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A three-dimensional spheroid-specific role for Wnt-β-catenin and Eph-ephrin signaling in nasopharyngeal carcinoma cells

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Original submission

First decision letter

MS ID#: JOCES/2020/256461

MS TITLE: A 3D-specific role of Wnt/β-catenin and Eph-Ephrin in Epstein-Barr virus-positive nasopharyngeal carcinoma cells

AUTHORS: Canhui Yi, Sook Ling Lai, Chi Man Tsang, Margarita Artemenko, Maggie Tang, Stella Pang, Kwok Wai Lo, Sai Wah Tsao, and Alice Sze Tsai Wong
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript. First, I apologize for the extended delay. We had a exceptionally challenging time arranging reviewers for this manuscript, likely due to the large outbreaks of COVID-19 in many countries this fall. Every effort was made by myself and the staff to arrange expert review as rapidly as possible but this was a very long review period.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. Reviewer 2 is particular is concerned that a number of the experiments are under-described to the point that it is difficult to understand which comparison were done, that some of the conclusions with regards to the role of EBV positivity are not fully supported, and that there is room to improve the depth of analysis with regards to the specific effects of the 3D environment on cell behavior and drug resistance. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating

where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this study, Yi and colleagues have reported a novel 3D model of EBV-positive NPC cells, and compared in multiple parameters with their 2D counterparts. These researchers have discovered that 3D cultures possessed such features as stronger migratory capacity, stemness and hypoxia gene expression, and higher resistance to chemodrugs. Endothelial cell coculture effectively enhanced 3D spheroid dissemination. Further, RNA-seq analyses of both 2D and 3D cultured cells showed significant differences in key gene/signaling between these two modes of cultures. Notably, known signalings such as VEGF/AKT/mTOR and previously unidentified signalings such as Wnt/beta-catenin, Eph-Ephrin were reported, followed by testing the responses to specific inhibitors of these signaling pathways that are in clinical trials. These key findings demonstrate the usefulness of the 3D culture model and potential applications in preclinical studies.

Comments for the author

Overall this study is clear and well designed, yet there are several issues to be resolved or answered.
(1) In Figure 1, in the flow cytometry analysis of cell cycle for both 2D and 3D cultured cells, the percentage of cell clusters in 3D cultures appeared to be high, and might interfere the interpretation of results, such as cell populations in G1, S or G2 phases. Could there be a better cell dispersion protocol to yield as many single cells as possible for analysis?
(2) In Figure 2, the font sizes for the bar graph are too small. And in Figure 2B, the lines overlapped with the scale bar. Please reposition the scale bar.

Reviewer 2

Advance summary and potential significance to field

Culture environment (dimensionality and co-culture) affect migration of EBV+ nasopharyngeal carcinomas (NPCs).

Comments for the author

This manuscript focuses on nasopharyngeal carcinomas (NPC) that are Epstein-Barr virus (EBV) positive as many papers have focused on EBV- NPCs. Significant concerns mentioned below limit my enthusiasm for moving forward. In particular, I do not understand why the authors play up the concern that studies are typically EBV- and they are using EBV+ cells but without a control to relate their work back to the body of literature in EBV- cells (see concern 1). This and other concerns would seem to be an important yet missed opportunity for the authors.

1. Throughout the manuscript, the authors use two EBV+ cell lines that recapitulate NPC characteristics especially when switching from 2D to 3D culture methods. What the authors do not establish is if these characteristic changes are specific to the lines themselves or if it is a result of

EBV positivity. Thus it is not clear how universal these behaviors are, i.e. the authors should establish if the 2D/3D effects occur with EBV- NPC for ALL experiments throughout the manuscript.

2. Calling the methods from Vinci et al a "3D spheroid" vs. "2D cell" migration assay is a bit deceptive to the average non-expert scientist. Saying "3D" implies to most people that the environment is 3D, but all that Figure 2 shows are either preformed spheroids or single cell suspensions plated onto a 2D substrate. The authors should remove the use of the terms 2D and 3D from this discussion entirely. Also the spheroids were "preformed" for an unknown number of days, and over that unspecified amount of time, the spheroids could make their own matrix. Thus when plated, they could remodel the additional matrix and use it to migrate onto the substrate; again, it is not the environment that was provided but the head start provided to those cells by culture in a spheroid (and who knows which ECM proteins they are making to impart this advantage - that could be examined with function blocking antibodies). The 2D equivalent doesn't exist since cells are trypsinized and replated. Finally, what is the logic behind each ECM protein concentration used? None is provided. Were these optimized in any way. Are there references in the literature to other optimization experiments using these exact cells?

