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Construction and Use of an Electrical Stimulation Chamber for Enhancing Osteogenic Differentiation in Mesenchymal Stem/Stromal Cells In Vitro

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Cover Letter

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Date: 19. September 2018

Lyndsay Troyer
Science Editor

RE: Submission of manuscript entitled "*In vitro* method for enhancing osteogenic differentiation in mesenchymal stem/stromal cells by means of electrical stimulation".

Dear Lyndsay Troyer,

On behalf of my coauthors herewith I submit our above manuscript invited for publication in your prestigious journal. In this manuscript we describe the construction of a cell culture chamber designed to expose cells to various types of electrical stimulation, and its use treating mesenchymal stem cells to enhance osteogenic differentiation.
We believe that the methods described in our paper will be of great interest to your readership.

I thank you in advance for considering our work for publication in your journal and I look forward to your positive response.

Sincerely,

Liudmila Leppik, PhD

TITLE:

Construction and Use of an Electrical Stimulation Chamber for Enhancing Osteogenic Differentiation in Mesenchymal Stem/Stromal Cells In Vitro

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KEYWORDS:

Electrical stimulation chamber, cell culture, MSC, osteogenic differentiation, direct current, 6-well plate

SUMMARY:

Here we present a protocol for the construction of a cell culture chamber designed to expose cells to various types of electrical stimulation, and its use in treating mesenchymal stem cells to enhance osteogenic differentiation.

ABSTRACT:

Mesenchymal stem/stromal cells (MSCs) have been used extensively to promote bone healing in tissue engineering approaches. Electrical stimulation (EStim) has been demonstrated to increase MSC osteogenic differentiation in vitro and promote bone healing in clinical settings. Here we describe the construction of an EStim cell culture chamber and its use in treating rat bone-marrow-derived MSC to enhance osteogenic differentiation. We found that treating MSCs with EStim for 7 days results in a significant increase in the osteogenic differentiation, and importantly, this pro-osteogenic effect persists long after (7 days) EStim is discontinued. This approach of pretreating MSCs with EStim to enhance osteogenic differentiation could be used to optimize bone tissue engineering treatment outcomes and, thus, help them to achieve their full therapeutic potential. In addition to this application, this EStim cell culture chamber and protocol can also be used to investigate other EStim-sensitive cell behaviors, such as migration,

proliferation, apoptosis, and scaffold attachment.

INTRODUCTION:

An increase in trauma and/or disease-induced bone defects are being treated using different combinations of cell therapy and regenerative medicine technologies. MSCs are the cell of choice in such treatments, due to their relatively high osteogenic activity, isolation and expansion efficiency, and safety[1]. To maximize their osteogenic activity and, thus, optimize their therapeutic effectiveness, several methods have been introduced to manipulate MSCs prior to their use in these treatments (as reviewed by Mauney et al.[2]). One such method is EStim, which has been shown to enhance MSC osteogenic differentiation in vitro[3] and promote bone healing in vivo[4]. Despite the growing number of studies focusing on treating MSCs with EStim, an optimal regimen for maximizing EStim's pro-osteogenic effect has yet to be defined.

Other in vitro methods using EStim utilize salt bridges submerged in the culture medium, which separates cells from metallic electrodes[5]. The advantage of this is that delivering EStim through salt bridges eliminates the introduction of chemical byproducts (*e.g.*, corrosion of metallic 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 when using the two systems. Setups that deliver EStim via metallic or carbon electrodes fixed inside the cell culture wells (as reviewed by Hronik-Tupaj and Kaplan[6]) 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 the 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 MSCs[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.

Here we describe the construction of an EStim cell culture chamber; then, we demonstrate its use by treating MSCs with different regimens of EStim and measuring the resulting effect on osteogenic differentiation. MSC osteogenic differentiation is assessed via calcium deposition, alkaline phosphatase activity, and osteogenic marker gene expression. Importantly, in past experiments that used this setup, we observed that these pro-osteogenic effects persist long after the EStim treatment was discontinued.

