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Endonuclease G depletion may improve efficiency of first generation adenovirus vector DNA replication in HeLa cells

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Abstract

First generation adenovirus (Ad5 ΔE1,E3) vectors are able to replicate their DNA in many tumour cells and can be used for oncotherapy. Highest rates of viral DNA replication occur in the G2/M transition of the cell cycle. In this study, we tried to increase the efficiency of Ad5 ΔE1,E3 DNA replication in the cervical carcinoma HeLa cells by using RNA interference (RNAi) to target endonuclease G (EndoG) whose depletion leads to an accumulation of cells in the G2/M transition. Targeting of EndoG by an shRNA encoded on an Ad5 ΔE1,E3 vector resulted in an early proliferation defect of cervical carcinoma HeLa cells. This effect coincided with enhanced DNA replication and encoded transgene expression of an Ad5 ΔE1,E3 vector. Applied in high concentrations, the EndoG-targeting Ad5 ΔE1,E3 vector showed enhanced HeLa cell killing ability relative to control Ad5 ΔE1,E3 vectors. These effects are most likely the result of EndoG depletion, which causes cells to accumulate in the G2/M transition of the cell cycle and extends favourable cellular conditions for Ad5 ΔE1,E3 DNA replication. Targeting of EndoG by RNAi may be a viable strategy for improving both the levels of transgene expression and the oncolytic properties of first generation adenovirus vectors.

Key words: Adenovirus vectors, oncolytic virotherapy, endonuclease G, transgene expression, RNA interference.

Introduction

The high efficiency of gene transfer into a wide variety of dividing and non-dividing mammalian cells, coupled with a relatively low risk of transformation, have made adenovirus type 5 (Ad5) derived vectors a staple tool for successful gene delivery *in vitro* and *in vivo* (1). In particular, the first generation Ad5 vectors, which have the entire E1 region of the viral genome deleted (Ad5 ΔE1,E3), have been used extensively for the transduction of therapeutic and cytotoxic genes into carcinoma cells.

An important consideration for the use of Ad5 ΔE1,E3 vectors is that they are considered replication incompetent, owing to the dependence of Ad5 on the products of E1 genes for the initiation of events that lead to productive viral replication (2). However, a number of studies have demonstrated that Ad5 ΔE1,E3 DNA is replicated in human tumour cells, which may be important in the context of their application in treatment of human malignancies (3-10). Namely, replication of viral DNA is correlated with increased expression of virally encoded genes that mediate cytotoxicity (4, 11). Furthermore, viral DNA replication can lead to productive replication of the virus, which results in host cell death and the production of new virions with the ability to repeat this process (3, 9). Therefore, it is important to study factors that may enhance this process, as Ad5 ΔE1,E3 could potentially have oncolytic properties that may be applied for treatment of human malignancies.

In the case of infection with wildtype Ad5, quiescent cells are made to progress to the S phase, however, Ad5 ΔE1,E3 may require different cellular factors to replace the transactivating properties of adenovirus E1A proteins and hence require a different cellular environment that can provide them. This is supported by the observation that infection of cultured cells with Ad5 ΔE1,E3 causes a partial G2/M arrest or delay in cell cycle progression, which becomes more apparent in later stages of infection, due to the inappropriate expression of cyclin A, cyclin B1, cyclin D and cyclin-dependent kinase $p34^{cdc2}$ which is induced by viral E4 genes (other than orf6) (3, 4, 9, 11). In fact, Steinwaerder et al. (9) and Bernt et al. (3) demonstrated that highest levels of Ad5 ΔE1,E3 DNA replication occur in the G2/M phase and that prolongation of G2/M leads to increased efficiencies of Ad5 ΔE1,E3 DNA replication in the human cervical carcinoma HeLa cells. Importantly, cells artificially arrested in the G2/M phase at time of infection with Ad5 ΔE1,E3 exhibit the most efficient virus internalization and viral DNA replication, relative to other points in the cell cycle, and exhibit multiplicity of infection (MOI) dependent cytopathic effects (CPE).

Endonuclease G (EndoG), a nuclear-encoded mitochondrial endonuclease, is a conserved apoptosis regulator in eukaryotic organisms (12). In addition to its mitochondrial localization, EndoG has been identified in nuclei of healthy non-apoptotic cells suggesting a need for this enzyme in processes that are not related to cell death (13, 14). Apart from its role in programmed cell death, EndoG has been implicated in vital roles in the cell, including mitochondrial DNA replication, DNA recombination and cell proliferation (15-19). Importantly, reduction of cellular levels of EndoG by RNA interference (RNAi) leads to the accumulation of both yeast and mammalian cells in the G2/M phase of the cell cycle without any additional stimulus (15, 16, 20, 21). Since exogenous expression of EndoG-GFP (EndoG-green fluorescent protein) can rescue this phenotype, the delay in G2/M transition is a specific effect

of EndoG silencing (21). Therefore, depletion of EndoG may prolong the G2/M delay associated with Ad5 ΔE1,E3 infection of tumour cells and thus increase viral DNA replication in a similar manner to what has been observed when tumor cells are chemically arrested in the G2/M phase (3, 9).

With respect to the application of Ad5 ΔE1,E3 vectors for viral oncolytic therapy, maximizing replication of Ad5 ΔE1,E3 DNA should enhance cytotoxicity of these vectors and result in higher efficacy with respect to cancer cell killing. Therefore, we tested whether an Ad5 ΔE1,E3 vector which encodes an RNAi effector against EndoG expression would exhibit augmented viral DNA replication and CPE in the human cervical carcinoma HeLa cells.

