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TITLE: Prostate-Specific and Tumor-Specific Targeting of an Oncolytic HSV-1
Amplicon/Helper Virus for Prostate Cancer Treatment

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14. ABSTRACT Oncolytic virotherapy is a promising approach for treating advanced cancers. A major focus in developing oncolytic viral vectors is enhancement of tumor-specificity and reduced toxicity to normal tissues. Recently discovered microRNAs (miRNAs) have provided a new opportunity for more stringent regulation of tumor-specific viral replication. In the present study, we incorporated multiple copies of miRNA complementary target sequences (for miR-143 or miR-145) into the 3' untranslated region (3'UTR) of a herpes simplex virus-1 (HSV-1) essential viral gene, ICP4, to create CMV-ICP4-143T and CMV-ICP4-145T amplicon viruses. Our results indicated that while miR-143 and miR-145 are highly expressed in normal tissues, they are significantly downregulated in prostate cancer cells. We further demonstrated that miR-143 and miR-145 inhibited the expression of the ICP4 gene at the translational level by targeting the corresponding 3'UTR in a dose-dependent manner. This enabled selective viral replication in prostate cancer cells. When mice bearing LNCaP human prostate tumors were treated with these miRNA-regulated oncolytic viruses, a >80% reduction in tumor volume was observed with significantly attenuated virulence to normal tissues in comparison to control amplicon viruses not carrying these 3'UTR sequences. Our study is the first to show that inclusion of specific miRNA target sequences into the 3'UTR of an essential HSV-1 gene is a viable strategy for restricting viral replication and oncolysis to cancer cells while sparing normal tissues.					
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INTRODUCTION

Prostate cancer remains the second leading cause of cancer death in men, and current treatment options are not effective in treating advanced states of the disease (1). Current treatments, including androgen withdrawal therapy, are not curative for advanced, metastatic disease and new treatment strategies are urgently required (2). In this regard, oncolytic virotherapy offers a new treatment modality for managing advanced cancer as many oncolytic viral agents have been proven safe in a number of clinical trials (3, 4). However, the efficacy is still quite limited due to the extensive deletions of viral genes, resulting in substantial attenuation of the viruses. To enhance the potency of viral oncolysis, the integrity of the viral genome should be preserved as much as possible. Hence, the goal of this project is to generate a prostate-cancer specific oncolytic HSV-1 by changing only the regulatory elements flanking an essential viral gene, without any deletions of the viral genome, for the treatment of locally advanced, recurrent and metastatic disease. The main hypothesis is that an oncolytic HSV-1 virus can replicate in a tumor-specific fashion through translational regulations of an essential viral gene, *ICP4*, leading to selective HSV-1 replication within and lysis of prostate cancer cells. To this end, we demonstrated that endogenous miRNAs can be exploited to regulate expression of an essential HSV-1 viral gene in a sequence-specific manner, resulting in viruses which are selectively oncolytic for tumor cells while leaving normal tissues unharmed. This new targeting strategy could provide a basis for the development of safe therapeutic viral vectors to treat locally advanced and metastatic cancers and may greatly enhance tumor-specific targeting when combined with existing strategies.

BODY

Translational regulation is a fairly new targeting strategy in oncolytic virotherapy. With the recent discovery of miRNAs in 2001, we are one of the first groups to demonstrate that differentially expressed tumor suppressor miRNAs can be utilized to control the replication of an oncolytic DNA virus in a tumor-specific fashion. We provided the first proof of principle that an HSV-1 amplicon/helper virus containing specific miRNA target sequences (miR-143 or miR-145) can be used for oncolytic virotherapy through tumor-specific expression of the *ICP4* gene (5). Tumor suppressor miRNAs such as miR-143 and miR-145 are ideal candidates to be incorporated into oncolytic viral vectors in that they are globally downregulated in tumor cells relative to normal tissues, thereby offering a wide applicability towards the treatment of various cancer types (6-8). Our work has recently been published in *Clinical Cancer Research* (5), and a copy is attached in the Appendices.

In vitro screening using cell lines that express varying levels of tumor-related miRNAs offer a quick and easy way to identify the best complementary miRNA target sites to incorporate into the viral vectors. Accordingly, we examined miR-143 and miR-145 expression levels in various normal and malignant prostate cancer cell lines attempting to find two representative cell lines with high and low miR-143 or miR-145 levels to test our viral constructs, which carry the specific target sequences in the 3'UTR of the *ICP4* gene. Unfortunately, the expression of miR-143 and miR-145 was lost in BPH-1 and PNT1B normal prostate cell lines (Figure 1). Similar results have previously been reported as well and it is most likely an oddity/abnormality that has occurred only in cell lines as miR-143 and miR-145 are highly expressed in normal human tissues (Figure 2). We did not screen miR-143 and miR-145 expression in normal non-prostate cell lines because they are less relevant to this study. Instead, we created two stable cell lines, LNCaP-143 and LNCaP-145, which express endogenous miR-143 or miR-145. The expression level of miR-143 in LNCaP-143 cells is equivalent to that seen in the normal mouse tissues, but the expression level of miR-145 in LNCaP-145 is >80 times less than in normal mouse tissues (Figure 1). Since both of these miRNAs are normally downregulated in tumor cells, and have been suggested to function as tumor suppressors, their re-expression in tumor cells may impart detrimental effects on cellular growth and may change cellular phenotypes. In this regard, miR-143 and miR-145 have been shown to inhibit tumor cell growth by targeting ERK5 and the insulin receptor substrate-1, respectively (9, 10). Subsequently, we infected these stable cell lines with our modified miRNA-mediated amplicon viruses and examined cell viability using the MTT assay. Our results showed that LNCaP cells were protected from CMV-ICP4-143T viral infection by miR-143 with 70% of the cells still alive at 120 hrs post-viral infection, whereas only 30% of the parental LNCaP cells were alive (Figure 3). In contrast, protection of LNCaP cells by endogenous miR-145 only lasted 96 hours post-viral infection with the CMV-ICP4-145T amplicon virus. Furthermore, our *in vivo* results showed

minimal toxicity in the gastrointestinal (GI) tract (eg. stomach, intestines) of mice treated with CMV-ICP4-143T or CMV-ICP4-145T amplicon viruses. These findings suggest that the efficiency and degree of protection by miRNAs is directly related to the amount of intracellular miRNAs present. Fortunately, a major advantage of this targeting strategy is that several copies of one or multiple miRNA species can be incorporated into the viral genome to further enhance tumor-specificity. In cases where the normal cells do not express a sufficient amount of a particular miRNA, a combination of more than one tumor-specific or tissue-specific miRNA can be used to enhance tumor-specificity (eg. 143T+145T). Another approach is to incorporate an additional tissue-specific miRNA from the organs that are normally more susceptible to viral infections. For example, since HSV-1 is a neurotropic virus and the neuron-specific miR-124 is highly expressed in the human brain and in the eyes and brain of nude mice (Figure 2 & 4), the incorporation of miR-124T into the 3'UTR can be beneficially more effective in eliminating viral spread throughout the nervous system, thereby reducing unwanted cytotoxicity in normal tissues.

This miRNA-mediated translational targeting strategy can also be used in conjunction with pre-existing targeting approaches. For instance, we have generated two prostate-specific and tumor-specific amplicon viruses, ARR₂PB-ICP4-143T and ARR₂PB-ICP4-145T (Figure 5), and tested their efficacies *in vivo*. A greater than 60% reduction in tumor size and luciferase (reporter protein) activity was observed in mice treated with ARR₂PB-ICP4-143T and ARR₂PB-ICP4-145T at 28 days post-viral injection (Figure 6 & 7). As with the CMV-ICP4-143T and CMV-ICP4-145T amplicon viruses, the ARR₂PB-ICP4-143T and ARR₂PB-ICP4-145T amplicon viruses efficiently reduced the tumor burden *in vivo* albeit with an initial delay in response. Quantitative real-time PCR analysis of various organs from the treated mice showed that the majority of the virus was detected in the tumors and only small traces were found in the stomach (Figure 8). This residual toxicity in the GI tract has always been an issue in our amplicon/helper system, and can partly be explained by the fact that an amplicon virus carries greater than 15 copies of the *ICP4* gene, which can easily overwhelm the regulatory controls. The best way to eliminate this problem would be to generate a recombinant oncolytic virus for the purpose of clinical applications. Nevertheless, our results demonstrate the potential applicability of dually regulating viral replication both at a transcriptional level by the prostate-specific promoter and at a translational level by miRNAs.

Our research has demonstrated that oncolytic viruses can be subjected to endogenous miRNA control, thus providing a foundation for the development of a novel and safe targeting strategy towards the treatment of prostate and other cancers. This would create a new category of oncolytic viruses, miRNA-mediated oncolytic viruses, which are safer and more efficient in tumor-specific destruction. This strategy is particularly applicable towards the development of cancer vaccines and virotherapeutics.

KEY RESEARCH ACCOMPLISHMENTS

- We tested the hypothesis that differential expression levels of endogenous microRNAs (miRNAs) can be used to control herpes simplex virus type 1 (HSV-1) viral replication at the translational level through regulation of the expression of an essential viral gene (ICP4).
- We demonstrated that, by incorporating specific miRNA target sequences, which are abundant in normal tissues but low or absent in prostate cancer cells, into the 3'UTR of the viral ICP4 gene, viral replication was restricted to tumor cells only.
- In both *in vitro* and *in vivo* tumor model systems, the miRNA-regulated viral replication showed strong selective tumor lysis.
- To our knowledge, this is the first proof of principle that an HSV-1 amplicon/helper virus containing specific miRNA target sequences (miR-143 or miR-145) can be used for oncolytic virotherapy through expression of an essential viral gene in a tumor-specific fashion.

REPORTABLE OUTCOMES

Publication

- Lee CY, Rennie PS, Jia WW. MicroRNA regulation of oncolytic herpes simplex virus type 1 for selective killing of prostate cancer cells. *Clinical Cancer Research*. 2009; 15(16): 5126-5135.

Conference Abstracts/Award

- Lee CY, Rennie PS, Jia WW. MicroRNA-mediated targeting of oncolytic Herpes Simplex Virus type 1 to prostate tumors. The Fifth International Meeting on Replicating Oncolytic Virus Therapeutics, Banff, AB, March 18-22, 2008. Abstract S16 (Oral presentation).
- Lee CY, Rennie PS, Jia WW. MicroRNA-mediated targeting of oncolytic Herpes Simplex Virus type 1 to prostate tumors [Abstract]. In: Proceedings of the 100th Annual Meeting of the American Association for Cancer Research; 2009 Apr 18-22; Denver, CO. Philadelphia (PA): AACR; 2009. Abstract #3777 (Poster presentation).
Won AACR-Qiagen Scholar-in-Training Award 2009.
- Lee CY, Rennie PS, Jia WW. MicroRNA regulation of oncolytic herpes simplex virus type 1 for selective killing of prostate cancer cells. The 10th Annual Pathology Day, Vancouver, BC, May 2009. Abstract #20. (Oral presentation)
- Lee CY, Rennie PS, Jia WW. MicroRNA-mediated targeting of oncolytic herpes simplex virus type 1 to prostate tumors. 3rd Annual Lorne D. Sullivan Lectureship and Research Day, Vancouver, BC, June 9, 2009. Abstract p13. (Oral presentation)

Degree

- I have obtained my PhD degree at the University of British Columbia (Convocation date: 11/25/2009) while supported by this award.

Current position

- I am now a postdoctoral scholar at the Stanford Cancer Center and Institute of Stem Cell Biology and Regenerative Medicine.

CONCLUSION

Even though virotherapy for cancer treatment is still in its infancy, significant progress has been made in the last two decades (11-14). The field of oncolytic virotherapy has evolved around one central theme - capitalizing on the differential cellular properties between normal and tumor cells. Based on data from clinical trials, it is widely recognized that the current oncolytic viral vectors can be safely administered to patients at high doses, but their efficacy is seldom seen. This could be explained by the attenuation of viruses through gene deletions, clearance of virus by the immune system, and ineffective intratumoral spread. However, the anti-tumor efficacy of oncolytic viruses is significantly enhanced when used in combination with chemotherapy or radiation therapy. To improve the effectiveness of an oncolytic viral vector, a major focus has been the enhancement of tumor-selective targeting without compromising viral cytotoxicity. To this end, we have successfully demonstrated that an oncolytic HSV-1 virus can be engineered to target tumor cells specifically without extensive collateral damage to normal tissues through both transcriptional and translational regulation of viral replication. Our results demonstrated that the utilization of the tissue-specific promoter and the miRNA-mediated 3'UTRs in a targeted virotherapy is a viable approach with significantly enhanced anti-tumor efficacy and specificity. This type of oncolytic virus can be armed with immunostimulatory cytokines to elicit immune responses against tumor antigens. Ultimately, this research provides a fundamental basis for the development of novel oncolytic viruses for the treatment of various cancers through both local and systemic deliveries.

