10-May-2021

Dear Editor,

In response to review comments for manuscript titled '**A 3D collagen-based scaffold model of the neuroblastoma microenvironment'.** Firstly, we would like to sincerely thank all reviewers for their time and expertise in evaluating our work. We appreciate the comprehensive and constructive appraisal of our manuscript and have addressed their concerns. We believe that our revisions suggested by the reviewers have significantly improved the data presentation and interpretation, and the quality of our manuscript.
We have comprehensively revised our main manuscript text in relation to sentence structure, mistypes, figure mentions and text clarity. To facilitate navigation through the revised manuscript, we used red text to track our changes.

Reviewer comments

Editorial request:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Author's response: The authors have proofread the manuscript and are confident that there are no spelling or grammatical issues.

2. Please revise the following lines to avoid previously published work: 85-86, 92-97, 100-102.
Author's response: The authors have restructured the sentences from these lines to avoid previously published work and have referenced previous work appropriately.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Author's response: The authors have ensured there is no use of personal pronouns in the methodology section of the manuscript. However, we feel it is appropriate to keep some personal pronouns in the discussion section (e.g., "Here we described", "We aimed", etc.)

4. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., HYPERflask, Corning, Falcon, Eppendorf, Alamar Blue, PicoGreen, QIAzol, miRNeasy, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.
Author's response: The authors have removed commercial language from the manuscript, substituting with appropriate generic terms, as below:
HYPERflask – Multilayer cell culture flask (or multilayer flask)
Falcon/Eppendorf - Centrifuge tube
Alamar Blue – Cell viability assay (reagent)
PicoGreen – Fluorescent double-stranded (ds)DNA stain
QIAzol - Phenol/guanidine-based cell lysis reagent
MiRNeasy - an appropriate RNA extraction kit

5. 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.
Author's response: The authors have adjusted numbering and have not used letters/bullets/dashes.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling

someone how to do the technique (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. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
Author's response: The authors have ensured the protocol section is written in the imperative tense.

7. Line 149-151: Please ensure that the Protocol section consists of numbered steps. We cannot have non-numbered paragraphs/steps/headings/subheadings.
Author's response: The authors have ensured that protocol sections consist of numbered steps with short “Notes” at the top of the subsection if additional information is required.

8. Please include a one-line space between each protocol step and then 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.
Author's response: A one-line space has been added between each step in the protocol and 3 pages have been highlighted for the video: from section 4.3 to 5.1 inclusive.

9. Please include the limitations of the protocol and the troubleshooting techniques in the discussion section.
Author's response: A discussion of the protocol limitations has been added to the discussion at page 22, lines 916-931.

10. 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: Copywrite permission has been requested to reuse some components of Figure 10 from previously published work by Curtin C. et al, 2018.
This permission will be uploaded to the Editorial Manager account once we receive it. Meanwhile, we uploaded the link to the editorial policy. The figure has been appropriately cited in the manuscript, page 21 line 872 “This figure has been adapted from Curtin et al., 2018 [17], an open access article under the CC BY-NC-ND license 4.0.”

11. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).
Author's response: In-text citations have been modified to appear as numbered superscripts.

12. Please do not use the &-sign or the word “and” when listing authors. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. End the list of authors with a period. Example: Bedford, C. D., Harris, R. N., Howd, R. A., Goff, D. A., Koolpe, G. A. Quaternary salts of 2-[(hydroxyimino)methyl]limidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).
Author's response: The bibliography has been modified to meet these requests.

13. Figure 5/ 6/7: Please replace the commercial terms (Alamar Blue, QIAzol, etc.) by generic terms.
Author's response: Commercial terms have been removed from figures and figure legends.

14. Figure 8: Please replace the commercial terms (PicoGreen, etc.) by generic terms.

Author's response: Commercial terms have been removed from figures and figure legends.