Materials and methods

Cell Culture

Both HeLa cells and HEK 293 cells were maintained in 150 mm, 24-well or 6-well standard culture dishes, growing in a monolayer. Culture medium was minimum essential medium (MEM, Gibco, USA) supplemented with 10% (v/v) foetal bovine serum (FBS) (PAA Laboratories, Austria), antimycotic-antibiotic (1X, Invitrogen, USA), GlutaMAX™ (1X, Invitrogen, USA) and with sodium bicarbonate (3.7 g/l, Invitrogen). Cultures were incubated in a 5% CO_2/air mixture at 37°C.

Construction of Ad5 ΔE1,E3 vectors

First generation (Ad5 ΔE1,E3) adenovirus vectors, AdsiEndoG and AdsiCAD, were constructed by the FLP-frt recombination system, which is based on the co-transfection of two plasmids with complementary viral DNA components into the E1-producing HEK 293 cells (22, 23). The pBHGfrtΔE1,3FLP vector (35,552bp) was a circularized human adenovirus type 5 (Ad5) genome with the following modifications: a substitution in the viral E1 region that encoded the yeast site specific recombinase FLP under the control of the CMVie promoter/enhancer and a *frt* recombination sequence; a deletion of the viral E3 region; and a deletion of the viral packaging sequence (Ψ). The pDC-CG-U6-EndoGshRNA and pDC-CG-U6-CADshRNA shuttle vectors (5,354bp) were pUC19-based plasmids that contained Ad5 inverted terminal repeats (ITRs), Ad5 packaging sequence, U6 promoter-regulated shRNA expression cassette (vector specific), CMVie promoter/enhancer-regulated AcGFP expression cassette with SV40 poly A signal, and a *frt* recombination sequence. The pDC-CG-U6-EndoGshRNA vector encoded a shRNA targeting the following sequence of EndoG mRNA: 5′-GAAUGCCUGGAACAACCUGGA-3′. The design of shRNAs, in the 5` to 3` orientation, was as follows: sense strand, which was homologous to the targeted region within EndoG mRNA; a short loop region (UUCAAGAGA); and the antisense strand, which was perfectly complementary to the target sequence. The pDC-CG-U6-CADshRNA vector was engineered in an analogous manner to pDC-CG-U6-EndoGshRNA, except the shRNA expressed by this vector targeted the caspase activated DNase (CAD) mRNA at the following sequence: 5′-GAGAAGUGGACUGGGAGUA-3′.

Transfection of HEK293 cells was carried out using Lipofectamine 2000 (Invitrogen, USA) as per manufacturer's instructions. The pBHGfrtΔE1,3FLP and pDC-CG-U6-shRNA vectors were mixed to a final quantity of 5 μg of DNA, at equal molar ratios, and co-transfected into nearly confluent HEK293 cells. Cultures were maintained for 10-15 days following transfection until visible plaques formed in the monolayer. Homologous recombination mediated by the site specific recombinase FLP between *frt* sequences on the pBHGfrtΔE1,3FLP and pDC-CG-U6-shRNA vectors resulted in the generation of Ad5 ΔE1,E3 DNA vectors, which contained all the viral elements required for replication, except genes encoded in the viral E1 region. The E1 region was replaced by the U6 promoter-regulated shRNA expression cassette (vector specific) and a CMVie promoter/enhancer-regulated AcGFP expression cassette. The HEK293 cells provided the E1 gene products *in trans* facilitating production of infectious virions, detected by the onset of cytopathic effects (CPE). When ~70% of cells exhibited visible CPE, a rubber policeman was used to scrape cells of the surface of the culture dish. The culture medium (about 2 mL) that contained infected cells was collected and sequentially frozen and thawed three times to release virions from cells that were still intact. The cell suspensions were centrifuged at 12,000 g for 5 min to settle cell debris. The supernatant was collected and stored at -70°C.

A control Ad5 ΔE1,E3 vector, AdGFP, was also used in this study, which contains the same CMVie promoter/enhancer regulated GFP expression cassette as AdsiEndoG and AdsiCAD, but is missing the U6 promoter regulated shRNA expression cassette.

Confirmation of Ad5 ΔE1,E3 vectors

To confirm the sequences of Ad5 ΔE1,E3 vectors, viral DNA was isolated and following digestion with a restriction endonuclease it was analyzed by agarose gel electrophoresis. In addition, PCR was used to confirm absence of wild type Ad5 contamination. One hundred μL of viral suspensions were mixed with an equal volume of a 1.2% SDS solution supplemented with 20 μL of 20 mg/mL Proteinase K (Sigma-Aldrich, USA). The mixtures were incubated at 55°C for 1 h. The resulting lysates were mixed with the binding solution of a commercially available Genomic DNA purification kit (Norgen Biotek, Canada) and processed on a spin column assembly, as per manufacturer's instructions.

Five μL of eluted DNA was mixed with 1 μL of *Hind* III restriction endonuclease (New England Biolabs, USA), 2 μL of the following buffer: 50 mM NaCl (Bio Basic, Canada), 10 mM Tris-HCl (Bio Basic, Canada), 10 mM $MgCl_2$ (Bio Basic, Canada), 100 μg/ml BSA (Sigma-Aldrich, USA), pH 7.9 and 12 μL of DNase/RNase-free water (Ambion, USA). Reaction was incubated at 37°C for 3 h and subsequently examined by 1% agarose gel electrophoresis. The resultant bands were sized by comparison to a DNA sizing ladder (HighRanger 1 kb DNA Ladder, Norgen Biotek, Canada). The digestion patterns matched with theoretical predictions based on an *in silica* model (Vector NTI, Invitrogen, USA). PCR on 2 μL of eluted DNA was performed using E1 region specific primers F 5′-ATTCACGTAGCCAGCCACTC-3′ and R 5′-TCGGTCACATCCAGCATCAC-3′. Absence of amplification confirmed that Ad5 ΔE1,E3

vector preps were free of contamination with wild type Ad5.

