Review of: "Blocking Gi/o-coupled signaling eradicates cancer stem cells and sensitizes breast tumors to HER2-targeted therapies to inhibit tumor relapse"

John Algernon Hassell[1]

1 McMaster University

Potential competing interests: The author(s) declared that no potential competing interests exist.

Overview:

The manuscript contains little new data compared to a previous publication by the same authors in JCI Insight (hereafter referred to as JCI), which was published in 2021. In fact, most of the experiments and outcome data are similar, and the conclusions are essentially identical between the manuscript and the publication except for a new spin attributing the effect of pertussis toxin on breast cancer stem cells (BCSCs) as defined by sphere forming assays. It is noteworthy that sphere forming assays were also described in the JCI article, but the effect of pertussis toxin on the frequency and volume of the spheres was not attributed to an effect on BCSCs.

Because there is little new data in the manuscript coupled with the fact that the authors seem to be unaware that similar data has been published years ago by others using a related transgenic mouse model and human breast cancer cell lines (see **References**), I do not recommend publication of the manuscript because it lacks novelty and only marginally expands beyond their JCI article.

General Comments:

In the Introduction the authors state that GPCRs of the $G_{i/0}$ subtype are functionally redundant because there are many (over 100) such GPCRs whose activity is reduced by pertussis toxin. However, the authors statement does not consider the fact that a single GPCR of the $G_{i/0}$ subtype might be required for sphere formation and tumorigenesis. In this regard It is noteworthy that several publications have shown that highly selective antagonists of individual GPCRs that signal via $G_{i/0}$ subtype as well as other G proteins target BCSCs as demonstrated by a reduction in the frequency of tumor sphere forming cells in vitro, and a reduction in tumor growth rate and volume in vivo (**References**). Hence, the assumption that pertussis toxin targets multiple GPCRs and that GPCRs of the $G_{i/0}$ subtype regulate the activity of BCSCs is not the only explanation for the findings reported in the manuscript.

In general, the description of the experiments whose outcome is presented in the figures is very poor. For example, the authors do not state whether the regulation of pertussis toxin in the bi-transgenic mice employs the Tet ON or OFF

system. I had to read the JCI article to learn that doxycycline is required to turn off expression of pertussis toxin.

It is not clear how long doxycycline was removed from the medium before the in vitro experiments were performed. It behooves the authors to briefly outline how the various assays were performed in the Results section because the Methods are at the end of the manuscript and frankly need to be rewritten to clearly describe how the experiments were carried out.

Specific comments:

1. The capacity FACS-purified tumor cell fractions from "pre-malignant" mammary glands from 4.5-month-old Neu transgenic mice and Neu/Pertussis toxin bi-transgenic mice were used to prepare cell populations for analysis of their capacity to form tumor spheres in vitro (Fig 1).

The authors do not explain the rationale for using pre-malignant mammary tissue nor do they describe the nature of this tissue. As a minimum, histological sections of the premalignant tissues from the transgenic and bi-transgenic mouse strains should be presented in any revised manuscript.

The purity of the cell fractions (Fig. 1 A; luminal, luminal progenitors and basal) were not assessed by transplantation into syngeneic mice. I raise this point because all tumor cell fractions formed spheres, and curiously those of the luminal and luminal progenitor fractions did so at a higher frequency than those of the basal fraction, which is expected to comprise the BCSC-enriched fraction.

Hence the nature of the cells comprising the various fractions are uncertain and needs to be verified by the authors using limiting dilution transplantation experiments in mice. If pertussis toxin affects the BCSCs, then that should be reflected by its effect on the frequency of these tumor cells in the various FACS fractions.

The statistical significance of the data in Fig. 1B was not stated. The latter should be corrected, assuming the data is statistically significant. It is curious that pertussis toxin had little effect on sphere formation by the luminal, luminal progenitor or basal cell fractions. No explanation was provided for the latter finding.

2. No data is presented to demonstrate that the tumor spheres arise from the self-renewal and proliferation of a single cell and are thus of clonal origin (Fig, 1D). Indeed, the evidence presented in the manuscript strongly suggests that the spheres are aggregates of individual tumor cells as shown in Figure 1D, 3A, and 4E. In this regard, the Methods section

state that 5-10 randomly chosen fields were examined; these fields from each of the figures should be provided as Supplemental data should a revised manuscript be submitted.

3. The data shown in Figure 2 panel A is not statistically significant (p=0.81) and should be removed from any revised manuscript.

4. The method used to assess the effect of pertussis toxin on tumor volume (Fig. 2B and Fig. 2D) and tumor-free survival (Fig. 2C) and is not described. The latter needs to be addressed should a revised manuscript be submitted.

5. The data shown in Fig. 3D is similar to that published in the JCI article (Fig. 3D). The authors should acknowledge this in the manuscript.

6. The data shown in Fig. 4-6 partially recapitulate previous findings shown in the JCI publication and should be acknowledged by the authors in the Results section.

References:

https://doi.org/10.18632/oncotarget.10614

https://doi.org/10.18632/oncotarget.16646

https://doi.org/10.1186/s12885-020-07193-6