

## ORIGINAL ARTICLE

RNA interference-mediated knockdown of p21<sup>WAF1</sup> enhances anti-tumor cell activity of oncolytic adenoviruses

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The ability of oncolytic adenoviruses to replicate in and lyse cancer cells offers a potential therapeutic approach. However, selectivity and efficacy of adenovirus replication need to be improved. In this study, we present that loss of p21<sup>WAF1</sup> promotes adenovirus replication and more effective cell killing. To test our hypothesis, we took HCT116 colon cancer cell lines carrying deletions of either p21<sup>WAF1</sup> or p53, and infected these cell lines with wild-type adenovirus (WtD) or the oncolytic adenoviruses, ONYX-015 and Delta-24. We found that WtD, ONYX-015 and Delta-24 induced stronger cytopathic effects in HCT116 p21<sup>−/−</sup> cells compared with HCT116-WT cells. This was accompanied by increased virus production. siRNA-mediated knockdown of p21<sup>WAF1</sup>, and similarly of p27<sup>KIP1</sup>, in HCT116-WT cells also enhanced replication of and cell killing by these viruses. Furthermore, we found that TE7, an esophageal carcinoma cell line, also showed a strong cell-killing effect and virus production when p21<sup>WAF1</sup> expression was suppressed by RNA interference before adenoviruses infection. Also, H1299 and DU-145 cells transfected with p21<sup>WAF1</sup> siRNA showed higher virus production after ONYX-015 and Delta-24 infections. These observations suggest that p21<sup>WAF1</sup> plays a role in mediating replication of oncolytic viruses with potential implications for adenoviral therapy of cancer.

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## Introduction

Replication-selective oncolytic adenoviruses are promising new therapeutic anti-cancer agents that selectively replicate in and lyse cancer cells while leaving normal cells unharmed.<sup>1–3</sup> One strategy for achieving tumor selectivity is to mutate or partially delete *E1* genes that are required for viral replication in normal cells but are dispensable in cancer cells.<sup>4</sup> For example, the adenovirus mutant dl1520/ONYX-015 does not express the E1B-55K protein, which theoretically restricts its replication to p53-defective tumor cells.<sup>5</sup> Although indeed aberrations within the p53 pathway seem to contribute to ONYX-015's selectivity, it is now clear that additional, p53-independent effects are important for its tumor selectivity.<sup>6,7</sup> Another oncolytic adenovirus, Delta-24, contains a 24-bp deletion in the viral *E1A* gene, which prevents binding of *E1A* to the RB protein.<sup>8</sup> Normal cells with functional RB are thus unable to support Delta-24 replication, whereas tumor cells with non-functional RB support viral replication.

The replicative potential of oncolytic viruses is an important factor determining the therapeutic efficacy of this approach, as mathematical modeling has shown.<sup>9</sup> To improve oncolytic potency and efficacy of therapeutic adenoviruses, enhancing the replicative potential, whereas maintaining tumor selectivity, would therefore be important. A detailed understanding of the molecular mechanisms dictating oncolytic virus replication in tumors cells is a prerequisite for reaching this goal.

p21<sup>WAF1</sup> and p27<sup>KIP1</sup> are members of the CIP/KIP family of cyclin-dependent kinase inhibitors (CKIs) that show a high degree of sequence homology at their N-terminal portions, enabling them to bind to both cyclin-dependent kinases (Cdks) and cyclins. Although CIP/KIP inhibitors bind and inhibit complexes containing cyclin D, E and A, the main target seems to be the cyclin E-Cdk2 complex. Binding of CKIs to this complex blocks progression from G1 to S phase.<sup>10,11</sup> In agreement with the role of p27<sup>KIP1</sup> as a negative regulator of cell cycle progression, its loss or cytoplasmic mislocalization usually correlates with increased tumor aggressiveness and a poor clinical outcome in various types of human cancers, with rare exceptions, for example in some breast cancers, where overexpression of p27<sup>KIP1</sup> has been correlated with rapidly proliferating carcinomas.<sup>12,13</sup>

Oncolytic adenoviruses such as ONYX-015 replicate most efficiently when the target cells are in the S-phase of the cell cycle.<sup>14</sup> It is therefore conceivable that host

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proteins important for cell cycle regulation affect the efficacy of virus replication. It is therefore interesting to note that recently valproic acid, a commonly used anti-epileptic drug, was found to have potential anti-cancer activity<sup>15</sup> and to inhibit adenoviral replication and viral spread by inducing p21<sup>WAF1</sup> expression.<sup>16</sup> Here, we investigate the role of p21<sup>WAF1</sup> and p27<sup>KIP1</sup> as regulators of replication of wild-type adenovirus and the therapeutic oncolytic adenoviruses, ONYX-015 and Delta-24, in cancer cells and explore the possibility to increase virus replication through inhibition of these CKIs.

## Materials and methods

### Cell lines

HCT116 wild-type (HCT116-WT) cells and their derivatives, HCT116 p21<sup>-/-</sup><sup>17</sup> and HCT116 p53<sup>-/-</sup> (clone 379.2),<sup>18</sup> carrying homozygous deletions of *p21* and *p53*, respectively, were kindly provided by Dr Bert Vogelstein (John Hopkins Cancer Center, Baltimore, MD). HCT116 cells were cultured in McCoy's 5A medium (UCSF Cell Culture Facility, San Francisco, CA) supplemented with 10% fetal bovine serum (FBS) (Valley Biomedical Products, Winchester, VA). The pancreatic cancer cell line MIA PaCa-2 was generously provided by Dr Martin McMahon (Cancer Research Institute, UCSF Comprehensive Cancer Center, San Francisco, CA). The lung cancer cell line, H1299, was a gift from Dr David M Jablons (Cancer Research Institute, UCSF Comprehensive Cancer Center). The prostate carcinoma cell line, DU-145, was a gift from Dr Colin Collins (Cancer Research Institute, UCSF Comprehensive Cancer Center). HEK-293 (human embryonic kidney), DU-145 and MIA PaCa-2 cells were grown in Dulbecco's Modified Eagle's Medium (UCSF Cell Culture Facility) supplemented with 10% FBS. The esophageal carcinoma cell line TE7 and H1299 were maintained in RPMI-1640 (UCSF Cell Culture Facility) supplemented with 10% FBS.