Amplification of infectious Ad5 ΔE1,E3 vectors

Nearly-confluent HEK293 cells, cultured in 150 mm standard tissue culture plates were independently infected with AdsiEndoG, AdsiCAD and AdGFP. Infection was carried out by aspirating culture medium and overlaying the monolayer with the Ad5 ΔE1,E3 vector of choice diluted in 2 mL of 1X PBS++ (1X PBS (Gibco, USA), supplemented with 1 mM of $MgCl_2$ (Bio Basic, Canada) and $CaCl_2$ (Bio Basic, Canada)). After 45 min of incubation in a 5% CO_2-air mixture at 37°C, medium (18 mL) was resupplied to the cells.

When ~70% of cells exhibited visible CPE (usually 48 h post-infection), the cells were scraped by a rubber policeman and the medium was collected and pooled. The cell suspension was centrifuged at 2000 g for 10 min and supernatant removed. One ml of PBS++ was added to the cell pellet for every 150 mm dish scraped and the cells were resuspended by vortexing. Three cycles of freezing with liquid nitrogen and thawing at 37°C in a water bath were sequentially carried out to lyse the cells and release virions. The cell suspension was centrifuged at 12,000 g to pellet cellular debris and supernatant was collected.

Determining titer of Ad5 ΔE1,E3 vectors

Viral titer was determined using the plaque assay standard protocol. Medium was aspirated from confluent HEK293 cells growing in a monolayer and cultured in 6-well culture dishes. Ad5 ΔE1,E3 vectors were serially diluted in 500 µL of PBS++ and several dilutions in the expected titer range were used to infect HEK293 cells cultured in 6-well tissue culture dishes. After 45 min of incubation in a 5% CO_2-air mixture at 37°C, PBS++ was aspirated and cells were overlaid with a liquid medium mixture of 0.6% UltraPure™ Agarose (Invitrogen, USA) and MEM supplemented with 5% FBS (PAA Laboratories, Austria). Plates were left at room temperature for 15 min to allow agar medium to solidify and they were subsequently incubated in a 5% CO_2-air mixture at 37°C. Visible plaques in the cell monolayer developed within 5 to 10 days and were counted. Viral titer was determined by multiplying the visualized plaque forming units (PFU) in a given well by the dilution factor of the initial viral suspension.

Infection of HeLa cells

HeLa cells were seeded at a density of 50,000 cells/well of a 24-well tissue culture plate one day before infection. On day of infection, medium was aspirated and 100 µL of PBS++ -diluted Ad5 ΔE1,E3 vector was added to the monolayer. After 45 min of incubation in a 5% CO_2-air mixture at 37°C, 500 µL of medium was added per well of cells. Next day, medium was replaced by fresh medium.

DNA/RNA isolation

Medium was aspirated and HeLa monolayers were washed twice with 1X phosphate buffered saline (PBS, Gibco, USA), pH 7.4. A solution from a commercially available kit (RNA/DNA/Protein Purification Kit, Norgen Biotek, Canada) was used to lyse cells directly on the plate. Entire lysate was collected and applied to a purification column supplied by the manufacturer and recommended protocol was followed as specified. DNA and RNA were eluted in separate fractions and examined for quality and quantity by agarose gel electrophoresis and spectrophotometry, respectively.

Quantitative PCR analysis

Quantitative PCR (qPCR) was performed with the Bio-Rad iCycler thermocycler (Bio-Rad, USA) on 3 µL of each DNA elution using the iQ SYBR Green Supermix (Bio-Rad, USA) with 300 nM of primers (Integrated DNA Technologies, USA) specific for the E2A gene encoded on the viral vector, F 5′-ACACTCAGCGGGTTCATCAC-3′ and R 5′-AGATGTGGCGCTACAAATGG-3′. The human 5S gene (Fwd primer 5′-GCCATACCACCCTGAACG-3′ and Rev primer 5′-AGCCTACAGCACCCGGTATT-3′) was used for normalization and the comparative threshold method was used to assess the relative abundance at each time point. The total reaction volume per sample was 20 µL, and the PCR protocol was as follows: 15 min at 95°C for enzyme activation, then 40 cycles of 15 s at 95°C, 30 s at 60°C and 45 s at 72°C. Melt curve analysis was performed by relative fluorescence assessment at 0.5°C increments with a 10 s duration, starting at 57°C and continuing for 80 cycles.

Reverse transcription followed by quantitative PCR

Three µL of the total RNA elution was used in the reverse transcription (RT) reaction using the Superscript III system (Invitrogen, USA). In the first step, RNA was mixed with 0.5 µL of 100 mM oligo(dT) 18-mer primer (Integrated DNA Technologies, USA) and the reaction volume was completed to 10 µL using DNase/RNase free water (Ambion, USA). Denaturing at 70°C for 5 min was followed by cooling at 4°C for 5 min. At this step, 0.1 µL of the reverse transcriptase Superscript III (SSIII, Invitrogen, USA) was added to the reaction in a mixture with 4 µL of the 5X First Strand Buffer, 2 µL of 0.1 M Dithiothreitol, 1 µL of 10 mM dNTPs (Bio Basic, Canada) and 2.9 µL of water. After the reaction mixture was completed, temperature was increased to 25°C for 5 min and then increased again to 42°C for 60 min, followed by 15 min at 70°C and an indefinite hold at 4°C.

