TUMOR-SPECIFIC TARGETING USING ONCOLYTIC HERPES SIMPLEX VIRUS TYPE 1 FOR PROSTATE CANCER TREATMENT

by

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ABSTRACT

Prostate cancer is the most common non-skin 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. This project aims to provide a proof-of-principle that an oncolytic herpes simplex virus type-1 (HSV-1) virus can be engineered to replicate in a tissue- and tumor-specific fashion through both transcriptional and translational regulation of an essential viral gene. Using the amplicon/helper system, we first generated a prostate-specific amplicon virus by inserting the ARR_2PB promoter in front of the *ICP4* gene, and two tumor-specific amplicon viruses by incorporating multiple copies of miR-143 or miR-145 complementary target sequences in the 3' untranslated region (3'UTR) of *ICP4*. Finally, we generated two prostate tumor-specific recombinant viruses using the bacterial artificial chromosome and the recombineering method. Our results showed that an amplicon system containing a prostate-specific promoter upstream of an essential viral gene can complement a helper virus for lytic replication specifically within prostate tumor cells. We also showed both *in vitro* and *in vivo* that viral replication and cell killing occurred only in tumor cells with abundant eIF4E protein or low levels of miR-143/miR-145 when viral gene expression was controlled by a modified 5'UTR or 3'UTR, respectively. Nude mice bearing LNCaP tumors treated with local or systemic injections of these tumor-specific viruses had a >85% reduction in tumor size at day 28 post-viral injection and without extensive toxicity to normal tissues. Quantitative real-time PCR analysis also showed that the majority of the virus was detected in the tumor mass and not within normal tissues. In conclusion, the incorporation of a tissue-specific promoter or a tumor-specific element in an oncolytic HSV-1 virus is a viable strategy to restrict viral replication and oncolysis selectively to tumor cells while sparing normal tissues. This work provides a basis for the development of novel oncolytic HSV-1 viruses for local and systemic treatment of locally advanced and metastatic prostate cancer.

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To my parents

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1 Introduction

1.1 Prostate cancer

Prostate cancer is the most commonly diagnosed non-skin cancer and the second leading cause of cancer death in men (1). It is estimated that over 211,000 men in North America will develop prostate cancer and approximately 33,000 will die from this disease. With the widespread use of prostate-specific antigen (PSA) screening and the rapid increase in the aging population, the prostate cancer incidence is likely to continue rising (2).

1.1.1 Epidemiology of prostate cancer

The risk factors for prostate cancer include age, race, family history and diet. Familial clustering of prostate cancer has been suggested by several studies and it has been shown that the risk of developing prostate cancer is twice as high in men with first-degree relatives with prostate cancer compared to men without affected relatives, and this risk increases with an increasing number of affected relatives (3, 4). To further understand the nature of this familial aggregation, multiple segregation analyses were performed and suggested that rare autosomal dominant high-risk genes were predicted to account for 43% of early-onset prostate cancer before age 55 and approximately 9% of all prostate cancer cases (5). Subsequent genome-wide screens of polymorphic DNA markers revealed several regions of linkage (6), and genetic mapping studies identified *RNASEL*, a candidate hereditary prostate cancer (HPC1) gene, and macrophage scavenger receptor 1 (*MSR1*) as potential prostate cancer susceptibility genes (7, 8).

A wide geographic variation is observed in the incidence and mortality rates of prostate cancer, with the highest rates in North America and Western Europe and the lowest rates in Africa and Asia (2). This variation can be best explained by lifestyle influences, as migratory studies showed that Asian immigrants in North America typically adapt to western diets and thereby adopt higher prostate cancer risks (9-11). The western diet is generally rich in animal fats and meats, and low in fruits and vegetables, and several studies have shown that dietary fats and consumption of red meats are associated with increased prostate cancer risks (12-14). On the other hand, consumptions of antioxidant micronutrients, such as tomatoes that contain lycopene, and vegetables that contain sulforaphan are associated with reduced risks (15, 16). It

has also been shown that the risk of developing prostate cancer is about 60% higher in African American men than in white American men (17). The exact reason for this discrepancy is unclear but may be attributable to a variety of factors including nutrition, genetic predisposition, socioeconomic status, and hormones (18).

1.1.2 Screening and diagnosis

Prostate cancer is rarely symptomatic at an early stage, because the majority of the adenocarcinomas (~70%) arise from the peripheral zone that is distant from the urethra. As the cancer progresses into the urethra or the bladder neck, urinary symptoms of obstruction or irritations can occur, and the spread of prostate cancer outside the prostate capsule can cause erectile dysfunction due to damaged branches of the pelvic plexus (19). Currently, prostate cancer is being detected at an earlier stage than in the past as a result of widespread combination of digital rectal examination (DRE) and serum PSA screening (20, 21). DRE is a useful tool to assess the shape, symmetry, firmness and nodularity of the prostate and it is evident that DRE can detect prostate cancer in 14-30% of men having a normal PSA level of 4ng / mL or less (22). However, DRE has low reproducibility depending on the experience of the examiners (23), and when used alone, can miss 23-45% of the cancers that are subsequently detected by prostate biopsies performed as a result of serum PSA elevations or transrectal ultrasound abnormalities (24, 25).

PSA is an androgen-regulated serum protease and a member of the human kallikrein family encoded by genes located on chromosome 19 (26). It is produced primarily by the epithelial component of the prostate gland (27). Numerous studies have shown that serum PSA values are correlated directly with prostate cancer risk (28), and are more sensitive than DRE. However, diagnostic specificity with regard to cancer is lacking as an elevation of serum PSA levels can occur during both benign (prostatitis, benign prostatic hyperplasia) and malignant prostate diseases (prostate cancer) and also as a result of prostate manipulation (prostate examination and prostate biopsy) (29). Traditionally, prostate biopsies are performed in patients with serum PSA levels greater than 4.0 ng/mL. However, there are problems with total PSA as it lacks sensitivity and specificity, resulting in a high number of false negative and false positive test results. For example, it has been reported that approximately 20-50% of clinically significant prostate cancer occurs in men with PSA levels less than 4 ng/mL. Hence, it is now

widely recognized that there is no serum PSA value below which prostate cancer can be excluded (30), and serum PSA testing should only be used as part of a risk assessment that also takes other factors, such as age, race, and family history, into consideration (31, 32). Nonetheless, the implementation of PSA screening with DRE has dramatically increased the detection of early-stage, organ-confined prostate cancer, which is more treatable. As a result of routine PSA screening, the lead time (time by which a cancer diagnosis is advanced) has been estimated to be around 10 years (33, 34).

The standard grading of prostate cancer is based on the Gleason grading system, which was first described by Gleason and Mellinger in 1974 (35). The system is used to determine the prognosis of prostate cancer based on its microscopic appearance and the differentiation state. The pathologist assigns a Gleason grade based on the two most common Gleason patterns seen on the prostate samples taken either from a biopsy or after radical prostatectomy. The grade ranges from 1 (least aggressive) to 5 (most aggressive), and the primary and secondary grades are then added together to give the Gleason grade ranging from 2 to 10. Another system used to determine the stage and extent of prostate cancer is the the TNM classification, which is based on T (primary tumor), N (lymph nodes) and M (metastases) categories (36). The T category is based on clinical examination, imaging, endoscopy, biopsy and biochemical tests, and is assigned when the tumor is still confined within the prostate gland or the adjacent seminal vesicles. The N category indicates that the disease has invaded and spread to lymph nodes, and is based on clinical examination or imaging. The M category is based on clinical examination, imaging, skeletal studies and biochemical tests, and is used when metastasis is detected at distant sites.

1.1.3 Prevention

Preventive measures against prostate cancer are desirable in light of the high lifetime risks of prostate cancer development, the treatment-associated morbidities, and the inability to cure metastatic disease. Thus far, several large randomized clinical trials are examining the preventive effects of two classes of agents, 5α-reductase inhibitors and antioxidant micronutrients. Since androgens play an important role in the growth of both normal and abnormal human prostates, a decrease in androgen levels should reduce the risk of developing prostate cancer. Testosterone, the main source of androgen, is produced by Leydig cells in the testes, and is converted to

dihydrotestosterone (DHT) in the prostate by 5-α reductase (type 1 and type 2) (37, 38). DHT has a five-fold higher affinity for the androgen receptor (AR) than testosterone, and is the predominant agonist of the AR pathway leading to the growth and maintenance of the prostate (39). These data suggest that prostate cancer occurrence and progression may be reduced by inhibiting the activity of 5-α reductase. There are three isoforms of 5-α reductase: type 1 is most prevalent in extraprostatic tissues, type 2 is the predominant form in the prostate, and type 3 is just recently discovered (40-42). Thus far, two 5-α reductase inhibitors have been studied in clinical trials. Finasteride is an inhibitor of type 2 5-α reductase, and dutasteride inhibits both types 1 and 2 5-α reductase (43, 44). In the Prostate Cancer Prevention Trial (PCPT), it was shown that finasteride reduced the risk of prostate cancer by 24.8% relative to a placebo over a 7-year period; however, aggressive forms (Gleason grade 7-10) were more common in the finasteride group (45). In addition, prostate cancer was detected in 18.4% of the men treated with finasteride versus 24.4% of men receiving placebo, and 30% of the patients in finasteride group discontinued the treatment due to undesirable side effects, including reduced ejaculate volume, erectile dysfunction, loss of libido, and gynecomastia (45, 46). As a result, the trial was terminated 15 months before the anticipated completion.

Recently, it has been reported that finasteride increases the sensitivity of both DRE and PSA for prostate cancer detection, which may partially explain the increased detection of high-grade tumors in the for-cause biopsies (47, 48). In addition, selective inhibition of low-grade prostate tumors in patients with both low- and high-grade components may have contributed to the increased detection of high-grade tumors in the finasteride group in the PCPT (49). In a bias-adjusted model, the estimated high-grade prostate cancer rate was 8.2% in the placebo group versus 6.0% in the finasteride group, indicating that finasteride treatment did not induce high-grade prostate cancer progression (50). However, it has also been shown recently that continuous finasteride treatment for patients with BPH caused a significantly higher incidence in high-grade (Gleason score >6) tumors (44.5%) when compared to intermitted therapy (25%) and controls (18.2%) (51). Careful considerations should be given before undertaking finasteride treatment. Currently, a phase III clinical trial is investigating the effects of dutasteride, which has been shown to suppress serum DHT by more than 90% in comparison to the 70% seen with finasteride (41, 43, 52), and may prove to be more effective than finasteride in prostate cancer prevention.

Several epidemiologic studies have shown that the intake of both selenium and vitamin E might reduce prostate cancer risks (53-55), and currently a phase III randomized, placebo-controlled Selenium and Vitamin E Cancer Prevention Trial (SELECT; *N* > 32,400) is investigating the effects of these antioxidant micronutrients in preventing prostate cancer for a follow-up of 7-12 years (56). However, a recent report of the SELECT study at a median overall follow-up of roughly 5.5 years showed that selenium, vitamin E, alone or in combination at the tested doses and formulations did not prevent prostate cancer in healthy men (57). These data indicated that the search for new, effective agents for prevention of prostate cancer is urgently required.

1.1.4 Treatment

Men with localized prostate cancer face a number of treatment choices, including active surveillance, radical prostatectomy, and radiation therapy. Each treatment option has its side effects, and factors such as age, life expectancy, medical history, Gleason score, and serum PSA level should all be taken into consideration when choosing a treatment with the intent to cure. Although there are no age limits for radical prostatectomy, it is used to treat men who have a life expectancy of at least 5 years, and men aged above 75 years rarely undergo such a treatment (58). Reports from a randomized controlled trial of active surveillance versus radical prostatectomy showed that surgery reduced the risk of metastases and local progression, and improved the disease-specific survival in a group of men aged under 65 years (59). In addition, a study from the Surveillance, Epidemiology, and End Results (SEER) Medicare database, comprising men aged 65-80 years and diagnosed with prostate cancer (Gleason 2-7), showed that after a 12-year study period, 37% of the active surveillance group in comparison with 23.8% of the treatment group undergoing either radiation therapy or surgery had died (60). On the other hand, it has also been suggested that radical prostatectomies have greater adverse impacts on the quality of life, such as sexual incompetence and urinary incontinence, and no effect on overall survival (61, 62).

Radiation therapy is administered via external beam radiation therapy (EBRT), interstitial brachytherapy or a combination of the two (63). EBRT is usually delivered with techniques that produce a highly conformal dose to the regions of interest (prostate) and minimize dose to a given volume of other critical organs (bladder, rectum) (64-67). However, it has been reported that the likelihood of second malignancies of the bladder

and rectum increased slightly after EBRT (68). Brachytherapy is the implantation of radioactive sources into the prostate under transrectal ultrasound guidance using either low-dose rate implants, such as iodine-125 or palladium-103, or high-dose rate sources, such as iridium-192 (69, 70). The short- and long-term sequelae of brachytherapy for prostate cancer differ from those of EBRT and radical prostatectomy. The most common side effects were nocturia (80%) and dysuria (48%) at 2 months after implantation, and these figures declined to 45% and 20%, respectively, at 12 months after implantation (71-73). Rectal morbidities, such as change in bowel habits, rectal bleeding or ulceration, were also observed after brachytherapy (71, 74). However, an advantage of this treatment is the lower incidence of treatment-associated erectile dysfunction in comparison to EBRT or radical prostatectomy (75-77).

Even though prostate cancer is frequently curable by radical prostatectomy or radiotherapy at an early stage when still confined to the organ, many patients diagnosed with locally advanced, recurrent or metastatic disease have concomitantly fewer curative treatment options and a median life expectancy of less than 3 years (78, 79). The primary treatment is usually androgen ablation therapy by either medical castration using LHRH (luteinizing hormone-releasing hormone) agonists, or surgical castration (80, 81). Despite an initial response rate of 80-90% to androgen deprivation therapy, almost all patients advance to an androgen-independent or castration-independent state manifested by increasing PSA levels, and this is often accompanied by high resistance to other chemotherapeutic drugs at 18-24 months after castration (82-85). At this terminal stage, the median survival for patients with metastatic, hormone-refractory prostate cancer is about 10-12 months, and current chemotherapeutic treatments are relatively ineffective (86). For instance, in a 1993 review of 26 chemotherapy trials, it was reported that the objective response rate was only 8.7%, and the median survival was only 6-10 months with no survival benefit for any agent (87).

Randomized clinical trials have demonstrated that systemic treatment with mitoxantrone plus prednisone or hydrocortisone reduces bone pain and improves quality of life, but does not prolong survival (88-90). Recently, two clinical trials showed that the median survival can be extended briefly by two months when docetaxel is given every three weeks in combination with prednisone or estramustine (91, 92). As a result of these studies, the role of systemic chemotherapy in hormone-refractory prostate cancer is well established. Several completed trials and ongoing studies are examining

the benefits of chemotherapy in patients with locally advanced or metastatic disease in a neoadjuvant or adjuvant setting to improve treatment outcomes (93-98). Thus far, single agent treatment of docetaxel followed by radical prostatectomy in patients with locally advanced prostate cancer demonstrated no complete pathological response (95, 97).

1.1.5 Androgen receptor

The androgen receptor (AR) has an important role in the development and progression of prostate cancer (99). It is a 110 kilodalton (kDa) phosphoprotein and functions as the central nuclear receptor transcription factor that mediates androgenic signals in the prostate gland (100). Upon activation by DHT binding to the ligand-binding domain, ARs form homodimers, which translocate to the nucleus and bind to androgen-responsive elements (ARE) to activate the expression of AR-regulated genes (101). It has been demonstrated that most hormone-refractory tumors express higher levels of AR than do androgen-dependent prostate cancer (102, 103), and that AR levels are higher in bone metastasis than in primary tumors (102). An increase in the AR-regulated PSA protein in serum is observed in the progression of prostate cancer towards an androgen-independent state (104). It was further demonstrated that the continuous activation of the AR signaling pathway in the absence of androgen is a result of excessive recruitment of coactivators (105). More recently, Snoek *et al*. showed that knocking down ARs using a tetracycline inducible shRNA results in the complete regression of castration-resistant C4-2 tumors as well as a reduction of serum PSA levels in 50% of mice (106). These findings suggest that the AR signaling pathway is intact in AR-expressing hormone-refractory tumors and that the knockdown of ARs is a viable strategy to treat castration-resistant prostate cancer (107).

1.2 Herpes simplex virus type 1

Herpes simplex virus type 1 (HSV-1) is a double-stranded DNA virus with a genome size of approximately 152 kilobase pairs (kb) encoding more than 84 genes. The viral genes are divided into three categories: immediate early (IE), early (E) and late (L) genes. There are five immediate-early (IE) genes: *ICP0*, *ICP4*, *ICP22*, *ICP27*, and *ICP47*. Of these genes, *ICP4* and *ICP27* are absolutely essential for viral replication. ICP4 is a sequence-specific DNA-binding protein that activates the transcription of E and L genes, induces viral DNA synthesis, and downregulates IE gene expression (108,

109). ICP27 is a multifunctional protein involved in the cooperative transcriptional activation of viral genes with ICP4, the shut-off of host gene expression, the inhibition of precursor mRNA splicing and the promotion of nuclear export of viral transcripts (110).

The virus enters host cells via cell receptor-mediated endocytosis through an initial attachment of viral glycoproteins gB and gC with heparin sulfate on the cell surface, followed by the binding of gD to viral entry receptors such as herpesvirus entry mediator (HVEM) and nectins (111). Following entry into host cells, the tegument protein (VP16) and the viral DNA genome are transported into the nucleus where viral genes are expressed in a coordinately regulated and sequentially ordered manner (112). Inside the nucleus, VP16 stimulates the transcription of IE genes, whose products are involved in transcription regulation and stimulation of the expression of E genes, which primarily encode enzymes involved in nucleotide metabolism and viral DNA replication. Upon viral DNA replication initiation, expression of L genes commences. L genes primarily encode for structural components of the virion (113). At the end of the replication cascade, mature viral particles are assembled and released from the host cell, resulting in cell lysis and cell death.

HSV-1 has a number of favorable features that make it an attractive viral vector for cancer treatment: 1). it infects a broad range of cell types; 2). it is cytolytic in nature; 3). up to 30kb of the viral genome can be replaced allowing accommodation of multiple therapeutic transgenes (114); 4). availability of the antiherpetic drug ganciclovir (115); 5). it remains as an episome, eliminating concern over insertional mutagenesis (116); 6). it has a much slower mutation rate as a DNA virus in contrast to RNA viruses (117); 7). lack of liver toxicity making it superior to adenoviral vectors.

1.3 Oncolytic virotherapy

Oncolytic virotherapy has emerged as a promising approach in the treatment of cancer. The rationale for using oncolytic viruses is that a tumor-specific replication-competent virus can preferentially replicate and amplify in tumor cells, causing selective oncolysis while leaving normal surrounding cells unharmed (Figure 1.1). Currently recognized oncolytic viruses are divided into three categories: 1). first-generation, naturally occurring viruses with inherent tumor-specificity (eg. Newcastle diseases virus, vesicular stomatitis virus, reovirus) (118-121); 2). second-generation virus mutants with deletions in genes essential for replication in

normal cells but not in cancer cells (eg. adenovirus Onyx-015, herpes virus G207) (122, 123); 3). third-generation virus mutants with viral genes regulated by tissue-specific elements (eg. adenovirus CV706) (124). Several oncolytic agents have been tested in clinical trials, and two agents have been extensively studied, Reolysin from Oncolytics and Onyx-015 (125-127). Results from these clinical trials showed that they were selective for tumors and well-tolerated by the treated patients; however, anti-tumor potency was still limited. Hence, further improvements in efficacy, specificity and delivery of oncolytic viruses are needed. In order to maximize the destruction of tumor cells while minimizing collateral damage to normal tissues, the design of oncolytic viral vectors has been focused on enhancing tumor-specific targeting and killing.

1.3.1 Deletion mutants

One targeting approach is to delete viral genes that are required for efficient replication in normal cells but dispensable in tumor cells. One example is the first virus used in clinical trials, adenovirus Onyx-015, which has a deletion of the *E1B* gene (128). Since the *E1B* gene product can bind to p53, leading to p53 inhibition or degradation, the theory is that Onyx-015 would be unable to propagate through the inactivation of p53 in normal cells, but would remain active in tumor cells lacking a functional p53 protein. This feature was appealing because 40% of human cancers contain *p53* mutations (129). To date, Onyx-015 has been extensively studied in 18 phase I and II clinical trials and is the first virus to undergo clinical trials combined with chemotherapy (126, 127, 130-133). Results from these studies showed that the administration of doses up to 2 x 10^{12} virus particles (10^{11} pfu) daily for 5 consecutive days was well tolerated with no toxicity. In some cases, transient anti-tumor response was observed; however, there was no improvement in long-term survival. Thus far, the clinical data with Onyx-015 have shown that concern over toxicity is unwarranted but that its efficacy is questionable. As a result, better strategies to enhance potency are required. In fact, a derivative of Onyx-015 that expresses cytosine deaminase and thymidine kinase have been studied in phase I trials for the treatment of prostate cancer alone or in combination with radiation therapy (134, 135). Again, no long term responses were observed. The greatest achievement in oncolytic virus field was in November 2005 when China approved the world's first oncolytic virus, a genetically modified adenovirus H101 (Shanghai Sunway Biotech) that is almost identical to Onyx-015, for the treatment

of head and neck cancer (136).

Another example is the multimutated HSV-1 G207 virus, which has both copies of *ICP34.5* deleted and the *ICP6* gene inactivated via insertion of *the E. coli LacZ* gene (123). The *ICP34.5* gene product blocks the shut-off of host cell protein synthesis induced by the cellular kinase PKR in virus-infected cells (137). The deletion of *ICP34.5* genes ensures a lack of neurovirulence, allowing replicative targeting in tumor cells (138), whereas inactivation of ICP6 offers an additional targeting mechanism for tumor cells with p16 tumor suppressor defects (139). G207 was initially tested in animal models to treat malignant glioma and has been shown to be efficacious and safe (123). It was subsequently shown to cause significant tumor regression in the treatment of a variety of solid tumors, including melanoma, breast, colon, bladder, gastric, head and neck, ovarian, pancreatic, and prostate cancers (140). Based on these promising results, a clinical phase I trial was initiated to test its safety, and it was shown that up to 3×10^9 pfu (plaque forming units) of G207 was safely inoculated into human brain tumors (141). More recently, a phase Ib clinical trial further demonstrated that multiple dose deliveries of 1×10^9 pfu of G207 can be safely inoculated directly into the brain tissue surrounding the tumor resection cavity without development of toxicity, and evidence of *in vivo* viral replication was also provided (142).

More recently, the anti-tumor efficacy of ICP0 mutant HSV-1 has been investigated extensively. The interferon signaling pathway plays a very important role in antiproliferative, antiviral and immunomodulatory activities. Interferons were initially used to treat cancer patients by blocking cell growth and promoting apoptosis (143). It was soon realized that many mutations within cancer cells deregulated the interferon signaling pathway (144), rendering cancer cells incapable of mounting antiviral response during viral infection. Mossman *et al*. demonstrated that HSV-1 mutants lacking ICP0 are hypersensitive to the antiviral effects of interferon in normal cells (145). It was subsequently demonstrated that the ICP0 mutant KM100 completely eradicates breast tumor cells in 80% of the treated mice and significantly increases survival using an immunocompetent murine model (146). The KM100 virus elicited strong adaptive immune response to tumor antigens as indicated by the high levels of serum IgG1 antibody specific to PyMT tumor antigen. This demonstrates that ICP0-based double mutants may provide a safe and efficacious platform for the development of oncolytic virotherapy and cancer vaccines.

1.3.2 Altering viral tropism

One major challenge for the systemic delivery of oncolytic viruses is the non-specific infection of both normal and tumor cells. To avoid unnecessary toxicity in normal tissues, the natural tropism of the virus can be ablated and engineered to infect only target cells. For instance, genetic fusion of the coxsackie and adenovirus receptor (CAR) to a single-chain antibody against human carcinoembryonic antigen (CEA) resulted in detargeting in the liver by more than 90% after systemic delivery, and retargeting to CEA-positive tumors and hepatic metastases of colon cancer in nude mice (147). Zhou *et al.* constructed a recombinant HSV-1 virus, R5111, which has binding sites for heparan sulfate proteoglycans in *gB* and *gC* genes ablated, and two copies of *IL-13* inserted in *gC* and *gD* genes (148). They showed that the virus can use the IL13Rα2 receptor for entry and that it replicates efficiently in a variety of cell lines that express this receptor. Another mutant virus, KgBpK^{-}gC^{-}, deleted for the *gC* and partial *gB* genes, was engineered to express gC:preS1 fusion molecules (149). PreS1 is the molecule that human hepatitis B virus uses to bind to hepatocytes specifically, and the virus indeed replicated efficiently and specifically in the hepatoblastoma cell line, HepG2. Recently, Menotti *et al*. inserted into the HSV-1 viral envelope protein gD a single-chain antibody against the human epidermal growth factor receptor 2/neuregulin (HER2/neu), and showed that this virus can only infect HER2/neu receptor positive cells (150). Altogether, these results provide evidence that viral tropism can be manipulated to infect tumor cells that express specific surface receptors.

1.3.3 Transcriptional targeting

Since deletions of viral genes, such as *E1A* and *E1B* of adenovirus or *ICP34.5* of HSV-1, showed significant attenuation of the virus with reduced cytotoxic effects in tumor cells, attempts have been made to incorporate tissue- or tumor-specific promoters in front of these genes to enhance anti-cancer efficacy and specificity. This strategy was used in the first adenovirus designed for oncolytic virotherapy, CV706, which is an adenovirus 5 (Ad5) that contains the enhancer and promoter of the PSA gene driving the expression of *E1A* gene (151). It has been shown that viral replication and oncolysis were highly specific to PSA-producing LNCaP cells. *E1A* is the first gene expressed from the viral genome, and regulates the expression of other viral genes. The incorporation of the PSA promoter in front of *E1A* restricted viral replication to

PSA-expressing cells. In a phase I trial, patients with locally recurrent prostate cancer after radiation therapy were treated with intraprostatic injection of 10^{11}-10^{13} vp of CV706 viruses (124). The virus was well tolerated and a greater than 50% reduction in PSA was observed in patients treated with the highest doses. Subsequently, several improved versions of CV706 were generated with tissue- or tumor-specific promoters regulating *E1A*, *E1B*, *E4*, and *E2*. CV764 is an adenovirus variant that has been engineered to contain a prostate-specific enhancer from PSA driving the *E1A* genes and the human glandular kallikrein (hk2) enhancer/promoter driving the *E1B* genes; it has been shown to replicate efficiently in prostate tumor cells but is attenuated in nonprostate cells (152, 153). In a clinical phase I trial, patients with hormone-refractory metastatic prostate cancer were treated with systemic administration of CG7870, which has *E1A* under the probasin promoter and *E1B* under the PSA promoter (154). The results showed that the virus was well tolerated and a 25-49% reduction in serum PSA was observed.

Other promoters have also been tested in various preclinical studies. Matsubara *et al.* constructed an adenovirus using the promoter of a noncollagenous matrix protein, osteocalcin, to drive the *E1A* gene, and showed that the virus was effective in eradicating pre-existing androgen-independent prostate tumors from the bone without adverse effects in mice (155). It has been shown that an adenovirus containing PSES (a chimeric enhancer of PSA and prostate-specific membrane antigen enhancer elements) upstream of the *E1A* and *E4* genes was able to dramatically inhibit the growth of CWR22rv prostate tumors after intratumoral or intravenous injections into nude mice (156). The most effective conditionally replicating adenovirus (CRA) examined so far is the telomerase-dependent CRA, which utilizes the human telomerase reverse transcriptase (hTERT) promoter to drive *E1A*, *E1B*, and *E4* gene expression, and has been shown to exhibit strong anti-tumor selectivity and potency (157-159). More recently, a *survivin* promoter driving the adenovirus *E1A* gene has been shown to be superior to the hTERT promoter in terms of anti-cancer specificity (160).

Similar targeting strategies have also been incorporated into oncolytic HSV-1 viruses. G92A was constructed by inserting an albumin enhancer/promoter upstream of the *ICP4* gene in the thymidine kinase gene locus of a mutant virus d120 that has both copies of *ICP4* deleted. This virus has been shown to replicate efficiently only in human hepatoma cells both *in vitro* and *in vivo* (161). rQNestin34.5 is an HSV-1 mutant with *ICP34.5* gene under control of a synthetic nestin promoter, and has been found to

replicate within and kill glioma cells efficiently in animal models (162). Kanai *et al.* used a replication-defective vector containing the musashi1 promoter driving the *ICP34.5* gene with G207 as a helper virus, and showed that this virus exhibited enhanced therapeutic efficacy compared with G207 alone both *in vitro* and *in vivo* for the treatment of brain tumors and non-small cell lung cancer (163, 164). All of these strategies capitalize on the overexpression of proteins in tumor cells in relation to normal cells so that their promoters can be used to regulate essential viral gene expression and thus viral replication in order to target tumor cells specifically.

1.3.4 Combination Therapies

Conventional therapies such as chemotherapy or radiotherapy has thus far proven to be ineffective in treating advanced cancers due to the narrow range of the therapeutic index, tumor cell resistance and limiting high-dose toxicities. Moreover, tumor cells are known to be genetically heterogeneous and any single agent treatment would select for the resistant phenotypes. One distinctive feature of oncolytic virotherapy is that its maximal effect increases with time as the virus replicates, whereas the activities of drugs decline with time. Since oncolytic virotherapy has proven to be safe and has shown efficacies in many cases, it is feasible to utilize a combination of oncolytic virotherapy with chemotherapy or radiotherapy to achieve synergistic or additive anti-tumor efficacy. In a xenograft mouse model, Chahlavi *et al*. demonstrated that a combination therapy of G207 plus the chemotherapeutic agent cisplatin resulted in 100% cures in contrast to 42% with G207 or 14% with cisplatin alone in cisplatin-sensitive head and neck tumors (165). A replication-selective HSV-1 ICP34.5 mutant (1716) has been tested in combination with each of four chemotherapeutic agents: mitomycin C, cis-platinum II, methotrexate, or doxorubicin (166). It was demonstrated that the combination of 1716 with common chemotherapeutic agents augmented the effect of HSV-based therapy in the treatment of human non-small cell lung cancer in a murine xenograft model. Ionizing radiation therapy has also been shown to enhance the lytic activity of NV1020, a mutant with multiple gene deletions including one copy of ICP34.5, in human glioma and hepatoma xenograft mouse models (167, 168). It has also been demonstrated that NV1020, when used in combination with 5-fluorouracil, caused inhibition of tumor growth and prolongation of survival when compared with either monotherapy alone in a subcutaneous syngeneic CT-26 tumor

model in BALB/c mice (169). This accumulating evidence suggests that utilizing oncolytic virotherapy in combination with chemotherapy or radiotherapy can greatly enhance the anti-tumor efficacy of each therapeutic agent in a synergistic or additive manner.

