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Improved anti-tumor therapy based upon infectivity enhanced adenoviral delivery of RNA interference in ovarian carcinoma cell lines

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Abstract

Background—Hec1 (Highly-Expressed-in-Cancer) has recently been shown to play an important role in the proper segregation of chromosomes during mitosis. Recently, an Adenovirus delivery system carrying RNA interference (RNAi) of Hec1 has been reported in a cervical adenocarcinoma model. Adenoviral delivery systems, however, have the main limitation of poor viral infectivity due to lack of the native receptor, Coxsackie-Adenovirus Receptor (CAR), on the surface of tumor cells. We hypothesize that the viral infectivity of the Adenovirus vector would be enhanced via a CAR-independent pathway by altering the targeting tropism, thus increasing the knockdown effect of Hec1 expression in ovarian carcinoma cells.

Methods—Two Adenoviruses (Ad-siRNA-Hec1 and Ad-siRNA-Hec1.F5/3), along with a negative control (Ad-siRNA-GAPDH.F5/3) were created using homologous recombination. HEY and SKOV3.ip1 cell lines were used to perform experiments. The following assays were then used to determine RNAi knockdown efficiency: 1.Quantitative PCR (QPCR), 2.Western blot, 3.MTS Assay, 4.Annexin V-FITC FACS, 5.Crystal violet staining. In all experiments a negative control served as a baseline measure.

Results—QPCR demonstrated a 2 log viral infectivity enhancement with Ad-siRNA-Hec1.F5/3 over Ad-siRNA-Hec1. QPCR at 72 hours revealed mRNA knockdown induced by Ad-siRNA-Hec1 and Ad-siRNA-Hec1.F5/3 in SKOV3.ip1 and HEY cells, respectively (71%/60%, and 32%/78% mRNA knockdown compared to negative control). Western blot revealed translational inhibition induced by both Hec1 Ads with the least knockdown seen with Ad-siRNA-GAPDH.F5/3. FACS analysis revealed increased Annexin V positivity in RNAi infected cells, suggesting a higher rate of apoptosis. MTS assay indicated increased cell death 8 days post-infection with Ad-siRNA-Hec1 and Ad-siRNA-Hec1.F5/3 in SKOV3.ip1 and HEY cell lines, respectively (75% vs. 35% and 43% vs.

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12% viable cells). Crystal violet staining revealed increased cell death with Ad-siRNA-Hec1.F5/3 in all tested cell lines.

Conclusions—RNAi against Hec1 results in gene expression knockdown and apoptosis *in vitro*. The infectivity enhanced adenovirus as delivery mechanism shows potential application in future gene therapy models of RNAi in ovarian cancer.

Keywords

RNA interference; adenovirus vector; infectivity enhanced adenovirus; ovarian cancer

Introduction

The aggressive nature and poor prognosis associated with advanced and recurrent ovarian carcinoma have led investigators to pursue alternative therapeutic strategies. Gene therapy as an alternative strategy in ovarian carcinoma has shown promise in preclinical models and in phase 1 trials. Gene therapy strategies that have been employed in the past include replacement of defective tumor suppressor genes (p53), molecular chemotherapy, alteration of drug sensitivity, and targeted oncolytic viral therapy [1]. The discovery of RNA interference (RNAi) has opened the door to a new world of potential application of gene therapy in cancer therapy.

The Hec1 gene, located on chromosome 18, has been shown to play a critical role in the proper segregation of chromosomes throughout the mitotic cycle [2]. RNAi of Hec1 mRNA expression has been shown to induce apoptosis *in vitro* through mitotic catastrophe. Also, it has been shown that incorporation of Hec1 siRNA (short interfering RNA) into an Adenoviral vector can result in gene expression knockdown and apoptosis in a cervical adenocarcinoma and glioma cell lines [3]. Our laboratory has also shown that Hec1 is overexpressed in our experimental ovarian carcinoma cell lines relative to a human fibroblast cell line. (Figure 1) Thus it is possible that RNA interference of Hec1 could lead to apoptosis and cell death in ovarian carcinoma cells, making it an attractive therapeutic target in ovarian carcinoma.