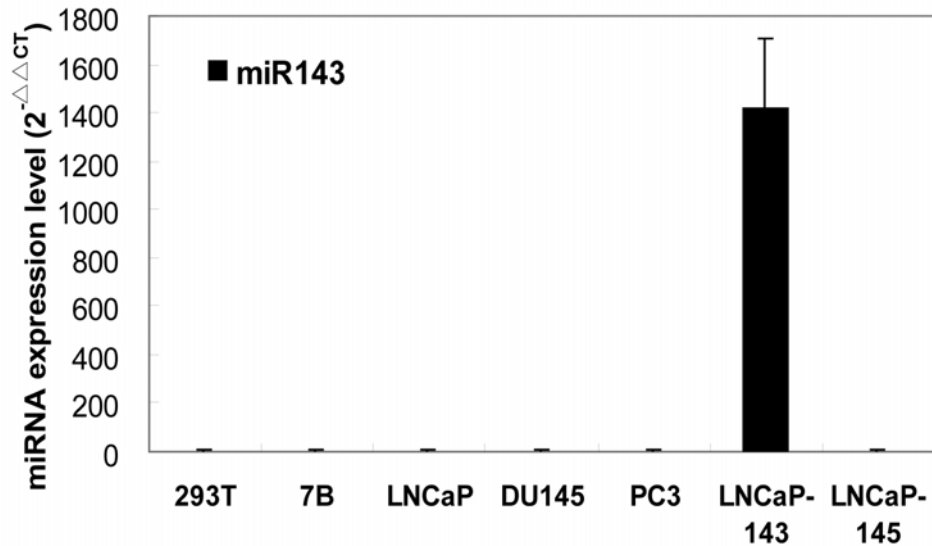
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APPENDICES

Figures

A



B

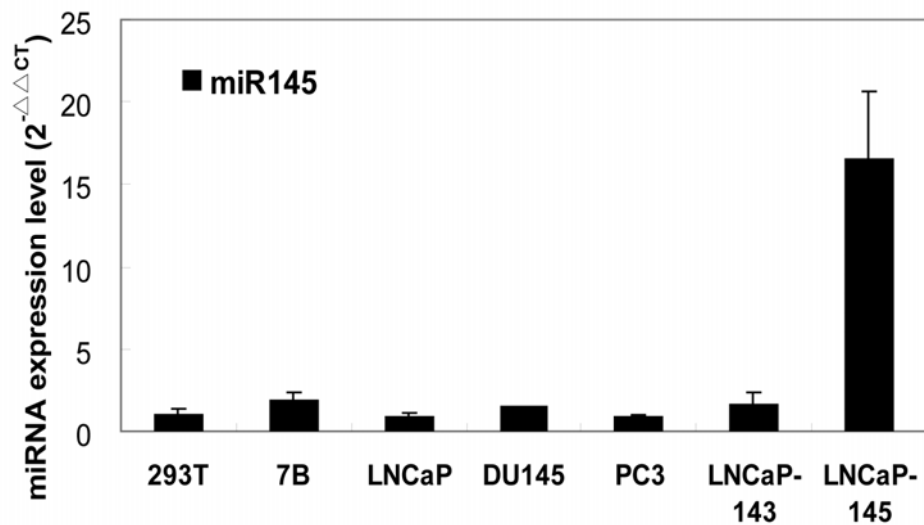


Figure 1. The expression of miR-143 and miR-145 in LNCaP stable cell lines. LNCaP-143 and LNCaP-145 are stable cell lines that constitutively express miR-143 (A) and miR-145 (B) from a CMV promoter. The expression of both miRNAs was lost in the normal prostate epithelial cell lines, BPH-1 and PNT1B. The expression level of miR-143 in LNCaP-143 cells is equivalent to that seen in the normal mouse tissues, but the expression level of miR-145 in LNCaP-145 is >80 times less.

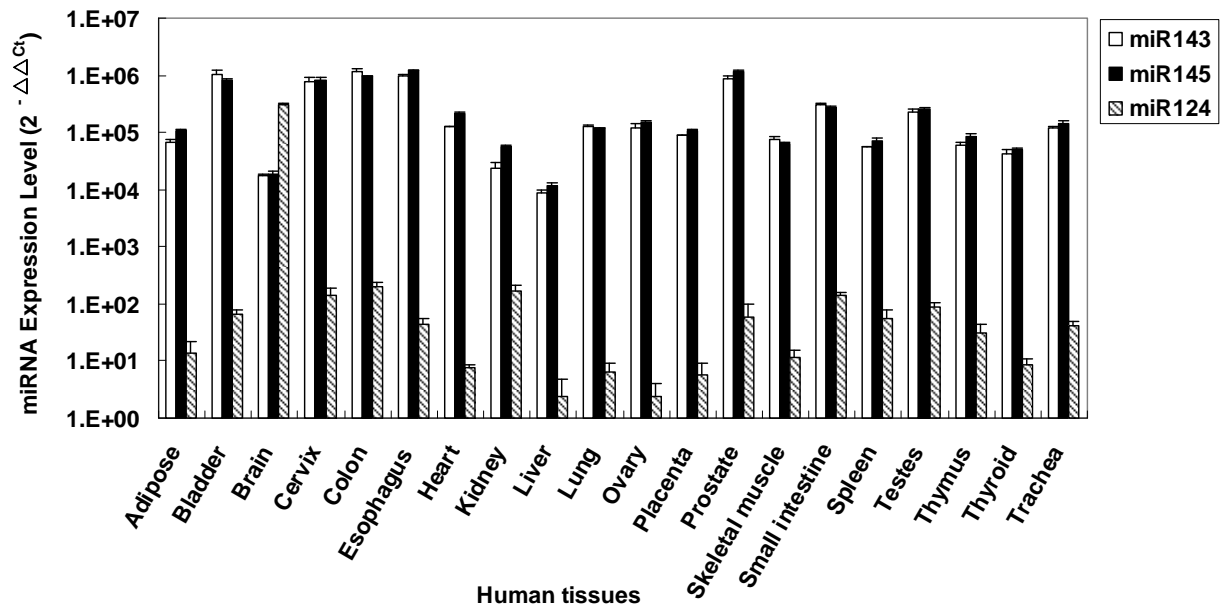


Figure 2. MiRNA expression levels in human normal tissues. The expression levels of miR-143, miR-145 and miR-124 in human tissues were determined by quantitative RT-PCR assays and normalized to U6 RNA. Human tissue RNAs were purchased from Ambion. Ten ng of RNA was reverse transcribed to cDNA followed by TaqMan real-time PCR assays. MiR-143 and miR-145 are highly expressed in all normal tissues examined here, whereas miR-124 is a neuron-specific miRNA that is only abundant in the brain.

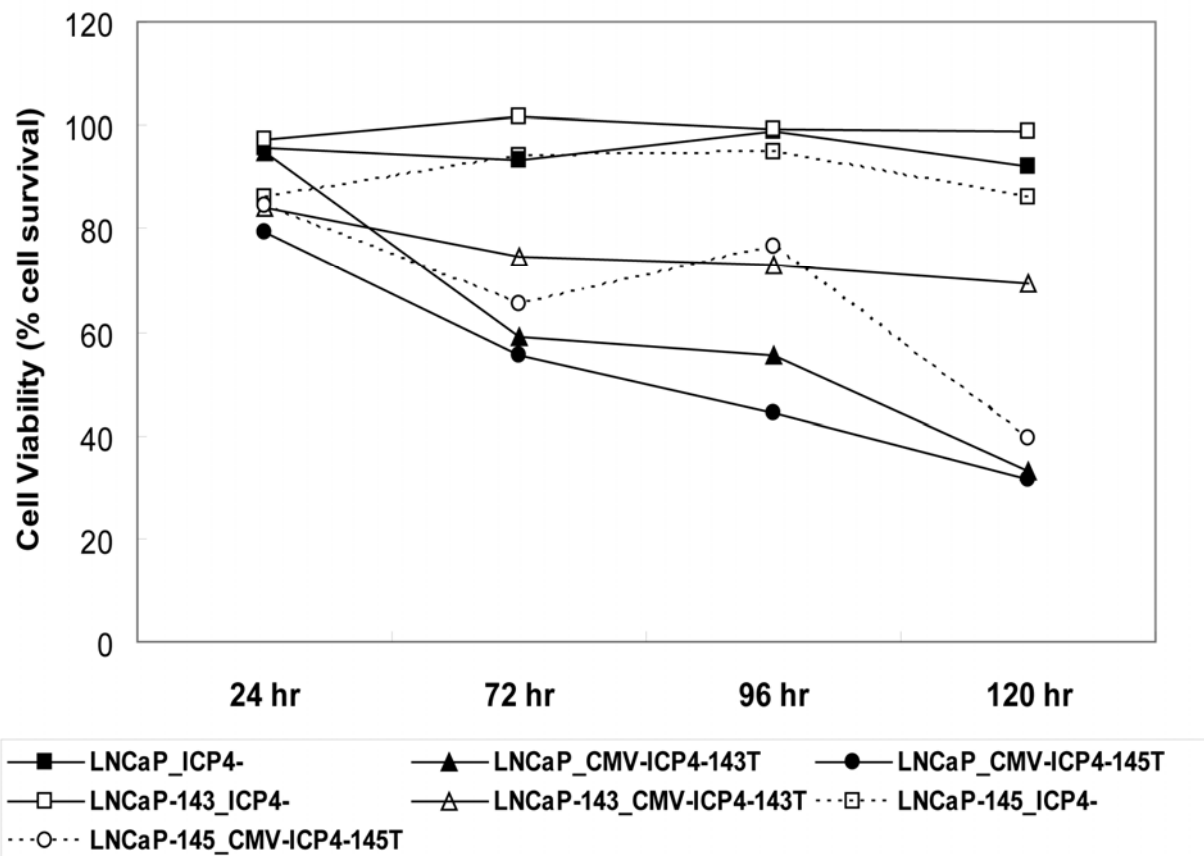


Figure 3. MiR-143 and miR-145 reduced cytotoxicity in LNCaP cells infected with miRNA-regulated amplicon viruses. To demonstrate whether miR-143 and miR-145 could protect normal tissues from miRNA-regulated amplicon virus infections, LNCaP, LNCaP-143 and LNCaP-145 cells were infected with the ICP4⁻ helper virus and amplicon viruses at an MOI of 1, and cell viability was determined by MTT assays. The results showed that $68 \pm 7\%$ of the LNCaP cells were killed by CMV-ICP4-143T and CMV-ICP4-145T amplicon viruses at 120 hr post-viral infection, whereas ICP4⁻ helper virus infection showed no significant cytotoxicity. On the other hand, $70 \pm 6\%$ of the LNCaP-143 cells infected with CMV-ICP4-143T amplicon virus were still alive at 120 hr post-viral infection ($p = 0.0005$) and $76 \pm 7\%$ of the LNCaP-145 cells survived CMV-ICP4-145T amplicon virus infection at 96 hr post-viral infection ($p < 0.005$). These results indicated that CMV-ICP4-143T and CMV-ICP4-145T amplicon viruses were able to kill LNCaP cells *in vitro* and that cell cytotoxicity was reduced when the infected LNCaP cells expressed the corresponding miRNA. In other words, miRNAs were able to protect cells from miRNA-regulated virus infections by inhibiting viral replication at a translational level through 3'UTR.