The qPCR detection of GFP transgene expression levels was carried out using the human β-actin gene for normalization and the comparative threshold method was used to assess the relative abundance of GFP mRNA at each time point. The primers used for detection of GFP cDNA were F 5′-GATCACATGAAGCAGCACGA-3′ and R 5′-GATGTTGCCATCCTCCTTGA-3′ and for β-actin cDNA F 5′-GCCGAGGACTTTGATTGCAC-3′ and R 5′-ACCAAAAGCCTTCATACATCTCA-3′. The iQ SYBR Green Supermix (Bio-Rad, USA) was used for the qPCR with 300 nM of primers, as described above for DNA analysis. Levels of EndoG mRNA were also interrogated at each time point using the primers: F 5′-GACGACACGTTCTACCTGAGCAACGT-3′ and R 5′-CCAGGATCAGCACCTTGAAGAAGTG-3′. The qPCR protocol for EndoG cDNA amplification varied slightly to protocol described previously due to the requirement of a higher primer annealing temperature, which was set at 65°C.

Cell counting

Medium was aspirated from HeLa cells cultured in 24-well standard tissue culture plates and monolayers were washed three times with 1X PBS (Gibco, USA). Cells were lifted with 200 µL of Gibco's 0.05% Trypsin-EDTA (1X, Gibco, USA) and counted on the haemocytometer counting chamber using the Olympus CK2 inverted microscope (Olympus, Japan) for magnification.

Results

Construction of recombinant Ad 5 vector for EndoG-silencing

In order to test whether targeting of EndoG by RNAi would augment viral DNA replication in HeLa cells, we constructed AdsiEndoG, an Ad5 ΔE1,E3 vector carrying an expression cassette for a shRNA directed against EndoG mRNA. In addition, a control Ad5 ΔE1,E3 vector with the same backbone sequence was constructed, but encoding a shRNA against CAD, which is an apoptotic nuclease with no known roles outside of apoptosis and which is not functional in non-apoptotic cells by way of chaperone-mediated inhibition (AdsiCAD).

The FLP recombinase system was employed to generate Ad5 ΔE1,E3 vectors with substitutions in the E1 gene region. The pBHGfrtdel1,3FLP plasmid encoded the FLP recombinase as well as the entire Ad 5 sequence, without viral E1 and E3 genes, and the Frt recombination site. This plasmid was co-transfected with a shuttle plasmid pDC-CG-U6-shRNA into HEK 293 cells, which have been transformed with the Ad E1 gene. Recombinations between the two plasmids at Frt and Ad5 ITR sites, created recombinant adenoviruses with the EndoG- or CAD-directed shRNA and GFP expression cassettes subbed in at the E1 position.

Replication of AdsiEndoG DNA in HeLa cells

To test whether knockdown of EndoG affects Ad5 ΔE1,E3 DNA replication rates, HeLa cells were infected at MOI 10 PFU/cell with AdsiEndoG, an Ad5 ΔE1,E3 encoding a shRNA expression cassette directed against EndoG and two control Ad5 ΔE1,E3 vectors: AdsiCAD, targeting the caspase activated DNase, and AdGFP.

Figure 1 is a summary of the relative Ad5 ΔE1,E3 DNA levels, as assessed by qPCR, at five time points post-infection and normalized with the cellular 5S rRNA gene. It is important to note that normalizing with the 5S rRNA gene resulted in the determination of only approximate Ad5 ΔE1,E3 DNA levels with respect to cell number, as differences in cell cycle distribution of the infected HeLa cells may have resulted in differences with respect to the number of 5S rRNA copies per individual cell. Nonetheless, differences in relative Ad5 ΔE1,E3 DNA levels with respect to the 5S cellular gene were observed between AdsiEndoG and one of or both of the control Ad5 ΔE1,E3 vectors at 24, 48 and 72 h post-infection, with AdsiEndoG DNA being relatively higher compared to the control vectors at these time points. These results indicated enhanced AdsiEndoG DNA replication relative to control Ad5 ΔE1,E3 vectors.

To examine if the same trend was observed when normalization with cellular DNA was disregarded, Ad5

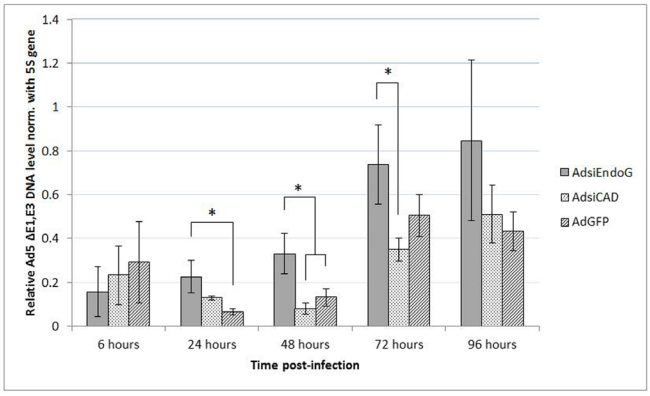

Figure 1. Effect of EndoG targeting on the replication efficiency of an adenovirus vector in HeLa cells. Relative levels of AdsiEndoG, AdsiCAD and AdGFP DNA over a 4 day time-course in HeLa cells infected at MOI of 10 PFU/cell (N=3). Ad5 ΔE1,E3 DNA level was inferred by qPCR analysis of the Ad5 E2A gene and normalized by the amplification of the cellular 5S gene, using the equation $2^{\Delta(Ct\ 5S - Ct\ E2A)}$. Significant differences were observed at 24, 48 and 72 h between AdsiEndoG and one or both of the control vectors (Student's *t* test, p values <0.05). HeLa cells were seeded at a density of 40,000-50,000 cells/well prior to infection and allowed to replicate over the next four days. Error bars indicate SD.