As with any therapeutic cancer model, effective delivery to the proper target is the rate limiting factor. This holds true for RNAi. RNAi strategies that have been employed in cancer gene silencing strategies include delivery of double stranded RNA complexes (siRNA), the use of double stranded DNA vectors encoding short hairpin containing RNA sequences (shRNA), or the use of viral vectors containing double stranded DNA encoding for shRNA sequences. Viral vectors are particularly attractive because they have been shown to induce long term knockdown of gene transcripts *in vivo*. Thus, the use of RNAi in a stable viral vector system, such as the Adenovirus, is a possible strategy for stable gene knockdown.

In the context of ovarian carcinoma, the use of the unmodified Adenovirus is limited by its ability to infect ovarian cancer cells, which are generally depleted of the human Coxsackie-Adenovirus receptor (CAR) [4]. Advances in infectivity have been made in the past by genetically modifying the Adenovirus to enhance viral transductional efficiency. This has resulted in enhanced transduction of the human Adenovirus into ovarian carcinoma cell lines [5,6]. We therefore postulate that by using the infectivity enhanced Adenovirus as a vector for RNAi delivery, we can improve the efficiency of Adenovirus transduction leading to enhanced mRNA knockdown.

Materials and Methods

Cell lines and primary ovarian tissue samples

The ovarian carcinoma cell lines HEY and SKOV3.ip1 were used in our experiments. HEY cells were obtained from American Type Culture Collection (Manassas, VA). The cell line SKOV3.ip1 was a kind gift from Dr. Judith Wolf (University of Texas M.D. Anderson Cancer Center, Houston, TX). 293 cells, which were used for viral propagation, were purchased from the American Type Culture Collection. All cell lines were maintained in recommended media in the presence of fetal bovine serum (FBS), and grown at 37°C in a humidified atmosphere of 5% CO₂.

Construction of Ad-siRNA

The Ad-siRNA-Hec1 (AdHec1) and the shuttle vectors, Shuttle-RNAiHec1 and Shuttle-RNAiGAPDH, were kind gifts from Dr. Esteban Gurzov (Universidad Autonoma de Madrid). AdHec1 was directly used in this study; Shuttle-RNAiHec1 and Shuttle-RNAiGAPDH, which contain the DNA sequences corresponding to the shRNA for Hec1 and GAPDH genes described by Dr. Gurzov [3], respectively, were used to construct Ad-siRNA-Hec1.F5/3 (AdHec1F5/3) and Ad-siRNA-GAPDH.F5/3 (AdGAPDH) vectors. Both vectors were modified by using a chimeric fiber composed of domains of Ad5 knob and Ad3 knob. The viral infectivity of these Ad vectors was enhanced by tropism modification (F5/3) via a CAR-independent pathway [5]. The AdGAPDH was used in this study as a negative control compared to the siRNA-Hec1-based Ad vectors, AdHec1 and AdHec1F5/3.

The Ad-siRNA.F5/3 genomes were constructed via homologous recombination in *Escherichia coli* as described previously [7]. A 524bp DNA fragment was amplified from the Shuttle-RNAiHec1 and Shuttle-RNAiGAPDH, respectively, by using PCR method and one pair of oligos (5' gatcgggtacctgtctagggagagatccggtacca and 5' ccgcaagcttctcagcaccctccagatctgc) which were engineered by restriction site *KpnI* and *HindIII*, respectively. The PCR products contained the CMV promoter, a siRNA, and a polyA terminator. After cutting with restriction enzymes, *KpnI* and *HindIII*, the PCR products were subcloned into AdEasy pShuttle vector (Quantum, Appligene) by using the same sites to generate AdpShuttle-siRNA-Hec1 and AdpShuttle-siRNA-GAPDH.