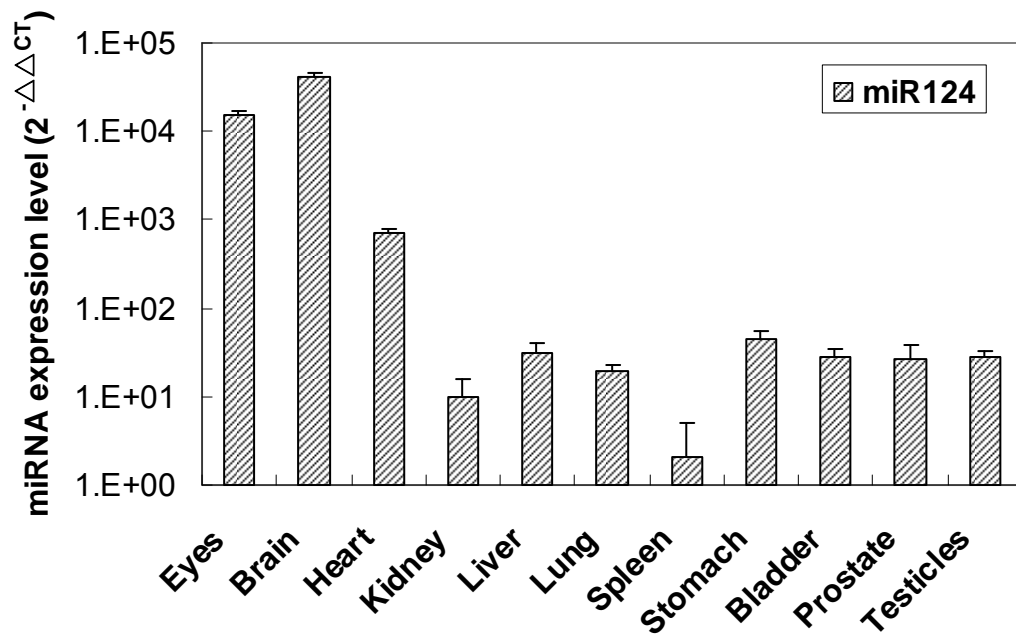


Figure 4. MiR-124 expression levels in normal mouse tissues. The expression levels of miR-124 in normal mouse organs were determined by quantitative RT-PCR assays and normalized to snoRNA234 RNA. Total RNA from the organs was extracted using the Trizol reagent and ten ng of RNA was reverse transcribed to cDNA followed by TaqMan real-time PCR assays. The results showed that miR-124 is highly expressed in the eyes and brain of nude mice.

1. ARR2PB-ICP4-143T



2. ARR2PB-ICP4-145T



Figure 5. S structure of the two prostate-specific and tumor-specific amplicon plasmids used to package replication-competent amplicon viruses. ARR₂PB-ICP4-143T and ARR₂PB-ICP4-145T are both prostate-specific and tumor-specific miRNA-regulated complementing amplicons. ARR₂PB-ICP4-143T amplicon contained five tandem copies of miR-143 complementary sequences (143Tx5) and ARR₂PB-ICP4-145T amplicon contained four tandem copies of miR-143 complementary sequences (145Tx4) after the *ICP4* gene.

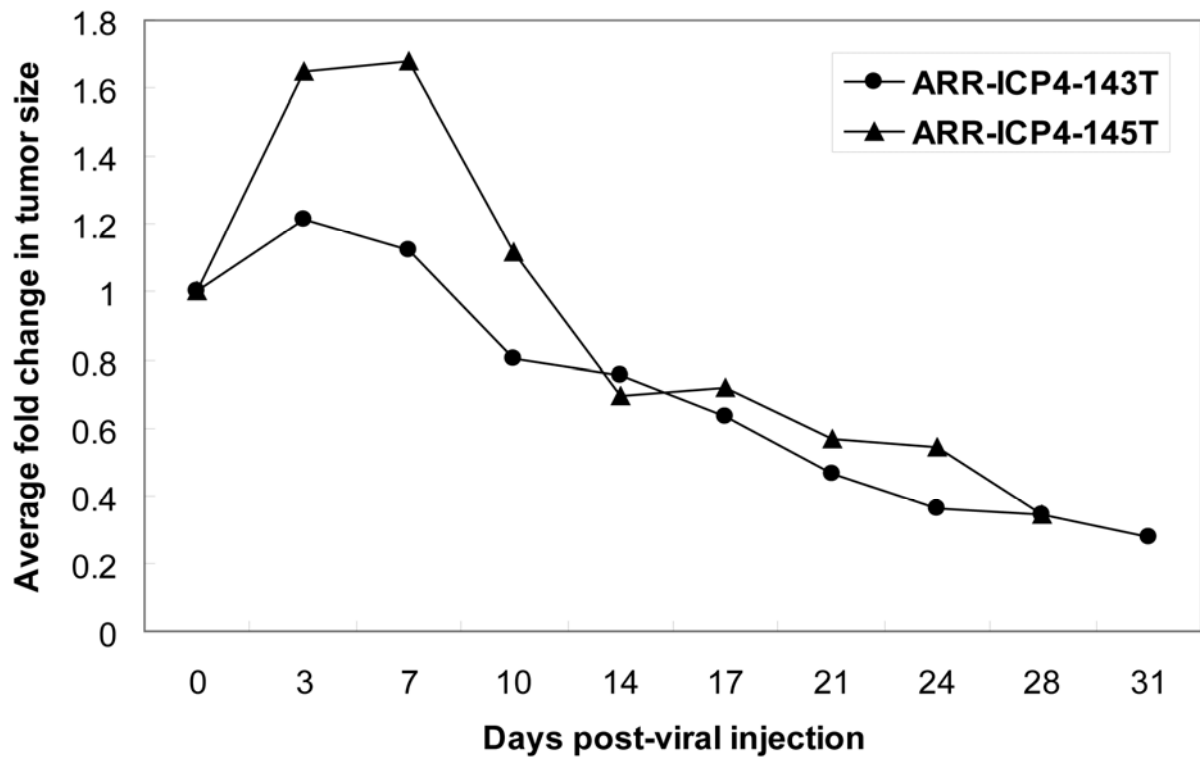


Figure 6. ARR₂PB-ICP4-143T and ARR₂PB-ICP4-145T amplicon viruses inhibited LNCaP-Luc tumor growth in xenograft mouse model. Nude mice with subcutaneous LNCaP-Luc tumors ($\sim 140 \pm 70 \text{ mm}^3$) were treated with two intratumoral injections (Day 0 and Day 7) totalling 2×10^6 pfu of the ARR₂PB-ICP4-143T or ARR₂PB-ICP4-145T amplicon virus. Continued growth of the tumors was observed initially during the first week after viral treatment. Regression of tumors was observed shortly after the second viral injection with tumor size decreasing steadily thereafter. At day 28 post-viral injection, a >65% reduction in the tumor size was observed in both treatment groups. In one of the three mice treated with the ARR₂PB-ICP4-145T amplicon virus, the virus did not reduce the tumor size but was able to inhibit further growth. Nonetheless, in comparison with the CMV-ICP4-143T and CMV-ICP4-145T amplicon viruses, the dually regulated ARR₂PB-ICP4-143T and ARR₂PB-ICP4-145T amplicon viruses were able to significantly inhibit tumor size efficiently.

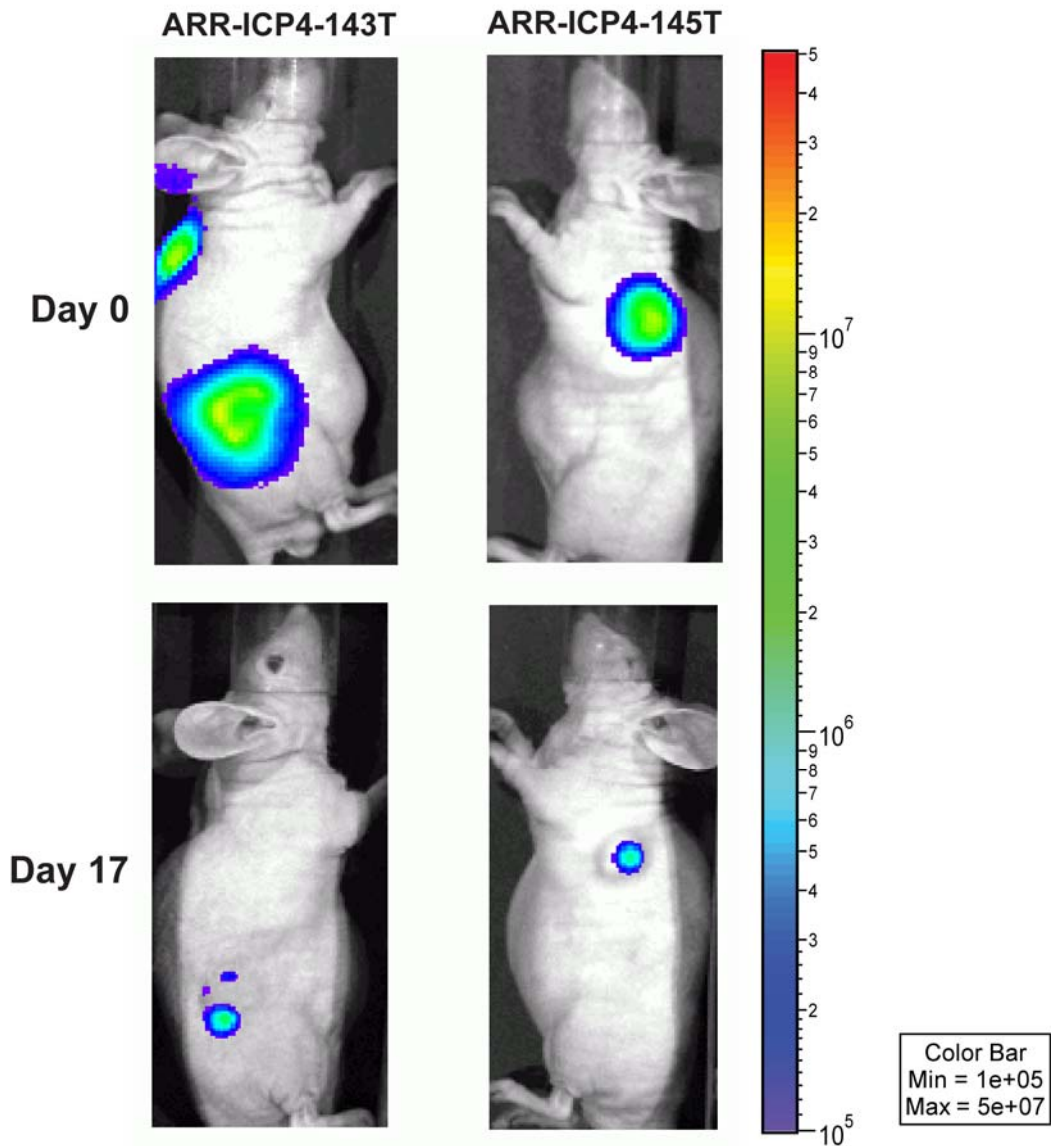


Figure 7. Representative IVIS images of the mice treated with the ARR₂PB-ICP4-143T and ARR₂PB-ICP4-145T amplicon viruses. The size of the tumor was also visualized by the IVIS imaging system. Luciferase activity was expressed as p/sec/cm²/sr. A >80% reduction in luciferase activity was observed in mice treated with the prostate-specific and miRNA-mediated amplicon viruses at day 17 post-viral injections.