ΔE1,E3 DNA levels were examined per well of infected cells rather than per cell. Equal proportions of total DNA elutions were analyzed by qPCR and raw Ct values for the E2A gene were converted to relative values based on the equation $2^{\Delta(Ct)}$ and the data was presented in Figure 2. The relative level of AdsiEndoG DNA per well of infected cells was statistically significantly higher than one or both of the control Ad5 ΔE1,E3 vectors at 24, 48, 72, and 96 h post-infection (24, 48, and 96 h relative to AdsiCAD and 24, 72, and 96 h relative to AdGFP, t test, p values <0.05). In absolute terms, at the conclusion of the experiment (96 h post-infection), there was 50 times more viral DNA in cells infected with AdsiEndoG, relative to its initial amount at time of infection, compared to only 14 and 9 times more for AdsiCAD and AdGFP, respectively.

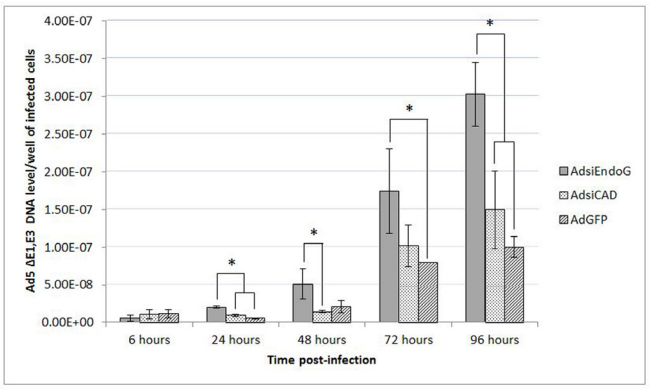

Figure 2. Effect of EndoG targeting on the replication efficiency of an adenovirus vector in HeLa cells disregarding normalization of data against cellular DNA. Relative content of AdsiEndoG, AdsiCAD and AdGFP over a four day time-course in HeLa cells infected at MOI of 10 PFU/cell (N=3). Ad5 ΔE1,E3 DNA content was inferred by qPCR analysis of the Ad5 E2A gene $2^{\Delta(Ct)}$. Significant differences were observed at 24, 48 and 72 and 96 h between AdsiEndoG and one or both of the control vectors (Student's *t* test, p values <0.05). HeLa cells were seeded at a density of 40,000-50,000 cells/well prior to infection and allowed to replicate over the next four days. Error bars indicate SD.

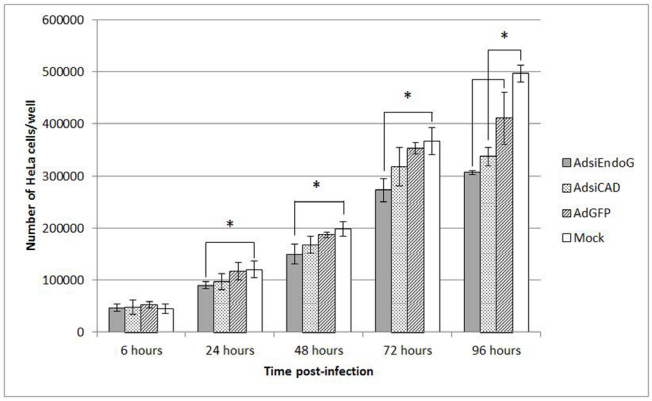

Figure 3. Effect of EndoG targeting on HeLa cell proliferation. Average number of HeLa cells infected with AdsiEndoG, AdsiCAD and AdGFP at MOI of 10 PFU/cell (N=3) or mock-infected over a four day time-course. HeLa cells were seeded at a density of 40,000-50,000 cells/well prior to infection and allowed to replicate over the next four days. At each time point, cells were washed twice with PBS and lifted by trypsin. Cell counting was done on the haemocytometer. Error bars indicate SD.

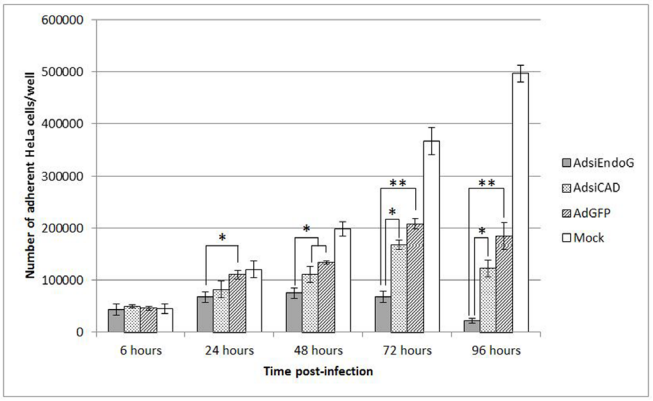

Figure 4. Effect of EndoG targeting on levels of adenovirus vector-induced CPE in HeLa cells. Average number of adherent HeLa cells infected with AdsiEndoG, AdsiCAD and AdGFP at MOI of 50 PFU/cell (N=3) or mock-infected over a four day time-course. HeLa cells were seeded at a density of 40,000-50,000 cells/well prior to infection and allowed to replicate over the next four days. At each time point, cells were washed twice with PBS and lifted by trypsin. Cell counting was done on the haemocytometer. Error bars indicate SD.

Effect of AdsiEndoG infection on HeLa cell proliferation

A technical repeat of the above experiment was done in order to assess replication rates of infected cells over the same four day time-course (Fig. 3). Cells were washed with PBS twice and subsequently lifted by trypsinization and counted by haemocytometer. At each time point after the initial assessment at 6 h post-infection, there were significantly fewer cells in wells infected with AdsiEndoG relative to the mock infected control (Student's *t* test, p values <0.05).

