Author's response to reviews

Title: The molecular basis of phytoestrogen Genistein induced mitotic arrest and exit of self-renewal in embryonal carcinoma and primary cancer cell lines

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Author's response to reviews: see over
Dear Dr. Dunckley,

Thank you for processing our manuscript and forwarding the reviewers’ comments. We have addressed their comments in our revised version of the manuscript- “The molecular basis of genistein induced mitotic arrest and exit of self-renewal in embryonal carcinoma and primary cancer cell lines” by Regenbrecht et al., manuscript number 1840865241194786 for publication in BMC Medical Genomics.

We have reformatted the manuscript according to the format of the journal and corrected minor typos as suggested by both reviewers and the editor.

Reviewer 1:

Comment:
- title: please remove the word ‘phytoestrogene’ (it is misspelled and unnecessary); instead use ‘genistein-induced’ (without capitals, also change throughout the text)

Response:
As suggested by the reviewer we have changed the title to “The molecular basis of genistein induced mitotic arrest and exit of self-renewal in embryonal carcinoma and primary cancer cell lines“
Comment:
- abstract: for clarity, please add a statement or two to the background section, place the methods in a separate section, and add 1 statement to the conclusion addressing the impact of your study

Response:
In the abstract we added a paragraph to the background section (see pg.2), explaining how we conceived the study. The methods used, are briefly explained (see pg.2) and a concluding remark on the impact of our study is given (see pg. 3).

Background

The issue of genistein as a potential anti-cancer drug has been addressed in some papers, but comprehensive genomic analysis to elucidate the molecular mechanisms underlying the effect elicited by genistein on cancer cells have not been performed on primary cancer cells, but rather on transformed cell lines.

Methods

We treated primary cancer cells and NCCIT cells with 50µM genistein for 48 h. We compared the mitotic index of treated versus untreated cells and investigated the protein expression of key regulatory self renewal factors OCT4, SOX2 and NANOG. We then used gene expression arrays (Illumina) for genome-wide expression analysis and validated the results for genes of interest by means of Real-Time PCR. Functional annotations were then performed using the DAVID and KEGG online tools.

Conclusions

The results of the present study, together with the results of earlier studies show that genistein specifically targets genes involved in the progression of the M-phase of the cell cycle. In this respect it is of particular interest that this conclusion cannot be drawn from the comparison of the individual genes found differentially regulated in the datasets, but by the rather global view of the pathways influenced by genistein treatment.

Comment:
- methods: this section should be placed in between background and results,
according to the journal format

Response:
We have reformatted the manuscript according to the journal’s layout

Comment:
- discussion: please elaborate on the significance of the changes observed in the expression of OCT4, SOX2, and NANOG (data presented in Fig. 1)

Response:
We have elaborately discussed the significance of changes observed for expression of OCT4, SOX2, and NANOG as presented in Fig. 1. (see pg. 12, 13)

The direct effect of genistein on GADD45 gene expression has been shown before [36]. In this study, we have verified this effect for GADD45G and GADD45A. Furthermore, GADD45G has been shown to be a negatively regulated, direct downstream target of OCT4 [27, 39, 40]. Indeed, genistein treatment of NCCIT cells led to the induction of GADD45A and GADD45G expression, as shown previously with other cancer types. Additionally, we noticed a reduction in NANOG transcription but not that of OCT4 and SOX2. A reduced level of NANOG could not be linked to a differentiation phenotype, but rather to reduced proliferation in NCCIT cells [27]. As shown before, down-regulation of OCT4 leads to the down-regulation of NANOG, we assume that our observed decrease in the transcript level of NANOG is a downstream effect of genistein-induced depletion of OCT4 protein [41].

Comments:
- page 7, line 5: please cite reference for ‘DAVID pathway annotation analysis’ (or refer to the website http://david.abcc.ncifcrf.gov)
- page 7, line 21: ‘fulfil’ is misspelled
- page 9, line 3-4: the references ‘Andrews et al. 2005’ and ‘Greber et al. 2007’ were not numbered
- page 9, line 5: the reference ‘Yin J et al. 2005’ was not numbered
- page 12, line 12: the reference ‘Bergan et al. 2008’ was not numbered

Response:
Minor corrections in the numbering of citations have been made.
Reviewer 2:
Comment:
There are several similar papers in the literature (see below), it would be more informative if experiments such as siRNA for specific protein be carried out to confirmed the effect of genistein. At a minimum the authors may consider comparison to existing databases of published results to identify similarity or differences between cell lines etc. and discussed accordingly.