The Ad backbone vector, pVK500F5/3 [a kind gift from Dr. V. Krasnykh (M.D. Anderson, Houston, TX)] contains both the Ad5 genome and a capsid modified F5/3 in its fiber region. After cleavage with *Pme I*, the AdpShuttle vectors were recombined with pVK500F5/3 to generate an Ad-siRNA-Hec1.F5/3 and Ad-siRNA-GAPDH.F5/3 genome. The resultant Adenovirus vectors encoded the siRNAs, Hec1 and GAPDH, respectively, in deleted E1 region and carried a F5/3-modified fiber. The generated Ad vectors were linearized with *Pac I* and transfected into 911 cells using lipofectamine (Invitrogen, Carlsbad, CA). Viruses were propagated in 911 cells, and purified by double CsCl density gradient centrifugation, followed by dialysis against phosphate-buffered saline (PBS) containing 10% glycerol. The viruses were titrated by TCID₅₀, and vp number was determined spectrophotometrically based on absorbance at a wavelength of 260nm.

The shRNA inserts in each virus are located in the E1 deleted region of the genome. A CMV promoter was used to initiate DNA transcription and a polyA tail was also used for purposes of transcriptional termination. The CMV promoter was used because an RNA Pol II promoter appears to be somewhat more time and tissue specific than RNA Pol III promoters such as the U6 and H1 promoters used in some RNAi experiments [8]. All viruses used were replication incompetent.

Quantification of Infectivity Enhancement of the F5/3 Modified Reagent using Quantitative Polymerase Chain Reaction (qPCR)

The experimental cell lines were plated in 12 well plates in their recommended media. At 90% confluence, each cell line was infected with AdHec1 and AdHec1F5/3 at a multiplicity of infection (MOI) of 100 vp/cell. After a three hour infection period, the cells were washed three times with PBS to remove all uninternalized virus. Next, genomic DNA was purified using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Quantitative PCR for the E4 region of the viral genome was then performed to determine the number of infectious particles internalized. The primers used for amplifying the E4 region were forward 5'-GGAGTGC GCCGAGACAAC-3' and reverse 5'-ACTACGTCCGGCGTTCCAT-3' and detected with the probe 6FAM-TGGCATGACACTACGACCAACAC-TAMRA. Human β -actin, a housekeeping gene, was amplified to normalize the Adenovirus gene copies within the cells.

Quantification of mRNA Knockdown of Ad-siRNA-Hec1 and Ad-siRNA-Hec1.F5/3 using Real-Time Quantitative Polymerase Chain Reaction (RT-PCR)

For determination of mRNA knockdown of Hec1, all experimental cell lines were plated into 12 well plates and grown in 2% FBS media until 75-90% confluent. The cells were then infected with AdHec1, AdHec1F5/3, or AdGAPDH at a MOI of 500 vp/cell. Three wells were left uninfected to serve as a negative control. All infections were performed in triplicate. After twelve hours, the viral containing media was removed and replaced with fresh media containing 2% FBS. After a 72 hour infection period, the cells were collected by trypsinization and total RNA was isolated with RNeasy kit (Qiagen, Valencia, CA) using the manufacturer's recommended protocol. Next, purified RNA was analyzed to determine the level of mRNA knockdown of Hec1 using TaqMan primers and probes designed by the Primer Express 1.0 software and synthesized by Applied Biosystems (Foster City, CA). The sequences to amplify the Hec1 gene were forward primer AACCAAGGACCTGGAAGCTGA, reverse primer TGTTTCAATCGCTTCTTTGCC and probe 6FAM-TGGCATATTTTAACTCCTCATTCCACAATTCTG-TAMRA. Human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene for internal control. The sequences to amplify GAPDH gene were forward primer GGTTTACATGTTCCAATATGATTCCA, reverse primer ATGGGATTTCCATTGATGACAAG and probe 6FAM-CGTTC TCAGCCTTGACGGTGCCAT-TAMRA. The PCR experiments were then carried out as previously described [7].