### Adenoviruses

Viruses included wild-type adenovirus (WtD), the E1B-55K-deficient adenovirus mutant ONYX-015<sup>19</sup> and Delta-24, which carries a 24-bp deletion in the viral *E1A* gene<sup>8</sup> (kindly provided by Dr Juan Fueyo, University of Texas MD Anderson Cancer Center, Houston, TX). Adenoviruses were amplified in HEK-293 cells, purified using the Adenopure Purification Kit (Puresyn, Malvern, PA) and their titers determined using the Adeno-X Rapid Titer Kit (Clontech, Mountain View, CA). Ad-Luc, a firefly luciferase encoding non-replicating adenovirus, was used for quantification of virus uptake.<sup>20</sup>

### Quantification of Adeno-fiber gene copies by quantitative real-time PCR

For the quantification of cellular uptake of the virus, HCT116-WT or HCT116 p21<sup>-/-</sup> cells were infected with Ad-Luc for 24 and 48 h. DNA was purified from cell lysates by using the DNeasy Tissue Kit (Qiaagen, Valencia,

CA), and adenovirus fiber and GAPDH genes were subjected to Taqman PCR (performed by the University of California, San Francisco, Comprehensive Cancer Center Genome Core; San Francisco, CA). The adenovirus fiber probe (5'-FAM-AACCCCGTGTATCCATA TGACACGGAAA-TAMRA-3') was designed to anneal to the target between the sense primer (5'-CATGTTG TTGCAGATGAAGCG-3') and the anti-sense primer (5'-GGCACAGTTGGAGGACCG-3'). For GAPDH, the probe (5'-FAM-ATGGCACCGTCAAGGCTGAGAAC G-BHQ1-3') was used in combination with the sense primer (5'-ATTCCACCCATGGCAAATTC-3') and the anti-sense primer (5'-TGGGATTTCATTGATGAC AAG-3').

### Cell viability

For this assay, HCT116 cell lines were seeded in 96-well plates overnight and infected with WtD, ONYX-015 or Delta-24 at multiplicities of infection (MOIs) of 0, 1, 3, 5, 10 or 30. Cell viability was measured by using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI) at 2–4 days post infection. Cell viability was expressed as percentage of the untreated medium control (that is, MOI = 0). Microphotographic pictures were taken at 72 h after infection with WtD, ONYX-015 or Delta-24 at MOI of 1.

### siRNA experiments

HCT116-WT, HCT116 p21<sup>-/-</sup> and HCT116 p53<sup>-/-</sup> cells were transfected with pre-designed ON-TARGET-plus siRNAs (Dharmacon, Chicago, IL). For p21<sup>WAF1</sup>, 30 nM of CDKN1A cat # J-003471-11 and J-003471-12 (oligonucleotides (oligos) #11, #12 hereafter) and for p27<sup>KIP1</sup> 30 nM of CDKN1B cat # J-003472-05 and J-003472-08 (oligos #5, #8 hereafter) were used. H1299, DU-145, MIA PaCa-2 and TE7 cells were transfected with 50 nM of p21<sup>WAF1</sup> siRNA #12. siCONTROL non-targeting siRNA (Dharmacon) was used as negative control. Reverse transfections were carried out by first adding the siRNA/Lipofectamine complexes into empty wells of 6-well plates followed by the addition of the cells. Transfections were carried out according to the guidelines for the Lipofectamine RNAiMax reagent (Invitrogen, Carlsbad, CA). At 48 h after transfection, cells were lysed for immunoblotting or were re-seeded in 96-well plates for infection with WtD, ONYX-015 or Delta-24 at an MOI of 1. Thereafter, viability of HCT116 cells was quantified by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) (Promega) as described above. A *t*-test was used for statistical analysis.

### Viral replication assays

HCT116 cell lines were infected with WtD, ONYX-015 or Delta-24 at an MOI of 1 in 6-well plates. At 72 h after infection, HCT116 cells were scraped into culture medium and lysed with three cycles of freezing (dry ice-ethanol bath) and thawing (37°C water bath). The cell lysates were cleared by centrifugation. Supernatants were serially diluted in cell culture medium containing 10% FBS and used to infect HEK-293 cells (passaged no more than six

times before infection). Viral titers were calculated at 48 h after infection by immunostaining for a viral protein (hexon) with the AdEasy Viral Titer Kit (Stratagene, La Jolla, CA) following the manufacturer's instructions.

HCT116-WT, HCT116 p53<sup>-/-</sup> cell lines were transfected with p21<sup>WAF1</sup> or p27<sup>KIP1</sup> for 48 h and infected with WtD, ONYX-015 or Delta-24 at MOI of 1 before virus replication assay. A *t*-test was used for statistical analysis.

### Immunoblotting

Cells were lysed in RIPA buffer (Sigma-Aldrich, St Louis, MO) supplemented with Complete/Mini protease inhibitor cocktail (Roche, Indianapolis, IN). Cell lysates were separated on NuPAGE 4–12% polyacrylamide Bis-Tris gels/MES-SDS buffer (Invitrogen) and transferred onto Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). Membranes were blocked in 5% milk/TBS containing 0.1% Tween 20 (TBST). Primary mouse monoclonal antibodies used were anti-p21 (sc-6246, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-p53 (sc-126, Santa Cruz Biotechnology) and anti- $\beta$ -actin (Sigma-Aldrich). Primary rabbit polyclonal antibody used was anti-p27 (sc-776, Santa Cruz Biotechnology). Both primary and secondary antibodies were diluted in 0.5% milk/TBST. Secondary antibodies were horseradish peroxidase-conjugated and reactive proteins were visualized with enhanced chemiluminescence (ECL) (Amersham Pharmacia, Piscataway, NJ).

## Results

### Effect of loss of p21<sup>WAF1</sup> on cell killing by oncolytic adenoviruses

To determine the effect of p21<sup>WAF1</sup> on cell killing, HCT116-WT, HCT116 p21<sup>-/-</sup> and HCT116 p53<sup>-/-</sup> cells were infected with different adenoviruses and cell viability was assessed. Dose-escalation experiments showed that WtD and ONYX-015 at MOIs of 1–30 induced a clearly stronger cytopathic effect in the HCT116 p21<sup>-/-</sup> cell line compared with HCT116-WT cells (Figure 1). As observed in other cell types, the stronger cell-killing potential of WtD compared with ONYX-015 was preserved in this setting.<sup>21</sup> By three days after WtD infection at an MOI of 1, the viability of HCT116 p21<sup>-/-</sup> cells was reduced to 11% compared with 38% of HCT116-WT cells (Figures 1a and d). For ONYX-015, 3 days after infection with an MOI of 1, the viability of HCT116 p21<sup>-/-</sup> cells was reduced to 23% compared with 69% of HCT116-WT cells (Figures 1b and d). The viability of HCT116 p53<sup>-/-</sup> cell line showed similar results as HCT116-WT cells (Figures 1a–d), suggesting that the cell-killing effect may be independent of p53. Delta-24 adenovirus also showed a more potent cell-killing effect on cells lacking p21<sup>WAF1</sup> with 32% of viable HCT116 p21<sup>-/-</sup> cells compared with 72% of viable HCT116-WT cells at MOI of 1 (Figures 1c and d).

