Reviewer’s report

Title: Multicellular tumor spheroid models to explore cell cycle checkpoints in 3D.

Version: 1 Date: 26 September 2012

Reviewer: Vicky Avery

Reviewer’s report:

Major essential revisions:

Results
The authors state that “we selected two representative spheroid growth stages” Could hey please provide reasons for why they selected these. What is the significance of the two growth stages that they chose? How many days do they cells / spheroids need to be in culture to reach the diameters reported? Is this reproducible?

A critical point would be the definition of the centre of a spheroid as this forms the basis of the majority of the studies performed. The authors state: “spheroid sections that were identified as closely passing through the spheroid center”. How was the centre of the spheroid defined both from the imaging and the analysis perspectives, and how was this confirmed?

The comment : “displayed an expression pattern of all cyclins that mirrored the proliferation gradient reported above”. Requires more explanation. Perhaps the authors could provide more detail of the cyclin organisation in large spheroids.

The statement: “while a clear gradient of both Fucci-red and Fucci-green is detected in large spheroids” is not supported by the data. The figures show a partial lack of staining in the centre of the spheroid where there are no cells or a decline in cell population. When you take this into consideration the pattern of staining is not gradient, in fact it looks dispersed at even intervals across the spheroid. Thus this is not convincing data… This is also evident in the Fucci-green results. There are ‘clearly’ less cells present in the middle of the spheroid otherwise there is a very even distribution of this stain throughout the spheroid.

Have the authors checked the actual numbers of cells in the centre of the spheroid and whether these are dead, or apoptotic?

There are no statistics provided to support graphic quantification in Fig 3B and D. In Fig 3D the graphs indicate that there is less staining occurring at ~ 130 µM – is this because there are simply less cells?

The data presented in fig. 3C visually looks similar whether a small or large spheroid, thus is not convincing.

There is no reference to small spheroids in the Fig 3 legend – only referenced in
There does not appear to be a “clear” gradient present in Fig 4A control for Fucci-red.

No statistics are provided for the data presented in Fig 4B; Fig 5B

The authors have chosen several known drugs to validate their system. One of these is lovastatin. Could the authors please clarify their choice of 60µM of lovastatin which they used in the 2D assays and how this correlates to the literature values for observable effects with this drug. The therapeutic dose of lovastatin is 50 nM to 500 nM, thus the concentration used is substantially higher. Literature precedent does indicate that concentrations #50 µM inhibit Ras, Rho, and Rap prenylation but 50 nM to 500 nM do not. However, what other effects does such a high concentration have on the cell’s general health?

The authors need to provide the compound / drug exposure times for all experiments – required here for lovastatin.

Given that the study used 60µM lovastatin for 2D cell assays, it was surprising that the concentrations used for the 3D study are significantly lower, and that an effect was observed at 5µM. The authors indicate cell death and spheroid collapse with high concentrations – what are the higher concentrations used? Generally speaking it is observed that the concentrations required to have an effect in 3D are greater than those in 2D and / or the exposure times, for the obvious reasons of penetration and size. It is important that the authors explain the discrepancies mentioned above, and provide evidence and experimental reasoning for their study.

The vehicle used for drug delivery has not been provided nor the data demonstrating to ill effects associated with it. This data is necessary.

Again, in the studies performed with etoposide the authors do not explain the concentration chosen. Please provide the basis for your decisions.

The sentence (Pg 13) : “As is the case with many other cell lines………..” requires re-writing. For example: Twenty-four hours after 1 hr treatment ……..

Have the authors identified the level of cell death / apoptosis under the conditions they are testing drugs?

More details are required regarding how the analysis was performed, namely the algorithms and reproducibility

Discussion

Page 15: define what is considered a “tolerable” concentration of lovastatin.

How would the authors confirm that the issues with lovastatin are associated with lack of penetration?

What are the impacts of continual drug exposure compared with limited exposure and removal of a drug?
Minor compulsory revisions:

Abstract:
Grammatical concerns
Example 1: “We describe in details the changes”

Background:
Use of non-scientific jargon
Example 1: “new antiproliferative drugs that fills the gap between monolayer cultured cells and animal models”

Also grammatical concerns with this sentence.

Grammatical concerns:
Some of the grammatical errors result in incorrect implications:
Example 1 - “is to target cell cycle checkpoint that ensure maintenance of the genome integrity”
Do the authors mean to infer that there is only one cell cycle checkpoint?

Poorly constructed sentences:
Example 1: “How are the overall duration of cell cycle…. remains poorly known.”
Example 2: “Here we report how the characterization of cell cycle parameters changes in growing pancreatic MCTS by various type of cues in association with new technological developments can be used to explore the regionalization and to monitor the activation of cell cycle checkpoints in 3D”.

Methods:
Please indicate source of cell lines.
More detail is required in the methods section.
On page 5 the authors refer to mAG-Geminin and mKO2-Cdt1 in the cell culture methods section but there is no indication to the reader that these are Fucci-green and Fucci-red. The authors need to provide this information rather than assume the reader knows this. The first mention of Fucci#Red and/or Fucci#Green is when they appear in the image acquisition and analysis sections without prior explanation.

There is also a tendency to interchange Cdt1-mKO2 with mKO2-CDt1, and Geminin-mAg with mAG-Geminin (Pg 9). The authors need to be consistent with terminology.

Have the authors compared spheroid formation and growth in reduced FCS%? This may be important for pancreatic cancer.

Grammatical concerns:
Example 1: CO2 should be CO2
Example 2: “EGF was removed by washing 400µm in diameter spheroids” – should read µl

Suppliers should be provided for reagents, for example DAPI stain.

More detail on spheroid formation would have been useful such as cell number and volume, rather than having to find the original manuscript (ref14) describing this to have any idea of the procedure used.

The authors refer to Ki-67 immunofluorescence staining, and again assume the reader knows all the details. A reference and brief description would be useful to include. The authors need to ensure that they use the same terminology consistently: Ki-67 interchanged with KI-67 (P8)

The authors refer to the use of known drugs but provide no details of how these were prepared or the solvent / vehicle used. This needs to be included.

For both the proliferation and Hypoxia studies it would be good to know which day of spheroid growth these are undertaken on.

Results
Grammatical concerns:

P.8 2nd paragraph 2nd sentence – should read For this aim, not “To this aim”.
In addition, the last 2 sentences in this paragraph need to be re-written

Are there alternative cell cycle markers which could help to confirm their results?

The authors indicate that: “Pimonidazole staining is observed in large spheroids and its regionalization matches those of the proliferation parameters”. Whilst the intent is understood, the use of the word “match” is not appropriate. It would read better if the authors were to clearly state that the marker for hypoxia is evident in the inner regions of the spheroid while proliferation exists in the outer regions.

Grammar “We therefore explored systematically cyclins expression,”
P9 1st paragraph last sentence needs to be re-written

There is no reference to small spheroids in the Fig 3 legend – only referenced in the text.

P13 spelling mistakes “Strickingly”; quantization;

Discussion
The frequent use of non-scientific jargon needs to be curtailed.
Use of words such as ‘precisely’ should be avoided.

Page 14: (ie away from nutrients, oxygen, ……..) please correct and rewrite sentence.

The authors have a tendency to overstate the outcome with no statistical
backing.

Conclusions:
The conclusions need to be more informative, summing up all of the research discussed.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

'I declare that I have no competing interests'