Wnt-luciferase reporter construct pBAR and the control reporter pfuBAR, were subsequently treated with siRNA targeting Dkk1 expression or scrambled control RNA. A significant reduction in luciferase activity was observed in the siRNA transfected cells when compared to scrambled control cells (p<0.001; Student’s t-test) in the pBAR reporter cells, whilst the luciferase activity in the pfuBAR reporter cells remained unchanged during treatment. (Results, paragraph 9, 10, Figures 5a, 5b,5c).

In discussion, the author should summarize the novel findings in the study.

Primary human osteoblasts exposed to dexamethasone in vitro display a reduction in alkaline phosphatase activity over a 72h time course when compared to control osteoblasts. These phenotypic changes are driven by a Wnt/β-catenin-dependent mechanism as clearly demonstrated by both alterations in β-catenin trafficking and changes in TCF/LEF-mediated transcription. Silencing Dkk1 expression rescues dexamethasone-induced suppression of primary human osteoblast function. Increased alkaline phosphatase activity was displayed by primary human osteoblasts treated with siRNA targeting Dkk1 expression when compared to scrambled control cells over a 72h time course of dexamethasone exposure. (Discussion, paragraphs 4,5)

The protein level of Dkk1 should be measured after silencing Dkk1 expression in human osteoblast cells. This is now easy, since there is a commercial available kit (R & D system).

Quantitative RT-PCR was used to confirm gene knockdown, however, the efficiency of Dkk1 RNA interference was assessed when the Dkk1 concentration of cell supernatant was analysed using ELISA. A 72% reduction in Dkk1 concentration was seen in the cell supernatant of siRNA transfected cells relative to untransfected control cells. This represented a significant reduction when compared to both scrambled control and transfection control cells (Results, paragraph 8; Figure 4b).

In my printed MS, all figures missed labelled numbers (such as figure 1, 2.). The author should label them in order.

This has been corrected.

Reviewer #3 Comments: (John Shaughnessy)

Since the experiments design for determining the Dex on osteoblastic differentiation is different from standard methods (the exposure to Dex was too short to determine the ALP activity and RSA), the author should prolong the time for ALP to at least 72 hours or longer or, for ARS, at least 2 or 3 weeks.

As also highlighted by Reviewer 2, we have now re-examined the effects of dexamethasone treatment on ALP activity in primary human osteoblasts over a new prolonged time course of extended to 72h (the 4h time point has been removed). We have also examined the effects of silencing Dkk1 on ALP activity in osteoblasts exposed to dexamethasone over this new prolonged time course of 72h. The original figures dealing with Alizarin Red staining have
been removed from the revised manuscript eliminating the source of conflict which the
reviewers have expressed (Results, paragraphs 2,10, Figures 1, 6).

Experiments should be done to confirm that is silencing of Dkk1 has an effect on Wnt
signaling (TCF transcriptional activity measured by luciferase report assay and β-catenin
protein alteration).

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primary human osteoblasts, we firstly examined β-catenin trafficking using
immunofluorescence analysis. Primary human osteoblasts were grown to confluency and
transfected with siRNA targeting Dkk1 expression or scrambled control RNA. Scrambled
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reduction in luciferase activity was observed in the siRNA transfected cells when compared
to scrambled control cells (p<0.001; Student’s t-test) in the pBAR reporter cells, whilst the
luciferase activity in the pfuBAR reporter cells remained unchanged during treatment.
(Results, paragraph 9, 10, Figures 5a, 5b.5c).

Editorial Requests:

Please clarify whether ethical approval was required for this study. Please put a statement to
this effect into the Methods section of the manuscript.

Institutional board review was received by the Mater Misericordiae University Hospital
Research Ethics Committee for this work under the project title ‘The identification of key
genes and gene clusters underpinning osteoporosis’. This has now been included in the
manuscript (Methods Section, paragraph 1).

We recommend that you copyedit the paper to improve the style of written English.

We have significantly corrected this paper and feel that the style of English of the revised
submission is of a high standard and suitable for publication.

We hope that the aforementioned corrections to our original manuscript have sufficiently
satisfied the editorial board of the BMC Musculoskeletal Disorders of this manuscript’s
suitability for publication.

If you have any further queries regarding this manuscript do not hesitate to contact me.

Kind Regards,

Dr. Joseph S. Butler