Answers to the reviewers' specific comments

Reviewer: 3

General Comments: The authors show that metastatic clones of PDAC expressed higher myoferlin level than the corresponding low metastatic ones. Human pancreatic cells expressing high myoferlin level have higher migratory potential than cells with a low myoferlin abundance and the migration rate is dependent on OXPHOS activity. Moreover, myoferlin silencing leads to a migration decrease associated with a reduction of mitochondrial respiration.

Specific comments

1. The Fig. 1. Panel A seems to be the same reported in fig. 2A of Rademaker et al., Oncogene 2018. The panel should be replaced with another one

Authors thank the reviewer for his/her careful observation and we apologize for our inattention. The western-blot Fig. 1A was replaced and correlation between migration speed and mean myoferlin abundance was recalculated in accordance.

2. The authors compared the protein level of myoferlin in different human pancreatic cell lines and associated the protein level with the migration rate. However, the cell lines were cultured in different medium, as reported in "methods" section. Can the different culture conditions affect the myoferlin expression level?

The reviewer's suggestion is very pertinent. Indeed, culture conditions are slightly different between cell lines and are summarized in the table below. Thanks to the supplementation described in the material and methods section, only the glutamine concentration differs from one cell line to the others.

	PANC-1	***BxPC-3***	***Patu8988T***	***MiaPaCa-2***
Myoferlin expression	***++***	***+++***	***+***	***-***
Medium	*DMEM*	*RPMI1640*	*DMEM*	*DMEM*
Serum	*10%*	*10%*	*10%*	*10%*
Glucose	*4.5 g/L*	*4.5 g/L*	*4.5 g/L*	*4.5 g/L*
Glutamine	*2 mM*	*2 mM*	*2 mM*	*4 mM*

Table 1: Culture conditions for PANC-1, BxPC-3, Patu8988T and MiaPaCa-2 cell lines.

We are currently investigating the impact of culture conditions, mainly glucose and glutamine availability, on myoferlin expression (Fig 1). We noticed that myoferlin expression is only slightly increasing when cells are cultured in low glutamine condition (1mM). As such, we can reasonably assume than the relative myoferlin abundance in the studied cell lines is not the result of culture conditions. For confidential reasons, this information will not be included in the manuscript.

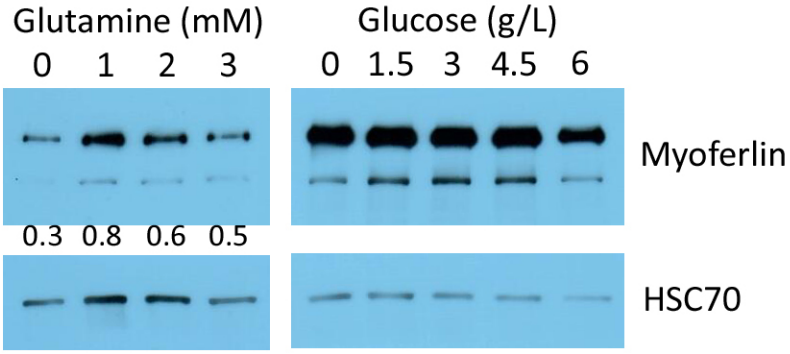


Fig 1: Myoferlin expression in PANC-1 cell line according to culture conditions.

3. Figs 2 and 3. The authors show that the higher migration rate in high myoferlin expressing cell lines is dependent on OXPHOS activity and that the myoferlin silencing induced a reduction of migration and OXPHOS activity. This does not exclude that the same mechanism can occur in cell lines with a lower migration rate and a lower myoferlin level. The same experiments, perhaps at 48 h, should be performed in PaTu8988T and MiaPaCa-2 cell lines.

We thank the reviewer for his/her very interesting suggestion. However, a 48h migration assay will require the use of an anti-mitotic compound (mitomycin) in order to discriminate the proliferation from the migration. This assay requires first the titration of mitomycin in order to determine its optimal non-toxic concentration. Due to the short deadline (10 days), we feel unable to performed this experiment adequately.

4. The OXPHOS activity should be also performed in PaTu8988T and MiaPaCa-2 and compared with BxPC-3, Panc-1, even if, again, different culture conditions can affect the OXPHOS activity.

