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Adenovirus-Mediated Delivery of Decoy Hyper Binding Sites Targeting Oncogenic HMGA1 Reduces Pancreatic and Liver Cancer Cell Viability

Faizule Hassan,[1] Shuisong Ni,[1] Tyler C. Arnett,[1] Melanie C. McKell,[1] and Michael A. Kennedy[1]

[1]Department of Chemistry and Biochemistry, Miami University, Oxford, OH 45056, USA

High mobility group AT-hook 1 (HMGA1) protein is an oncogenic architectural transcription factor that plays an essential role in early development, but it is also implicated in many human cancers. Elevated levels of HMGA1 in cancer cells cause misregulation of gene expression and are associated with increased cancer cell proliferation and increased chemotherapy resistance. We have devised a strategy of using engineered viruses to deliver decoy hyper binding sites for HMGA1 to the nucleus of cancer cells with the goal of sequestering excess HMGA1 at the decoy hyper binding sites due to binding competition. Sequestration of excess HMGA1 at the decoy binding sites is intended to reduce HMGA1 binding at the naturally occurring genomic HMGA1 binding sites, which should result in normalized gene expression and restored sensitivity to chemotherapy. As proof of principle, we engineered the replication defective adenovirus serotype 5 genome to contain hyper binding sites for HMGA1 composed of six copies of an individual HMGA1 binding site, referred to as HMGA-6. A 70%–80% reduction in cell viability and increased sensitivity to gemcitabine was observed in five different pancreatic and liver cancer cell lines 72 hr after infection with replication defective engineered adenovirus serotype 5 virus containing the HMGA-6 decoy hyper binding sites. The decoy hyper binding site strategy should be general for targeting overexpression of any double-stranded DNA-binding oncogenic transcription factor responsible for cancer cell proliferation.

INTRODUCTION

High mobility group AT-hook 1 (HMGA1) proteins are architectural transcription factors[1,2] that belong to the high-mobility group family of proteins.[3] HMGA1 proteins are intrinsically disordered, being composed of three "AT-hook"[1] DNA binding motifs that allow them to bind to the minor groove of DNA in AT rich regions.[4–6] HMGA1 proteins are referred to as architectural transcription factors because of their ability to bend or unwind DNA upon binding, thus changing the architecture of the DNA and proteins in the context of chromatin structure.[7] Due to the effect of HMGA1 binding on DNA and chromatin structure, HMGA1 proteins are recognized as non-histone chromatin proteins that play an essential role in chromatin remodeling required for regulation of gene transcription.[8,9] Mechanistically, the bending and unwinding of chromosomal DNA facilitates recruitment of conventional transcription factors to promoter sites, allowing for the establishment of protein complexes referred to as enhanceosomes,[10] that regulate gene expression.[8,11] The interferon (IFN)-β enhancer complex is recognized as the archetypical enhanceosome in which HMGA1 binding to the DNA along with other transcription factors was observed to cause a reversal in intrinsic DNA bending.[12–14] To date, more than 75 transcriptional targets of HMGA1 have been identified.[8] Structurally, HMGA1 proteins have also been reported to cross-link directly to DNA, indicating a potential role in higher order structuring of DNA and chromatin in the nucleus.[15]

In its normal role, HMGA1 is essential and critical to embryonic development; therefore, *HMGA* is expressed at high levels in embryonic tissues.[16] HMGA1 is normally expressed at very low levels in healthy differentiated somatic adult cells,[9] and its expression is usually upregulated only transiently in adult cells during certain adaptive immune responses where HMGA1 plays a role in the formation of enhanceosome complexes[17] that regulate gene expression in response to infection.[18] Normal HMGA1 function is involved in both positive and negative regulation of genes responsible for apoptosis, cell proliferation, immune response, and DNA repair,[18] among others, as discussed in a recent review by Sumter et al.[8]

The correlation between elevated HMGA1 expression and cancer was first discovered by Giancotti et al.[19] in 1985. Since then, elevated levels of high mobility group AT-hook 1 (HMGA1) protein have also been reported in almost every type of human cancer[8,20] and high levels of *HMGA*1 expression have been linked to metastases[21] and poor patient prognosis.[22] Overexpression of *HMGA*1 is correlated with increased cell proliferation and contributes to tumor growth,[8,20] in part due to suppression of the tumor suppressor protein, p53, causing inhibition of apoptosis.[23] HMGA1 contributes to tumorigenesis at both the transcriptional and post-transcriptional level through expression of onco-microRNA (miRNA) and interference in DNA repair and
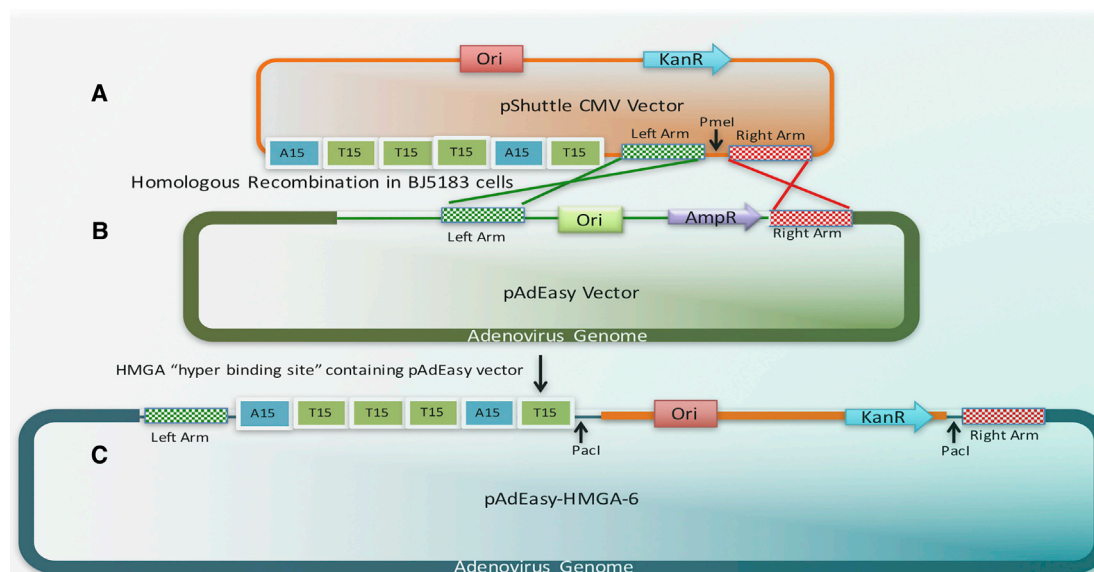
Figure 1. Schematic Depiction of the Design of the HMGA-6 Hyper Binding Site and Its Insertion into a Shuttle Vector Needed for Incorporation into the Virus Genome

