Author's response to reviews

Title: Synergetic downregulation of 67kDa laminin receptor by the green tea (Camellia sinensis) secondary plant compound epigallocatechin gallate: a new gateway in metastasis prevention?

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Author's response to reviews: see over
“Synergetic downregulation of 67kDa laminin receptor by the green tea (Camellia sinensis) secondary plant compound epigallocatechin gallate: a new gateway in metastasis prevention?”

**Changes in the revised manuscript:**

- Line 117: "MC" changed to "media control"
- Lines 203 – 206, sentences changed: "Thus, the extent of the synergetic effect evoking 63% of relative residual 67LR mRNA expression as a mean value from ten independent experiments was revealed. In contrast the other treatment combinations where EGCG was applied in absence of the 67LR knockdown did not lead to substantial changes in relative expression."
- Lines 254 – 255, sentence changed: "Reasonably the anti metastatic potential of green tea as a daily diet develops best outgoing from the gastrointestinal tract"
- Abbreviations changed:
  - "ET" changed to "EGCG" in lines: 116, 2 x in 120, 190, 2 x in 217, 222, 2 x in 223, 227, 228, 276, 385, 396, 400
  - "MC" changed to "MEDIA" in lines: 116, 2 x in 120, 188, 216, 217, 222, 2 x in 223, 226, 2 x in 228, 276, 385, 396, 401
- All Figures changed according to the new abbreviations & resulting legends!
Answers to Reviewer #1

>>> Minor Essential Revisions

Reviewer 1 - Query #1:

1. Though I understand the meaning of it, it is not completely clear to me why the authors apply the Principal Component Analysis. What are the benefits compared to the bar graphs?

Authors reply:

We initially and routinely used the normalization from Figure 1a for our knockdowns, due to another aim of the antecedent work. At this point of time we did not expect the effect or intend to find it. After repeated observation over a long period of experiments we applied the reference point from figure 1b also to the 67LR gene (a knockdown target) due to a suspicious repetition of tendencies. That way the effect was exposed explicitly. Principally you are right. The information in the PCA is the same as in the bar graphs. But that is logical or even stringent, as all three graphs are based on the same raw data set. The scientist always faces the task how to process and present the raw data best. An advantage of the PCA is the evaluation independent from any kind of normalization and statistics. For physiological matters a very weak but significant regulation is not imperatively important. The PCA delivers evaluation beyond any p-values and avoids further steps of data processing what always represents a source of error, especially in large data sets like gene expression data. The synergetic receptor-/ligand dependency is here shown without a potentiation of technical artifacts by exposing the maximal variation within the dataset. Normalization on the other hand always forces a “polarized” view on the data.

Reviewer 1 - Query #2:

2. The present study combines two kinds of treatment: Drug treatments in different concentrations and co-treatments with different RNAi-knockdowns. However, within these treatment scenarios I miss the combination when EGCG is applied alone and hence no siRNA-expression is induced.

Authors reply:

This case is actually implied within the limits of the assay. The treatment variants labeled with NV (MC/NV & ET/NV) represent the samples where no siRNA is expressed. NV stands for Non-Targeting virus. Hence, these samples solely differ by the applied drug treatment (as MC = media control and ET = EGCG treated). The “NV-virus” represents the infection control and does not lead to any RNA interference. This ubiquitous adenovirus presence was necessary due to the side effects of the viral infection which should be present in all samples due to comparability (see Müller et al., as cited in the manuscript). A direct comparison in gene expression between infected and uninfected samples under different drug treatments is not possible within this knockdown model. As an inevitable effect of the knockdown application is viral toxicity, what must be co-simulated.

Reviewer 1 - Query #3:

3. In Line 250/251 the authors declare green tea to be most effective in the gastrointestinal tract. Because EGCG has not been tested with other cell-types in the present study, I would suggest the authors not to trivialize the observed effects.