1.4 MicroRNAs

1.4.1 Discovery

The prototypic microRNA (miRNA) was first described 16 years ago when *lin-4* was found to be expressed at specific developmental stages in *C. elegans* (170). The *lin-4* product was identified to be a 21-nt RNA containing complementary sequences, which bind to the 3'UTR region of the *lin-14* mRNA, and therefore has a negative regulatory effect on the level of LIN-14 proteins by promoting mRNA instability and inhibiting translation (171). The next miRNA to be identified was *let-7*, which is 21-nt in length and is expressed specifically during the developmental stages of *C. elegans* from larvae to adult. It negatively regulates the expression of transcripts that have complementary sequences at the 3'UTR end (172, 173). Since then, extensive studies have been carried out in search of additional previously unknown small RNAs. Eight years ago, a newly emerging class of small RNAs, named miRNA, was characterized and identified in worms, flies and humans by three groups simultaneously (174-176). Together, they uncovered nearly 100 previously unknown 21-23-nt miRNAs from non-coding regions of the genome, and potential regulatory roles in gene expression were implicated. The Tuschl lab unexpectedly found 16 and 21 novel miRNAs in *Drosophila* and Hela cells, respectively, while searching for the endogenous products of the RNA interference (RNAi) mechanism (174). The Bartel (175) and Ambros (176) groups uncovered 60 novel miRNAs in the nematode *C. elegans*, which had previously been reported to have two short RNAs known to control development. To date, more than 500 miRNAs have been identified in the human genome, and at least 7 DNA viruses have been found to encode miRNAs. As a group, miRNAs are estimated to regulate 30% of the genes in the human genome (177).

1.4.2 Biogenesis and function

MicroRNAs are generally transcribed by RNA polymerase II (pol II) in the nucleus to

form a long primary transcript known as primary-miRNAs (pri-miRNAs) (178). The pri-miRNAs are capped, polyadenylated, and are usually hundreds of bases or several kilobases in length, and might contain monocistronic or polycistronic miRNAs (179, 180). These transcripts are often spliced and many miRNAs are located within intergenic or intronic segments (Figure 1.2). The miRNA is contained within a 60-80 nucleotide sequence of the pri-miRNA that can form a stem-loop hairpin structure. Inside the nucleus, the stem-loop is cleaved by Drosha to liberate a shorter, roughly 60 nt hairpin, termed a pre-miRNA that contains a 2 nt 3′ overhang (181). Subsequently, the pre-miRNAs are exported to the cytoplasm by the nuclear export factor exportin 5 which depends on the cofactor Ran-GTP (182-184). Further cytoplasmic processing of the pre-miRNA by a second RNase III enzyme, Dicer, generates a 18-24 nucleotide duplex miRNA-miRNA*, bearing 2 nt 3′ overhangs, which is similar in both structure and function to the short interference RNA (siRNA) duplexes that result from Dicer cleavage of long double-stranded RNAs (dsRNAs) (185). After cleavage, the miRNA duplex is loaded onto a large protein complex known as the RNA-induced silencing complex (RISC), which includes the Argonaute proteins (Ago1-4 in humans). A single-strand of the mature miRNA duplex remains stably associated with RISC, whereas the opposite strand, known as the passenger strand (miRNA*), is degraded (186-188). The mature miRNAs then act as guides that direct RISC to target mRNAs (reviewed in (189, 190)), and the degree of complementarity between a miRNA and its target determines the mechanism of regulation. When a miRNA binds to the target mRNA with perfect complementarity, the target mRNA is cleaved by RISC, thereby blocking protein expression, whereas imperfect base pairing between the miRNA and its target leads to translational silencing of the target. However, imperfectly complementary miRNAs can also reduce the number of target mRNAs to varying degrees via degradation (191).

1.4.3 MicroRNA and cancer

The first evidence suggesting a correlation between abnormal miRNA expression and cancer was the deletion and downregulation of both miR-15 and miR-16 in the majority of all B cell chronic lymphocytic leukemia (B-CLL) cases. Further analysis showed that miR-15 and miR-16 are located at chromosome 13q14, a region deleted in more than half of B-CLL (192). Subsequently, two papers reported reduced accumulation of miR-143 and miR-145 in colorectal neoplasia (193), and

overexpression of precursor miRNA-155/BIC RNA in children with Burkitt's lymphoma (194). It was further found that human miRNA genes are frequently located at fragile sites and genomic regions involved in cancers (195).

A correlation between abnormal miRNA expression and lung cancer was first reported by Takamizawa *et al.* (196). They showed that reduced expression of let-7 miRNA in human lung cancers was associated with poor postoperative survival, and that overexpression of let-7 in the A549 lung adenocarcinoma cell line inhibited lung cancer cell growth *in vitro*. They further reported that reduced expression of Dicer was associated with a poor prognosis of lung cancer (197). Subsequently, the 3'UTRs of the human RAS genes were identified to contain multiple let-7 complementary sites (LCSs), allowing let-7 to regulate RAS expression. Ras is a well known oncogene, and many human cancers have been found to have elevated levels of RAS expression (198). A lower level of let-7 expression is detected in lung tumors when compared to normal lung tissues, whereas RAS protein is significantly higher in lung tumors, implying a regulatory role of let-7 in tumorigenesis (199).

Recently, several groups have demonstrated the regulatory roles of miRNAs in cell proliferation. An anti-apoptotic role of miR-21 was suggested to contribute to malignancies in brain cancer (200). They showed a strong overexpression of miR-21 in the highly malignant human brain tumor, glioblastoma. Knockdown of miR-21 in cultured glioblastoma cells triggered activation of caspases and led to increased apoptotic cell death. A pro-apoptotic role for miRNA has also been demonstrated by the inversely correlated expression of miR-15 and miR-16 and Bcl2 expression in CLL (201). In addition, miR-15a and miR-16-1 have been shown to target *CCND1* (encoding cyclin D1) and *WNT3A* genes to promotes survival, proliferation and invasion of prostate cancer cells (202). Organ or tissue-specific miRNAs have also been reported to regulate cell differentiation. For example, Sempere *et al*. (203) identified a subset of brain-specific miRNAs whose expression behavior is conserved in both mouse and human differentiating neurons, implicating the regulation of mammalian neuronal development or function by miRNAs.

Xi *et al*. (204) were the first group to report possible regulatory mechanisms of miRNA expression. They investigated the regulation of miRNA expression by p53 because p53 functions as a transcription factor, and they demonstrated that the downregulation of p53 via siRNAs abolished the effect of p53 in regulating miRNAs in

the human colon cancer cell line, HCT-116 (wt-p53). In addition, they showed that approximately half of the 326 putative promoters for miRNAs contained p53 binding sites. The oncogene c-myc has also been proposed to directly activate a cluster of six miRNAs, two of them negatively regulating the transcription factor E2F1, suggesting a new mechanism by which c-myc tightly controls cell proliferation in a cell-type specific manner (205). In summary, there is accumulating evidence that suggests a regulatory role for miRNAs in cell proliferation, cell differentiation and tumorigenesis of human cancers. In fact, miRNAs have been proposed to function either as an oncogene or a tumor suppressor (Figure 1.3), and overexpression or downregulation of miRNAs have been reported in the tumorigenesis of a variety of human cancers including brain cancer (200), lung cancer (196, 197, 199), breast cancer (206, 207), colorectal cancer (193, 204, 208), ovarian cancer (209), cervical cancer (210), and prostate cancer (207, 211-213).

1.4.4 MicroRNA expression in prostate cancer

MiRNA profiles have been shown to be surprisingly informative, reflecting the developmental lineage and differentiation state of tumors. Lu *et al*. reported a general downregulation of miRNAs in tumors compared with normal tissues, and that miRNA expression profiles are more accurate in classifying poorly differentiated tumors than mRNA expression profiles (214). High-throughput microarray analysis of the clinical samples obtained from needle core biopsies of prostate cancer and breast cancer exhibited differential miRNA gene profiles between normal and malignant cells (207). Volinia *et al*. performed a detailed microarray analysis of 540 samples including 363 tumor tissues and 177 normal tissues covering 6 solid tumor types: breast, colon, lung, pancreas, prostate (56 cancer, 7 normal samples), and stomach cancer (215). The miRNA signatures for colon, prostate, stomach and pancreas cancers are most similar with one another, whereas breast and lung cancers are represented by relatively different signatures. They identified 39 upregulated miRNAs and 6 downregulated miRNAs in prostate cancer. Porkka *et al*. examined the expression of 51 miRNAs in 6 prostate cancer cell lines, 9 prostate cancer xenograft samples, 4 BPH, and 9 prostate carcinoma samples using an oligonucleotide array hybridization method (212). They reported 37 downregulated and 14 upregulated miRNAs in carcinoma samples. Another study by Ozen *et al*. examined the expression of 480 miRNAs in clinically localized

prostate cancer (16 samples) relative to benign peripheral zone tissues (10 samples), and showed a widespread downregulation of miRNAs in tumors (213). Their findings are consistent with the observation that global repression of miRNA expression significantly enhances tumorigenesis (216). Furthermore, it was shown that the androgen responsive LNCaP cells and the androgen unresponsive PC3 cells displayed differential miRNA expressions. Galardi *et al*. demonstrated that miR-221 and miR-222 are highly expressed in PC3 cells but almost undetectable in LNCaP cells (217). MiR-125b has also been found to stimulate androgen-independent growth of prostate cancer cells by targeting the pro-apoptotic gene, *Bak1* (218). In addition, they showed that both miRNAs negatively regulate the cyclin-dependent kinase inhibitor $p27^{Kip1}$, and that their overexpression may contribute to the growth and progression of prostate cancer to a more aggressive hormone-refractory form. Recently, it was shown that circulating miRNAs are stable blood-based markers for cancer diagnosis as miR-141 expression is readily detectable in the serum of patients with prostate cancer (219). These studies have shown that differential miRNA expression signatures can represent diagnostic and/or prognostic biomarkers for cancer, and are very informative with regards to the specific stage of tumor progression.

1.4.5 MicroRNA-mediated targeting

Effective and stringent tissue-specific gene expression can now be achieved due to the discovery of miRNAs. Brown *et al.* (220) constructed a lentiviral vector by inserting multiple copies of target sequences for miR-142-3p into the 3'UTR region of a green fluorescent protein (GFP) reporter gene, and demonstrated endogenous miRNA regulation of transgene expression. A differential expression of GFP regulated by the 3'UTR at a translational level was observed between hematopoietic lineages and nonhematopoietic cells. This enabled tissue-specific delivery of therapeutic genes under the control of the 3'UTR region without provoking an immune response, thereby increasing the duration of transgene expression, which is a favorable feature for gene therapy. In addition, this tissue-specific targeting by 3'UTR elements may be more effective than the leaky tissue-specific promoters. As a result, this provides new opportunities in the exploration of a miRNA-mediated targeting strategy for transgene expression or oncolysis (Figure 1.4).

1.5 Eukaryotic translation initiation factor 4E

1.5.1 Function

Translation is the last step in the process of transferring our genetic makeup, DNA, into functional proteins, and is divided into three distinct phases: initiation, elongation, and termination. Since the flow of information from genes to proteins is slow and time consuming, eukaryotes cope with this problem by maintaining a pool of mRNAs that are readily available and can be rapidly translated into proteins that are required for cell growth or survival when given the right stimuli. Under most circumstances, the rate-limiting step in this process is the initiation phase (221). A key player in this phase is the eukaryotic translation initiation factor 4E (eIF4E), which is part of the eIF4F complex that consists of three subunits: eIF4E, the cap binding protein; eIF4A, an ATPase and RNA helicase; eIF4G, a scaffolding protein (222, 223). eIF4E recognizes and binds to the 5' cap 7-methylguanosine (m^7G) that is present on all nuclear transcribed cellular mRNAs (224), and together with the eIF4F complex, unwinds the secondary structures within the 5' untranslated region (5'UTR) to facilitate the loading of the 40S ribosomal subunit to the mRNA (222). Under normal cellular conditions, eIF4E is sequestered from interaction with eIF4G by the inhibitory 4E binding protein (4EBP). Upon mitogenic activation, 4EBPs are phosphorylated at multiples sites by both the ras-ERK and PI3 kinase/AKT signaling pathways, resulting in the release of eIF4E and thus translational initiation (225). Under normal cellular conditions, there is a limited amount of free eIF4E present within a cell and therefore mRNAs have to compete with each other to gain access to the eIF4F complex. Most cellular mRNAs, such as the housekeeping genes, are strong mRNAs that contain short, unstructured 5'UTRs, and they can be translated with ease even when the presence of eIF4Es is limited. In contrast, weak mRNAs, particularly those of growth factor or proto-oncogenes, contain lengthy, G+C rich, 5'UTRs that form extensive secondary hairpin structures, and which are translated less efficiently, especially when eIF4E levels are low (223, 226).

1.5.2 eIF4E and cancer

The link between translational control and tumorigenesis was first established based on the observation that the overexpression of eIF4E promotes cellular transformation and tumorigenesis in immortalized normal cell lines (NIH3T3, CREF or

MM3MG), and that eIF4E expression is elevated in many malignant cells (227). Subsequently, it was found that eIF4E elevation causes overexpression of proteins that are known to contribute to the malignant transformation of tumors as a result of the enhanced translation of mRNAs that are normally translationally suppressed by their lengthy 5'UTRs. These mRNAs usually encode for proto-oncogenes such as c-myc, cyclin D1, and ornithine decarboxylase, angiogenesis factors such as fibroblast growth factor (FGF-2) and vascular endothelial growth factor, and enzymes that promote tumor invasiveness such as matrix metalloproteinase-9 (228). Furthermore, eIF4E was demonstrated to be elevated in various types of cancer, including breast, head and neck, lung, prostate, colorectal, bladder, cervical cancer, and lymphoma (229). More recently, it has been shown that eIF4E is most dramatically upregulated in prostate cancers with a Gleason score of greater than 7, and that increased eIF4E expression is associated with poor patient survival rates (230).

1.5.3 Translational targeting by 5'UTR

With an increased understanding of the mechanisms by which eIF4E, together with 5'UTR, regulates gene expression through translational controls, and the observation that normal and tumor cells display differentially eIF4E levels, De Benedetti and colleagues developed a new targeting strategy of gene therapy. They inserted the 5'UTR of rat FGF-2 in front of the HSV-1 thymidine kinase (TK) gene, and tested its specificity and efficacy in combination with the pro-drug ganciclovir as a suicide-gene therapy. After systemic administration of this vector through liposomal vehicles, a reduction in subcutaneous breast tumors and lung metastases (>90%) was observed with improved mean survival and no toxicity (231). Subsequently, the utilization of the 5'UTR in combination with suicide-gene has been incorporated into viral based vectors. For example, Yu *et al*. inserted both the prostate-specific promoter and the 5'UTR from rat FGF-2 upstream of the *TK* gene in a lentiviral vector, and demonstrated tumor-specific killing of prostate cancer cell lines after ganciclovir treatment (232). Additionally, Mathis *et al*. used the same strategy in an adenovirus vector and the specific killing of human breast cancer cell lines was also observed (233). They also showed, in a mouse model, that the expression of TK was found primarily in subcutaneous breast tumors with significantly reduced expression in normal liver. Clearly, incorporating the 5'UTR into a viral vector can enhance the specificity of gene

therapy and it may also be applicable to oncolytic virotherapy (Figure 1.5).

1.6 Summary

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. For new therapies to have a major impact on patient survival, improvement of anti-cancer efficacy and specificity is urgently needed. 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. 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 tissue-specific and tumor-specific fashion through both transcriptional and translational regulations of an essential viral gene, *ICP4* or *ICP27*, leading to selective HSV-1 replication within and lysis of prostate cancer cells while sparing normal cells. The specific aims are to determine: 1). whether the prostate-specific expression of an essential viral gene can render an oncolytic HSV-1 tissue-specific; 2). whether the 3'UTR region carrying tandem repeats of complementary sequences to a specific miRNA can regulate gene expression at a translational level as determined by the abundance of the specific miRNA present in a cell type; 3). whether the 5'UTR region can regulate gene expression at a translational level as determined by the abundance of endogenous eIF4E; 4). whether tissue- and tumor-specific targeting of an oncolytic virus can be achieved through both transcriptional and translational regulations.

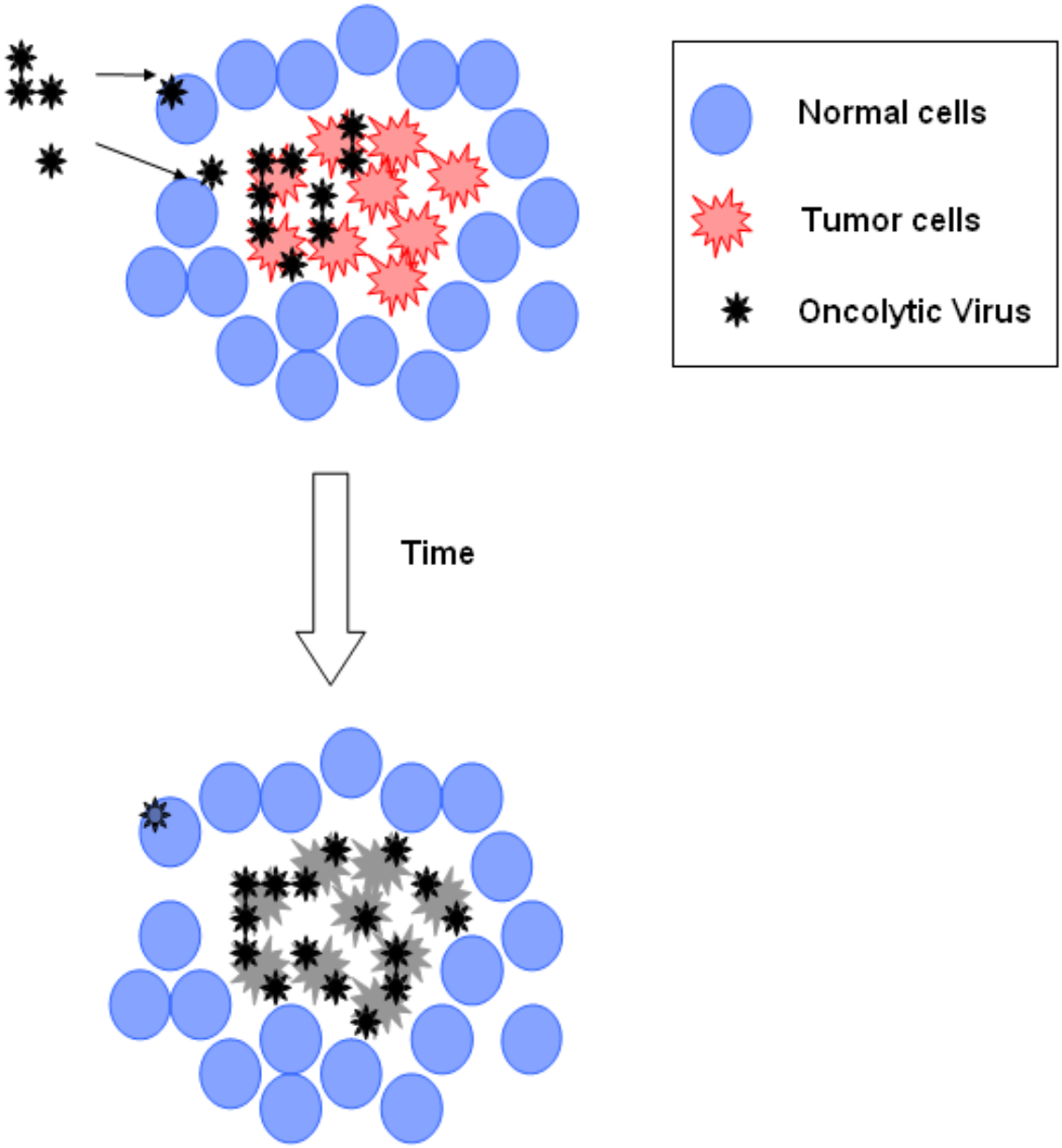

Figure 1.1 A schematic diagram of the theory behind oncolytic virotherapy for cancer treatment. A tumor-specific replication-competent virus (oncolytic virus) preferentially replicates and multiply in tumor cells, releasing more viral progenies that can initiate another round of lytic cycle in tumors cells. This eventually leads to the selective lysis of tumor cells while leaving normal surrounding cells unharmed.

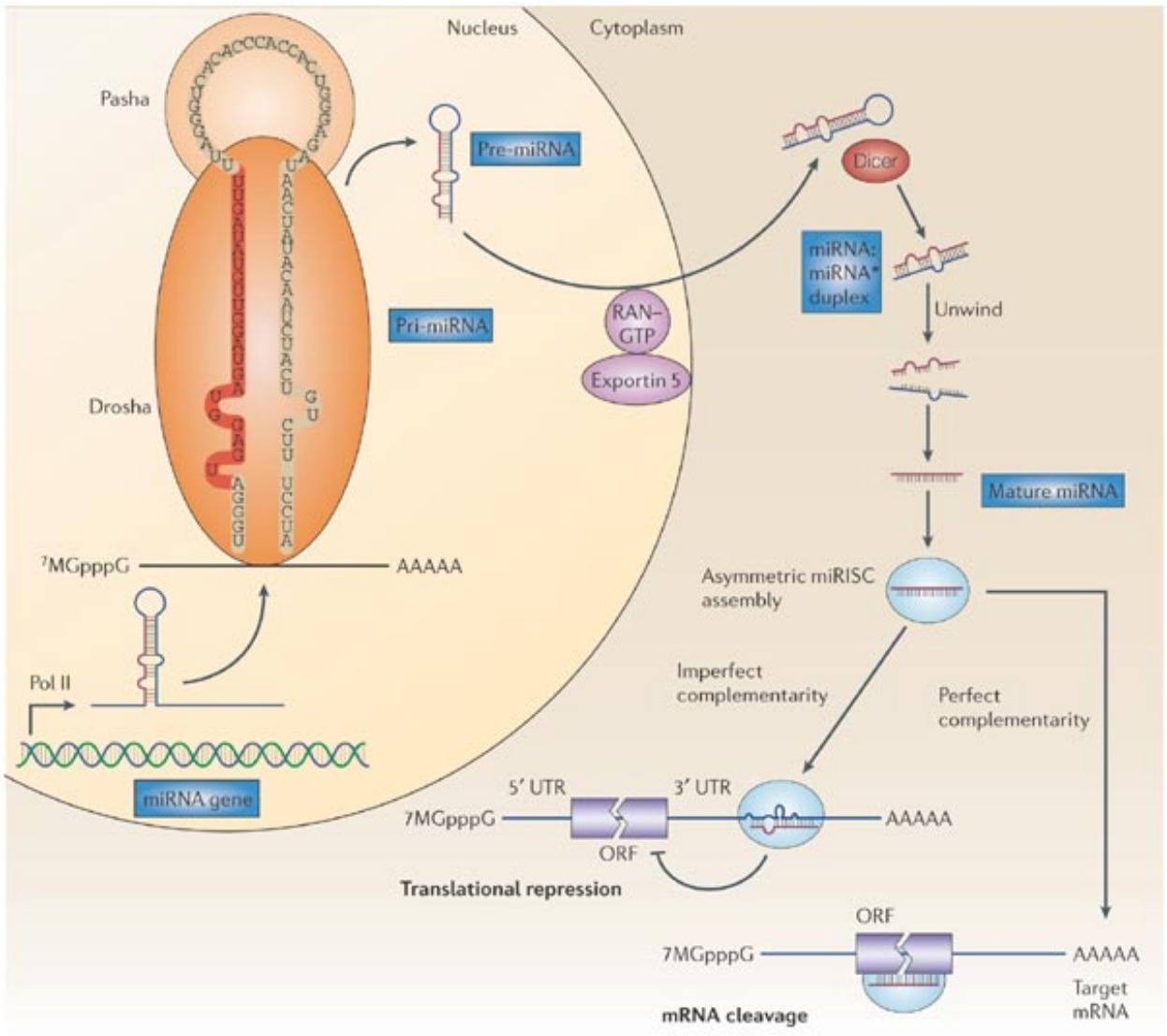

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Figure 1.2 The biogenesis of microRNAs. MicroRNAs are transcribed by Pol II in the nucleus to form pri-miRNA, which is further processed by Drosha and Pasha to form pre-miRNA. Pre-miRNA is then transported into the cytoplasm by Exportin 5. Inside the cytoplasm, Dicer cleaves pre-miRNA into mature miRNA, which is then incorporated into RISC to temporarily suppress the expression of their target mRNAs in mammalian cells.

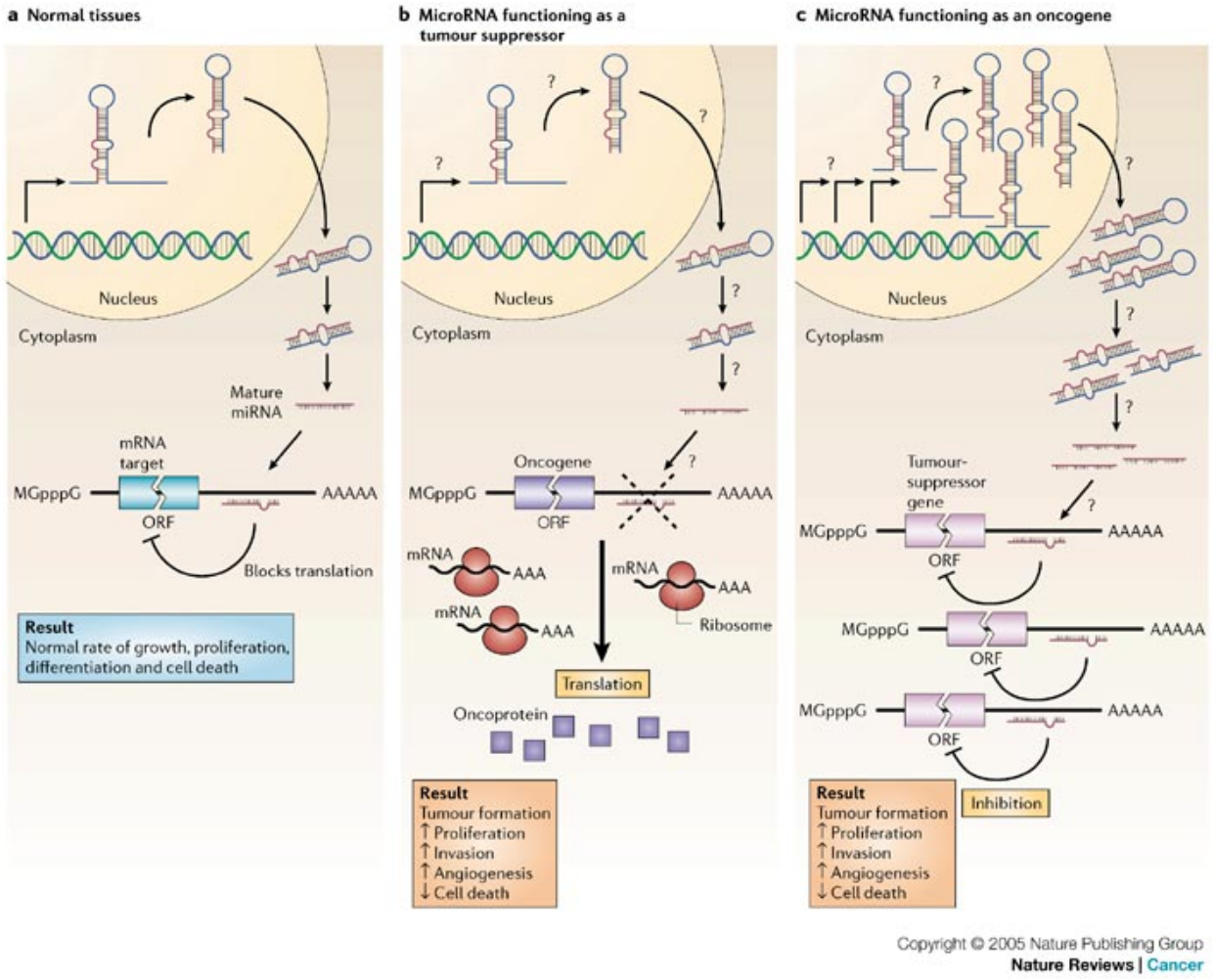

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Figure 1.3 The role of microRNAs as oncogenes or tumor suppressors. (A) In normal cells, microRNAs function to target mRNAs to maintain a balance between cell proliferation, differentiation and cell death. (B) In tumor cells, a downregulation of tumor suppressor microRNAs causes an overexpression of their target mRNAs (oncogenes), resulting in increased rate of tumorigenesis. (C) In tumor cells, oncogenic microRNAs are upregulated leading to suppression of their target mRNAs (tumor suppressors), again promoting tuomrigenesis.

