REFEREE’S REPORT

Author: We would like to thank the Reviewer for a useful insight of our paper. We agree with most of the comments and addressed them accordingly.

# Does the manuscript contain enough significant original material? YES

The idea to test arsenic trioxide, lovastatin and vitamin D3, as inhibitors of mesenchymal stem cells into osteoblasts is original, and the advantage of this work.

# Is the manuscript clearly and concisely written? NO

In general, the manuscript is confusing. However, it is not necessary to rewrite it, but just to rearrange certain parts, in order to achieve a smoother text.

The first sentence of the abstract is absolutely unnecessary.

Author: The sentence is removed in our new version.

The second and the third paragraphs of Introduction are cumbersome, too many words, but little said. Innappropriate MSC differentiation into osteoblasts is the most important issue, and the sentence “Heterotopic ossification results from ectopic osteoblast differentiation of mesenchymal stem cells (MSC)” (lines 5-56) should be the highlight of this part. Innappropriate differentiation leads to desease, but controlled differentiation might be used for body implants (this is written in the text, but should be rearranged). A health issue of HO should be metioned (just short for information, not in details): how frequent is this disease/condition, when and why prophylaxis is indicated, treatment options (i.e. operation) problems.

Author: Sentence from 55-56 is moved up front, at the beginning of the second paragraph, line 45

Second paragraph is now rearranged. Frequency of the acquired form of HO is added, line 37. Frequency of the genetic form of HO is added, line 43.

“we explored the efficacy and mode of action”

The efficacy and mode of action are not truly investigated within this work; rather effect on two parameters was investigated.

Author: We agree with a reviewer and removed the “efficacy of action for these three compounds as this will be subject of future studies. The suggested wording is replaced in line 75.

Other notes in introduction section are indicated within the manuscript.

Author: We thank our reviewer for the valuable comments. The suggested changes in the Introduction were accepted in the new version of the manuscript.

The Experimental section contains several obscurities:

Line 89: “recombinant Mouse Prolactin protein 1ng/ml”: what is the purpose of adding?

Author: the reference (26) was added to the method as an addition of Prolactin was found recently to promote osteogenic differentiation properties of BMSC cells per Tsai TL et al.

Line 92: “hBMSC” ATCC ref number shoud be inidicated (probaly it is ATCC®PCS-500-012™), as it was for basal medium.

Author: Correct number for hBMSC was added, line 106

Cultivation and experimental conditions are not clear at all and are very confusing. Basal cultivation conditions for mouse and human BMSC differ, but upon reachin confluency, both type of cells are cultivated under the same conditions (in OM)? Were cells (beeing confluent when changed to OM) repassaged during OM replacing? For how long were cells (confluent) maintained in OM under treatment, 2, 3, 4 or 6 days? If experiments were repeated three times, duration of treatment must be the same in all experiments. Also, the cultivation and treatment conditions should be exactly the same for proten (AP) and gene expression (AP and Gli1) studies.

Author: Basal cultivation condition for mouse and human cell differ. Human cells were grown in the media suggested by the manufacturer (ATCC) and mouse cells in the media previously used (reference 8) to propagate mouse BMSC. Once either culture reaches confluence, they are switched to the OM media. Cells were not repassaged during OM replacing.

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. We didn’t see significant AP expression in human cells after four days only. 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. We thank the reviewer for these valuable suggestions.

It is also unclear why are different concentrations were used in single treatment (5 and 10 μM) and combination. There is no dose-dependent study for tested compounds, how are testing concentrations selected?

Author: The initial doses for ATO (10uM), Vit D (10uM) and Lovastatin (1uM) are selected based on previously validated and published *in vitro* data showing effective inhibition of Hh pathway at these inhibitor concentrations (references 8 and 18 in the revised manuscript). This important information is now included in revised version of the manuscript (line 164-166). We then tested if the similar inhibitory effect on osteogenesis is possible to achieve by a significant (half) reduction in inhibitors concentrations for increased efficacy. The data suggest that cooperative or synergy-like effect is present, but the full assessment of drug combination synergism requires a completely new study.

*The vehicles are different for all three tested compunds, did vehicle included in respective control?* Vehicles were used appropriately in the control (Ethanol for VitD and Lovastatin and PBS for ATO). This is now clarified in the method section (line 115).

Cytotoxity was not tested, is the effect reliable, or might be due to difference in cell number? AP was measures inside the cells, but not in cell conditioned media. Why not?

All results were expressed and normalized based on cell counting (AP staining) or RNA amount (qRT PCR), thus the observed effects clearly cannot be due to any difference in the cell number. Also, we did not observe any major toxic effect of the compounds used, based on our microscopic (morphological) observations. Per standard protocol in the field, we measured AP inside the cells as a proxy of osteogenic activity, a focus of our study. It is unclear what a change in the secreted AP levels would indicate, if any.

