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our reference

your reference

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pages 9

to Dr. Amit Krishnan
Review editor
Journal of Visualized Experiments

Dear Dr. Amit Krishnan,

Thank you for the input into our manuscript "*In vitro* three-dimensional sprouting assay of angiogenesis using mouse embryonic stem cells for vascular disease modeling and drug testing". We are submitting a revised manuscript in which we have addressed all points raised by yourself and the referees and include a point by point response.
We hope that you and the referres will agree and we look forward to your response.
Best regards
Franck Lebrin

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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We apologize for the spelling and grammar errors. They have been corrected.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

This has been corrected as suggested.

3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

Please refrain from using bullets or dashes.

This has been corrected as suggested.

4. Line 115-125: The Protocol should contain only action items that direct the reader to do something. For example: “Prepare conditioned medium using supplement 1X Glasgow MEM (G-MEM BHK-21) medium 116 with 10% (vol/vol) heat-inactivated Fetal Bovine Serum (FBS), 0.05 mM β-mercaptoethanol, 117 1X non-essential amino acids (NEAA 10x), 2 mM L-glutamine and 1 mM sodium pyruvate”.

This has been corrected as suggested.

5. Please include a one-line space between each protocol step and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Corrected.

6. Please title case and italicize journal titles and book titles in the Reference section. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

We apologize. This has been corrected.

7. Figure 3: Please include scalebars in all the images of the panel. Include the details of the magnification in the Representative Results section to make it more informative.

Thank. Figure 3 has been modified as requested.

Reviewer #1:

This paper by Galaris and colleagues aimed to describe the putative use of mouse ESC-derived embryoid bodies for vascular specific phenotypic assays like sprouting or characterisation of the endothelial specification. The authors suggest that these assays can be useful assets for drug discovery and development. They also argue for the benefits of vascularised (mouse) EBs as a 3D model. Although I do not share the authors' enthusiasm for rodent lines in human drug development; nevertheless, the protocols are well written with convincing figures. Specific question:

1. The authors claim that this protocol is "an unbiased, robust and reproducible three-dimensional EB-based assay". Therefore, if any steps of the protocol may be scalable or require manual steps? Was there any difference in mESC lines that the authors used here to manufacture a large number of homogenous EBs?

No. Our lab has worked with multiple 129/Ola mESC lines over the past decade. All wild-type lines studied have been differentiated as described in this protocol to form endothelial sprouts in collagen I gel. As far, they showed similar ability to form EBs (size and shape evaluated by light microscopy), produced the same yield of endothelial cells and have similar ability to sprout when plated in collagen I. Moreover, we have also characterized the CD31+ population isolated from day-9-old EBs generated from different 129/Ola mESC lines by RNA sequencing and found that these cells had similar mRNA expression profiles (not included in the present protocol).

2. Was there a potential difference between lines? By this protocol, was the size and structure of EBs reproducible?

Yes. We have not observed obvious difference in term of size and EB shape between lines. For clarity, photos are now included in figure 2.

3. Is there a reliable way to count cells in 3D constructs? Chamber does not seem to appropriate.

No. We don’t count EBs in 3D but dissociate them for FACS analysis. We apologize for the lack of clarity. A protocol is now included in page 5, lines 246-265.

4. Line 438. It is not entirely clear how this platform is suitable for genetic screening studies. The obvious difference in species may cast doubt on its value.

Sorry for the lack of clarity. This refers to the paper published by Elling U. *et al.,* (Nature, 2017; 550(7674): 114-118) that has generated the larger haplobank of hemi/homozygous mutant mES cells by reversible mutagenesis and did show how this approach was useful to screen for genes implicated in sprouting angiogenesis.

This reference is now included and the following sentence has been added:

“to be used for genetic screening as recently described by Elling U. *et al.* that have generated a large haplobank of hemi/homozygous mutant mESC (Elling et aL., 2017, Nature) and for phenotypic drug discovery program”.