(A) The HMGA-6 hyper binding site is depicted by six consecutive boxes labeled A15 or T15. The site was integrated into the pShuttle CMV vector in preparation for homologous recombination with the pAdEasy vector (B). The regions of sequence homology are designated as the "Left Arm" and the "Right Arm" common to both vectors. Successful homologous recombination resulted in insertion of the HMGA-6 hyper binding site into the adenovirus genome as indicated in (C).

apoptosis.[8,20,24] HMGA1 interferes with apoptosis by repressing p53 promoter activity, delocalizing the pro-apoptotic activator HIPK2 from the nucleus to the cytoplasm, and decreasing transcription of p53 activators BAX and cyclin-dependent kinase inhibitor 1A.[20,24] HMGA1 interaction with p53 also activates the transcription of MDM2, which is an inhibitor of p53.[20] HMGA1 activation of inflammatory pathways further promotes cancer progression by increasing cell proliferation, angiogenesis, and invasion.[25] High HMGA1 levels are also responsible for chemotherapy resistance in cancer cells,[26,27] including in pancreatic cancer cells.[28] Collectively, because of its role in promoting cancer and its ability to render cancer cells resistant to chemotherapy, HMGA1 is recognized as a biomarker for diagnosis and a therapeutic target for treating pancreatic cancer.[29] Suppression of *HMGA*1 expression by small interfering RNA (siRNA)[28] and lentivirus-mediated RNA interference with HMGA1[30] has been reported to restore sensitivity to gemcitabine. We have previously shown that transfection of human pancreatic cancer cells with phosphorothioate-substituted DNA aptamers containing single 15-base pair HMGA1 decoy binding sites reduced cancer cell viability and increased sensitivity to chemotherapy treatment.[31] Adenovirus-mediated suppression of HMGA1 protein synthesis has also been reported as a potential therapy for treating human malignant neoplasia.[32,33]

Here, we have tested the ability of a non-naturally-occurring hyper binding site for HMGA1 to suppress its oncogenic action in human cancer cells. The HMGA1 hyper binding site, which consisted of six copies of a single HMGA1 binding site, is referred to as HMGA-6. We intended to use HMGA-6 as a decoy for binding and sequestering excess HMGA1 in the nucleus of HMGA1-positive cancer cells. The vector for the replication defective adenovirus serotype 5 virus (i.e., Ad5), with the commercial name AdEasy, was engineered to incorporate HMGA-6, which consisted of six consecutive segments of 15 adenine or thymine residues. The engineered AdEasy vector containing the decoy HMGA-6 hyper binding site is referred to as AdEasy-HMGA-6. Virus particles containing the HMGA-6 hyper binding site were prepared by transfection of the linearized engineered genomic DNA into a complemented AD293 cell line that supported virus synthesis. The AdEasy-HMGA-6 virus was tested on four human pancreatic cancer cell lines (MIA PaCa-2, PANC-1, AsPC-1, and BxPC-3), a human liver cancer cell line (Hep-G2), and a non-cancerous immortalized human pancreatic duct epithelial cell line (E6E7). Infection of the cancer cells with AdEasy-HMGA-6 resulted in a significant reduction in cell viability for all of the cancer cell lines tested and caused no loss of cell viability in a non-cancerous human pancreatic ductal epithelial cell line. All of the cancer cell lines tested exhibited an increased sensitivity to the chemotherapy agent gemcitabine following infection with AdEasy-HMGA-6.

RESULTS

Design and Construction of HMGA-6 Hyper Binding Sites

The HMGA1 hyper binding site was designed to contain six tandem 15-base-pair double-stranded DNA binding sites for HMGA1 (thus the hyper binding site is referred to as HMGA-6). Each of the six HMGA1 binding sites contained a run of 15 consecutive adenines reading from the 5′- to -3′ direction in either forward or reverse direction (Figure 1A) so that both segments were long enough to achieve
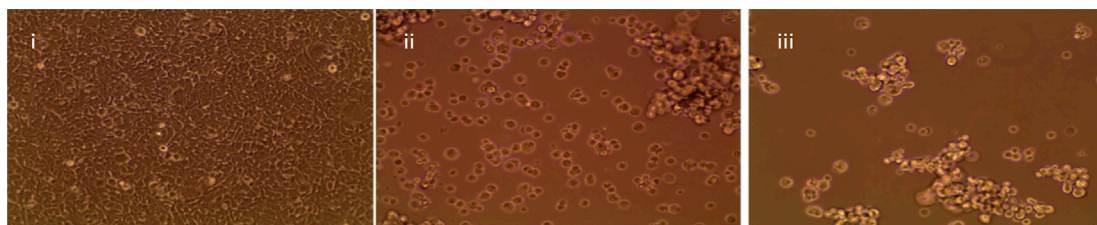
Figure 2. Cytotoxic Effects Caused by Viral Infection
(i) Negative control, AD293 cells transfected with the pUC-GFP plasmid DNA. (ii) Infection with AdEasy DNA caused detachment and clumping of cells characteristic of cytotoxicity associated with viral replication. (iii) Infection with the AdEasy-HMGA-6 DNA also resulted in a cytotoxic effect. All images were taken with a 20× objective lens.

the narrow minor groove structure required for tightest HMGA1 binding.[1,7] The HMGA-6 hyper binding site was inserted into a shuttle plasmid in preparation for homologous recombination (Figure 1A). Successful homologous recombination was carried out by transfecting both the shuttle plasmid containing the HMGA-6 hyper binding site (Figure 1A) and the pAdEasy vector containing the replication defective adenovirus serotype 5 genome (Figure 1B) into the homologous recombination competent *E. coli* BJ5183 strain, resulting in an engineered viral genome containing the HMGA-6 hyper binding site (Figure 1C), which is referred to as AdEasy-HMGA-6.