Authors reply:

I agree the expression fails and hence, we have fixed this passage in the revised manuscript in order to clarify the expression.
Reviewer 1 - Query #4:

4. According to the available literature EGCG is an intensively studied compound throughout many scientific disciplines. Therefore I would suggest the authors may replace the term "ET" in figures by EGCG. Furthermore the "T" represents on the one hand "Treatment" (ET) on the other hand "Targeting" (TV). This may be misleading for readers.

Authors reply:

In order to optimize the abbreviations we applied the following changes:

ET >>> EGCG
MC >>> MEDIA
targeting virus = TV
nontargeting virus = NV
Answers to Reviewer #2

>>> Major Compulsory Revisions

Reviewer 2 - Query #1:

1. The term media control is misleading since it indicates an EGCG control without virus treatment. It would be more convenient (and also easier for the reader) to indicate directly to which sample the respective treatments have been normalized to (for example in the Figure legend).

   Figure 1a: MC/TV was normalized to MC/NV. ET/TV was normalized to ET/NV.
   Figure 1b: ET/TV was normalized to MC/TV. ET/NV was normalized to MC/NV.

Authors reply:

We implied the reviewer’s suggestions for the figures legends as these really might be easier to understand for the reader. Additionally we combined that change with a suggestion from one of the other reviewers concerning the abbreviations used for the treatments. The resulting indexing is as follows:

Abbreviations:

ET >>> EGCG
MC >>> MEDIA
Targeting virus = TV
nontargeting virus = NV

Legends:

Figure 1a: MEDIA/TV normalized to MEDIA/NV - EGCG/TV normalized to EGCG/NV
Figure 1b: EGCG/TV normalized to MEDIA/TV - EGCG/NV normalized to MEDIA/NV

Reviewer 2 - Query #2:

2. For further approval the authors show the measured effect by applying a descending concentration series of EGCG. Why are the given results distributed in two plots?

Authors reply:

The two shown plots (Figures 3a/b) originate from two independently cast experiment series (time-lag of about a year). Two different scientists where working on the generation of this data. To the time the first one (Figure 3a) was conducted we where not aware of the effect shown in this publication. Nevertheless, we decided to present both plots, as the independent finding is a further proof for the existence of the effect. You are right it would be more demonstrative if all EGCG concentration would be united in one plot. And we have tried to present the data like this. The problem was that the fluctuations between the experiment series where to high for a pooled graph. There is certainly a dose dependency as it is indicated in Figure 3b. Therefrom, one future task is to evaluate the definite correlation. However, our focus is, as our institute does not work on the direct development of pharmaceutical medications, to elucidate involved physiological pathways and thereby it is mostly necessary to use concentrations beyond a pharmacological relevant dose.

Reviewer 2 - Query #3:

3. The passage around line 250 describes the anti-metastatic potential of green tea to be most effective in the gastrointestinal tract. Is it not possible to postulate this thesis on the base of this study.

Authors reply:
I agree the expression fails and hence, we have fixed this passage in the revised manuscript in order to clarify the expression.

Reviewer 2 - Query #4:

4. Why does treatment with EGCG “alone” show absolutely no effect and why is the effect only present after the lentiviral knockdown? This should also be included in the discussion.

Authors reply:

Actually EGCG alone evokes very little effects, what is present in Figure 2b (MEDIA/NV vs. EGCG/NV). We amended this in the revised manuscript (lines 205-206). Besides, it is not obligatory that a gene is regulated by a treatment. However, you are right a virus free sample is not included in the assay. All samples contain the vector (TV or NV). This was technically necessary as we had to co-simulate the effects of the viral mediated knockdown. Preliminary experiments (among the work of Müller et al., as cited in the manuscript) showed that samples without virus application couldn’t be directly compared with samples containing the viral vector. We had to take this compromise in order to have the ability of silencing genes. And now to your question “...why is the effect only present after the adenoviral knockdown?”. This finding was exactly the reason for this publication. We did not expect the EGCG-receptor expression itself to shift after EGCG application. A response to the treatment was expected for downstream genes, which where in focus of our initial questioning (several immune markers).