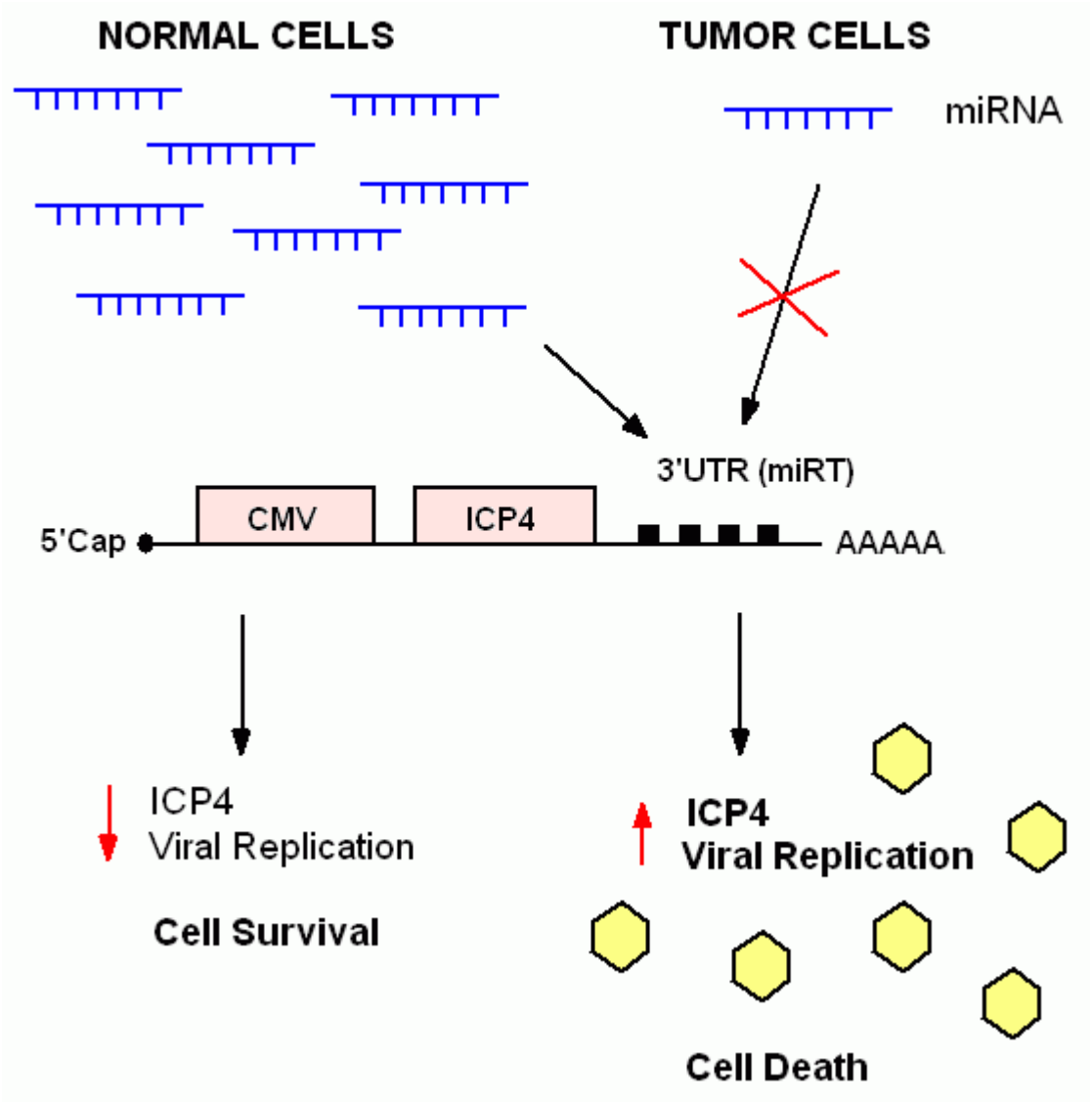

Figure 1.4 Selective destruction of tumor cells by microRNA-mediated oncolytic HSV-1. Tumor-specific targeting of an oncolytic virus can be achieved by incorporating multiple copies of the complementary target sequences to a microRNA in the 3'UTR of an essential viral gene, *ICP4*. The candidate microRNA is downregulated in tumor cells but highly expressed in normal tissues. In normal tissues, the miRNAs bind to the target regions within 3'UTR, leading to destruction of *ICP4* mRNA transcript. As a result, there is no viral replication and the normal cells survive. In tumor cells, ICP4 is expressed due to the low levels of miRNAs, leading to lytic viral replication and subsequent cell death.

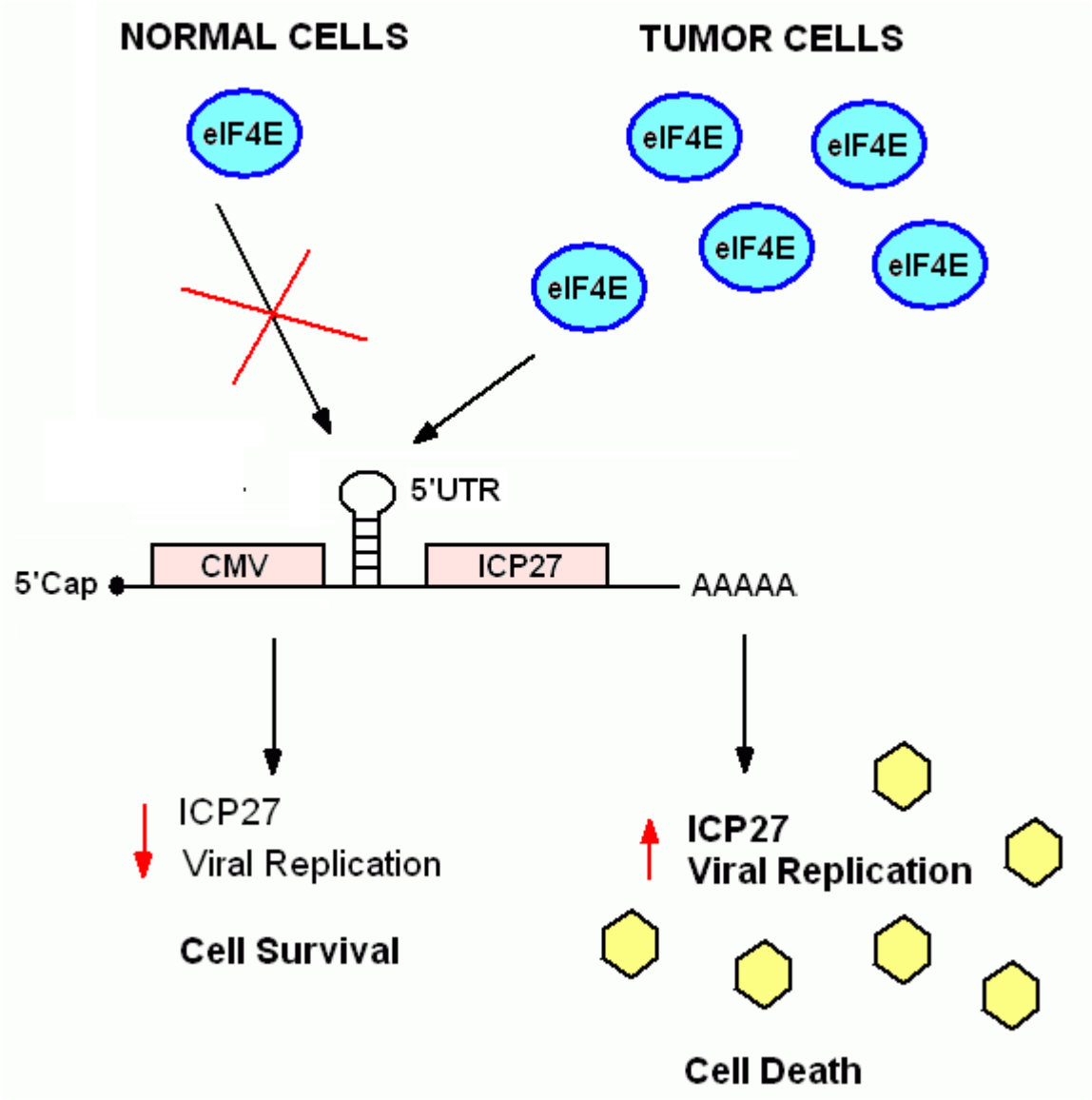

Figure 1.5 Selective destruction of tumor cells by eIF4E-mediated oncolytic HSV-1. Tumor-specific targeting of an oncolytic virus can be achieved by incorporating a complex 5'UTR in front of an essential viral gene, *ICP27*. In normal tissues where the eIF4E level is limiting, weak mRNAs carrying extensive 5'UTR are not translated efficiently. As a result, ICP27 is not expressed, leading to no viral replication and the normal cells survive. In tumor cells where eIF4E is overexpressed, the 5'UTR is unwound efficiently, resulting in ICP27 expression, lytic viral replication and the subsequent cell death.

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2 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*

2.1 Introduction

Prostate cancer is the most commonly diagnosed non-skin cancer in men and the second leading cause of cancer death in men (1). It is estimated that over 234,000 men in North America will develop prostate cancer and approximately 27,000 will die from this disease (2). If caught early, when still confined to the prostate, it is frequently curable by surgery or radiotherapy. However, patients who present with metastatic disease have concomitantly fewer curative treatment options and a median life expectancy of <3 years (3-5). The primary treatment for locally advanced, recurrent or metastatic prostate cancer is usually some form of androgen ablation therapy (6, 7). Unfortunately, the effectiveness of androgen withdrawal therapy in advanced prostate cancer is restricted to a duration of about 18-24 months due to progression of tumor cells (8-10). At this lethal end phase, even with the most effective chemotherapy regimens, median survival is only around 19 months (11, 12). Clearly, if we are to have an impact on patient survival, more effective, new approaches for dealing with advanced disease are required. In this regard, gene therapy offers a promising therapeutic option for treating locally advanced, recurrent and metastatic AI prostate cancer (for reviews (13-15)). Prostate cancer lends itself to gene therapy since the prostate is not essential for sustaining life and therefore can be ablated, plus intraprostatic administration of gene therapy via transurethral, transperineal and transrectal routes is technically easy (16). Another advantage is that there are several prostate-specific promoters available which allow for tissue specific targeting.

Herpes simplex virus type 1 (HSV-1) has a number of favorable features that make it an attractive viral vector for cancer treatment, and various forms of replication-defective or replication-conditional vectors have been developed for cancer therapy (17). G207, an example of this type of recombinant HSV-1, has been shown to

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be efficacious in treating malignant glioma (18) and is being evaluated in clinical trials (19). Other replication-competent HSV-1 vectors are also currently being tested in human clinical trials for treatment of malignant melanoma (20), breast cancer (21), head and neck cancer (22), and metastasis from colorectal cancer (23). Results from the above phase I clinical trials have demonstrated that HSV-1 viral therapy was well tolerated by patients and have shown efficacy in some cases.

HSV-1 virus is a double-stranded DNA virus with a genome size of approximately 152 kb encoding more than 84 genes. The viral genes are divided into three categories: immediate early (IE), early (E) and late (L) genes. There are five immediate-early (IE) genes: *ICP0*, *ICP4*, *ICP22*, *ICP27*, and *ICP47*. Among them, *ICP4* and *ICP27* are absolutely essential for viral replication. The virus enters host cells via cell receptor-mediated endocytosis and once in the cell, the tegument protein (VP16) and the viral DNA are transported into the nucleus where viral genes are expressed in a coordinately regulated and sequentially ordered manner (24). Inside the nucleus, VP16 stimulates the transcription of IE genes, whose products are involved in transcription regulation and stimulation of the expression of E genes, which primarily encode enzymes involved in nucleotide metabolism and viral DNA replication. Upon viral DNA replication initiation, expression of L genes commences. L genes mainly code for structural components of the virion (25). At the end of the replication cascade, mature viral particles are assembled and released from the host cell, resulting in cell lysis and cell death.

Tissue-specific and tumor-specific control of the expression of viral genes essential for replication could potentially regulate oncolytic virus replication differentially and therefore result in selective killing of cancer cells. We hypothesized that a replication defective HSV-1 mutant lacking one of the essential genes may become oncolytic when the missing gene is supplemented by a co-existing amplicon expressing this viral gene in a cell-type specific fashion. To test this hypothesis, an HSV-1 amplicon structure was constructed containing a prostate-specific promoter inserted in front of the *ICP4* gene (26). The helper virus used in this study is a replication-deficient recombinant ICP4^{-} helper virus (CgalΔ3) which has had both copies of the *ICP4* gene deleted and an insertion of the bacterial β-galactosidase gene in an intergenic site of the short unique region (27). The helper virus carries all other necessary genes for viral replication except the *ICP4* gene, and therefore it cannot replicate except in the presence of a

complementing cell line transfected with *ICP4* gene or co-infected with an amplicon virus expressing this gene. The amplicon virus carries both a viral origin of replication and a packaging signal, which allow the construct to replicate and be packaged by the helper HSV-1 virus in host cells (28). ARR_2PB is a small composite promoter derived from the prostate-specific rat probasin promoter that has been reported to be expressed in a highly prostate-specific manner in transgenic mice (26, 29, 30). This androgen-responsive promoter has also been shown to direct prostate-specific expression of EGFP and herpes thymidine kinase in human prostate cancer cell lines both *in vitro* and *in vivo* using a lentiviral vector system (31, 32). In the present study, our results demonstrated that expression of the *ICP4* gene under a prostate-specific promoter in an amplicon virus complemented the $ICP4^{-}$ helper virus to enable viral replication and subsequently lead to oncolysis of LNCaP human prostate cancer cells both *in vitro* and *in vivo*.

2.2 Materials and Methods

2.2.1 Plasmid preparation

The ARR_2PB-ICP4 plasmid was constructed and described by Yang *et al* (33). The CMV-ICP4 plasmid was made by replacing the ARR_2PB promoter (465 bp) of the ARR_2PB-ICP4 plasmid with the CMV promoter (605 bp), which was excised from the plasmid pHR-CMV-EGFP at ClaI-BamHI sites. The structure of the two amplicon plasmids is represented in Figure 1.

2.2.2 Cell culture conditions

Vero and its derivative cell line, 7B (transfected to express *ICP4* and *ICP27*), cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% FBS and antibiotics (penicillin and streptomycin). LNCaP prostate cancer cells were grown in DMEM supplemented with 10% FBS and antibiotics. For androgen-regulation experiments, LNCaP cells were maintained in DMEM with 10% charcoal-stripped FBS with or without the addition of 10nM R1881, a synthetic androgen. All cells were maintained in a 5% CO_2 incubator at 37℃.

2.2.3 Viral preparation

The replication-deficient ICP4$^-$ helper virus, CgalΔ3 (27) was a gift from Dr. Paul Johnson (Neurovir Inc., Vancouver, BC, Canada) and was propagated and tittered in 7B cells (33, 34). Briefly, 7B cells were infected with ICP4$^-$ helper virus at an MOI of 0.1. Two days after viral infection, cells were scraped from the plates and the medium containing both ICP4$^-$ helper virus and 7B cells was subjected to three freeze-thaw cycles in order to lyse 7B cells and to release intracellular viral particles. The cell debris was then precipitated by centrifuging at 3,200 x g for 5 minutes (Thermo IEC Centra CL3/CL3R) and the viral supernatant tittered on 7B cells. For amplicon virus production, 7B cells were grown in 100 mm cultural dish containing DMEM with 10% FBS without antibiotics one day before transfection. Monolayer of 90%~95% confluent 7B cells were transfected with 24 μg of the CMV-ICP4 or ARR$_2$PB-ICP4 plasmid DNA using the Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacturer's instruction. At 24 h after transfection, the 7B cells were superinfected with the ICP4$^-$ helper virus at an MOI of 1. Three days after the superinfection, viral supernatant was collected as described above and subjected to further viral amplification by infecting more plates of 7B cells. Finally, the stock virus produced was a mixture of helper and amplicon viruses, and are referred to as CMV-ICP4 amplicon/helper virus and ARR$_2$PB-ICP4 amplicon/helper virus. The virus titer was determined using 7B cells. The helper/amplicon ratio was determined by quantitative real-time PCR analysis using *ICP0* primers (5'-TTACGTGAACAAGACTATCACGGG-3' and 5'-TCCATGTCCAGGATGGGC-3') for determination of the amount of helper virus, and *ICP4* primers (5'-GGCCTGCTTCCGGATCTC-3' and 5'-GGTGATGAAGGAGCTGCTGTT-3') for determination of the amount of amplicon virus present in the viral mixture.

2.2.4 Viral infection

LNCaP cells (35) were seeded in 6-well plates at a density of 3.5 x 10^5 cells/well in 2 ml of DMEM medium with 10% charcoal-stripped FBS. After three days, the LNCaP cells were infected with ICP4$^-$ helper virus, CMV-ICP4 amplicon/helper virus, or ARR$_2$PB-ICP4 amplicon/helper virus at an MOI of 1 and incubated at 37℃ for 1 hour. The medium was then replaced with 2 ml DMEM plus 10% charcoal-stripped FBS with or without R1881 (10 nM). The supernatant was collected and tittered for virus production on 7B cells at 48 hours or 72 hours after infection as described above. The

total RNA was extracted from the infected cells at 16 hours after infection by Trizol and subjected to one-step RT-PCR analysis.

2.2.5 Cell viability assay

LNCaP cells were seeded in 96-well plates at a density of 3 x 10^4 cells/well in 50 µL medium and then infected with CMV-ICP4 or ARR_2PB-ICP4 amplicon/helper virus at helper virus MOI of 1 on the following day. After 48, 72, 96 and 120 hours of viral infection, live cells were measured by an MTT assay (Cell Proliferation Kit 1; Roche Diagnostics, Indianapolis, IN) for cell viability according to the manufacturer's instructions (36).

2.2.6 LNCaP xenograft experiments

Athymic nude mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and LNCaP cancer cells were implanted subcutaneously by injecting 5 x 10^6 cells in 100 µL of medium with matrigel at four different sites over the shoulders and hind legs on both sides. Animals were monitored for tumor development two times a week by palpation of the injection sites. When detected, tumor volumes were determined by caliper measurements and calculated using the formula volume = width x length x thickness x π/6. When the tumor volume reached approximately 100 mm^3 (at ~1 - 1.5 month after tumor inoculation), the mice were treated with two intratumoral injections (second injection at 1 week after first injection) of medium alone, or of 2 x 10^6 pfu of the $ICP4^-$ helper virus, CMV-ICP4 amplicon/helper virus, or ARR_2PB-ICP4 amplicon/helper virus. Blood was collected by tail-vein bleeding method at days 0, 7 and 14, and the PSA level from the serum was determined using the Abbott's kits for total PSA MEIA assays on the IMx system. At the end of the treatment period, mice were sacrificed using CO_2 asphyxiation, and selected organs (kidney, liver) and the tumors were removed and analyzed. Statistical significance between different treatment groups was determined by Student's t-test (33). For the study of ARR_2PB-ICP4 amplicon/helper virus replication *in vivo*, a single intratumoral injection of 2 x 10^6 pfu ARR_2PB-ICP4 amplicon/helper virus was administered and tumors were excised from the mice at day 2 (early stage, n = 4) and day 5 (late stage, n = 3). The virus was liberated from the tumors by homogenization in PBS and the virus titer was determined by plaque forming assays on 7B cells. All experimental procedures were approved by the UBC Animal Care

Committee and followed the guidelines and policies of the Canadian Council on Animal Care.

2.2.7 DNA, RNA isolation and RT-PCR

Total RNA was extracted from LNCaP cells infected with ARR_2PB-ICP4 amplicon/helper virus using Trizol (Invitrogen) following manufacturer's protocol. Two μg of RNA was then converted into cDNA in the one-step RT-PCR reactions. Organs collected from mice were subjected to three freeze-thaw cycles, cut into small pieces and homogenized in 1x PBS buffer using a 2 ml dounce homogenizer. DNA was extracted from the homogenate using the phenol-chloroform extraction method (37). Oligonucleotide primers for *ICP0* gene (5'-TTACGTGAACAAGACTATCACGGG-3' and 5'-TCCATGTCCAGGATGGGC-3'), *ICP4* gene (5'-GGCCTGCTTCCGGATCTC -3' and 5'-GGTGATGAAGGAGCTGCT GTT-3'), and *β-actin* gene (5'-ACGAGGCC CAGAGCAAGAG-3' and 5'-TCTCCATGTCGTCCCAGTTG-3') were designed for one-step RT-PCR and quantitative real-time PCR using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). All real-time PCR reactions were performed 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 and then compared to the control group (LNCaP infection with $ICP4^{-}$ helper virus). DNA copy number was normalized to a standard curve prepared from the stock viral DNA according to the manufacturer's protocols.

2.2.8 Histochemical staining

Harvested tissues were embedded in OCT and 10 μm sections were subjected to X-gal staining (38). Briefly, the sections were fixed with 4% paraformaldehyde for 15 minutes, washed once with 1 x PBS for 5 minutes, and then incubated with 4% X-gal substrate (Invitrogen) in LacZ buffer overnight at 37℃. The following day, the sections were stained with Eosin and pictures were taken using a light microscope.

2.3 Results

2.3.1 Prostate-specific expression of ICP4 complements the replication of HSV-1 ICP4$^-$ helper virus in LNCaP prostate cancer cells *in vitro*

To test the androgen-responsiveness and the complementing ability of the amplicons to aide in ICP4$^-$ helper virus replication, LNCaP human prostate cancer cells (35) were infected with ICP4$^-$ helper virus alone or ARR_2PB-ICP4 (Figure 2.1) at an MOI of 1 (an input virus amount of ~5 x 10^5 pfu) in the presence or absence of 10 nM of the synthetic androgen, R1881. The virus titer at 72 hours post-infection was determined by plaque forming assays on 7B cells (Figure 2.2A). The results showed that the ICP4$^-$ helper virus alone was incapable of replicating in LNCaP cells, in keeping with the fact that it lacks the essential IE gene *ICP4*, whereas both the CMV-ICP4 and ARR_2PB-ICP4 amplicons were able to complement the ICP4$^-$ helper virus to enable its replication in LNCaP cells. Addition of 10 nM of the potent androgen R1881 significantly increased virus titers (t-test, $p<0.01$) by >2-fold from 2.30±1.69 x10^6 pfu/ml to 5.88±0.85 x10^6 for experiments with the ARR_2PB-ICP4 amplicon/helper virus but had no effect when the CMV-ICP4 amplicon/helper virus was used. Consistently, *ICP4* mRNA was >3-fold higher in the presence of 10 nM R1881 (41.1 ± 2.5) as compared to no androgen treatment (13.4 ± 2.0) (Mean ± SD, n = 4, $p<0.01$). This is in keeping with the fact that the ARR_2PB promoter contains two copies of androgen response elements and is thus very androgen responsive (26).

We next determined whether viral replication was dependent on the ratio of helper virus to amplicon. LNCaP cells were infected with ARR_2PB-ICP4 amplicon together with helper virus at various helper virus to amplicon ratios (50:1, 100:1, 200:1, 400:1, 800:1, 1600:1) at a final helper virus MOI of 1 in the presence of 10 nM R1881. As shown in Figure 2.2B, virus replication was acutely dependent on the helper to amplicon ratio with an approximately 3.6 and 6-fold increase in the virus titer at a ratio of 50:1 as compared to the virus titer at ratios of 100:1 and 200:1, respectively (5.88±0.85 x10^6 versus 1.64±0.49 x10^6 and 0.95±0.27 x10^6 pfu/ml; $p<0.001$).

It was necessary to confirm that replication of ICP4$^-$ helper virus was solely dependent on the ARR_2PB-ICP4 amplicon and not caused by a reversion to wild-type virus through a recombination event due to the presence of homologous regions (such as OriS and packaging sequence) contained in both the ARR_2PB-ICP4 amplicon

construct and the ICP4- helper virus. Accordingly, we infected 7B and Vero cells with the ARR_2PB-ICP4 amplicon/helper virus stock at various MOI's from 1 to 10^{-6}. The $ICP4^-$ helper virus can grow in 7B cells but not in Vero cells, while any recombinant wild-type revertants will grow in both. We speculated that since the amplicon and helper virus must be in the same cell to allow the helper virus to replicate, then by reducing the MOI, the probability of co-existence of both in the same cell would decline to near zero. We found that approximately 1 in 10 amplicons from a stock with a helper to amplicon ratio of 50:1 will infect the same cells with the helper virus enabling the formation of plaques. As expected, no plaques were seen when cells were infected with viral stock at an MOI $<10^{-5}$ (data not shown). These results support the conclusion that replication of helper virus was entirely dependent on the presence of the amplicon and that no recombinant virus was generated during the preparation of viral stock solutions. Taken together, our results indicated that prostate-specific expression of ICP4 by an amplicon complemented the $ICP4^-$ helper virus, enabling it to replicate in LNCaP cells, and that with the ARR_2PB-ICP4 amplicon, viral replication was androgen-responsive and dependent on the relative amount of amplicon present in the viral infectant.

2.3.2 ARR_2PB-ICP4 amplicon virus with helper virus showed substantial cell kill of the LNCaP cancer cells grown *in vitro*

Having found that the ARR_2PB-ICP4 amplicon/helper virus was able to complement replication of $ICP4^-$ helper virus in LNCaP cells, we next used MTT cell kill assays to determine whether the amplicon with helper virus could actually kill the LNCaP cells. With ARR_2PB-ICP4 and CMV-ICP4 amplicons approximately 15% and 30% of infected LNCaP cells were killed in the first 72 hours post-infection respectively (Figure 2.3A). By comparison, 75% and 85% of the cells were killed at 96 and 120 hours post-infection respectively, with either amplicon. The delayed cell killing by the amplicons/helper viruses was consistent with the >10-fold increase in the virus titer in LNCaP cell infections. At 48 hours post-infection, the virus titers for ARR_2PB-ICP4 and CMV-ICP4 were $2.45\pm0.041 \times 10^5$ and $1.875\pm0.177 \times 10^5$ pfu/ml respectively (Figure 2.3B). More than a 10-fold increase in the virus number was measured at 72 hours post-infection, with titers of $5.875\pm0.854 \times 10^6$ and $2.188\pm0.178 \times 10^6$ pfu/ml for ARR_2PB-ICP4 and CMV-ICP4 amplicons, respectively.

2.3.3 ARR_2PB-ICP4 amplicon/helper virus inhibits growth of LNCaP tumor xenografts

Since the ARR_2PB-ICP4 amplicon/helper virus combination was able to replicate efficiently and kill LNCaP cells *in vitro*, we next tested the therapeutic efficacy of using this amplicon and helper virus *in vivo* with the LNCaP xenograft tumor/nude mouse model. When subcutaneous LNCaP tumors grew to approximately 100 mm^3 on the flanks of nude mice, two intratumoral viral injections, totaling 2 x 10^6 pfu viruses, were administered. Subsequently, the size of each tumor was monitored by caliper measurements once every two days, and the fold change in tumor size for each group was determined by comparing the tumor size at each day to the original size at Day 0 (Figure 2.4A). For the control groups treated with either DMEM medium alone or $ICP4^-$ helper virus alone, a 3 and 3.5-fold increase in tumor size was observed, respectively, at day 22 post-viral treatment. In contrast, mice treated with the CMV-ICP4 and ARR_2PB-ICP4 amplicon/helper viruses showed greater than 75% reduction in tumor size at day 16 and at day 20 post-viral treatment, respectively ($p < 0.01$). These results were consistent with the changes in serum PSA levels (Figure 2.4B), with an approximately 2.5-fold increase in mice treated with $ICP4^-$ helper virus and a >80% reduction in the serum PSA of mice treated with ARR_2PB-ICP4 amplicon/helper virus ($p < 0.05$).

The CMV-ICP4 amplicon/helper virus was highly toxic to the mice as the animals exhibited herpetic lesions on the skin around the viral injection area and had severe hemorrhage in the stomach. As well, two mice in this group died on day 10 and the remainder died on day 16 post-inoculation. By comparison, considerably less toxicity was observed in ARR_2PB amplicon/helper virus treated animals. No skin lesions were seen in any of these animals, but gastritis was evident in two out of five animals, resulting in delayed death on day 16 and day 20 post-infection.

To assess viral replication within the LNCaP tumor mass *in vivo*, a total of 2 x 10^6 pfu of ARR_2PB-ICP4 amplicon/helper virus was administered intratumorally in a single dose. Tumors were excised from the mice at day 2 (early stage) and day 5 (late stage) post-viral injection, and the virus was extracted from homogenized tumors. Virus titer (pfu/mg tissue) was determined by plaque assays on 7B cells (Figure 2.4C). At an early stage after viral injection, there was a 2-fold decrease in the amount of virus within LNCaP tumors compared to the amount of input virus, whereas a >10 fold increase was

observed at a later stage relative to input virus ($p < 0.05$). This indicated that ARR_2PB-ICP4 amplicon/helper virus was able to replicate within the LNCaP tumors *in vivo*.

2.3.4 Distribution of the ARR_2PB-ICP4 amplicon/helper virus in tumor bearing mice

Tissue sections were examined by X-gal staining to determine the presence of viral activity (Figure 2.5). The $ICP4^-$ helper virus carries the bacterial *LacZ* gene that encodes β-galactosidase, which can convert the X-gal substrate into a blue form detectable under the microscope. In the tumors, high levels of β-galactosidase activity were detected in animals treated with either CMV-ICP4 or ARR_2PB-ICP4 amplicon/helper virus. As well, substantial amounts of X-gal staining were evident in both kidney and liver from animals treated with the CMV-ICP4, but not with the ARR_2PB-ICP4 amplicon/helper virus (Figure 2.5).

Viral DNA in kidney and liver, as well as in the tumor mass, were also identified and quantified by real-time quantitative PCR using the organs collected from mice treated with amplicons and helper viruses at the termination of the experiments (Table 2.1). Viral sequences were detected by real-time PCR in the kidneys and livers from animals infected with CMV-ICP4 or ARR_2PB-ICP4 amplicon/helper viruses. While there was a large variation in estimates of viral DNA copy number by quantitative PCR analysis among the samples, higher copy numbers were consistently found in CMV-ICP4 amplicon/helper virus treated animals.

2.4 Discussion

The main objective of this study was to determine whether we could selectively direct or restrict the oncolytic activity of a mutated herpes virus to prostate cancer cells by combining it with an amplicon virus which expressed the complementing wild-type gene under a prostate-specific promoter. To achieve this, we exploited the HSV-1 helper virus/amplicon system by inserting the prostate-specific and AR-regulated ARR_2PB promoter in front of *ICP4*, an essential viral gene, in an amplicon to limit its expression to only prostate-derived cells, so that in the presence of $ICP4^-$ helper virus, lytic viral replication and selective cell killing could only occur in these cells. The HSV-1 amplicon virus contains an HSV-1 replication origin (oriS), a packaging signal sequence, a

transgene of interest, and can be packaged into infectious HSV-1 virions in the presence of a transactivating helper virus (28, 39). We constructed two amplicon viruses: CMV-ICP4, a constitutively active, non-tissue selective complementing amplicon; and ARR_2PB-ICP4, an AR-enhanced, prostate-specific complementing amplicon (Figure 2.1).

We tested the complementing ability of the amplicon viruses to aide in viral replication of the helper virus in LNCaP cells in the presence or absence of the potent, synthetic androgen, R1881. As previously reported (40), the replication-defective $ICP4^{-}$ helper virus alone was unable to replicate in the LNCaP cells, but either of the amplicon viruses, CMV-ICP4 or ARR_2PB-ICP4, was able to complement the replication of the helper virus in LNCaP cells. In LNCaP cells grown *in vitro*, CMV-ICP4 amplicon/helper virus was refractory to the addition of androgen, whereas the ARR_2PB-ICP4 amplicon/helper virus infection was androgen responsive such that at 72 hours post-infection, the virus titer increased by approximately 2.5-fold in the presence of supplemented androgen (Figure 2.2A). Furthermore, replication of the ARR_2PB-ICP4 amplicon/helper virus was dependent on the relative amount of amplicon virus in the viral mixture, as determined by the virus titer obtained from the LNCaP infections with the ARR_2PB-ICP4 amplicon/helper virus at various helper:amplicon ratios (Figure 2.2B). To further test this, both 7B (ICP4+) and its parental cell line Vero cells ($ICP4^{-}$) were infected with the same stock of CMV-ICP4 amplicon/helper virus and the helper:amplicon ratios in both cells were measured 48hrs after initial infection with the same titer. Interestingly, the ratio of helper:amplicon was significantly reduced in Vero cells but not in 7B cells (data not shown). It is apparent that the dependency of ICP4-expressing amplicon for viral replication generates a selection pressure that is in favor of growth of amplicon since there were approximately 50 helper virus for each amplicon in the initial virus stock and only those cells infected with both helper and the amplicon were permissive for viral replication. Given the reverse correlation between the ratio of helper:amplicon and the oncolytic cytotoxicity of this virus (Figure 2.2 and 2.3), the progressively increased proportion of the amplicon after initial inoculation may enhance the efficiency of the helper/amplicon mixture for tumor lysis when the amplicon complements the helper virus replication in a tumor specific fashion. To determine the potential therapeutic efficacy and relative toxicity of the ARR_2PB-ICP4 amplicon/helper virus treatments, we performed *in vivo* studies using the LNCaP xenograft/nude mouse

model (31). The ARR_2PB-ICP4 amplicon/helper virus was as effective as the CMV-ICP4 amplicon/helper virus in terms of inhibiting the LNCaP tumor growth, whereas when used alone, the $ICP4^-$ helper virus had no effect, which is consistent with the fact that the $ICP4^-$ helper virus is a replication-defective virus incapable of killing tumor cells (Figure 2.4A, 2.4B). The relative amount of virus within the LNCaP tumor mass was found to be 7-fold higher at late stage of viral infection as compared to the input virus, supporting the conclusion that the amplicon was able to complement the replication deficiency in $ICP4^-$ virus in tumor mass. Together, these results indicate that killing of the tumor cells was a result of viral replication rather than mere cytotoxicity of the virus (Figure 2.4C).