Confirmation of Virus Synthesis and Observation of Cytotoxicity due to Viral Replication

The expected cytotoxic effect of cell death and cell clumping caused by viral replication was observed when the AD293 cells (a derivative of HEK293 that complements missing genes in AdEasy required for viral replication) were transfected with linearized native AdEasy or AdEasy-HMGA-6 DNA, which indicated virus synthesis and replication (Figure 2). Virus synthesis was directly confirmed using immunocytofluorescence assays probing for virus hexon proteins (Figure 3). Since cells were not infected with virus, but transfected with linearized DNA encoding the viral genome, positive probing for viral coat proteins indicated virus synthesis inside cells.

Confirmation of HMGA1 Expression in Various Human Pancreatic and Liver Cancer Cell Lines

HMGA1 expression was measured in four human pancreatic cancer cell lines (MIA PaCa-2, AsPC-1, PANC-1, and BxPC-3), the human liver cancer cell line, HepG2, and the non-cancerous human pancreatic ductal epithelial cell line E6E7 (Figure 4). Western blot analysis confirmed HMGA1 expression in all of the cancer cell lines and lack of HMGA1 expression in the non-cancerous human pancreatic ductal epithelial E6E7 cell line (Figure 4).

Determination of Cell Viability after AdEasy-HMGA-6 Infection of Human Pancreatic and Liver Cancer Cells with and without Gemcitabine Treatment

The number of living MIA PaCa-2 cells determined by a cell viability assay was measured 72 hr after infection with replication defective AdEasy or AdEasy-HMGA-6 viruses in the presence or absence of gemcitabine (Figure 5A). MIA PaCa-2 cells were infected with three different viral doses (0.33, 3.3, and 33.3 virus particles per cell) and

treated with four different gemcitabine doses (0 nM, 1 nM, 10 nM, and 100 nM). There was no statistically significant reduction in the number of viable cells post infection with the AdEasy virus at any virus dose (Figure 5A; Table S1). A significant reduction in the number of viable cells was observed after AdEasy-HMGA-6 virus infection compared to infection with AdEasy virus at all gemcitabine doses based on calculated p values (Figure 5A; Table S1). Since both viruses were replication defective, the decreased number of viable cells following infection with AdEasy-HMGA-6 compared to infection with AdEasy was solely due to the additional presence of the HMGA-6 hyper binding site and not due to viral replication. In the absence of gemcitabine, infection of MIA PaCa-2 cells at a dose of 0.33 particles per cell (ppc) (i.e., one virus particle per three cells), resulted in a >60% reduction in the number of viable cells, and infection at doses of 3.3 or 33.3 virus particles per cell resulted in a nearly 80% reduction in the number of viable cells. This indicated that infection of HMGA1-positive cancer cells with viruses containing decoy HMGA-6 hyper binding sites significantly decreased proliferation, increased cytotoxicity, or increased apoptosis. The p values for comparison of the number of viable cells post AdEasy-HMGA-6 infection relative to post AdEasy infection or compared to untreated cells ranged from 10^{-6} to 10^{-9}. Whereas increasing the dose of AdEasy infection resulted in no significant reduction in the number of viable cells after 72 hr, increasing the dose of AdEasy-HMGA-6 caused an additional statistically significant reduction in the number of viable cells after 72 hr, with the p value for the 0.33 ppc dose compared to untreated cells equal to 1.4×10^{-6} and the p value for the 3.3 and 33.3 ppc doses reducing to $2–3 \times 10^{-9}$ (Table S1). Infection with AdEasy-HMGA-6 virus significantly increased sensitivity to gemcitabine, as indicated by a more than 2-fold reduction in the number of viable cells compared to gemcitabine treatment alone at all doses (Figure 5A; Table S1). Infection of the cancer cells with the AdEasy-HMGA-6 virus combined with gemcitabine treatment had an additive effect, resulting in a 4- to 8-fold decrease in the number of viable cells, depending on the viral dose compared to treatment with 10 nM gemcitabine alone. A table of p values for the comparison of all cell viability assays shown in Figure 5A can be found in Table S1.

The effect of AdEasy-HMGA-6 virus infection on cell viability was tested on three additional human pancreatic cancer cell lines (PANC-1, AsPC-1 and BxPC-3) and one human liver cancer cell
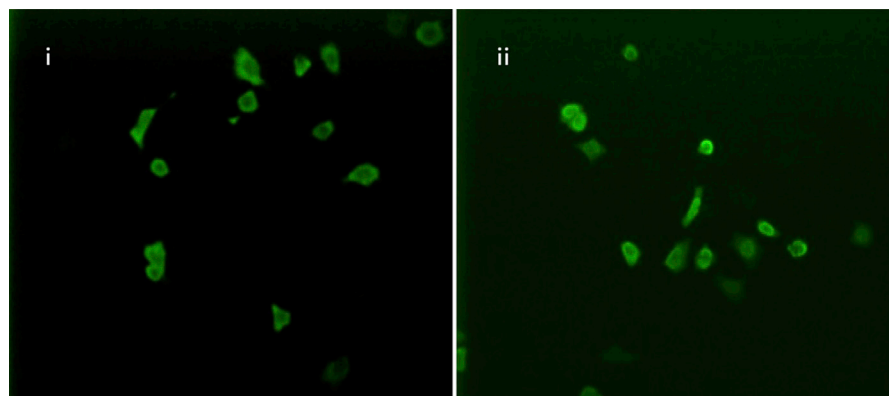
Figure 3. Immunocytofluorescence Assays for Viral Coat Proteins in Infected AD293 Cells
(i) Fluorescence images of AD293 cells infected with linearized native AdEasy DNA. (ii) Fluorescence images of AD293 cells infected with linearized AdEasy-HMGA-6 DNA. Assays for uninfected cells exhibited no fluorescence (data not shown).