Minor:

1. The manuscript needs to be checked for multiple typos, spelling errors, English language and formatting.

We apologize and have corrected them.

2. Line 275: "(red channel)" and "(green channel)" both need rephrasing.

This has been corrected. The following sentence is now included:
“Measure the total cellular area of the sprout occupied separately by PECAM-1 (endothelial cells labeled in red) and by **α**-SMA (mural cells labeled in green) positive cells”.

3. Line 413. "Use high quality mESCs". This statement is rather vague and may require further instructions.

The referee is correct that this is vague. We have therefore added the following sentence:

“Use high quality mESCs. They should robustly express well accepted markers of the pluripotent state such as Nanog, Oct4, Sox2 and SSEA-1. It is essential to monitor carefully their growth, mES cell shape and the size and morphology of mES colonies. Because, karyotypic stability is stochastic in cultured cells, it should also be reassessed after extensive passaging”.

4. Regarding reference 35 (consensus guidelines on angiogenesis), I would suggest referring to this better and describing novelties/similarities versus the already available other platforms.

We thank the referee for his comment. However, we believe that the recent review summarizing the various angiogenesis models including advantages and limitations for each method by Nowak-Sliwinska, P. *et al.* nicely summarize issues regarding each

model of angiogenesis including mice and microfluidics combined with vascularized OoC. Off course, it was not our intention to ignore the recent data on vascularized OoC in fact these systems are one of the most interesting discoveries on angiogenesis models. We have therefore included additional references related to these specific technologies.

Reviewer #2:

In this manuscript, Galaris et al. explain the embryoid body model derived from mouse embryonic stem cells (mESCs) to recapitulate key features of in vivo sprouting angiogenesis, which has been reported previously, both in mouse and human models. The main relevance of this work is the detailed method in which the authors show its relevance in exploiting to screen for drugs and genes modulating angiogenesis. The manuscript is well written and focused. I have only minor concerns with this work.

Line 77. Whist? Whilst?
The referee is correct, it should read whilst and not whist.

Line 79. Please, reason why human organoids and organ-on-chip model strongly rely on data from animal models to assess their efficacy and safety.

Sorry for the lack of clarity, we refer to bringing drugs into new clinical practice that rely to animal models to have access to PK and PD data. We have now modified the text to clarify this point. The following sentence has been added:

“Whilst human organoid and organ-on-chip models can provide important insights into disease complexity and to the identification of novel drugs, bringing drugs into new clinical practice also strongly rely on data from animal models to assess their efficacy and safety”.

Line 113. It is inconvenient to have the title with the number 1 and the subtitles same, 1, 2, 3…
Would at least be better to write Protocol 1. Medium and reagents for mESC cell culture (is "cell" not excessive in this case, since the C in mESC is abbreviation for cell?). Same goes for Protocol 2 etc…

The numbering and the text have been modified as requested.

Line 141. "1X106" - X missing

Corrected

Line 164. " stain in suspension" - stay? in suspension

Yes, this has been corrected

Line 184. "as an example"

Corrected

Line 192 and 193. "use a and b instead of 1 and 2 again"

Yes, this has been corrected

Line 197. "The medium can be stored it at 4…" - delete it.

This has been deleted

Line 203. Here and throughout the manuscript basic fibroblast growth factor is abbreviated b-FGF or even bFGF2 (in flow chart and table of materials). To my knowledge it is either bFGF or FGF2.

We apologize for these inconsistencies and have now corrected them.

Line 203. "in the presence of … "

Corrected

Line 213 . "NAOH" - NaOH

Corrected

Line 232. Protocol 8-2: This is an interesting approach to dehydrate the gel - researchers may be more interested in this procedure as the dehydration is often problematic. Please, address this method in more detail or have a figure legend in Fig1. In addition, the table of materials used does not include the items used.

This is a good point. We have included in the table of materials all information and we will use the movie to clearly describe the procedure to dehydrate the gel.

Line 236. "Wash the slides 3 times".