With respect to toxicity, the CMV-ICP4 amplicon/helper virus exhibited high levels of toxicity to the treated mice, causing skin lesions, severe hemorrhage in the stomach, and death of all mice within 10 days after the second viral injection. By comparison, the ARR_2PB-ICP4 amplicon/helper virus was much less toxic to the mice, although some mild gastritis developed at 10 days after the second viral injection. In order to assess the extent of viral infection to other tissues, histochemical analyses of cross-sections of tumors and normal organs were performed by incubation with X-gal to indicate the presence of the $ICP4^-$ helper virus, which carries the *LacZ* gene. Beta-galactosidase activity from both the CMV-ICP4 and ARR_2PB-ICP4 amplicon/helper viruses were found extensively throughout the LNCaP tumor mass; with CMV-ICP4 amplicon/helper virus also detected in the normal organs, but not with the ARR_2PB-ICP4 amplicon/helper virus (Figure 2.5). However, the relatively more sensitive quantitative real-time PCR substantiated CMV-ICP4 amplicon/helper virus histochemical results and revealed a comparatively small amount of the ARR_2PB-ICP4 amplicon/helper virus present in normal tissues, such as liver and kidney (Table 2.1). The reduced activity of ARR_2PB-ICP4 amplicon/helper virus in non-prostate normal tissues was unlikely caused by contamination of wild-type virus created by recombination between the amplicon and helper virus during virus production as we have extensively tested this possibility and demonstrated that it was not the case. More likely, the non-prostate specific viral replication may be attributed to the leakage of ARR_2PB promoter in the amplicon. Our previous study has shown that HSV-1 ICP0 may interfere with the specificity of some cellular promoters (33), hence, the ICP0 expressed from the helper virus may override the prostate specificity of the ARR_2PB promoter to cause low level expression of ICP4 in

some cells, resulting in non-prostate specific replication of the virus. To avoid this risk, it may be necessary to create a helper virus with an ICP0^{-} backbone, since it has been reported that *ICP0* deletion makes the virus capable of replicating in a tumor-specific fashion in some cancer cells (41).

This study provides a proof in principle that a tissue-specific promoter driving an essential viral gene in a separate viral construct can complement a replication deficient virus to restrict oncolytic killing to cancer cells derived from the target tissue. The advantages of this approach compared to using recombinant HSV-1 with an essential gene regulated by a tissue-specific exogenous promoter are that 1) the amplicon/helper system is much easier and versatile to create than recombinant HSV-1 viruses; 2) since the regulatory elements of a tissue specific promoter is located outside of the viral genome, less interference from the viral genomic structure may be expected for better control of the transgene expression; and 3) since the oncolytic replication of the helper virus is solely dependent on the presence of amplicon, adjusting the ratio of helper to amplicon can determine the potency of the viral stock for the treatment. Since typically a helper/amplicon ratio from 3 passages is in a range of 1:3 to 3:1, a final ratio for lytic viral replication (50:1 in the present study) can be easily achieved by diluting the stock with various amounts of pure helper virus. In addition, high helper/amplicon ratios reduce the probability of homologous recombination between the amplicon and helper virus. Although outside the scope of the present investigation, to further eliminate the risk, a hybrid viral system could be used (42). For instance, an HSV-1 virus with a complete deletion in the *ICP4* region may be used to package an adenovirus-associate virus that carries ARR$_2$PB-ICP4. In this system, the homologous region will be completely eliminated.

In conclusion, this study demonstrated that prostate-specific expression of the *ICP4* essential gene by an amplicon virus was capable of complementing the replication of an ICP4^{-} helper virus in the LNCaP cells, achieving selective killing of the prostate cancer cells both *in vitro* and *in vivo*, and that HSV-1 viral replication could be restricted and regulated by incorporating a tissue-specific or tumor-specific element in front of an essential viral gene to achieve oncolytic selectivity.

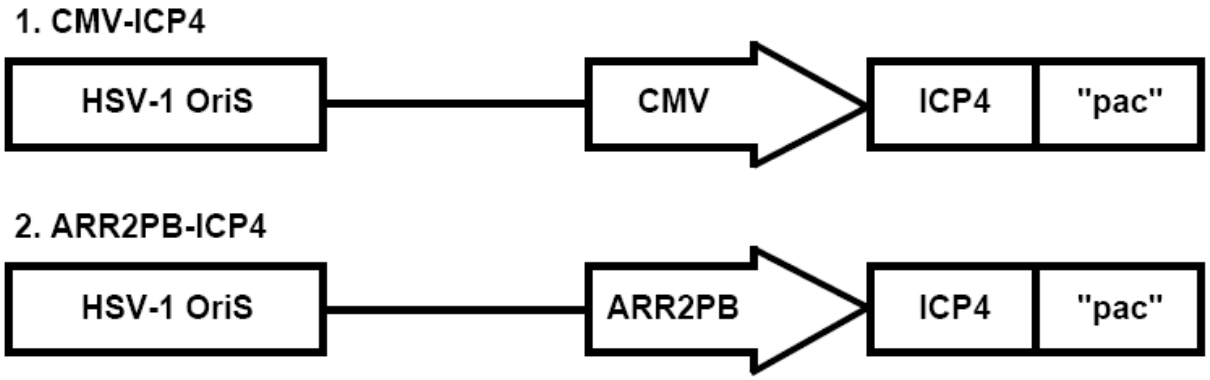
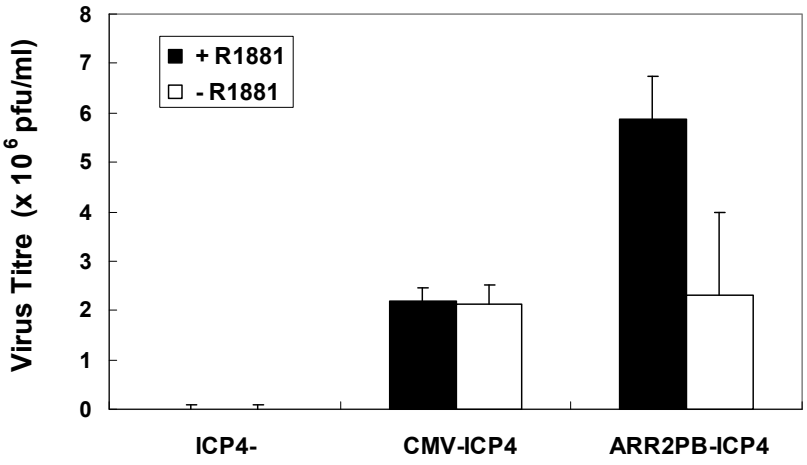


Figure 2.1 Structure of the two amplicon plasmids used in these studies. The CMV-ICP4 amplicon is a constitutive, non-tissue selective complementing amplicon whereas the ARR_2PB-ICP4 amplicon is an androgen-regulated, prostate-specific complementing amplicon.

A



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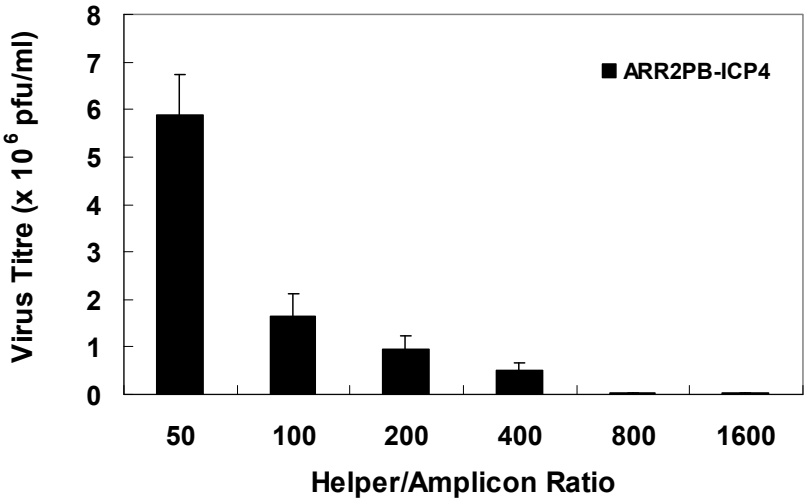
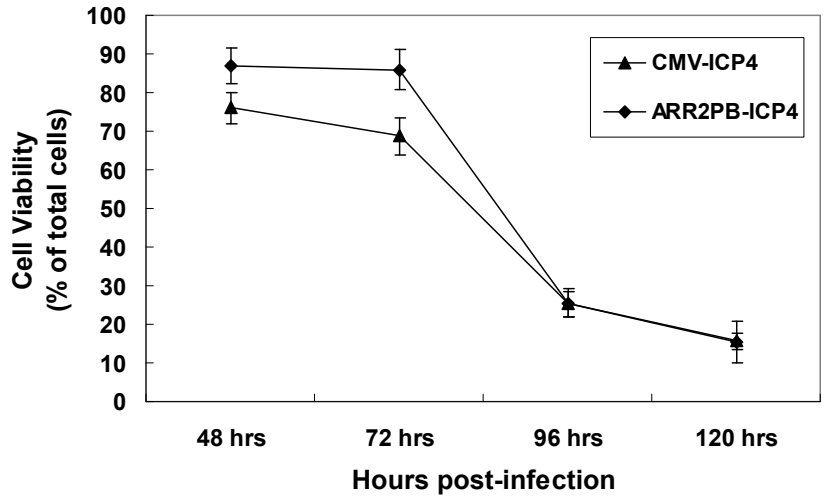


Figure 2.2 Prostate-specific expression of ICP4 complements the replication of the ICP4$^-$ helper virus in LNCaP cancer cells in an androgen-responsive and amplicon-dependent manner. (A) LNCaP cells were infected with ICP4$^-$ helper virus, CMV-ICP4 or ARR$_2$PB-ICP4 amplicon/helper virus with a helper/amplicon ratio of 50:1 at an MOI of 1 and the virus titer was determined at 72 hours post-infection in the presence or absence of 10 nM R1881. (B) LNCaP cells were infected with ARR$_2$PB-ICP4 amplicon/helper virus at different helper/amplicon ratios in the presence of 10 nM R1881, and the virus titer was determined at 72 hours post-infection. The mean plaque forming units/ml ± SD were calculated from four independent determinations.

A



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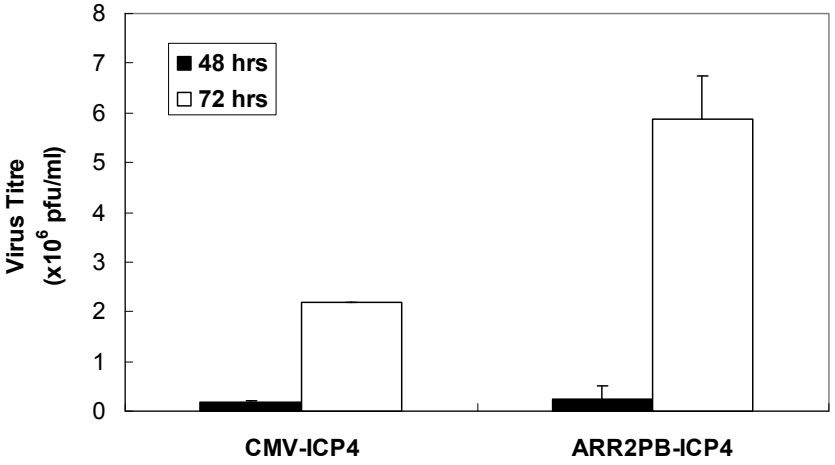
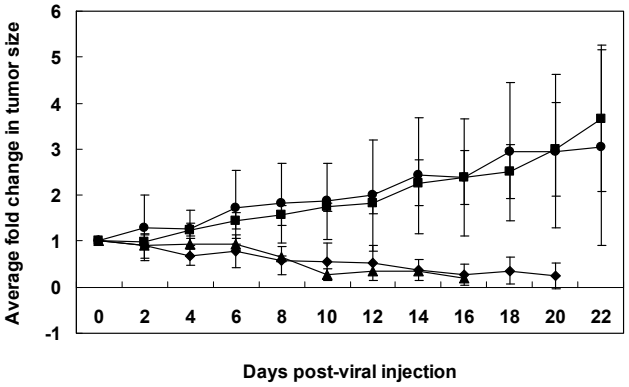


Figure 2.3 CMV-ICP4 and ARR$_2$PB-ICP4 amplicon/helper viruses showed substantial cell killing of the LNCaP tumor cells *in vitro*. (A) LNCaP cells were infected with the CMV-ICP4 and ARR$_2$PB-ICP4 amplicon/helper viruses at a helper/amplicon ratio of 50:1 with an MOI of 1. Results from MTT assays for cell viability, performed at 48, 72, 96, and 120 hours post-infection, are expressed as mean ± SD of six determinations. (B) LNCaP cells were infected with CMV-ICP4 or ARR$_2$PB-ICP4 amplicon/helper virus with a helper/amplicon ratio of 50:1 at an MOI of 1 in the presence of 10 nM R1881. The virus titer was determined at 48 and 72 hours post-infection. The mean plaque forming units/ml ± SD were calculated from four independent determinations.

A



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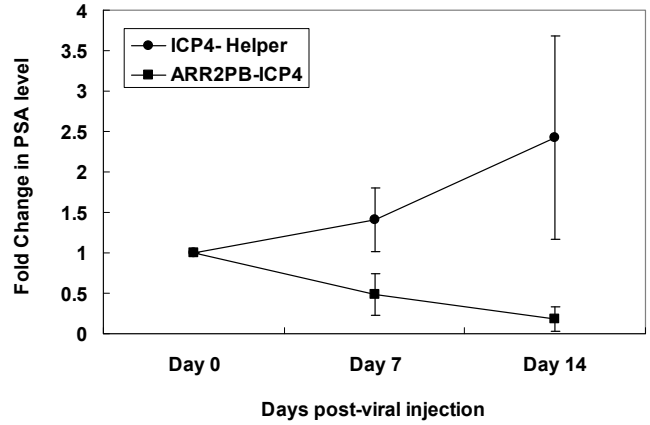

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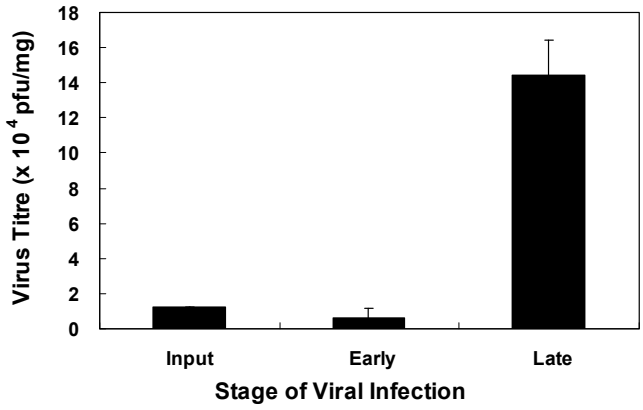

Figure 2.4 Efficient tumor growth suppression by intratumoral injections of amplicons and helper virus using the LNCaP xenograft model. (A) Nude mice with subcutaneous LNCaP tumors were treated with two intratumoral viral injections (Day 0 and Day 6) of medium alone (—●—), ICP4^{-} helper virus alone (—■—), CMV-ICP4

amplicon/helper virus (), or ARR_2PB-ICP4 amplicon/helper virus () (at a helper/amplicon ratio of 50:1) for a total of 2 x 10^6 pfu. The mean fold change in tumor size was determined by comparing the tumor size from each day to the original size at Day 0 and the average was plotted on the graph (n = 3 - 5 for each group). (B) PSA levels (ng/ml) of the mice treated with $ICP4^-$ helper virus and ARR_2PB-ICP4 amplicon/helper virus were determined by tail vein bleeding at Day 0, 7, and 14 and the sera were subjected to ELISA assays for PSA. The results were expressed as the mean of the fold changes in PSA level as compared to Day 0 ($ICP4^-$, n = 3; ARR_2PB-ICP4, n = 5). (C) Virus was extracted from LNCaP tumors treated with 2 x 10^6 pfu of ARR_2PB-ICP4 amplicon/helper virus at Day 2 (Early, n = 4) and Day 5 (Late, n = 3) and the mean titer ± SD was determined by plaque forming assays on 7B cells.

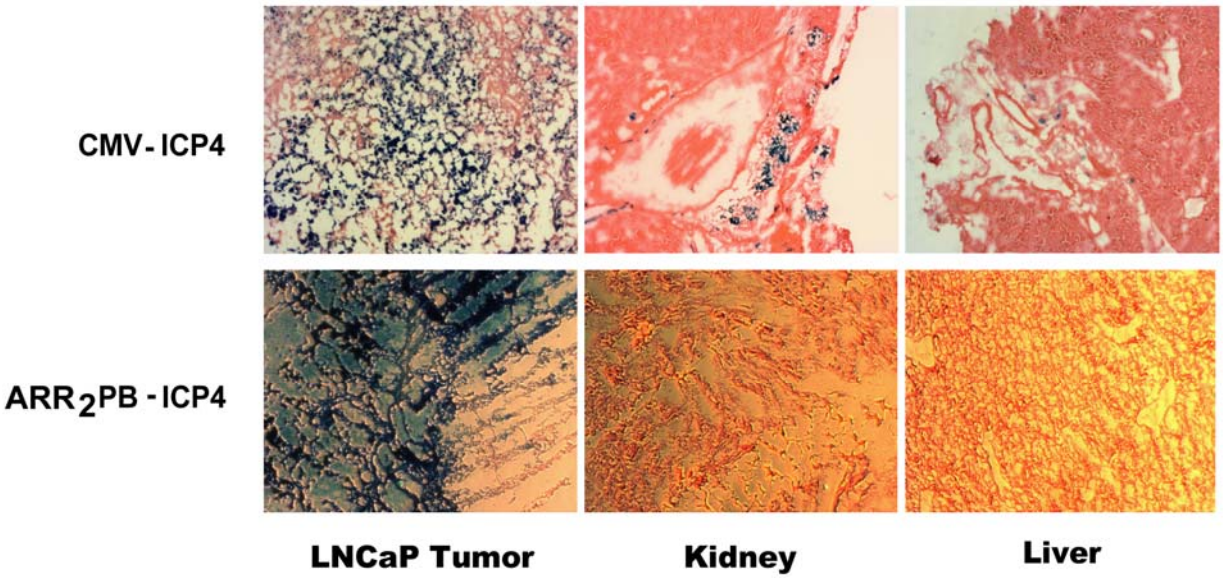

Figure 2.5 Prostate-specific targeting of the ARR_2PB-ICP4 amplicon/helper virus *in vivo*. Organs were collected at the time when mice had to be sacrificed due to morbidities or at day 22 post-viral injection. Histochemical analysis of the 10 μm sections of LNCaP tumor, kidney and liver, collected from mice treated with either CMV-ICP4 or ARR_2PB-ICP4 amplicon/helper virus were analyzed by X-gal staining for the presence of the $ICP4^{-}$ helper virus, which is indicated by the dark blue color.

Table 2.1 Relative copy number of the *ICP0* gene per µg of total cellular DNA as an index for the amount of virus present within normal tissues and LNCaP tumors

Mouse	CMV-ICP4			Mouse	ARR_2PB-ICP4		
	Kidney	Liver	Tumor		Kidney	Liver	Tumor
1	16.63	1.63	8,464	**1**	11.29	0.33	3,702
2	157.09	0.09	6,570	**2**	0.001	0.01	8,878
3	15.10	33.33	ND	**3**	2.23	1.04	2,224
				4	12.17	5.90	2,921
Mean	**62.94**	**11.68**	**7,517**		**6.42**	**1.82**	**4,431**

ND Not determined

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3 MicroRNA regulation of oncolytic herpes simplex virus type 1 for selective killing of prostate cancer cells*

3.1 Introduction

Prostate cancer is the most commonly diagnosed non-skin cancer in men and one of the leading causes of cancer-death (1). While 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 impact 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 type-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 demonstrated 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, ARR_2PB (16). While others have also attempted to incorporate tissue-specific promoters in front of suicide and/or

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essential viral genes to achieve specific killing of cancer cells, nonspecific toxicity to normal tissues, due to leaky promoters has remained a problem (17, 18) and suggests 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 (miRNAs).

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'UTR of the targeted mRNAs and thereby promoting either mRNA cleavage or repression of gene expression at the posttranscriptional 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 downregulated in several cancer cells as compared to 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 downregulated 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 demonstrate tumor-specific targeting of miRNA-regulated oncolytic HSV-1 viruses for killing prostate cancer cells both *in vitro* and *in vivo*.

3.2 Materials and Methods

3.2.1 Plasmid constructs

Five tandem copies of miR-143 complementary sequences (143T) and four copies of miR-145 complementary sequence (145T) were generated by PCR reaction from the template 5'-CTCGAGCGGTTAATTAACGTGAGCTACAGTGCTTCATCTCACGATTGAGCTACAGTGCTTCATCTCAGATCTGAGCTACAGTGCTTCATCTCAGTCATGAGCTACAGTGCTTCATCTCAGTCATGAGCTACAGTGCTTCATCTCAGCTATCGATGCAGTCTAGA-3' and 5'-CTCGAGCGGTTAATTAACGAAGGGATTCCTGGGAAAACTGGACCGATAAGGGATTCCTGGGAAAACTGGACGATCAAGGGATTCCTGGGAAAACTGGACGTCAAAGGGATTCCTGGGAAAACTGGACGCTATCGATGCAGTCTAGA, respectively (Integrated DNA Technologies, Coralville, USA). The primers used to PCR amplify these miRT fragments were 5'-GACGCTCG AGCGGTTAATTAACG-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 (Figure 3.1).

3.2.2 Cell cultures

LNCaP (36), DU145, PC-3, 293T human embryo kidney cells, and Vero cells were obtained from American Type Culture Collection (ATCC; Manasas, VA), and 7B was kindly provided by Dr. William Goins (University of Pittsburgh School of Medicine). All cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and antibiotics containing penicillin and streptomycin. Vero and 7B cells are monkey kidney cells used to package amplicon viruses as previously described (16). LNCaP, DU145 and PC-3 are human prostate cancer cell lines and LNCaP-Luc is a derived stable cell line that expresses luciferase.

3.2.3 Viruses

The replication-deficient ICP4$^-$ helper virus (CgalΔ3) and all of the amplicon viruses were packaged, propagated and tittered in 7B cells as previously described (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 hr after transfection, cells were superinfected with ICP4⁻ helper virus at a multiplicity of infectivity (MOI) of 1 and the virus was collected three days after superinfection. The amplicon viruses were then amplified and propagated by infecting more 7B cells. Since the stock virus produced was a mixture of helper and amplicon viruses, virus titer and the helper/amplicon ratio was determined by tittering the virus both in 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.

3.2.4 Transfection of precursor miRNA and viral infection

LNCaP cells were seeded in 12-well plates at a density of 3 x 10^5 cells/well in 2 ml DMEM medium with 10% FBS and no antibiotics. The next day, cells were co-transfected 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, Texas, USA) at a concentration of 0 nM, 0.1 nM, 1 nM, 5 nM, 10 nM, 20 nM, and 50 nM using Lipofectamine™ 2000. The same cells were then superinfected with ICP4⁻ helper virus at an MOI of 1 after 24 hours co-transfection. At 24 hour post-superinfection, protein samples and total RNA were extracted and subjected to Western blot and one-step real-time RT-PCR analysis. Viral supernatant was also collected at 48 hours post superinfection and titered on 7B cells. To study the effect of miRNA on viral replication, LNCaP cells were transfected with 0 nM or 20 nM of miR-143 or miR-145 and 24 hour later the same cells were infected with CMV-ICP4, CMV-ICP4-143T or CMV-ICP4-145T amplicon virus at an MOI of 0.1 (input virus = 10^4 pfu). At 72 hours post-viral infection, virus was collected and titered in 7B cells.

3.2.5 Western blotting

LNCaP cells were lysed with 2X sample buffer (100 mM Tris-HCL, 4% SDS, 20% glycerol, 357 mM 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% non-fat milk (Bio-Rad Laboratories, Ontario, Canada) at room temperature for 1 hour and then incubated with primary antibodies overnight at 4°C. Next day, the membrane was washed with Tris-Buffered

Saline Tween-20 (TBST), incubated with secondary antibody at room temperature for 1 hour, and then washed with TBST again before imaged using ECL reagent (Perkim Elmer, Boston, MA) and VersaDoc imaging system (Bio-Rad). Primary antibodies were prepared in 5% BSA in TBST with the following dilutions: anti-ICP4 (EastCoast Bio Inc., North Berwick, ME) at 1:800 dilution (37), anti-ICP27 (Virusys Corporation, Sykesville, MD) at 1:800 dilution (37, 38), and anti-β-actin (Cell Signaling, Danvers, MA) at 1:1000 dilution. Anti-mouse and anti-rabbit secondary antibodies were prepared in TBST at a 1:2000 dilution (Cell Signaling). Band density was determined using ImageJ software (NIH).

3.2.6 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 previously described (16). Total RNA was extracted from the cell lines and mouse organs using Trizol (Invitrogen) following manufacturer's protocol. To determine *ICP4* mRNA levels in co-transfection studies, 200 ng RNA was used in one-step real-time RT-PCR using the same primers as previously described (16). All RT-PCR reactions were performed in 25 µL SYBR-green mixture containing the MultiScribe Reverse Transcriptase using the ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). *ICP4* mRNA level was first normalized to *β-actin* mRNA level ($\Delta CT = CT_{ICP4} - CT_{Actin}$) and then compared to the negative control group where LNCaP cell was 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 to negative control where miRNA was undetectable, to get ΔΔCT values. Reverse transcription (RT) and real-time PCR were performed 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. One µL of the RT product 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 sec. and 60°C for 1 min.

3.2.7 LNCaP xenograft mouse model

Athymic nude mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and LNCaP-Luc cells were inoculated subcutaneously by injecting 5 x 10^6 cells in 100 µL of medium with matrigel at two different sites on the flanks. Tumor volumes were determined by caliper measurements and calculated using the formula volume = width x length x thickness x π/6. Once the tumor size reached approximately 100 mm^3, mice were treated with two intratumoral injections (at Day 0 and Day 7) of 1 x 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 CO_2 asphyxiation and several organs (brain, heart, kidneys, liver, lung, spleen, stomach, prostate, bladder, 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 minutes prior to imaging with the IVIS 200 system (Caliper Life Sciences, Ontario, Canada). All experimental procedures were approved by UBC Animal Care Committee and followed the guidelines and policies of Canadian Council on Animal Care.

3.2.8 Histochemical staining

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

3.2.9 Statistical analysis

Statistical significance (p-value <0.05) is determined using student's t-test and data are presented as mean ± SD.

3.3 Results

3.3.1 MiR-143 and miR-145 are downregulated in LNCaP cells, but highly expressed in normal mouse prostates

MiR-143 and miR-145 have been reported to be downregulated 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 downregulated in LNCaP, DU145 and PC-3 cells (Figure 3.2A). 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 (Figure 3.2B). 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).

3.3.2 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 the 3'UTRs (Figure 3.1). 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 co-transfected 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 (Figure 3.1), in a concentration-dependent manner (Figure 3.3A & B). A 50% reduction in ICP4 protein level was observed when CMV-ICP4-143T was co-transfected with 5 nM pre-miR143

and when CMV-ICP4-145T was co-transfected with 10 nM pre-miR145 as compared to the controls ($p<0.05$). In both cases, there was a ~60-70% reduction in ICP4 protein level in the presence of higher miRNA concentrations (20 nM and 50 nM). 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; not a result of reduced viral infectivity. Since 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 (RISC). Indeed, a 62% reduction in *ICP4* mRNA level was observed when CMV-ICP4-143T was co-transfected with 10 nM pre-miR143 (Figure 3.3C; $p < 0.05$) and a 37% reduction in *ICP4* mRNA was observed when CMV-ICP4-145T was co-transfected with 20 nM pre-miR145 (Figure 3.3D; $p < 0.05$). Reciprocally miR-143 had no effect on CMV-ICP4-145T nor did miR-145 on CMV-ICP4-143T; indicating that both microRNAs suppress ICP4 expression in a sequence-specific manner.

3.3.3 MiR-143 and miR-145 inhibit replication of miRNA-regulated amplicon viruses in a dose-dependant manner

To demonstrate that miRNA could control viral replication through the regulation of ICP4 expression, virus titers were measured in LNCaP cells first pre-transfected 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 pre-transfected with 20 nM miR-143 or miR-145 as compared to no miRNA transfection (Figure 3.4A). With the CMV-ICP4-143T amplicon virus, the virus titer was 6-fold and 3-fold less when LNCaP cells were pre-transfected with 20 nM miR-143, as compared to 0 nM or 20 nM of miR-145 ($p < 0.05$). With the CMV-ICP4-145T amplicon virus, the virus titer was 3-fold less when LNCaP cells were pre-transfected with 20 nM miR-145 relative to 0 nM or 20 nM 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 (Figure 3.4B and C). MiR-143 was able to inhibit the complementing ability of CMV-ICP4-143T amplicon, but not CMV-ICP4-145T (Figure 3.4B). The virus titer was 6-fold less when

CMV-ICP4-143T was co-transfected with 20 nM and 50 nM of pre-miR143 relative to 0.1 nM of this miRNA. Pre-miR-143 showed no effect on the CMV-ICP4-145T amplicon's complementing ability. Similarly, a 4-fold reduction in virus titer was observed when CMV-ICP4-145T was co-transfected with 20 nM and 50 nM of pre-miR145 as compared to the titer at 0.1 nM of this miRNA. Pre-miR-145 had no inhibitory effect on CMV-ICP4-143T amplicon (Figure 3.4C). 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-miR143, 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 hr post infection, whereas 70% of the parental LNCaP cells were dead (data not shown). These results demonstrate 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.