line (HepG2) (Figures 5B–5E). While the efficacy of AdEasy-HMGA-6 treatment varied with the cell line, infection with AdEasy-HMGA-6 virus significantly reduced the number of viable cells 72 hr post infection and increased sensitivity to gemcitabine in all tested cell lines, based on the p values reported in Tables S2–S5. The AsPC-1 cell line was even more affected compared to MIA PaCa-2 cells, with the number of viable cells 72 hr post infected reduced by more than 70% compared to the control even at the lowest viral dose (Figure 5B). BxPC-3 was somewhat less sensitive compared to MIA PaCa-2 cells, with the number of viable cells 72 hr after infection dropping to 50% compared to the untreated cells (Figure 5C). The PANC-1 cells responded similarly to the MIA PaCa-2 cells (Figure 5D). While AdEasy-HMGA-6 virus infection also reduced the number of viable HepG2 liver cells 72 post infection, the HepG2 cell line was the least responsive, displaying a less than 20% drop in the number of viable cells at the lowest viral dose and reaching a maximum reduced cell number of about 70% with the highest viral dose of 33.3 ppc (Figure 5E). Combination of gemcitabine treatment with AdEasy-HMGA-6 virus infection caused a significant reduction in the number of viable cells 72 post infection compared to gemcitabine treatment alone in all of the cell lines tested. Viral doses of 3.3 and 33.3 ppc resulted in about a 40% and 70% reduction in the numbers of viable cells in the absence of gemcitabine treatment and resulted in a 2- to 5-fold reduction in the number of viable cells in combination with a 10 nM dose of gemcitabine. The most dramatic example of the effect of combined treatment on reduced cell viability can be seen in the HepG2 cell line, where the cell viability of about 80% was observed for cells treated with 10 nM gemcitabine was reduced to about 20% when combined with 33.3 ppc AdEasy-HMGA-6 virus treatment, representing about a 4-fold reduction in the number of viable cells. The p values for all comparisons for all cell types are included in the Supplemental Information. Tables of p values for comparison of all cell viability assays shown in Figures 5B–5E can be found in Tables S2–S5.

The effect of AdEasy-HMGA-6 infection on the non-cancerous E6E7 cell line (immortalized pancreas ductal epithelial cells) was also measured (Figure 5F; Table S6). AdEasy-HMGA-6 virus infection had no statistically significant effect on the number of viable E6E7 cells 72 hr post infection at any viral dose. In contrast, the number of viable E6E7 cells 72 hr after administration of gemcitabine dropped significantly at doses of 10 nM or 100 nM (Table S6). The absence of reduced E6E7 cell viability after AdEasy-HMGA-6 virus infection suggests that the AdEasy-HMGA-6 virus has the potential to selectively reduce the viability of HMGA1-positive cancer cells and increase their sensitivity to chemotherapy treatment while leaving non-cancerous HMGA1-negative cells unaffected, an observation which is important for eventual clinical application. A table of p values for comparison of all cell viability assays shown in Figure 5F can be found in Table S6.

Confirmation that AdEasy-HMGA-6 Infects the Non-cancerous E6E7 Human Pancreatic Epithelial Cells
It was possible that addition of AdEasy-HMGA-6 virus to E6E7 cells failed to cause reduced viability because AdEasy-HMGA-6 was unable to infect the E6E7 cells because they lacked the Coxsackievirus and adenovirus receptor (CAR) required for adenovirus infection. To experimentally address this possibility, we performed plaque assays by adding conditionally replicative Ad5ΔΔ virus engineered for selective replication in cancer cells to E6E7 cells and observed that they failed to replicate or cause lysis in E6E7 cells (Figures 6A–6C). We then added replication-competent wild-type Ad5 virus to the E6E7 cells and observed the expected cell death associated with viral infection followed by viral replication and subsequent cell lysis (Figures 6D–6F). These observations confirmed that adenovirus was capable of infecting E6E7 cells, and, consequently, the E6E7 cells must express the CAR required for adenovirus infection. Therefore, we can conclude that the AdEasy-HMGA-6 virus infected the E6E7 cells but did not cause reduced E6E7 cell viability, as observed in Figure 5F.
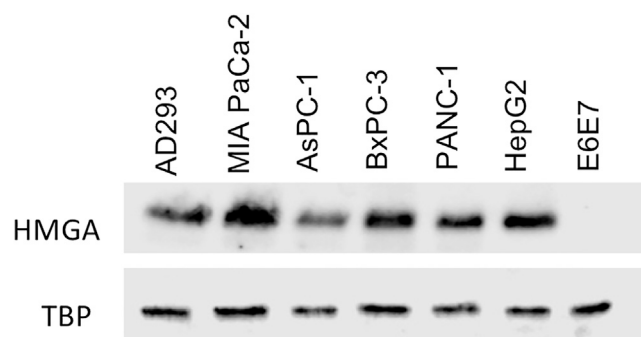
Figure 4. Western Blot Analysis of HMGA1 Expression Levels in Different Cancerous and Non-cancerous E6E7 Cell Lines

20 µg of total nuclear protein was loaded in each well, transferred to membrane and probed with rabbit anti HMGA1 antibody. TATA binding protein was used as loading control.

Confirmation that AdEasy-HMGA-6 Did Not Undergo Replication in Uncomplemented Cells

It was also possible that the reduced cell viability observed upon addition of AdEasy-HMGA-6 in each of the human cancer cell lines was due to unintentional and unanticipated replication of the virus in those cells. This possibility was unlikely given that the AdEasy vector from which the genome encoding the AdEasy-HMGA-6 virus was engineered to introduce the HMGA-6 hyper binding site lacked the E1 and E3 regions required for replication from the outset. Notwithstanding, the plaque assay experiments shown in Figure 6 confirmed that adenovirus was capable of infecting the E6E7 cells, and this also confirmed that AdEasy-HMGA-6 infected the E6E7 cells in the experiment for which the results are reported in Figure 5F. The data presented in Figure 5F illustrate that addition of AdEasy-HMGA-6 virus to E6E7 cells did not cause reduced cell viability, thereby indicating that AdEasy-HMGA-6 did not replicate in the E6E7 cells. This result confirmed that AdEasy-HMGA-6 does not replicate in uncomplemented cells, thus supporting that the reduced cell viability observed in each of the cancer cell lines expressing HMGA1 upon addition of AdEasy-HMGA-6 was due to the introduction of the HMGA-6 hyper binding site into the nucleus of those cells and not due to unintentional replication of the AdEasy-HMGA-6 virus.

Apoptosis and Necrosis Assays following AdEasy-HMGA-6 Infection of Human Pancreatic MIA PaCa-2 Cells

In an effort to study the mechanism of cell death, MIA PaCa-2 cells were infected with either AdEasy-HMGA-6 (Figures 7A and 7C) or AdEasy (Figures 7B and 7D) at four different doses (10, 20, 40, and 80 ppc) and hallmarks of apoptosis (Figures 7A and 7B) or necrosis (Figures 7C and 7D) were monitored for 120 hr following treatment. As shown in Figure 7A, at all of the doses of AdEasy-HMGA-6, the luminescence signal, which measured the apoptotic hallmark, sharply increased within 6 hr of infection and then started to decrease, returning to the baseline levels by 30 hr post-infection. However, when the cells were infected with

the AdEasy virus, no significant increase of either luminescence or fluorescence signal was detected (Figures 7B and 7D). Likewise, there was no sign of apoptosis in the cells that did not receive any treatment (either virus). However, the increase of fluorescence signal that indicated cell membrane disintegration (necrosis hallmark) did not start to increase until 24 hr in AdEasy-HMGA-6 infected cells (Figure 7C). This type of delayed increase in necrosis hallmark is called secondary necrosis, which is a normal phenomenon that can be observed in the absence of phagocytic cells in the case of apoptosis.