PROTOCOL:

1. Construction of electrical stimulation cell culture chamber

1.1. To build the EStim chamber, collect two lids of standard 6-well cell culture plates; 99.99% platinum wire, 60 cm in length with a diameter of 0.5–1 mm; silver-coated copper wire, 70 cm in length with a diameter of 0.6 mm; cutting pliers; soldering iron kit; one tube of superconductive glue; one wire terminal block connector, six small 2.2 V LEDs (optional); one tube of noncorrosive silicone adhesive coating (optional); one roll of black electrical insulation tape; standard, flexible,

insulated copper electric wire (0.14 mm^2), 2 m in length (**Table of materials**).

1.2. In a 6-well plate lid, mark and then drill two holes (with a diameter of ~1 mm), 25 mm apart, near the outer edge of each of the six wells (12 holes in total), as shown in **Figure 1A,B**.

1.3. Cut twelve 5 cm lengths of platinum wire with cutting pliers. Bend each of the wires manually into an L-shape, leaving one end 3 cm long and the other 2 cm. Cut the silver-coated wire into two 35 cm lengths.

1.4. Insert the longer (3 cm) bent end of one platinum wire (from inside to out) into each drilled hole, leaving 1–2 mm protruding from the outside of the lid to be soldered. Secure the platinum wires in the lid holes with superconductive glue and leave it to dry for around 6 h.

1.5. Solder the tips of all six platinum wires (that will later serve as cathodes, see **Figure 1A**) protruding from the lids to one of the silver-coated wires. Repeat the same procedure, soldering the remaining six platinum wires (that will later serve as anodes) to the other silver-coated wire.

1.5.1. Add LEDs in the circuit between each of the six anode-cathode platinum electrode pairs, to confirm functionality during the experiments (optional). Place a piece of black insulation tape under each LED to prevent exposing the cells in the culture plates to the LED light (**Figure 1C**).

1.6. Glue the wire terminal block connector to the top left corner of the 6-well plate lid and connect both silver-coated wires to the input terminals, as shown in **Figure 1C**.

1.7. Cut out a 20 mm x 20 mm section from the top left corner of a second 6-well plate lid (**Figure 1B**, Lid Nr. 2) to accommodate the terminal block connector on the first lid. Cover the first lid, equipped with the electrodes, with the second lid and tape them together with adhesive tape.

1.7.1. To improve the bonding of the two lids, use silicone adhesive coating (optional). To do so, cover the lid with the silver electrodes with a 3–5 mm layer of silicone adhesive and cover it with the other lid, allowing 12 h for the adhesive to dry.

1.8. Connect one end of the two standard insulated copper wires to the output terminals of the wire connector and the other ends to banana male connectors (4 mm).

NOTE: The length of these wires depends on the distance from the shelf in the incubator, where the cells will be kept, to the power supply, outside the incubator (**Figure 1D**).

1.9. Adjust the dosage (voltage) and regimen of EStim delivered to the cells by regulating the DC power supply.

1.9.1. Turn on the power supply by pressing the **ON/OFF** button on the front panel. Activate channel 1 by pressing button **1**.

1.9.2. Press button Nr. **4** (V-set) to set the voltage. Press buttons **2** and **5** to set the load output at 2.5 V. Press **Enter**.

1.9.3. Ensure that the load output of 2.5 V (2,500 mV) corresponds to an EStim of 100 mV/mm, according to the following, simplified equation.

$$V\text{EStim} = \frac{V}{d}\text{; or}$$
$$V = V\text{EStim} \times d$$

Here, V = the power supply voltage output in millivolts; d = the distance between electrodes in millimeters; V_{EStim} = the stimulation voltage in millivolts per millimeter.

NOTE: This simplification applies only in case of constant DC voltage. The resistance between the medium and the cells is negligible as electrodes are not in direct contact with the cells; instead, EStim is delivered to the cells through the medium.

2. Mesenchymal stem cell culture in osteogenic medium

2.1. Purchase and store commercially available rat MSCs (see **Table of Materials**) in liquid nitrogen until the day of the experiment. Alternatively, isolate MSCs from other animals according to protocols published elsewhere[7,8] in accordance with local institutional regulations for the use of experimental animals.

2.2. On the day of the experiment, remove one vial (1 x 10^6 cells) of MSCs from the liquid nitrogen storage, and quickly (within 1 min) thaw the cells in a water bath preheated to 37 °C.

2.2.1. Under sterile conditions in a laminar flow hood, pipette the vial content into a 50 mL falcon tube and add 9 mL of normal medium (NM) prewarmed to 37 °C, consisting of Dulbecco's modified Eagle's medium (DMEM; 1x) with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin solution. Pellet the cells for 5 min by centrifugation at 300 x *g*.