### Increased virus production in HCT116 p21<sup>-/-</sup> cells

As the increase in killing of HCT116 p21<sup>-/-</sup> cells could have been a result of enhanced apoptosis and not of virus-mediated cell lysis, we examined the ability of adenovirus to replicate and to produce viable progeny in HCT116-WT, HCT116 p21<sup>-/-</sup> and HCT116 p53<sup>-/-</sup> cells. At 72 h after cells were infected with WtD, ONYX-015 and Delta-24 at an MOI of 1, virus production was determined by using the AdEasy Viral Titer Kit (Figure 2). For WtD, ONYX-015 and Delta-24, HCT116 p21<sup>-/-</sup> infection resulted in 2.5, 4.5 and 2.1-fold, respectively, increase in viral production compared with HCT116-WT cells, respectively. HCT116 p21<sup>-/-</sup> cells had approximately twofold more viruses produced compared with HCT116 p53<sup>-/-</sup> for all viruses used in this study.

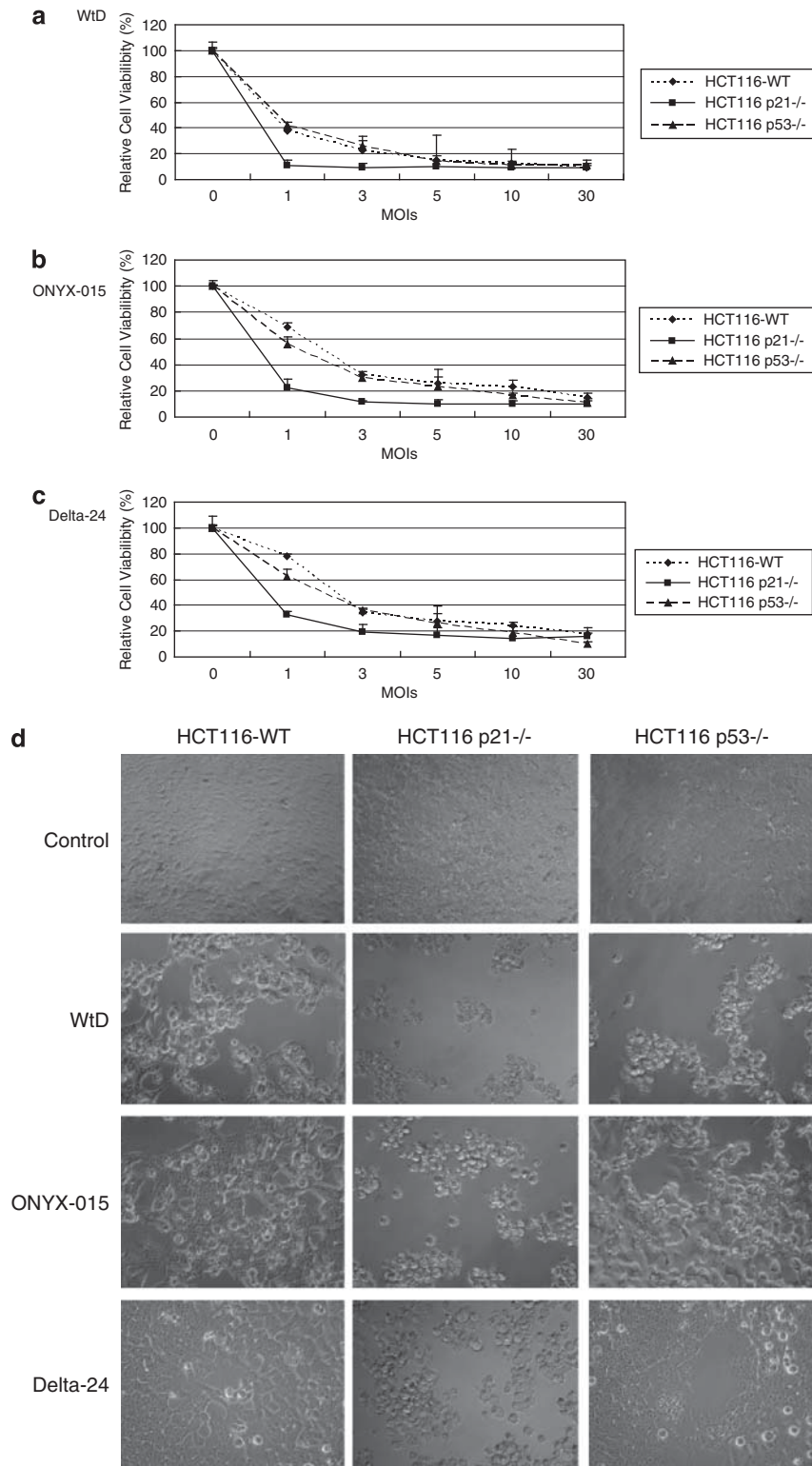
### Adenovirus entry into cancer cells is not affected by loss of p21<sup>WAF1</sup>

To ensure that differences in cell killing between HCT116-WT and HCT116 p21<sup>-/-</sup> cells were not related to differences affecting the viral entry mechanism (for example, differences in receptor expression), we compared uptake of the non-replicating Ad-Luc adenovirus<sup>19</sup> in these cell lines. As the constitutive promoter driving the luciferase reporter gene in principle could be differentially expressed in the different HCT116 cell lines, we quantified the cellular uptake of adenovirus in terms of copy numbers of the adenovirus fiber gene, normalized by GAPDH gene copy numbers, using TaqMan PCR. We found that the number of copies of adenovirus fiber taken up by the cells was the same for both cell lines after 24 and 48 h after infection (data not shown), thus indicating that loss of p21<sup>WAF1</sup> does not affect adenoviral entry into these cell lines.