DISCUSSION

While the precise mechanistic role that HMGA1 plays in cancer is not yet fully understood, elevated HMGA1 levels in cancer cells are known to cause cancer cell proliferation due to misregulation of affected genes and increased chemotherapy resistance.[8,20,24] Consequently, HMGA1 has been suggested as a potential biomarker for tumor progression.[29] Also, since high levels of HMGA1 expression are generally associated with poorer prognosis and outcome, HMGA1 is now being considered a drug target for cancer drug therapy development.[29] In this paper, we have introduced a new strategy of delivering decoy HMGA1 hyper binding sites into the nucleus of cancer cells using an engineered adenovirus. Cellular infection with an adenovirus typically occurs when a fiber domain on the adenovirus binds to a CAR on the outside of the cell and to associated integrins, which promotes endosomal-facilitated internalization of the virus into the cell. Once internalized inside the cell, the fiber domains of the virus are shed and the endosomal-encapsulated virus is transported to the vicinity of a nuclear pore. At this time, the virus escapes the endosomal capsule and binds to the nuclear pore, resulting in passage of the viral DNA into the nucleus of the cell. The rationale for the decoy strategy is that delivery of non-naturally occurring hyper binding sites for HGMA1 to the nucleus of cancer cells will cause sequestration of the overexpressed HMGA1 architectural transcription factors at the decoy binding sites due to competition of the hyper binding sites for binding to HMGA1 with the naturally occurring binding sites on the genomic DNA. We anticipate that competitive binding of HMGA1 to the decoy hyper binding sites should result in a benign outcome because the decoy binding sites are not involved in the regulation of any genes. This suggestion was supported by our observation that infection of the non-cancerous E6E7 human pancreatic epithelial cells with the AdEasy-HMGA-6 virus did not exhibit reduced cell viability. Our hypothesis is that sequestration of excess amounts of nuclear HMGA1 at the decoy hyper binding sites should mitigate the oncogenic consequences of overexpressed HMGA1 and cause the cells to be less oncogenic and also reduce the HMGA1-associated chemotherapy resistance. It has been recently demonstrated that colon cancer cells and anaplastic thyroid cancer cells expressing high levels of HMGA1 are more resistant to chemotherapeutic drug 5-fluorouracil and doxorubicin compared to the same cells that do not express HMGA1.[26,27] Our results suggest that sequestration of HMGA1 with the hyper binding sites increases sensitivity to gemcitabine in all tested cancer cell lines.
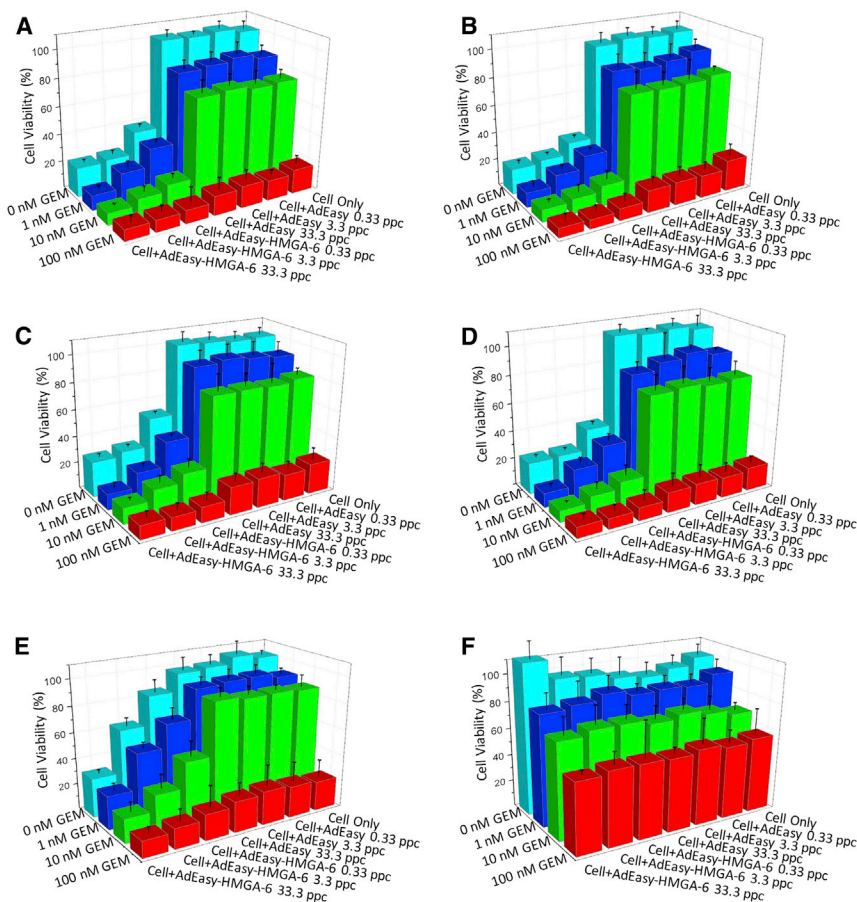
Figure 5. Cell Viability Assays following Treatment of Pancreatic and Liver Cancer Cells with AdEasy-HMGA-6 DNA

(A–F) The effect of AdEasy-HMGA-6 infection on cell viability was determined by comparing virus treated and untreated cell lines in the presence or absence of chemotherapy drug gemcitabine (GEM). 3 × 10³ cells were seeded in a 96-well plate 24 hr before infection for (A) MIA PaCa-2, (B) AsPC-1, (C) BxPC-3, (D) PANC-1 (E) HepG2, and (F) E6E7. Cells were infected at three different viral doses (0.33, 3.3, and 33.3 ppc) of AdEasy or AdEasy-HMGA-6 virus in the presence or absence of four different doses of GEM (0 nM, 1 nM, 10 nM, and 100 nM). Cell viability was measured 72 hr after infection and/or gemcitabine treatment using the CellTiter 96 Aqueous one solution cell proliferation assay kit (Promega). Results were averages of two experiments performed in triplicate (n = 6). The data points represent the mean of the measurement. The error bars represent ± SD.