3.3.4 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 (~120 ± 14 mm^3), which are a LNCaP-derived cell line expressing luciferase. The animals were treated with intratumoral injections of 2 x 10^6 pfu amplicon viruses. Tumor volume was determined by calliper 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-oncolytic $ICP4^-$ 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 (Figure 3.5A). 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 and CMV-ICP4-145T treated animals, although some gastritis developed 28 days after the viral treatment. Furthermore, tumors were almost completely eradicated in three out of the seven mice treated with CMV-ICP4-143T and two out 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 (Figure 3.5B). These results showed that the miRNA-regulated amplicon viruses were able to selectively kill LNCaP tumor cells.

3.3.5 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 (Figure 3.6A). 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 (Figure 3.6B). The extent of amplicon virus infection is indicated by the relative copy number of *ICP4* (per μg 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 >3300-fold less in mice treated with CMV-ICP4-143T than in mice treated with CMV-ICP4 amplicon virus.

3.4 Discussion

The microRNAs, miR-143 and miR-145, have been reported to be downregulated in various cancer types (27-35). This is consistent with our findings in the present study (Figure 3.2). Based on these findings, we cloned multiple tandem copies of their complementary target sequences into the 3'UTR region of the viral essential gene, *ICP4* (Figure 3.1). 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 4 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 (Figure 3.3).

MiR-143 and miR-145 inhibited the complementing ability of the amplicon virus and viral replication in a sequence-specific and dose-dependent manner (Figure 3.4). Interestingly, the virus titers of CMV-ICP4 amplicon virus, which did not carry any 3' miRNA target sequences were also slightly lower in cells pre-treated with 20 nM 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 downregulated 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 downregulated 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 demonstrated 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 and miR-145-regulated oncolytic viruses may be particularly useful in a wide range of cancers since they are generally downregulated 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 since the whole virus contains only two copies of *ICP4* gene, whereas owing to the fact that approximately 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*. Since 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 (Figure 3.6).

MiRNA regulated oncolytic viral replication has also been shown recently using a RNA virus, a let-7-sensitive vesicular stomatitis virus (VSV), $VSV^{let\text{-}7wt}$ (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:RISC 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 region since 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 regions, 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.

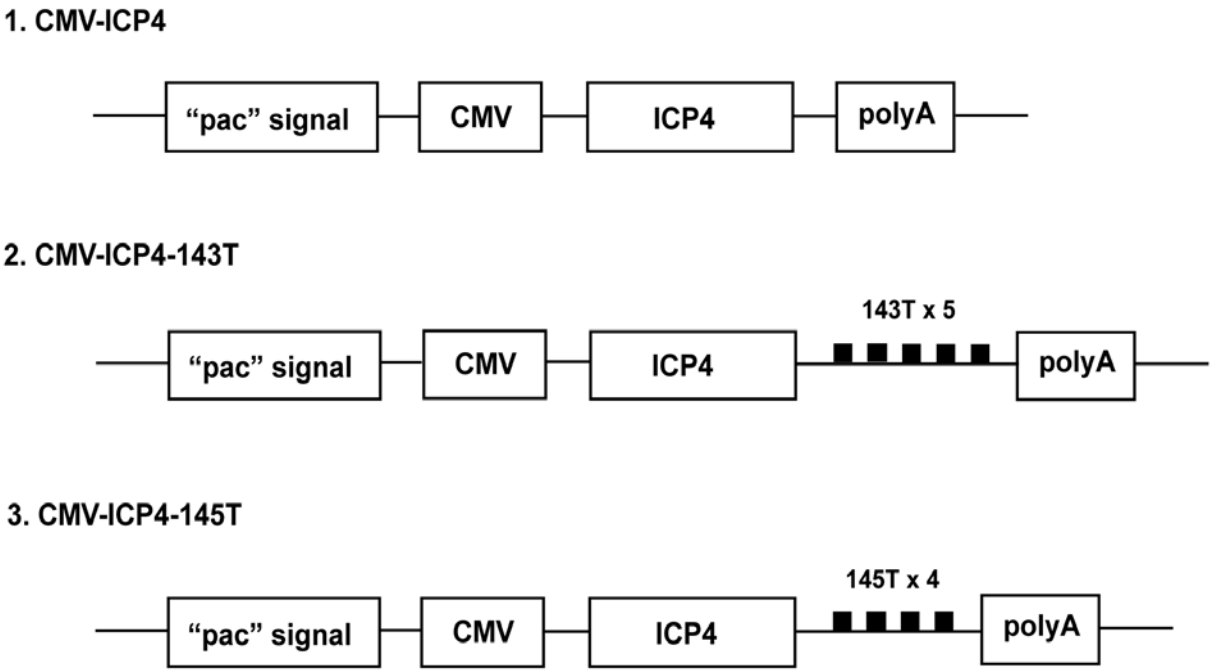

Figure 3.1 Structure of the three amplicon plasmids used to package replication-competent amplicon viruses. CMV-ICP4 amplicon was a constitutive, non-tissue selective complementing amplicon, whereas CMV-ICP4-143T and CMV-ICP4-145T amplicons were tumor-specific miRNA-regulated complementing amplicons. 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).

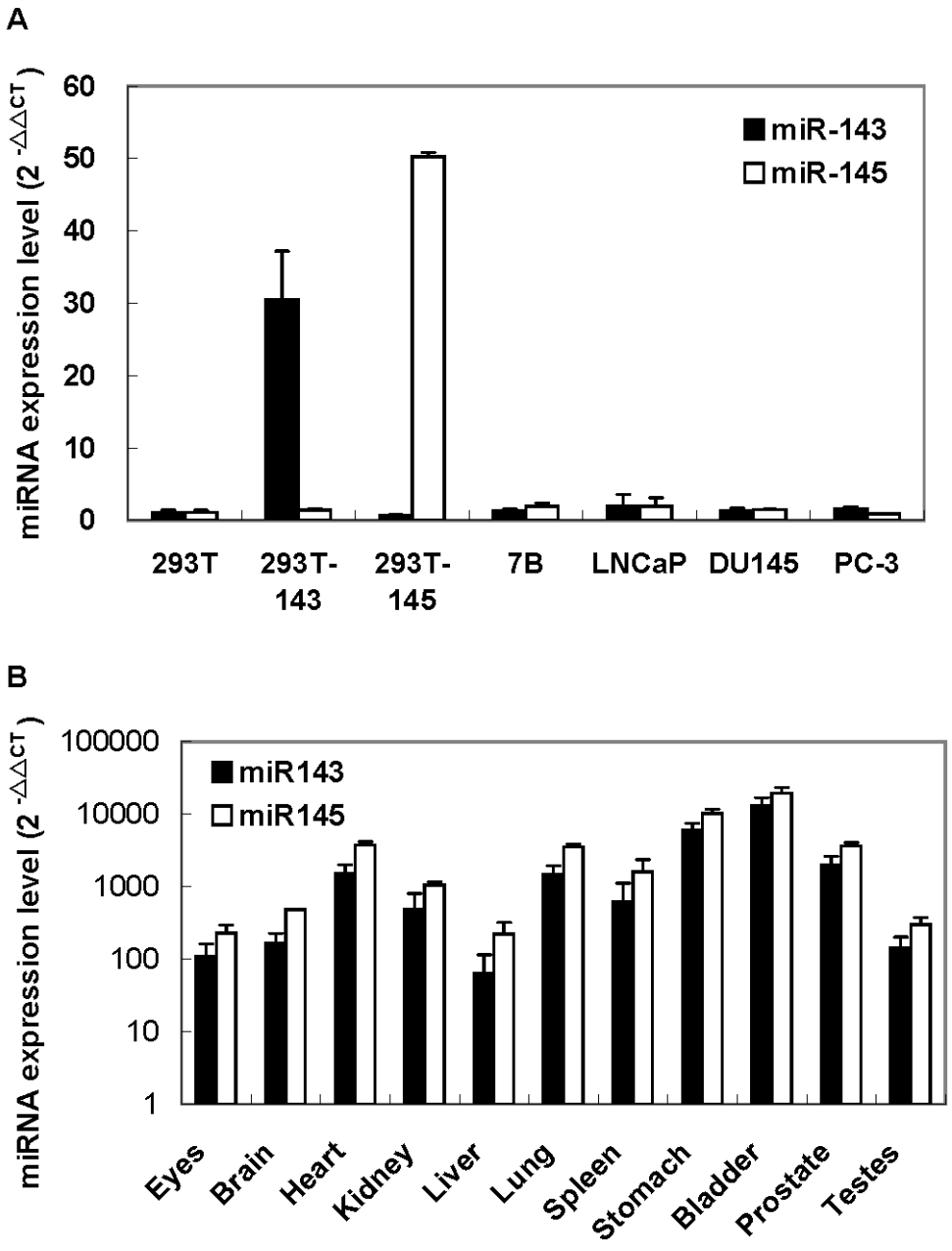

Figure 3.2 MiR-143 and miR-145 expression levels in cell lines and mouse tissues. The expression levels of miR-143 and miR-145 in various cell lines (A) and a panel of organs (B) harvested from nude mice were determined by quantitative RT-PCR assays. 293T-143 and 293T-145 are 293T cells transiently transfected with 20 nM pre-miR143 and pre-miR145 respectively as positive controls. Total RNA from the cells and organs were extracted using the Trizol reagent and 10 ng of RNA was reverse transcribed to cDNA followed by TaqMan real-time PCR assays.

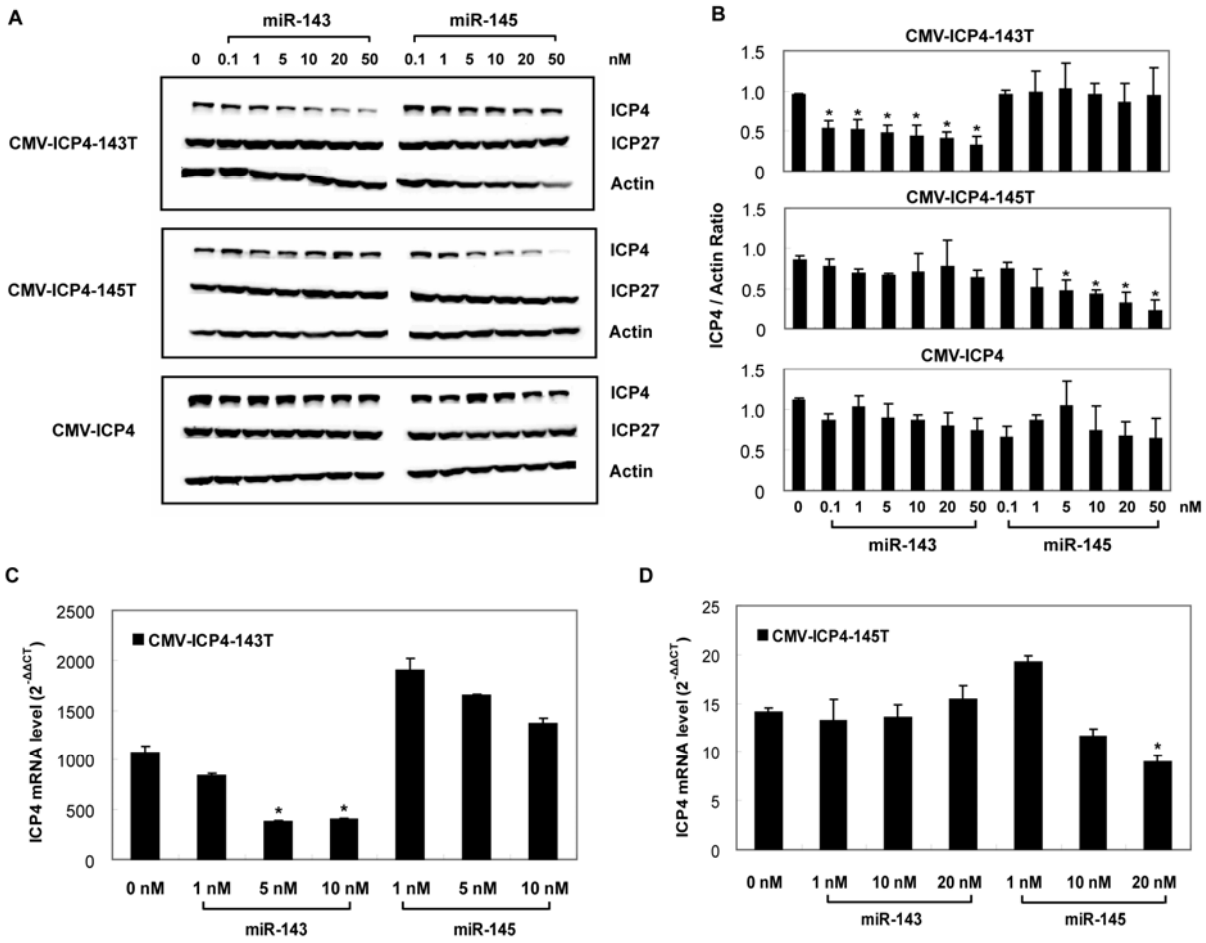

Figure 3.3 MiR-143 and miR-145 inhibit ICP4 expression at a translational level by targeting the 3'UTR in the amplicons. LNCaP cells were co-transfected with amplicon plasmid (CMV-ICP4, CMV-ICP4-143T, or CMV-ICP4-145T) and pre-miR143 or pre-miR145 at a concentration of 0 to 50 nM. 24 hours after transfection, LNCaP cells were superinfected with ICP4^{-} helper virus at an MOI of 1. Total protein and RNA were extracted at 24 hours 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 (means ± SD, n = 3; * indicates $p<0.05$ compared to 0 nM control).

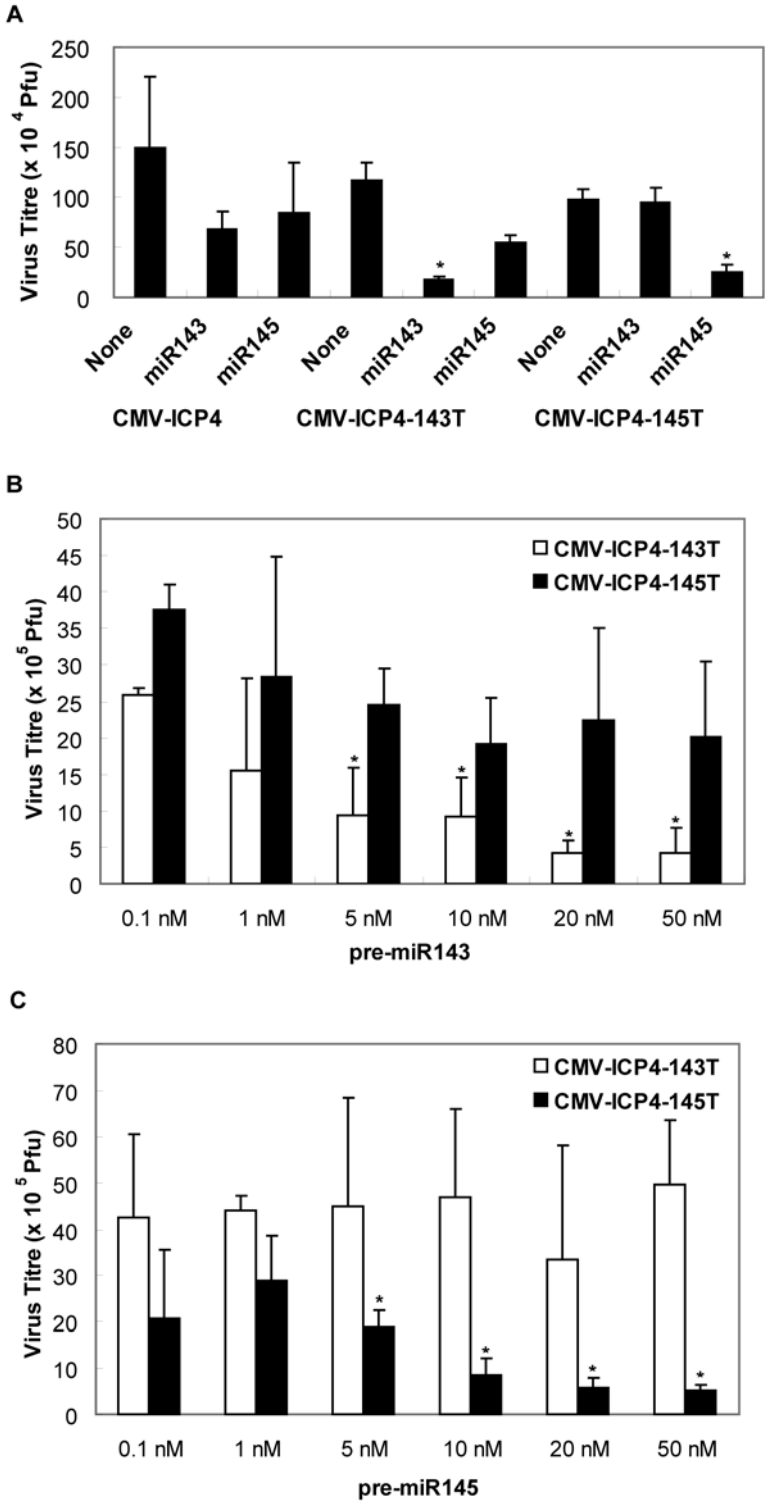

Figure 3.4 MiR-143 and miR-145 suppress amplicon viral replication in LNCaP cells. (A) MiR-143 and miR-145 inhibited viral replication of the CMV-ICP4-143T and CMV-ICP4-145T amplicon viruses, respectively. LNCaP cells were transfected with miR-143 or miR-145 at a concentration of 0 nM or 20 nM. At 24 hours after transfection,

cells were infected with CMV-ICP4, CMV-ICP4-143T or CMV-ICP4-145T amplicon virus at an MOI of 0.1 (input virus = 10^4 plaque forming units (pfu). At 72 hours post-viral infection, virus was collected and titered in 7B cells. The mean pfu ± SD were calculated from four independent determinations (* denotes $p<0.05$ relative to 0 nM 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 co-transfected with amplicon plasmid (CMV-ICP4, CMV-ICP4-143T, or CMV-ICP4-145T) and pre-miR143 or pre-miR145 at a concentration of 0.1 to 50 nM. 24 hours after co-transfection, LNCaP cells were superinfected with $ICP4^{-}$ helper virus at an MOI of 1. At 48 hours post-superinfection, virus was collected and titered on 7B cells.

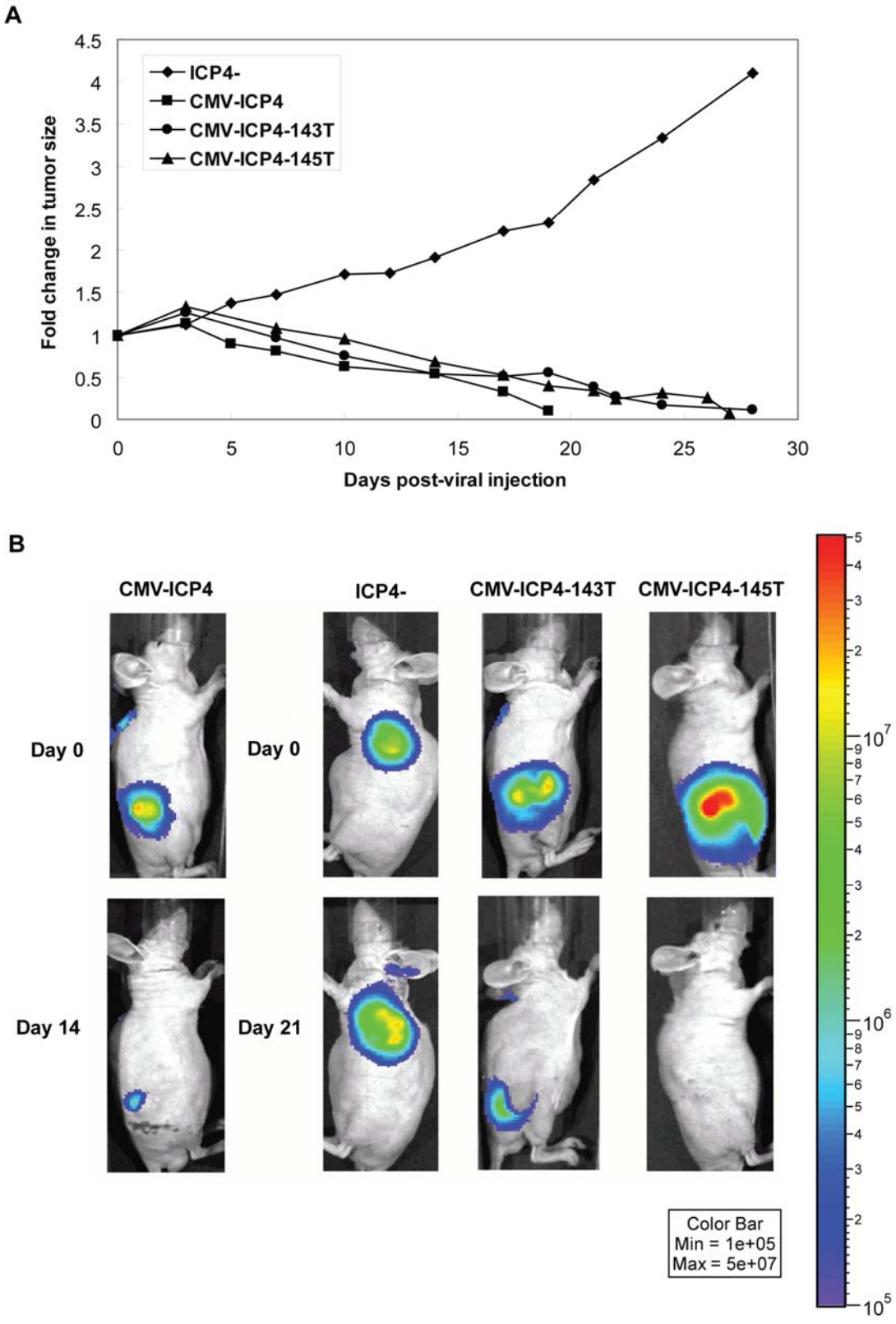

Figure 3.5 MiRNA-regulated amplicon viruses suppress prostate tumor growth in a human xenograft mouse model. (A) Intratumoral injections of miRNA-regulated amplicon viruses inhibited tumor growth in LNCaP xenograft mouse model. Nude mice

with subcutaneous LNCaP-Luc tumors (~120 ± 14 mm^3) were treated with two intratumoral injections (at Day 0 and Day 7) of 1 x 10^6 pfu $ICP4^-$ helper virus only (♦, n = 5), CMV-ICP4 amplicon virus (■, n = 3), CMV-ICP4-143T 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/sec/cm^2/sr.

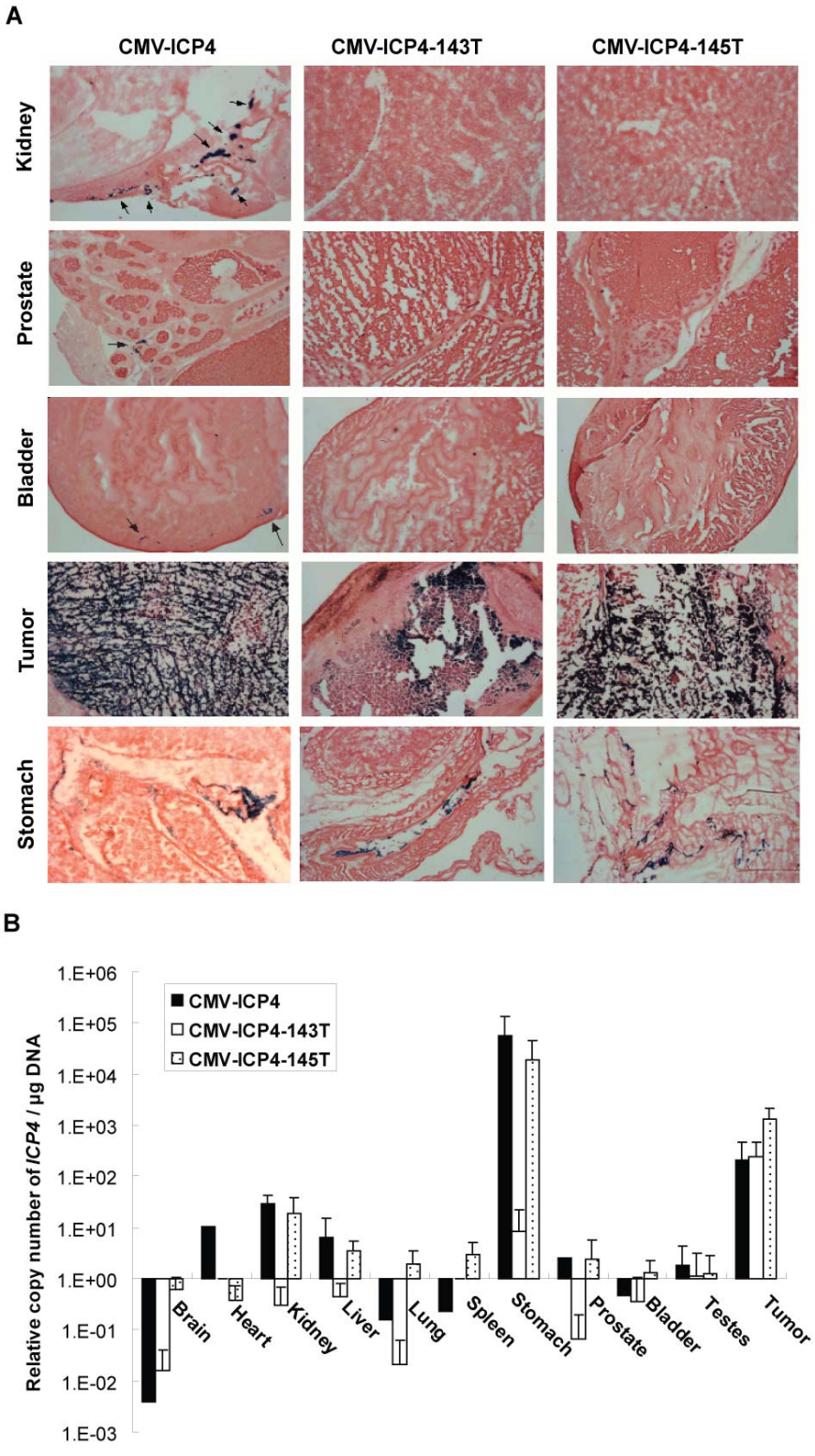

Figure 3.6 MiRNA-regulated amplicon viruses specifically target tumor cells *in vivo*. Various organs were harvested from the mice treated with amplicon viruses at the end of the experiment. (A) Organs were embedded in OCT and cut into 10 µm sections

which were then subjected to x-gal staining. Blue color indicated the 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.

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4 Local and systemic delivery of oncolytic herpes simplex virus type-1 for targeting prostate tumors*

4.1 Introduction

Prostate cancer remains the most commonly diagnosed non-skin cancer in men and the second leading cause of cancer death in men (1). Early stage, locally confined prostate cancer is frequently curable by radical prostatectomy or radiation therapy; however, no curative treatment option is available for patients with locally advanced or metastatic disease (2, 3). While hormone withdrawal therapy is the only effective treatment for advanced/metastatic prostate cancer, the disease will eventually recur and become castration-resistant (hormone refractory). Although promising results have been reported from recent clinical trials using docetaxel-based combination therapies for treatment of hormone-refractory prostate cancer, the median survival is still only ~18 months (4, 5). Hence, more effective, new treatment regimens are urgently needed to improve patient survival.

Oncolytic viruses are promising therapeutic agents for treatment of various cancers since they have the potential to specifically replicate in and lyse tumor cells while sparing normal tissues (6). There are several features of herpes simplex virus type 1 (HSV-1) that make it a favorable oncolytic vector for cancer treatment (7). Clinical trials of malignant glioma using G207, a mutant oncolytic HSV-1 virus with deletions of both copies of the *ICP34.5* gene and an inactivating insertion of the *LacZ* gene into the *ICP6* locus, showed no maximum tolerated dose while multiple deliveries seemed to be safe (8, 9). However, further improvement of antitumor efficacy observed anecdotally in some virus treated patients is required. Current oncolytic HSV-1 viral vectors in clinical trials contain deletions of genes that are involved in neurovirulence, which may result in a much more attenuated virus with far less viral replication efficiency. Hence, it may be better to maintain as much of the viral genome as possible by modifying only the regulator elements flanking essential viral genes to achieve tumor-specific replication.

Replication of the HSV-1 virus relies on cellular transcriptional and translational machinery. Previous studies by our group and others have shown that, by replacing the

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promoter of an essential viral gene with a tissue-specific promoter, viral replication can be regulated in a tissue-specific fashion (10-12). In searching for more stringent regulatory elements to increase tumor specificity, we hypothesize that HSV-1 replication can be further regulated at the translational level by employing the 5'-untranslated regions (5'UTR) that form complex secondary structures (12, 13). The 5'UTR from genes that are commonly associated with malignant progression and metastasis, such as *c-myc*, *ODC*, *cyclin D1*, *VEGF*, *FGF-2* and some matrix metalloproteases, are rich in GC residues and can form extensive secondary hairpin structures to repress the translation of their downstream mRNAs in normal cells (13-15). These secondary hairpin structures can be unwound in the presence of sufficient eIF4E/eIF4F complexes, leading to translation initiation of the mRNA (16). The eIF4E protein, part of the eIF4F complex, plays a crucial role in the initiation of translation process by binding to the 5'cap (7-methylguanosine) of mRNA transcripts. Its overexpression has been reported in various cancer types, including prostate cancer, and can lead to increased translation of cancer-associated genes to enhance cell proliferation, suppress apoptosis, and induce malignant transformation (16-20). Recent data also suggest that elevated eIF4E activation is common in advanced prostate cancer and is associated with progression to a hormone-refractory state resulting in reduced patient survival (20, 21). These observations point towards a new tumor-specific targeting strategy that could be incorporated into current viral vectors. Indeed, our group and others have shown that when the 5'UTR from the *rFGF-2* gene is inserted in front of a suicide gene (HSV-1 thymidine kinase) in a lentiviral vector or an adenoviral vector, the expression of thymidine kinase gene was significantly enriched in tumor cells only, leading to increased tumor-specific killing in combination with ganciclovir (GCV) treatment (20, 22).

In the present study, our aim was to demonstrate that through dual regulation of an essential viral gene at both the transcriptional and translational level by the prostate-specific promoter ARR_2PB and the *rFGF-2* 5'UTR, respectively, a recombinant HSV-1 virus would show high selectivity for tumor cells, resulting in tumor-specific oncolysis. To test our hypothesis, we generated two recombinant viruses, ARR_2PB-ICP27 (A27) and ARR_2PB-5'UTR-ICP27 (AU27), by inserting the ARR_2PB promoter in front of the essential *ICP27* gene with or without the 5'UTR from *rFGF-2* and tested their efficacy and toxicity both *in vitro* and *in vivo*. Our results showed that dual

regulation of HSV-1 viral replication significantly enhances tumor specificity while retaining high efficiency of tumor lysis.