15. Figure 9: Please include scale bars for all the images in the panel (eg., B iii)
Author's response: Appropriate scale bars have been added to Figure 9. Legend corresponding to figure 9 has been adjusted to inform reader of the appropriate scaling of images, page 20 line 836-850..

16. Figure 10: Please revise the Y axis legend to include the units within parenthesis.
Author's response: Units on the Y axis have been included within parenthesis (ng/ml).

17. Please maintain a single space between the numeral and (abbreviated) unit, except in cases of %, x, and ° (i.e., the degree sign; excluding temperature) in the Table of Materials. Examples: 5 mL, 10%, 3°, 100 °C, 3x SSC
Author's response: Spaces have been used before units as requested, with the exception of %, x, and °.

Reviewer 1

Summary: in the article "A 3D collagen-based scaffold model of the neuroblastoma microenvironment" the authors described a number of standard lab methodologies that can be applied for the analysis of 3D scaffolding model of neuroblastoma. Although the title of their work is very interesting and inviting for reading, the methodologies described are lowering the value of the title.

Major Concerns:
The title of the manuscript needs to be adopted to its context. What one would expect is to see how the authors produced, managed and analysed the 3D scaffolding system and its microenvironment created after 14 days of neuroblastoma cell growth while comparing it with the native metastatic neuroblastoma milieu. Instead, the focus of the work is viability and morphological assessments of cell lines grown in 3D conditions.
Author's response: The authors have taken this into consideration and re-titled our manuscript, 3D in vitro biomimetic model of neuroblastoma using collagen-based scaffolds. This is a methodology paper that demonstrates how some already extensively characterised 3D bio-engineered platforms can be adopted or repurposed to study a different tissue pathology in a minimal format. We believe that it is sufficient to incorporate a more detailed collagen-based scaffolds features and manufacturing in order to describe this acellular component of the proposed 3D in vitro platform (Haugh et al., 2010; Fergal J. O'Brien et al., 2004) and focus more on characterisation of the cellular component as this is essential to study neuroblastoma biology and/or carry out drug screening. A table with the physical properties of the two scaffolds has been added to the text at page 4, line 133. Additional manufacturing information has been added also to the introduction section page 3 line 116-131. Both scaffolds are manufactured with identical blending, freeze-drying, sterilisation and crosslinking protocols.

Minor Concerns:
The reviewer is wondering why the scaffolds are kept first in adherent and only after 24h moved to low-adhesion plates? In order to allow that all the cells attach to the scaffold, wouldn't it be better to place them immediately in these types of plates?
Author's response: We appreciate this comment. This was our oversight. The authors can confirm non-adherent plates are used throughout the whole experiment. They are used immediately when plating cells at Day 0 and scaffolds are transferred to new non-adherent plates at Day 1. This has been addressed in the text, page 10 line 402 and page 11 line 432 - 436, to better clarify to the reader they are used for the entirety of the experiment.

Reviewer 2

Minor Concerns:
Two different scaffold compositions are considered, but in the protocol, it isn't considered the physical characterization of scaffolds as porosity, young modulus, or swelling. Would be interesting for other scientists to include a section of scaffolds characterisation
Author's response: We appreciate this comment. The proposed 3D model has acellular and cellular components. Since the focus is on the cellular component, the paper would benefit additional information regarding acellular component. A table with the physical properties of the two scaffolds has been added to the text at page 4, line 133. Additional manufacturing information has been added also to the introduction section page 3 line 109-131. Both scaffolds are manufactured with identical blending, freeze-drying, sterilisation and crosslinking protocols. Making scaffolds reproducible. Scaffolds only differ in the bio-composite additions to the scaffolds.

Reviewer 3

Summary: The manuscript by Gallagher et al aims to reverse engineer the neuroblastoma microenvironment using 3D collagen scaffolds functionalized with nHA and GAG. The manuscript is well written and provides sufficient step-by-step protocols which can be easily followed.

