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October 1, 2015

In Reply Refer To: 691-151

Dr. Fang-Fang Ji  
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Dear Dr. Ji:

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Thank you for reviewing our manuscript. We appreciate the helpful comments from the reviewers and have updated and improved the manuscript according to the suggestions of the reviewers:

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**1) Reviewer 1 comments:**

**"In their manuscript "Induction of CXC Chemokines in Human Mesenchymal Stem Cells by Stimulation with Secreted Frizzled-Related Proteins through Non-Canonical Wnt Signaling" Bischoff et al. investigate how MSC respond to canonical and non-canonical Wnt signaling. They show that non-canonical Wnt5a protein and frizzled-related proteins (sFRPs) that are known inhibitors of both canonical and non-canonical Wnt signaling stimulate the p44/42 ERK and phospholipase C pathways operating through the non-canonical frizzled (Fzd) receptors 2 and 5 in MSC. A number of published reports had previously shown that SFRP1 is not only a suppressor but also an activator of wnt signaling, which somewhat limits the novelty of the study. The authors provide evidence that this signaling results in elevated expression of CXCL5 and CXCL8 by MSC and speculate that these chemokines contribute to blood vessel formation during osteogenesis in bone repair.**

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**The manuscript is well written and may have important implications for the field of MSC biology.**

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**However, not all conclusions are supported by results. The core of the manuscript is Wnt signaling. It is not clear what the chemokines regulated by it do, if anything. Cherry picking two chemokines out of all the soluble factors that could be regulated by the Wnt pathway seems unfounded. For instance, VEGF, the bona fide angiogenesis regulator, is induced by Wnt signaling <http://cancerres.aacrjournals.org/content/61/16/6050.long>"**

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**Specific questions:**

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**"What is the direct evidence that CXCL5 and CXCL8 functionally adds to bone formation through angiogenesis? Could previously**

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**reported bone development defect in CXCR-null mice be due to the lack of MSC proliferation / migration / differentiation defect? And could the vascular bone phenotype of these mice be a consequence, rather than the cause, of MSC function deficiency?"**

*These are good points. We are pursuing studies to address the role of the CXC chemokines in the bone formation be it an angiogenic role or a role in MSC functions. We have previously characterized the bone defect in the mCXCR2<sup>-/-</sup> mice and showed that one of the phenotypes is decreased blood vessel density in newly formed bone (cranial defect model) suggesting an angiogenic role; but realize that the chemokines may also have a role in proliferation and chemotaxis of the MSCs (or in the in vivo case inflammation which is needed for bone and wound repair in general). We have addressed some of these other functions in previous reports showing that conditioned medium from MSCs undergoing osteogenic differentiation can 1) stimulate MSC chemotaxis which can be prevented using a CXCR2 neutralizing antibody; and 2) stimulate endothelial tube formation in an in vitro angiogenesis assay.*

**"The study is incomplete without an assay measuring a functional change in MSC properties in the context of their speculated angiogenesis/osteogenesis implication. If CXCL5 and CXCL8 do promote angiogenesis, how do they do that? By signaling through CXCR1 and/or CXCR2? In what cells? Endothelial? Or MSC themselves? Are these receptors expressed on MSC? These are some of the questions that the authors should try to answer. Unless the data on CXCL5 and CXCL8 expression relevance are provided, the title and the conclusions should be restricted to the observations on Wnt signaling and the chemokine data – grouped in one figure and discussed with reservation."**

*As indicated in the Introduction, in humans, CXCL8 signals through both CXCR1 and CXCR2 whereas CXCL5 only signals through CXCR2. Angiogenesis has only been associated with CXCR2 signaling. Since CXCL5 is also upregulated and believed to signal only through CXCR2 this may suggest an angiogenic role. Both receptors are present on MSCs; although, levels exhibit variability between donors. The initial purpose of this study was to look at Wnt control of CXC chemokine expression in MSCs which are upregulated in response to treatment with Wnt5a. We unexpectedly found that sFRP1, which should inhibit canonical Wnt signaling, actually stimulated CXC chemokine expression. Our intent with this report is to characterize the expression of the CXC chemokine and the signaling involved with the upregulation as indicated in the title, not to demonstrate the functional role of this upregulation. We have revised portions of the text to reflect the purpose of the study and to match the title as suggested.*

