Dear Dr. Vineeta Bajaj,

On behalf of my coauthors I thank you for your and the reviewer’s helpful comments and valuable suggestions. We have addressed each comment individually both in the below list (blue font) and we highlighted the corresponding edits, additions, corrections throughout the text in our revised manuscript. We believe the suggested revisions have greatly improved our manuscript and hope that this revised form it is acceptable for publication in your journal.

**Editorial comment:** *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

Author’s response: Done.

Page 7, line 258: in vitro was changed to *in vitro*

Page 8, line 290:” in vitro and for in vivo” were changed to “*in vitro* and for *in vivo”*

**Editorial comment:** *Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”*

Author’s response: Done.

**Editorial comment:** *Keywords: Please provide at least 6 keywords or phrases.*

Author’s response: The keyword phrase “six well plate” was added.

**Editorial comment:** *Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.*

Author’s response: Done. We added the following to the introduction:

Other methods used *in vitro* to expose cells to EStim utilize salt bridges submerged in the culture medium which separate cells from metallic electrodes 5. The advantage of the former is that delivering EStim through salt bridges eliminates the introduction of chemical byproducts, from corrosion of metalic electrodes, that may be cytotoxic. Despite this advantage salt bridges are cumbersome to work with and the EStim they deliver differs from that delivered in *in vivo* models making it difficult to correlate results obtained using the two systems. In the case of setups that deliver EStim via metalic or carbon electrodes fixed inside the cell culture wells (reviewed in 6), these better simulate devices used *in vivo*, however these devices are difficult to clean/sterilize between uses and the number of cells that can be studied per experiment is limited. We designed the EStim chamber presented here specifically to address limitations of these other setups. While most of our experience using this EStim chamber has been with 2D and 3D cultures containing bone marrow- and adipose tissue- derived MSC 3,4, a major benefit of this chamber is that it is versatile and with relatively minor changes can be adapted to study other cell types under a variety of different conditions.

**Editorial comment:** *Please remove commercial language from the manuscript: GlutaMax.*

Author’s response: Done.

**Editorial comment:** *Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol.*

Author’s response: We did so in the Protocol and included the following corrections:

Section 1.5.1: “At this point LEDs can be added” was changed to “Add LEDs”

Section 1.7.1: The sentence “Silicone adhesive coating can be used to improve the bonding of the 2 lids (optional)” was changed to “To improve bonding of the 2 lids use silicone adhesive coating (optional)”.

**Section 1.8.1: “**load output should be at 2.5 V” was changed to “ set load output at 2.5 V”

**Editorial comment:** *Any text that cannot be written in the imperative tense may be added as a “Note.”*

Author’s response: “Note” was added at the end of step 1.8.1

**Editorial comment:** *Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly, and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.*

Author’s response: Safety procedures and use of the hood were added in the protocol as follows:

Section 2.2: “under sterile conditions in a laminar flow hood “

**Section 2.4: “**Note: perform all operations except centrifugation and incubation under steril conditions In a laminar flow hood.”

Section 3.2: “In a laminar flow hood”

Section 3.6: “under sterile conditions”

**Editorial comment:** *1.8: Please split into two steps.*

Author’s response: Section 1.8 was divided into 2 by adding a subsection: “1.8.1”.

**Editorial comment:** *2.6: Please specify incubation conditions.*

Author’s response: Specifications were added in section 2.6“incubate at 37 °C, 5 % CO2, 5 % O2”.

**Editorial comment:** *Figure 1: Please include a space between numbers and their corresponding units (20 mm, 25 mm, etc.)*

Author’s response: Done.

**Editorial comment:** *Table of Equipment and Materials: Please remove trademark (™) and registered (®) symbols. Please sort the items in alphabetical order according to the Name of Material/ Equipment.*

Author’s response: Done.  
  
**Reviewer #1:**  
  
**Reviewers' comments:** *The authors carried out the study on effect of EStim in influencing osteogenic differentiation of MSCs by setting up a ES chamber and applying 100 mV/mm on cells (seeded on TCPS) 1 h per day for 0-7 days. The experimental design is primary with limited novelty and incomplete characterizations. Although JoVE is a methods journal that novelty is not so important, some basic requirements to clarify the topic are still necessary.*Author’s response: In preparing this manuscript the authors tried to adhere to JoVe journal’s main focus “text protocols which facilitate scientific reproducibility and productivity”. In doing so we were forced to leave out some details, however we believe/hope that our descriptions are of sufficient detail to “facilitate scientific reproducibility and productivity” for readers. For the interested reader we provide references to our previous publications (Mobini et al. 2017; Eischen-Loges et al. 2018; Leppik et al. 2018) in which we describe the materials and methods in great detail.