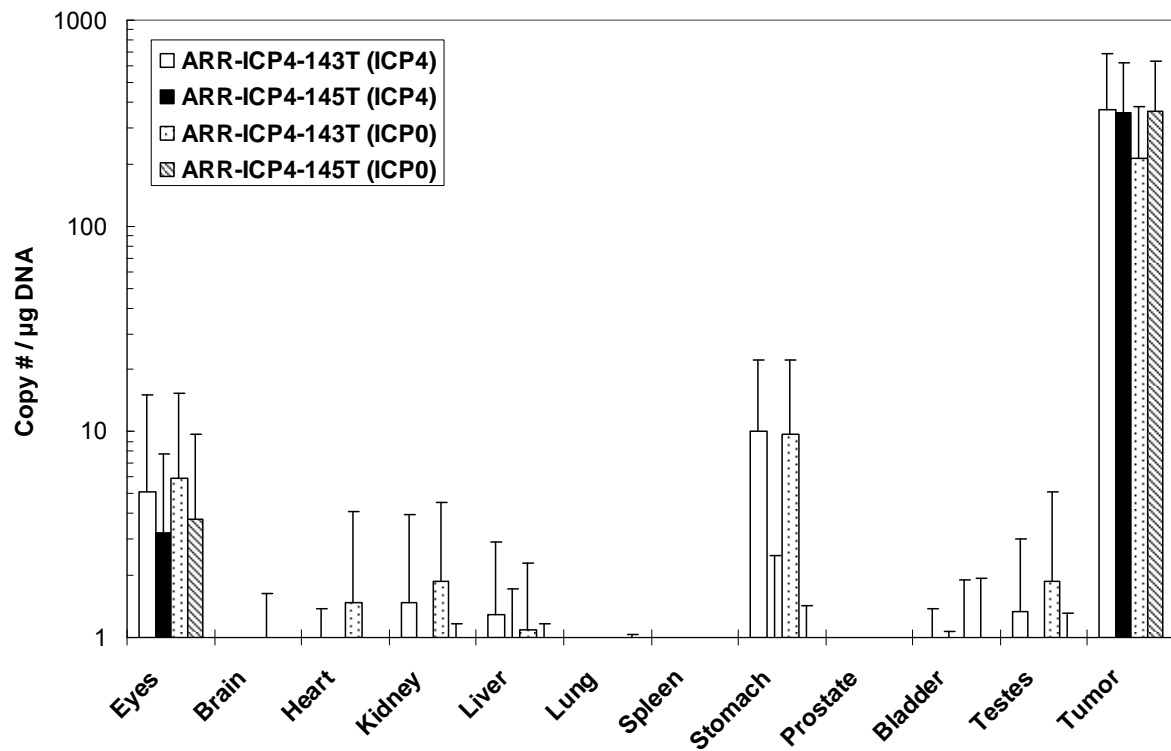


Figure 8. Biodistribution of the ARR₂PB-ICP4-143T and ARR₂PB-ICP4-145T amplicon viruses. Total DNA was extracted from the organs and the copy number of *ICP4* and *ICP0* was determined by real-time PCR assays. The results were expressed as relative copy number / μg of DNA. The presence of the amplicon and helper viruses was indicated by the copy number of *ICP4* and *ICP0*, respectively. The majority of the virus was detected in the tumors with over 200 copy numbers of *ICP4* or *ICP0* genes. A very small amount of the viruses was also detected in the eyes (less than 5 copies) and the stomach (less than 10 copies) of the treated mice.

CURRICULUM VITAE

Cleo Yi-Fang Lee

Education

Sept. 1999 – May, 2003 B.Sc. Honors Biochemistry & Molecular Biology
University of British Columbia

Sept. 2003 – Sept. 2009 Ph.D. Pathology & Laboratory Medicine
University of British Columbia

Awards and Distinctions

<u>Name/Type</u>	<u>Dates</u>	<u>_____</u>
British Columbia Provincial Scholarship	1999-2000	
Simon Fraser University Entrance Scholarship	1999-2000	
Canada Millennium Scholarship Foundation Bursary	2001-2002	
Ph.D. Tuition Fee Award, UBC	2004-2008	
The Canadian Student Health Research Forum (CSHRF) Travel Award	2007	
CIHR - Institute of Cancer Research Silver Poster Award	2007	
US Army DOD pre-doctoral award (3 years)	2008-2010	
AACR-Qiagen Scholar-in-Training Award	2009	

Research Expertise

- Prostate cancer
- Cancer gene therapy
- Targeted virotherapy
- Viral vector mediated oncolysis
- MicroRNA regulation
- HSV-1 amplicon and recombinant viruses
- Molecular cloning
- Cell culture/gene transfection
- Xenograft mouse model
- Histology

Research Experience

<u>Position</u>	<u>Organization/Department</u>	<u>Supervisor(s)</u>	<u>Dates</u>
Undergrad student (Honors Thesis) ¹	University of British Columbia Biochemistry & Molecular Biology	Dr . Ross MacGillivray	Sept. 2002 – Apr. 2003
Research student ²	University of British Columbia Division of Infectious Disease, VGH	Dr . Michael Grigg	May-Aug. 2003
Ph.D. student (Directed Study) ³	University of British Columbia Pathology & Laboratory Medicine	Dr . Christopher Ong	Sept. 2003 – Apr. 2004
Ph.D. student ⁴	University of British Columbia Pathology & Laboratory Medicine	Dr . Paul Rennie Dr . William Jia	Sept. 2003 – Aug. 2009

¹ Honors Thesis: Analysis of human coagulation factor IX gene mutations causing hemophilia B in two B.C. families.

² Published article: The SRS superfamily of *Toxoplasma* surface proteins.

³ Project Title: Generation of translationally controlled tumor-specific reporter transgenic mice by lentivirus mediated transgenesis.

⁴ Ph.D. Thesis title: Tumor-specific targeting using oncolytic herpes simplex virus type1 for prostate cancer treatment.

Competitive Grant

1. The Prostate Cancer Research Foundation of Canada (PCRFC) Research Grant for one year (Co-Investigator, Dr. William Jia is PI), Oncolytic HSV-1 with a 3' UTR regulatory element for prostate cancer specific virotherapy (2007-2008).

2. US Army Department of Defense Prostate Cancer Pre-Doctoral Training Award for three years, Prostate-Specific and Tumor-Specific Targeting of an Oncolytic HSV-1 Amplicon/Helper Virus for Prostate Cancer Treatment (2008-2010).

Publications

1. Jung C*, Lee CY*, Grigg ME. The SRS superfamily of Toxoplasma surface proteins. *International Journal for Parasitology* 2004; 34: 285-296. *CJ and CYL contributed equally to this work.
2. Lee CY, Bu LX, Rennie PS, Jia WW. An HSV-1 amplicon system for prostate-specific expression of ICP4 to complement oncolytic viral replication for *in vitro* and *in vivo* treatment of prostate cancer cells. *Cancer Gene Therapy* 2007; 14(7): 652-660.
3. Lee CY, Rennie PS, Jia WW. MicroRNA regulation of oncolytic herpes simplex virus type 1 for selective killing of prostate cancer cells. *Clinical Cancer Research* 2009; 15(16): 5126-5135.
4. Lee CY, Bu LX, DeBenedetti A, Williams BJ, Rennie PS, Jia WW. Local and systemic delivery of oncolytic herpes simplex virus type-1 for targeting prostate tumors (submitted).

Abstracts/Conference Presentations

1. Lee CY, Bu L, Yan H, Rennie PS, Jia WW. Prostate-specific expression of ICP4 complements ICP4(-) HSV-1 virus for oncolysis of prostate cancer cells [abstract]. In: Proceedings of the 97th Annual Meeting of the American Association for Cancer Research; 2006 Apr 1-5; Washington, DC. Philadelphia (PA): AACR; 2006; 47: Abstract nr 4025. (Oral Presentation in a Minisymposium session)
2. Lee CY, Bu LX, Rennie PS, Jia WW. An HSV-1 amplicon system for prostate-specific expression of ICP4 to complement oncolytic viral replication for *in vitro* and *in vivo* treatment of prostate cancer cells. 18th Annual Canadian Student Health Research Forum (CSHRF), Winnipeg, MB, June 5-8, 2007. Abstract p100. (Invited Poster Presentation: Won CIHR - Institute of Cancer Research Silver Poster Award)
3. Lee CY, Bu L, Jia WW, Rennie PS. An HSV-1 amplicon system for prostate-specific expression of ICP4 to complement oncolytic viral replication for *in vitro* and *in vivo* treatment of prostate cancer cells. Prostate Cancer Conference (CPCRI & CPRN), Toronto, ON, September 28-30, 2007. (Poster Presentation)
4. Lee CY, Jia WW, Bu L, Rennie PS. Prostate-specific oncolytic HSV-1 viruses for treatment of prostate cancer. 2nd Annual Lorne Sullivan Urology Research Day, Vancouver, BC, June 10, 2008. Abstract p21. (Oral presentation in the Oncology Session)
5. Lee CY, Jia WW, Bu L, Rennie PS. Prostate-specific oncolytic HSV-1 viruses for treatment of prostate cancer. 4th International PacRim Breast and Prostate Cancer Meeting, Whistler, BC, August 12-16, 2008. Abstract p49. (Poster presentation)

6. **Lee CY**, Rennie PS, Jia WW. MicroRNA-mediated targeting of oncolytic herpes simplex virus type 1 to prostate tumors. The Fifth International Meeting on Replicating Oncolytic Virus Therapeutics, Banff, AB, March 18-22, 2009. Abstract S16. (Oral presentation)
7. **Lee CY**, Rennie PS, Jia WW. MicroRNA-mediated targeting of oncolytic herpes simplex virus type 1 to prostate tumors [abstract]. In: Proceedings of the 100th Annual Meeting of the American Association for Cancer Research; 2009 Apr 18- 22; Denver, CO. Philadelphia (PA): AACR; 2009. Abstract nr 3777. (Poster presentation)
8. **Lee CY**, Rennie PS, Jia WW. MicroRNA regulation of oncolytic herpes simplex virus type 1 for selective killing of prostate cancer cells. The 10th Annual Pathology Day, Vancouver, BC, May 2009. Abstract #20. (Oral presentation)
9. **Lee CY**, Rennie PS, Jia WW. MicroRNA-mediated targeting of oncolytic herpes simplex virus type 1 to prostate tumors. 3rd Annual Lorne D. Sullivan Lectureship and Research Day, Vancouver, BC, June 9, 2009. Abstract p13. (Oral presentation)

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MicroRNA Regulation of Oncolytic Herpes Simplex Virus-1 for Selective Killing of Prostate Cancer Cells

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Abstract Purpose: Advanced castration-resistant prostate cancer, for which there are few treatment options, remains one of the leading causes of cancer death. MicroRNAs (miRNA) have provided a new opportunity for more stringent regulation of tumor-specific viral replication. The purpose of this study was to provide a proof-of-principle that miRNA-regulated oncolytic herpes simplex virus-1 (HSV-1) virus can selectively target cancer cells with reduced toxicity to normal tissues.

Experimental Design: We incorporated multiple copies of miRNA complementary target sequences (for miR-143 or miR-145) into the 3'-untranslated region (3'-UTR) of an HSV-1 essential viral gene, *ICP4*, to create CMV-ICP4-143T and CMV-ICP4-145T amplicon viruses and tested their targeting specificity and efficacy both *in vitro* and *in vivo*.

Results: Although miR-143 and miR-145 are highly expressed in normal tissues, they are significantly down-regulated in prostate cancer cells. We further showed that miR-143 and miR-145 inhibited the expression of the *ICP4* gene at the translational level by targeting the corresponding 3'-UTR in a dose-dependent manner. This enabled selective viral replication in prostate cancer cells. When mice bearing LNCaP human prostate tumors were treated with these miRNA-regulated oncolytic viruses, a >80% reduction in tumor volume was observed, with significantly attenuated virulence to normal tissues in comparison with control amplicon viruses not carrying these 3'-UTR sequences.

Conclusion: Our study is the first to show that inclusion of specific miRNA target sequences into the 3'-UTR of an essential HSV-1 gene is a viable strategy for restricting viral replication and oncolysis to cancer cells while sparing normal tissues. (Clin Cancer Res 2009;15(16):5126–35)

Prostate cancer is the most commonly diagnosed nonskin cancer in men and one of the leading causes of cancer death (1). Although prostate cancer is frequently curable in its early stage by surgical or radiation ablation, many patients will present with locally advanced or metastatic disease for which there are currently no curative treatment options (2–4). Although androgen withdrawal therapies, which block the growth-promoting effects of androgens, are often used to treat advanced disease, progression to a castration-resistant state is the usual

outcome, giving rise to a median survival of ~18 months (5). Brief survival extensions can sometimes be achieved using current docetaxel-based chemotherapy protocols (6). However, to have any major effect on current survival rates, more effective new treatment strategies need to be developed.

In this regard, oncolytic virotherapy offers a promising therapeutic option for treating locally advanced as well as recurrent, metastatic, castration-resistant forms of prostate cancer (7, 8). In particular, herpes simplex virus-1 (HSV-1) has proven to be an excellent viral vector with various forms of replication-defective or replication-conditional vectors having been developed to treat different types of cancer (9, 10). The replicative and oncolytic nature of these viruses permit *in situ* viral multiplication and spread of the viral infection throughout the tumor mass causing lytic cell death. Several phase I clinical trials have shown that HSV-1 viral therapy was well tolerated by patients and in some cases showed considerable efficacy (11, 12).

