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 amplon 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 amplon 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 amplon 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
miRNAs are overexpressed and some are down-regulated in various cancers (22, 23). Recent studies have also shown that some are promising biomarkers for prognosis and diagnosis and several miRNA signatures within the genome have been shown by microarray analysis of clinical samples. miRNA expression profiles between normal and cancer cells have been studied at the post-transcriptional level (19). miRNAs 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′-CTCGAGCGGT-TAATTAACGTGACTAAGCCTCACTCAGATGATGATGATGATGATGATGATGATGATGATG-3′ (forward) and 5′-CGATTGAGCTACAGTGCATCGATAGC-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 embryonic 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 tissue-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).
all of the amplicon viruses were packaged, propagated, and titrated in ICP4-helper virus at a multiplicity of infectivity (MOI) of 1 and the Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The gene, and helper virus titer was determined in 7B cells, which express ICP4 by plaque-forming assay in Vero cells, which does not complement the virus in both Vero and 7B cells. Amplicon virus titer was determined from virus titer and the helper/amplicon ratio were determined by titrating virus was then amplified and propagated by infecting more 7B cells. Because virus was collected 3 days after superinfection. The amplicon viruses were then amplified and propagated by cutting out 100 nmol/L ICP4-143T or ICP4-145T, 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 = 10^8 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 imaging using enhanced chemiluminescence reagent (PerkinElmer) 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).

**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 cells 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-PCR 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 (ΔCT = CTICP4 - C TActin) and then compared with the negative control group, where LNCaP cells were infected with ICP4 helper virus only (ΔΔCT = ΔCTCICP4 - ΔCTICP4). The results were expressed as 2-ΔΔCT. 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 ΔΔCT 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 / 6. Once the tumor size reached 100 mm^3, 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 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-infection, 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-infection and titered on 7B cells. To study the effect of miRNA on viral replication, LNCaP cells were transfected with 0 or 20 nmol/L ICP4-143 or ICP4-145, 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 = 10^8 plaque-forming units (pfu)]. At 72 h post-viral infection, virus was collected and titered in 7B cells.

**Viruses.** The replication-deficient ICP4 helper virus (CpaLa3) 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 × 10^4 per well in 2 mL of 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 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
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 or miR-145 compared with no miRNA transfection (Fig. 4A). 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 co-transfected 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
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

**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 (∼120 ± 14 mm³) were treated with two intratumoral injections (at days 0 and 7) of 1 × 10⁶ pfu ICP4 helper virus only (♦; n = 5), CMV-ICP4 amplicon virus (▪; n = 7), or CMV-ICP4-145T amplicon virus (▴; 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.
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 \(2 \times 10^6\) 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 noncolytic 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 \(\mu\text{m}\) 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/\(\mu\text{g}\) 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 advance 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, VSVlet-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 tumor 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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