4.2 Materials and Methods

4.2.1 Construction of recombinant virus

The ARR_2PB-ICP27 (A27) and ARR_2PB-5'UTR-ICP27 (AU27) recombinant viruses were generated by the recombineering method (23), which replaced the endogenous *ICP27* promoter with the ARR_2PB promoter ± the 5'UTR (619 bp) of rat *FGF-2* gene (24) in BAC.p25 (Figure 4.1). The Bacterial Artificial Chromosome (BAC) was inserted into the thymidine kinase (*TK*) locus by homologous recombination to generate BAC.p25 containing the whole HSV-1 genome (25). This recombineering technique generated two BACs: BAC.AR27 and BAC.ARU27. In order to restore TK activity and remove the BAC from the viral genome, the *TK* gene was then rescued by homologous recombination in 2-2 cells with Hypoxanthine Aminopterine Thymidine (HAT) selection (26). The packaged A27 and AU27 viruses were then plaque-purified three times in 2-2 cells to ensure successful TK rescue. The presence of the *TK* gene was then confirmed by PCR and western blotting for TK expression. The recombinant viruses were then propagated in 2-2 cells. The wild-type virus (KOS strain) was propagated in Vero cells and the replication-defective virus $ICP27^{-}$ was grown in 2-2 cells. Virus titer was determined by plaque forming assay as previously described (11).

4.2.2 Cell culture

RWPE-1 (27), LNCaP (28), DU145 (29), PC-3 (30), H460 (31), MCF-7 (32) and Vero cells were obtained from American Type Culture Collection (ATCC; Manasas, VA). BPH-1 (33) and PNT1B (34) cells were gifts from Dr. Simon Hayward (Vanderbilt University) and Dr. Norman J. Maitland (York, UK), respectively. The 2-2 cell line was kindly provided by Dr. Priscilla A. Schaffer (University of Pennsylvania School of Medicine). All cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics containing penicillin and streptomycin except for RWPE-1 cell line, which was maintained in Keratinocyte Serum Free Medium (K-SFM) with 0.05 mg/ml bovine pituitary extract and 5 ng/ml human recombinant epidermal growth factor (Invitrogen). PNT1B and LNCaP cells are

human normal and tumor prostate cancer cell lines, respectively. They are positive for androgen receptor and are both androgen-responsive. The 2-2 cell line (transfected with *ICP27* gene) is a derivative cell line generated from Vero cells. For the androgen-responsive experiments, PNT1B and LNCaP cells were maintained in DMEM with 10% charcoal stripped serum (CSS; HyClone, VWR, West Chester, PA) for two days before infection and then 10 nM R1881 (Perkin Elmer, Boston, MA) was added to the medium 1 hour after viral infection.

4.2.3 Western blotting

PNT1B and LNCaP cells were infected with wild-type, A27 and AU27 viruses at a multiplicity of infectivity (MOI) of 3, and total proteins were collected at 8 and 12 hours post-infection. PNT1B and LNCaP cells were lysed with 2X sample buffer (100 mM Tris-HCL, 4% SDS, 20% glycerol, 357 mM β-mercaptoethanol, 0.04% bromophenol blue) and boiled for 5 minutes. Protein samples were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was blocked in 5% non-fat milk (Bio-Rad Laboratories, Ontario, Canada) at room temperature for 1 hour and then incubated with primary antibodies overnight at 4°C. On the next day, the membrane was washed with Tris-Buffered Saline Tween-20 (TBST; 20 mM Tris, 500 mM NaCl, 0.1% Tween-20), incubated with secondary antibody at room temperature for 1 hour, and then washed with TBST again before being imaged using the ECL reagent (Perkin Elmer, Boston, MA) and the VersaDoc imaging system (Bio-Rad). Primary antibodies were prepared in 5% BSA in TBST at the following dilutions: anti-ICP4 (EastCoast Bio Inc., North Berwick, ME) at 1:800 (35), anti-ICP27 (Virusys Corporation, Sykesville, MD) at 1:800 (35, 36), anti-TK (a gift from Dr. William C. Summers, Yale University) at 1:500, anti-eIF4E (BD Transduction Laboratories, Mississauga, ON) at 1:1000, and anti-β-actin (Cell Signaling, Danvers, MA) at 1:1000. Anti-mouse and anti-rabbit secondary antibodies were prepared in TBST at a 1:2000 dilution (Cell Signaling). Band intensities were quantified using ImageJ software (NIH).

4.2.4 DNA, RNA extraction and RT-PCR

Total RNA was extracted from cell lines and mouse organs using Trizol (Invitrogen) following the manufacturer's protocol. To determine *ICP27* and *β-actin* mRNA levels in co-transfection studies, 200 ng RNA was used in one-step real-time RT-PCR as

previously described (11). All RT-PCR reactions were performed in 25 µL SYBR-green mixture containing the MultiScribe Reverse Transcriptase, using the ABI prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The *ICP27* mRNA levels were first normalized to the *β-actin* mRNA level ($\Delta CT = CT_{ICP4} - CT_{Actin}$) and then compared to the negative control group where LNCaP cell was infected with ICP27¯ virus only ($\Delta\Delta CT = \Delta CT - \Delta CT_{ICP27-}$). The results were expressed as $2^{-\Delta\Delta CT}$. DNA was extracted from the organs of the mice treated with viruses using the phenol-chloroform and *ICP27* copy number was determined by quantitative real-time PCR using the *ICP27* primers (5'-GTCTGGCGGACATTAAGGACA-3' and 5'-TGGCCAGAATGACAAACACG-3').

4.2.5 Virus growth assays

PNT1B and LNCaP cells were infected with viruses at an MOI of 0.01 with or without 10 nM R1881 treatment. Total virus was collected at 24 hr, 48 hr and 72 hr post-infection, and virus titer was determined by the plaque-forming assay in 2-2 cells.

4.2.6 LNCaP xenograft mouse model

Athymic nude mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and were inoculated with LNCaP cells subcutaneously by injecting 5 x 10^6 cells in 100 µL of medium with matrigel at two different flank sites. Tumor volumes were determined by caliper measurements and calculated using the formula volume = width x length x thickness x π/6 (11). Once the tumor size reached approximately 100 mm^3, mice were treated with a single intratumoral injection of 2 x 10^6 pfu of wild-type, ICP27¯, A27, or AU27 virus at Day 0. For dose response experiment, mice were treated with one single intravenous injection of 5 x 10^7 pfu of ICP27¯, AU27 or 5 x 10^5 pfu of AU27 virus at Day 0. At the end of the experiment, mice were sacrificed using CO_2 asphyxiation, and organs (eyes, brain, heart, kidneys, liver, lung, spleen, stomach, intestines, prostate, bladder, testes) and the tumors were removed and analyzed by real-time PCR. All experimental procedures were approved by UBC Animal Care Committee and followed the guidelines and policies of the Canadian Council on Animal Care.

4.2.7 Statistical analysis

Statistical significance (p-value <0.05) was determined using Student's t-test and the

results are presented as mean ± SD. Kaplan-Meier survival curves were created using GraphPad Prism 5 software and the log-rank (Mantel-Cox) test was used to determine statistical significance.

4.3 Results

4.3.1 eIF4E is overexpressed in prostate cancer cells

As we previously reported (20), the eukaryotic initiation factor (eIF)4E expression level was higher in cancer cells than in non-cancerous cells with the highest expression levels observed in LNCaP prostate and MCF-7 breast cancer cell lines (Figure 4.2). When compared to that in LNCaP cells, a 6-fold and 4-fold reduction in eIF4E levels was observed in BPH-1 and PNT1B cells, respectively ($p<0.002$). While both BPH-1 and PNT1B cells exhibited low eIF4E expression, non-malignant PNT1B cells were chosen as a control cell line to study the transcriptional and translational regulation of ICP27 expression since PNT1B cells express the androgen receptor (AR), which is necessary for ARR_2PB promoter activity.

4.3.2 ICP27 expression is regulated at both transcriptional and translational level by the ARR_2PB promoter and the rFGF-2 5'UTR respectively

a) Transcriptional regulation. We have previously demonstrated prostate-specific expression of another essential viral gene, *ICP4*, in an amplicon/helper system using the highly androgen/AR-responsive ARR_2PB promoter (11). To test the specificity of the ARR_2PB promoter and its regulation of the *ICP27* gene in the context of a whole virus, we first examined *ICP27* mRNA levels by quantitative RT-PCR analysis in LNCaP cells infected with A27 and AU27 viruses in the presence of 0 nM or 10 nM R1881. Consistent with our previous findings (11), ICP27 mRNA levels were >2-fold higher for both A27 and AU27 infections in the presence of 10 nM R1881 when compared with no androgen treatment at 4 hr, 8 hr and 24 hr post-infection (Figure 4.3, A; $p<0.03$). Therefore, these findings support transcriptional regulation of *ICP27* gene expression within the HSV-1 viral genome by the ARR_2PB promoter in an androgen/AR-dependent fashion.

b) Translational regulation. We next determined ICP27 protein expression level in both normal PNT1B and LNCaP cancer cells infected with A27 and AU27 viruses by

Western blot analysis. For A27 virus infections, addition of 10 nM R1881 resulted in a >80% increase in the ICP27 protein in both cell lines at 8 hr post-infection (Figure 4.3, B & C; $p<0.04$). Similar results were also observed for AU27 virus infections in LNCaP cells, with a ~4-fold increase in ICP27 protein level in the presence of 10 nM R1881 at 8 hr post-infection (Figure 4.3, B). However, A27 and AU27 behave quite differently in PNT1B cells. At the same MOI, the ICP27 protein level was >60% higher after A27 infection than after AU27 infection at 8 hr post-infection (Figure 4.3, C). The expression of another HSV-1 immediate-early gene, *ICP4* with its endogenous promoter, remained unchanged in both cells lines. Clearly, the expression of ICP4 was neither dependent on eIF4E level nor AR.

To verify that reduced ICP27 expression from AU27 viral infected PNT1B cells was not due to a reduction in transcriptional activity, quantitative RT-PCR analysis of *ICP27* mRNA levels was performed in PNT1B and LNCaP cells infected with wild-type, A27, or AU27 viruses. Surprisingly, levels of *ICP27* mRNA were consistently higher in PNT1B cells than in LNCaP cells (Figure 4.3, D). Thus, the reduction in ICP27 protein levels in PNT1B cells is consistent with down-regulation at the translational level. Together these results demonstrate that viral *ICP27* gene expression from the AU27 virus can be transcriptionally and translationally regulated by the ARR_2PB promoter and the 5'UTR element, respectively. Furthermore, while the ARR_2PB promoter was able to direct the expression of the *ICP27* gene in an androgen/AR-responsive fashion in the presence of a complex 5'UTR, this transcriptional upregulation by androgen could not be translated into increased protein production in cells with low levels of eIF4E.

4.3.3 Replication of AU27 virus is androgen/AR-regulated and tumor-specific

To demonstrate that the AU27 virus can differentially replicate in normal and tumor cells, we compared viral growth in PNT1B and LNCaP cells. Wild-type (KOS) virus was able to infect and replicate in both LNCaP and PNT1B cells with similar efficiency when infected at an MOI of 0.01 (Figure 4.4, A). Despite a slight delay in viral growth in PNT1B cells for the first 24 hours, there was no significant difference in viral titers between PNT1B and LNCaP cells by 48 hours post-infection. Hence, both cells have similar infectivity and viral replicability for wild-type HSV-1 virus.

The A27 viral titer was approximately 2-fold higher in the presence of 10 nM R1881 when compared to the absence of R1881 in both PNT1B and LNCaP cells, which is

consistent with androgen regulation of ICP27 expression from this virus. Interestingly, for the AU27 virus, adding 10 nM R1881 caused an approximately 2-fold increase in virus titer in LNCaP cells, but not in PNT1B cells (Figure 4.4, B & C; $p<0.05$). Thus, the replication of both viruses was androgen/AR-responsive in cancer cells, but this androgen-driven transcriptional up-regulation in PNT1B cells was overridden by translational suppression by the complex 5'UTR in the AU27 virus in cells with low eIF4E levels, resulting in a 700-fold lower viral titer in PNT1B cells than in LNCaP cells. The 5-10 fold difference in the ICP27 mRNA levels in LNCaP cells infected with A27 and AU27 viruses observed between Figure 4.3A and Figure 4.3D reflects the variations within qRT-PCR reactions carried out at different times. Although the magnitude is different, the experimental trend is the same. Regardless, this demonstrates that the AU27 virus can differentially replicate in LNCaP cells due to differences in endogenous eIF4E level while sparing normal cells with low eIF4E levels. The therapeutic index of this targeted virotherapy is therefore significantly increased.

4.3.4 Intratumoral injection of AU27 virus reduces tumor size and prolongs survival

To study the efficacy and toxicity of the A27 and AU27 viruses, nude mice with established LNCaP xenograft tumors (>100 mm^3) were treated with one intratumoral injection of 2 x 10^6 pfu of ICP27$^-$, wild-type, A27, or AU27 viruses. In comparison to day 0 post-viral injection, a >2.5 fold increase in tumor size was observed in mice treated with ICP27$^-$ virus at day 31 post-viral injection, while a >90% reduction of tumor volumes was evident in mice treated with wild-type, A27 or AU27 virus (Figure 4.5, A). In many cases, the tumors became barely palpable and complete eradication of the tumors was observed in three out of six mice treated with A27 virus and in four out of eight mice treated with AU27 virus. The treated mice were monitored for two months after initial viral injection. Approximately 60% of the mice treated with ICP27$^-$ were sacrificed by day 50 post-treatment due to aggressive tumor growth. By comparison, 80% of the mice treated with wild-type virus exhibited signs of toxicity in the gastrointestinal tract and were sacrificed by day 31 post-viral injection, whereas 83% of the mice treated with A27 virus survived 31 days of treatment period (Figure 4.5, B). However, only 33% survived for two months due to similar virulence in the gastrointestinal tract. On the other hand, 88% of the mice treated with AU27 virus exhibited no signs of morbidity and survived for

two months, at which time the experiments were terminated. Only one out of eight AU27 treated mice showed toxicity in the intestine at day 28 post-viral injection and was sacrificed early. Thus, the AU27 virus was effective in tumor eradication with remarkably reduced toxicity when compared to wild-type and A27 viruses, resulting in a significantly improved survival ($p<0.01$).

To examine the extent of viral biodistribution, *ICP27* gene copy number (per μg of DNA) in various organs was determined by quantitative real-time PCR analysis. The majority of the virus was detected in the tumors of all treatment groups (Figure 4.5, C). In mice treated with wild-type virus, a significant amount of virus was detected in the eyes and small traces were also found in the brain, kidneys and stomach. Similar results were observed in mice treated with A27 virus with detectable virus in the eyes (in 1 of 5 mice) and brain (in 2 of 5 mice). By comparison, no virus was detected in the normal tissues of mice treated with AU27 virus, except that a trace of virus was detected in the stomach and intestine of one mouse that showed virus-related toxicity at day 28 post-viral injection. Interestingly, when the AU27 virus was injected into only one tumor on one flank, the virus was also detectable in the tumor on the other flank in two cases. This suggests that the virus could leak into the blood stream and still specifically target distant tumor cells.

4.3.5 Systemic administration of AU27 virus resulted in tumor-specific targeting and killing

Since AU27 virus treatment showed maximal reduction in tumor size with minimal toxicity to normal tissues through intratumoral injections, we next tested its efficacy and dose-dependency through systemic administration. Nude mice with LNCaP tumors were treated with one intravenous injection of low (5×10^5 pfu) or high (5×10^7 pfu) doses of AU27 virus. In the control group, 5×10^7 pfu replication-defective ICP27$^-$ virus was injected intravenously and a >3-fold increase in tumor size was observed at day 28 post-viral injection (Figure 4.6, A). In contrast, a >60% and >85% reduction in tumor size was observed in mice treated with a single intravenous injection of 5×10^5 pfu and 5×10^7 pfu of AU27 virus, respectively. This demonstrated that systemic targeting of the virus may be dose-dependent. No sign of morbidity or mortality was observed in any of the three treatment groups, which is most likely due to quick clearance of the virus through systemic delivery. Quantitative real-time PCR analysis of various organs

harvested from the treated mice showed that the virus was found significantly higher levels in the tumors (Figure 4.6, B). A very small amount of virus was detected in the intestine of one mouse treated with 5 x 10^7 pfu of AU27 virus, but there was no noticeable toxicity.

4.4 Discussion

Prostate cancer patients with locally invasive and metastatic disease have relatively few treatment options, and even these are more palliative than curative in intent (37). Systemic delivery of chemotherapeutic drugs may be limited by toxicity to normal tissues, whereas targeted oncolytic virotherapy can greatly enhance the therapeutic index when the virus is engineered to specifically replicate in and kill tumor cells while sparing normal tissues. In this study, we demonstrated the targeting specificity of a recombinant oncolytic HSV-1 virus through both transcriptional and translational regulation of the essential *ICP27* viral gene. We found that the eIF4E protein is overexpressed in LNCaP cells by at least 4-fold when compared to a non-malignant, normal epithelial prostate cell line, PNT1B (Figure 4.2). In addition, androgen stimulation resulted in a consistent ~2-fold increase in *ICP27* mRNA transcripts and virus titers when LNCaP cells were infected with A27 or AU27 virus (Figure 4.2 & 4.3). However, some leaky ICP27 expression from the ARR_2PB promoter in the absence of androgen was observed and was most likely due to the activity of the promiscuous viral transactivator ICP0 protein, which may interact non-specifically with a variety of viral genes (38, 39). *ICP27* mRNA was transcribed efficiently in both PNT1B and LNCaP cell lines, but protein expression was hindered by the complex *rFGF-2* 5'UTR only in PNT1B cells (Figure 4.3). Moreover, AU27 virus replicated well in LNCaP cells, but not in PNT1B cells irrespective of androgen status (Figure 4.4), demonstrating differential replicability of the virus in normal versus tumor cells. We also showed that after *in vivo* administration, the AU27 virus was very effective in killing LNCaP xenograft tumor cells and extending survival without causing extensive toxicity to normal tissues (Figure 4.5 & 6). Wild-type virus was very toxic to the mice, causing a major impact on the gastrointestinal system, while AU27 was considerably safer and amplified mainly inside the tumor.

Interestingly, the transcriptional upregulation of *ICP27* expression by androgen stimulation of the ARR_2PB promoter was overpowered by translational suppression by

the complex 5'UTR in non-cancer cells. This dominance of translational regulation over transcription provides a significant implication in oncolytic virus design; in that viral gene expression and replication can be selectively enhanced through transcriptional upregulation in tumor cells, as evidenced by the increase in viral titer in the presence of activated androgen receptor, while at the same time being translationally repressed in normal tissues. Previous clinical studies have reported that progression of prostate cancer to a castration-resistant state is frequently accompanied by over-expression or over-activation of the androgen receptor, which allows tumor cell proliferation in the absence of androgen (3, 40-42). In such cases, the oncolytic effect of AU27 virus can be augmented in tumor cells that retain elevated androgen receptor activity without compromising its safety in non-cancer tissues due to translational override. This finding provides a new avenue to solve a long existing conflict in oncolytic viral vector design - a balance between the aggressiveness of an oncolytic virus for cancer cell killing and the safety concern of uncontrolled viral lysis.

While a >4-fold elevation of eIF4E levels is observed in LNCaP cells when compared to non-malignant BPH-1 and PNT1B cells, there may be cases where the level of eIF4E is not as dramatically elevated in tumor cells relative to normal tissues [~2-fold increase (22)]. In such cases, other tumor-specific elements can also be used in combination with this targeting strategy to enhance specificity. For instance, recent strategies utilizing endogenous microRNAs to regulate oncolytic viral replication using various viral vectors also showed great promise (43, 44). In our present approach, we have demonstrated that employment of a complex 5'UTR element could confer stringent tumor-specificity and enhance tissue-specificity of an oncolytic HSV-1 virus. This targeting strategy may be particularly useful in treating the most aggressive cancers, as recent data indicate that elevated activation of eIF4E is associated with poor patient survival (21). Furthermore, the advantage of using oncolytic HSV-1 is the lack of liver toxicity, making it superior to adenoviral vectors since liver damage is the major clinical limitation of adenoviral therapy.

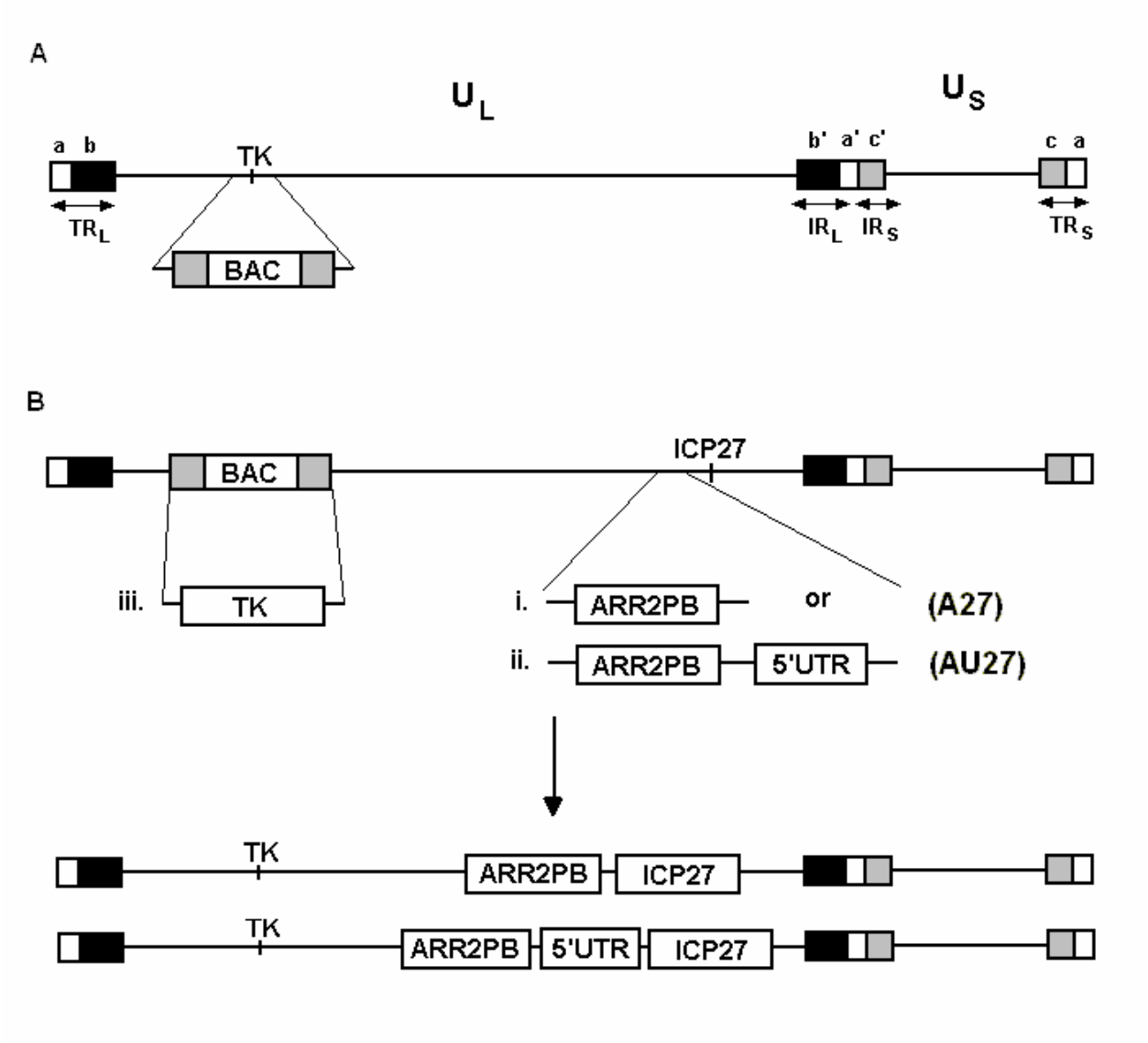


Figure 4.1 Schematic diagrams showing the construction of the recombinant viruses ARR$_2$PB-ICP27 and ARR$_2$PB-5'UTR-ICP27. (A) BAC was inserted into the *TK* locus by homologous recombination. (B) ARR$_2$PB-ICP27 (A27) and ARR$_2$PB-5'UTR-ICP27 (AU27) recombinant viruses were generated by recombineering method which replaced the endogenous *ICP27* promoter with ARR$_2$PB promoter (i) or ARR$_2$PB promoter + 5'UTR from *FGF-2* (ii). *TK* gene was then rescued by homologous recombination in 2-2 cells with HAT selection (iii).

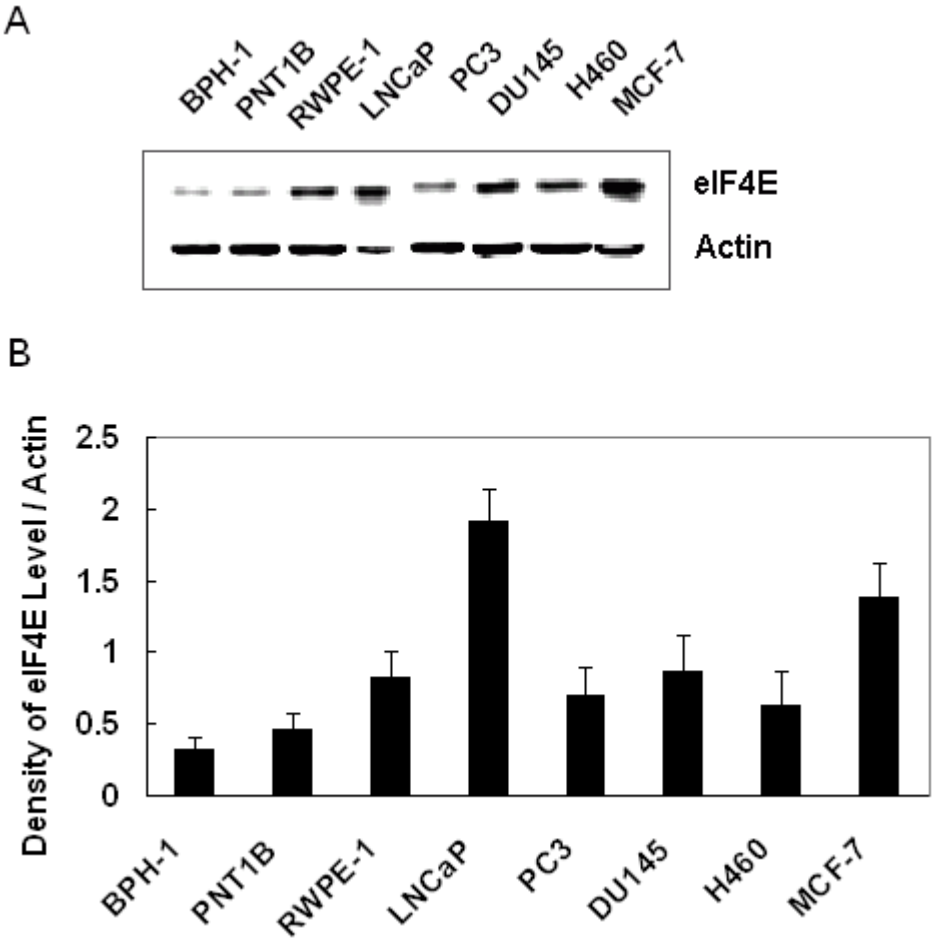


Figure 4.2 eIF4E expression levels in cell lines. Total protein was extracted from normal human epithelial prostate cell lines (BPH-1, PNT1B, RWPE-1), human prostate cancer cell lines (LNCaP, PC3, DU145), lung caner cell line (H460), and breast cancer cell line (MCF-7). (A) eIF4E and β-actin expression was determined by Western blot analysis. (B) Density ratio of eIF4E levels normalized to β-actin (means ± SD, n = 3).

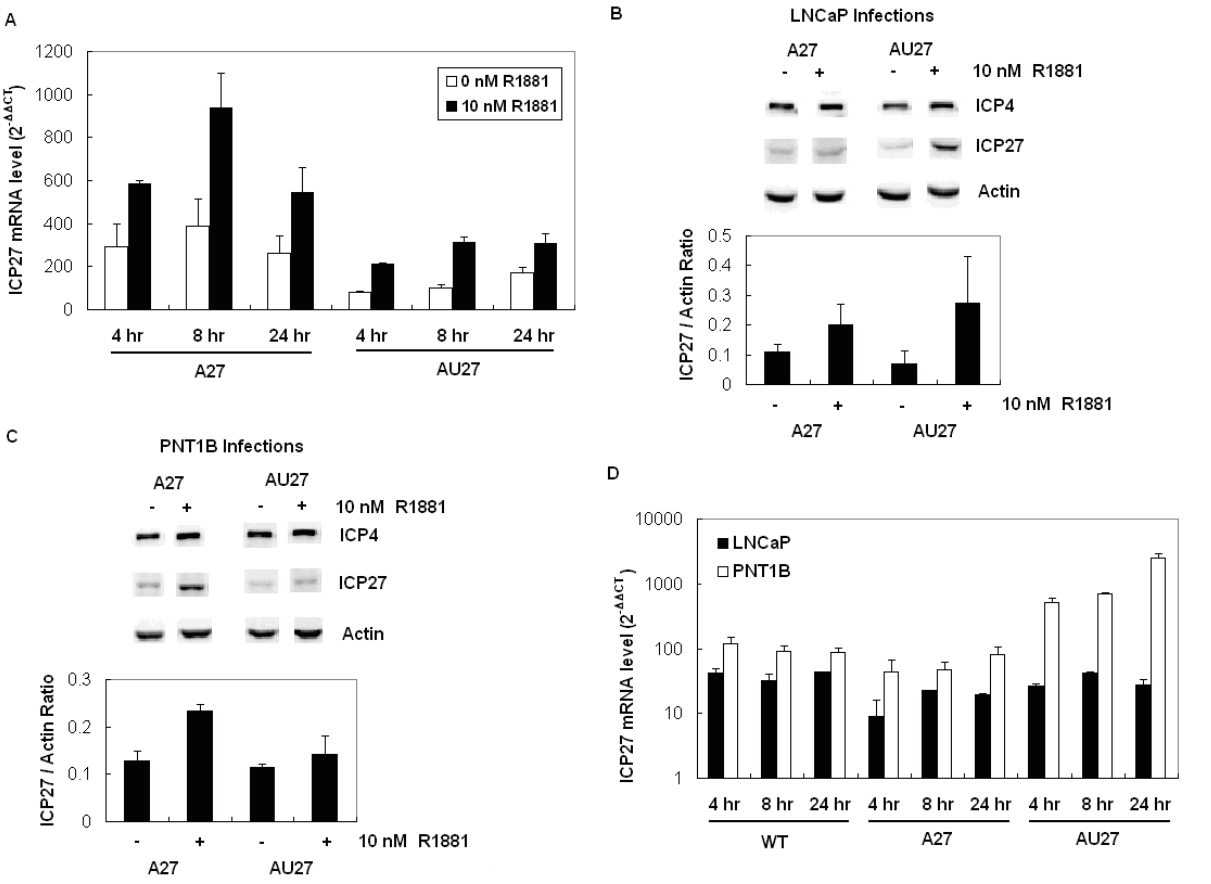


Figure 4.3 Transcriptional and translational regulation of ICP27. (A) LNCaP cells were infected with A27 and AU27 viruses at an MOI of 3 in the presence of 0 nM or 10 nM R1881. Total RNA was extracted at 4 hr, 8 hr and 24 hr post-infection and *ICP27* mRNA level was determined by quantitative RT-PCR and normalized with β-actin. (B) LNCaP and (C) PNT1B cells were infected with A27 and AU27 viruses as described above. Total protein was extracted at 8 hr post-infection, and ICP4, ICP27, and β-actin expression were determined by western blot analysis. Density ratio of eIF4E levels normalized to β-actin (means ± SD). (D) *ICP27* mRNA level at 4 hr, 8hr and 24 hr post-infection was determined by quantitative RT-PCR using *β-actin* as an endogenous control.