Conversely, the only time point where the difference in cell number reached statistical significance between the other infection conditions, with AdsiCAD and AdGFP, and the mock infected control was at 96 h post-infection. CPE was not observed in any of the wells at any time point after infection with all of the Ad5 ΔE1,E3 vectors, suggesting that differences in cellular abundances were not a result of virus-associated lysis, but due to unequal cell replication rates. Therefore, targeting of EndoG resulted in a HeLa cell proliferation defect, which coincided with increased Ad5 ΔE1,E3 DNA replication over the same time-course.

Infection of HeLa with AdsiEndoG at high MOI

Since replication of Ad5 ΔE1,E3 DNA can lead to proportional CPE when HeLa cells are infected at high initial MOI, we tested whether previously observed differences in Ad5 ΔE1,E3 DNA replication rates between AdsiEndoG and AdsiCAD or AdGFP would lead to differential CPE in cells infected with these vectors. Therefore, HeLa cells were infected at MOI of 50 PFU/cell, which was 5 times higher than in the initial experiment.

CPE resulted in a morphological change in cell shape that lead to the detachment of cells from the surface of the culture dish and only adherent cells were counted at each time point of the experiment. At MOI of 50 PFU/cell, CPE was observed in all of the Ad5 ΔE1,E3 infection conditions, which reduced the amount of adherent cells per well compared to the mock infected control. The degree of observed CPE was highest in wells of HeLa cells infected with AdsiEndoG relative to AdsiCAD and AdGFP infected cells, which was reflected in the lowest adherent cell counts at 24, 48, 72 and 96 h post-infection (Student's t test *p* values <0.05, Fig. 4). At 96 h post-infection, less than 5% of cells infected with AdsiEndoG were still adhered to the plate, relative to mock infected control, compared with 24.7% and 37.2% of cells infected with AdsiCAD and AdGFP, respectively.

Effect of EndoG targeting on Ad5 ΔE1-encoded transgene expression

As AdsiEndoG and AdsiCAD exhibited differences in replication levels in HeLa cells infected at MOI of 10 PFU/cell, while also showing different levels of induced CPE in cells infected at MOI of 50 PFU/cell, we tested whether these differences had an effect on the expression of a GFP transgene both of the vectors encoded. HeLa cells were infected with MOI of 10 PFU/cell and relative GFP mRNA expression assessed (Fig. 5). At 72 h post-infection, cells infected with AdsiEndoG

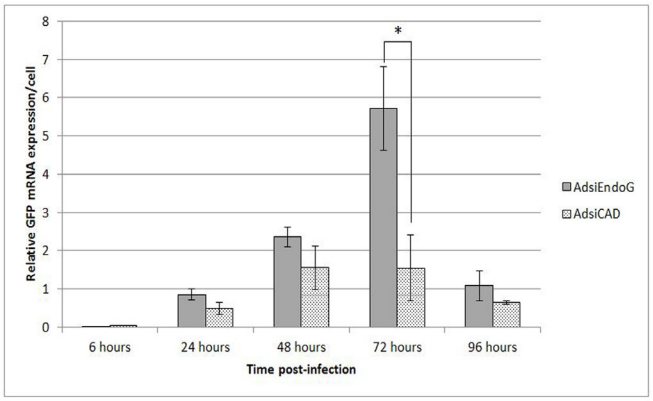

Figure 5. Effect of EndoG targeting on adenovirus vector-encoded reporter gene mRNA expression. Relative GFP mRNA expression over a four day time-course in HeLa cells infected with AdsiEndoG, AdsiCAD and AdGFP at MOI of 10 PFU/cell (N=3). GFP mRNA expression was inferred by RT-qPCR analysis and normalized with the expression of the cellular β-actin gene using the equation $2^{\Delta(Ct\ \beta\text{-actin} - Ct\ GFP)}$. Significant difference in GFP mRNA expression between AdsiEndoG and AdsiCAD was observed at 72 h post-infection (Student's *t* test, p values <0.05). HeLa cells were seeded at a density of 40,000-50,000 cells/well prior to infection and allowed to replicate over the next four days. Error bars indicate SD.

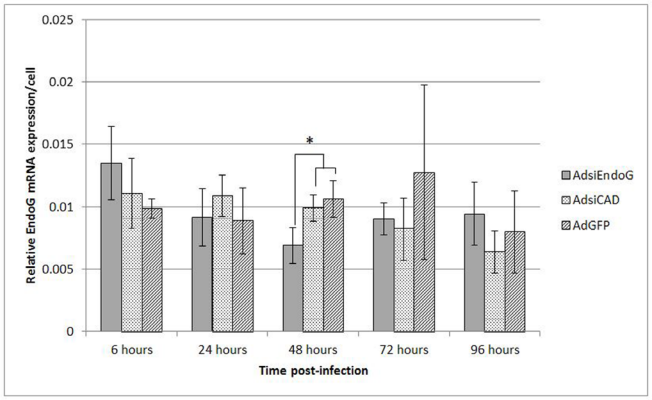

Figure 6. Effect of adenovirus vector-mediated EndoG targeting on cellular EndoG mRNA expression. Relative EndoG mRNA expression over a four day time-course in HeLa cells infected with AdsiEndoG, AdsiCAD and AdGFP at MOI of 10 PFU/cell (N=3). EndoG mRNA expression was inferred by RT-qPCR analysis and normalized the expression of the cellular β-actin gene using the equation $2^{\Delta(Ct\ \beta\text{-actin} - Ct\ ENdoG)}$. Significant differences in EndoG mRNA expression between AdsiEndoG and AdsiCAD and AdGFP were observed at 48 h post-infection (Student's *t* test, p values <0.05). HeLa cells were seeded at a density of 40,000-50,000 cells/well prior to infection and allowed to replicate over the next four days. Error bars indicate SD.

expressed significantly more GFP mRNA compared to cells infected with AdsiCAD (p value <0.05).

