The authors investigated ectopic formation of bone in muscle and other soft tissues, by a process known as heterotopic ossification. Based on the published studies that suggested the role of hedgehog signaling in heterotopic ossification, the authors studied the roles of some of the known inhibitors of hedgehog signaling on ossification in cultured cells.

Overall, the data presented do not support conclusions of the study and there are several issues the authors will need to address.

CRITIQUE

1. The overall rationale for the study is not clear. If the goal is to inhibit osteogenesis in therapeutic mesenchymal cells, such inhibition will also affect normal homeostasis in bone. If the goal is to learn more about the mechanisms of inhibition, the authors should have done some targeted blocking studies.

Author: We revised our statement: We explored the effect of very well known (ATO) and other less known (Lovastatin and Vitamin D3) Hh inhibitors on the regulation of osteogenesis in bone marrow derived MSC. (line 75-77)

The data suggest that cooperative or synergy-like effect for these three molecules combination is present, but the full assessment of drug combination synergism requires a completely new study.

We are fully aware and agree with a reviewer that inhibiting osteogenesis for therapeutic application systemically is not a feasible solution for HO but rather targeted and controlled drug release combination product (hydrogel, Depofoam or even 3D printed drug)

1. In general, the experimental protocols should be disclosed with much more detail.

Author: We thank our Reviewer for the suggestion, and improved our Experimental section. Chemicals preparation was moved up front in the section.

All mouse BMSC were cultured for 4 days in OM media and harvested at the same time for gene expression and AP staining. Duration of the treatment was the same for all replicate experiments.

All human BMSC were culture for 6 days in OM media and harvested at the same for gene expression and AP staining. Duration of the treatment was the same for all replicate experiments.

The above information was added in the method section (line 124) to improve clarity of our manuscript.

1. For quantitative data, the numbers of independent experiments and biological replicates need to be specified.

Author: Number of replicates was stated on a couple of places: line *147, 179, 214*

1. The experiments were done both with mouse and human mesenchymal stromal cells, which makes it impossible to compare experimental data within a single coherent set.

Author: we used human BMSC to test if Hh-mediated osteogenesis in murine progenitor cells is unique or rather conserved differentiation process.

1. Data in Figure 1 need to be better explained. The control group does not appear like normal healthy mesenchymal cells. All other groups also appear heterogeneous and unhealthy. In addition, it is not clear how the alkaline phosphatase expression was interpreted, as the expression varies from one cell to another and it is not obvious which structures are cells and which are just debris.

Author: As stated in the method section of the revised manuscript (line 92), we adopted a common approach in the bone field (ref. 8) for isolation and propagation of BMSC cells. Our method simply use the culture medium to select the plastic adherent MSCs . The majority of isolated BMSC cells were successfully transdifferentiated into osteoblasts using OM media suggesting that the majority of starting stem cells were of mesenchymal origin. All results were expressed and normalized based on cell counting (AP staining) or RNA amount (qRT PCR). Also, we did not observe any major toxic effect of the compounds used or cell death in control cells based on our microscopic (morphological) observations.

1. I also do not understand data in Figure 2. Is it expected that both the hedgehog and the alkaline phosphatase expressions decrease in parallel? Are there any statistically significant differences between the groups?

Author: Initially it was hypothesized that Gli1 expression will follow same trend as AP expression (since all three molecules are Hh inhibitors), but treatment with Vit D seems inconsistent. Vitamin D alone did not suppress the Hh pathway, at least not at *Gli1* readout level. This result suggests that alternative, Hh-independent, pathway such as vitamin D receptor (VDR) may possibly downregulate osteogenic activity in MSC cells treated with VitD. This was clarified on page (lines 234-241).

Statistical significance has been added in graphs in figures 2 and 4 per reviewer’s recommendation.

1. Data for human cells in Figure 3 and Figure 4 are also difficult to interpret. It should be specified how exactly were the alkaline phosphatase cells identified and counted, in how many sections and from how many experiments. The resulting decreases in alkaline phosphatase expression are note, but the differences are rather small – inhibition is only partial. It is unclear if this level of inhibition would prevent heterotypic ossification, and this very important question has not been studied. Alkaline phosphatase is an early, transient marker.

Author: We thank Reviewer for the comment. Our results clearly demonstrate a reproducible and significant inhibitory effect of ATO, Vid D and Lovastatin on both, AP activity and HO in vitro. We agree with the reviewer that additional studies are needed to confirm the efficacy of these agents on HO process *in vivo*. To the best of our knowledge, this is the first study which shows that combined therapy of Hh inhibitors may be beneficial against HO.

We are aware that AP is an early and exclusively transient marker, therefore for comparison purposes duration of the treatment was the same for all replicate experiments and AP measurement represent a snapshot in time, as described above.