3. There is no labeling strategy to prove that HUVECs were in "direct cell-cell contact" when in co-culture. Morphology differences between the cell types along is not good enough to prove beyond a reasonable doubt that the two cells are indeed touching. Would conditioned media induce the same effect? If so, its paracrine mediated and does not require direct contact. I would guess that the authors have a system where paracrine cytokines drive the response. Also in Figure 2B, the authors do not show C666-1 cells. Why? Is this same effect present with single cells in "2D."

4. Quantifying PCR bands from a gel is problematic due to differences in exposure times, image saturation (and these appear to have actin bands that are saturated), assay linearity, etc. Why did the authors choose this method in Figure 2C/D? The errors are so small and the bar graph values in the quantification so similar yet there are obvious differences between NPC43 and C666-1, e.g. BMI-1. Thus I must question this data.

5. No time course information is provided for when cells were lysed for RT-PCR in Figure 2C. How long had they been in culture on the substrates? How did those conditions change the response from baseline (i.e. cells in a TCP flask)? How long were the cells in Figure 2D under hypoxia? How much was each line affected vs. its normoxic control?

6. 2D v.s 3D drug resistance differences should be normalized to proliferative differences in Figure 3A. Moreover no experimental details are apparent in the methods section for these assays (my apologies if I missed it). So I am not sure how long they've been in culture. If they were plated and immediately assayed for 72 hours, their structural differences could play a role, but I would only feel comfortable if the authors could show that these behaviors moderated with time as the spheroid spread out into a 2D monolayer. Alternatively the spheroids could be trypsinized and plated as a single cell suspension to show if there was something non-cell autonomous about being in 3D.

7. Again the authors are vague and short on details for the RNA-sequencing assay. When was this performed relative to plating the single cell suspension vs. spheroid? RNA-seq for both lines should be performed (and a control EBV- line would be lovely too). Does "abundance" in Figure 4A mean FPKM? Or some other normalization? Specificity is lacking. The Venn diagram of "the two comparisons" in Figure 4C isn't obvious.
Figures 4A-B was a 2D/3D comparison. What is 4C comparing?

8. Why is only "3D' data shown in Figure 5? Isn't the point that dimensionality has an effect on pathways and thus drug efficacy?

Minor:
1. Line 29 - "Despite....in NPC" should be "Although..." for the correct tense in the clause.
2. Figure 2A - Matrigel panel - 2D image series does not appear to the he same cell culture over time.

3. I am not sure that I would agree that the authors' analyses are "comprehensive" on line 135. Its semantics but comprehensive is subjective. I would posit that comprehensive would include mouse models for example.

First revision

Author response to reviewers' comments

Below we address the reviewers' comments point by point. Page, line, and figure number refer to the revised manuscript.

Reviewer #1
1. In Figure 1, in the flow cytometry analysis of cell cycle for both 2D and 3D cultured cells, the percentage of cell clusters in 3D cultures appeared to be high, and might interfere the interpretation of results, such as cell populations in G1, S or G2 phases. Could there be a better cell dispersion protocol to yield as many single cells as possible for analysis?
Response: We appreciate the suggestion. We have repeated the experiments by passing our dissociated samples through cell strainers (70 μm) to obtain uniform single-cell suspensions for the flow cytometry analysis (Fig. 1B) (Materials and methods p. 12 , lines 288-292; Results p. 4 , lines 85-88).

2. In Figure 2, the font sizes for the bar graph are too small. And in Figure 2B, the lines overlapped with the scale bar. Please reposition the scale bar.
Response: The font sizes for the bar graph are enlarged (Fig. 2). Figure 2B has been replaced with new results following the suggestion by Reviewer #2 to repeat the experiment with CellTracker (in which Fig. 2B is now new Fig. 3A).