**Reviewers' comments:** *In the Introduction, the authors declare that "an optimal regimen for maximizing EStim's pro-oeteogenic effect has yet to be defined" is not true. Please refer to: Siqi Zhu, et al., Time-dependent effect of electrical stimulation on osteogenic differentiation of bone mesenchymal stromal cells cultured on conductive nanofibers, J Biomed Mater Res A: 105A: 3369-3383, 2017..*

Author’s response: In the citied paper (Zhu et al. 2017) the authors divided osteogenic differentiation into 3 stages and investigated at which of these EStim had maximum effect on osteogenesis. Accordingly, these experiments did not define *"an optimal regimen for maximizing EStim's pro-oeteogenic effect”.* The EStim chamber we describe in the present “methods” paper makes it possible to test different EStim dosages and regimens so that readers can determine the optimal regimen for the different type of cells s/he is studying.

**Reviewers' comments:** *The authors describe their ES setting up in a detailed way for reproduction, however, can the authors put some words on explaining any special design or feature in your design to show advantages over devices used in other reports?*

Author’s response: Done. We edited the discussion section of our manuscript and formulated more specifically the advantages:

~~Our EStim chamber makes it possible to simultaneously deliver EStim to 6 individual wells seeded with cells, it is reusable, and it is simple to clean/sterilize and maintain between multiple experiments. “~~The EStim setup does not require special equipment/knowledge and can be performed in a standard laboratory by junior researchers. Cells are cultured in standard 6-well plates and can be used after EStim treatment in other protocols or in vivo. Electrodes fixed on the lid of the 6-well plate make it easy to clean and sterilize the device between experiments and to re-use it. Simultaneous stimulation of cells in 6 wells provides ample material for analysis and reproducibility.”

**Reviewers' comments:** *How did the authors determine the 100 mV/mm, 1 h / day being proper for cell proliferation and differentiation? Under the present EStim parameters, it turns out to be 7 days being the strongest in promoting osteogenic differentiation. If the EStim parameters are changed, will it still be 7 days? More investigation on this issue is necessary and required to draw the conclusion.*

Author’s response: We determined 100 mV/mm, 1 hr/day to be the optimal stimulation dosage and regimen in studies in which we exposed cells to a variety of higher (150 mV/mm) and lower (50 mV/mm) voltages which we found to be toxic and non-effective, respectively (Mobini et al. 2016; Mobini et al. 2017). In addition, in separate experiments we tested different treatment regimens and found 7 days 1hr/day EStim to be optimal (Eischen-Loges, M, et al. 2018). This experience and the corresponding references are cited in the present paper.

**Reviewers' comments:** *Cell viability under continuous EStim should be evaluated.*

Author’s response: Yes, we performed cell viability tests in previous studies in which we applied continuous EStim (Mobini et al. 2017; Eischen-Loges et al. 2018).   
**Reviewers' comments:** *To culture the cells for differentiation study, what kind of medium is used? The normal culture medium, or osteoinductive medium? Please clarify.*

Author’s response: We use “osteoinductive” medium, as described in the protocol in Section 2.6.

**Reviewers' comments:** *…the effects of EStim on cell activities are quite limited if the cells are cultured on non-conductive substrates. Therefore, conductive substrates are commonly applied when cells are investigated under EStims. Therefore, the authors should discuss this point in setting up their device that how conductive substrates will be fixed in the chamber.*

Author’s response: While we have not used conductive substrates in our studies, they can certainly be studied in the EStim chamber we present in our paper. Text addressing this point was added to the discussion: ” With minimal modifications the EStim chamber can be used with 3D ceramic scaffold material 4 or conductive substrates 11, by simply placing the materials seeded with cells between the electrodes”.  
  
**Reviewer #2:**  
  
**Reviewers' comments:** *In figure 3, the time at harvesting cells for analysis should be clearly mentioned in figure legends.*Author’s response: We added the evaluation time points to the figure legend: Osteogenic marker gene expression (measured with RT-qPCR at days 3, 7 and 14 of culture) in cells treated with EStim for 3, 7, and 14 days, or non-treated.

Also, the word “day” was added to the abscissa in figure 3.

**Reviewers' comments:**  *It would be great if authors would inform the comparative results in the representative results section showing why 1 hour of ES would be optimal.*

Author’s response: We address this point in the above response to the same question posed by Reviewer #1.   
  
**Reviewers' comments:**  *In general, MSC tends to start differentiation after confluent cell density. In the method authors described, initial cell number of plating seems not to reach at confluency 1 day after plating. In case of non-treated cells, it might be hard to start to be differentiated under the supplements of stimulating chemicals for bone differentiation at the next day of cell plating with this cell density. If any specific reasons, it would be nice to describe why.*

Author’s response: We applied osteogenic medium once the cells reached approximately 60-70% confluency, according to the manufacture’s recommendations.

**Reviewers' comments:**  *It would be better to more clearly specify the tolerable window of ES intensity with ES time per day. In the protocol, it was shortly described as "DC power supply (0-30 V/0-3A)". Of course ES intensity would vary on the purpose of experiments (migration, proliferation…etc) and should be optimized each experiment. However, at least for the purpose of in vitro osteogenic differentiation, the window for ES intensity with ES time per day should be shortly but clearly mentioned in the protocol or discusion sections if authors have performed experiments for optimization. Especially in JoVE method journal this would be much helpful for readers to potentially apply this method.*

Author’s response: We clarified this point in our revision under Protocol sections 1.8.1” To apply 100 mV/mm of EStim…set load output at 2.5V” and 3.3 “Set the power supply to 2.5 V load output and treat the cells with EStim for 1 hour”.