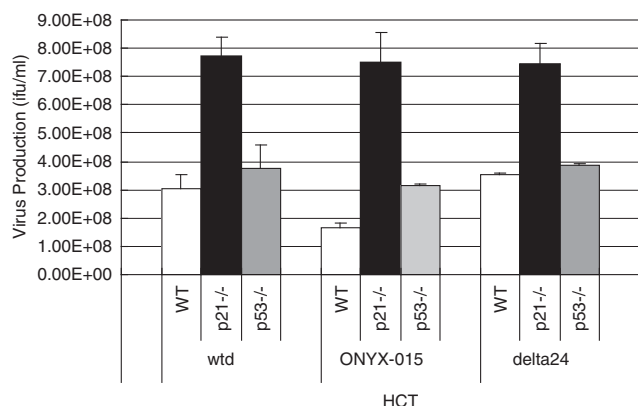
### Enhanced tumor cell killing by oncolytic adenoviruses following knockdown of p21<sup>WAF1</sup> or p27<sup>KIP1</sup> by small-interfering RNAs

To determine if indeed the loss of p21<sup>WAF1</sup> promotes adenovirus-mediated cell killing and is not an artifact related to the HCT116 p21<sup>-/-</sup> cell line, we silenced p21<sup>WAF1</sup> in HCT116-WT cells and evaluated whether cell killing and virus production were also enhanced in this system. As p21<sup>WAF1</sup> is functionally related to p27<sup>KIP1</sup><sup>10</sup> we expanded our analysis also by knockdown of p27<sup>KIP1</sup>.

The HCT116-WT, HCT116 p21<sup>-/-</sup> and HCT116 p53<sup>-/-</sup> cell lines were transfected with p21<sup>WAF1</sup>-specific siRNAs, p27<sup>KIP1</sup>-specific siRNAs or non-targeting control siRNAs, then infected with adenoviruses. Efficient knockdown of p21<sup>WAF1</sup> or p27<sup>KIP1</sup> was confirmed by Western blot analysis (Figures 3a–d), whereas transfection of non-targeting control siRNAs had no effect. To test the effect of the p21<sup>WAF1</sup> or p27<sup>KIP1</sup> siRNAs on virus-mediated cell killing, cell viability was measured following virus infection in the presence or absence of the siRNAs. At 72 h after WtD infection, the viability of HCT116-WT cells transfected with p21<sup>WAF1</sup> siRNA (oligo #11) decreases to 54% compared with the non-targeting negative siRNA control. Viability of HCT116-WT cells transfected with another p21<sup>WAF1</sup> siRNA (oligo #12) or



**Figure 1** Determination of cell viability. HCT116-WT, HCT116 p21<sup>-/-</sup> and HCT116 p53<sup>-/-</sup> cells were infected at multiplicities of infection (MOIs) of 0–30 for 72 h with wild-type adenovirus (WtD) (a), ONYX-015 (b) or Delta-24 (c). Cell viability was measured using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) assay at different times and expressed as percentage of the untreated medium control. Data represent means of triplicate experiments. Error bars indicate s.d. Cytopathic effect (d) in HCT116-WT, HCT116 p21<sup>-/-</sup> or HCT116 p53<sup>-/-</sup> cells infected with WtD or ONYX-015 or Delta-24 at an MOI of 1 for 72 h.



**Figure 2** Virus production in HCT116-WT, HCT116 p21<sup>-/-</sup> and HCT116 p53<sup>-/-</sup> cells. Viral titers were determined at 72h after infection with wild-type adenovirus (WtD), ONYX-015 or Delta-24 at an multiplicity of infection of 1. The values represent averages of viral yield measurements determined in duplicate. Error bars indicate s.d.

p27<sup>KIP1</sup> siRNA (oligos #5 and #8) decreased to ~65% after WtD infection (Figure 3e). Similarly to WtD, the oncolytic effect of ONYX-015 increased when p21<sup>WAF1</sup> was silenced (Figure 3f), although p27<sup>KIP1</sup> knockdown showed no effect on cell killing by ONYX-015 (Figure 3f). Delta-24 reduced the viability of the HCT116-WT cells to 65% when p21<sup>WAF1</sup> siRNAs (oligos #11 and #12) or p27<sup>KIP1</sup> siRNA (oligos #5 and #8) were used (Figure 3g).

HCT116 p53<sup>-/-</sup> transfected with p21<sup>WAF1</sup> siRNAs (oligos #11 and #12) (Figures 3h–j) showed no oncolytic effect with any of the adenoviruses. It suggests that it may have a p53-dependent cell-killing effect when p21<sup>WAF1</sup> are silenced in this cell line. Only a reduction of the viability to 60% after Delta-24 was observed after transfection of p27<sup>KIP1</sup> siRNA (oligos #5 and #8) in HCT116 p53<sup>-/-</sup> cells.

As expected, the viability of HCT116 p21<sup>-/-</sup> cells transfected with p21<sup>WAF1</sup> siRNA (oligo #12) did not change after infection with any of the adenoviruses, compared with the non-targeting negative control siRNA (data not shown), confirming that the observed effects are resulting from target knockdown and not from off-target effects. Also, all three viruses had no change in cell killing compared with non-targeting negative control in HCT116 p21<sup>-/-</sup> cells transfected with p27<sup>KIP1</sup> siRNAs (data not shown).

#### *Increased viral particle production following knockdown of p21<sup>WAF1</sup>*

To confirm that the increase in cell killing was indeed related to increased virus production, a measure of virus replication, production of viral particles was determined using the AdEasy Viral Titer kit (Stratagene). In HCT116-WT, we found an increase of WtD, ONYX-015 and Delta-24 production (Figures 4a–c), after transfection with p21<sup>WAF1</sup> siRNA (oligos #11 and #12) compared with the non-targeting negative siRNA control. Similarly, an increase in production of all viruses was found after transfection of p27<sup>KIP1</sup> siRNA (oligos #5 and #8). These

results suggested that p21<sup>WAF1</sup> and p27<sup>KIP1</sup> are playing a role in modulating efficacy of virus replication.