A key issue in developing a safe and effective oncolytic virotherapy is the achievement of maximal killing of tumor cells while maintaining tumor specificity of viral targeting (13–15). We have recently shown that a replication-defective HSV-1 helper virus (CgalΔ3), lacking the essential *ICP4* gene, became oncolytic in a tissue type-specific fashion when the *ICP4* gene was provided by an amplicon expressing this gene under the regulation of a prostate-specific promoter, ARR₂PB (16). Although others have also attempted to incorporate tissue-specific promoters in front of suicide and/or essential viral genes to

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Translational Relevance

Prostate cancer is the most common cancer and the second leading cause of cancer death in men. Current treatments, including androgen withdrawal therapy, are not curative for advanced, metastatic disease and new treatment strategies are urgently required. In this regard, oncolytic virotherapy offers a promising new approach. A major focus in developing oncolytic viral vectors is enhancement of tumor specificity and reduced toxicity to normal tissues. In this study, we showed that endogenous microRNAs can be exploited to regulate expression of an essential herpes simplex virus-1 viral gene in a sequence-specific manner, resulting in viruses that are selectively oncolytic for tumor cells while leaving normal tissues unharmed. This new targeting strategy could provide a basis for the development of safe therapeutic viral vectors to treat locally advanced and metastatic cancers and may greatly enhance tumor-specific targeting when combined with existing strategies.

achieve specific killing of cancer cells, nonspecific toxicity to normal tissues due to leaky promoters has remained a problem (17, 18) and suggests that improved stringency of oncolytic viral targeting needs to be developed. One approach to achieve this is to take advantage of unique tissues-associated patterns of expression of microRNAs (miRNA).

miRNAs are ~22 nucleotides, noncoding small RNAs that are known to play important regulatory roles in cell proliferation, cell differentiation, apoptosis, and tumorigenesis by binding to the 3'-untranslated region (3'-UTR) of the targeted mRNAs and thereby promoting either mRNA cleavage or repression of gene expression at the post-transcriptional level (19–21). Differential miRNA expression profiles between normal and cancer cells have been shown by microarray analysis of clinical samples and several miRNA signatures within the genome have been proven to be promising biomarkers for prognosis and diagnosis of cancers (22, 23). Recent studies have also shown that some miRNAs are overexpressed and some are down-regulated in

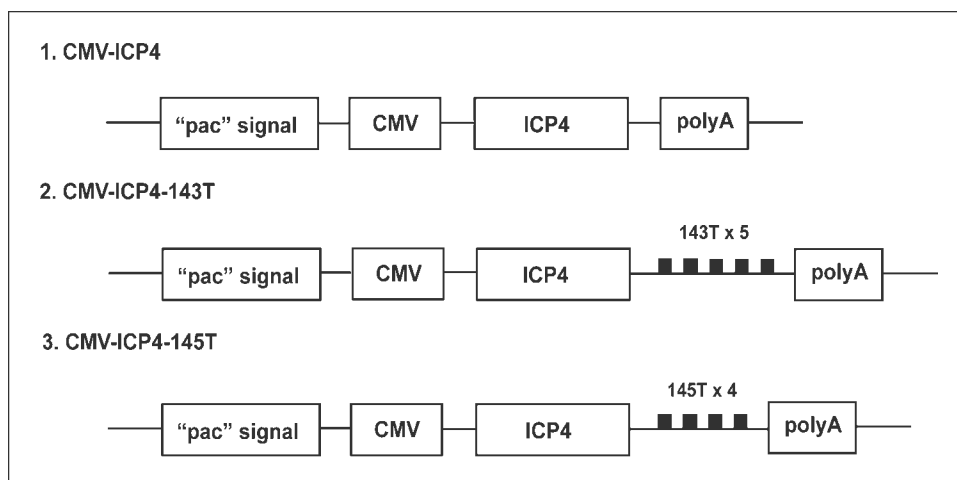
several cancer cells compared with their normal tissues of origin, suggesting that these miRNAs may play a role as oncogenes or tumor suppressors in the tumorigenesis of various human cancers (24–26). Based on these observations, we have developed a novel strategy for targeting tumor cells by taking advantage of the differential miRNA expression level between normal and cancer cells. We hypothesized that tumor-specific targeting of oncolytic HSV-1 virus could be achieved at the translational level by incorporating multiple copies of miRNA target sequences into the 3'-UTR of the essential viral gene, *ICP4*. To test this hypothesis, we constructed an *ICP4*-expressing amplicon containing 3'-UTR target sequence of miR-143 and miR-145, which are expressed in normal tissues but generally down-regulated in many types of cancer cells and clinical samples including those from breast cancer (27), colorectal cancer (28, 29), cervical cancer (30), ovarian cancer (31), liver cancer (32), B-cell malignancies (33), and prostate cancer (34, 35). In principle, this should permit unimpeded translation of the *ICP4* gene in cancer cells and subsequent oncolysis but enable protection of normal cells due to degradation of the amplicon transcript by miR-143 or miR-145. In the present study, we show tumor-specific targeting of miRNA-regulated oncolytic HSV-1 viruses for killing prostate cancer cells both *in vitro* and *in vivo*.

Materials and Methods

Plasmid constructs. Five tandem copies of miR-143 complementary sequences (143T) and four copies of miR-145 complementary sequence (145T) were generated by PCR from the template 5'-CTCGAGCGGTAAATTAACGTGAGCTACAGTGCTTCATCTCACGATTGAGCTACAGTGCTTCATCTCAGATCTGAGCTACAGTGCTTCATCTCAGTCATGAGCTACAGTGCTTCATCTCAGTCATGAGCTACAGTGCTTCATCTCAGCTATCGATGCAGTCTAGA-3' and 5'-CTCGAGCGGTTAATTAA-CGAAGGGATTCTGCGAAAAGTGGACCGATAAGGGATTCTGCGAAAAGTGGACGATCAAGGGATTCTGCGAAAAGTGGACGTCAAAGGGATTCTGCGAAAAGTGGACGCTATCGATGCAGTCTAGA-3', respectively (Integrated DNA Technologies). The primers used to PCR amplify these miRT fragments were 5'-GACGCTCGAGCGGTTAATTAACG-3' (forward) and 5'-GCAGTCTAGACTGCATCGATAGC-3' (reverse). The *ICP4* gene (~4 kb) and the miRT fragments, excised by *XhoI* and *XbaI* digestion, were then cloned into the pcDNA3.0-neo vector, which contains the viral packaging signal and deletion of the neomycin gene, to generate CMV-*ICP4*-143T and CMV-*ICP4*-145T plasmids (Fig. 1).

Cell cultures. LNCaP (36), DU145, PC-3, and 293T human embryo kidney cells and Vero cells were obtained from American Type Culture

Fig. 1. Structure of the three amplicon plasmids used to package replication-competent amplicon viruses. CMV-*ICP4* amplicon was a constitutive, non-tissue-selective complementing amplicon, whereas CMV-*ICP4*-143T and CMV-*ICP4*-145T amplicons were tumor-specific miRNA-regulated complementing amplicons. CMV-*ICP4*-143T amplicon contained five tandem copies of miR-143 complementary sequences (143Tx5) after *ICP4* gene and CMV-*ICP4*-145T amplicon contained four tandem copies of miR-145 complementary sequences (145Tx4).



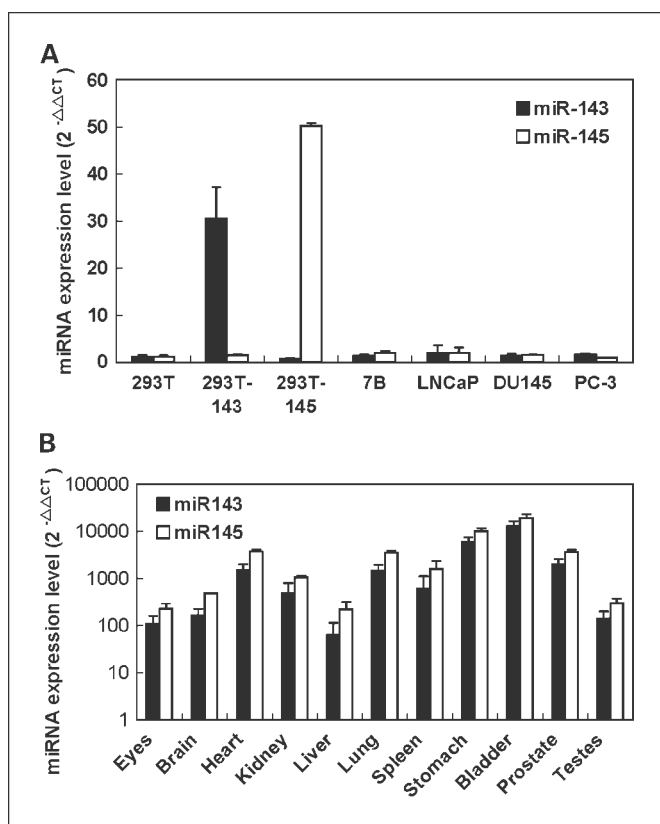


Fig. 2. miR-143 and miR-145 expression levels in cell lines and mouse tissues. The expression levels of miR-143 and miR-145 in various cell lines (A) and a panel of organs (B) harvested from nude mice were determined by quantitative RT-PCR assays. 293T-143 and 293T-145 are 293T cells transiently transfected with 20 nmol/L pre-miR-143 and pre-miR-145, respectively, as positive controls. Total RNA from the cells and organs were extracted using the Trizol reagent and 10 ng RNA was reverse transcribed to cDNA followed by TaqMan real-time PCR assays.

Collection, and 7B was kindly provided by Dr. William Goins (University of Pittsburgh School of Medicine). All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics containing penicillin and streptomycin. Vero and 7B cells are monkey kidney cells used to package amplicon viruses as described previously (16). LNCaP, DU145, and PC-3 are human prostate cancer cell lines and LNCaP-Luc is a derived stable cell line that expresses luciferase.

Viruses. The replication-deficient ICP4⁻ helper virus (CgalΔ3) and all of the amplicon viruses were packaged, propagated, and titered in 7B cells as described previously (16). Briefly, monolayer of 7B cells were transfected with 24 μg of the amplicon plasmid DNA using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. At 24 h after transfection, cells were superinfected with ICP4⁻ helper virus at a multiplicity of infectivity (MOI) of 1 and the virus was collected 3 days after superinfection. The amplicon viruses were then amplified and propagated by infecting more 7B cells. Because the stock virus produced was a mixture of helper and amplicon viruses, virus titer and the helper/amplicon ratio were determined by titrating the virus in both Vero and 7B cells. Amplicon virus titer was determined by plaque-forming assay in Vero cells, which does not complement ICP4 gene, and helper virus titer was determined in 7B cells, which expresses the ICP4 gene.

Transfection of precursor miRNA and viral infection. LNCaP cells were seeded in 12-well plates at a density of 3×10^5 per well in 2 mL DMEM with 10% fetal bovine serum and no antibiotics. The next day, cells were cotransfected with 300 ng of the amplicon plasmid (CMV-ICP4, CMV-ICP4-143T, or CMV-ICP4-145T) and precursor

miRNA (pre-miR-143 or pre-miR-145; Ambion) at a concentration of 0, 0.1, 1, 5, 10, 20, and 50 nmol/L using Lipofectamine 2000. The same cells were then superinfected with ICP4⁻ helper virus at a MOI of 1 after 24 h cotransfection. At 24 h post-superinfection, protein samples and total RNA were extracted and subjected to Western blot and one-step real-time reverse transcription-PCR (RT-PCR) analysis. Viral supernatant was also collected at 48 h post-superinfection and titered on 7B cells. To study the effect of miRNA on viral replication, LNCaP cells were transfected with 0 or 20 nmol/L miR-143 or miR-145, and 24 h later, the same cells were infected with CMV-ICP4, CMV-ICP4-143T, or CMV-ICP4-145T amplicon virus at a MOI of 0.1 [input virus = 10^4 plaque-forming units (pfu)]. At 72 h post-viral infection, virus was collected and titered in 7B cells.