2.2.2. In a laminar flow hood, remove the supernatant and carefully resuspend the cell pellet in 12 mL of NM prewarmed to 37 °C. Transfer the resuspended cells to a T-75 cell culture flask.

2.3. Culture the cells at 37 °C, 5% CO_2, 5% O_2 until they reach an 80%–90% confluence (after approximately 3–5 days).

2.4. Passage the cells.

NOTE: Perform all operations except centrifugation and incubation under sterile conditions in a laminar flow hood.

2.4.1. After reaching an 80%–90% confluence, retrieve the cells with cell detachment solution.

Aspirate the cell culture medium, wash 2x with 1x phosphate-buffered saline (PBS), add 5 mL of 1x cell detachment solution, and return the cells to the incubator for 5 min.

2.4.2. Once the cells are detached, add an equal amount of culture medium to inactivate the detachment solution. Collect the cells in a 50 mL falcon tube and spin them at 300 x *g* for 5 min.

2.4.3. Discard the medium and resuspend cells in 1 mL of fresh normal medium. Assess the number of viable cells with trypan blue stain.

2.4.4. Seed 1 x 10^6 cells in a new T-75 flask with 12 mL of prewarmed NM. Culture the cells at 37 °C, 5 % CO_2, 5 % O_2 until they reach an 80%–90% confluence.

NOTE: Cell passaging (steps 2.4.1–2.4.4) can be repeated a few times until the needed number of cells is obtained. Do not use cells older than passage 8.

2.5. Seed 9 x 10^4 cells in 3 mL of NM (10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, DMEM) in each well of a 6-well culture plate. Incubate the cells for 1 day at 37 °C, 5 % CO_2, 5 % O_2.

2.6. The next day, aspirate culture medium and apply 3 mL of osteogenic differentiation medium (OM; normal medium supplemented with 10^{-7} M dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate).

2.7. Place the 6-well plate with cells in the incubator and incubate at 37 °C, 5% CO_2, 5% O_2 overnight.

3. Treating MSCs with EStim

3.1. On the day the cells are treated with EStim, sterilize the electrodes in 70% ethanol solution for 30 min; then, dry them under UV light in a safety cabinet for an additional 30 min.

3.2. In a laminar flow hood, cover the 6-well plate containing the cultured MSCs with the lid equipped with the electrodes, making sure that the electrodes are completely submerged in medium (if necessary, add medium). Transfer the covered 6-well plate (EStim chamber) with the cells to the incubator and connect its wires to the power supply.

3.3. Set the power supply to 2.5 V load output and treat the cells with EStim for 1 h[3,9].

3.4. After stimulation, disconnect the power supply and remove the EStim chamber from the incubator. Under sterile conditions, exchange the lid equipped with electrodes with a standard 6-well plate lid.

3.5. Return the cells to the incubator and leave them overnight. Clean the electrodes, first with PBS and then with 70% ethanol solution. Clean the accumulated corrosion products from the

electrode surface with fine sandpaper.

3.6. Repeat steps 3.1–3.5 for 6 consecutive days. On day 4, prior to applying EStim and under sterile conditions, change the culture medium by aspirating 1.5 mL of medium and replacing it with 1.5 mL of prewarmed fresh OM.

3.7. After applying EStim for 7 consecutive days, maintain the cells in culture for an additional 7 days, exchanging the medium every 3–4 days.

4. Osteogenic differentiation measurements

4.1. Analyze cell morphology changes under a microscope.

4.2. To assess the effect of EStim on MSC osteogenic differentiation, measure calcium deposition, alkaline phosphatase activity, and osteogenic marker gene expression, as described elsewhere[3,9].

REPRESENTATIVE RESULTS:

To evaluate the effect of 100 mV/mm of EStim on the osteogenic differentiation of MSCs, cells treated with EStim for 3, 7, and 14 days or nontreated (control) were analyzed at day 14 of culturing by assessing morphological changes and calcium deposition (**Figure 2**). This was done by imaging cells using bright-field microscopy (morphology changes) or by fixing cells in 4% paraformaldehyde solution, staining them with 0.02% alizarin red solution and then imaging them using bright-field microscopy (calcium deposition analysis).

