UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI
SCIENZE BIOMEDICHE PER LA SALUTE

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Dear Editor,

I would like to thank you and the reviewers for the careful revision of our manuscript and for the useful suggestions.

According to reviewer's comments, we prepared a revised version of the manuscript. Changes in the text are highlighted in red in the final version. We also enclose our itemized response to your comments.

We hope that the quality of our manuscript has been significantly improved.

Best regards
Nicoletta Gagliano

N. Gagliano

Reply to Reviewer's comments

Author's responses are highlighted in bold.

Reply to Reviewer 02543990

This is an interesting study that investigated the cellular features of and EMT related marker expression in pancreatic cancer cell lines under 2D and 3D culture conditions. The experiments are well designed and very nice images are provided. However, there are still a few issues need to be addressed to improve the quality of this manuscript.
We would like to thank the Reviewer for the encouraging comments.

1.Statistical results should be presented in Figures 8A, 8B, and 8D.
We thank the Reviewer for the suggestion. Since the number of samples in each experimental group was low, we did not analyze our data by inferential statistic but we aimed at measuring differences between cells grown in 2D-monolayers or in 3D-spheroids by calculating the size effect according to Cohen (Cohen's d). In the revised manuscript we showed in Figures 8A, 8B and 8C when Cohen's d suggests a large size effect. Figure 8 and the relative figure legend were modified accordingly.

Dipartimento di Scienze biomediche per la salute
Nome Via, n° - CAP Milano, Italy
Tel - Fax - indirizzomail@mail.it

2. For the cell line of HPAC, the expression of E-cadherin at mRNA level (Figure 8A) doesn't agree with the protein level (Figure 8B &8C), what is the potential mechanisms behind?
The potential mechanisms responsible for the differences in gene and protein expression profile can be based on a lower stability of mRNA, that is degraded and not translated, and/or on posttranslational modifications of the protein. In this specific case, some posttranslational modifications of E-cadherin were described, Responsible for the proteolytic cleavage of the protein and the release of protein fragments from the plasma membrane. This proteolytic cleavage of E-cadherin can be exerted by γ-secretase or caspase-3, and is catalyzed by matrix metalloproteinases (please see David et al. 2012; Ferber et al., 2008). Due to these posttranslational mechanisms, high levels of mRNA do not correspond to high protein levels.
Our results in Figure 8 are consistent with this mechanism, as showed in the Western blot analysis revealing more evident low molecular weight fragments originated from E-cadherin proteolysis in PDAC cells grown in 2D- monolayer.

3. Compared to 2D culture condition, cells cultured in 3D condition tended to express similar or reduced level of αSMA in both HPAF-II and HPAC cell lines, but it was drastically opposite in PL45 cells, how to explain it?
αSMA is a key marker of EMT, highly expressed in HPAF-II, HPAC and PL45 cells. These three cell lines exhibit a very similar epithelial-like phenotype, although some differences were reported. HPAF-II cells were described as well-differentiated cells (Gagliano et al. Anti-cancer Drugs 2012; Rajasekaran et al. Pancreas 2004), HPAC as moderately well-differentiated (Deer et al. Pancreas 2010). By contrast, we characterized PL45 in our previous work and we described a less differentiated phenotype on the basis of acting cytoskeleton arrangement (diffuse cytoplasmic actin and no evident cortical actin). We can hypothesize that higher αSMA expression in PL45, compared to HPAF-II and HPAC cells, could be consistent with their less differentiated epithelial-like phenotype.

Reply to Reviewer 02439200

The manuscript entitled "Epithelial-to-mesenchymal transition in pancreatic ductal adenocarcinoma" addresses an important aspect of tumorigenesis, namely EMT. Although this is an important area of investigation with potential applications to the development and progression of PDAC, I have some significant concerns about this manuscript.

Major concerns:
1) The authors state that E-cadherin is down regulated in PDAC yet they show that the expression of E-cadherin is increased in 3-D cultures.
The loss of E-cadherin is universally considered the key event of EMT. Many papers focusing on PDAC and PDAC cells describe the loss of E-cadherin in this carcinoma.

