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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Version: 2 Date: 13 April 2010

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

The manuscript has been extensively revised to: 1) accommodate all of the reviewers’ comments (see below point-by-point responses to their concerns), 2) more clearly demonstrate the novelty and significance of the results described, and 3) conform to the journal style. When reconsidering the manuscript for publication, we urge you to consider the following points: 1) our results represent the first and only demonstration of oncolytic HSV-mediated induction of RNAi, which until now was not considered possible due to the highly oncolytic properties of HSV, 2) although this is a proof-of-concept study, when adapted to target tumour-related genes, our approach has the potential to improve response rates to oncolytic HSV treatment in clinical situations, and 3) all three reviewers agree that our study is novel and interesting with exciting potential future applications.

Best regards,

Anna-Maria Anesti
Guy Simpson

Reviewer 1: Oliver Ebert

1.) The rationale for expressing RNAi from a replicating virus is not entirely clear. Replicating viruses kill susceptible tumor cells through a cytopathic effect, and therefore these viruses would not benefit much from the added potency provided by therapeutic gene silencing. The authors argue that this strategy would be useful for improving efficacy in selected tumor cells which are only “moderately” susceptible to HSV infection. Could the authors better define what they mean by “moderately”? Does the virus replicate in and kill these cells? Rat 9L glioma cells are used for the in vivo study; are these cells representative of most glioma cell lines in terms of their relative susceptibility to HSV infection?

Moderately susceptible tumour cells allow HSV viral entry and replication, but at a much lower rate than that required for effective tumour killing. As a result, a lower number of cells in a tumour will get infected by the virus at any given dose than in more susceptible cell types. While the virus will eventually kill infected cells, the process is slower. The lower level of viral replication will also result in a lower number of infectious particles being released to infect neighbouring tumour cells. When the kinetics of tumour cell killing do not exceed tumour growth, expression of shRNA/miRNA in combination to the viral cytopathic effect may be beneficial. We have demonstrated that in the time that is required for the oncolytic HSV to kill a moderately susceptible tumour cell, shRNA/miRNA can be expressed and also induce silencing in that cell (figure 2c&d), albeit at moderate levels. When this shRNA/miRNA is engineered to target proteins secreted by tumour cells, which aid their growth or promote local immune suppression, moderate silencing
in infected cells may be sufficient to promote cell death in both infected and non-infected cells in a tumour. We agree with the reviewer that the rationale needed to be better explained and it has been made clearer in the revised manuscript.

As demonstrated in fig 2a and previously shown by others, the virus used in this study infects 9L cells with moderate efficiency (Simpson et al 2005, Aghi 1999 Cancer Res 59 p3861, Jacobs et al 2001, Cancer Research p2983). This is further demonstrated by comparing revised fig 1f and 2b, which show cell death in a highly susceptible and moderately susceptible cell line, respectively. At MOI 1.0, 72hrs, the majority of 9L cells are still healthy, whereas the majority of BHK cells are killed by the virus. We have also previously shown in vivo that Oncovex replication inhibits the growth of 9L tumours in the flanks of nude F344 rats, but does not cure the tumours (Simpson et al 2005). Similar results have been obtained with this virus in RG2 glioma cells in vitro and in vivo. In contrast, hU87-MG cells (glioblastoma astrocytoma) are much more susceptible to the virus (Simpson et al 2005). Expression of prodrug activation genes from this oncolytic backbone in 9L cells enhanced the cytopathic effect of the virus (Simpson et al 2005). Thus, 9L cells are a suitable model to demonstrate the potential of this approach. This has been discussed in the revised manuscript.

2.) In Figure 1, #-gal expression levels in OncU6shLacZ and Onc MiR-LacZ infected cells were compared with those infected with the negative controls, U6shGFP and miR-neg. However, in the figure, there is only 1 bar labeled "control". Which of the two controls does this refer to? Furthermore, an additional control representing uninfected cells is needed in order to account for the fact that many of these cells are likely dead due to the virus infection, resulting in a drop in LacZ expression. To this end, it would also be quite important to include cell survival and viral titer data at these time-points.