A detailed analysis of osteogenic marker gene expression changes was performed at days 3, 7, and 14 of culturing (**Figure 3**). This was done by measuring the relative expression of genes *RunX2*, *Collagen I*, *Osteopontin*, and *Osterix* by means of RT-qPCR and the comparative delta Ct (threshold cycle values) method[10], where housekeeping genes *Rplp1* and *Ywhaz*[11] were used for normalization.

Exposing MSCs to 100 mV/mm of EStim for 3 days (1 h per day) had no effect; however, 7 days of exposure resulted in an increase in osteogenic differentiation, as determined by morphology changes (**Figure 2A–C**), calcium deposition (**Figure 2E–G**), and osteogenic marker gene expression changes (**Figure 3**) in comparison to a time-matched control without EStim. Prolonged EStim exposure (14 days) did not further enhance the osteogenic differentiation beyond that seen after 7 days of treatment (**Figure 2D,H** and **Figure 3**).

As shown in **Figure 2**, cells treated with EStim for 7 and 14 days appeared more condensed (**Figure 2C,D**) than those treated with EStim for 3 days or nontreated cells (**Figure 2 A,B**) and showed an increased calcium deposition (**Figure 2G,H**) compared to those treated for only 3 days or nontreated controls **(Figure 2E,F**). Analysis of the osteogenic marker expression at 3, 7, and 14 days of culturing confirmed the enhanced osteogenic differentiation in cells treated with EStim for 7 and 14 days (**Figure 3**). The expression of osteogenic-differentiation-related marker genes[12] *RunX2*, *Collagen I*, *Osteopontin*, and *Osterix* were the highest in cells treated with EStim for 7

days.

FIGURE AND TABLE LEGENDS:

Figure 1: EStim cell culture chamber. (**A**) Electric circuit diagram of the EStim chamber showing the anodes (black), cathodes (red), and LEDs connected to the DC power supply. (**B**) Image of the marked 6-well plate lids and L-shaped platinum electrodes (bottom view) incorporated into the 6-well plate lid. (**C**) Assembled EStim chamber (top view) with wire connector, LEDs, and electrical insulation tape that shields the cells from the LED light (arrows). (**D**) EStim cell treatment setup with the EStim chamber in the incubator connected to the DC power supply, on the outside.

Figure 2: Effect of EStim on MSC morphology and calcium deposition. Cells in osteogenic culture medium, exposed and not exposed (controls) to 100 mV/mm of EStim for 3, 7, and 14 days (1 h/day). (**A**–**D**) Morphology and (**E**–**H**) calcium deposition (alizarin red staining) on day 14 of culturing. Significant changes in cell morphology and calcium deposits were visible in cells treated with EStim for (**C** and **G**) 7 and (**D** and **H**) 14 days (10x magnification; the scale bar = 200 µm). This figure was modified from Eischen-Loges et al.[9].

Figure 3: Effect of EStim on MSC osteogenic marker gene expression. The osteogenic marker gene expression (measured with RT-qPCR at days 3, 7, and 14 of culturing) in cells treated with EStim for 3, 7, and 14 days, or nontreated. (**A**) At day 7 of culturing, the *RunX2* expression was significantly higher in cells treated with EStim for 7 days. (**B**) The *Colla1* expression was significantly higher in cells treated for 7 days, measured at day 14 of culturing. (**C**) The expression of *Osteopontin* was significantly increased in EStim-treated cells at days 3, 7, and 14 of culturing. (**D**) The *Osterix* expression was absent in control cells at all time points and was seen only at 7 and 14 days of culture in cells exposed to EStim. Different letters on the bars indicate significant ($p < 0.05$) differences among groups at the same time point. The asterisk indicates significant ($p < 0.05$) differences between time points within the same group. This figure was modified from Eischen-Loges et al.[9].

DISCUSSION:

Here we describe the construction of a chamber and a method for treating mesenchymal stem cells with EStim that results in enhanced osteogenic differentiation.