Then we got aware of the effect under combined 67LR-knockdown and EGCG treatment. This was really unreckoned. If your knockdown candidate is co-regulated in dependency from your treatment, you face a big problem in your loss-of-function setting. And therefore we couldn’t ignore that synergy and hence investigated it explicitly.

At this point of time we can only describe a finding and propose a thematic background. We can proof the correlation by statistical testing of the data and additionally by applying the PCA (beyond any normalization). But why a newly described effect exists always has to be elucidated after its discovery.

Reviewer 2 - Query #5:

5. Why is/are no target gene(s) of the 67LR pathway included in the study? This would be good to confirm the effect and also to shed light on the underlying mechanism.

Authors reply:

The characterization of the 67LR as a receptor for EGCG is fairly young. Since the basic discovery by Tachibana et al. (as cited in the text) there is, to our knowledge only one physiological pathway postulated in this association (Umeda et al., cited as well). Surely, as also mentioned in the text, there is a long history of findings referring to the 67LR. However, we did deep going literature research concerning the 67LR after we read the article of Tachibana. What we found is that there is ubiquitous occurrence of this gene product in a partially really “enigmatic” way. What Tachibana found is in contrast a very precise correlation. Therefrom this new finding, linked to the tea catechin, is in the spotlight of our research.

I agree that genes involved in 67LR signaling must be investigated. However, as the 67LR and its pathways are not yet entirely understood, potential target genes are not clear at this point of time. Umedas postulation is promising, but aims for a slightly other context. In order to identify the right candidates much more basic research must be conducted in this field. For us, this represents our work for follow up studies.

>>> Minor Essential Revisions

Reviewer 2 - Query #1:

1. The title might be improved by using the more common synergistic instead of synergetic.

Authors reply:
First of all I must say that I am not a native English speaker. For this manuscript we used a professional language editing service (http://www.edanzediting.com/, as recommended by Biomed Central) in order to improve the quality. According to this “synergetic” seems to be an acceptable expression. I did some search on this matter but could not find a difference between the two words synergistic / synergetic.

Reviewer 2 - Query #2:

2. It is misleading to postulate a new gateway in metastasis prevention since the enhanced inhibitory aspects of green tea is only found after initial artificial down-regulation of 67LR which might be difficult to achieve in patients.

Authors reply:

From the view of pharmaceutical application you are right. We more think in the dimension of a chronic consumption of green tea in the synergy with gene regulations mediated by small RNAs. EGCG is certainly not a medication that can block or arrest metastasis formation or even heal cancer. Nevertheless, the here monitored effect may contribute to the life prolonging properties of the Camellia sinensis infusion reported in several epidemiological studies.

Reviewer 2 - Query #3:

3. In Figure 1a the y-ordinate is labeled as “knockdown”. Would it not be more precise to use the term knock-down efficiency?

Authors reply:

This is a question of definition I think. Due to the two different kinds of normalization versus the “drug treatment”-control on the one hand and the knockdown-control on the other, we had to make some decisions. Here we differ between “knockdown” and “gene regulation”. But within the definition of “knockdown” a second determination must be considered. You can give the knockdown as a value x or as 1-x (here e.g. 20% & 80%). Hence, we defined “knockdown” for the direct value x and the terminology “knockdown-efficiency” for 1-x.

Reviewer 2 - Query #4:

4. Figure 2 could be improved by indicating the groups in the Figure legend and by using independent Figure legends for each graph.

Authors reply:

We have adapted the legend for Figure 2ab to our revised abbreviations as indicated in “Major Compulsory Revisions: Reviewer 2 - Query#1”. Additionally we made individual legends for 2a & 2b and further grouped the symbols by roman numerals. Please note that for Figure 2a the 67LR gene is not included into the dataset for the PCA and hence the black square and the black diamond do not separate the two clouds (as anticipated).