In HCT116 p53<sup>-/-</sup> cells, increased production of WtD was measured after suppression of p21<sup>WAF1</sup> and p27<sup>KIP1</sup> by siRNAs (Figure 4d). Although less efficient than WtD, increased virus production was observed when p21<sup>WAF1</sup> and p27<sup>KIP1</sup> were silenced and infected with ONYX-015 or Delta-24 for 72 h (Figures 4e and f). These results suggest that p53 plays an important role in virus replication and oncolytic effect after knockdown of p21<sup>WAF1</sup> and p27<sup>KIP1</sup>. The exact mechanism by which p21<sup>WAF1</sup>/p27<sup>KIP1</sup> suppress replication of oncolytic adenoviruses remains unclear and is under investigation. In agreement with our observations made in cell-killing effect in HCT116 p21<sup>-/-</sup> cells, knockdown of p21<sup>WAF1</sup> and p27<sup>KIP1</sup> did not affect virus production (data not shown).

#### *p21<sup>WAF1</sup> regulates replication of adenoviruses in human tumor cell lines*

To find out whether the observed effects resulting from p21<sup>WAF1</sup> knockdown on adenovirus replication are specific for HCT116 cells, we expanded our analysis to TE7 cells, an esophageal carcinoma cell line, as this tumor type is a possible target for adenoviral therapies. Viability of this cell decreases to 28, 71 and 59% after p21<sup>WAF1</sup> knockdown (oligo #12) and adenovirus WtD, ONYX-015 and Delta-24 infection, respectively, compared with untreated cells (Figures 5a and b).

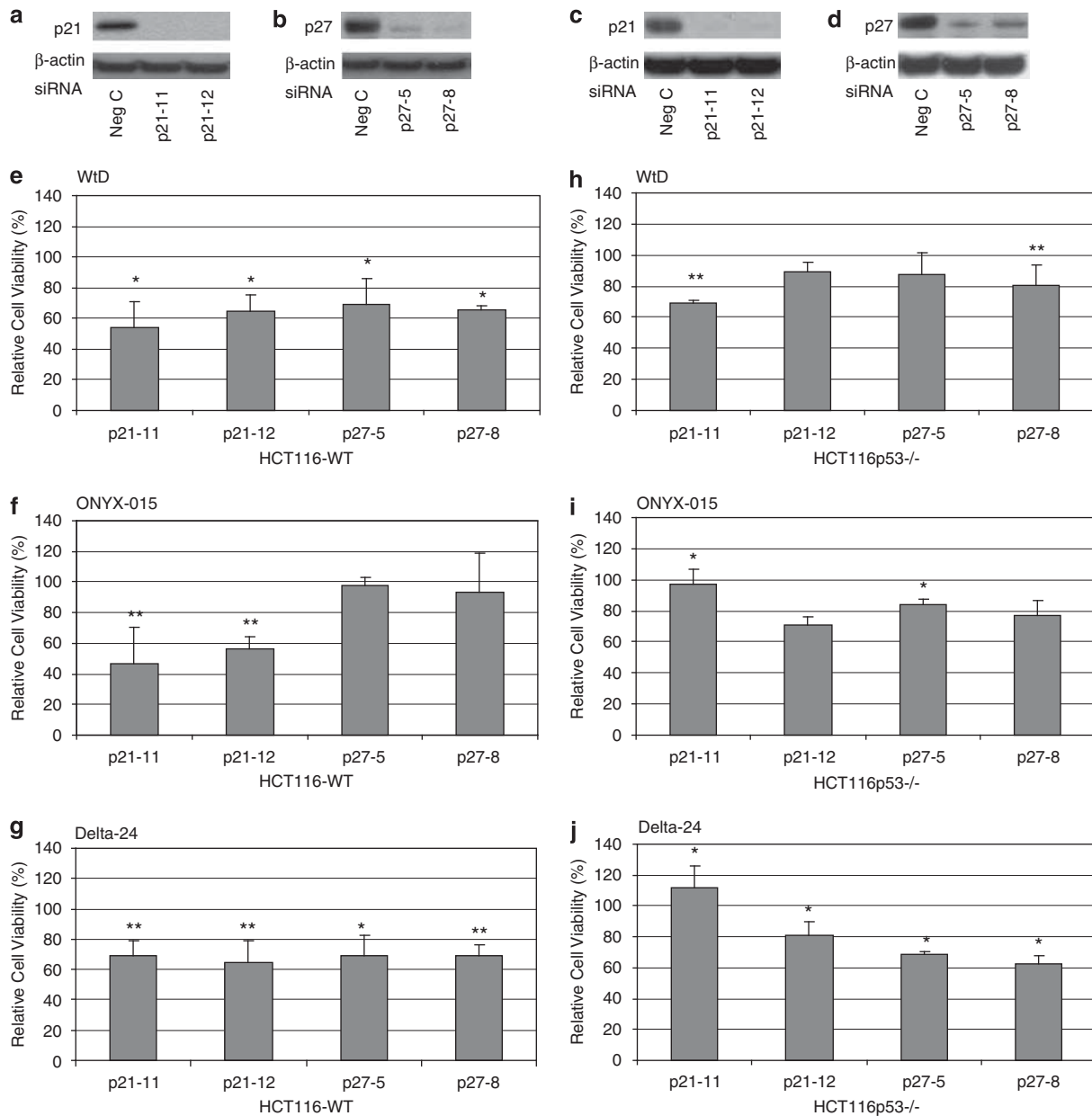
To address the role of p53 in cells lacking functional p53 expression, we used H1299, p53 null, and DU-145, non-functional p53. Interestingly, no effect of p21<sup>WAF1</sup> siRNA on cell killing or virus production were observed for both cells lines infected with WtD (Figures 5a, c, d and 6). For H1299 cells infected with oncolytic adenoviruses, ONYX-015, no increase in cell-killing effect was observed, although there was an increased viruses production after p21<sup>WAF1</sup> knockdown (oligo #12) (Figures 5c and 6). For DU-145 cells, a slightly decrease in the viability and increase in virus production were observed after p21<sup>WAF1</sup> knockdown (oligo #12) and infection of ONYX-015 and Delta-24 (Figures 5d and c).

MIA PaCa-2 cells, a human pancreatic cancer cell line that does not express p21<sup>WAF1</sup>,<sup>22</sup> showed extensive cell killing when infected with WtD regardless of the presence or absence of p21<sup>WAF1</sup> siRNA (oligo #12) (Figures 5a, e and 6).