The EStim setup presented does not require special equipment/knowledge and can be performed in a standard stem cell biology/biochemistry laboratory by junior researchers. However, when building and using the EStim chamber, special care must be taken in a few critical steps. When handling the platinum electrodes, extra care must be taken as this metal is very malleable and delicate. While other metals, such as steel or tungsten, can be used, these are not recommended as they are prone to corrosion, which can be cytotoxic[13]. Also, cleaning/sterilizing the lid with the electrodes must be performed as precisely as described in the protocol since this method has been tested repeatedly and found to be effective in eliminating problems with contamination. Finally, while this chamber could be used to study other EStim-sensitive cell activities like migration[14,15], proliferation, apoptosis[16], cell membrane voltages[17], and scaffold adhesion[19], the

EStim protocol we describe here (100 mV/mm, 1 h/day, 7 days) focused only on osteogenic differentiation in rat MSCs. Special attention should be given if higher voltages (≥150 mV/mm) and/or longer durations of EStim (>4–5 h) are applied since cytotoxic products resulting from electrolysis can accumulate in the medium. In this case, the medium must be carefully monitored and exchanged accordingly. To study other parameters and/or other cell types and conditions, we recommend that separate dosage (voltage) and regimen titration studies be conducted as these changes can affect how cells respond to EStim.

Possible malfunctioning of the EStim chamber can be due to breaks in the electrical circuitry after repeated use and can be monitored via the added LED lamps. In case of breakage (indicated by nonilluminated LED light[s]), the lid can be removed and easily disassembled to identify and repair the break. In addition, the simple design of the EStim chamber makes it easy to modify it according to the needs of different experimental setups and methods of the analysis. Examples include using different sizes of cell culture plates, by simply varying the length of and the distance between the electrodes or adding different (3D) culture conditions with ceramic scaffold material[4] or conductive substrates[18], by simply placing these materials seeded with cells between the electrodes.

As in all in vitro experiments, cell behaviors and functions observed/induced in the EStim chamber are not always directly transferable to in vivo models. Accordingly, when interpreting the EStim-induced cell behaviors/functions in the chamber, researchers must always take this into consideration.

The setup and method presented here have many advantages over other in vitro methods used to expose cells to EStim (systems with salt bridges[5] and systems with electrodes incorporated in cell culture wells[19]). An important advantage of the system described here is that, since the cells are cultured in standard 6-well plates, they can be used after EStim treatment in other in vivo or in vitro protocols. In addition, the fact that electrodes are fixed on the lid of the 6-well plate makes it easy to clean and sterilize the device between experiments and to reuse it. Finally, the ability to simultaneously stimulate cells in six wells provides ample material for analysis and reproducibility.

To gain a better understanding of the mechanisms at the cellular membrane level by which EStim affects cell activities in ongoing studies using the chamber described here, we are exploring the relationship between externally delivered EStim and cell membrane potential (V_{mem})[17]. In addition, based on previous findings[9], in future studies, we will pretreat the cells with EStim in the chamber, alone and with different 3D scaffolds, and with conductive substrates, to stimulate specific cell functions; then, we will implant them into animal models to determine if they retain the observed enhanced functions in vivo. These studies will contribute to the growing body of information about the mechanisms by which EStim regulate cell function and, in doing so, could contribute to optimizing cell therapy approaches in regenerative medicine and tissue-engineering-based treatments.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