HMGA1 has been shown to decrease cellular apoptotic pathways by: (1) preventing the p53-mediated apoptosis by translocating the HIPK2, a p53 proapoptotic activator, from nucleus to cytoplasm; (2) inhibiting transcriptional suppression of the anti-apoptotic gene Bcl-2; (3) decreasing the expression of the Bcl-2-specific miR-34; and (4) regulating the p53 expression by suppressing its promoter activity.[26,27] Consistent with previous studies, our results showed that sequestration of HMGA1 by the hyper binding sites increased apoptotic cell death in a dose-dependent manner in the MIA PaCa-2 cancer cell line. It is our further hypothesis that sequestration would also restore normal mechanisms of apoptosis, leading to death of the cancer cells, and provide more time for the chemotherapy agents to cause cell death. Our study indicates that sequestration of overexpressed oncogenic HMGA1 in pancreatic and liver cancer cells by decoy hyper binding sites targeting HMGA1 significantly reduces the number of viable cancer cells 72 hr post infection. A significant drop of ∼60% in the number of viable cancer cells was observed even with an infection dose corresponding to one virus particle per three cells. The reduction in the number of viable cells post infection could be due to reduced cell proliferation, increased apoptosis, or a combination of both. The observation of a nearly two-thirds decrease in the number of viable cells when only one cell out of three was

infected likely indicates a non-linear reduction in cell proliferation, possibly due to a bystander effect. Our data suggested that the apoptosis stimulated by infection with AdEasy-HMGA-6 promoted secondary necrosis, which likely represents a bystander effect that could explain the significant reduction in cell viability, even at sub-stoichiometric doses of virus numbers per cell. Such a secondary bystander effect caused by apoptosis has been reported in the literature and referred to as "contagious apoptosis."[34] Higher doses of three virus ppc and 33 virus ppc further dramatically decreased the cancer cell viability to only 20% compared to uninfected cells. Mechanistically, the dose versus response is not clear at this time. At 33 virus ppc, this would correspond to 204 HMGA1 binding sites added to the nucleus. One competitive advantage that the HMGA-6 hyper binding sites have over the naturally occurring genomic binding sites for HMGA1 is that they are easily accessible to nuclear HMGA1 because the adenovirus DNA is not bundled in chromosomes like the genomic DNA. Bundling of the genomic DNA in chromosomes may shield many naturally occurring HMGA1 binding sites from binding to HMGA1; however, when a binding site is exposed upon opening of the chromosomal DNA in preparation for gene expression or DNA replication, there would be high pressure for excess HMGA1 binding to the exposed HMGA1 binding site, which could significantly alter the gene expression regulated by that specific HMGA1 binding site. In such a case, the availability of ∼200 decoy binding sites could significantly compete for HMGA1 binding.

As a first step toward elucidating the underlying mechanism responsible for reduced cell viability following infection with AdEasy-HMGA-6 virus, we also studied the mechanism of cell death to assay for apoptosis and necrosis. As an early apoptosis marker, we measured the exposure of phosphatidylserine on the outer layer of the
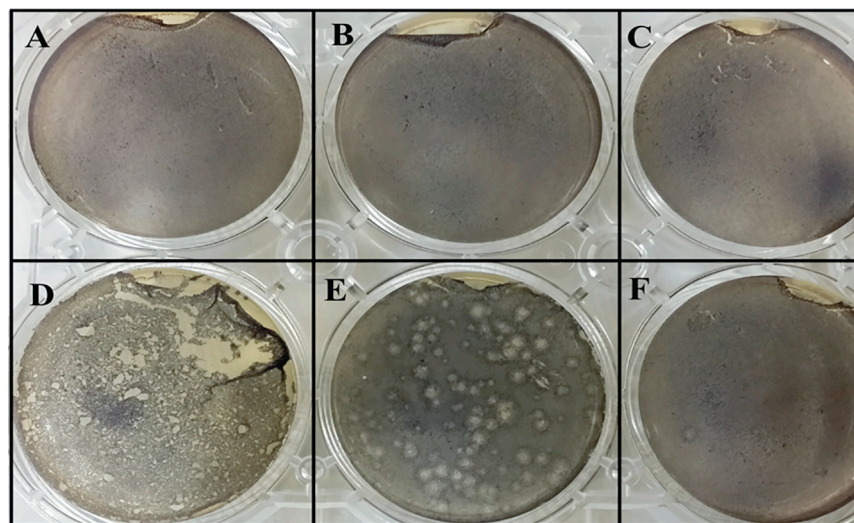
Figure 6. Plaque Assay Confirming Adenovirus Infection of E6E7 Cells

(A–F) Monolayers of 100% confluent non-cancerous E6E7 cell line (immortalized pancreas ductal epithelial cells) were infected with Ad5ΔΔ (A–C) or Ad5 (D and E) virus in semi-solid agarose media. The following dilutions of virus were used: (A) 10^{-1}, (B) 10^{-2}, (C) 10^{-3}, (D) 10^{-5}, (E) 10^{-6}, and (F) E6E7 cell only.

CAAAAAAAAAAAAAAAATTTTTTTTTTTTTT TTAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAATTTTTTTTTTT TTTTTAAGCTTCGTCGACGCGTGACTGAT CTAAGC-3′. The oligonucleotide was designed to contain six tandem 15-base pair stretches of consecutive adenines (A) or thymines (T). The HMGA-6 oligonucleotide was PCR amplified by using the forward primer (5′-TAGCACTCT GATACCGTCGACGGGTACC-3′) and reverse primer (5′-GCTTA GATCAGTCACGCGTCGACGAAGCTT-3′) and cloned into the KpnI and HindIII site of the pShuttle-CMV vector (Agilent Technologies). Linearized (by PmeI) HMGA-6 DNA containing pShuttle-CMV vector was co-transformed with the circular pAdEasy vector (replication-deficient adenoviral vector) (Agilent Technologies) in the *E.coli* BJ5183 strain, which facilitated the transfer of the HMGA-6 DNA to the pAdEasy vector by homologous recombination.

