Peer Review File

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Reviewer Comments

Mathew et al. set out to identify how Early B-cell Factor-2 (Ebf2) controls differentiation properties of pancreatic cancer cells. The authors show that the increased expression of Ebf2 may induce transdifferentiation of cancer cells into more mesenchymal and/or lipid-rich cells. This transition correlated with differences in cell proliferation and engraftment in vivo. Mechanistically, overexpression of Ebf2 may impact PI3K signaling pathway. While there is significant effort to understand the signaling mechanisms downstream of Ebf2, the lack of clarity with regards to which cell lines are being used in any given experiment (Miapaca or otherwise?), make it difficult to follow. The observations themselves are interesting, however, there are several conceptual and technical points that need to be addressed, especially with regard to the potential of established cancer cell lines to transdifferentiate into distinct lineages is overstated. The manuscript is written in a rather convoluted fashion and would benefit from grammar/phrasing edits.

Specific points:

1. The rationale for exploring overexpression of Ebf2 to begin with is not clear.
   It is well established that pancreatic tumor cells are plastic and, a significant proportion of cells have stem cell properties. This study aims to test whether suppression of stem cell-related factors and activation of cell differentiation inhibits pancreatic tumor growth. Ebf2 is not expressed in normal adult pancreatic cells whereas, Ebf1 and Ebf3 protein aberrations were found in PDAC(Ref.#17). Studies have shown that adipogenic differentiation inhibits cancer cell evolution leading to healthy progenitor cell development (Ref. # 1). Here, we show that Ebf2 plays a significant role in pancreatic cancer stem cell differentiation and tumor suppression. Additional information is provided on pages 4&5, lines 93-100.
2. The protocols described are used to isolated and expand Lrg5+ primary epithelial cells that eventually expand into organoids. These are not CSC expansion protocols, and the authors did not sufficiently demonstrate stem cell markers, such as CD44 and CD24. Keratin and ZO-1, etc. are epithelial lineage markers that do not signify stemness. Instead, markers of lineages are used to define stem cell characteristics. Formation of a spheroid in 3D culture does not signify stemness characteristics.
   CD44 and CD24 are expressed in pancreatic cancer cells but not a specific marker for PDAC stem cells. Therefore we used asymmetric stem cell division as a marker to identify cancer stem cells as shown by us (Fig.1 and supplementary Fig.1) and others Pine et al., Proc Natl Acad Sci U S A2010 Feb 2;107(5):2195-200.
3. Western blots in Figure 2 are not quantified, and it is not clear which cell line is being used.

We have edited the Figure 2 figure legends to reflect the cell lines used.

4. One should be extremely careful with labeling subclones as endothelial or adipose – just because cancer cells acquire certain characteristics, such as increased lipid uptake etc. It is not clear what proportion of cells become adipose-like versus endothelial-like, this differentiation pattern could be better quantified.

   We characterized the endothelial and adipose-like cells using endothelial and adipose-specific markers as shown in Supplemental Figure 2. We agree with the reviewer regarding quantifying the progenitor cell subtypes such as endothelial or adipose type. Although this can add additional information in cell culture studies, quantifying the exact subpopulation of cells in vivo is challenging.

5. Figure 3, it is not clear which cell line is being worked with here. And what is control? Large variability in tumor sizes, for example, endothelial-like, could mean that the purity of this subclone is variable. Statements of duct or acinar lineage are difficult to discern from H&E, lineage specific markers should be used.

   For clarity, we edited the Figure 3 legend (page 27, lines 614 and 619). To reflect the control group, please see the edits on page 7, line 166. In Figure 3a. Statistical analysis has shown the large variability in tumor size in tumors derived from control and polyclonal cells. Figure 3b shows the representative tumors only. The number of tumors, n=11, in endothelial subsets. As noted by the reviewer, using lineage-specific markers is an excellent idea to show the particular lineages. However, in tumor histology, we show a clear differentiation pattern in Ebf2-expressing tumors compared to controls.