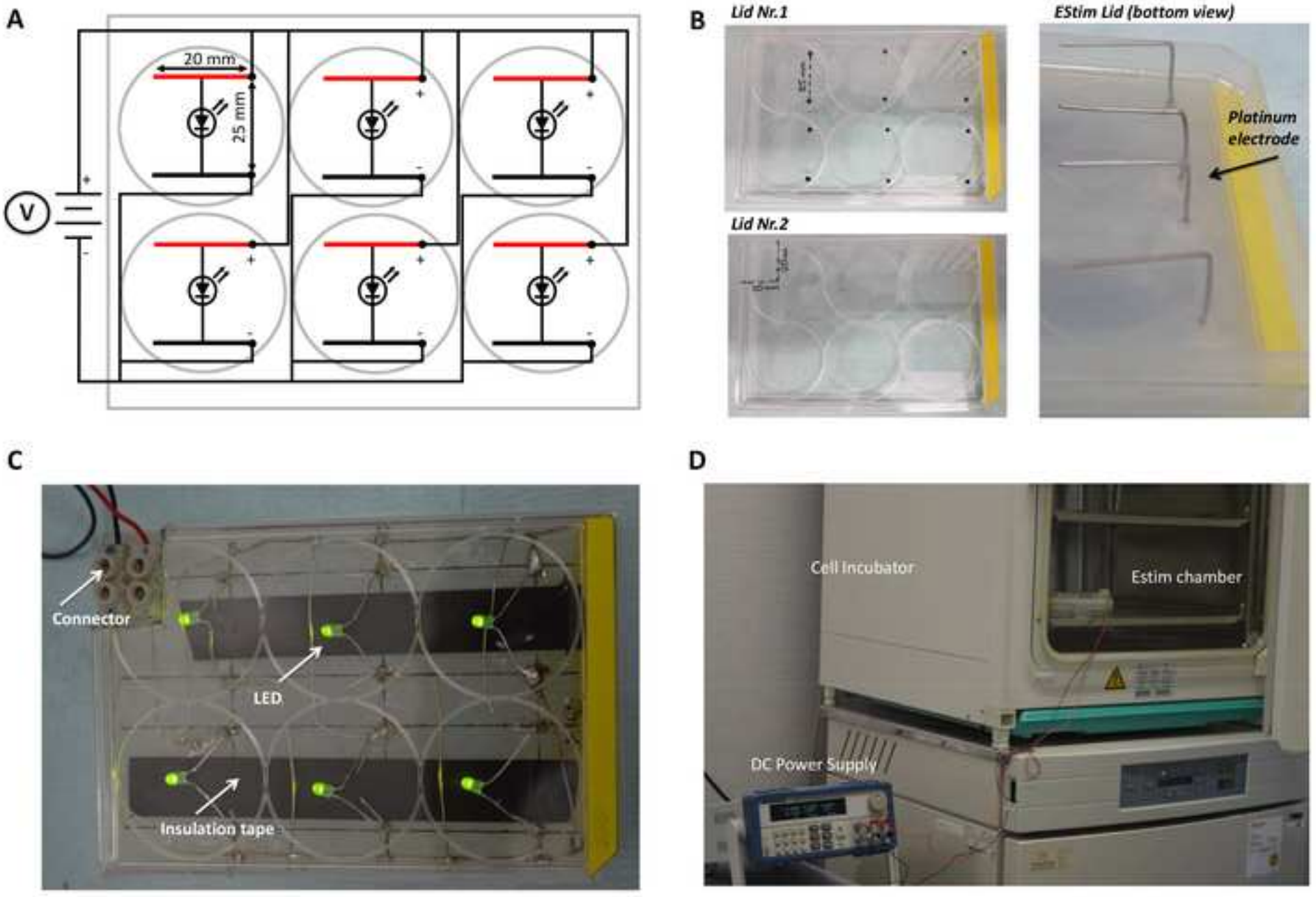


Figure 2