All PCR experiments were carried out using a LightCycler™ System (Roche Molecular Biochemicals, Indianapolis, Indiana). Thermal cycling conditions were subjected to 30 minutes at 48 °C, 10 minutes at 95 °C and 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Data was analyzed with LightCycler software 3.

Determination of Hec1 translational inhibition using Western blotting technique

All experimental cell lines were plated in 6 well plates and allowed to grow to 75% confluence. At this point, each cell line was treated with AdHec1, AdHec1F5/3, AdGAPDH at a MOI of 500 vp/cell. A negative control (MOCK) was also used in the experiment. After twelve hours, the viral containing media was removed and replaced with fresh media containing 10% FBS. After 96 hours, the cells were washed in phosphate buffered saline (PBS) and lysed in Laemmli buffer and stored at -20°C until use. Equal amounts of protein were separated by SDS-PAGE gel electrophoresis and transferred onto a PVDF membrane. The membrane was blocked overnight in 5% nonfat dry milk at 4°. The next morning, the membrane was washed for thirty minutes in TBS-Tween and then incubated with a 1:1000 dilution of Gene-Tex mouse anti-Hec1 monoclonal antibody (Abcam, Cambridge, MA) and mouse anti- β actin monoclonal

antibody (Sigma, St. Louis, MO) in TBS-Tween for one hour. The membrane was again washed in TBS-Tween and then incubated with a secondary peroxidase-conjugated antibody (dilution 1:2000) (Sigma, St. Louis, MO) for 1 hour. Antibody binding was then detected using a chemiluminescence detection system. Western blot films were then developed and analyzed for the level of Hec1 translational inhibition. β -actin was used to ensure equal loading of protein.

Determination of apoptosis induction using Annexin V-FITC flow cytometry

Induction of apoptosis was determined using the Annexin V-FITC (fluorecein isothiocyanate)/PI (propidium iodide) method (Pharmigen, San Diego, CA). All ovarian cancer cell lines were grown in 25 mm flasks in 10% FBS media until 90% confluent. Then, each cell line was infected with AdHec1 or AdHec1F5/3 at a MOI of 500 vp/cell in 2% FBS media. Twelve hours after infection, the media was removed and replaced with fresh media with 2% FBS. Four days after infection, cells were collected by trypsinization, washed three times with PBS, and assayed for the presence of extracellular phosphatidylserine using a conjugated Annexin V-FITC antibody with Propidium Iodine (PI) used to identify necrotic cells. All experiments were compared to a negative control group of cell lines which were left uninfected (MOCK). All experiments were performed at the University of Alabama at Birmingham Core FACS facility.

Determination of cell death using MTS cell viability assay and crystal violet staining

Cell viability experiments were performed using the MTS ([3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium]) dye reduction assay (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI). The experimental cell lines were plated at 1×10^4 cells/ well in 96 well plates in recommended media and allowed to adhere overnight. The next day, cells were infected with AdHec1, AdHec1F5/3, or AdGAPDH at a MOI of 500 vp/cell. To serve as a baseline negative control, three wells for each cell line were left uninfected (MOCK). All experiments were performed in triplicate. Infections were allowed to carry out for 2, 4, 6, and 8 days. At each time point, MTS solution was added to the wells in a 1:5 ratio and allowed to incubate for 3 hours at 37°C in 5% CO₂ humidified atmosphere. Next, the wells were analyzed with a plate reader to detect absorbance at 490 nm. The results were plotted against a previously derived standard curve to generate cell viability. All cell viability results are expressed as a percentage of viable cells compared to uninfected control at each time point.

