Author’s response to reviews

Title: Hypoxic enhancement of exosome release by breast cancer cells

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Version: 3 Date: 23 August 2012

Author’s response to reviews: see over
Dear Professor Stoeltzing

Thank you for asking us to submit a revised version of our paper MS: 6414064577288208 ‘Hypoxic enhancement of exosome release by breast cancer cells’ to BMC Cancer. We attach a revised manuscript, which responds to the reviewers’ comments as follows:

Referee 1
We thank the referee for his kind comments and are pleased he feels the hypothesis is clear, that the data are well-controlled and support the conclusions drawn and that the article is of importance in its field. In respect to the suggested discretionary revision, we agree that examining the influence of exosomes secreted by hypoxic cells on tumour proliferation is an important topic, which we are currently studying and also examining the effects on vascular and lymphatic cells. However, the data are not sufficiently secure to be submitted for publication.

Referee 2
The referee is incorrect in assuming that ‘the study was set up for demonstrating the superiority of a commercial assay for the isolation of exosomes from cultured cells as compared with conventional approaches.’ The study was designed to examine the hypothesis that hypoxia enhances exosome release by cancer cells. The use of the Exoquick reagent proved superior to ultracentrifugation as a method for exosome isolation so formed an important part of the protocol for obtaining exosomal purification, however the technique itself was not a focus of the study and we have removed reference to it from the abstract to prevent such an impression.

Whilst the yield of exosomes is higher by the Exoquick method, the quantity of material is still relatively limited such that Western blots are being utilised to detect very low amounts of protein in comparison to whole cells. To enable such detection we have utilised an antibody to the exosomal marker CD63 which provided the greatest sensitivity of detection (see Figure 1D), however as stated in the methods this antibody requires non-reducing conditions which reduces the sharpness of the bands in Western blotting analysis, as can be seen in the detection of CD63 in exosomes in the work of Kosaka et al (2010), Logozzi et al (2009) and Koumangoje et al (2011). As requested, we have presented additional CD63 and CD9 immunoblot quantification in several different breast cancer lines that support our conclusion of hypoxically induced exosome release.

We believe the central novelty in these observations is our evidence that hypoxia substantially increases the number of directly quantified exosomes released by several different breast cancer cells. Additional novelty is provided by the evidence for the HIF dependence of this process and the increased expression of miR-210 in exosomes released by hypoxic cells. The senior author has not previously published on enhancement of exosome release by hypoxia. The paper (Ref 42) that is referred to demonstrated enhanced miR-210 levels in whole cells under hypoxic conditions. In Ref 24 Svensson et al (2011) demonstrated the hypoxic enhancement of secretion of specific proteins, many of which are likely exosomal, but this work did not actually identify whether there is enhancement of exosome release by hypoxia, exosomes were not directly examined or quantified. Similarly, the work of Gutwein et al and Ngora et al monitored the effects of hypoxia on proteins presumed to be exosomal but did not directly quantify exosomal release in response to hypoxia. As suggested we have reworded the results, discussion and conclusion sections and incorporated discussion of these papers in order to make this clearer.
Referee 3
We thank the referee for her kind comments, that this is well organized and written manuscript dealing with a topic of considerable interest and an article of importance in its field.

Jonathan Gleadle

Additional references