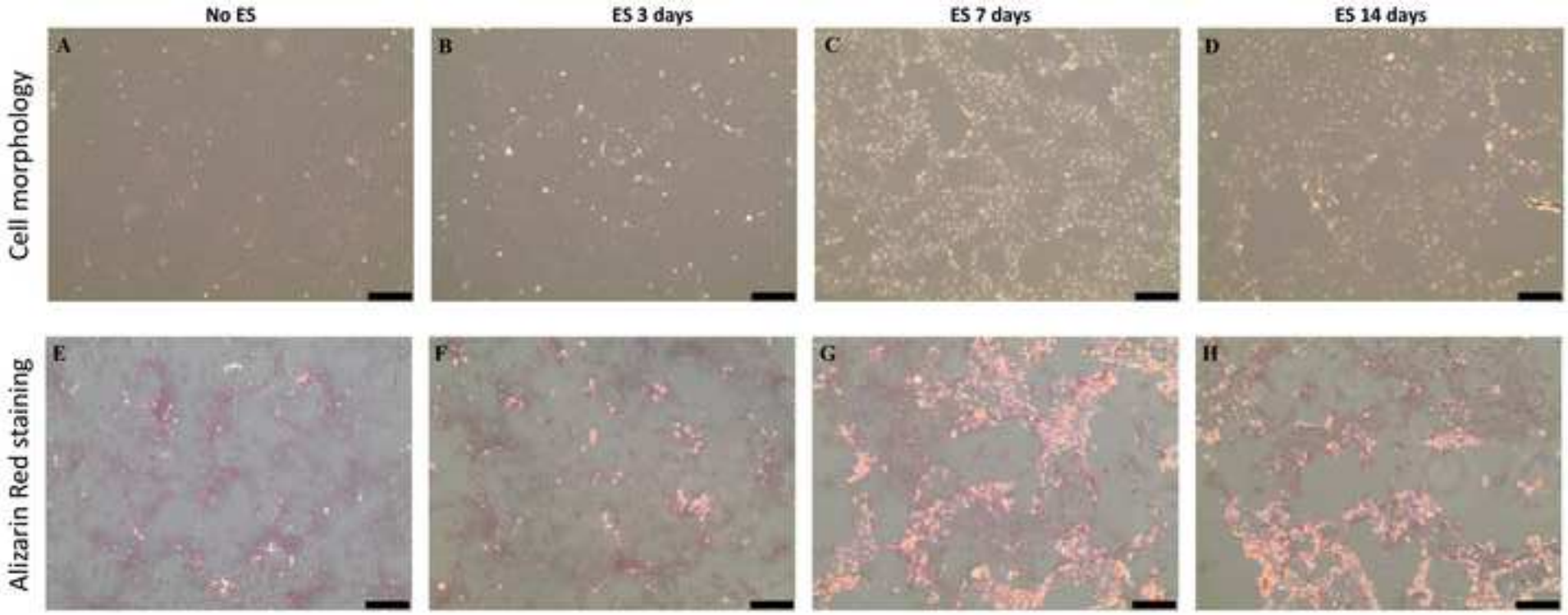
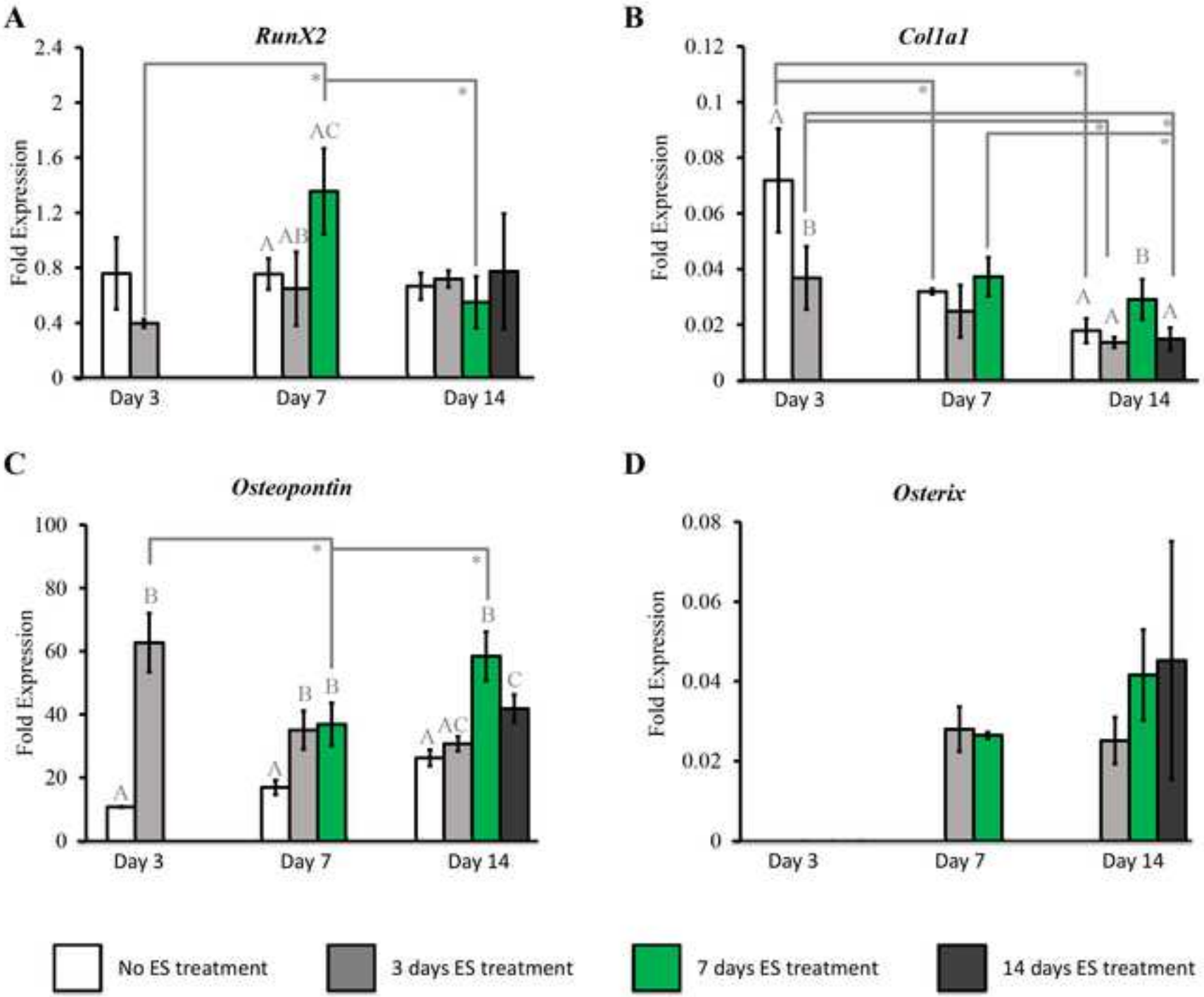