Cell Culture

Human pancreatic adenocarcinoma cell lines (MIA PaCa-2, AsPC-1, BxPC-3, PANC-1) and the liver cancer cell line (Hep G2) were purchased from the American Type Culture Collection (ATCC). The AD293 cell line was purchased from Agilent Technologies. AD293, Hep G2, MIA PaCa-2, and PANC-1 cells were grown in DMEM medium (containing 4.5 g/L glucose, 110 mg/L sodium pyruvate, and 4 mM L-glutamine) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. AsPC-1 and BxPC3cells were grown in RPMI medium supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin. Cells were maintained in 5% CO_2-humidified atmosphere at 37°C. The human pancreatic duct epithelial cell line (E6E7)[37] was provided by Dr. Ming-Sound Tsao from the University Health Network research hospital associated with the University of Toronto. The E6E7 cells were cultured as described by Furukawa et al.[38] Briefly, E6E7 cells were cultured in 25mL of keratinocyte serum-free (KSF) medium supplemented with 50 mg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor (Thermo Fisher Scientific, USA) in a T-75 flask. The medium was replaced every 3 days. For subculturing, the monolayer of E6E7 cell line was detached with the HBSS containing 0.025% trypsin (Thermo Fisher Scientific, USA), 0.265 mmol/L EDTA (Thermo Fisher Scientific, USA), and 0.5% polyvinylpyrrolidone (Thermo Fisher Scientific, USA). After neutralization of the trypsin with

cell membrane. Translocation of phosphatidylserine to the outer leaflet of the lipid bilayer occurs very early in the apoptosis.[35] The exposed phosphatidylserine binds with a luciferase-tagged Annexin V fusion protein, which results in increased luminescence signal in response to increased cellular apoptosis. The assay also makes use of a cell-impermeable fluorescent DNA dye, which detects necrosis. Therefore, increased fluorescent signal was proportional to the increased necrosis-induced cell membrane damage. We observed a time-dependent increase in luminescence within 6 hr of AdEasy-HMGA-6 infection followed by a delayed increase in fluorescence signal in the MIA PaCa-2 cell line. This kinetic difference in the development of the signals is the trademark of an apoptotic phenotype (https://www.promega.com/-/media/files/resources/application-notes/glomax-discover/an316-using-the-realtime-glo-annexin-v-apoptosis-and-necrosis-assay-on-the-glomax-discover-system.pdf?la=en).[36]

While we have demonstrated that sequestration of oncogenic HMGA1 by decoy hyper binding sites engineered into the adenovirus serotype 5 genome is capable of significantly reducing the viability of HMGA1-positive cancer cells, the decoy hyper binding site strategy should be generally applicable to targeting any overexpressed oncogenic transcription factor that binds double-stranded DNA. The composition of the hyper binding sites simply needs to be changed to include multiple copies of the consensus-binding sequence for the targeted traditional transcription factor. Experiments assessing toxicity and biodistribution of the engineered adenovirus containing the HMGA1-5 decoy hyper binding site in mice are currently underway in our laboratory.

MATERIALS AND METHODS
Cloning of the HMGA-6 Hyper Binding Site in the pAdEasy Vector
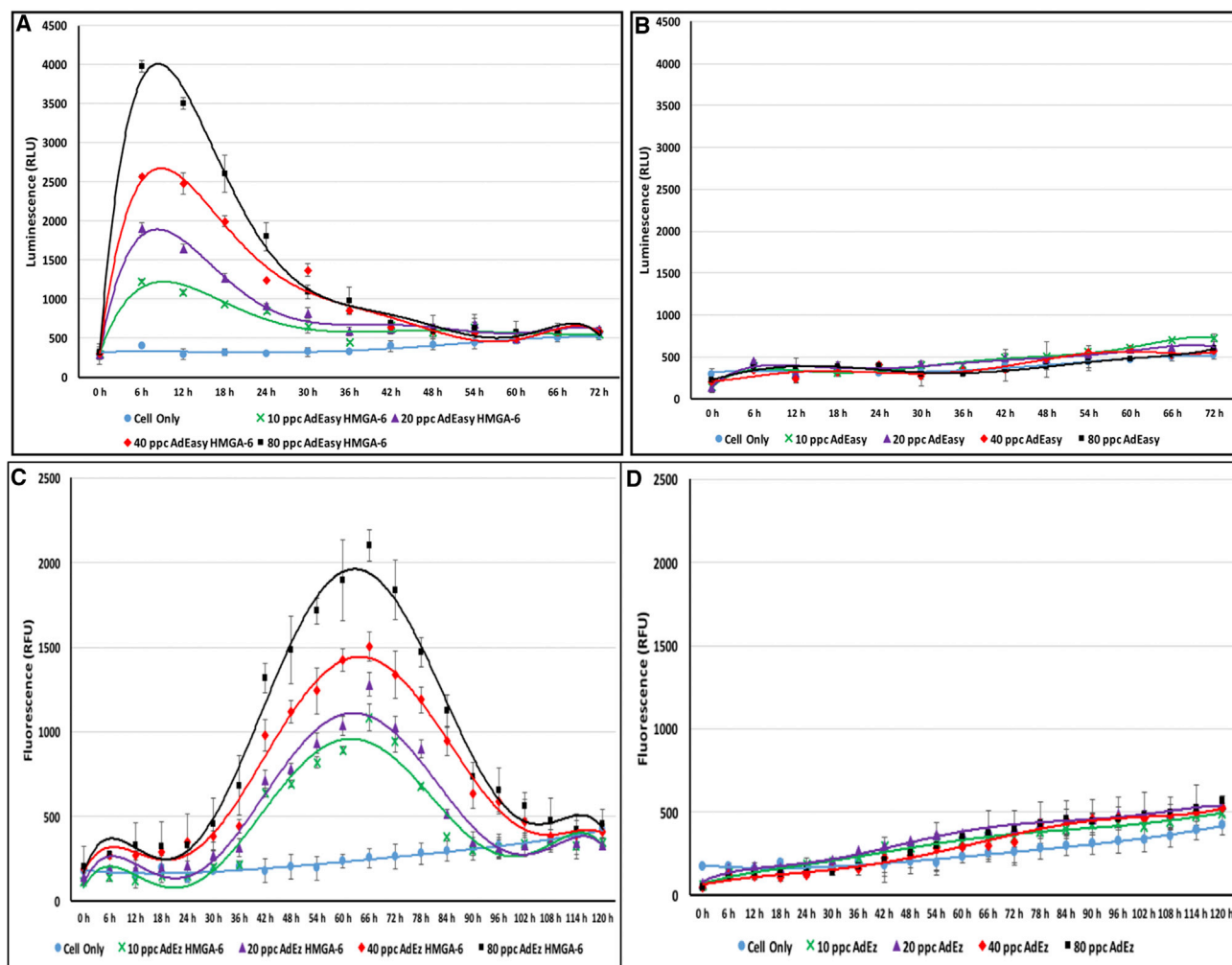
The synthetic 148-base pair linear HMGA-6 hyper binding site oligonucleotide was purchased from Integrated DNA Technologies (Coralville, IA). The sequence of the synthetic HMGA-6 *hyper binding site* was: 5′-TAGCACTCTGATACCGTCGACGGGTAC