B-galactosidase levels are expressed as a percentage of the expression measured in the cells infected with the negative controls (%LacZ). The negative control for Onc U6shLacZ is Onc U6shGFP and the negative control for Onc miR-neg is Onc miR-LacZ. However, since both negative control bars are 100%, we have simplified the graph by using a single bar for both controls. This has been made clearer in the revised manuscript.
Although we agree with the reviewer that it would be interesting to demonstrate the level of cell survival by including uninfected cells, we do not feel that this is a relevant negative control in this experiment. The number of cells and concentration of virus (i.e. control, Onc U6shLacZ, and Onc miR-LacZ) added to each culture remain the same in each one of the conditions. Since silencing of LacZ does not affect the cytopathic effect of the virus, the number of cells killed by each of these viruses is expected to be the same at each MOI and timepoint tested. Silencing is calculated as a percentage of the negative controls in order to compare silencing between conditions with different levels of cell survival. To take into account any variability in the number of dead cells, all experiments were repeated 4 times. This has been made clearer in the revised manuscript. Instead, to demonstrate cell survival, we have included figure 1f to the revised manuscript. Since all BHK-LacZ cells express lacZ, β-galactosidase levels are a good indication of the number of cells that have survived at each MOI and timepoint. Fig1f clearly shows that the numbers of dead cells increase with MOI and time.

3.) Growth curves of the recombinant siRNA- and miRNA-expressing vectors in comparison with the parental OncovEX vector should be included. Though it is unlikely that the insertion of the short RNAi sequences would alter the viral kinetics, this should be demonstrated.

The cassettes were inserted into the LAT region that is involved in the maintenance and establishment of latency, and reactivation from latency, and plays no role in the lytic phase of HSV infection. Furthermore, there is no reason to suspect that expression of shRNA/miRNA would affect growth of the virus. Such an effect has not been observed when these cassettes were inserted into disabled HSV vectors (Anesti et al. 2008) or during the production of high titre preparations of these oncolytic viruses. This has been discussed in the revised manuscript. Furthermore, figure 1f demonstrates that the number of dead cells in cultures infected with virus expressing either shRNA or miRNA is almost the same, indicating that viral kinetics (i.e. growth and cytotoxicity) are not affected by insertion of different RNAi inducing cassettes in the HSV genome.

Reviewer 2: John C Bell

(1) Page 7….xenograph should be xenograft
This has been corrected in the revised manuscript.
(2) The authors argue that this strategy could be used to enhance the replication of an OV in target cells. What sort of targets are they contemplating? How would this type of genetic manipulation maintain tumour specific replication? For instance, if the vector was engineered to dampen the anti-viral response would the resultant virus have enhanced virulence? This should be discussed.

It has not been argued that this strategy will enhance replication. We have only suggested that induction of RNAi may enhance the tumour killing properties of an OV by targeting proteins secreted by tumour cells, which aid their growth or promote local immune suppression.

(3) I like the idea that the vector could be programmed to down-regulate factors secreted normally by tumours to dampen anti-tumour immune responses. Can the authors expand on this concept?

Oncolytic HSV vectors expressing RNAi triggers could be programmed to downregulate factors normally secreted by tumours which increase angiogenesis and dampen anti-tumour immune responses. Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis. Up-regulation of VEGF is important in blood vessel formation in solid tumours (Plate et al., 1992) and also mediates tumour evasion of immune surveillance by inhibiting the development of dendritic and other hematopoietic cells (Ohm et al., 2001). In recent years, numerous studies have demonstrated that silencing of VEGF by non-viral-mediated delivery of siRNA leads to reduction in tumour size of up to 90% (Reich et al., 2003; Zhang et al., 2003; Detwiller & Kwon, 2005; Senn, 2005). To improve delivery of RNAi triggers against VEGF, non-replicating viruses were generated (Wang 2008, Lombardi 2009) and more recently, replication-competent adenoviruses expressing shRNA against VEGF and Interleukin-8 were shown to affect angiogenesis and inhibit tumour growth (Yoo et al., 2007, 2008). Other factors secreted by tumours, such as Interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), are attractive targets for knockdown using oncolytic HSV. IL-10 is an anti-inflammatory, immunosuppressive cytokine that is involved in tumour escape from immune surveillance (Yue et al., 1997). Elevated IL-10 levels have been found in a variety of human malignant tumours and are an independent prognosis factor for decreased response to chemotherapy in patients with advanced gastrointestinal malignancies (O'Garra, 1992, 2008). In a malignant B-1 cell line derived from a murine model for CLL, induction of RNAi against IL-10
resulted in anti-proliferative and pro-apoptotic effects (McCarthy et al., 2004). TGF-β is a multifunctional polypeptide which switches its role from a tumour suppressor in normal cells to a tumour promoter in advanced cancers (Wel et al., 2008). TGF-β protein-receptor interactions promote processes such as immune suppression, tissue remodelling, and formation of blood vessels, which lead to the growth and metastasis of cancer cells (Nagathihalli et al., 2010). TGF-β is the prototypic member of a large superfamily of secreted proteins that include three TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3). Antisense oligonucleotides against TGF-β1 developed for the treatment of non-small cell lung carcinoma (NSCLC), colorectal and prostate carcinomas have shown efficacy in preclinical development (Saunier et al., 2006). Antisense oligonucleotides against TGFβ2 have been tested in a number of clinical trials against various cancers, with promising results (Hau et al., 2007).

