Author's response to reviews

Title: Expression of RNA interference triggers from an oncolytic herpes simplex virus results in specific silencing in tumour cells in vitro and tumours in vivo.

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Author's response to reviews: see over
Dear Editor,

We would like to thank reviewer 2 for recommending our manuscript for publication. As requested by reviewer 1, we have performed two additional experiments and have revised the manuscript and figures accordingly. The MTS assay (figure 1g) and growth curves (figure 1h) included in the revised manuscript, in addition to our response to the reviewer’s first point, we believe address all of the reviewer’s comments. We therefore hope that the reviewer is satisfied and that the manuscript can now be accepted for publication.

Best regards,

Anna-Maria Anesti
Guy Simpson

Reviewer’s comments:

1. The authors argue that since the direct oncolytic effect of HSV is impaired by its attenuated ability to infect and replicate in some tumors, the therapy can be improved by incorporating an RNAi targeted to a secreted tumor protein. However, the copy number of the RNAi trigger would also be inhibited in these cells due to low/moderate levels of infectivity/replication. Therefore, the question of whether or not this strategy would be effective in these tumors remains to be answered. The only way to sufficiently answer this question would be to incorporate one of the proposed therapeutic RNAi triggers into the virus.

We explain that expression of shRNA and miRNA from the LAT region of an oncolytic HSV is not dependant on viral replication (page 10). Insertion of the same shRNA/miRNA expression cassettes in the LAT region of replication-defective HSV viruses resulted in efficient expression of RNAi triggers in BHK cells in the complete absence of viral replication (Anesti et al., 2008). Moderately susceptible tumour cells allow HSV entry and replication, but at a much lower rate than that required for effective tumour killing. Thus, expression of RNAi triggers from the LAT region, which is independent of viral replication and results in the production of numerous shRNA/miRNA molecules from a single viral genome copy, has the potential to improve the oncolytic properties of HSV by compensating for low viral particle numbers. Furthermore, in such tumours, the oncolytic process is slower thus allowing sufficient time for the silencing molecules to accumulate and induce effective silencing of targeted genes before infected cells are killed by the virus. Figure 2 demonstrates that despite the low level of HSV infectivity/replication in 9L cells, expression of miR-LacZ is sufficient to induce silencing of lacZ which is strongly expressed in 9L tumours. When the miRNA is engineered to target proteins secreted by tumour cells, which aid their growth or promote local immune suppression, moderate silencing in a proportion of cells in a tumour may be sufficient to promote cell death in both infected and non-infected cells in the tumour thus intensifying the effect of the treatment. We have explained this better in the revised manuscript.
2. It is unclear why the authors use quantification of b-gal concentration as a measure of cell survival. MTT assays are well established and standardized and would provide much more informative data than relying on the amount of b-gal expression of the stable cell lines to reflect cell viability. It has been demonstrated that HSV infection results in a shut-down of translation of host cell proteins, and even if this shut-down is only partial, I would expect a shift in translation levels to favour anti-viral responses. Therefore, a drop in a b-gal expression in virus-infected cells (increasing over time and in direct proportion to MOI) does not necessarily indicate cell death, but perhaps the cellular response to infection.

As requested by the reviewer, we have performed an MTS assay. Figure 1g of the revised manuscript demonstrates that cell death in BHK-LacZ cells increases with time and MOI of virus. Since β-galactosidase levels in BHK-LacZ cells infected with negative controls decrease with time and MOI of virus (Figure 1f) as a result of cell death induced by the oncolytic backbone (Figure 1g) and neither the cytotoxicity nor the growth of oncolytic HSV are affected by expression of RNAi triggers (Figure 1g and 1h), a significant further reduction in the levels of β-galactosidase in cells infected with oncolytic HSV expressing either shLacZ or miR-LacZ (Figures c, d and e) can only be attributed to an RNAi-specific effect.

3. The argument that “there is no reason to suspect” that insertion of short RNAi sequences would influence the growth or cytotoxicity of the virus, is not really scientifically acceptable. When presenting scientific data it is always important to include the necessary controls, even when the results are predicted. To include an additional panel representing the control vector would involve a minimal amount of work and answer some questions. Just because the kinetics of the shRNA- and miRNA-encoding vectors result in similar kinetics does not mean that they are similar to that of the backbone.

As requested by the reviewer, we have investigated the properties of oncolytic HSV expressing shRNA or miRNA by performing an MTS cell viability assay at 24, 48 and 72 hours on BHK-LacZ cells infected with the oncolytic backbone virus OncoVEX, Onc U6shLacZ or Onc miR-LacZ at MOIs 1.0, 0.1 and 0.01. Furthermore, we have prepared growth curves for these viruses. Figures 1g and 1h demonstrate that expression of shRNA or miRNA from the LAT region does not significantly influence the cytotoxicity and growth of oncolytic HSV, respectively.