Knockdown of EndoG mRNA by AdsiEndoG

Relative EndoG expression was also assessed by RT-qPCR in HeLa cells infected with AdsiEndoG, AdsiCAD and AdGFP at MOI of 10 PFU/cell (Fig. 6). At 48 h post-infection EndoG mRNA abundance is statistically significantly lower in cells infected with AdsiEndoG relative to AdsiCAD and AdGFP infected cells (p value <0.05).

Discussion

Human adenovirus type 5 derived first generation vectors (Ad5 ΔE1,E3) are routinely used for the transduction of exogenous genes into human tissues and cells, both *in vitro* and *in vivo* (1, 24). Owing to the deletion/substitution of the viral E1 region, which encodes the E1A and E1B genes, these vectors are considered replication incompetent in normal, quiescent cells (2). However, Ad5 ΔE1,E3 DNA replication has been demonstrated in some proliferating, cultured cells and in tumour derived cells lines (3-10). The efficiency of Ad5 ΔE1,E3 DNA replication has been positively correlated to the development of cytopathic effects (CPE) in infected cells, and higher intranuclear concentration of Ad5 ΔE1,E3 DNA is related to increased expression of viral genes that mediate cytotoxicity (4, 9, 11, 25, 26). Enhanced cell killing ability of Ad5 ΔE1,E3 vectors is desired for their potential use in viral oncolytic therapy, therefore it is important to develop strategies that can enhance their replication in tumour cells (27, 28).

Other studies have established the importance of cell cycling for efficient Ad5 ΔE1,E3 DNA replication and identified the G2/M transition in the cell cycle as the optimal cellular environment for this process (3, 9, 11). Prolongation of the G2/M transition or chemically induced cell cycle arrest in this phase results in enhanced Ad5 ΔE1,E3 internalization, viral DNA replication and development of CPE in the cervical cancer cell line HeLa (3, 9).

The mitochondrial apoptosis regulator EndoG exerts control of the cell cycle in physiological circumstances (15, 16, 20, 21). RNAi induced silencing of EndoG expression results in a cell proliferation defect that is characterized by an accumulation of cells in the G2/M transition without any additional stimuli (21). Therefore, we hypothesized that an Ad5 ΔE1,E3 virus that encodes an RNAi effector against EndoG mRNA would exhibit augmented DNA replication and CPE in HeLa cells compared to control Ad5 ΔE1,E3 vectors.

Using an *in silica* method we identified a suitable region within EndoG mRNA for targeting by RNAi. To facilitate the knockdown, a vector for shRNA expression was constructed, which also encoded an autonomous cassette for the expression of a GFP reporter gene. Using the FPL-frt recombination system in HEK 293 cells, we engineered AdsiEndoG, an Ad5 ΔE1,E3 vector capable of knocking down EndoG levels in HeLa cells by RNA Pol III U6 promoter driven expression of shRNA. We also engineered the control vector AdsiCAD, which was homologous to AdsiEndoG, except it encoded a shRNA template directed against the caspase activated DNase (CAD). CAD is expressed in a completely inactive form in the cell and has no known functions outside of apoptosis, therefore its knockdown has no effect on cells under physiological circumstances. In addition we made use of a previously constructed Ad5 ΔE1, AdGFP, which did not code for any RNAi effectors, but encoded a homologous GFP expression cassette.

Infection of HeLa cells at MOI of 10 PFU/cell with AdsiEndoG and the control AdsiCAD and AdGFP vectors resulted in detectable viral DNA replication. This finding confirmed previous studies that demonstrated Ad5 ΔE1,E3 DNA replication in HeLa even when cells are infected at a relatively low MOI (3, 9). Furthermore, low levels of Ad5 ΔE1,E3 DNA replication did not result in cell killing, as CPE was not observed in any of the infected cells, which was, again, an expected outcome based on previous reports (3, 9). However, the levels of Ad5 ΔE1,E3 DNA replication varied significantly between cells infected with the AdsiEndoG and the two controls. Despite similar levels of viral DNA internalization, measured at 6 h post-infection, levels of AdsiEndoG DNA were significantly higher relative to control vectors at 24 h post-infection and this trend continued to the conclusion of the experiment. In fact, at 96 h post-infection, AdsiEndoG DNA accumulated to levels that were on average 50 folds higher than the initial concentration used to infect cells. In comparison, over the same time-frame, the DNA of the two control vectors, AdsiCAD and AdGFP, increased 14 and 9 folds, respectively, without showing a statistically significant difference between them. Therefore, AdsiEndoG DNA was replicated at the highest levels, relative to the two control vectors.

We hypothesized that differences in levels of Ad5 ΔE1,E3 DNA replication rates between AdsiEndoG and the controls arose from cell cycle deregulation caused by EndoG depletion in AdsiEndoG infected HeLa cells. To test this claim, proliferation of HeLa cells infected with Ad5 ΔE1,E3 or mock-infected cells was monitored

over the entire course of the experiment. Cells infected with AdsiEndoG exhibited a cell proliferation defect, unrelated to development of CPE, which was observed 24 h following infection and at every time-point assayed thereafter. We did not assess cell cycle by a biochemical approach, so we cannot conclusively say that the cells were arrested in the G2/M phase transition, however, in addition to the daily cell counts, this conclusion is supported by previous reports that showed a similar effect of EndoG knockdown on Vero, 293T and yeast cell proliferation, which was characterized by an accumulation of cells in the G2/M transition of the cell cycle (15, 16, 21). Therefore, increased rates of AdsiEndoG DNA replication, which coincided with a measurable decrease in EndoG mRNA levels and cellular proliferation, most likely resulted from a prolongation of the G2/M transition and, hence, the temporal extension of a favourable cellular environment for Ad5 ΔE1,E3 DNA replication. This finding is in agreement with observations made from Ad5 ΔE1,E3-infected HeLa cells chemically arrested at the G2/M transition phase of the cell cycle (3, 9).