To further determine cell death using a crystal violet assay, the three experimental cell lines were grown in 24 well plates in media containing 2% FBS to a confluency of 50%. At this point, the cells were infected in media containing 2% FBS with AdHec1, AdHec1F5/3, or AdGAPDH at a MOI of 1000 vp/cell, 100 vp/cell, 10 vp/cell, 1 vp/cell, 0.1 vp/cell, and 0 vp/cell. Twelve hours after infection, the viral containing media was removed and replaced with fresh media containing 2% FBS. After 8 days, the media was removed, the cells were fixed in 10% formalin, and then stained with 1% crystal violet to visualize cell death.

Statistical Analysis

Results were analyzed by t-test using SPSS software (Chicago, IL). Statistical testing was used to determine if AdHec1F5/3 improved transduction efficiency, mRNA knockdown, and cell death compared to AdHec1. A p-value < 0.5 was considered statistically significant.

Results

The goal of the following experiments was twofold: to determine if infectivity enhancement improved the efficiency and efficacy of Adenovirally mediated RNA interference against Hec1 and to determine if RNA interference against Hec1 mRNA leads to apoptosis, making it a

potential therapeutic target in ovarian carcinoma cell lines. The following experiments tested AdHec1 versus AdHec1F5/3. Although in most experiments there was an uninfected set of cells (MOCK) used as a negative control, the virus AdGAPDH was also used to control for viral infection itself.

Infection with Ad-siRNA-Hec1.F5/3 Results in Enhanced Infectivity in Ovarian Cancer Cells

After a three hour infection with Ad-Hec1 or AdHec1F5/3, qPCR was used to determine if the F5/3 capsid modification will enhance viral infectivity. (Figure 2) Detection of the E4 segment of the Adenoviral genome was used as a surrogate for viral transduction. In SKOV3.ip1 cells, there were 5.95×10^2 E4 copies/ ng DNA in cells infected with AdHec1 versus 2.64×10^4 E4 copies/ ng DNA in cells infected with AdHec1F5/3 ($p < .05$). In HEY cells, there were 3.85×10^2 E4 copies/ ng DNA in cells infected with AdHec1 versus 1.44×10^4 E4 copies/ ng DNA in cells infected with AdHec1F5/3 ($p < .05$). There was a 2 log increase in viral transduction with the infectivity enhanced Adenovirus.

Hec1 mRNA Expression Knockdown is Achieved with Ad-siRNA-Hec1 and Ad-siRNA-Hec1.F5/3

To test for Hec1 mRNA expression inhibition, qPCR was used to determine if RNAi directed against Hec1 transcripts resulted in mRNA knockdown in ovarian cancer cell lines after 72 hours. (Figure 3) In HEY cells, the number of Hec1 copies/ ng RNA was: 13.2 (+/- 4.17), 8.93 (+/- 2.36), 2.94 (+/- .69), and 7.75 (+/- 2.07) for MOCK, AdHec1, AdHec1F5/3, and AdGAPDH respectively. In Skov3.ip1 cells, the number of Hec1 copies/ ng RNA was: 10.5 (+/- 2.62), 3.02 (+/- .66), 4.18 (+/- 1.41), and 8.56 (+/- 2.52) for MOCK, AdHec1, AdHec1F5/3, and AdGAPDH respectively. This translated to 32% and 78% mRNA knockdown in HEY cells and 71% and 60% in Skov3.ip1 cells with AdHec1 and AdHec1F5/3 respectively. The infectivity enhanced Adenovirus did enhance mRNA knockdown in the HEY cell line, but not the SKOV3.ip1 cell line ($p=ns$ for both cell lines).

Hec1 Protein Expression is Inhibited by Infection with Ad-siRNA-Hec1 and Ad-siRNA-Hec1.F5/3

A Western blot experiment was performed after 96 hours to determine the level of translational inhibition induced by virally mediated RNA interference of Hec1. Western blot of protein lysates are depicted in figure 4. Translational inhibition of Hec1 is noted in tested cell lines, with minimal knockdown seen with cells infected with AdGAPDH. In the HEY cell line, there appears to be enhanced knockdown with AdHec1F5/3 infected cells versus infection with the unmodified virus. In SKOV3.ip1 cells, there appears to be minimal benefit to the infectivity enhanced Adenovirus versus the unmodified virus in terms of translational inhibition.