Western blotting. LNCaP cells were lysed with 2× sample buffer (100 mmol/L Tris-HCl, 4% SDS, 20% glycerol, 357 mmol/L β-mercaptoethanol, 0.04% bromophenol blue) and boiled for 5 min. Protein samples were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat milk (Bio-Rad Laboratories) at room temperature for 1 h and then incubated with primary antibodies overnight at 4 °C. Next day, the membrane was washed with TBS-Tween 20, incubated with secondary antibody at room temperature for 1 h, and then washed with TBS-Tween 20 again before imaged using enhanced chemiluminescence reagent (Perkin-Elmer) and VersaDoc imaging system (Bio-Rad). Primary antibodies were prepared in 5% bovine serum albumin in TBS-Tween 20 with the following dilutions: anti-ICP4 (EastCoast Bio) at 1:800 dilution (37), anti-ICP27 (Virusys) at 1:800 dilution (37, 38), and anti-β-actin (Cell Signaling) at 1:1,000 dilution. Anti-mouse and anti-rabbit secondary antibodies were prepared in TBS-Tween 20 at a 1:2,000 dilution (Cell Signaling). Band density was determined using ImageJ software (NIH).

DNA, RNA extraction, and RT-PCR. DNA was extracted from the organs of the mice treated with amplicon viruses using the phenol-chloroform extraction method and ICP4 copy number was determined by quantitative real-time PCR using the same ICP4 primers and method as described previously (16). Total RNA was extracted from the cell lines and mouse organs using Trizol (Invitrogen) following the manufacturer's protocol. To determine ICP4 mRNA levels in cotransfection studies, 200 ng RNA was used in one-step real-time RT-PCR using the same primers as described previously (16). All RT-PCRs were done in 25 μL SYBR Green mixture containing the MultiScribe Reverse Transcriptase using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). ICP4 mRNA level was first normalized to β-actin mRNA level ($\Delta CT = CT_{ICP4} - CT_{Actin}$) and then compared with the negative control group, where LNCaP cells were infected with ICP4⁻ helper virus only ($\Delta\Delta CT = \Delta CT - \Delta CT_{ICP4^-}$). The results were expressed as $2^{-\Delta\Delta CT}$. TaqMan miRNA assay kit (Applied Biosystems) was used to determine the expression level of miR-143 and miR-145, which were normalized to U6 (endogenous control for human cells) or snoRNA234 (endogenous control for mouse tissues) to give ΔCT values and then compared with negative control, where miRNA was undetectable, to get $\Delta\Delta CT$ values. RT-PCR and real-time PCR were done as instructed by the company. Briefly, 10 ng RNA was first converted into cDNA in 96-well optical plate at the following conditions: 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. The RT product (1 μL) was then subjected to real-time PCR assay in 96-well optical plate at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

LNCaP xenograft mouse model. Athymic nude mice were purchased from Harlan-Sprague-Dawley and LNCaP-Luc cells were inoculated subcutaneously by injecting 5×10^6 in 100 μL medium with Matrigel at two different sites on the flanks. Tumor volumes were determined by caliper measurements and calculated using the formula: volume = width × length × thickness × π / 6. Once the tumor size reached ~100 mm³, mice were treated with two intratumoral injections (at days 0 and 7) of 1×10^6 pfu of the ICP4⁻ helper virus, CMV-ICP4, CMV-ICP4-143T, and CMV-ICP4-145T amplicon virus. At the end of the experiment, mice were sacrificed using CO₂ asphyxiation and several organs (brain, heart, kidneys, liver, lung, spleen, stomach, prostate, bladder, and testes) and

the tumors were removed and analyzed by histochemical and real-time PCR analysis. For imaging the tumors, mice were injected with D-luciferin intraperitoneally at a concentration of 150 µg luciferin/g of body weight 15 min before imaging with the IVIS 200 system (Caliper Life Sciences). All experimental procedures were approved by University of British Columbia Animal Care Committee and followed the guidelines and policies of Canadian Council on Animal Care.

Histochemical staining. Harvested tissues were embedded in OCT (Sakura Tissue-Tek) and 10 µm sections of the organs were prepared on slides, fixed with 4% paraformaldehyde for 30 min, washed with PBS, and then subjected to X-gal staining using a commercial kit (Novagen) overnight at 37°C. Next day, the slides were counterstained with eosin. Pictures of the slides were taken at ×5 magnification using a light microscope and detection of virus was indicated by a dark blue color.

Statistical analysis. Statistical significance ($P < 0.05$) is determined using Student's *t* test and data are presented as mean ± SD.

Results

miR-143 and miR-145 are down-regulated in LNCaP cells but highly expressed in normal mouse prostates. miR-143 and

miR-145 have been reported to be down-regulated in many prostate cancer cells (26, 28, 34, 35). Using quantitative RT-PCR, we verified that the expression of miR-143 and miR-145 are down-regulated in LNCaP, DU145, and PC-3 cells (Fig. 2A). We also confirmed that miR-143 and miR-145 are not expressed in 7B cells. Their absence is essential for efficient packaging of the amplicon viruses containing miR-143 and miR-145 target sequences. Despite their expression being lost in many cell lines (data not shown), an abundance of miR-143 and miR-145 was found in most normal organs including mouse prostate (Fig. 2B). The expression pattern of miR-143 and miR-145 was similar in all organs examined, with the highest levels in bladder and stomach, moderate levels in prostate, heart, lung, spleen, and kidney, and relatively lower expression levels in eyes, brain, liver, and testes. A similar expression pattern of miR-143 has also been reported in human tissues (39).

Regulation of ICP4 gene expression through inclusion of miR-143 and miR-145 target sequences in the 3'-UTR. We generated three amplicon constructs carrying the ICP4 gene under the

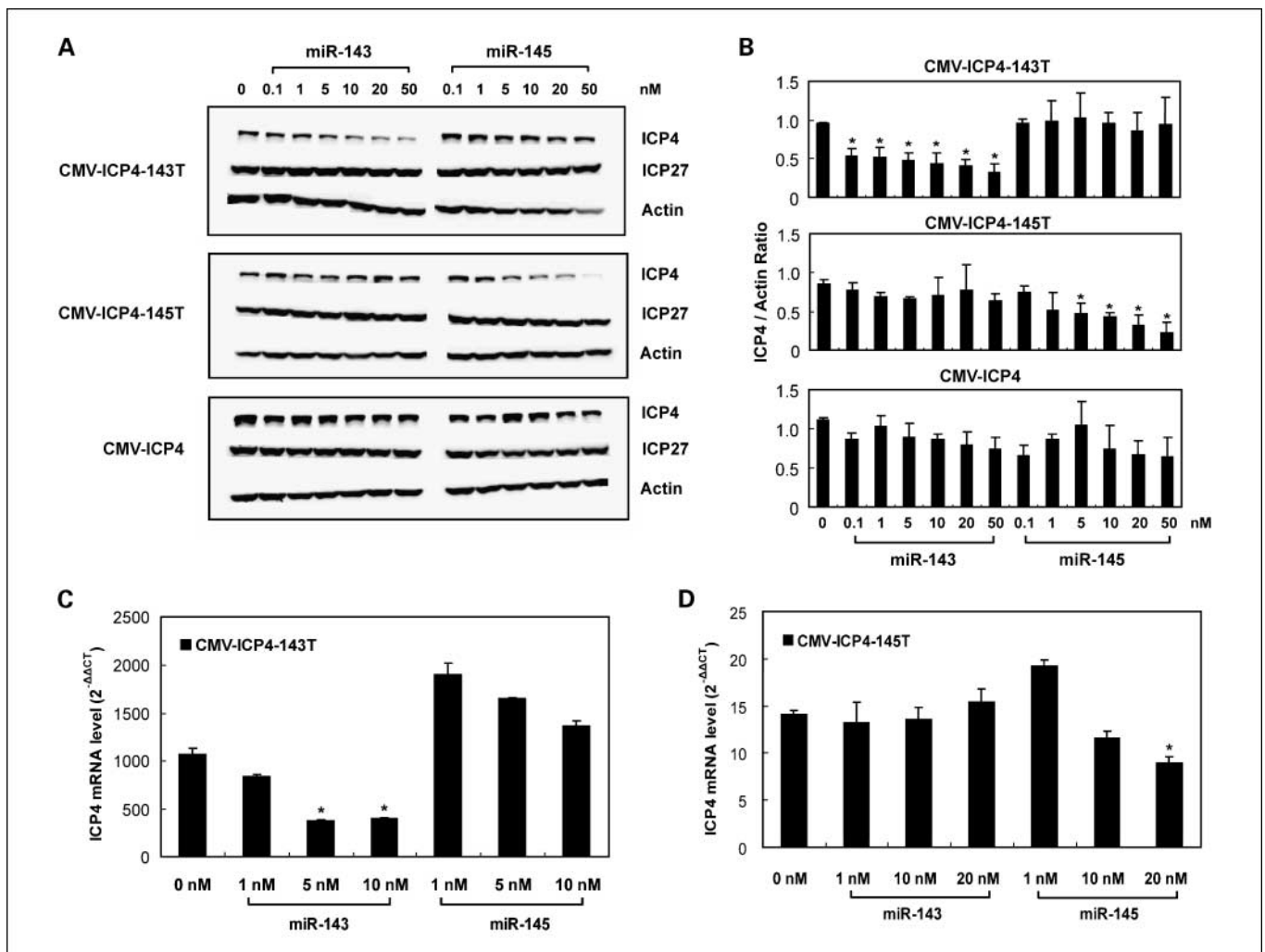


Fig. 3. miR-143 and miR-145 inhibit ICP4 expression at a translational level by targeting the 3'-UTR in the amplicons. LNCaP cells were cotransfected with amplicon plasmid (CMV-ICP4, CMV-ICP4-143T, or CMV-ICP4-145T) and pre-miR-143 or pre-miR-145 at a concentration of 0 to 50 nmol/L. At 24 h after transfection, LNCaP cells were superinfected with ICP4 helper virus at a MOI of 1. Total protein and RNA were extracted at 24 h post-superinfection. A, ICP4, ICP27, and β-actin protein levels were determined by Western blot analysis. B, density ratio of ICP4 level was normalized to β-actin. C and D, ICP4 mRNA level was determined by quantitative RT-PCR analysis. Mean ± SD ($n = 3$). *, $P < 0.05$, compared with 0 nmol/L control).

CMV promoter with either five copies of miR-143 or four copies of miR-145 complementary target sequences in 3'-UTR (Fig. 1). The amplicons also contained a viral origin of replication and a packaging signal, which facilitated replication and packaging of

the amplicon virus by ICP4⁺ helper virus in host cells. The helper virus used to package the amplicon viruses in this study was a replication-deficient recombinant ICP4⁺ helper virus (CgalΔ3), which lacked the *ICP4* gene and could not replicate by itself unless in a complementing cell line or in the presence of a complementing amplicon (16, 40).