We have quantified western blots using the scion image software which measures band density, and this revealed a 20% reduction in β-galactosidase levels (p=0.04). However, because we agree with the reviewer that it can be difficult to convincingly demonstrate moderate reduction in protein levels using western blot, knockdown of β-galactosidase in the same tumours was confirmed using β-gal enzyme activity assay, and this revealed a highly significant 49% knockdown of β-galactosidase (P =0.0001). B-gal enzyme activity assay is quantitative and considerably more sensitive than western blot. Furthermore, β-galactosidase, which is highly expressed in 9L tumour cells, has a very long half-life resulting in high background on western blots, which makes quantification using band density very difficult. B-gal staining of sections is not quantitative and would fail to demonstrate silencing in the tumour as a whole. Furthermore, we can safely conclude that there is some degree of co-localisation between virus and β-gal since GFP (expressed by the virus) was detected in tumours by RT-PCR and every cell in the tumour expresses LacZ.
As the reviewer has rightly pointed out, Fig 2a demonstrates that at an MOI 1.0 only a proportion of 9L cells in the culture are infected by the virus at the time of the assay. As demonstrated in Fig 2a and previously shown by others, the virus used in this study infects 9L cells with moderate efficiency, i.e. 9L cells allow HSV viral entry and replication, but a lower number of cells in a tumour or culture will get infected by the virus at any given dose than in more susceptible cell types, and whilst the virus will eventually kill infected cells, the process is slower. In a culture dish, as the MOI and timepoints increase and the cell growth rate decreases, the virus will eventually infect all the cells in the culture and CPE can be achieved. However, in vivo, the kinetics of tumour killing do not exceed tumour growth and we have shown that Oncovex replication inhibits the growth of 9L tumours in the flanks of nude F344 rats, but does not cure these tumours (Simpson et al., 2005). This has been better explained in the revised manuscript.

To address the reviewer’s comment, we have revised Fig 2b to show that at the time of the assay a higher number of cells are killed by the virus at MOI 5.0 than MOI 1.0. To further demonstrate this, we have also included data for MOI 10.0. 5 times more cells are killed by the virus at an MOI 5.0 compared to MOI 1.0 and 1.5 times more cells are killed by the virus at MOI 10.0 compared to MOI 5.0. At MOIs 5.0 and 10.0, the majority of cells are dead and thus the reason we photographed the culture at MOI 1.0, where toxicity is minimal and silencing is optimal. We therefore demonstrate that balancing cytotoxicity against expression of RNAi triggers is critical in achieving optimal silencing. Although infecting cultures with oncolytic HSV at a higher MOI results in more infected cells and more viral particles per cell and thus more RNAi triggers being expressed, the cells will be killed faster thus allowing less time for the RNAi triggers to act. This point is further demonstrated in revised figure 1c-f. At an MOI 1.0, silencing in BHK cells (which are highly susceptible to HSV infection) is optimal at 48hrs where almost half of the cells are still healthy, whereas at 72hrs the majority of cells are killed by the virus and thus silencing is minimal. At an MOI 0.01,
however, minimal silencing is achieved at 24hrs, due to the low number of RNAi triggers being expressed, whereas optimal silencing is achieved at 72 hrs when the majority of cells are still healthy. This has been explained better in the revised manuscript.