Apoptosis is Induced by RNA Interference of Hec1 in Ovarian Cancer Cell Lines

To determine if virally mediated RNAi directed against Hec1 resulted in increased apoptosis, our experimental cell lines were infected with Ad-Hec1, AdHecF5/3, and AdGAPDH as described in Materials and Methods. Our experimental cell lines displayed increased Annexin V positivity when infected with both Hec1 Ads. When compared to cells infected with AdHec1, cells infected with AdHec1F5/3 displayed increased Annexin V positivity (12.4% vs. 44.8% in HEY cells and 13.49% vs. 22.22% in SKOV3.ip1 cells). (Figure 5)

RNA interference of Hec1 Induces Cell Death in Ovarian Cancer Cell Lines

Given that RNAi induced knockdown of Hec1 led to transcriptional and translational inhibition and led to induction of apoptosis, it would be expected that cell death might be induced in our experimental cell lines. MTS cell viability assays were utilized to determine if mRNA knockdown of Hec1 lead to cell death in ovarian carcinoma cells. Infected cell lines were

assayed at 2, 4, 6, and 8 days post infection; a mock infection (no viral infection) was used as a baseline measure of cell viability. (Figure 6) In all tested cell lines, RNA interference of Hec1 led to cell death. In AdHec1 infected cells, the percentage of viable cells remaining after 8 days was 43% and 75% in HEY and SKOV3.ip1 cells respectively. In cells infected with the infectivity enhanced Adenovirus (AdHec1F5/3), the percentage of viable cell remaining after 8 days was 12% and 35% in HEY and SKOV3.ip1 cells respectively. In both tested cell lines, AdHec1F5/3 improved cell death over AdHec1 after 8 days ($p < .05$ for tested cell lines).

Cell killing was evaluated by standard crystal violet staining method after an 8 day period. (Figure 7) Intensity of staining in cells directly correlates with the number of viable cells present in each well. In the HEY cell line, cell kill is significantly enhanced by the infectivity enhanced Adenovirus over the unmodified Adenovirus (2 log improvement in cell kill). In the SKOV3.ip1 cell line, the cell kill induced by AdHec1F5/3 is slightly better than the unmodified AdHec1 virus.

Discussion

The application of RNA interference has become one of the most widely used methods of gene silencing since its discovery in 1998 [9]. The details by which RNAi causes gene specific degradation of mRNA are still being elucidated. However, it is known that double stranded RNA segments (dsRNA) are processed into 20 to 25 nucleotide short interfering RNA (siRNA). These siRNA's are incorporated into a complex that binds homologous target RNA's, which ultimately undergo cleavage, leading to gene silencing. The specificity by which RNAi binds mRNA in a sequence specific manner has opened the door to the possibility of using this technique in a model of cancer therapeutics [10-12].

RNA interference as a therapeutic model in cancer therapy has only recently experienced a wealth of discovery. In the realm of ovarian cancer, the literature is limited but growing. For example, Duan et al. demonstrated that the use of RNAi directed against MDR1, a gene associated with resistance to paclitaxel, can increase sensitivity of the drug when used to treat cell lines expressing MDR1 [13]. siRNA knockdown of Her-2/neu has also been shown to decrease the expression of VEGF and increase the expression of thrombospondin, a potent antiangiogenic factor [14]. RNA interference strategies have also been used to target genes associated with the development of cervical and endometrial carcinoma [15,16].