## **Discussion**

Oncolytic viruses are designed to replicate in and lyse cancer cells on the basis of genetic changes present in tumor but not normal cells. A variety of such agents were developed. For example, H101, a variant of ONYX-015, has been approved for the use in head-and-neck cancer by the Chinese authorities.<sup>23</sup> Theoretically, this therapeutic platform has the advantage of great flexibility. For example, oncolytic adenoviruses can be modified ('armed') to express therapeutic genes or changed in their

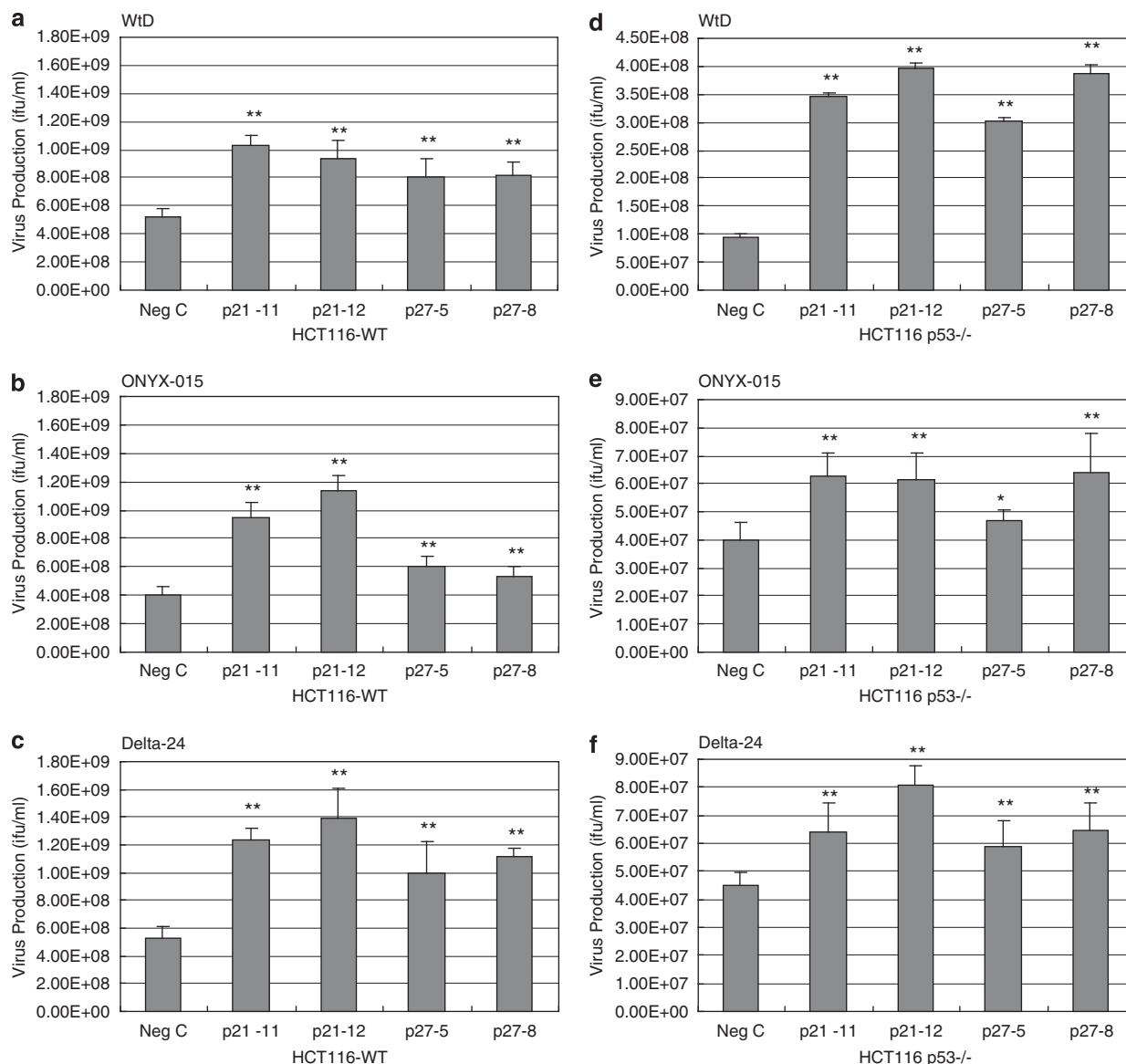




**Figure 3** Viability of HCT116 cell lines transfected with p21<sup>WAF1</sup> or p27<sup>KIP1</sup> siRNAs before adenovirus infection. HCT116-WT or HCT116 p53<sup>-/-</sup> were transfected with p21<sup>WAF1</sup> or p27<sup>KIP1</sup> siRNAs. Adenovirus infections were carried out 48 h later. For HCT116 cells, viability was assessed 72 h after addition of the wild-type adenovirus (WtD) or Delta-24 adenoviruses or 94 h for ONYX-015. For HCT116 p53<sup>-/-</sup> cells, viability was assessed 72 h after addition of the WtD or 94 h for ONYX-015 and Delta-24. Western blot analysis of p21<sup>WAF1</sup> (a and c) or p27<sup>KIP1</sup> (b and d) expression in HCT116-WT cells transfected with p21<sup>WAF1</sup> (oligos #11 and #12), p27<sup>KIP1</sup> (oligos #5 and #8), or non-targeting control siRNAs.  $\beta$ -actin was used as loading control. Cell viability of p21<sup>WAF1</sup> or p27<sup>KIP1</sup> siRNA-transfected HCT116-WT (e–g) or HCT116 p53<sup>-/-</sup> (h–j) cells followed by WtD (e and h), ONYX-015 (f and i) or Delta-24 (g and j) infection. Viability is expressed as a relative percentage of cells transfected with non-targeting siRNA control. Error bars indicate s.d. \* $P < 0.05$ , \*\* $P < 0.005$  compared with negative control.

cell tropism.<sup>2,24</sup> These approaches rely on viral replication, the capacity of the virus to self-amplify and spread in the tumor. Our data show that loss of p21<sup>WAF1</sup> promotes adenovirus replication and more effective cell killing compared with cells expressing p21<sup>WAF1</sup>. We took advantage of a derivative of the human HCT116 colon cancer cell line harboring a homozygous deletion of p21<sup>-/-</sup>, and found that wild-type adenovirus and

ONYX-015 replicate more efficiently in tumor cells lacking p21<sup>WAF1</sup> expression compared with wild-type cells (Figure 1). This finding was confirmed through our experiments using small-interfering RNAs directed against p21<sup>WAF1</sup> and p27<sup>KIP1</sup>, which increased virus-mediated cell killing and production of viable viral particles in HCT116 cells expressing wild-type p53 (Figures 3 and 4). In agreement with our findings, Hoti