We then investigated the effect of miR-143 and miR-145 on ICP4 expression in LNCaP cells. When pre-miR-143 or pre-miR-145 was cotransfected with the amplicon plasmid into LNCaP cells, miR-143 and miR-145 inhibited ICP4 expression driven by CMV-ICP4-143T and CMV-ICP4-145T amplicons (Fig. 1) in a concentration-dependent manner (Fig. 3A and B). A 50% reduction in ICP4 protein level was observed when CMV-ICP4-143T was cotransfected with 5 nmol/L pre-miR-143 and when CMV-ICP4-145T was cotransfected with 10 nmol/L pre-miR-145 compared with the controls ($P < 0.05$). In both cases, there was a ~60% to 70% reduction in ICP4 protein level in the presence of higher miRNA concentrations (20 and 50 nmol/L). The expression of ICP27 protein, another essential viral gene (endogenous control), was not affected by the expression of miR-143 or miR-145, indicating that the reduction of ICP4 protein was a direct effect of miR-143 and miR-145 activity on their 3'-UTR targets and not a result of reduced viral infectivity. Because miR-143 and miR-145 bind to the target sequences in the 3'-UTR of CMV-ICP4-143T and CMV-ICP4-145T with perfect complementarity, *ICP4* mRNA would be subjected to degradation by RNA-induced silencing complex. Indeed, a 62% reduction in *ICP4* mRNA level was observed when CMV-ICP4-143T was cotransfected with 10 nmol/L pre-miR-143 (Fig. 3C; $P < 0.05$) and a 37% reduction in *ICP4* mRNA was observed when CMV-ICP4-145T was cotransfected with 20 nmol/L pre-miR-145 (Fig. 3D; $P < 0.05$). Reciprocally miR-143 had no effect on CMV-ICP4-145T nor did miR-145 on CMV-ICP4-143T, indicating that both miRNAs suppress ICP4 expression in a sequence-specific manner.

miR-143 and miR-145 inhibit replication of miRNA-regulated amplicon viruses in a dose-dependent manner. To show that miRNA could control viral replication through the regulation of ICP4 expression, virus titers were measured in LNCaP cells first pretransfected with miR-143 and miR-145 and then followed by infection with amplicon viruses. Approximately a 2-fold decrease in virus titer was observed when LNCaP cells, infected with CMV-ICP4 amplicon virus, were pretransfected with 20 nmol/L miR-143 or miR-145 compared with no miRNA transfection (Fig. 4A). With the CMV-ICP4-143T amplicon virus, the virus titer was 6- and 3-fold less when LNCaP cells were pretransfected with 20 nmol/L miR-143 compared with 0 or 20 nmol/L miR-145 ($P < 0.05$). With the CMV-ICP4-145T amplicon virus, the virus titer was 3-fold less when LNCaP cells were pretransfected with 20 nmol/L miR-145 relative to 0 or 20 nmol/L miR-143 ($P < 0.05$). These results indicate that viral replication is greatly reduced when the infected LNCaP cells express the appropriate miRNA. This inhibition of viral replication was miRNA sequence-specific and dose-responsive with increasing miRNA concentration (Fig. 4B and C). miR-143 was able to inhibit the complementing ability of CMV-ICP4-143T amplicon but not of CMV-ICP4-145T (Fig. 4B). The virus titer was 6-fold less when CMV-ICP4-143T was cotransfected with 20 and 50 nmol/L pre-miR-143 relative to 0.1 nmol/L of this miRNA. Pre-miR-143 showed no effect on

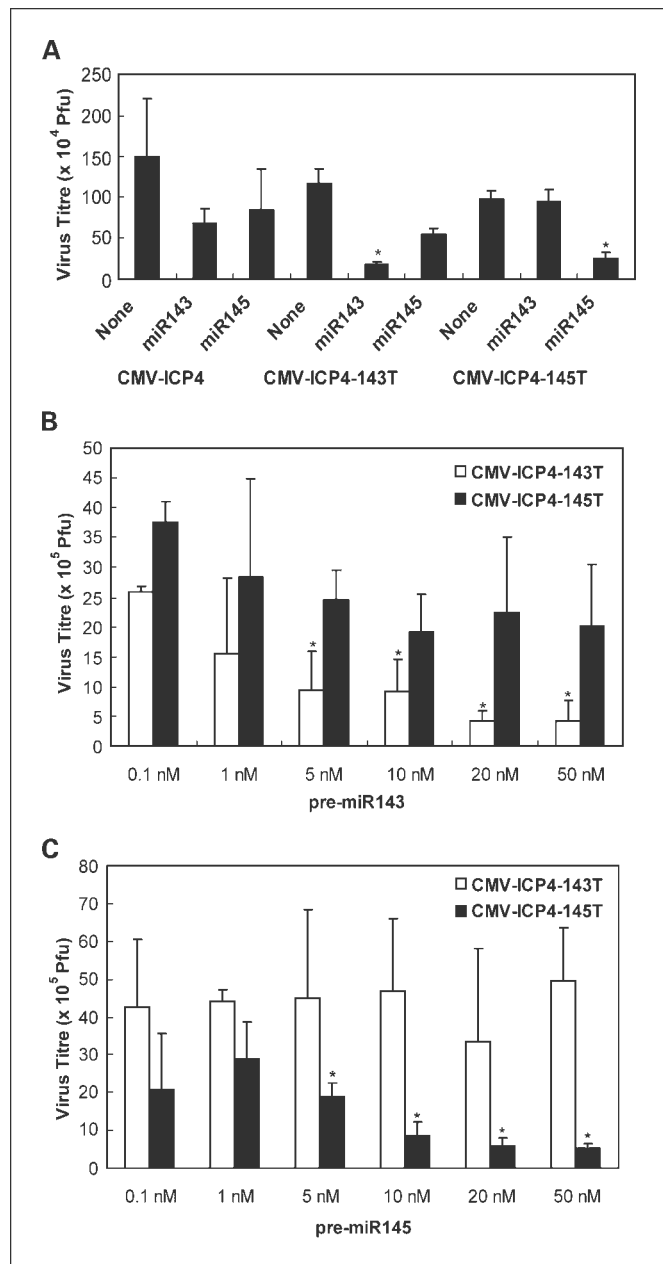
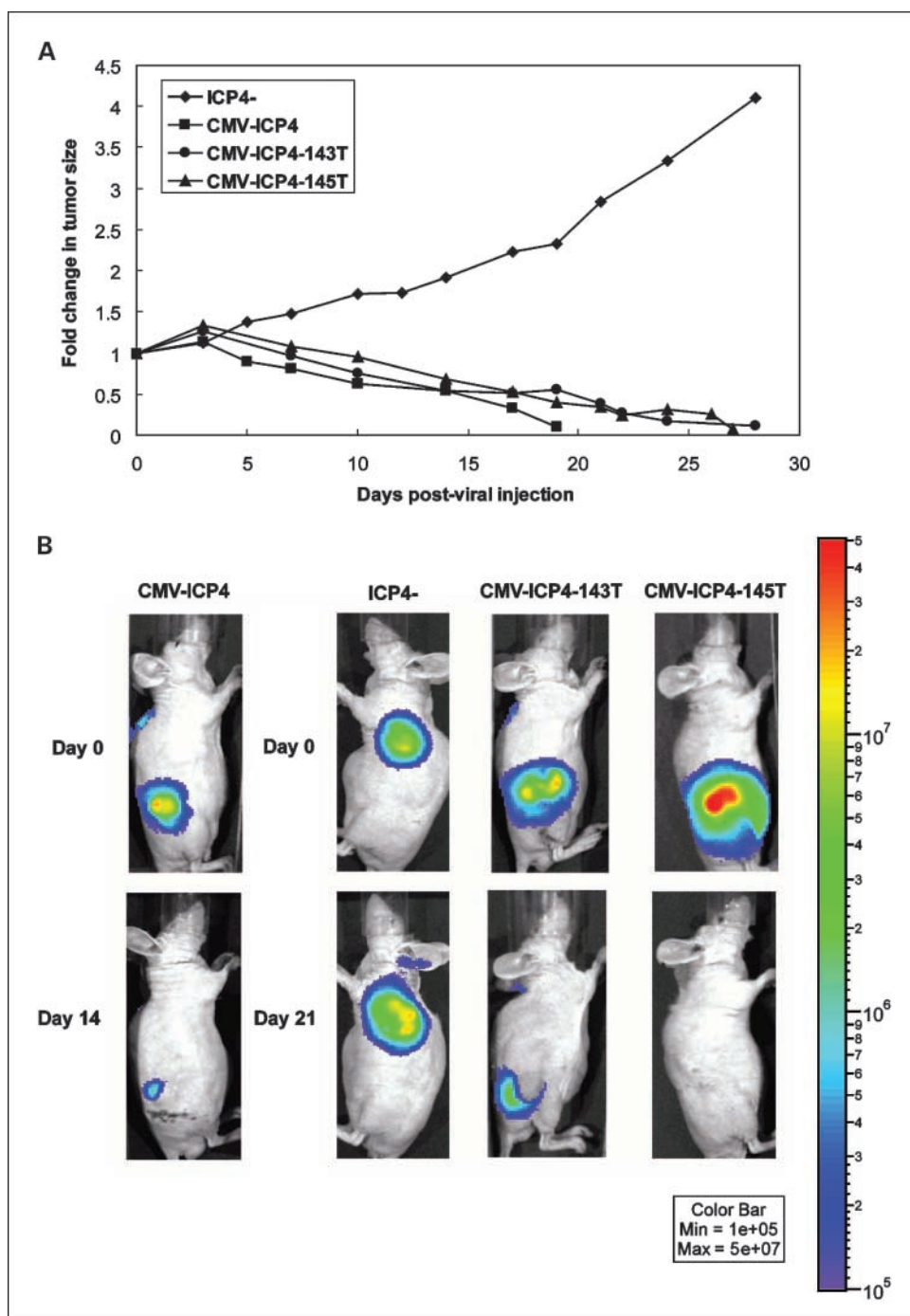


Fig. 4. miR-143 and miR-145 suppress amplicon viral replication in LNCaP cells. **A**, miR-143 and miR-145 inhibited viral replication of the CMV-ICP4-143T and CMV-ICP4-145T amplicon viruses, respectively. LNCaP cells were transfected with miR-143 or miR-145 at a concentration of 0 or 20 nmol/L. At 24 h after transfection, cells were infected with CMV-ICP4, CMV-ICP4-143T, or CMV-ICP4-145T amplicon virus at a MOI of 0.1 (input virus = 10⁴ pfu). At 72 h post-viral infection, virus was collected and titered in 7B cells. The mean \pm SD pfu was calculated from four independent determinations. *, $P < 0.05$, relative to 0 nmol/L controls. miR-143 (**B**) and miR-145 (**C**) inhibited the ability of amplicons to complement replication of ICP4⁺ helper virus in a dose-dependent manner. LNCaP cells were cotransfected with amplicon plasmid (CMV-ICP4, CMV-ICP4-143T, or CMV-ICP4-145T) and pre-miR-143 or pre-miR-145 at a concentration of 0.1 to 50 nmol/L. At 24 h after cotransfection, LNCaP cells were superinfected with ICP4⁺ helper virus at a MOI of 1. At 48 h post-superinfection, virus was collected and titered on 7B cells.

Fig. 5. miRNA-regulated amplicon viruses suppress prostate tumor growth in a human xenograft mouse model. **A**, intratumoral injections of miRNA-regulated amplicon viruses inhibited tumor growth in LNCaP xenograft mouse model. Nude mice with subcutaneous LNCaP-Luc tumors ($\sim 120 \pm 14 \text{ mm}^3$) were treated with two intratumoral injections (at days 0 and 7) of 1×10^6 pfu ICP4' helper virus only (\blacklozenge ; $n = 5$), CMV-ICP4 amplicon virus (\blacksquare ; $n = 3$), CMV-ICP4-143T amplicon virus (\bullet ; $n = 7$), or CMV-ICP4-145T amplicon virus (\blacktriangle ; $n = 5$). Tumor volume was determined by caliper measurements and the results were expressed as mean fold change in tumor size. **B**, tumor was imaged using IVIS system and representative images were shown. The luciferase activity was expressed as p/s/cm²/sr.



the complementing ability of the CMV-ICP4-145T amplicon. Similarly, a 4-fold reduction in virus titer was observed when CMV-ICP4-145T was cotransfected with 20 and 50 nmol/L pre-miR-145 compared with the titer at 0.1 nmol/L of this miRNA. Pre-miR-145 had no inhibitory effect on CMV-ICP4-143T amplicon (Fig. 4C). The amount of transfected miRNAs may not reflect actual intracellular miRNA levels required for suppression of viral replication. However, we have created a cell line, LNCaP-miR-143, which expresses endogenous miR-143 at similar levels as in normal mouse tissues. Seventy percent of these cells were protected from CMV-

ICP4-143T viral infection at 120 h post-infection, whereas 70% of the parental LNCaP cells were dead (data not shown). These results show that miRNAs can inhibit the ability of amplicons to complement the replication of helper virus in a dose-dependent manner through the presence of specific 3'-UTR target sequences and thereby regulate viral replication at the translational level.

miRNA-regulated amplicon viruses can selectively suppress LNCaP tumor growth. The therapeutic efficacy of these miRNA-regulated amplicon viruses was next tested *in vivo* using immunocompromised nude mice bearing subcutaneous