Hec1 has recently emerged as an important component in cell cycle regulation. It has been shown to be a critical component in kinetochore-microtubule formation. Hec1 is believed to interact with checkpoint proteins that are essential in cell cycle progression [2,17]. Gurzov et al. demonstrated that RNAi against Hec1 inhibited cervical adenocarcinoma cell growth *in vivo* [3]. Consistent with data presented by Gurzov, the data we have presented demonstrated that suppression of Hec1 leads to cell death in ovarian carcinoma cell lines. Based on this, Hec1 could be an attractive target for cancer therapy and should be explored further.

As with any potential therapeutic model, delivery is the rate limiting step. One of the major obstacles in gene therapy for ovarian carcinoma is delivery to the target. Specifically in regards to Adenoviral gene therapy, the Coxsackievirus-Adenovirus receptor (CAR) plays a crucial role in providing an entry point of the virus into the tumor cell. This receptor, which plays a role in cell-cell adhesions, has been shown to be underexpressed in malignant cells, including ovarian carcinoma. Therefore, many Adenoviral strategies utilize CAR-independent pathways to enhance cell entry [18]. To this end, our vector has been tested in the past *in vitro* and *in vivo* in ovarian carcinoma and been shown to be effective as a vector [6].

Other authors have used alternative vehicles for delivery of RNAi. Sood et al. have shown efficient intraperitoneal transfer of siRNA using the neutral liposome 1,2-dioleoyl-sn-

glycero-3-phosphatidylcholine in an ovarian cancer model [19,20]. Other methods of delivery employed in the literature include the use of polyethylenimine [21] and protamine [22]. Other viral vectors including the Retrovirus and Adeno-associated virus have also been used for delivery [23,24]. Now that RNAi strategies are becoming the method of choice for *in vitro* gene knockdown, the technique of *in vivo* delivery will need to be perfected.

In regards to RNAi strategies, the F5/3 modified Ad has been tested in the past. Uchida et al. demonstrated significant *in vivo* tumor suppression in a glioma model when the Ad-F5/3 vector was used to direct RNAi against the anti-apoptotic protein survivin [25]. This vector has not, however, been tested in ovarian carcinoma and no direct comparison between the modified and unmodified vector has been reported in regards to RNAi strategies.

Our infectivity enhancement experiment (Figure 2) indicates that the infectivity enhanced Adenovirus displays higher tropism toward our experimental ovarian carcinoma cell lines. Although the mRNA knockdown experiments performed (Figure 3) were not very consistent, the apoptosis, MTS, and crystal violet assays (Figures 5, 6, and 7) do show a consistent improvement in cell kill with the infectivity enhanced Ad (AdHec1F5/3) over the unmodified Ad (AdHec1). Also, it is apparent that the SKOV3.ip1 cell line performs better in terms of infectivity (Figure 2) compared to the HEY line. However, this does not translate in terms of apoptosis. We hypothesize that there may be some mechanism of resistance of Hec1 knockdown observed in the SKOV3.ip1 cell line compared to the HEY cell line similar to resistance to traditional cytotoxic chemotherapy seen in ovarian cancer. When our experimental reagents were tested in an *ex vivo* tissue slice model, transductional efficiency was inconsistent and varied (data not shown). This may be reflective of the genomic heterogeneity seen in many solid tumors, including the variation in levels of CAR expression seen in ovarian carcinoma [26]. Also, it can be noted that there is some mRNA inhibition, translational inhibition, and cell death noted when the experimental cell lines are treated with the Ad-GAPDH reagent. We postulate that this inhibition may be reflective of some mild toxic effect encountered with the 5/3-modified Adenovirus alone. Infection with the adenovirus has been shown to cause profound changes in host-cell macromolecular synthesis. Virion fiber proteins inhibit macromolecular synthesis when applied directly to cells bearing the adenovirus receptor. Cell-specific proteins involved in macromolecular synthesis, export of cellular mRNA from the nucleus to the cytoplasm, and cell-specific translation are all inhibited after infection. [27] Certainly future *in vivo* models will need to address potential toxicity associated with the use of genetically modified viral agents as vectors for delivery of therapeutic agents.