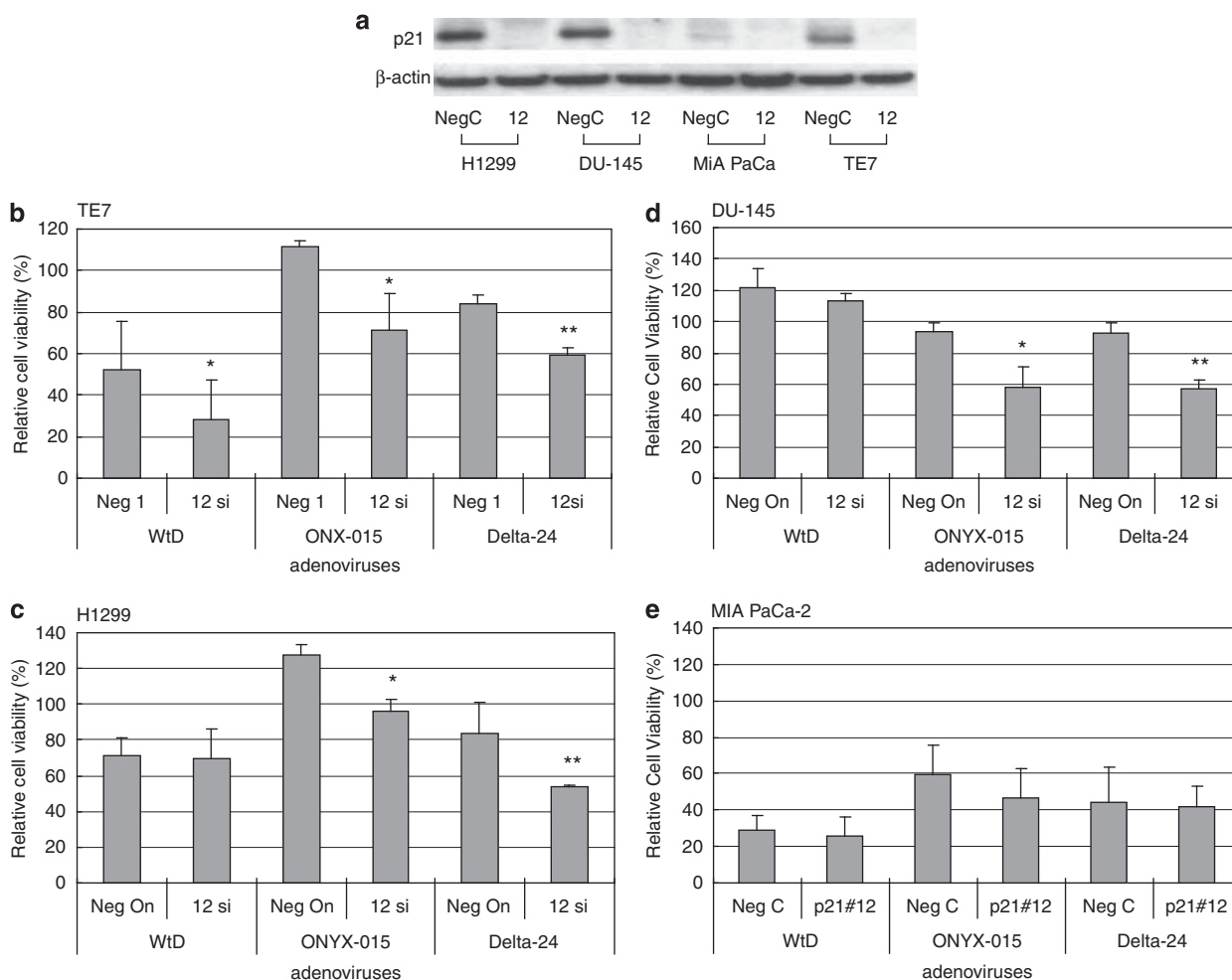


**Figure 4** Replication efficiency of adenovirus in HCT116-WT or HCT116 p53<sup>-/-</sup> cells transfected with p21<sup>WAF1</sup> or p27<sup>KIP1</sup> siRNAs. HCT116-WT (a-c) or HCT116 p53<sup>-/-</sup> (d-f) cells were transfected with p21<sup>WAF1</sup> or p27<sup>KIP1</sup> siRNAs for 48 h. The viral titers were determined 72 h after infection with wild-type adenovirus (WtD) (a and d) or ONYX-015 (b and e) or Delta-24 (c and f). Results are shown as means of duplicate experiments. Error bars represent s.d. \**P* < 0.05, \*\**P* < 0.005 compared with negative control.

*et al.*, 2006<sup>16</sup> recently showed that valproic acid upregulates p21<sup>WAF1</sup> expression resulting in inhibition of adenovirus replication. The exact mechanism by which CDK inhibitors mediate virus replication remains elusive. However, it is known that p21<sup>WAF1</sup> interacts with SET (also called template activating factor TAF1) and proliferating cell nuclear antigen (PCNA). SET stimulates the replication of the adenovirus DNA and PCNA increases DNA replication and repair. Thus, binding of p21<sup>WAF1</sup> to SET and PCNA may inhibit viral DNA replication.<sup>25,26</sup>

We examined the effects of CDK inhibitors on the oncolytic adenoviruses ONYX-015 and Delta-24, which have been developed as therapeutics for the use in

humans. These viruses differ in deletions within the adenovirus *E1*-gene region. ONYX-015 is characterized by a deletion of E1B-55K, whereas Delta-24 carries a deletion in the E1A region. Our results using p21<sup>WAF1</sup> or p27<sup>KIP1</sup> siRNAs in HCT116-WT cells show that both viruses behave similarly in regard to cell killing and virus production (Figures 3 and 4). HCT116 cells lacking p53 function, showed no difference in cell viability in the presence or absence of p21<sup>WAF1</sup> or p27<sup>KIP1</sup> siRNAs (Figure 3). However, replication of adenoviruses was more productive in these cells when p21<sup>WAF1</sup> or p27<sup>KIP1</sup> expression was suppressed by siRNA before infection. We expanded our studies using two different cell lines with non-functioning p53, H1299 and DU-145, and we found



**Figure 5** Viability of TE7 (b), H1299 (c), DU145 (d) and MIA PaCa-2 (e) cells transfected with p21<sup>WAF1</sup> siRNAs before adenovirus infections. Cells were transfected with p21<sup>WAF1</sup> siRNA for 48 h. Western blotting of p21<sup>WAF1</sup> (a) in cells transfected with p21<sup>WAF1</sup> siRNA or non-targeting siRNA. β-actin was used as loading control. Cell viability was measured at 72 h after infection with wild-type adenovirus (WtD), ONYX-015 or Delta-24. Data is represented as cell viability relative to untreated cells (equal to 100%). \**P* < 0.05, \*\**P* < 0.005 compared with negative control.