Reviewer #2
1. Throughout the manuscript, the authors use two EBV+ cell lines that recapitulate NPC characteristics, especially when switching from 2D to 3D culture methods. What the authors do not establish is if these characteristic changes are specific to the lines themselves or if it is a result of EBV positivity. Thus it is not clear how universal these behaviors are, i.e. the authors should establish if the 2D/3D effects occur with EBV- NPC for ALL experiments throughout the manuscript.
Response: We thank the Reviewer for the very useful comment. C666-1 and NPC43 are derived from undifferentiated NPC. There is currently no corresponding EBV-negative lines. The well-differentiated NPC cell lines: EBV-negative HK1 and its corresponding HK1-EBV (stably transfected with EBV) are included in the revised version (Materials and methods p. 12, lines 277-280). Our new data showed that there is little or no difference in cell cycle progression (Fig. 1B), migration (Fig. 2), stemness (Fig. 4A) and hypoxia (Fig. 4B & 4C) characteristics among EBV-positive C666-1, NPC43, HK1+EBV and HK1 (Materials and methods p. 12, 13, 14, lines 288-292, lines 296-306, lines 333-346; Results p. 4, 5, 6, lines 82-83, 85-88, 96-100, 119-124). While these characteristic changes might be independent of EBV status, it may not be conclusive due to the limited number of cell lines and their different histologic subtypes. Due to these limitations, we have toned down our claim on EBV positivity. However, this is surely a line of investigation that we would like to further pursue when undifferentiated EBV-negative NPC cells are available.

2. Calling the methods from Vinci et al a "3D spheroid" vs. "2D cell" migration assay is a bit deceptive to the average non-expert scientist. Saying "3D" implies to most people that the environment is 3D, but all that Figure 2 shows are either preformed spheroids or single cell suspensions plated onto a 2D substrate. The authors should remove the use of the terms 2D and 3D from this discussion entirely. Also the spheroids were "preformed" for an unknown number of days, and over that unspecified amount of time, the spheroids could make their own matrix. Thus when plated, they could remodel the additional matrix and use it to migrate onto the substrate; again, it is not the environment that was provided but the head start provided to those cells by culture in a spheroid (and who knows which ECM proteins they are making to impart this advantage - that could be examined with function blocking antibodies). The 2D equivalent doesn't exist since cells are

trypsinized and replated. Finally, what is the logic behind each ECM protein concentration used? None is provided. Were these optimized in any way. Are there references in the literature to other optimization experiments using these exact cells?
Response: We have studied these comments carefully and we thank the reviewer for the valuable inputs. We incorporated the suggestion accordingly where the terms 2D and 3D are revised as 2D monolayer and 3D spheroids to make them clear. All necessary information had been clarified in the Materials and methods where spheroids were pre-formed for 72 hours alongside with monolayer control which cultured for the same duration of time (Materials and methods p. 12, 13, lines 296-306). All monolayer cells and spheroids were then replated/ transferred onto new plates precoated with thin layer of ECM for immediate experiments in the following 6 days. We then tested the ability of tumour spheroids to disseminate and migrate out from those spheroids. This process mimics tumour cells migration from a small tumour cluster or micro-metastasis through engagements with host stromal matrix proteins.

3. There is no labeling strategy to prove that HUVECs were in "direct cell-cell contact" when in co-culture. Morphology differences between the cell types along is not good enough to prove beyond a reasonable doubt that the two cells are indeed touching. Would conditioned media induce the same effect? If so, its paracrine mediated and does not require direct contact. I would guess that the authors have a system where paracrine cytokines drive the response. Also in Figure 2B, the authors do not show C666-1 cells. Why? Is this same effect present with single cells in "2D."
Response: We thank the Reviewer again for the useful suggestion. Results of all four cell lines in this study are showed (Fig 2B is now new Fig. 3A). To track both cell populations in heterotypic condition, we now used tumor spheroids labelled with CellTracker Orange CMRA dye and unlabeled HUVECs (Materials and methods p. 13, line 309-315). Coculture assays (direct cell-cell contact) displayed a disperse state of all tumor spheroids, particularly to greater extent in C666-1 and HK1 spheroids, but less efficient in NPC43 and HK1+EBV spheroids (Fig. 3A). Our results also showed that conditioned medium from HUVECs (non-contact) induced similar effect (Fig. 3B, Fig. S3, Fig. S4) (Materials and methods p. 13, 14, lines 316-325). These findings, labelling strategy and conditioned medium, all demonstrate a contact-independent mechanism (Results p. 5, 6, lines 105-116).

