Author's response to reviews

Title: The effects of low dose X-irradiation on osteoblastic MC3T3-E1 Cells in Vitro

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Version: 2 Date: 17 April 2012

Author's response to reviews: see over
Reviewer's report
Title: The Effects of Low-dose X-irradiation on Osteoblast-like Cells in Vitro
Version: 1 Date: 16 January 2012
Reviewer: David Monroe
Reviewer's report:
Overview of Manuscript: This manuscript describes the effects of low dose X-irradiation on the bone-forming activities of osteoblasts using the MC3T3-E1 cell model. Overall this is a satisfactory manuscript, however a few issues need to be clarified (see below):

Major Compulsory Revisions:
- The quantitation of the RT-PCR and Western blots in Figures 6 and 7 need to include statistical analysis (p values).
  Statistical analysis (p values) has been added to Figure 6 and Figure 7. Meanwhile, semi-quantitative RT-PCR has been replaced by real time RT-PCR. See Figure 6 and Figure 7.

Minor Essential Revisions:
- There are a number of grammatical (subject/verb agreement, tense, pluralization, punctuation) and spelling mistakes scattered throughout the manuscript that need to be edited for proper English usage.
  A number of grammatical and spelling mistakes have been modified throughout the whole manuscript.

Discretionary Revisions:
- Figures 2 and 3 display completely negative data and do not add to the ideas presented in the manuscript in any significant way and just take up space. I would suggest removing these figures and referencing as "data not shown" in the Results section.
  Taking all the reviewer’s comments into consideration, Figure 2 and Figure 3 were modified, data of cell cycle was removed. See Figure 2-3.

Level of interest: An article of limited interest

Quality of written English: Needs some language corrections before being published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: I declare that I have no competing interests
Reviewer's report
Title: The Effects of Low-dose X-irradiation on Osteoblast-like Cells in Vitro
Version: 1 Date: 14 January 2012
Reviewer: John Hawse

Reviewer's report:
This manuscript describes the effects of low dose irradiation on osteoblast cells in vitro. The authors have found that low dose irradiation decreases proliferation rates in a dose dependent manner 5-6 days following exposure but does not alter the cell cycle profile or result in changes in apoptosis. However, low dose irradiation was shown to increase alkaline phosphatase positive cells and increase mineralization rates of MC3T3 cells. This was accompanied by significant increases in the expression of osteocalcin, Cbfa1 and OPG with a concomitant decrease in RankL expression levels. While this manuscript expands upon previous studies, provides insight into the effects of low dose irradiation on osteoblasts and addresses a clinically relevant subject, a number of revisions are necessary prior to publication.

Major Compulsory Revisions:
1). The authors state both in the introduction and in the discussion that previous reports have demonstrated that moderate to high doses of irradiation negatively affect osteoblast proliferation and differentiation and acknowledge that their present results are in contrasts to these published reports. The authors also state that identifying the range of irradiation which begins to negatively impact osteoblast cell populations is necessary. However, they do not provide any data to show that increasing doses of irradiation do negatively affect osteoblasts. For these reasons, the authors should add a few more increased doses of irradiation to the experiments provided in this manuscript to address these important issues and to confirm that low dose irradiation is actually beneficial.

This revised manuscript contained the dose of 2 Gy, which resulted in significant decrease of cell proliferation measured by MTT and BrdU. The results were consistent with previous reports mentioned in the discussion. This confirmed the doses used in the present study (0-1Gy) were actually beneficial. See Figure 1 and Figure 2.

2). It is not apparent, nor explained, why decreased proliferation was observed at day 5 and 6 following irradiation. Since no changes in the cell cycle or apoptosis levels were observed, these data do not make sense. Typically, if cells are not dying, it would be expected that the curve would eventually plateau, but not decrease. This issue should be explained.

We repeated the experiment of MTT and Brdu once again, and added the apoptosis on day 5-7 after irradiation (i.e. on day 6-8 after been seeded). In MTT and BrdU assay, cells were plated at a density of $1 \times 10^3$/well, instead of 3
$10^3$ well, and the results were similar to the original results. Taking all the reviewer’s comments into consideration, Figure 2 and Figure 3 were modified, data of cell cycle was removed. Formerly, we meant that no changes of apoptosis were observed between different doses of radiation. Here we explained the decreased proliferation might be associated with increased cell apoptosis and initiation of osteoblast differentiation. See Line 1-7 on Page 11, as well as Figure 1-3.