The scaffold has a dimension of 6 mm in diameter and 4 mm in thickness. This reviewer is wondering about the nutrient or drug diffusion into the scaffolds. Would the authors briefly comment on this?
Author's response: We would thank the reviewers for this consideration. We are confident that the collagen-based scaffolds used in this protocol have been optimised in depth to create manufacturing methods that create homogenous pore sizes and structure, conducive to allow cells to grow, infiltrate and allow nutrient and drug diffusion (Haugh et al., 2010; Fergal J. O'Brien et al., 2004). However, we have briefly commented on potential uneven nutrient distributions within the scaffolds on page 22, line 917-920. Curtin et al., 2018 describes both the Coll-I-GAG and Coll-I-nHA scaffolds required drug concentrations that matched clinically relevant values of that seen in vivo to mount a response on NB cell lines in 3D environment. The dose response of Kelly and kellyCis83 NB cell lines was 8-14 and 80-140 times higher, respectively, in the cell's grown in 3D scaffolds compared to 2D. Although the exact diffusion rates into scaffold are unknown, with this previously published data we can trust that the diffusion rates are to a higher physiologically relevant degree than 2D in vitro models.

In general, Alamar blue assay could be used to determine cell numbers. In my opinion, it would be more appropriate to perform a standard curve (cell number vs fluorescence signal) and calculate the fluorescence signal result back into „cell number", rather than % reduction. The author also mentioned on page 18 line743-746 that the Alamar Blue assay to be a simple and effective technique to support DNA quantification data. Do results (calculated cell number) from Alamar blue assay correlate to PicoGreen assay (Figure 7 vs 8)?
Author's response: We appreciate this comment, however we feel a standard curve is slightly more difficult given the 3D nature of this model. To generate a standard curve 2D plated cells would need to be used, and so the fluorescent signal may not be comparative to cells growing within 3D scaffolds. Adding to this, there is a possibility that some cells will have a higher metabolic rate than others, and so it is difficult to accurately convert to viable cell numbers. Therefore, we opted to look at the percentage reduction of the Alamar Blue reagent by cells, including a blank which contained Alamar Blue reagent diluted in full growth media, lacking only the cells in scaffolds. In the results on page 17, we have added a section comparing the results from Alamar Blue and PicoGreen assays from lines 711-

715. There were some differences in the growth trends for specific samples, however overall, the trends obtained from the two assays were consistent, and we feel that inclusion of both assays increases our confidence when examining these trends. Importantly, in many experimental strategies scientists look at relative changes rather than absolute, e.g. how metabolism on day 7 is different to day 1 or how cell viability changed in response to a drug? So, it is essential to run 2-3 different but robust assays to evaluate cell behaviour, gather data and accurately interpret the results.

Please also discuss that fluorescence interference from test compounds can be influenced the analysis of the Alamar Blue assay

Author's response: The authors have taken this point into consideration. They feel the appropriate controls are in place to account for inference from test compounds that may be influencing the cell viability assay. A brief overview of experimental design of the cell viability assay to account for inference; The cell viability assay was carried out under the same experimental protocols for each day of analysis. Flat bottom plates were used for each cell viability assay, as it is known that differing round and flat bottom plates can interfere with the cell viability assay reading. The blank used for the assay was the media that the cells were grown in, this eliminated interference from serums (e.g. FBS) (Goegan et al., 1995; Page et al., 1993).

The authors mentioned on Page 18 line 755 that the 3D models enable cell growth and infiltration visualization. Cell growth can be addressed using qualitative biochemical assays. This reviewer is wondering if the cell infiltration can be qualitatively analysed in this 4mm-thick scaffold

Author's response: Traditionally, to assess infiltration into scaffolds, scaffolds are fixed and undergo tissue processing. They can then be embedded in paraffin wax and cut into thin sections for histological staining and imaging. If the scaffold is embedded in a sideways orientation, it is possible to stain and image whole scaffolds from the side-view, enabling visualisation and qualitative assessment of cell infiltration and distribution throughout the scaffolds. Image data can then be converted to a % infiltration for quantitative assessment. We describe the preparation of scaffolds for histological staining in section 5.3, lines 568-658, and sample images of scaffold sections stained with H&E can be seen in Figure 9.