Corrected

Line 237. "Fixate" - fix.

Corrected

Line 261. "EB soma" - ?

We apologize for the lack of clarity. The following sentence has been added: “Define the base of the endothelial sprout starting at the EB core area and manually draw a line until the sprout tip end”.

Line 276. "colocalize" - merged?

Corrected

Line 287. "Collagenase A" - Collagenase A has not been mentioned before in the protocol - only TVP.

Line 288. "analyzed by FACS as described" - has FACS been described before?

The referee is correct. We have now included a short paragraph describing the FACS analysis and the cell dissociation method including the use of collagenase A. All antibodies and chemical compounds are now included in the supplementary table

Line 237. "mRNA expression levels of all markers that have been analyzed (….) were comparable" - What does this mean? They are not comparable but just present.

We apologize for the lack of clarify. We have now made clear that we do compare the expression levels of pan-endothelial cell markers between 3 independent mESC lines. We have now included the following sentence: “The mRNA expression levels of all analyzed markers (*Flk1*, *Flt1, Flt4, Eng, Tie2* and *Cdh5*) were comparable between lines and experiments confirming the robustness of the differentiation protocol (**Figure 2B)”**.

Line 237. "Activin like Kinase Receptor 1"- Activin Receptor-like kinase 1

This has been corrected

Line 323. "genotypic origing"- genotypic origin

This has been corrected

Line 329. "Binamy"- Binary

Corrected

Line 349. Please, use top right and bottom right to depict vehicle alone and DAPT, respectively.

The referee is correct. The text has been changed as follow: “On the top right panel, high magnification of endothelial sprouts from vehicle alone and on the bottom right panel, high magnification of endothelial sprouts from DAPT (0.5µmol.L^{-1}) condition.

Line 329. "12 days old"- 12 day old (more of this, please check in find)

We apologize and have now corrected them

Line 356. "Acvrl1…. models"- mice

Corrected

Line 367. "representation from representative wild-type EBs"- Delete representation

Corrected

Line 367. "representative12 days old EB"- representative 12 day old EB

Corrected

Line 380. "HUVECS"- HUVECs

Corrected

Line 384. "sprounting"- sprouting

Corrected

Line 387. "It also offer advantages to many three-dimentional angiogenesis"- "It also offers advantages to many three-dimentional angiogenesis models

Corrected

Line 392. "much more difficult to implement"- Please, explain why?

This refers to the complexity of the procedures when compared to most *in vitro* models. We have now modified the text as follow for clarity: “The *ex vivo* retina explant[31] or vascularized micro-organ assay[32] can recapitulate well all blood vessel formation steps but they have complex experimental procedures and are not suitable for high throughput drug or genetic screening”.

Line 398. "and do not produce"- and does not produce

Corrected

Line 417. "not let to become"- not allowed to become

Corrected

Line 419. "the best production of endothelial cells"- what is best production here? Quality of ECs or the best yield of ECs - or both?

The referee is correct that is vague. We have corrected and made clear that it was the yield.

Line 422. "in the presence"

Corrected

In general in discussions, it would be an advantage to mention similar approaches done

This point is well taken and the text has been modified to include recent data on human iPSCs as follow: “This protocol also nicely complement similar *in vitro* angiogenesis assays using human iPSCs allowing comparisons between mouse and human data. Although it is important to note that human iPSC-derived endothelial cells show less ability to sprout than the mouse cells (Belair G et al., acta Biomater.; Bezenah J. et al., 2018)”.

Line 430. "EBs are finally cultured onto collagen I gel"- is it onto or in?

Corrected.

Figure 2B - Pecam-1 vs Cdh5 - not consistent (Pecam-1, CD31), please, be consistent

Figure 2B refers to mouse gene and is therefore labeled *cdh5* and *Pecam1*

Figure 2C - might be nice to depict arterial vs venous genes on the Figure.

It has been modified as suggested and data are now provided as figure 2C and 2D.