Author's response to reviews

Title: Neoplastic transformation of rat liver epithelial cells is enhanced by non-transferrin-bound iron.

Authors:

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Author's response to reviews: see over
RE: MS 4448342141553270-Neoplastic transformation of rat liver epithelial cells is enhanced by non-transferrin-bound iron.

Dear Dr. Rohra,

Thank you for the excellent and thorough reviews of our paper. In response, please find attached a revised manuscript. It includes a revised Table 1, Table 2, Figure 1, Figure 2, Figure 3, and revised text. We have checked to ensure it conforms with the style formatting of BMC Gastroenterology. Our responses to the concerns raised by each reviewer are detailed below, using the reviewers' numbering system (or paragraph number of the critiques if not numbered by the reviewer).

In response to reviewer 1:
Major (5) points:

1. We clarified the distinction between transferrin-mediated and transferrin-independent mechanisms of iron overload (Background paragraph 2, p4). Our reasoning is based on the established observation that uptake of transferrin-iron in cells is regulated by the number of type 1 transferrin receptors at the cell surface. For example, uptake of transferrin iron is higher in bone marrow than liver, due to the higher number of transferrin receptors. However in diseases of iron overload these cells, as well as liver tumors (also with a high number of transferrin receptors), have lower iron loads than non-tumor liver tissue (with lower transferrin receptor numbers). This implicates non-transferrin receptor mechanisms in overload, and suggests that a key NTBI uptake or sequestration mechanism is altered in tumor cells.

2. We revised the background (5th paragraph, p6) with respect to the terms physiological and pathological. Ferric citrate is found in blood and so is physiological. As the reviewer points out, high levels have been reported under iron overload and may cause iron toxicity in liver, and so may also be pathological. We wanted to use terminology that emphasized the distinction from forms used previously for experiments of this type (e.g. ferric-NTA), which may also be pathological but are clearly non-physiological. To be most accurate we now describe FAC in the background and discussion as a "physiologically and pathologically relevant form of NTBI".

3. We performed statistical analyses of the cell proliferation data using a 2 tailed student t-test for samples of unequal variance, and included the results in Figure 2A. We found a significant (p<0.01) decrease in cell number after 7 days treatment with
200 μM FAC. This supports our statement that tumor promoting concentrations of FAC have anti-proliferative effects on normal T51B cells. A description of the statistical procedure was added under Methods (p7) and noted in the Figure legend. Relevant passages in the results (p13) and discussion (p14) were revised accordingly.

4. We added variance data and statistical information to Tables 1 and 2. These tables now list the means +/- sem of four soft agar dishes per point. Conditions found to be significant (p<0.01) or highly significant (p<0.001) are so noted.

5. Statistical evaluation of the data compiled for Figures 2 and 3 was added to each Figure, as described under points 3 and 4 (above) and the legends to these figures.

Minor (1) points:
1. Figure 1A was corrected.

In response to reviewer 2:

Major (6) points:
1. A goal of this study was to develop a cell culture based model useful for investigating how iron may contribute to HCC in humans. To address the reviewer's concerns with our experimental conditions, we commented further on why we used T51B cells and how this model is relevant to hepatocellular carcinoma (new paragraph 3 in the discussion, p15).

2. HCC associated with iron overload in humans may require decades to develop. It is common and well accepted to use high concentrations of test substances for shorter time periods both in vitro and in animal models for the purposes of evaluating potential human carcinogenicity. These choices are necessary, albeit imperfect, steps towards studying a disease that has no better in vitro models. Consequently, we now have a viable model for investigating mechanisms of iron-related cancer and pre-clinical screening of preventatives and therapeutics.

It is also worth noting that low steady state concentrations of iron citrate do not necessarily indicate low delivery rates of iron to the liver. In experimental animals, high clearance rates suggest iron citrate in the blood is a transient form of iron that is readily taken up by the liver. Although the steady-state concentration is low at any given time, the total flux of iron may be significant via this route. As reviewer 1 pointed out, iron citrate is thought to be pathological for this reason. Furthermore, the local concentration of iron citrate in an iron-loaded cirrhotic liver is unknown, and could be significantly higher than levels circulating in the blood. We clarified these points in the revised manuscript (new paragraph 3 in the discussion, p15).

3. We added information on potential NTBI uptake routes in T51B cells (new paragraph 4 in the discussion, p16). As for nearly all cell types, however, the specific pathway in T51B cells remains poorly defined.
4. We assessed iron load at later stages of cell transformation by measuring ferritin levels in T51B cells treated with 50, 200, and 500 µM FAC for 12 weeks. We found ferritin levels roughly comparable to those detected at 2 or 5 days FAC treatment, and noted this in the results (results paragraph 1, p11). Assuming ferritin levels are proportional to iron concentrations, it appears that iron does not accumulate at a constant rate, but levels off. Now that conditions of FAC treatment needed for a physiological response (tumor promotion) are established, it will be worthwhile to investigate the kinetics of iron accumulation in more detail in future studies.