The problem is that some PDAC cells, although they are highly malignant and invasive, retain E-cadherin expression and functional adhering cell junctions, therefore exhibiting an epithelial phenotype. This is the case of the three PDAC cell lines we used in this study.
The maintenance of an epithelial phenotype and the concomitant expression of invasive and malignant potential are not mutually exclusive but can be concomitant, as previously demonstrated (please see Gagliano et al. 2012).
HPAF-II, HPAC and PL45 PDAC cells, cultured in 3D-spheoids, not only retain E-cadherin expression, but also reveal an up-regulation of the protein. We feel this is the novelty of our study and a new information on PDAC. Also, this is a confirmation that PDAC cells are characterized by an epithelial phenotype. As a consequence, the stronger cell adhesion in 3D-spheroids mediated by higher levels of E-cadherin may be important for the collective migration and invasion of these carcinoma cells.

2) Additionally, the authors state that in 3-D cultures cells undergoing EMT dedifferentiate. Although some cells in 3-D cultures express α-SMA and Col-1, so do some cells in the 2-D cultures. These proteins are normally markers for activated pancreatic stellate cells. Are they expressed in metastatic PDAC cells?
Some EMT markers such as αSMA and COL-I are typically expressed by activated fibroblasts and pancreatic stellate cells in the stroma. αSMA is an EMT marker expressed also in metastatic PDAC cells, as previously demonstrated (Gagliano et al., Anti-cancer Drugs 2012; Li et al., Tumour Biology 2015). Also COL-I was detected in PDAC cells: its expression was demonstrated in AsPC-1, BxPC-3, CAPAN-1, CAPAN-2, PANC-1 (for reference see Lohr et al., Br.J.Cancer 1994; Yeon et al., PlosOne 2013).

3) It is not clear what the overall utility of 3-D culturing of PDAC cells is. There is tremendous variation within and between the various cell lines. It should be clearly pointed out how this study will facilitate investigation of PDAC initiation and progression.
Many studies demonstrated that 2D-monolayer cultures are characterized by limitations since they exhibit a remote resemblance to the *in vivo* condition. As a consequence, they do not mimic the living tissues and are a poor predictor of whether a given drug will ultimately yield clinical benefit. In contrast, 3D *in vitro* tumor models of human solid tumors mimic *in vivo* tumor conditions, including 3D architecture and cell–cell communication (please see Hicks et al. J Natl Cancer Inst 98: 1118–1128, 2006; LaBarbera et al., Expert Opin Drug Discov 7: 819–830, 2012; Howes et al. Mol Cancer Ther 6: 2505–2514, 2007; Yeon et al. PLoS ONE 8: e73345, 2013, and many others).
Our study, according to these previous suggestions, shows that PDAC cells cultured in 2D-monolayers and 3D-spheroids exhibit a different phenotype in relation to the expression of EMT markers, to the pattern of expression of proteins playing a key role in EMT (such as E-cadherin), and to the presence of cell structures (such as invadopodia) used for tumor invasion. Especially some EMT markers such as N-cadherin and COL-I, were almost undetectable in 2D-monolayers but were revealed in 3D-spheroids. We feel that all these experimental evidences strengthen the previously suggested importance to find an *in vitro* experimental model able to better mimic *in vitro* the behavior of cancer

cells, in order to obtain useful information understand the phenotype and the behavior of PDAC cells, and to test new therapeutic tools.

Minor concerns:

1) In general the micrographs are not large enough or of high enough quality to assess the validity of the authors interpretation of the micrographs.
Micrographs showing confocal and electron microscopy results were of high quality and support the results and their interpretation. Unfortunately, due to the submission procedure, these micrographs were embedded in the text and, very likely, some quality was lost. In the revised manuscript we tried to enlarge these micrographs to help their interpretation. If needed, we can provide the high quality micrographs.