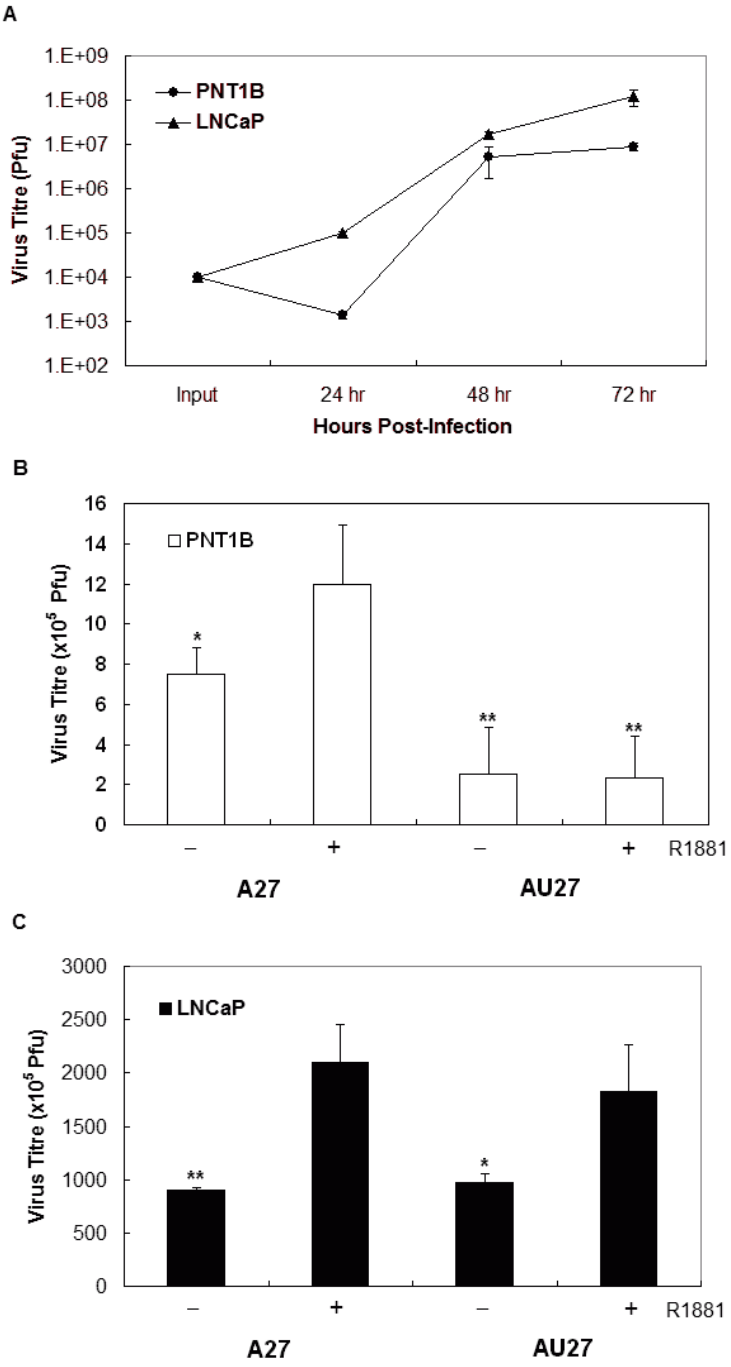
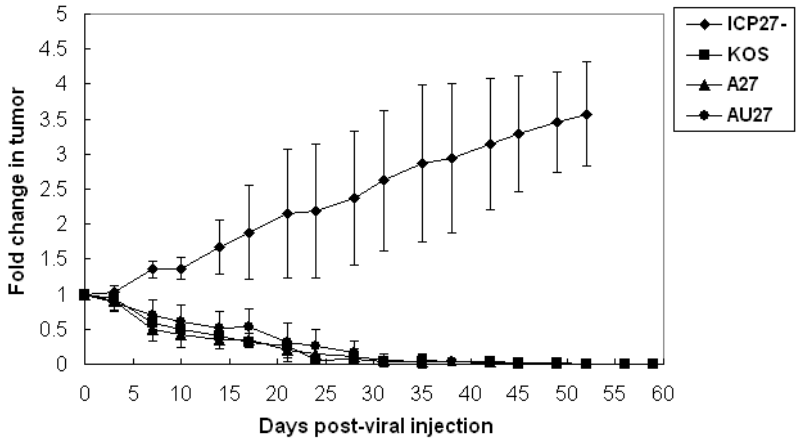
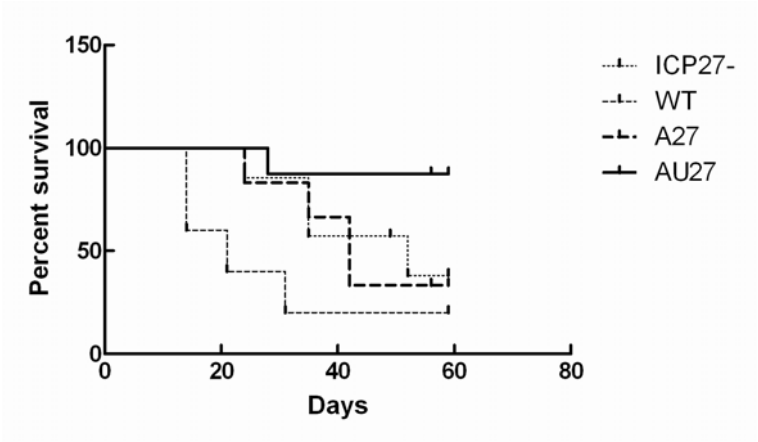


Figure 4.4 Tumor-specific replication of AU27 virus *in vitro*. Tumor-specific replication of AU27 virus *in vitro*. (A) PNT1B and LNCaP cells were infected with wild-type virus at an MOI of 0.01 (10^4 pfu) and virus titer was determined at 24 hr, 48 hr and 72 hr post-infection. (B) PNT1B and (C) LNCaP cells were infected with A27 or AU27 at an MOI of 0.01 with ±10 nM R1881 treatment and virus titer was determined at 48 hr post-infection. Virus titer was plotted as mean ± SD from four determinants (* = $p < 0.05$; ** = $p < 0.005$).

A



B



C

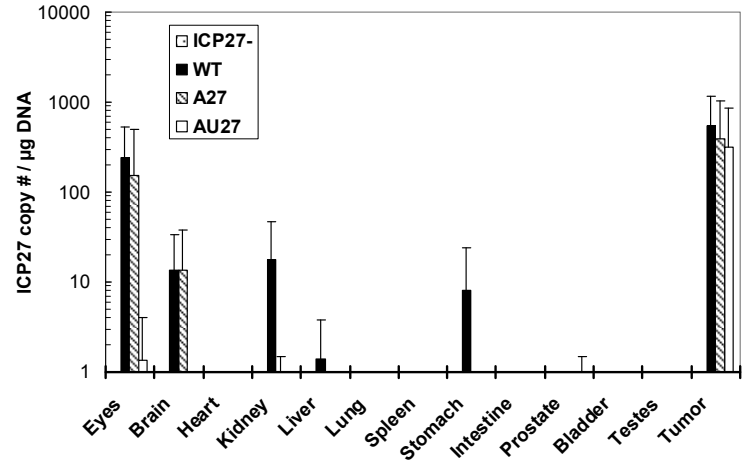


Figure 4.5 AU27 virus inhibited tumor growth without causing toxicity to normal tissues *in vivo*. (A) Nude mice with LNCaP tumors were treated with one intratumoral injection of 2 x 10^6 pfu of ICP27$^-$ (n = 7), WT (n = 5), A27 (n = 6) or AU27 (n = 8) at Day

0 when tumor size reached approximately 100 ± 20 mm^3. Tumor size was determined by caliper measurements. (B) Survival rate of the treated mice mentioned above was monitored for approximately two months and any mouse that showed signs of morbidity due to viral toxicity was sacrificed immediately. Mice in $ICP27^{-}$ treatment group were sacrificed due to tumor burden when tumor size exceeded 4-fold increase. (C) Total DNA was extracted from various organs of the treated mice at the end of the experiment. *ICP27* copy number (per μg of DNA) was determined by real-time PCR assays to indicate the presence of virus in the tissues.

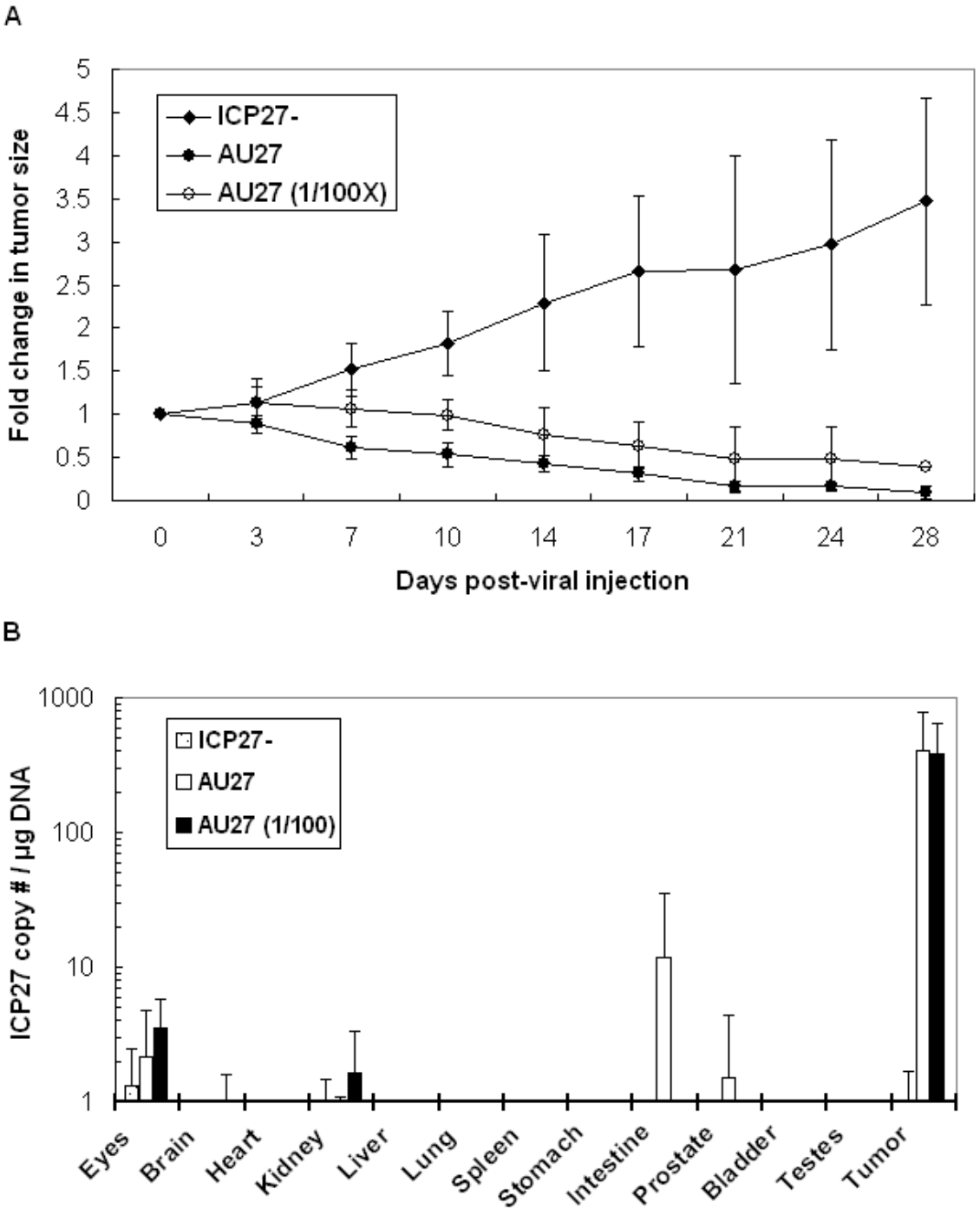


Figure 4.6 AU27 virus targeted and killed tumor cells after systemic administration. (A) Nude mice with LNCaP tumors were treated with a single intravenous injection of 5 x 10^7 pfu of ICP27$^-$ (n = 4), AU27 (n = 5) or 5 x 10^5 pfu (100x less) of AU27 (n = 4) at Day 0 when tumor size reached approximately 140 ± 65 mm^3. (B) At day 28 post-viral injection, total DNA was extracted from various organs of the treated mice. *ICP27* copy number (per µg of DNA) was determined by real-time PCR assays.

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5. Conclusions and future directions

5.1 Transcriptional targeting using tissue-specific promoters

Previous studies employing oncolytic HSV-1 targeting have focused mainly on the deletion of viral genes that are dispensable in tumor cells (1-3); however, this usually results in attenuation of the viruses, therefore compromising their efficiency to kill such cells. An alternative method to selectively target tumor cells is to transcriptionally control the expression of essential viral genes. The first attempt to control viral replication transcriptionally through the regulation of an essential HSV-1 viral gene was demonstrated by Miyatake *et al*., who generated a recombinant HSV-1 virus, G92A, by inserting the *ICP4* gene driven by the liver-specific albumin enhancer/promoter at the *TK* locus (4). The G92A virus replicates efficiently in hepatocellular carcinoma cell lines; however, its application is quite limited in humans because albumin is expressed in both normal and cancerous liver cells. Moreover, this virus is *TK*-negative and therefore cannot be controlled by antiviral drugs such as acyclovir. Additional research utilizing tumor-related promoters to drive *ICP4* gene expression to target tumor cells includes the use of the calponin promoter to target malignant human soft tissue and bone tumors (5), and the use of the β-catenin-dependent promoter to target colorectal cancers or cancers characterized by elevated activation of the β-catenin signaling pathway (6).

We are the first to describe an HSV-1 amplicon system that uses a prostate-specific promoter, ARR_2PB-ICP4, to control the expression of an essential HSV-1 viral gene, *ICP4*, which can then complement an $ICP4^-$ helper virus for efficient replication and destruction of prostate cancer cells both *in vitro* and *in vivo*. Previously, it was reported that basal activities of the ARR_2PB promoter could be seen in nonprostatic cell lines, but its activity was highly specific for prostatic epithelium in transgenic mice (7). Incidentally, using this promoter in our viral targeting system, we observed a level of viral toxicity in the stomach of mice treated with the ARR_2PB-ICP4 amplicon/helper virus, which, in some cases, led to delayed gastritis-related death. One explanation for this finding is that a low level of ICP4 could still be expressed from the leaky promoter and was therefore sufficient to initiate lytic viral replication. This residual toxicity can be completely eliminated by supplemental treatment with ganciclovir (GCV) (Appendix A). GCV can be phosphorylated by the HSV-1 TK enzyme into a monophosphate form that cellular kinases further convert into GCV-triphosphate, thus causing cell death by

inhibiting DNA replication (8). Similar to the ARR_2PB-ICP4 treatment group, an approximately 70% reduction in tumor size was observed in mice treated with the ARR_2PB-ICP4 amplicon/helper virus plus GCV at day 28 post-viral injection, however no morbidity or mortality was observed in these mice (Appendix B, Figure B.1). Thus, supplementary GCV treatment was able to eliminate the toxicity caused by the ARR_2PB-ICP4 amplicon/helper virus without compromising the efficiency of inhibiting the growth of LNCaP tumors. Furthermore, the additional GCV treatment can augment the tumor killing efficiency via the "bystander effect", which causes the death of neighboring cells due to the transfer of phosphorylated GCV through gap junctions (9, 10). This effect has been the principle rationale for suicide gene therapy. In summary, this research further demonstrates a favorable feature of oncolytic HSV-1 viruses whereby GCV can provide a safeguard against unintended viral spread to normal tissues.

5.2 MicroRNA-mediated translational targeting

Translational regulation is a fairly new targeting strategy in oncolytic virotherapy. With the recent discovery of miRNAs in 2001, we are the first group 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. 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 (11-13).

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 (Appendix B, Figure B.2). 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 (Appendix B, Figure B.3). 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 (Appendix A). 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 (Appendix B, Figure B.2). 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 (14, 15). Subsequently, we infected these stable cell lines with our modified miRNA-mediated amplicon viruses and examined cell viability using the MTT assays (Appendix A). 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 (Appendix B, Figure B.4). 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 (Appendix B, Figure B.3 & B.5), 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, ARR_2PB-ICP4-143T and ARR_2PB-ICP4-145T (Appendix B, Figure B.6), 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 ARR_2PB-ICP4-143T and ARR_2PB-ICP4-145T at 28 days post-viral injection (Appendix B, Figure B.7 & B.8). As with the CMV-ICP4-143T and CMV-ICP4-145T amplicon viruses, the ARR_2PB-ICP4-143T and ARR_2PB-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 (Appendix B, Figure B.9). 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. From a transcriptional regulation stand point, the chances for the *ICP4* gene to be non-specifically expressed from the ARR_2PB promoter in the amplicon virus are 15 times greater. In the case of translational control, there could potentially be 15 times more of the *ICP4* mRNA transcripts requiring degradation by the endogenous miRNAs. 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.

Recently, similar targeting strategies taking advantage of differentially expressed miRNAs have been reported by three separate groups. Two of these groups utilized tissue-specific miRNAs to retarget adenovirus and picornavirus, respectively, while one group used a tumor-specific miRNA to retarget the vesicular stomatitis virus (VSV). For example, Ylosmaki *et al*. constructed a recombinant adenovirus that carries three copies of complementary target sequences to the liver-specific miR-122 in the 3'UTR of the *E1A* gene and demonstrated that viral replication was inhibited in cell lines (Huh7) expressing high levels of miR-122, indicating that this approach could alter the tropism

of a virus (16). This application may be particularly useful in the current adenoviral vectors as liver toxicity has always been a concern when delivering these viruses systemically. Further, a similar approach has been demonstrated by Kelly *et al*., who inserted two copies each of miR-133 and miR-206 target sites into the 3'UTR of the coxsackievirus A21 (CVA21) (17). They showed that this virus inhibited the growth of multiple myeloma tumors and prolonged the survival of treated mice without causing muscle paralysis. On the other hand, and more similar to our approach, Edge *et al*. demonstrated tumor-specific replication of an oncolytic VSV virus by incorporating three repeats of the complementary target sequence to the tumor suppressor miRNA, let-7, in the 3'UTR of the VSV *M* gene, which encodes for the matrix protein and is essential for viral growth and replication (18). Let-7 has been shown to be downregulated in lung and breast cancer, and one of its target genes is the oncogene, *RAS*. Other tumor suppressor miRNAs such as miR-15 and miR-16 could also be tested to direct tumor-selective oncolytic viral targeting. The major disadvantage of employing a miRNA-mediated targeting strategy in an RNA virus is that mutations occurring in the 3'UTR region containing the miRNA target sites may enable the virus to escape from miRNA regulation and this has indeed been reported in one case (17). Altogether, miRNA-mediated targeting has proven to be a promising approach in harnessing viral tropism and transgene expression. Our research, along with others, 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.

5.3 EIF4E-regulated translational targeting

Another translational targeting strategy is to control the initiation of protein synthesis through eIF4E regulation. We are the first group to investigate the effect of transcriptional and translational regulation of the essential HSV-1 viral gene, *ICP27*, on viral replication and tumor-specific targeting. To do this, we replaced the endogenous promoter of the *ICP27* gene in a recombinant virus with the prostate-specific and androgen receptor-dependent promoter, ARR_2PB, plus the *FGF* 5'UTR to control viral

replication. We showed that both *ICP27* gene expression and viral replication are enhanced by elevated androgen receptor activity *in vitro*. However, this transcriptional upregulation of viral gene expression can be effectively suppressed in normal cells via translational regulation using a complex 5'UTR. Expression of a gene with this type of 5'UTR requires high levels of eIF4E/eIF4F complexes, which are overexpressed in tumor cells, but low in normal cells. Since eIF4E overexpression has recently been shown to be associated with progression to a hormone-refractory state and reduced patient survival, the utilization of 5'UTR as a tumor-specific targeting strategy may be particularly useful for the treatment of advanced disease (19). We found that translational regulation overrides the transcriptional upregulation of the viral gene by androgen and inhibits viral gene expression and viral replication in normal prostate cells, but not in prostate tumor cells. This effect is particularly favorable as the cytolytic activity of a virus can be enhanced in the tumors while at the same time being suppressed to a safe level in normal tissues. Furthermore, our *in vivo* data demonstrated that the AU27 virus exhibits strong oncolytic activity in prostate tumors while remaining inactive within normal tissues. In the LNCaP xenograft mouse model, a single intravenous injection as low as $5x10^5$ pfu of the AU27 virus was sufficient to cause a greater than 60% reduction in tumor size, whereas $5x10^7$ pfu was able to cause a greater than 85% reduction by day 28 post-viral injection. Furthermore, significant viral replication was found in the tumor mass, but not in any of the normal organs tested by quantitative real-time PCR analysis. Recently, a similar targeting approach has also been demonstrated in a conditionally replicative adenovirus (CRAds) that utilizes the *CXCR4* gene promoter plus the *FGF* 5'UTR to control the expression of the *E1A* gene for the treatment of breast cancer (20). It was shown that the virus effectively inhibited tumor growth in a breast cancer murine model, and that no liver toxicity was observed when using the human liver tissue slice model. Altogether, our results indicate that a dual transcriptional and translational targeting strategy for viral essential gene expression increases both viral lytic activity and tumor specificity. Moreover, this same approach can be easily adapted to other types of cancer by incorporating an appropriate tissue-specific promoter.

In contrast to the amplicon/helper system, no toxicity was seen in the GI tract of the mice treated with the AU27 recombinant virus. There are two possible explanations for this discrepancy: 1). different viral packaging systems, and 2). regulation of two different viral genes. As previously described, one major problem lies in the inherently different

properties between the amplicon virus and the recombinant virus in terms of the number of gene copies that they carry. Another possibility is that the *ICP27* gene may be a more favorable candidate than the *ICP4* gene. Unlike the ICP4 protein, ICP27 is a multifunctional protein that is involved in many processes during the viral life cycle, such as the transcription of viral genes as a co-activator (21), shutoff of host protein synthesis by inhibiting pre-mRNA splicing (22), and promoting viral mRNA processing and export (23). As a result, it is possible that the threshold for ICP27 expression to initiate lytic viral replication is much higher than that for ICP4. Therefore, low levels of ICP27 expression may not be as big of a concern as for leaky ICP4 expression. Caution should be taken when replacing the promoter of a viral gene as this may alter the normal kinetics of gene expression and viral replication. However, the concern over attenuating the virus is not necessary in the case of the *ICP27* gene, since it has been shown that alteration in the *ICP27* endogenous promoter merely causes a delay in viral gene expression and has no adverse effect on viral replicability (24). On the other hand, retaining the endogenous promoter of the *ICP4* gene may be beneficial because this promoter contains both positive and negative regulatory elements for its own protein, thus the ICP4 protein can negatively regulate its own expression through a negative feedback loop (25). By changing its promoter, the ICP4 protein will continue to accumulate and may cause cytotoxic effects in normal tissues. Further detailed studies of the biology of viral genes and its life cycle may reveal other ideal candidate genes for tumor-specific regulation.

Due to the systemic nature of most malignancies, the ultimate goal is to deliver oncolytic viral vectors intravenously to target the disseminated disease. Using the LNCaP xenograft mouse model, we have demonstrated that the AU27 virus was able to kill the subcutaneous LNCaP tumors efficiently after intravenous administration. To examine the ability of the AU27 virus in targeting metastases, future studies should test the efficacy of oncolytic viruses using immunocompetent mouse models such as the TRAMP (transgenic adenocarcinoma of mouse prostate) model (26) or the PTEN (phosphatase and tensin homolog deleted on chromosome 10) knockout model (27). Moreover, the use of immunocompetent mouse models can yield more insights into the effect of an immune response against the efficacy of oncolytic viral vectors. It has been shown that innate and adaptive immune responses can either hinder the spread and oncolytic effect of the virus by restricting viral distribution (28) or augment the anti-tumor efficacy through redirected targeting to tumor antigens (29). In addition, pre-existing

immunity has been shown to exhibit minimal effects on the efficacy of the oncolytic HSV-1 viruses (3, 30). In light of these observations, further investigation is required to fully understand the role of immunity in oncolytic virotherapy. Before proceeding towards clinical trials, the virus should be tested in immunocompetent mice with a goal set to determine doses, toxicity, route of administration of the virus and the immunological effect on the virus. Immunostimulatory cytokines or other therapeutic genes may also be incorporated into this oncolytic viral backbone to further improve therapeutic efficacy in that the cytolytic effect can be dramatically enhanced by the initial direct lysis of the infected tumor cells followed by the amplification and dissemination of the therapeutic genes encoding anti-angiogenic agents, cytokines, or prodrug-converting enzymes. This could be used in neoadjuvant or adjuvant settings in combination with chemotherapy or radiation therapy.

5.4 Conclusions

Even though virotherapy for cancer treatment is still in its infancy, significant progress has been made in the last two decades (1, 31-33). 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, the miRNA-mediated 3'UTRs, and the eIF4E-regulated 5'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.

5.5 References

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Appendix A

A.1 Supplementary material and methods

A.1.1 GCV treatment in LNCaP xenograft model

Athymic nude mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and LNCaP cancer cells were implanted subcutaneously by injecting 5 x 10^6 cells in 100 µL of medium with matrigel at four different sites over the shoulders and hind legs on both sides. Animals were monitored for tumor development two times a week by palpation of the injection sites. When detected, tumor volumes were determined by caliper measurements and calculated using the formula volume = width x length x thickness x π/6. When the tumor volume reached approximately 100 mm^3 (at ~1 - 1.5 month after tumor inoculation), the mice were treated with two intratumoral injections (Day 0 and Day 7) of DMEM medium alone, or of 2 x 10^6 pfu of the $ICP4^-$ helper virus, or ARR_2PB-ICP4 amplicon/helper virus. Starting at Day 10, ganciclovir (GCV) was administered intraperitoneally at a concentration of 50 µg/g once every two days (1, 2). The mean fold change in tumor size was determined by comparing the tumor size from each day to the original size at Day 0 and the average was plotted on the graph (n = 3 for each group). All experimental procedures were approved by the UBC Animal Care Committee and followed the guidelines and policies of the Canadian Council on Animal Care.

A.1.2 Generation of stable LNCaP-143 and LNCaP-145 cell lines

To make miRNA-expression vectors that express miR-143 or miR-145, two pairs of oligonucleotides miR-143S (5'-TGCTGTGAGATGAAGCACTGTAGCTCAGTTTTGGCCACTGACTGACTGAGCTACTGCTTCATCTCA-3'), miR-143AS (5'-CCTGTGAGATGAAGCAGTAGCTCAGTCAGTCAGTGGCCAAAACTGAGCTACAGTGCTTCATCTCAC-3') and miR-145S (5-'TGC TGGTCCAGTTTTCCCAGGAATCCCTTGTTTTGGCCACTGACTGACAAGGGATTTGGGAAAACTGGAC-3'), miR-145AS (5'-CCTGGTCCAGTTTTCCCAAATCCCTTGTCAGTCAGTGGCCAAAACAAGGGATTCCTGGGAAAACTGGACC-3') were annealed and cloned into the pcDNA6.2-GW-EMGFP vectors (Invitrogen, Vancouver, Canada) following the company's instructions. LNCaP-143 and LNCaP-145 are stable cell lines that constitutively express miR-143 and miR-145,

respectively, as well as GFP protein. To generate these cell lines, LNCaP cells were transfected with miRNA expressing vectors and grown in DMEM media containing 10% FBS and Blasticidin (10 μg / μL) for more than two weeks. All cells were maintained in a 5% CO_2 incubator at 37℃.

A.1.3 Cell viability assay

LNCaP, LNCaP-143 and LNCaP-145 cells were seeded in 96-well plates at a density of 1 x 10^4 cells/well in 100 μL medium. The next day, cells were infected with $ICP4^-$ helper, CMV-ICP4-143T or CMV-ICP4-145T amplicon virus at MOI of 1. Cell viability was then determined at 24, 72, 96 and 120 hours by MTT assay as previously described (3).

A.1.4 Amplicon plasmid constructs

ARR_2PB-ICP4-143T and ARR_2PB-ICP4-145T amplicon plasmids were generated by replacing the CMV promoter of the CMV-ICP4-143T and CMV-ICP4-145T amplicon plasmids with the ARR_2PB promoter (4), respectively.

A.1.5 LNCaP-Luciferase xenograft mouse model

Athymic nude mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and LNCaP-Luc cells were inoculated subcutaneously by injecting 5 x 10^6 cells in 100 μL of medium with matrigel at two different sites on the flanks. Tumor volumes were determined by caliper measurements and calculated using the formula volume = width x length x thickness x π/6. Once the tumor size reached approximately 100 mm^3, mice were treated with two intratumoral injections (at Day 0 and Day 7) of 2 x 10^6 pfu of the ARR_2PB-ICP4-143T (n = 3) or ARR_2PB-ICP4-145T (n = 9) amplicon virus. At the end of the experiment, mice were sacrificed using CO_2 asphyxiation and several organs (eyes, brain, kidneys, liver, lung, spleen, stomach, prostate, bladder, testicles) and the tumors were removed and analyzed by 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 minutes prior to imaging with the IVIS 200 system (Xenogen). All experimental procedures were approved by UBC Animal Care Committee and followed the guidelines and policies of Canadian Council on Animal Care.