Reviewer 3: Kevin A Cassady

1. Is the question posed by the authors well defined? There is no question posed. The authors state that “We aimed to express RNAi from oncolytic HSV, which has the potential to improve OncoVEX treatment by silencing tumor related genes in cells that are not directly killed by virus replication” This goal has not been achieved. The authors do not show any knockdown of a tumor specific gene other than their experimental transcripts (similar to that shown previously in the 2008 publication). Furthermore there is no evidence that this enhances indirect oncolytic effect altering tumor growth or animal survival.

The aim of this study was to show that it is possible to express RNAi triggers from an oncolytic HSV. Unlike oncolytic adenoviruses, which replicate at a very slow rate and have been already shown to induce RNAi in tumours, replicating HSV was not considered a suitable vector from which to express RNAi triggers, because it is robustly oncolytic. We show, for the first time, that it is possible to express RNAi triggers from an oncolytic HSV. Furthermore, we show that effective and specific silencing can be achieved in cells that are either highly susceptible or moderately susceptible to HSV infection. We then propose that since HSV is highly oncolytic, this approach may be particularly beneficial in the treatment of tumours that are moderately susceptible or unresponsive to HSV oncolysis. To this end, we have shown silencing in one such type of tumour in vivo. This is a proof-of-concept study that clearly demonstrates the potential to combine RNAi with oncolytic HSV, which has exciting potential clinical applications, but until now was not considered possible.

Figure 1. I recommend revising the schematic (currently shows LAT transcript in the wrong orientation). If the authors wish to show it in the sense direction then I suggest using the b’a’ repeats to show the transcript in the sense orientation. In addition the a’c’ flanking domain around the Us gene region were not included in their schematic representation of the HSV genome. The purpose of the schematic is so that a reader can identify the targeting domain of the plasmid and other potential mutations present in the construct. The image provided does provide the level of detail necessary (restriction sites near gene insertion points or absolute base numbers for gene insertion points in the LAT transcript and the impact on the antisense #0 transcript). This is especially frustrating to this reader because the virus is from a sequenced strain and these data are readily available.
Figure 1 has been completely revised to address the reviewer’s comments.

The fluorescent microscopy images are less convincing than in the previous 2008 publication. Were equivalent exposure times used for the Onc U6 shLacZ infected (upper) and OncU6shGFP infected samples (lower panel) (Figure 1b). There is no information provided on magnification of the image (I assume using a 10x objective). The LacZ quantitative data is sound and very convincing.

The exposure times were identical. Indeed, a 10x objective was used. This information has been added to the revised images.

Figure 2B LacZ knockdown. Is it possible that the authors reversed the statistical significance or MOI label? The high MOI (5) sample has less gene knockdown (greater LacZ expression) and greater standard deviation than the low MOI (1.0) cohort, yet according to the statistical analysis there is greater statistical certainty (a P value of 0.0001) in the sample with the greatest standard deviation. Also there are no error bars provided for the control samples and no discussion of the statistical methods used to validate the data.

In revised figure 2b, error bars have been added to the negative controls and all P values have been re-calculated using unpaired student t test.

Figure 2C: Loading controls show high variability in the # tubulin production in their samples. The authors loaded equivalent protein mass (20ug). Is # Tubulin subject to VHS-mediated degradation? Is there another explanation for the variability in the loading control?

There is no published data to suggest that tubulin is subject to vhs-mediated degradation and it is unlikely that vhs would target tubulin, as it dowregulates genes that are activated following infection and which can be deleterious to HSV infection. This variability is most likely due to a technical issue (more likely pipetting of the samples). For this reason, we have used enzyme activity assay, which is more sensitive, to confirm knockdown in these tumours.

Figure 2D: Authors show statistically significant difference between the Onc miR-neg and Onc miR-LacZ treated samples in terms of LacZ expression. There appears to be a significant difference between the untreated (120%) and Onc miR-neg treated sample. Was this analyzed? How is this explained (loss of cells through viral lysis in the virus treated sample)? Again there are no error bars
We have previously showed *in vivo* that the backbone HSV virus used in this study can inhibit the growth of 9L tumours in the flanks of F344 rats (Simpson et al 2005). Therefore, we can conclude, as the reviewer suggested, that the difference is due to oncolysis caused by the viral backbone. The P value is 0.005 and has been calculated using unpaired student t test. Each *in vivo* experiment was performed twice. This information is now provided in the revised manuscript. Error bars have been added to the negative control bars.