Author's response to reviews

Title: Role of malignant ascites on human mesothelial cells and their gene expression profiles

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Response to the reviewer’s comments

Reviewer 1: Please look carefully into Table 1 and 2. Sometimes comma is used instead of period for the fold changes.

Response: In the revised manuscript, we have corrected these errors.

Reviewer 1: I’d also suggest authors removing any genes without description in Table 1 and 2, since they are not informative.

Response: As suggested by the reviewer, we have removed genes that were not informative from the Table 1 and 2.

Reviewer 1: (Discretionary revision) – I’ll be very curious if cell growth will still be successfully inhibited by targeting TGF-β or NF-kB pathways.

Response: We are currently investigating factors in ascites associated with the proliferation and migration of HPMCs. We have a manuscript that we’ll submit within the next month demonstrating that HGF is a critical factor in ascites for the migration of HPMCs. Although we cannot rule out at this point a role for TGF-β in ascites, we do not believe that it plays a crucial role. Although levels of TGF-β were higher in malignant ascites, its relative levels in these ascites was low (< 1 ng/ml) whereas efficient concentrations of TGF-β to stimulate HPMC proliferation or migration are usually ≥ 5 ng/ml. Ascites activates the NF-kB pathway in ovarian cancer cells (unpublished data) but we have not assessed specific inhibitors of this pathway so far.

Reviewer 2: Images in Figure 1A, 1B and 1C need a scale bar.

Response: As requested by the reviewer, we have added a scale bar for these figures.

Reviewer 2: It would be interesting if the authors would demonstrate if the
addition of TGF-β would make the similar morphological changes in the HPMCs as it has been exerted by ascites.

Response: Although we cannot rule out at this point a role for TGF-β in ascites, we do not believe that it plays a crucial role in inducing morphological changes. Although levels of TGF-β were higher in malignant ascites, its relative levels in these ascites was low (< 1 ng/ml) whereas efficient concentrations of TGF-β to stimulate HPMC proliferation or migration are usually ≥ 5 ng/ml. There have been several reports demonstrating that recombinant TGF-β at concentration ≥ 5 ng/ml induces a myofibroblastic differentiation of HPMCs. Thus, there is no doubt that adding TGF-β will induce morphologic changes as those we have observed. Interestingly, in a manuscript that will be submitted within the next month, we have confirmed the myofibroblastic differentiation of HPMCs in the presence of ascites using fibroblastic marker α-SMA. Furthermore, we show that recombinant HGF, which is present at high levels in ascites (< 1 ng/ml) induces morphological changes similar to those induce by ascites.

Reviewer 2: Images in Figure 2A, 2B and 2C need scale bar.

Response: As requested by the reviewer, we have added a scale bar for these figures. The legend was corrected to include scale bar.

Reviewer 2: A P value should be added in the Figure 2D.

Response: As requested by the reviewer, we have calculated P values for the data presented in Figure 2D. The P values were included in the legend of Figure 2D.

Reviewer 2: The authors need to quantify the cell proliferation of the experiments depicted in Figure 2.

Response: As requested by the reviewer, we have quantified HPMC proliferation in the presence of ascites or benign fluid using XTT assay over 96 h. This new figure (figure 2D) has been added in the revised manuscript. Findings are consistent with those of figure 2A, 2B and 2C.

Reviewer 2: It would also be informative to show a time dependent and dose dependent increase in growth of these cells.

Response: We agree with the reviewer that this is a relevant experiment to perform. In the follow up manuscript to this one which will be submitted soon, we show that malignant ascites as well as HGF induce a concentration-dependent effect on HPMCs as well as a concentration-dependent activation of Akt and ERK1/2.

Reviewer 2: Addition of TGF-β antagonist to inhibit the cell proliferation induced by ascites would have been more appropriate to associate with the findings from Figure 1.

Response: For the reasons mentioned above, we do not believe that TGF-β plays a significant role in ascites-induced HPMC proliferation. In contrast to
TGF-#, we have found that HGF plays a critical role in ascites-induced migration of HPMCs and we have confirmed its role using blocking antibodies and inhibitor of c-Met receptor. These data will appear in the follow up manuscript mentioned above.

Reviewer 2: The authors should acknowledge that LPA may not have major role to play in the proliferation in the samples tested instead of categorically stating that “LPA may not be a critical factor for ascites-mediated proliferation of HPMC cells.

Response: As suggested by the reviewer, we have tempered our conclusion to acknowledge that the fact that these data are derived from a limited number of samples (see Conclusions – lane 366).

Reviewer 2: Figure 3C lacks data for the cell line OV401.

Response: As requested by the reviewer, the data for OV401 were added in Figure 3C in the revised manuscript and are consistent with those of OV370.

Reviewer 2: Additional data showing PARP cleavage would be important to support apoptosis data in Figure 3B.

Response: We have some doubts about the utility of confirming our quantitative data with a qualitative method. In our experience, measuring TRAIL-induced apoptosis with the Cell Death Detection ELISA kit has shown excellent correlation with other methods (caspase-3 activity, detection of active caspase-3 fragments, etc...) (Oncogene 2010;29:5523; Oncogene 2010;29:3519; J Ovarian Res 2013;6:62). In contrast, correlation between PARP cleavage and caspase-3 activity has been more variable and not very sensitive. We have found that massive activation of the apoptotic cascade by TRAIL is necessary to get cleavage of PARP in ovarian cancer cells and that its activation may vary according the experimental conditions (Mol Cancer Ther 2006;5:509). In addition, PARP cleavage can also occur with other processes such as necrosis. Based on the differences that were observed in Figure 3B and 3C, it is not clear that a qualitative assessment by measuring PARP cleavage would be sensitive enough to yield convincing results.

Reviewer 2: Bar diagrams in Figure 6 need the error bars and P value.

Response: As requested by the reviewer, error bars and P value have been added in the revised manuscript.

Reviewer 2: It is interesting that the VEGF pathway was downregulated when it is generally accepted that VEGF is the causal factor for ascites formation. Can the authors speculate as to why high VEGF levels usually present in the ascites had no role to play in the upregulation of specific pathways. Are receptors for VEGF not expressed on HPMC?

Response: We agree with the reviewer that ovarian cancer ascites contain VEGF (Am J Cancer Res 2012;2:566). VEGF is produced by both cancer and
mesothelial cells. VEGF is a key regulator of angiogenesis which drives endothelial cell survival, proliferation, and migration while increasing vascular permeability. By promoting tumor angiogenesis and enhancing vascular permeability, VEGF contributes to the development of peritoneal carcinomatosis associated with malignant ascites. It has been demonstrated that HPMCs expressed VEGF receptors (Flt, NRP-1) (Faseb J 2004;18:358, Cancer Lett 2006;239:212). However, the effect of VEGF on HPMC has not been. Although the VEGF present in ascites would be expected to stimulate VEGF receptor signalling, ascites may also contain factors that might inhibit this pathway. For example, TGF-β has been shown to down-regulate expression of the two VEGF main receptors in mesothelial cells (PLoS ONE 2013;8:e60776). In addition, ascites may contain sFlt-1 which inhibits VEGF-mediated signalling. Nonetheless, down-regulation of VEGF signalling in HPMCs by ascites remains unclear for the moment.

Reviewer 2: Provide a more detailed description of how the HPMCs were isolated and set up in culture.

Response: As requested by the reviewer, a provide more details on HPMC isolation and culture in the revised manuscript (lane 120-128).