3). With regard to the above comment, if the decrease in proliferation is real, a possible explanation would be increased cell death. However, the authors did not examine the levels of apoptosis at days 5 and 6 when these decreases were observed. Instead, they only looked at days 1-3. The authors should repeat the BrdU and apoptosis assays on days 4-6 to match their proliferation data. BrdU and apoptosis assays have been added to match the proliferation data. See Figure 2 and Figure 3.

Minor Essential Revisions:
1). The composition of the cell lysis buffer used for protein extraction should be described in the methods section. The composition of the cell lysis buffer used for protein extraction has been added. See Line 4-5 on Page 8.

2). Why were the protein levels of OPG and RankL not analyzed as was done for OCN and Cbfa1? This data would make a nice addition to confirm the gene expression results. OPG/RANKL system was mainly used to investigate the association between osteoblasts and osteoclasts. Here we mainly focused on osteoblasts. As a result, the datas about OPG/RANKL system were removed.

3). There are numerous grammatical errors throughout the manuscript that need to be addressed prior to publication. A number of grammatical and spelling mistakes have been modified throughout the whole manuscript.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Not suitable for publication unless extensively edited

**Statistical review:** No, the manuscript does not need to be seen by a
statistician.

**Declaration of competing interests:**
I declare that I have no competing interests
Reviewer's report
Title: The Effects of Low-dose X-irradiation on Osteoblast-like Cells in Vitro
Version: 1 Date: 23 January 2012
Reviewer: Lexie Holliday

Reviewer's report:
Major Compulsory changes
1. In my view the author's need to make more clear the rationale of the study:
To use low dose radiation to enhance bone healing clinically? To study the biology of the effects of radiation of on cells? Why is it important to do these studies?
The Background has been re-written. The present study was aimed to investigate the molecular mechanism of promoting fracture mineralization by radiation, through studying the biological effects of radiation on osteoblasts in this experiment. See Background.

2. The author's suggest that low dose radiation increase alk phos activity, bone nodule formation and change gene expression but there are no efforts to understand the underlying mechanism. How does low dose radiation cause these effects?
There were limited studies on the effects of LDI on the healing and remodeling of bone tissues. What we done was preliminary, Cbfa1 was one of the important factors involved in the mechanism, which could act as a transcriptional factor and bind with certain cis-acting elements of OCN genes to further enhance their transcriptional activities. Much more studies are needed to address this question.

3. The alk phos assay was not quantitative.
The quantitative ALP activity has been added. See Figure 4.

4. A crucial finding in this study was that low dose radiation induced changes in gene expression, but these changes were not measured by a qualitative technique, ie Real Time PCR, competitive RT-PCR or Northerns. The approach used is not quantitative.
Semi-Quantitative RT-PCR has been replaced by real time RT-PCR. See Figure 6.

Minor discretionary revisions
1. It would strengthen the report if primary osteoblasts were also tested. There is no guarantee that the effects of low dose radiation will be the same on them as the cell line. This is particularly important given that there are apparent discrepancies in the literature, as pointed out in the discussion, between studies using primary cells versus cell lines.
As multipotential cells, mesenchymal stem cells (MSCs) could be induced into
osteoblasts and had been long taken as important subjects of research. Among studies of moderate and high dose irradiation, some showed that radiation mainly suppressed the proliferation or cell cycle progression, while some showed that only the process of differentiation was suppressed, as well as some showed that proliferation and differentiation of MSCs were both suppressed. MSCs are heterogeneous in differentiation potential and comprise both progenitors and relative mature cells. These controversial conclusions may be associated with MSCs themselves besides the diversity of radiation dose and research models. See Line 10-18 on Page 12. Of course, we are aware of the limitations of the study. Much more studies are needed to address this question.

Level of interest: An article of limited interest

Quality of written English: Needs some language corrections before being Published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: I do not have any competing interests.