In contrast, HeLa cells infected with the control Ad5 ΔE1,E3 vectors, AdsiCAD and AdGFP, did not show augmented proliferation, relative to mock-infected cells, up to 72 h post-infection. However, at the very end of the experiment time-course, at 96 h post-infection, all of the Ad5 ΔE1,E3 vectors induced a measurable cell proliferation defect, which was most apparent in cells infected with AdsiEndoG. This was expected since basal expression of viral E4 genes, other than orf6, can induce the inappropriate expression of cyclin A, cyclin B1, cyclin D and cyclin-dependent kinase p34^{cdc2} which deregulate the cell cycle and cause a partial arrest at the G2/M transition (4, 9, 11).

Since EndoG knockdown in HeLa produced a cell proliferation defect and resulted in enhanced replication of Ad5 ΔE1,E3 DNA, we tested whether expression of the reporter gene GFP, encoded by all the vectors, was similarly affected. At 72 h post-infection, cells infected with AdsiEndoG expressed significantly more GFP mRNA than AdsiCAD-infected cells. The most obvious explanation for this result is that higher concentrations of vector DNA and, hence, DNA template availability, led to higher transcription rates of the reporter gene (29). Although this would explain the bulk of the effect, differential expression rates per vector copy could also be responsible for the observation. In fact, when GFP mRNA expression was normalized with cellular Ad5 ΔE1,E3 DNA content at 72 h post-infection, cells infected with AdsiEndoG still showed higher reporter expression relative to AdsiCAD infected cells. A possible explanation for the observation may lie in the inherent property of the cytomegalovirus immediate/early (CMVie) promoter/enhancer element, which was used to drive GFP expression, to increase in activity following the activation of the cellular DNA damage response, especially in tumor derived cell lines (30). In this case, knockdown of EndoG could have led to a higher accumulation of DNA damage, due to its proposed role in cellular DNA repair/recombination, which is consistent with a cell cycle arrest/delay at the G2/M transition (31), and hence, higher transcription rates of a gene under the control of the CMVie promoter/enhancer.

Although replication of Ad5 ΔE1,E3 DNA and encoded transgene expression were enhanced by EndoG targeting, the question still remained whether AdsiEndoG could induce higher levels of CPE in the cervical cancer HeLa cells. Since, initial vector copy number is a predetermining factor in Ad5 ΔE1,E3 DNA replication and subsequent CPE development, we tested whether increasing the MOI to 50 PFU/cell would augment the cell killing ability of AdsiEndoG. All of the Ad5 ΔE1,E3 vectors were able to reduce HeLa cell viability at this high MOI, however infection with AdsiEndoG brought on the appearance of CPE sooner and to a much higher extent relative to the two control Ad5 ΔE1,E3 vectors. At 96 h post-infection, relative to the mock-infected condition, only 5% of cells infected with AdsiEndoG were still attached to the culture dish surface, compared to 25% and 37% of cells infected with AdsiCAD and AdGFP, respectively.

These results are in agreement with the study performed by Bernt et al. (3), which demonstrated synergy between Ad5 ΔE1,E3 infection and administration of cytostatic drugs on reducing viability of HeLa cells growing in culture or a HeLa xenograft established in mice. Importantly, the only chemotherapeutics that showed this effect were ones that arrested the cell cycle at the G2/M transition, whereas G0-G1 arrest did not produce an effect. Therefore, increased CPE upon infection with high MOI of AdsiEndoG, observed in our study, is most likely a result of G2/M transition arrest or delay induced by EndoG knockdown.

Another important consideration of the study performed by Bernt et al. (3) is that cells were infected prior to administration of the cytostatic drugs, meaning that the virus was fully internalized upon drug treatment. Therefore, the observed effects of G2/M arrest on enhanced tumour cell killing ability were specific to increased rates of Ad5 ΔE1,E3 DNA replication and not increased virus internalization. This is supported by our findings, since knockdown of EndoG, and hence the associated cell proliferation defect, occurred after transcription of AdsiEndoG-encoded shRNA, which required prior internalization of the virus. Thus, the positive effects of EndoG-knockdown on the ability of AdsiEndoG to induce CPE in HeLa cells were not the result of higher virus internalization rates, but occurred due to enhanced viral DNA replication.

The data showed that EndoG knockdown induced a cell proliferation defect in HeLa cells, which coincided with increased replication of Ad5 ΔE1,E3 DNA and encoded transgene expression. In addition, at high MOI, an Ad5 ΔE1,E3 vector encoding shRNA against EndoG induced higher levels of CPE in the HeLa cervical cancer cells relative to traditional Ad5 ΔE1,E3 vectors. Taken together, targeting of EndoG or other cell cycle regulators may improve the utility of first generation adenovirus vectors in cancer therapy. Firstly, expression of therapeutic or cytotoxic genes may be increased even upon infection with relatively low Ad5 ΔE1,E3 doses due to increased viral DNA replication and second, the oncolytic ability of Ad5 ΔE1,E3 may be enhanced at higher doses. However, both the efficacy and the replication potential (production of new infectious virions) of EndoG-targeting Ad5 ΔE1,E3 viruses should be as-

sessed in other tumour cells and in normal cells.

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