A.2 References

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Appendix B

B.1 Supplementary data

Figure B.1 Efficient inhibition of tumor growth using a combination of ARR_2PB-ICP4 amplicon/helper and GCV treatment in LNCaP xenograft model. A >70% reduction in tumor size was observed at day 28 post-viral injection without any morbidity or mortality. Supplemental GCV treatment eliminated the residual toxicity caused by the ARR_2PB-ICP4 amplicon/helper virus without compromising anti-tumor efficacy.

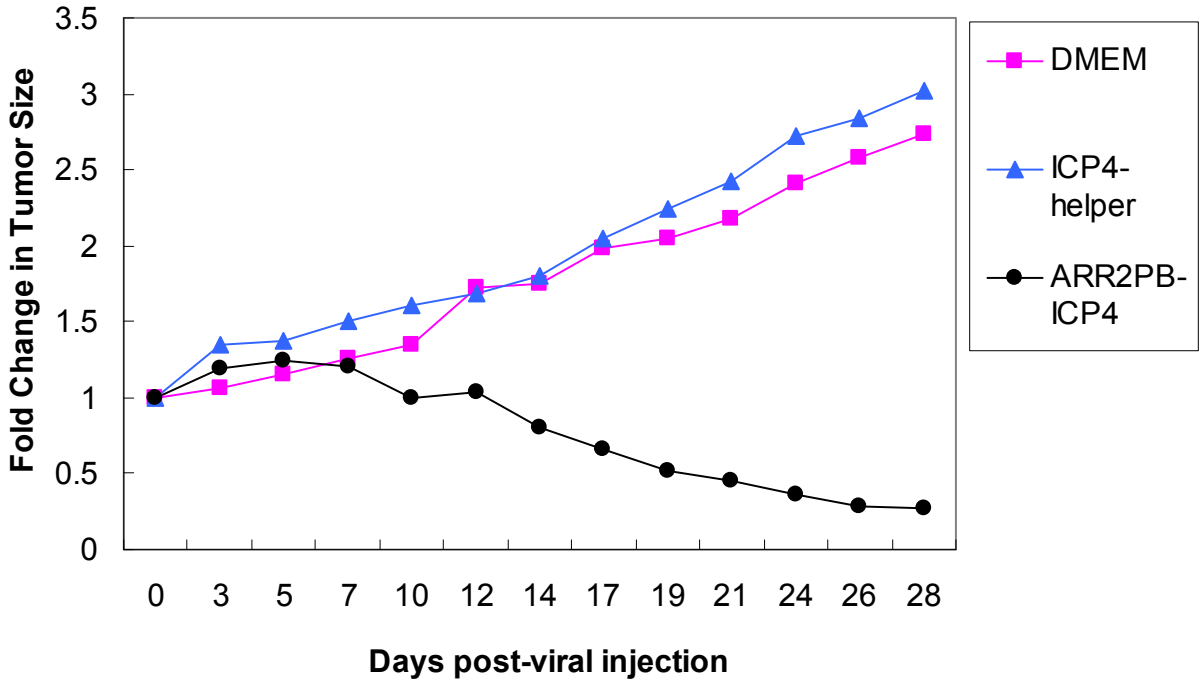


Figure B.2 The expression of miR-143 and miR-145 in LNCaP stable cell lines. LNCaP-143 and LNCaP-145 are stable cell lines that constitutively express miR-143 (A) and miR-145 (B) from a CMV promoter. The expression of both miRNAs was lost in the normal prostate epithelial cell lines, BPH-1 and PNT1B. 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.

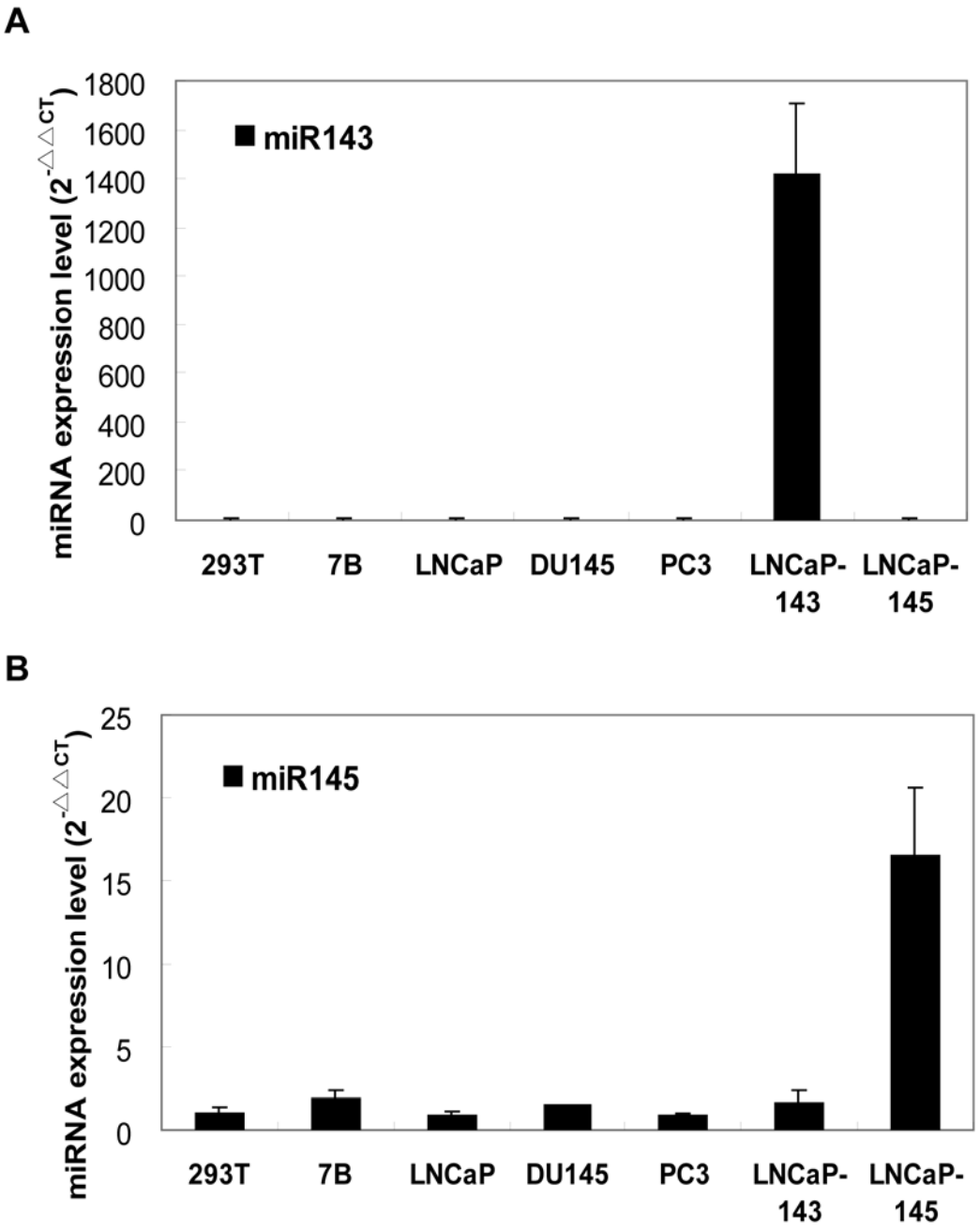


Figure B.3 MiRNA expression levels in human normal tissues. The expression levels of miR-143, miR-145 and miR-124 in human tissues were determined by quantitative RT-PCR assays and normalized to U6 RNA. Human tissue RNAs were purchased from Ambion. Ten ng of RNA was reverse transcribed to cDNA followed 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.

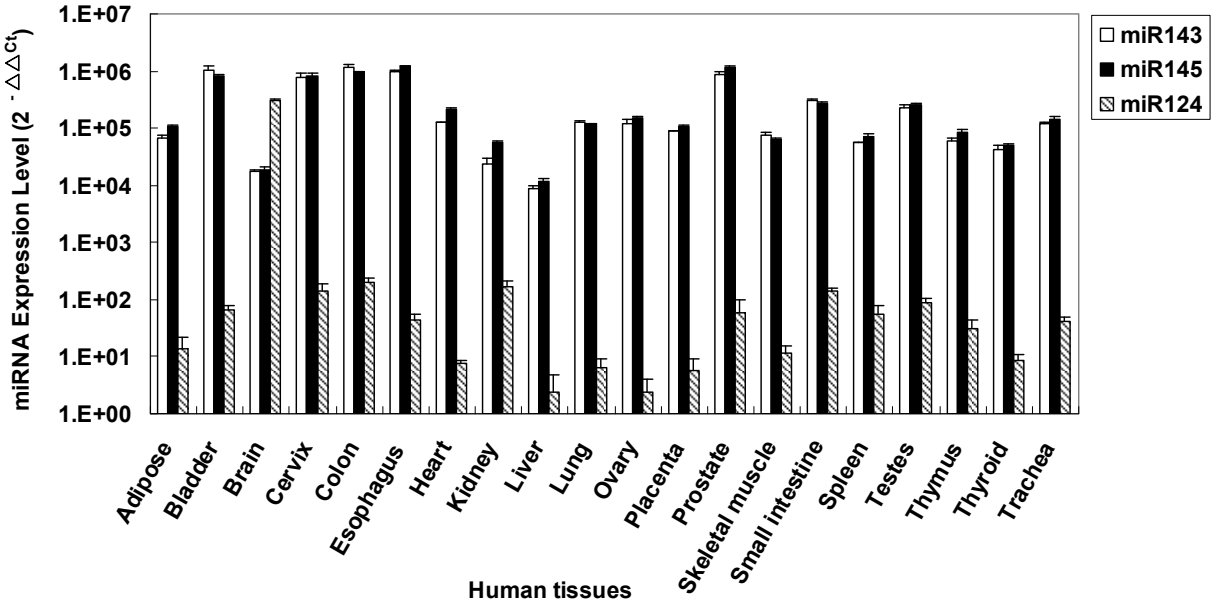


Figure B.4 MiR-143 and miR-145 reduced cytotoxicity in LNCaP cells infected with miRNA-regulated amplicon viruses. To demonstrate whether miR-143 and miR-145 could protect normal tissues from miRNA-regulated amplicon virus infections, LNCaP, LNCaP-143 and LNCaP-145 cells were infected with the ICP4^{-} helper virus and amplicon viruses at an MOI of 1, and cell viability was determined by MTT assays. The results showed that 68 ± 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 ± 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 ± 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.

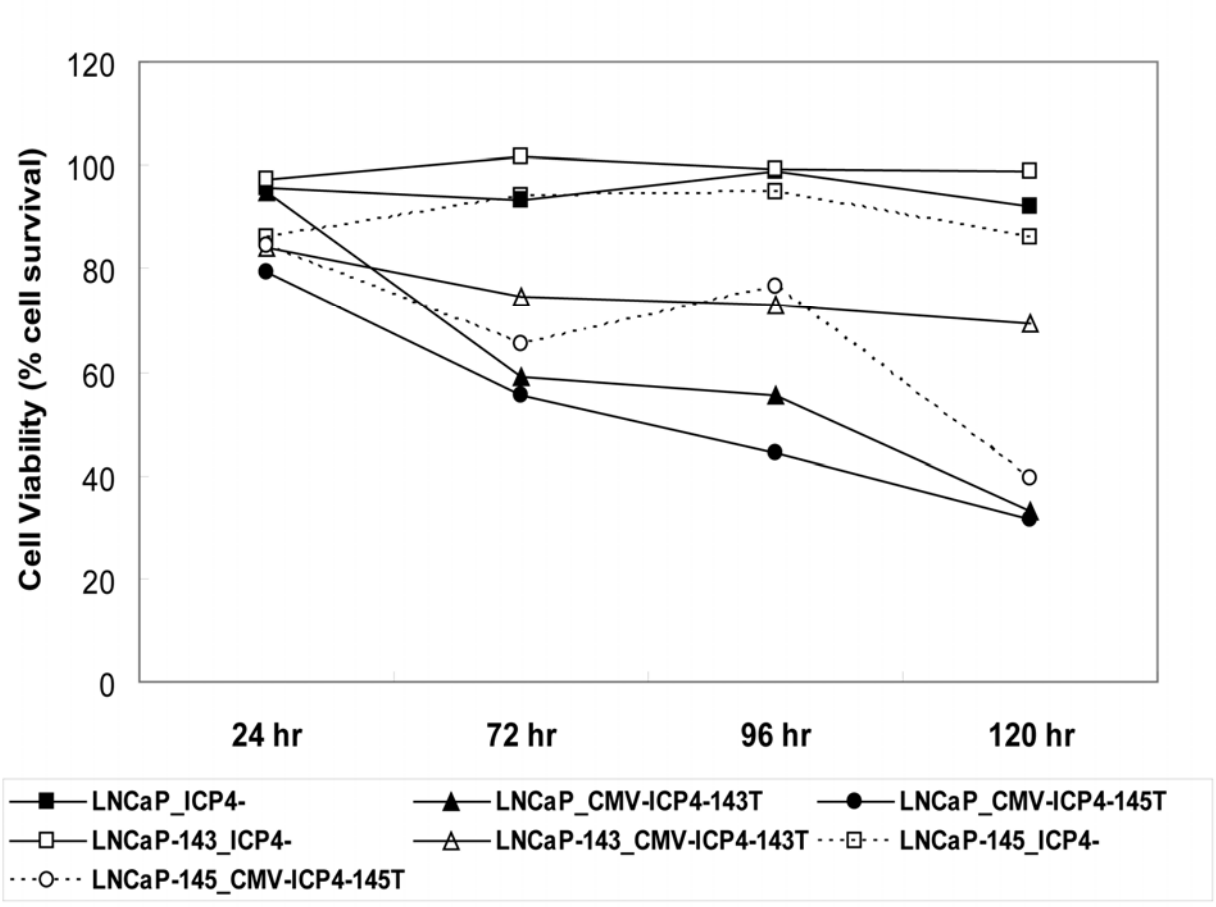


Figure B.5 MiR-124 expression levels in normal mouse tissues. The expression levels of miR-124 in normal mouse organs were determined by quantitative RT-PCR assays and normalized to snoRNA234 RNA. Total RNA from the organs was extracted using the Trizol reagent and ten ng of RNA was reverse transcribed 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.

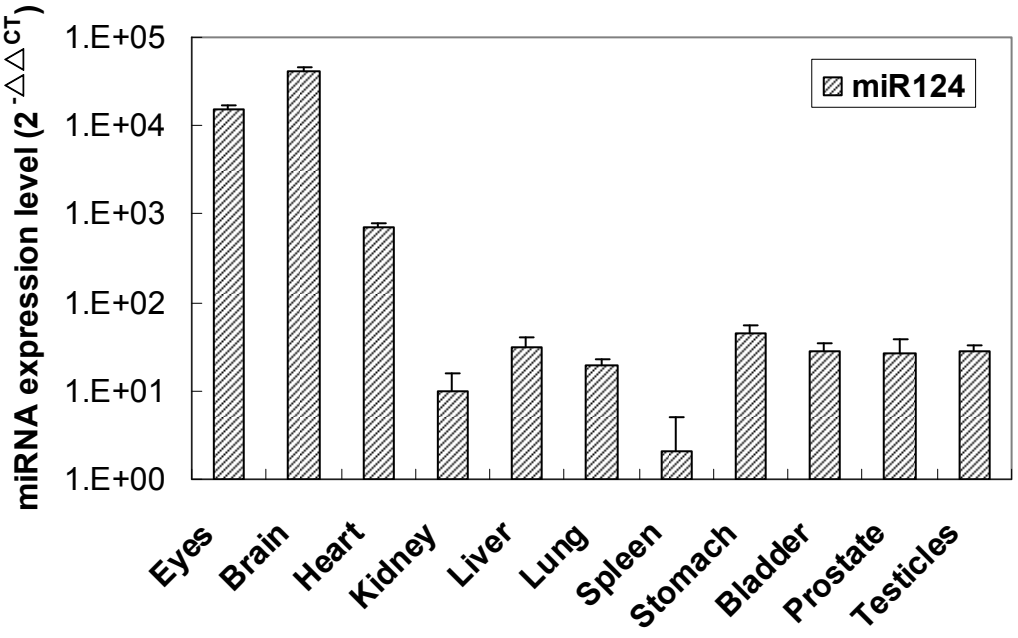


Figure B.6 Structure of the two prostate-specific and tumor-specific amplicon plasmids used to package replication-competent amplicon viruses. ARR_2PB-ICP4-143T and ARR_2PB-ICP4-145T are both prostate-specific and tumor-specific miRNA-regulated complementing amplicons. ARR_2PB -ICP4-143T amplicon contained five tandem copies of miR-143 complementary sequences (143Tx5) and ARR_2PB-ICP4-145T amplicon contained four tandem copies of miR-143 complementary sequences (145Tx4) after the *ICP4* gene.

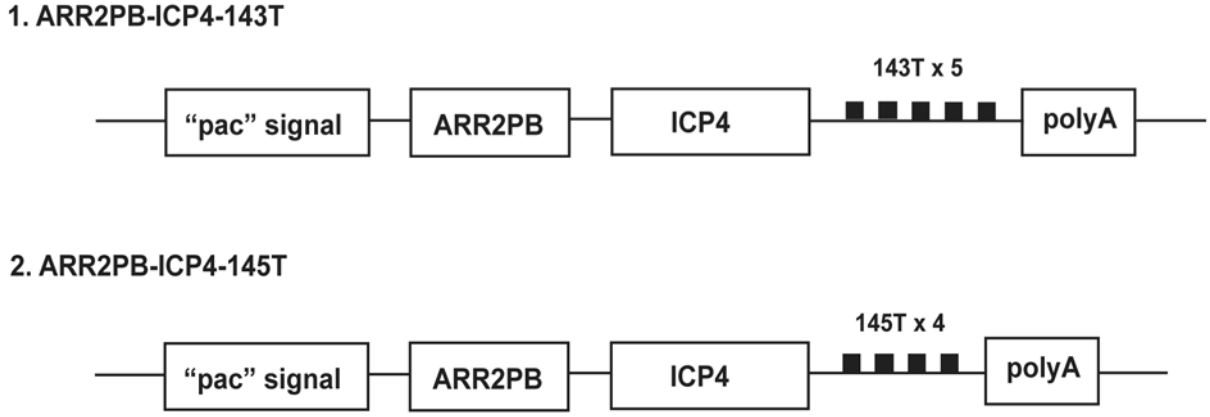


Figure B.7 ARR_2PB-ICP4-143T and ARR_2PB-ICP4-145T amplicon viruses inhibited LNCaP-Luc tumor growth in xenograft mouse model. Nude mice with subcutaneous LNCaP-Luc tumors (~140 ± 70 mm^3) were treated with two intratumoral injections (Day 0 and Day 7) totalling 2 x 10^6 pfu of the ARR_2PB-ICP4-143T or ARR_2PB-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 ARR_2PB-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 ARR_2PB-ICP4-143T and ARR_2PB-ICP4-145T amplicon viruses were able to significantly inhibit tumor size efficiently.

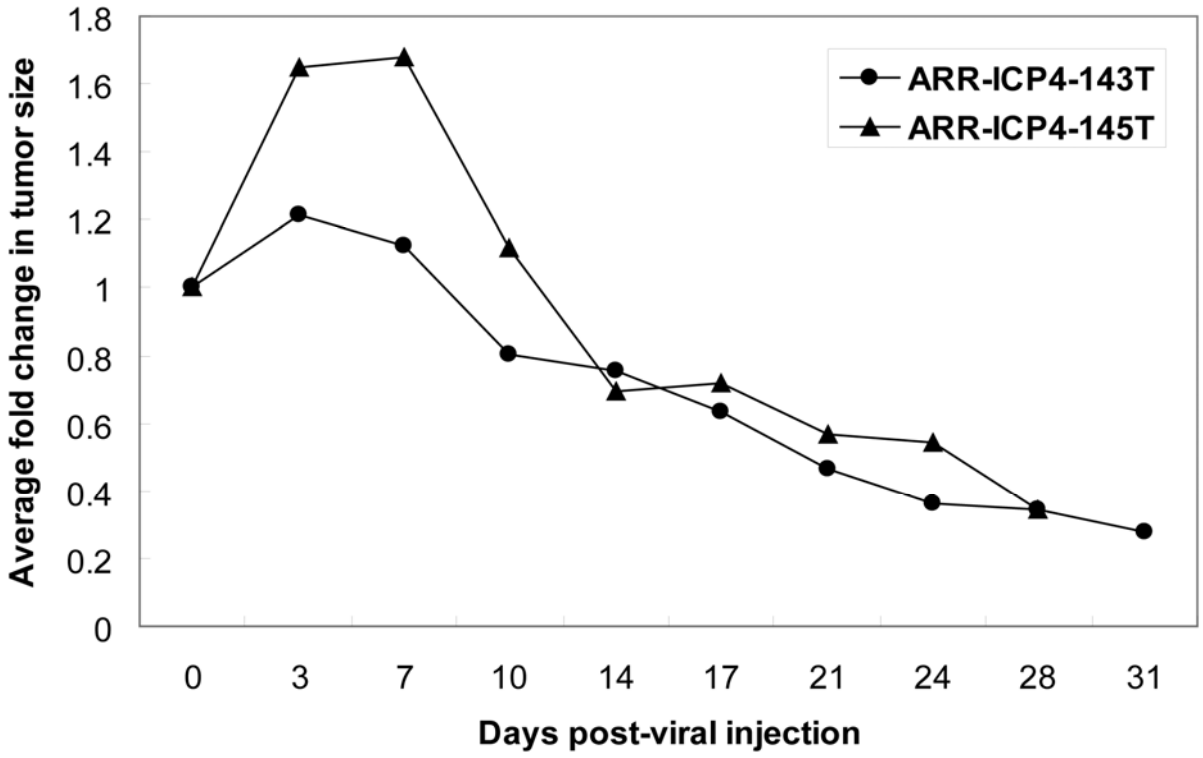


Figure B.8 Representative IVIS images of the mice treated with the ARR_2PB-ICP4-143T and ARR_2PB-ICP4-145T amplicon viruses. The size of the tumor was also visualized by the IVIS imaging system. Luciferase activity was expressed as p/sec/cm^2/sr. A >80% reduction in luciferase activity was observed in mice treated with the prostate-specific and miRNA-mediated amplicon viruses at day 17 post-viral injections.

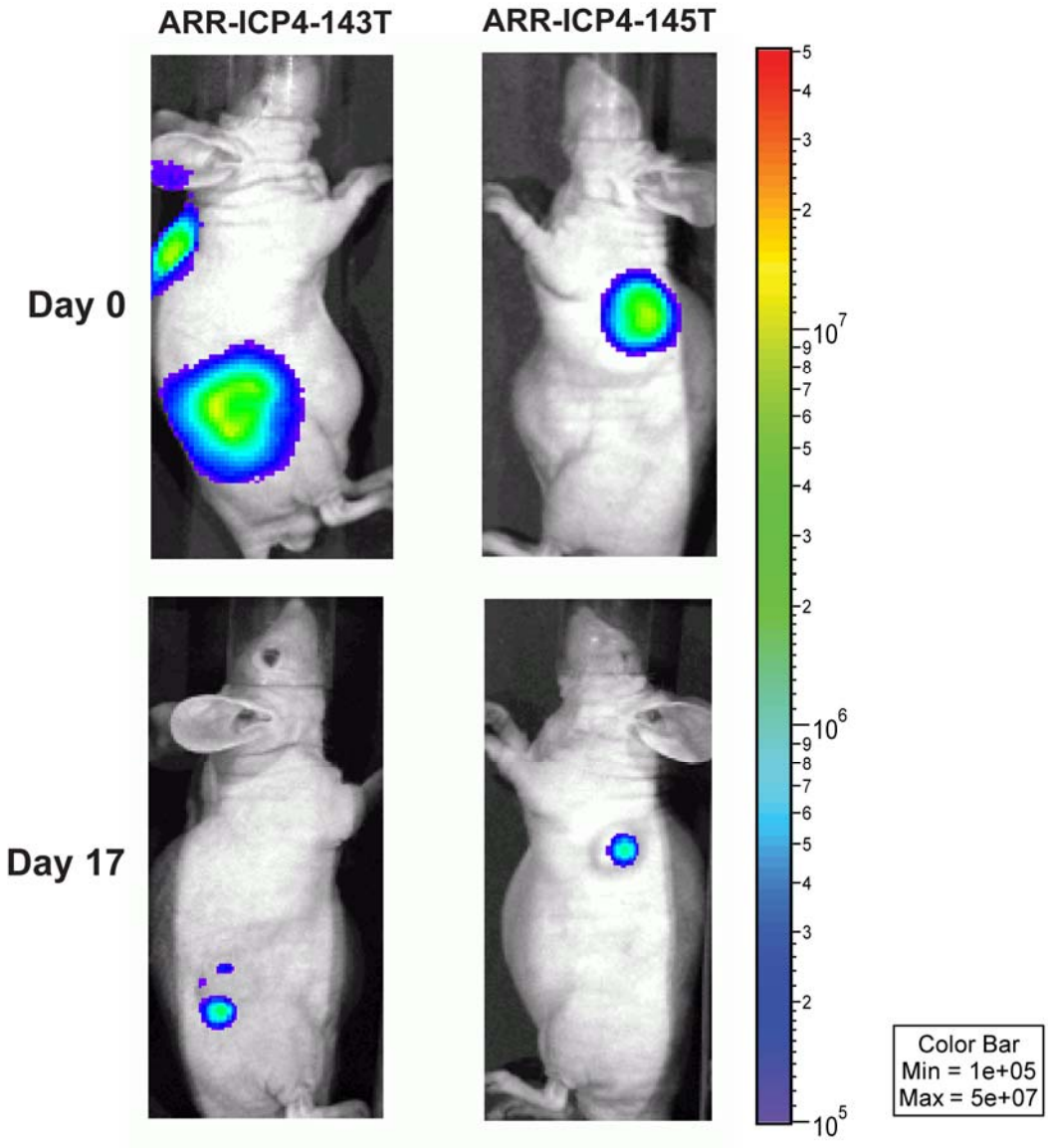
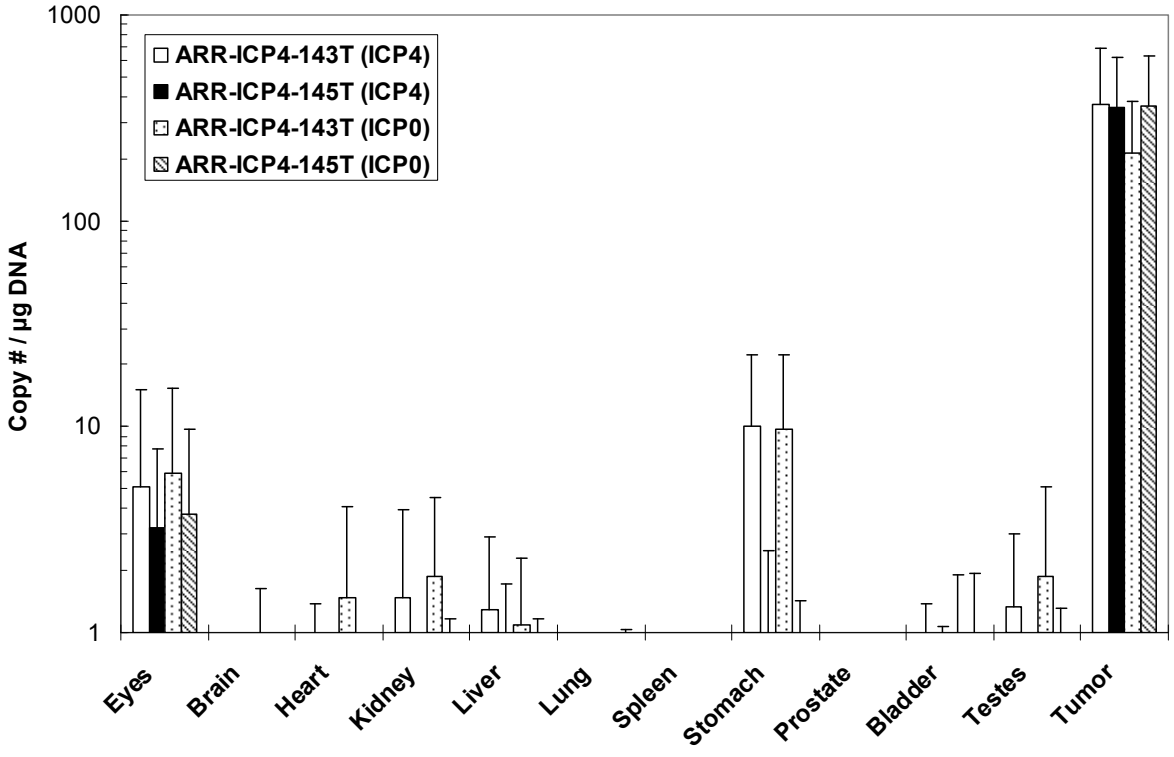


Figure B.9 Biodistribution of the ARR_2PB-ICP4-143T and ARR_2PB-ICP4-145T amplicon viruses. Total DNA was extracted from the organs and the copy number of *ICP4* and *ICP0* was determined by real-time PCR assays. The results were expressed as relative copy number / μg of DNA. The presence of the amplicon 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.



Appendix C

C.1 Biohazard approval certificate

The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: **H07-0164**

INVESTIGATOR OR COURSE DIRECTOR: **Jia, William**

DEPARTMENT: **Surgery**

PROJECT OR COURSE TITLE: **Oncolytic HSV-1 with a 3'UTR regulatory element for prostate cancer specific virotherapy**

APPROVAL DATE: **08-11-14**

APPROVED CONTAINMENT LEVEL: 2

FUNDING AGENCY: **Prostate Cancer Research Foundation of Canada**

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Wesbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 FAX: 604-822-5093

C.2 UBC animal care certificate

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A05-0967

Investigator or Course Director: Paul S. Rennie

Department: Surgery

Animals:

Mice Nude 700
Mice PTEN KO Transgenic mice, covered under Protocol #A04-0236 80
Mice Nude, female 6

Start Date: July 1, 2001 **Approval Date:** July 9, 2008

Funding Sources:

Funding Agency: Prostate Cancer Research Foundation of Canada
Funding Title: Oncolytic HSV-1 with a 3'UTR regulatory element for prostate cancer specific virotherapy

Funding Agency: Prostate Cancer Research Foundation of Canada
Funding Title: Oncolytic HSV-1 with a 3' UTR regulatory element for prostate cancer specific virotherapy

Funding Agency: Prostate Cancer Research Foundation of Canada
Funding Title: SiRNA knockdown of Androgen Receptors to Treat Androgen -independent Prostate Cancers

Funding Agency: National Cancer Institute of Canada
Funding Title: Program on prostate cancer progression

Unfunded title: n/a

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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