5. The transformation experiments were each examined at multiple (>3) time points; the details were added to the cell culture and transformation assays section of Methods (methods paragraph 4, p8). We chose 16 weeks for presentation in Figure 3 because this point contained the most replicates, and because these data fairly represent what was also observed at other times.

6. Our reasons for selecting FAC for this study are now described in the final paragraph of the Background (p6).

**Minor (1) points:**
1. TPA was changed to phorbol 12-myristate 13-acetate.

**In response to reviewer 3:**

**Major (6) points:**
1a. Concerns about the concentration of FAC were addressed for reviewer 2 (point 2), and in the revised paper (new paragraph 3 in the discussion, p15).

1b. With respect to whether our controls were adequate: The controls for all FAC treatments were either untreated cells (Table 1) or the same or higher concentration of ammonium citrate (Table 2 and Figure 3), with or without MNNG as appropriate. We feel these were adequate for the conclusions we made.

1c. Our purpose for using MNNG was to investigate whether FAC can act as a tumor promoter. An initiating dose of MNNG was followed for up to 20 weeks in culture (in parallel to cells also given FAC), to make sure that MNNG without iron had no significant effect. The methods section was revised to clarify this point (Methods paragraph 4, p8).

2a. Methods: The iron assay using bathophenanthroline sulfonate was based on the method of Scheiber-Mojdehkar et al 1999 (reference #25). We did not find a citation to an original method in that paper. Partly due to the comments of the reviewer, and partly because we found that method not very sensitive or robust, we repeated the iron measurements using a superior method based on ferrozine (as described in Methods paragraph 5, p9). These new data are presented in a revised Figure 1A.
2b. Methods: The reference provided by the reviewer (Hasinoff 2003) characterized the effect of iron on calcein fluorescence in a test tube environment designed to mimic the reducing conditions inside cells. Under these artificial conditions, the author provided evidence that calcein is degraded by iron, and concluded that absolute calcein fluorescence cannot be used as a quantitative measure of iron concentration. Even if degradation occurs in cells, we did not use calcein in this way. We used it as a yes/no indicator of iron uptake by T51B cells, which is valid even if the entering iron degrades calcein rather than merely quenching it. In our study, the calcein technique supplemented and extended the other indicators of iron uptake by showing that all the cells, rather than a subpopulation, internalize iron.

We offer 4 independent measures of iron (chemical assay, calcein fluorescence, ferritin H, and ferritin L) to support our statement (last sentence of Results paragraph 1, p 11): "iron given as FAC readily accumulates in T51B cells in a uniform fashion with expected effects on cellular pathways regulating iron metabolism". This seems sufficient to us.

3a. Results: We repeated the iron determinations using an improved assay, and presented these new data in a revised Figure 1A. The iron content of T51B cells is similar to that found in other cells by other investigators. For example in HepG2 cells (Parkes et al. ref #23), control cells had 1.4 +/- 0.7 nmol iron/mg protein, while after 7 days treatment with 180 µM FAC the reported value was 65 nmol iron/mg protein.

3b. Results: The reviewer is correct: the western blot shown in Figure 1B did not reveal ferritin H or L in untreated cells. Others have found ferritin to be "undetectable" in liver cells cultured without added iron (Hann et al. ref 13). Although a 10x longer exposure of our blot shown in Figure 1B displayed a faint band for ferritin H in untreated cells, this is at the detection limit of this technique, and offers little additional information. The important point is that both ferritins increase dramatically with iron treatment, clearly indicating iron uptake in the cells.

3c. Results: Effects of 200 µM FAC on cell proliferation were significant, as shown by the statistical evaluation of Figure 2A data. Including the data on 500 µM FAC creates a more complete concentration/effect curve and offers a likely explanation (high toxicity) for why tumor promotion decreased at this concentration. Finally, we should point out that the term "protein-free cell culture" describes none of our experiments.

4. For Figure 3, the scale (0-120 colonies from 25,000 cells) is linear and includes all data from the experiment. To further address the reviewer's concerns, we performed a statistical evaluation that shows MNNG and ammonium citrate had no significant effect compared to ammonium citrate alone, and that the most significant effect of FAC is as a tumor promoter. (See revised Figure 3).

Minor points: none
In response to reviewer 4:

Major (2) points:
1. As described above, the iron assay was repeated with a superior assay and the data are included in a revised Figure 1A. We have included variance data which is the limiting factor in determining sensitivity and accuracy. Our estimate of the limit of detection is described under *Biochemical measurements* (Methods paragraph 5, p. 9).

The reviewer indicates ferritin can be induced by cellular stress, which has been reported only for ferritin H. This is not true for ferritin L, which we also show to increase in T51B cells treated with FAC (Figure 1C). This point, in addition to the lack of ferritin induction by the controls (ammonium citrate alone or FAC + dfo), seems good evidence for iron being the important trigger, rather than cellular stress.

2. FAC toxicity was determined by MTT assay after 5 days treatment with FAC, and these data were added to the paper as a new Figure 2B.

Minor (2) points:
1. Figure 1A was corrected.

2. Information on s.e.m. and statistical significance was added to the tables.

Thank you once again for your assistance and we look forward to your reply.

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