Author's response to reviews

Title: Potential cancer-related role of circadian gene TIMELESS suggested by expression profiling and in vitro analyses

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Version: 2 Date: 12 September 2013

Author's response to reviews: see over
September 12, 2013

Dear Editor:

Attached please find the revised manuscript “Potential oncogenic role of circadian gene TIMELESS is suggested by expression profiling and in vitro analyses”, along with point-by-point responses to the comments made by the reviewer. Some responses reference the page numbers which contain changes to the revised manuscript.

We appreciate the suggestions and comments from the reviewers, and believe that the manuscript has been substantially strengthened.

Sincerely,

Yong Zhu
Authors' response to reviewers

Title: Potential oncogenic role of circadian gene *TIMELESS* is suggested by expression profiling and *in vitro* analyses

Authors:

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Response to Reviewer 1

Version: 1 Date: 17 June 2013

Reviewer: Filippo Tamanini

Reviewer's report:

General: Mao et. al. explore the role of the TIMELESS protein in cancer by analyzing its expression profile in online available gene expression databases and by characterizing the impact of its downregulation by siRNA on the rate of proliferation of breast (MCF-7) and cervical (HeLa) cancer cells.

The description of a priori chosen gene (Timeless) that is found differentially expressed in cancer specimens, does not say much about its functional and biological relevance relative to cancer, unless it is supported by strong experimental work. The required functional support is partly provided here by Timeless siRNA knockdown experiments, which however remain not sufficient and rather superficial in the interpretation of the results. Finally, some experiments presented in this manuscript are not properly controlled as detailed below.

Major issues:

1. The question about the expression level of TIMELESS relative to cancer prognosis is interesting, but overall it has been already addressed by the same authors with a similar approach and conclusion (Fu et. al Mol Carcinog. 2012). Therefore, the gene expression analysis is not novel, although it has been extended here to more cancer studies.

We appreciate the comment. While the idea of looking at TIMELESS expression in multiple cancers is not novel in itself, we felt its application to be a natural - and necessary - extension of the hypothesis-generating mono-cancer approach of the Fu et al. study, as the premise of the current study is that the relevance of TIMELESS expression patterns may not be restricted to breast cancer alone.

2. In the present manuscript the authors perform experiments using only one siRNA against TIMELESS. This is problematic, because any loss-of-function study requires to be performed with two independent siRNAs to compensate for background effects caused by off targeting.

We agree that there is the inherent danger of uncontrolled for off-target effects in the use of a single siRNA. Two TIMELESS siRNAs were initially purchased and both conferred a similar effect on cell proliferation in transfected HeLa cells (data not available). However, only the siRNA that conferred the greater initial phenotypic effect was chosen for subsequent assays. This is indeed a limitation of the current study, and we have noted as such in the amended Discussion (pg. 15).
3. The cell proliferation experiments are not convincing. Please, show whether, or not, the low level of proliferation may be caused simply by increased apoptosis in absence of TIMELESS. Moreover, it would be great for the article to extend this part of the study with more experiments that link the downregulation/overexpression of TIMELESS to in vitro cancer properties (cell mobility, invasion, colonies formation in soft agar, comet assays, etc), as it was shown previously by the authors for CRY2.

We thank the reviewer for this insight. There is indeed the possibility that a loss of cell viability resulting from apoptosis could have confounded our proliferation results. Looking at the proliferation curve, it is evident that proliferation of siRNA-transfected cells plateaus between the 48 h and 72 h time points and decreases thereafter, marking the period during which cell death most certainly contributed to the observed disparities in absorbance. We have noted this in the amended Discussion on pg. 15.

While performing the suggested assays may yield additional insights into TIMELESS's role in tumorigenesis, we feel doing so may be beyond the scope of our preliminary study. Measuring the capacity of TIMELESS to influence other potentially cancer-relevant pathways, however, will likely be the focus of a future investigation, and this is stated in the amended Discussion (pg. 15).

4. We know already by other studies (see Engelen et al PlosOne 2013) that TIMELESS expression is particularly elevated in cell/tissues undergoing active proliferation. Therefore, it is somehow not surprising that TIMELESS expression is high in cancer tissues, which are by nature highly proliferative. Please, provide some comments and references on this point.

We thank you for the comment. It was never our intention to portray increased TIMELESS expression as a cancer-exclusive phenotype. Although we agree that increased TIMELESS expression may be present in all highly proliferative cells, this relationship does not necessarily diminish TIMELESS's significance in cancer simply because heightened cellular proliferation can be an important driver of the cancerous state. Even if TIMELESS expression is elevated as a result of, rather than a precursor to, heightened proliferation, TIMELESS expression may represent a natural response to abnormal proliferative rates and its potential physiological significance in cancer cannot be discounted. Regardless, the reviewer raises an interesting point, and we have amended the manuscript with a brief discussion (pg. 14).

Minor issues:

1. Please provide the sequences of the siRNA oligos used, including the standard deviation and significance of Timeless expression by qPCR after downregulation by siRNA.

The siRNA oligo was originally purchased from Ambion, which does not provide sequence information. However, additional information regarding the siRNA, including the Ambion ID
(s17053) and the target exon (exon 11), has been added to the manuscript (pg. 6). Standard deviation values have also been added to the histogram of TIMELESS expression following siRNA transfection (Supplementary Fig. 1). TIMELESS knockdown was confirmed using quantitative RT-PCR. As indicated in Supplementary Fig. 1, TIMELESS mRNA levels were reduced by more than 90% following knockdown (P <0.01) (pg.10).

2. More discussion and potential experiments should be elaborated around the genes that are found differentially expressed after Timeless knockdown (which in my opinion is the most relevant and novel part of the study). Please, validate those genes by qPCR including standard deviations.

As requested, we have amended the manuscript with additional discussion of differentially expressed transcripts in the top IPA network (pgs. 13-14). We have also performed additional validation on the five genes exhibiting the most statistically significance expression differences in the top IPA network. We were able to confirm differential expression in all but one gene (GDF15). In general, newly validated genes exhibited lower magnitudes of change compared to microarray reads, which could partly be explained by loss of qPCR sensitivity brought about by RNA degradation. New validation information, along with error bars, have been added to Supplementary Fig. 2.
Response to Reviewer 2

Version: 1  Date: 27 June 2013

Reviewer: William Hrushesky

Reviewer's report:

General: First, I am familiar with the lab. It is essentially a molecular epidemiology, rather than a molecular biology, lab. It is a good molecular epidemiology laboratory and an adequate cancer molecular biology laboratory. This distinction is very important when deciding what to do with this paper. This paper must thereby, be considered as a cancer epidemiology paper...not as a strictly hypothesis driven mechanistic molecular biology of cancer paper.

Epidemiology papers make associations and correlations they do not strictly test molecular mechanisms. Good molecular Epidemiology papers add several levels of molecular correlation and association... THEY DO NOT AND CANNOT, by their nature, prove that a certain gene product causes a certain cancer or class of cancers.

This paper piles up a rather huge and convincing hill of molecular evidence that TIMELESS may be relevant to cancer cause, growth and spread....Across a wide range of human and experimental cancers.

I will not with your understanding, parrot all or even some of these more and less convincing observations, analyses and experiments. None are perfect BUT the sum total of evidence convinces me and will convince your readers that TIMELESS is of interest in cancer biology and control. This IS important, if not strictly novel.

We would like to express our sincere thanks for the reviewer's thoughtful insights and generous comments.