4. Quantifying PCR bands from a gel is problematic due to differences in exposure times, image saturation (and these appear to have actin bands that are saturated), assay linearity, etc. Why did the authors choose this method in Figure 2C/D? The errors are so small and the bar graph values in the quantification so similar yet there are obvious differences between NPC43 and C666-1, e.g. BMI-1. Thus I must question this data.
Response: We appreciate the suggestion. We have carried out RT-qPCR to precisely quantify the mRNA levels, which are now provided in new figure Fig. 4A & 4B. The newly obtained RT-qPCR data are consistent with previous RT-PCR results (Materials and methods p. 14, lines 333-346; Results p. 6, lines 119-124).

5. No time course information is provided for when cells were lysed for RT-PCR in Figure 2C. How long had they been in culture on the substrates? How did those conditions change the response from baseline (i.e. cells in a TCP flask)? How long were the cells in Figure 2D under hypoxia? How much was each line affected vs. its normoxic control?
Response: Following the Reviewer’s suggestion, all necessary details about the Materials and methods used have been provided in the revised version (Materials and methods p. 14, lines 333-346). All the spheroids and monolayer control have been cultured for 72 hours then harvested for RT-qPCR. No hypoxia culture condition is involved in our study.

6. 2D v.s 3D drug resistance differences should be normalized to proliferative differences in Figure 3A. Moreover no experimental details are apparent in the methods section for these assays (my apologies if I missed it). So I am not sure how long they've been in culture. If they were plated and immediately assayed for 72 hours, their structural differences could play a role, but I would only feel comfortable if the authors could show that these behaviors moderated with time as the spheroid spread out into a 2D monolayer. Alternatively, the spheroids could be trypsinized and plated as a single cell suspension to show if there was something non-cell autonomous about being in 3D.
Response: All necessary details about the Materials and methods used have been provided in the revised version (Materials and methods p. 15, lines 355-356). After preformed for 72 hours, the spheroids were immediately assayed for drugs treatment on agarose-coated plate to maintain the

spherical formation. 2D monolayer and 3D spheroids drug resistance differences is now normalised to their proliferative ratio. In concordance with our previous results, new analyses (survival percentage relative to DMSO control after normalised to 3D/2D proliferation ratio) show higher drug resistance in 3D spheroids compared to 2D monolayer cells (Fig. 5 and 6) (Results p. 6, lines 132-137).

7. Again the authors are vague and short on details for the RNA-sequencing assay. When was this performed relative to plating the single cell suspension vs. spheroid? RNA-seq for both lines should be performed (and a control EBV- line would be lovely too). Does "abundance" in Figure 4A mean FPKM? Or some other normalization? Specificity is lacking. The Venn diagram of "the two comparisons" in Figure 4C isn't obvious. Figures 4A-B was a 2D/3D comparison. What is 4C comparing?
Response: Necessary details for RNA-seq is now provided (Materials and methods p. 16 lines 376-383). The spheroids were preformed for 72 hours alongside with monolayer cells then harvested for RNA-seq. RNA-seq data comparison among 2D monolayer and 3D spheroids of all four cell lines employed in this study are now added (Fig. S6) (Results, p. 7, lines 143-152). Differentially expressed genes in 3D spheroids compared with 2D monolayer of newly presented genome-wide analysis on all 4 cell lines are in concordance with our previous reported data on C666-1. In addition, comparison of RNA transcriptome profiles between HK1 and its HK1+EBV revealed no significant differences. While these gene expression changes might be independent of EBV status, it may not be conclusive due to the limited number of cell lines and their different histologic subtypes. Due to these limitations, we have toned down our claim on EBV positivity. However, this is surely a line of investigation that we would like to further pursue when undifferentiated EBV-negative NPC cells are available.

8. Why is only "3D' data shown in Figure 5? Isn't the point that dimensionality has an effect on pathways and thus drug efficacy?
Response: We appreciate the suggestions. Both 2D monolayer control and 3D spheroids data are now shown in the particular figures after revised (now Fig. 5, 6 and 8) (Results p. 6, 7, 8 lines 132-137, 161-163, 165-166, 169-171).

Minor:
1. Line 29 - "Despite....in NPC" should be "Although..." for the correct tense in the clause.
Response: The tense is corrected (Abstract p. 2, now line 24).

