Author's response to reviews

Title: MDM2 negatively regulates the human telomerase RNA gene promoter

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Author's response to reviews: see over
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To The BioMed Central Editorial Team

RE: Revisions to MS: 1433508544740704 - MDM2 negatively regulates the human telomerase RNA gene promoter

Please find attached an electronic submission of the above revised manuscript that I hope you may now find suitable for publication.

We have reviewed the referees’ comments and feel we can address the issues you have raised in your Editorial letter. Clearly Referee’s 2 & 3 were very happy with the manuscript whilst Referee 1 required a bit more convincing.

Response to reviewer 1

MAJOR COMPLAURY REVISIONS

1. With regard to the reproducibility a little concern exists for the results of the chromatin immunoprecipitation; how reproducible are they as a weaker PCR product is seen when precipitating with MDM2 antibodies compared to Sp1 and TFIIIB precipitations and also some PCR product is formed when no antibody is used for the precipitations.

The chromatin immunoprecipitation experiments were performed three times and the PCR analysis of individual experiments was performed at least twice. The results were very reproducible and figure 1 shows a representative experiment. To clarify this point, the text has been modified in both the methods section (p.4) and in the legend for figure 1 (p.11). The reviewer also makes a point that the product generated from the MDM2 precipitation is less intense than those generated by Sp1 and TFIIIB antibodies. This is a relatively common phenomenon in chromatin IP (see, for example, J.Won et al, PNAS 2004, 101(31)11328) and presumably reflects a variety of factors including differing relative affinities of different antibodies for their antigens, relative concentrations of individual factors associated with the promoter and the availability of binding epitopes within larger complexes. We have not modified the text on this point. The reviewer also points to the presence of product in the “no-antibody lane”. The figure contains a negative control for the PCR, indicating that the “no-Ab” band is not PCR contamination. The presence of residual chromatin is also a relatively common feature of chromatin IP (see A.Clem et al, Gene 2003, 322, p.113) although it is often not shown. Although no antibody is included, this sample is however incubated with protein-A beads. The residual band presumably results from non-specific binding of chromatin associated proteins with protein-A. Non-specific binding of protein to beads is also a well known feature of conventional IP, as reflected by the existence of methodologies for pre-clearing lysate. The text has not been modified on this point.

2. In a previous study the authors showed different regulatory activities of the four Sp1 sites, two being positive regulators and two being negative regulators. As the RB control element (RCE) is only present at the Sp1.3 binding site, it can be speculated that when only analysing the effect of MDM2 on the four Sp1 binding sites (i.e. without the CCAAT and INR sequences) a negative regulation by MDM2 is seen on the Sp1.3 site. If not are the Sp1.1 and Sp1.4 sites which have been described as negative regulators themselves affected by MDM2? If not analysed the authors could speculate about the role of MDM2 on the individual Sp1 sites.
The reference to RCE in the text may cloud the point that the Sp1/pRb/MDM2 circuit is probably part of a more diverse set of mechanisms by which MDM2 might regulate the hTR promoter. The text has been modified on pages 3 and 6 to remove this reference. We have performed the gel shift assay for MDM2 binding to the Sp1.3, but did not observe an mdm2 specific supershift, suggesting that the mechanism of MDM2 action does not involve direct binding to this site. The text has also been modified on page 5 to indicate that this experiment was performed but this data is not shown. We also show in the paper that MDM2 can repress a reporter construct lacking functional Sp1 sites, indicating that the repressive effect is not wholly dependent on any of the individual Sp1 sites or specifically on the RCE motif. It is likely that other mechanisms are of equal importance. The CCAAT motif, for instance, is a key element in hTR promoter activation. We also show that MDM2 blocks NF-Y dependent transactivation, suggesting MDM2 might affect the NF-Y recruiting initiation transcription complex.

3. A more fundamental question concerns the biological meaning of the finding that MDM2 inhibits hTR promoter activity since both an amplification of MDM2 and hTR overexpression are frequent events in cancer cells. Moreover, is anything known about the endogenous expression levels of MDM2 in the 5637 cell line?

We have not directly examined MDM2 expression in 5637. However, according to K.M.Rieger et al British Journal of Cancer 1995, 72(3)683, the 5637 cell line expresses low levels of MDM2 but it is not amplified. The text has been modified on pages 2, 3, and 5 to indicate this and the reference has been included. In the same study, the authors find MDM2 amplification in only 2/12 bladder carcinoma cell lines. Since hTR amplification is well correlated with prognostic stage in bladder carcinomas (for review, see M. Muller, 2002, Oncogene 21(4) p650), the over-expression profiles of hTR and MDM2 are not necessarily overlapping. However, even in instances where both are over-expressed, it is likely that numerous other over-active pathways also combine to regulate hTR. It is possible that MDM2 regulation may be more physiologically relevant in the context of normal development.

MINOR ESSENTIAL REVISIONS

1. In Figure 4, which is very illustrative, the authors also speculate on a negative effect of MDM2 on the TATA box binding complex, whereas this is not formally proven by their studies.

On pages 6-7 we suggest that MDM2 may repress the hTR promoter by multiple mechanisms including interactions with Sp1, NF-Y and, possibly, the general transcription factors. We have modified the text on pages 6-7 to clarify that possible interactions with general transcription machinery remain speculative.

DISCRETIONARY REVISIONS

1. In line of the described findings it would be interesting to know whether in the here described context MDM2 binds to Sp1 and if a physical interaction is disturbed in the presence of pRb. Have the authors for example performed coimmunoprecipitations for MDM2 and Sp1, both in the absence and presence of pRB? Alternatively, if available the authors might want to add data on EMSAs using Sp1 binding site specific oligos in which a supershift of Sp1 binding is expected upon addition of MDM2.
We have not performed Sp1/MDM2 co-IP experiments, but, as indicated above we have performed gel shift analysis for MDM2 binding to Sp1.3. The results indicated that MDM2 did not directly bind the hTR promoter at this site and are now referred to in the text as “data not shown” on page 5.

Please do not hesitate to get in touch if I can be of help.

Yours sincerely,

W. Nicol Keith