**"There are too many figures with too little data. Group them in to panels."**

*We have combined some of the figures into panels as suggested and have rewritten the figure legends to reflect these changes.*

**"Secreted CXCL8 protein needs to be measured. VEGF and other**

**angiogenic factors should be measured along as controls.”**

*It should be noted that stimulation of angiogenesis by sFRP1 (Fz1A) independent of VEGF, bFGF2, or angiopoietin1 has been demonstrated (see Introduction and reference #55 in manuscript). Since CXCL5 is only known to signal through the CXCR2 (angiogenic receptor) not through CXCR1 we have only characterized protein secretion of CXCL5. As mentioned before, we are focusing on the upregulation of ELR<sup>+</sup> CXC chemokines and several potential roles in bone repair including a role in angiogenesis and therefore have not measured other known angiogenic factors such as VEGF.*

**“P44/42 p and PLC pathways are not a specific readout of Wnt signaling.”**

*As indicated in the Discussion, we chose to investigate the p44/42 pathway since it is known to be involved in expression of CXC chemokines; and the PLC pathway as it has been shown to stimulate osteogenesis thru non-canonical wnt signaling, is involved in G-protein coupled signal transduction pathways, and since the Fzd receptors (to which the sFRPs bind) are G-protein coupled receptors.*

**“The authors make it sound like there is only one murine CXCR receptor. However, mouse CXCR1 has also been cloned.”**

*We have revised the Introduction to mention the existence of the murine CXCR1.*

## **2) Reviewer 2 Comments:**

**“The paper found that CXC chemokine expression in hMSC is controlled in part by cFRPs signalling through non-canonical Wnt involving Fzd2/5 and the ERK and PLC pathways. The results are interesting.**

**As minor point it could be interesting to know if Authors have evaluated CXCL10. If not this point could be at least discussed.**

**See: Autoimmun Rev. 2014 Mar;13(3):272-80.”**

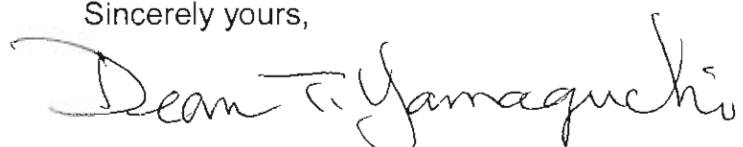
*Thank you for your suggestion. We have not looked specifically at CXCL10 as we have been focusing on the ELR<sup>+</sup> CXC chemokines which are induced upon osteogenic differentiation of MSCs, signal through CXCR2, and are angiogenic. CXCL10 is in a class of ELR<sup>+</sup> CXC chemokines that signal through the CXCR3 and are angiostatic which may have a role in remodeling of the bone after repair.*

## **3) Editorial Comments:**

*We have corrected the editorial changes indicated on the manuscript and have included the Biostatistics review and statement as requested. An audio core tip file has been uploaded as well as the manuscript in Microsoft word file (.doc) and figures in powerpoint (.ppt) format.*

*We are pleased that the manuscript has been improved and hope our responses to the reviewers' comments will be acceptable for publication in the World Journal of Stem Cells.*

Sincerely yours,

A handwritten signature in black ink that reads "Dean T. Yamaguchi". The signature is written in a cursive, flowing style. The first letter "D" is large and loops around. The "T" is simple and vertical. The "Yamaguchi" part is written with connected letters, ending in a small flourish.

Dean T. Yamaguchi, MD, PhD