One potential pitfall of the Adenovirus as a delivery mechanism is the off target effects observed in past studies, particularly *in vivo* hepatotoxicity. The liver has been shown to be the major organ responsible for clearance of the adenovirus *in vivo* [28,29]. Another potential problem is in regards to the neutralizing antibody response elicited *in vivo* in response to Adenoviral infection [30].

In conclusion, RNAi against Hec1 results in mRNA knockdown and apoptosis *in vitro*, making Hec1 a potential novel therapeutic target in the treatment of ovarian carcinoma. Also, based on our results, the infectivity enhanced adenovirus as delivery mechanism shows potential application in future therapeutic models of RNAi in ovarian cancer. Future research in regards to viral delivery of RNAi strategies will need to focus on development of *in vivo* models and refining the Adenovirus to circumvent off target effects of the Adenovirus *in vivo*.

Abbreviations

bp

base pair

CMV	cytomegalovirus
RNAi	RNA interference
MOI	multiplicity of infection
pfu	plaque-forming units
vp	viral particles
Hec1	Highly Expressed in Cancer gene 1

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Figure 1.

Hec1 mRNA Expression in Ovarian Carcinoma Cell Lines.

Hec1 mRNA is expressed in ovarian carcinoma cell lines compared to a positive control (HeLa) and a human fibroblast (HFBC) control.

Figure 2.

Viral Infectivity of Ad-siRNA-Hec1 and Ad-siRNA-Hec1.F5/3 in Ovarian Carcinoma Cell Lines

Infectivity enhancement of AdHec1F5/3 over AdHec1 in ovarian carcinoma cells. The infectivity enhanced Adenovirus displayed approximately two log improvement in viral transduction over the unmodified virus. All experiments performed in triplicate. Error bars represent +1 standard deviation (SD). ($p < .05$ in both cell lines)

Figure 3.

RNAi induced mRNA Knockdown of Hec1 in Ovarian Carcinoma Cell Lines

Transcriptional inhibition induced by RNA interference of Hec1 by AdHec1 and AdHec1F5/3 in HEY and SKOV3.ip1 cell lines. AdGAPDH was used as a negative control virus. One sided error bars indicate +1 SD. (p=NS for both cell lines)

Figure 4. Translational Inhibition Induced by RNA interference of Hec1 in Ovarian Cancer Cell Lines. Western blot showing translational inhibition induced by RNA interference against Hec1. After 96 h, the cell lysates were collected, and expression of Hec1 was detected by Western blotting using mouse anti-Hec1 monoclonal antibody and peroxidase conjugated goat anti-mouse secondary antibody. Cell lysates were also stained for β -actin to ensure equal protein loading.

Figure 5.

Induction of Apoptosis Manifested by Annexin V Positivity in Ovarian Carcinoma Cell Lines
 Represented below are dot plots of experimental cell lines assayed for the presence of extracellular phosphatidylserine after viral infection using a conjugated Annexin V-FITC antibody with Propidium Iodine. In each plot, the vertical line separates Annexin V positive from negative cells; the horizontal line separates PI positive and negative cells.

Figure 6.
 MTS Cell Viability Assays
 Viability of ovarian carcinoma cells at day 2, 4, 6, and 8 after infection (MOI 500) with AdHec1, AdHec1F5/3, or AdGAPDH. Two sided error bars indicate $\pm$ 1 standard deviation

Figure 7.**Crystal Violet Staining**

Crystal violet staining of ovarian carcinoma cell lines after infection with AdHec1, AdHec1F5/3, and AdGAPDH. A no viral infection lane (MOCK) served as a negative control. An enhanced lytic effect is noted with the infectivity enhanced virus, especially in the HEY cell line. The negative control virus, AdGAPDH, showed minimal to no lytic effect in any tested cell line.