2. Figure 2A - Matrigel panel - 2D image series does not appear to the he same cell culture over time.
Response: The figure has now been substituted with image from previously repeated experiment (new Fig. 2).

3. I am not sure that I would agree that the authors' analyses are "comprehensive" on line 135. Its semantics but comprehensive is subjective. I would posit that comprehensive would include mouse models for example.
Response: The word ‘comprehensive’ has been removed (Discussion p. 9, line 197-198).

Second decision letter

MS ID#: JOCES/2020/256461

MS TITLE: A 3D-specific role of Wnt/ß-catenin and Eph-Ephrin in nasopharyngeal carcinoma cells

AUTHORS: Canhui Yi, Sook Ling Lai, Chi Man Tsang, Margarita Artemenko, Maggie Tang, Stella Pang, Kwok Wai Lo, Sai Wah Tsao, and Alice Sze Tsai Wong
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but Reviewer 2 raised a couple of specific points that will require amendments to your manuscript. I hope that you will be able to carry these out because I would like to be able to accept your paper. Please describe your changes fully in the cover letter as I would like to be able to decide myself, without further peer review.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This reviewer is satisfied with the revision.

Comments for the author

This reviewer is satisfied with the revision.

Reviewer 2

Advance summary and potential significance to field

See prior comments

Comments for the author

I would like to thank the authors for the thorough response to my long list of concerns. I believe that the authors responded strongly to my first concern, which I felt was exceptionally important. Given the limitations with EBV- lines, I can understand their approach and believe that they answered the concern as best as possible.

With respect to the matrix present in the spheroids vs. monolayer, I appreciate the revisions made to the methods and results sections. However I think that it is an important discussion point to say that while the materials are coated with the same matrix when both cell system are added, the spheroid has had 72 hours to make matrix. Even though the monolayer culture has had the same time in culture, the trypsin-based replating method used on these cells (page 12) will make the niche's matrix state a bit different. I think that in the context of this work, that is at least an important point to mention in the discussion.

Again I would like to thank the reviewers for the attention to my other comments/concerns. I believe that those have been addressed.

Minor:
1. In their response, the authors updated Figure 1B, and I note that the figure legend now does not indicate what the green section is in the plots; red and blue sections are appropriately annotated.

Second revision

Author response to reviewers' comments

We sincerely thank the reviewer for the helpful comments. In response to Reviewer's comments, we have revised the manuscript that we think further improve the clarity of the manuscript.

Page and line numbers refer to the revised manuscript.

Reviewer #2
1. With respect to the matrix present in the spheroids vs. monolayer, I appreciate the revisions made to the methods and results sections. However I think that it is an important discussion point to say that while the materials are coated with the same matrix when both cell system are added, the spheroid has had 72 hours to make matrix. Even though the monolayer culture has had the same time in culture, the trypsin-based replating method used on these cells (page 12) will make the niche's matrix state a bit different. I think that in the context of this work, that is at least an important point to mention in the discussion.
Response: We are grateful to the reviewer's suggestion, and we have now mentioned "whether the 3D spheroids may secrete their own extracellular matrices within the particular spheroid formation duration mimicking the in vivo extracellular matrices in solid tissues, and whether the trypsin-based replating method applied to the 2D monolayer compared to its 3D spheroid counterpart has an impact in modifying cell surface cellular proteins and thus altering the cellular signalling warrant further investigations" in the discussion (p. 9, lines 213-217).

2. In their response, the authors updated Figure 1B, and I note that the figure legend now does not indicate what the green section is in the plots; red and blue sections are appropriately annotated.
Response: We apologise for the misannotation, and we have annotated the different sections. G0/G1-phase (blue area, indicates non-dividing cells); S phase (green area, indicates cells undergo DNA synthesis during replication) and G2/M-phase (red area, indicates cells growth and mitosis) (p. 24, lines 558-560).

Third decision letter

MS ID#: JOCES/2020/256461

MS TITLE: A 3D-specific role of Wnt/ß-catenin and Eph-Ephrin in nasopharyngeal carcinoma cells

AUTHORS: Canhui Yi, Sook Ling Lai, Chi Man Tsang, Margarita Artemenko, Maggie Tang, Stella Pang, Kwok Wai Lo, Sai Wah Tsao, and Alice Sze Tsai Wong
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.