Figure 7. Apoptosis and Necrosis Assays following Treatment of MIA PaCa-2 Cells with AdEasy-HMGA-6 DNA

(A–D) MIA PaCa-2 cells were seeded at a density of 2000 cells/well 24 h before infection with four different dosages (10, 20, 40, and 80 ppc) of either AdEasy-HMGA-6 (A or C) or AdEasy (B or D). Apoptosis and necrosis were measured using the RealTime-Glo Annexin V Apoptosis and Necrosis Assay (Promega) kits. Apoptosis (A and B) and necrosis (C and D) assays were measured in every 6 hr following virus infection and continued for 120 h. The data is only shown to 72 hr for the apoptosis assays. The data points represent the mean of 4 replicates. The error bars represent ± the standard deviation.

KSF containing 0.1% soybean trypsin inhibitor (Thermo Fisher Scientific, USA) and 0.1% bovine serum albumin (Thermo Fisher Scientific, USA), the cells were subcultured at 1:3 to 1:5 split ratios.

Preparation of Virus

The linearized pAdEasy or pAdEasy-HMGA-6 DNA was transfected into the AD293 cell line, a derivative of the HEK cell line, by using ViraPack Transfection Kit (Agilent Technologies) according to the manufacturer protocol. Viral production was confirmed by immuno-cytofluorescence assays. Cultures of AD293 cells transfected with linearized DNA encoding native AdEasy or encoding engineered AdEasy-HMGA-6 were probed for virus production using mouse anti-hexon primary antibodies (Abcam #ab8251) and Alexa fluor-coupled donkey anti-mouse immunoglobulin G (IgG) secondary

antibodies containing GFP (Invitrogen #A-21202). After 7–10 days of transfection, when cytopathic effects were visible, the primary viral stock was prepared by freezing-thawing the infected cells four times in a dry ice-methanol bath and a 37°C water bath.

Viral Amplification

1×10^6 AD293 cells were plated in a 60-mm plate the day before infection in order to obtain 50%–60% confluence. On the day of infection, old media was replaced by 0.5 mL of primary virus stock plus 0.5 mL of media (without FBS and antibiotics). After 2 hr of incubation at 37°C, 4 mL of media supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin were added and cells were incubated at 37°C for 7–10 days. When cytopathic effects were visible under the microscope, the secondary viral stock was prepared as above.

Plaque Assays

Monolayers of AD293 cells were infected with serially diluted AdEasy or AdEasy-HMGA-6 virus in six-well plates. After 4 hr of incubation at 37°C, the infected cells were overlaid with 2 mL of 0.4% agarose in DMEM media with 2% FBS. 5–7 days of post incubation or when the plaques were fully developed, the monolayers were stained for 3 hr at 37°C by adding MTT solution (final concentration is 0.5 mg/mL in PBS) (Sigma). The plaques were counted by naked eye and the viral titer was determined.

Western Blot Analysis

Cytoplasmic and nuclear protein extracts were prepared according to our previous study.[6] Briefly, proteins were extracted using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific), separated by SDS-PAGE gel, and transferred to immune-blot polyvinylidene fluoride (PVDF) membrane. The membrane was then probed with 1:30,000 dilution of rabbit anti-HMGA1 antibody (Abcam #ab129153) as primary antibody and 1:1,000 dilution of goat anti-rabbit IgG as secondary antibody (Abcam #ab205718). An enhanced chemiluminescence detection system (ECL, GE Health Care) was used to detect target proteins, and the image was captured by a LI-COR Odyssey Fc Imager system. To ensure equal loading of the proteins in each well, membranes were re-probed with anti-TATA binding protein antibody (Abcam #ab197874).

Cell Viability Assay

Cells were plated at a density of 3×10^3 cells per well in 96-well plates and incubated for 24 hr at 37°C incubator with 5% CO_2-humidified atmosphere. The reported cell density was measured from a single suspension of a quantified, i.e., counted, number of cells in a volume of media to achieve the desired cell density. The assays were repeated in triplicate for a cell density determined for a single cell suspension. The assay was repeated a second time, with a cell density determined from an independent suspension of counted cells. After 24 hr, cells were treated with the virus at three different doses (0.33, 3.3, and 33.3 virus ppc with the reported dose based on the initial estimate of the number of cells in each well) and/or gemcitabine (0, 1, 10, and 100 nM). It should be noted that it is difficult to determine an accurate initial cell count because of the tendency of these cancer cell lines to form clumps, and this introduces some systematic error into the reported values of the multiplicities of infection. After 72 hr of treatment, the cell viability assay was performed using the CellTiter 96 aqueous one solution cell proliferation assay kit (Promega). The percentage of the cell viability of the virus- and/or gemcitabine-treated cells was reported relative to the cell viability of the untreated cells. Each data point was generated from triplicate samples of two experiments. Note that the cell viability assay determines the relative number of live cells at the time of the measurement.

Apoptosis and Necrosis Assays

Cells were plated at a density of 2×10^3 cells per well in 96-well plates in 100 μL media and incubated for 24 hr at 37°C with 5% CO_2-humidified atmosphere. After 24 hr, media was replaced with 100 μL of fresh media, and the cells were treated with the virus at the dose of 10 ppc, 20 ppc, 40 ppc, or 80 ppc. The apoptosis and necrosis were measured using the RealTime-Glo Annexin V Apoptosis and Necrosis Assay kit (Promega) following the manufacturer's instructions. Luminescence (which measures the apoptosis hallmark) and fluorescence (which measures the necrosis hallmark) signals were measured every 6 hr following the treatment and continued to be monitored for 120 hr.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six tables and can be found with this article online at https://doi.org/10.1016/j.omto.2018.01.002.

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