LNCaP-Luc tumors ($\sim 120 \pm 14 \text{ mm}^3$), which are a LNCaP-derived cell line expressing luciferase. The animals were treated with intratumoral injections of 2×10^6 pfu amplicon viruses. Tumor volume was determined by caliper measurements and luciferase in the tumor mass was visualized using an IVIS imaging system. At 28 days post-viral injection, a >3.5 -fold increase in tumor volume was observed in mice treated with the non-colytic ICP4⁻ helper virus and a $>80\%$ decrease in tumor size

was observed in mice treated with CMV-ICP4, CMV-ICP4-143T, and CMV-ICP4-145T amplicon viruses (Fig. 5A). Mice treated with CMV-ICP4 amplicon virus developed herpetic skin lesions around injection sites, which caused death as early as 14 days after viral treatment. In contrast, no herpetic lesions were seen in CMV-ICP4-143T-treated and CMV-ICP4-145T-treated animals, although some gastritis developed 28 days after the viral treatment. Furthermore, tumors were almost

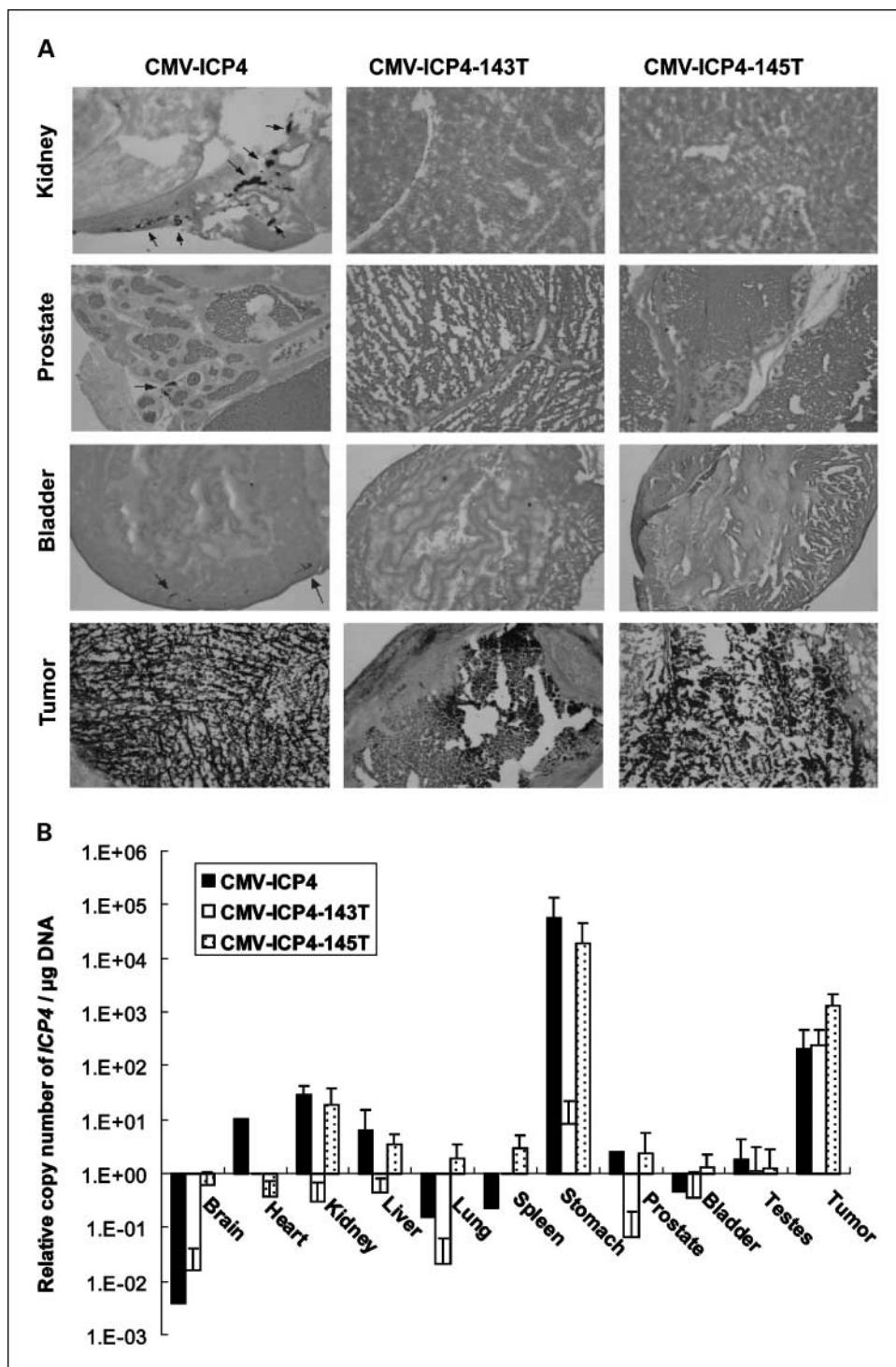


Fig. 6. miRNA-regulated amplicon viruses specifically target tumor cells *in vivo*. Various organs were harvested from the mice treated with amplicon viruses at the end of the experiment. **A**, organs were embedded in OCT and cut into 10 μm sections, which were then subjected to x-gal staining. *Arrows*, presence of virus. **B**, total DNA was extracted from the organs and ICP4 copy number was determined by real-time PCR assays. The results were expressed as relative copy number of ICP4/ μg DNA, which was indicative of the presence of amplicon.

completely eradicated in three of the seven mice treated with CMV-ICP4-143T and in two of the five mice treated with CMV-ICP4-145T. Using an IVIS imaging system, a >80% reduction in luciferase activity was observed in mice treated with the miRNA-regulated amplicon viruses (Fig. 5B). These results showed that the miRNA-regulated amplicon viruses were able to selectively kill LNCaP tumor cells.

miR-143 and miR-145 protect normal tissues from miRNA-regulated oncolytic viral infections. To examine the extent of viral replication outside the tumor mass, several organs were harvested from mice treated with miRNA-regulated amplicon viruses. Ten micrometer sections were stained with x-gal substrate for β -galactosidase activity, as an indicator of the presence of helper virus, which carried a *lacZ* reporter gene. High levels of β -galactosidase activity were detected in the tumor mass in all treatment groups. *LacZ* staining was also observed in other organs such as the kidney, prostate, and bladder in mice treated with CMV-ICP4 amplicon virus but not in mice treated with CMV-ICP4-143T and CMV-ICP4-145T amplicon viruses (Fig. 6A). The extent of viral spread was also examined by determining the presence of the *ICP4* gene in normal tissues and in LNCaP-Luc tumors using real-time PCR assays (Fig. 6B). The extent of amplicon virus infection is indicated by the relative copy number of *ICP4* (per microgram of total DNA) in each organ. With the exception of the stomach, the results indicate that the most amplicon virus was found in tumors for all three treatment groups. Some amplicon virus was also present in the heart, kidney, liver, and prostate of mice treated with CMV-ICP4 or CMV-ICP4-145T amplicon virus, whereas no virus was detected in these organs in mice treated with CMV-ICP4-143T amplicon virus, except in the stomach. Even in this organ, the *ICP4* copy number was >3,300-fold less in mice treated with CMV-ICP4-143T than in mice treated with CMV-ICP4 amplicon virus.

Discussion

The miRNAs, miR-143 and miR-145, have been reported to be down-regulated in various cancer types (27–35). This is consistent with our findings in the present study (Fig. 2). Based on these findings, we cloned multiple tandem copies of their complementary target sequences into the 3'-UTR of the viral essential gene, *ICP4* (Fig. 1). The number of copies of miRNA binding sites in the 3'-UTR was decided based on previously published work (41, 42), which indicated that approximately four copies may be sufficient. *In vitro*, the presence of 3'-UTR target sequences for miR-143 and miR-145 specifically and efficiently reduced both *ICP4* mRNA and protein expression levels (Fig. 3).

miR-143 and miR-145 inhibited the complementing ability of the amplicon virus and viral replication in a sequence-specific and dose-dependent manner (Fig. 4). Interestingly, the virus titers of CMV-ICP4 amplicon virus, which did not carry any 3' miRNA target sequences, were also slightly lower in cells pretreated with 20 nmol/L miR-143 or miR-145, suggesting that HSV-1 viral infection/replication was somewhat hindered in LNCaP cells transfected to express miR-143 or miR-145. One explanation was that miR-143 and miR-145 directly or indirectly down-regulated cellular genes that were required for efficient HSV-1 viral infection or viral replication (39, 43). Another possibility was that miR-143 and miR-145 directly down-regulated

viral genes carried by the helper virus, thereby causing reduced efficiency of viral replication. Nevertheless, the efficacy of using specific miRNA-regulated amplicon viruses to treat prostate tumors was clearly shown. CMV-ICP4-143T and CMV-ICP4-145T amplicon viruses effectively inhibited tumor growth (>80% reduction in tumor volume) without major toxicity to other normal tissues (Figs. 5 and 6). These results showed that incorporating miRNA target sequences in an oncolytic virus is a viable strategy for specifically targeting and killing cancer cells while sparing normal cells. miR-143-regulated and miR-145-regulated oncolytic viruses may be particularly useful in a wide range of cancers because they are generally down-regulated in many types of malignancies but are quite abundant in normal tissues.

In the present study, we focused on using HSV-1 amplicons instead of recombinant virus because amplicons are more easily constructed and therefore ideal for proof-of-concept. Regulation of viral gene expression and viral replication by miRNA would be expected to be more stringent in the context of a whole virus because the whole virus contains only two copies of *ICP4* gene, whereas owing to the fact that ~152 kb of viral genome was packaged into one viral particle, the amplicon viruses used in the present study would have ~15 copies of the amplicon plasmid (~10 kb) carrying the *ICP4* gene (44). As a result, many more *ICP4* mRNA transcripts would be generated from an amplicon virus than from a recombinant whole virus. This could result in a small amount of *ICP4* protein being expressed, which would be sufficient to initiate lytic viral replication. Hence, our current amplicon virus system may underestimate the regulatory capability of the 3'-UTR miRNA target and building the miRNA target sequences as well as other tissue-specific or tumor-specific elements into the whole HSV-1 viral genome should increase the safety margin substantially.

The major advantage of the amplicon/helper system is its tremendous flexibility, which offers a quick and easy way to test and fine-tune the efficiency of a specific regulatory element both *in vitro* and *in vivo*. Because a significant proportion of men with early prostate cancer are not cured by surgery or radiation therapy, local administration of amplicon viruses through intratumoral/intraprostatic injection may be beneficial for treating primary and high-risk localized or locally advanced prostate cancer while limiting the spread of virus to other tissues. This therapeutic approach can be used either alone or in combination with surgery or chemotherapy in a neoadjuvant or adjuvant setting to further enhance efficacy (45).

Intratumoral/intraprostatic injection is a feasible approach in that human prostate is easily accessible through transperineal and transrectal routes (46). One advantage of intratumoral injection is that local administration would ensure maximal uptake of virus by tumor cells, but the limitation is that it is probably not effective against treating metastatic tumors at distant sites. Another limitation is that the toxicity of the virus in normal tissues may not be evident because the virus is injected directly into the tumor cells. However, we have shown that mice treated with the wild-type virus, CMV-ICP4, exhibited skin lesions and the virus was detected in several normal tissues. On the other hand, extensive viral spread was not observed in mice treated with miRNA-regulated viruses (Fig. 6).

miRNA-regulated oncolytic viral replication has also been shown recently using a RNA virus, a let-7-sensitive vesicular

stomatitis virus, VSV^{let-7wt} (47). An advantage of using a DNA virus as opposed to a RNA virus with this miRNA targeting strategy is that replication of a DNA virus occurs at a much slower rate, such that saturation of the miRNA:RNA-induced silencing complex machinery due to efficient and robust growth of RNA viruses would not be a problem. Moreover, in the case of HSV-1, miRNA targeted sequences can be incorporated into the 3'-UTR of more than one viral gene to increase tumor specificity. DNA viruses also have much lower mutation rate than RNA viruses (48).

To further enhance tumor specificity and to improve regulation of oncolytic viral replication, the copy number of miRNA target sequences can be increased. In addition, target sequences of more than one miRNA species can be incorporated into the 3'-UTR because miRNA expression patterns differ across normal tissues of different origins. For instance, a combination of miRNA target sequences, such as miR-143T + miR-145T, could be incorporated to make the tu-

mor specificity more stringent while increasing the protection of normal tissues. The 3'-UTR of each viral construct could be tailored according to the miRNA expression pattern of the cell types that one wants to target. Through synergistic effects of various regulatory elements in the promoter (16), 5'-UTR (49, 50) or 3'-UTR, stringent regulation of viral gene expression and viral replication can likely be achieved to develop a highly effective and tumor-specific virotherapy for cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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