This reviewer would suggest some recent works that can be considered to add to the introduction (if the author feel that it actually adding value to their manuscript):

1. A general review of ECM characteristics and reverse-engineering of the tumor microenvironment can be considered to add in the introduction: Ouellette JN et al 2021 Bioengineering; Sapudom J et al 2018 Biomaterials Science.

2 On Page 3 line 94-97. The collagen-hydroxyapatite scaffold has also been used as a cell culture model of the tumor microenvironment (He F et al 2019 Biomaterials).

3. The author discussed GAG in the ECM. As short discussion of HA on cell behavior would be helpful. For example: It has been known that GAG exhibits molecular specific effects on cell behaviors (Kreger ST et al 2009 Matrix Biology, Sapudom J et al 2017 Acta Biomaterialia, Sapudom J et al 2020 Biomaterials Science).

4. A recent review on potential use of 3D models for drug screening can be considered to add in the introduction (Brancato V et al 2020 Biomaterials).

Author's response: Thank you for recommending these recent publications. We have assessed all of them, except for "He F et al 2019 Biomaterials" as we could not identify this paper.

In lines 75-78 when discussing the importance of the ECM in Cancer, we have added text referencing Brancato 2020 and Ouellette 2021 and their sources.

In lines 85-89 when discussing collagen in the ECM we have added text referencing Ouellette 2021.

In lines 90-93 when discussing the value of 3D matrices in assessment of cell response to chemotherapeutics we have added text referencing Sapudom 2018.

In lines 93-96 when discussing the correlation between 3D models and in vivo studies we have added text referencing Ridky 2010 (from Brancato 2020).
Some of the recommended texts focussed specifically on Collagen scaffolds supplemented with hyaluronan (Kreger 2009, Sapudom 2017, Sapudom 2020). In our manuscript, we mention supplementing collagen scaffolds with glycosaminoglycans (GAGs). While hyaluronan/hyaluronic acid is a category of GAG commonly used in scaffolds, we focus on the use of chondroitin-6-sulphate, another category of GAG. We have clarified this in lines 102-103, 112-115 and 118-119. We have maintained use of the abbreviation "Coll-I-GAG" throughout the document for ease of reading and as it has been used in this context in several previous publications referenced in the manuscript (O'Brien 2004, O'Brien 2005, Haugh 2011, Murphy 2010, Haugh 2009). We have not included the references on hyaluronan as it is not within the focus of this paper.

Reviewer 4
Summary: In this research, authors in detail described the protocol about studying neuroblastoma microenvironment using 3D collagen-based scaffolds. The manuscript is well presented and the optimizations of the method, particularly the dilution of cell, are very rigorous and detailed.

Minor Concerns:
Line 398 and 386, authors should pay attention to unit format.
Author's response: The authors have corrected all unit formatting throughout the text.

The difference between Coll-GAG and Coll-nHA, especially mechanical properties, should be discussed.
Author's response: Thank you for this consideration and we agree that additional information in the introduction on the background of the scaffolds should be given to the reader. A table with the physical properties of the two scaffolds has been added to the text at page 4, line 133. Additional manufacturing information has been added also to the introduction section page 3 line 109-131. Both scaffolds are manufactured with identical blending, freeze-drying, sterilisation and crosslinking protocols. Making scaffolds reproducible. Scaffolds only differ in the bio-composite additions to the scaffolds.

More explicit explanation of the statistical methods in manuscript is needed.
Author's response: The statistical methods used to analyse cell viability and DNA quantification results have been added as steps at the end of their respective sections (lines 501-509 and 554-561).

Once again, thank you all for taking the time to read and analyse the paper submitted. We hope all minor and major concerns have been addressed and look forward to your response.

Your sincerely,

Olga Piskareva
Corresponding author