Figure 3



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Estim fabrication			
Banana connector/Jack adaptor	Poppstars	1008554	2 pieces
Cutting pliers	Knipex	78 03 125	
DC power supply (0-30V/0-3A)	B&K Precision	Model 9130B	Any simular model could be used
Insulated flexible wires (0.14 mm^2)	Conrad Electronic International	604794 , 604093	2 pieces
Non-corrosive silicone rubber	Dow Corning	3140 RTV	*could be purchased by many stores
Platinum Wire (999,5/1000; 1mm ø)	Junker Edelmetalle	00D-3010	0.6 m needed for 1 Estim chamber
70% Ethanol solution	any		Sterilisation of Estim chamber
Silver coated copper wire (0.6 mm ø)	Conrad Electronic International	409334 - 62	≈70 cm needed for 1 Estim device
Soldering iron Set	Conrad Electronic International	1611410 - 62	Any simular model could be used
TPP 6-well plate lid	Sigma-Aldrich	Z707759-126EA	2 lids for Estim chamber
2.2V wired circular LEDs	Conrad Electronic International	599525 - 62	6 pieces
UHU Super glue	UHU GmbH & Co. KG	n/a	*could be purchased by many stores
MSC culture			
β-Glycerophosphate disodium salt hydrate	Sigma-Aldrich	G9422	osteogenic cell culture
DMEM, low glucose, GlutaMAX Supplement, pyruvate	Thermo-Fischer Scientific	21885025	cell culture
DPBS, no calcium, no magnesium	Thermo-Fischer Scientific	14190144	cell culture
Dexamethasone	Sigma-Aldrich	D4902	osteogenic cell culture
Fetal Bovine Serum	Thermo-Fischer Scientific	10500064	cell culture
50 ml Falcon tube	Sarstedt	62,547,004	cell culture
L-Ascorbic acid	Sigma-Aldrich	A4544	osteogenic cell culture

Penicillin/Streptomycin	Thermo-Fischer Scientific	15140122	cell culture
Sprague-Dawley (SD) rat mesenchymal stem cells, bone marrow origin	Cyagen	RASMX-01001	cell culture
Cell detachment solution	Thermo-Fischer Scientific	A1110501	cell culture, cell detachment
TC Flask, T75	Sarstedt	833911302	cell culture
TPP 6-well plates	Sigma-Aldrich	Z707759-126EA	cell culture
Trypan Blue Dye, 0.4% solution	Bio-Rad	1450021	cell count

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Title of Article: *In vitro* method for enhancing osteogenic differentiation in mesenchymal stem/stromal cells by means of electrical stimulation

Author(s): Liudmila Leppik, Mit B. Bhavsar, Karla M.C. Oliveira, Maria Eischen-Loges, Sahba Mobini and John H. Barker

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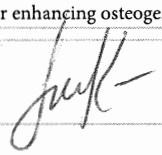
Signature:	 Date: 19.09.2018

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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 % CO_2, 5 % O_2".

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* chamber and then transplanted 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).

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Allen antworten

Fr 03.08, 16:01

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Dear Liudmila,

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Regards,

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