Response:
Upon the request of the reviewer, we have compared our data to that of the literature suggested by the reviewer. (see pg. 8, pg. 11, pg. 12, pg. 16, pg. 17)

[pg8] Comparison to datasets from selected publications
To compare our data with that of previous studies, we extracted all genes detected as differentially expressed in the respective studies and deleted duplicate genes names from the lists. Pathway and Gene Ontology analyses were carried out as described above.

[pg 11/12] Comparison with previously published data
We compared our data with that of previously published datasets related to genistein [29-31] dependent expression patterns. Because the different pre-requisites used to carry out these studies, we included all genes significantly differentially expressed, regardless if they were over- or under-expressed. This analysis revealed a common set of only three genes differentially expressed between the datasets. DCXR, NQO1 and SCD are involved in key metabolistic pathways, thus suggesting their important role in genistein-processing and translation of the stimulus into a cellular response. Another important finding of the comparison between these gene-sets, is that on a pathway level all gene-sets point towards the mitotic cell cycle (Fig.1), specifically towards the M-phase regulating genes.
As shown in Figure 1, the overlap of the genes found in our dataset compared to that of others [29-31] recovers only three genes. These three genes were DCXR, NQO1, SCD, which are all involved in metabolism. DCXR and NQO1 have been implicated in various tumors, thus not specifically linked to genistein treatment. On the other hand, Stearoyl-CoA desaturase (SCD) seems to be of particular interest in investigating the effects of genistein. SCD is an iron-containing enzyme that catalyzes a rate-limiting step in the synthesis of unsaturated fatty acids and has been implicated in the regulation of cell growth and differentiation through effects on cell membrane fluidity and signal transduction [66, 67].

A comparison of the Gene Ontology of the other datasets to ours revealed an astonishing similarity between the studies. For example, the percentage-distribution of genes accompanying the various phases of the cell cycle is more or less identical, with about 75% of genes involved in M-phase transition.

But more importantly, this study provides insights into the molecular mechanisms underlying the morphological changes elicited by genistein treatment of embryonal carcinoma and distinct primary and transformed cancer cell lines. From the comparisons of distinct datasets obtained under various conditions in terms of concentration and induction-time of genistein, as well as varying cell culture conditions, it seems that the molecular mechanisms triggered by the treatment are very robust and universal.

In conclusion, genistein may be a potent cell-cycle regulating drug targeting the M-phase, both in cell lines and primary patient-derived cancer cells from various tumor entities. But still, enthusiasm has to be dampened, because these doses will not be attained pharmacologically. However, if this pitfall of high dose levels can be overcome - for example by adjuvant administration of other compounds making cancer cells more sensitive towards genistein treatment, genistein may well justify emerging phase I and II trials of this potent cell-cycle regulating compound in the treatment of cancer patients.

Figure 3 and 5 have been revised and the dataset from the literature has been compared to our dataset. (see figures 3 and 5, figure legend pg. 25)
This comparison revealed comparable results regarding the cellular processes involved in response to genistein treatment. With our data backed up by those studies, we are convinced that further siRNA analysis as recommended by the reviewer is not necessary.

Comment:
The concentration used also of concern, as 50 microM may not be achievable even at pharmacological dose and need to be discussed.

Response:
A statement on the concentration of genistein used has been added to the discussion (pg. 17)
But still, enthusiasm has to be dampened, because these doses will not be attained pharmacologically. However, if this pitfall of high dose levels can be overcome - for example by adjuvant administration of other compounds making cancer cells more sensitive towards genistein treatment, genistein may well justify emerging phase I and II trials of this potent cell-cycle regulating compound in the treatment of cancer patients.

Comment:
There are also typos such as “phytoestrogen” in the title need to be corrected.
Response:
Typos were corrected (see Referee 1)

We hope that you will find this revised version of the manuscript now suitable for publication in your journal.

Sincerely,
Dr. J. Adjaye