Line 149 “active form of Vitamin D”: There are two forms of vitamin D, D2 and D3. In both cases, active form is 1,25 dihydroxy form. Could BMSC hydroxylate D3 to 1,25 (OH)D3?

Author: We removed “active form” from line 149 for accuracy.

Line 160 “Alkaline phosphatase levels, reflecting osteogenic activity” AP is widely expressed, but not exclusively bone enzyme. Did AP activity tested before differentiation?

Author: AP is widely used in the bone field as an early marker for osteogenesis. AP was not tested before differentiation as we are capturing relative AP levels, compared to the control, untreated cells at the same time point.

Line 184: “significantly reduced (up to 90%) mRNA levels” Gene expression is expressed as relative, and it is not appropriate to convert it in percentage.

Author: Correction were made according to the suggestion above, throughout the text.

# Are the conclusions adequately supported by the data? NO

Discussion and conclusions are partly based on something that was not the subject of the study. Gli1 activation might be triggered by Hh pathway, but other pathways might be also involved. So, Gli1 suggest possible involvement of Hh signaling, but does not confirm it. Within this study, inhibitory effect of vitamin D3 on Gli1 expression is not shown, but on the contrary, vitamin D3 induced stimulation was observed. This was not commented in discussion (but on the contraty, vitamin D3 was assumed as Hh inhibitor, lines 245-246). The Gli1 was monitored ony on gene expression, but not at the protein level. Similarly, AP was measured within the cells, but not in cell conditioned media. Based on results showed here, the involvment oh Hh pathway in osteogenesis can not be confirmed, but only assumed.Combination of all three tested compounds does not provide complete cumulative effect, suggesting that different mechanisms of their action are involved.

Author: To the best of our knowledge Gli1 is the best reliable readout of an active Hh pathway. We are aware of recent findings that other pathways may crosstalk with Hh pathway, especially in cancer cells. Published data demonstrate that Hh dependent pathway is predominantly involved in stem cells differentiation, while in case of cancer both Hh dependent and Hh independent pathway exist. Lack of the Hh pathway inhibition by Vit D and the fact that the combination of all three tested compounds do not provide complete cumulative effect-this suggest that these inhibitors inhibit osteogenesis of MSC via both Hh-dependent and Hh-independent pathways. This is now discussed and added in discussion section of the revised manuscript (lane 235-239).

Conclusion (last sentence, lines 258-260): conclusion must be drawn from own results, but not based on literature data; Gli inhibitors for cancer treatment were not tested here, but might be used in similar study like this one.

Author: Arsenic trioxide, is one of the Gli inhibitors, with a commercial name “ Trisenox” approved for cancer treatment. That was added to the conclusion section, for a better clarity (line 267-268).

Dose-dependent study and cytotoxicity testing are missing.

Author: While initial efficacy and toxicity of these drugs alone or in combination were tested here for the first time, the full dose-dependent and toxicity experiments are envisioned for the sequel studies.

# Does the manuscript give appropriate credit to related recent publications? YES/NO

In some parts of the manuscript, references are missing (as indicated in the text).

Author: The missing references has been added (line 58, line 60, line 69, line 70)

# Are the references appropriate and free of important omissions? YES

Citing is appropriate.

# Is the length of the manuscript appropriate? YES

The length of the manuscript is optimal.

# Does the manuscript need condensation or extension? YES

*Rearrangement of some parts is needed (suggestions are given above and in the manuscript).* **Is the quality of the figures (including legends and axes labelling) satisfactory? NO** *Figure 1 legend should be removed from line 172 to line 166 (below figure).*

Author: Corrected as advised.

In general, labeling in the figures, should be exactly the same as in the text (for example figure 3, page 9: Cholecalciferol, VitD or VitD3?).

Author: Labelling is corrected to be uniform- Vitamin D3 or abbreviation VitD

Statistical signifficant differences are not labeled in graphs in figures 2 and 4; omitted or there is no signifficance?

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

# Are the nomenclature and units in accordance with SI? NO

Nomenclature should be uniformed within the text (for instance either vitamin D3 or VitD or cholecalciferol; Lovastatin or Lov). Also, the label in the figure must follow the text. Once shorcut is used, it shoud be further used through text.

Line 99 “10-4 M L-ascorbic acid 2-phosphate and 10 mM β-glycerol phosphate” This should be uniformed, either 10 -4 M and 10-2 M or 100 μM and 10 mM.

Author: We thank the reviewer for these suggestions. Nomenclature is now uniform throughout the text (lines 113 and 114)

Line 98 “100 U/mL” and Line 118 “100 U/mL” : should be mL in both cases. Similar differences are are also present within the text and should be corrected.

Author: Penicillin Streptomycin commercial mixture (100x) contains 10,000 units of penicillin (base) and 10,000 µg of streptomycin (base)/ml. Unites for Penicillin are U/ml and for Streptomycin are µg/ml.

# Are the English grammar and syntax satisfactory? YES

Some comments are indicated within the manuscript.