Authors thank the reviewer for this relevant comment. Oxygen consumption rate (OCR) of BxPC-3, Panc-1, PaTu8988T and MiaPaCa-2 cell lines were compared on a pairwise fashion in the same culture medium without prior adaptation. Results were included as Fig1E, and showed significant decrease of basal OCR in cell lines according to their low migratory abilities. Interestingly, the basal OCR correlates highly with the myoferlin abundance.

5. Pag. 9 line 173: Rather than “Myoferlin Increases OXPHOS Activity in HM Clones” Reduction of Myoferlin is associated with a decrease of OXPHOS activity… or the Myoferlin silencing decreases OXPHOS activity …

Authors thank the reviewer for the sounding suggestion. Title of section 2.5 was amended according to reviewer’s suggestion.

6. Fig. 5, panel B. An evaluation of some OXPHOS component in addition to IDH should be performed

We want to thank the reviewer for his/her sound comment. We performed a western blot analysis of NADH dehydrogenase subcomplex NDUFB5 (complex 1) and cytochrome C oxidase COXIV (complex 4) in LM1-3 and HM1-3 clones. Unfortunately, NDUFB5 antibody gave us no

signal while COXIV showed no significant difference between conditions. COX IV western blot was added as Fig. 5C.

7. Fig. 5, panels A and C. The average values of the same clone are different in the panels. For example HM3 has a basal OCR of around 25 in the left panel and 40 in the right panel; the same for LM3 clone comparing panel A and panel C.

Authors thank the reviewer for his/her relevant remark. Pairwise comparisons of OCR were performed using an XFp seahorse and normalized by Hoechst incorporation as previously described [1]*. This normalization procedure is relative and not absolute, explaining why the unit of the OCR axis is pmol/min/A.U. (arbitrary unit). Indeed, the Hoechst incorporation and fluorescence emission are highly depending of several factors including time, temperature, fixation, cell density, … The 4 panels showed in Fig. 5A are biological independent experiments, explaining why the basal OCR levels are different, even in the same clone.*

8. The paper can be strongly improved by evaluating the enzymatic activity of OXPHOS complexes, the aerobic ATP synthesis in basal condition and in myoferlin silencing cell lines.

Authors thank the reviewer for his/her sounding suggestion. Unfortunately, our laboratory has not acquired the expertise in performing OXPHOS complex activity yet. As such, this assay was not feasible within the short delay given for this revision. However, we add western blot showing 2 mitochondrial complex proteins in myoferlin-silenced Panc-1 cell line (Supplemental Figure 1D). The myoferlin silencing did not modify the abundance of these proteins. ATP synthesis was previously measured in Panc-1 cell line upon myoferlin silencing and published in Oncogene [1]*.*

9. In the discussion section the authors could added a speculation on the mechanism by which myoferlin modulates OXPHOS activity.

We are grateful to the reviewer for allowing us to expose our hypothesis about the mechanism by which myoferlin controls the mitochondrial function. Of course, we cannot ignore our previous observation demonstrating the disorganization of the mitochondrial network in absence of myoferlin [1]*. Obviously, the connection between the bioenergetics and the mitochondrial morphology was previously established* [2]*, but this only moves the problem to determine the link between myoferlin and mitochondrial morphology. As the physiological function of myoferlin is related to membrane biology, it is likely that the mechanism by which myoferlin controls OXPHOS is also related to membrane processes among which we can propose iron uptake or mitochondrial fusion. This aspect was added to the discussion section.*

References

1. Rademaker, G.; Hennequière, V.; Brohée, L.; Nokin, M.-J.; Lovinfosse, P.; Durieux, F.; Gofflot, S.; Bellier, J.; Costanza, B.; Herfs, M.; Peiffer, R.; Bettendorff, L.; Deroanne, C.; Thiry, M.; Delvenne, P.; Hustinx, R.; Bellahcène, A.; Castronovo, V.; Peulen, O. J. Myoferlin controls mitochondrial structure and activity in pancreatic ductal adenocarcinoma, and affects tumor aggressiveness. *Oncogene* **2018**, *66*, 1–15.
2. Alirol, E.; Martinou, J. C. Mitochondria and cancer: is there a morphological connection? *Oncogene* **2006**, *25*, 4706–4716.