2) The authors continually interpret the micrographs regarding subcellular structures and organelles but do not provide evidence that these structures and organelles are in fact what the authors believe they are.
Electron microscope micrographs we submitted are representative and summarize the results obtained on the ultrastructure of PDAC cells grown in 2D and 3D experimental conditions. To give an overview of the ultrastructure, some images were obtained at low magnification but we confirm the identification of the structures we describe. In fact, the description of our results and our conclusions are derived after observing many samples at TEM under the appropriate magnification. This careful observation gave us the possibility to recognize the specific structures we described. For example, tight junctions have been identified on the basis of the presence of focal fusion between the adjoined cells, adherens junctions have been identified on the basis of their morphology and of the presence of microfilaments coming from the cell cortex. Finally, desmosomes have been identified for their typical structure and on the basis of the presence of intermediate filaments attached to a plaque located on the cytoplasmic side of the plasma membrane, as shown at high magnification in Figure 3, representing HPAC ultrastructure.

Additionally, the magnification does not always seem to be right.
We thank the Reviewer for his comments about some magnifications. As suggested, we carefully checked for electron microscope micrographs magnification and we verified the scale bars. Some figure legends were corrected accordingly.

The nuclei under similar conditions are not the same size.
We agree with the observation that nuclei, at the same magnification, are not of the same size. This is a characteristic of PDAC cells exhibiting a nuclear polymorphism of different degrees, as described by Sipos (Sipos et al., Virchows Arch 2003).

Dipartimento di Scienze biomediche per la salute
Nome Via, n° - CAP Milano, Italy
Tel - Fax - indirizzomail@mail.it

3) The data presented in this manuscript would be much stronger if the co-localization of the various proteins was investigated.
Confocal microscopy results show the expression of many different proteins in PDAC cells. E-cadherin, β-catenin and cortical actin are located at cell boundaries ad are expressed at high extent in all the considered cells. Therefore, we feel that is easier to understand their localization and expression if they are shown separately. This is particularly evident when describing acting arrangement in relation to the presence of invadopodia. In relation to the remaining markers analyzed by confocal microscopy, they are unrelated for their localization and some of them expressed at very low levels. Therefore, we feel that a co-localization micrograph could not be able to highlight and describe the differences of PDAC cells in the two different experimental conditions.

4) The 2-D cultures are shown in longitudinal sections whereas the 3-D cultures are shown in vertical cross-sections. It is impossible to compare these images to each other. How long have these cells been culture before fixation.
The section (and therefore images) of cells grown in 2D or 3D cultures is dependent on the different experimental conditions. When cells are cultured in 2D-monolayers, they grow flat and attached on the substrate forming a very thin single layer. To analyze these cells by electron microscopy it is only possible to obtain a section showing the cell attached on the plastic substrate. In contrast, cells cultured in 3D-spheroids grow in 3D free floating aggregates and they are cut on a different plane. In this case the orientation of the cut is not relevant since the morphology of cells is always the same.
However, although the section is different, in both situations is possible to clearly observe the general morphology, the presence of cell junctions and other characteristics useful to understand if the phenotype of cells grown in 2D and 3D cultures is different.

5) In figure 8 it is unclear what bands are being quantified. Are the authors referring to all bands or just the 120 kD band?
The bands obtained by Western blot analysis that were quantified in bar graphs in Figure 8B are the 120 kDa band, corresponding to the full length E-cadherin. This was described in the results section.

6) This manuscript is full of jargon and needs to be carefully proofread.
To improve the manuscript, the manuscript was carefully revised for the English language.

Reply to Reviewer 00227509

This manuscript describes EMT phenomena in 3-D cell culture, using 3 kinds of pancreatic cancer cell lines. The authors investigated ultrastructural characterization of EMT with transmission electron microscopy and expression of EMT-associated protein such as α SMA and E-cadherin. A marked EMT phenomenon was observed in 3-D cell culture, compared to 2-

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Tel - Fax - indirizzomail@mail.it

D culture. The results are very interesting. The authors should change a few points before publication as described below.
We would like to thank the Reviewer for the positive comments.

1.The authors should describe phonotype of 3 pancreatic cancer cell lines and discuss the relationship between EMT potentials and character of each cell.
We thank the Reviewer for the suggestion. We inserted in the Discussion a comment on the possible relationship between PDAC phenotype and EMT potential. Since new references were added, the reference list was modified accordingly.

2.Conclusion is too long. The authors should make the conclusion shorter.
According to Reviewer's suggestion, in the revised manuscript the conclusion was shortened.

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