**Reviewer #3:**  
  
**Reviewers' comments:** *The use of electrical probes adds risk of cell contamination and, while low, is still at risk of corrosion.*

Author’s response: To minimize the risk of contamination and corrosion the electrodes are located on the removable lid and not inside the wells. Accordingly, the wells, containing the cells, are replaced for each new set of experiments. Cleaning/sterilizing the lid with the electrodes (described in Section 3.5) between experiments results in our not having experienced problems with contamination and/or corrosion.

**Reviewers' comments:** *Why use probes when electromagnetic field produces similar results? (See Ross et. al. 2018, J Cell, Stem Cells & Regenerative Medicine), and radiates through cell plates*?

Author’s response:

-While the results in experiments using DC EStim (with electrodes) and electromagnetic fields (without electrodes) have been described as being similar, whether the mechanisms are the same using these different methods is debated (Balint et al. 2013).

-We use DC EStim in our *in vivo* experiments and therefore we prefer to use the same in our *in vitro* experiments.

-DC EStim provides focused treatment (to the cells between the electrodes) in *in vitro* models, as well as in *in vivo* models, whereas in electromagnetic treatments EStim exposure is diffuse. In our *in vivo* applications we treat bone injuries and prefer to focus the treatment in the injury itself and not the surrounding tissues (muscle, skin, nerves, subcutaneous).

-In vitro experiments with electromagnetic treatments are complicated by the presence of environment-generated “noise” electromagnetic fields generated by devices like cell incubator/smartphones etc (Kohane und Tiller 2001). The use of Faradic cages prevents influence of these “noise” fields but makes the experimental set up more complicated.   
  
**Reviewers' comments:** *How could a multi-probe device be used in IN VIVO studies? Particularly how is this method translational to humans?*

Author’s response:

The EStim chamber presented here is designed as an easy method for performing *in vitro* experiments. For *in vivo* studies, cells could first be pre-treated with EStim in our *in vitro* chamberand thentransplanted into animal models.   
  
**Reviewers' comments:** *From which anatomical part of the rat were MSCs harvested? Bone marrow? Adipose tissue? Wharton's Jelly?*

Author’s response: The cells used in these experiments are bone marrow derived MSC .

The words: “bone marrow origin” were added to the table of materials  
**Reviewers' comments:** *Why did the authors choose 2.5 V as the EStim therapeutic voltage?*

Author’s response: The therapeutic voltage used in this paper was 100 mV/mm, based on previous experiments (Mobini et al. 2017). 2.5 V was the voltage emitted by the DC power supply in order to achieve 100 mV/mm between the electrodes in our EStim chamber (see section 1.8.1 Note).

References:

Eischen-Loges, Maria; Oliveira, Karla M. C.; Bhavsar, Mit B.; Barker, John H.; Leppik, Liudmila (2018): Pretreating mesenchymal stem cells with electrical stimulation causes sustained long-lasting pro-osteogenic effects. In: *PeerJ* 6, e4959. DOI: 10.7717/peerj.4959.

Kohane, M. J.; Tiller, W. A. (2001): Biological processes, quantum mechanics and electromagnetic fields: the possibility of device-encapsulated human intention in medical therapies. In: *Medical hypotheses* 56 (6), S. 598–607. DOI: 10.1054/mehy.2000.1263.

Leppik, Liudmila; Zhihua, Han; Mobini, Sahba; Thottakkattumana Parameswaran, Vishnu; Eischen-Loges, Maria; Slavici, Andrei et al. (2018): Combining electrical stimulation and tissue engineering to treat large bone defects in a rat model. In: *Sci. Rep.* 8 (1), S1. DOI: 10.1038/s41598-018-24892-0.

Mobini, Sahba; Leppik, Liudmila; Barker, John H. (2016): Direct current electrical stimulation chamber for treating cells in vitro. In: *BioTechniques* 60 (2), S. 95–98. DOI: 10.2144/000114382.

Mobini, Sahba; Leppik, Liudmila; Thottakkattumana Parameswaran, Vishnu; Barker, John Howard (2017): In vitro effect of direct current electrical stimulation on rat mesenchymal stem cells. In: *PeerJ* 5, e2821. DOI: 10.7717/peerj.2821.

Zhu, Siqi; Jing, Wei; Hu, Xiaoqing; Huang, Zirong; Cai, Qing; Ao, Yingfang; Yang, Xiaoping (2017): Time-dependent effect of electrical stimulation on osteogenic differentiation of bone mesenchymal stromal cells cultured on conductive nanofibers. In: *Journal of biomedical materials research. Part A* 105 (12), S. 3369–3383. DOI: 10.1002/jbm.a.36181.