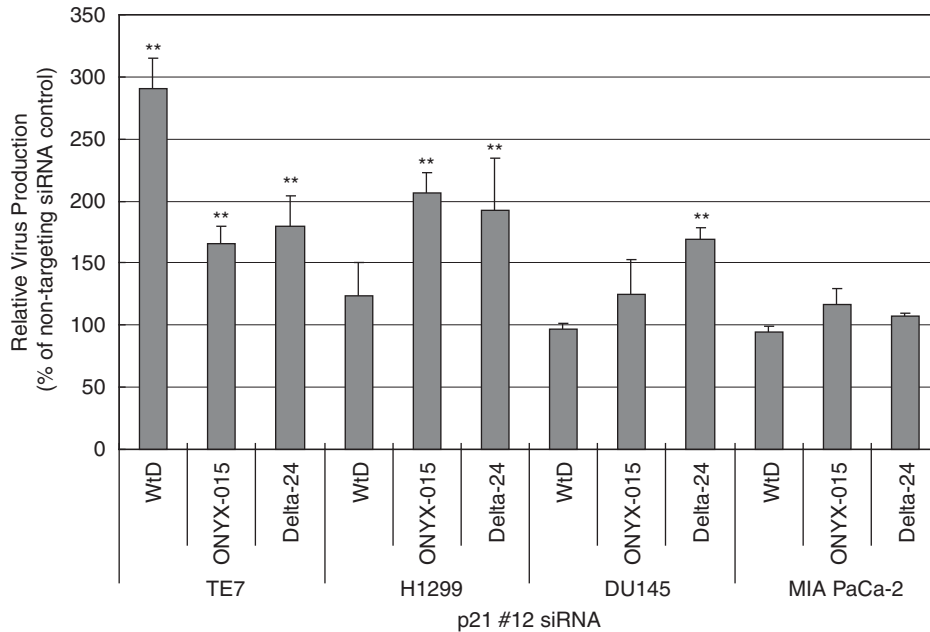
that again virus production but not cell-killing effect were enhanced after p21 knockdown in cells infected with oncolytic adenoviruses, ONYX-015 or Delta-24. This suggest that p53 plays a role in cell killing or virus production when its downstream, p21, is silenced. Earlier reports have shown the requirement of p53 in adenovirus-induced cell death. Georger *et al.*<sup>27</sup> reported that wild-type p53 was associated with increased anti-tumor activity of ONYX-015 in human malignant glioma xenografts. Given the complexities of virus replication and cancer cell environment, understanding the mechanism that increase oncolytic virus replication and cell killing is under investigation to provide information relevant to cancer therapy.

We found that TE7, an esophageal carcinoma cell line harboring wild-type p53, also showed enhanced viral cell killing when p21<sup>WAF1</sup> expression was suppressed by siRNA before infection. The pancreatic cancer cell line, MIA PaCa-2, does not express p21<sup>WAF1</sup> and was very susceptible to infection and cell killing by adenoviruses in the presence or absence of p21<sup>WAF1</sup> siRNA.

Delta-24 carries a deletion within the CR2 region of *E1A*, a region necessary for interaction with and inactivation of RB. Fueyo *et al.*<sup>28</sup> showed that D54MG and U-251MG cells treated with replication-deficient adenoviral vectors expressing either Rb or p21<sup>WAF1</sup> and infected the cells with Delta-24-RGD had less cell death. This finding also confirms that expression of p21<sup>WAF1</sup> decreases the cytopathic effects of the Delta-24 adenovirus. Delta-24 has been extensively studied pre-clinically as a promising therapy for the treatment of gliomas and ovarian cancer. Recently, a phase I trial of Delta-24RGD have been approved (Dr J Fueyo, personal communication).

It is interesting to note that p21<sup>WAF1</sup> has shown to regulate replication of other viruses. For example, p21<sup>WAF1</sup> was found to restrict human immunodeficiency virus type 1 (HIV-1) infection of hematopoietic stem cells. Zhang *et al.*<sup>29</sup> showed that p21<sup>WAF1</sup> blocked viral infection by complexing with HIV-1 integrase and aborting chromosomal integration.





**Figure 6** Replication efficiency of adenovirus in TE7, H1299, DU145 and MIA PaCa-2 cells transfected with p21<sup>WAF1</sup> siRNAs for 48 h. The viral titers were determined at 72 h after infection with wild-type adenovirus (WtD), ONYX-015 or Delta-24. Results are shown as means of duplicate experiments. Data are represented as relative percentage of cells transfected with non-targeting siRNA control (equal to 100%). Error bars represent s.d. \*\* $P < 0.005$  compared with negative control.

Taken together, our data illustrate an important role for p21<sup>WAF1</sup> as modulator of replication of oncolytic adenoviruses with potential implications for the design of future clinical trials and improved oncolytic adenoviruses. p27<sup>KIP1</sup> also reduced killing of HCT116 cells by the oncolytic adenoviruses used, particularly by WtD and Delta-24.

p21<sup>WAF1</sup> expression may represent a predictive marker for efficacy of virus replication in human cancer which could be useful for selecting patients with the highest likelihood of tumor response. Furthermore, improved oncolytic adenoviruses expressing p21<sup>WAF1</sup> siRNA under the control of tumor or tissue-specific promoters or liposomal delivery of p21<sup>WAF1</sup> siRNAs might represent interesting new approaches. Interestingly, antisense oligonucleotides directed against human p21<sup>WAF1</sup> have been described as a means of improving cancer radiotherapy.<sup>30</sup>

In summary, this study highlights the role of p21<sup>WAF1</sup> as regulators of oncolytic adenovirus replication in cancer cells. Our findings are potentially relevant for patient selection as well as for the development of new therapeutic strategies that include suppression of p21<sup>WAF1</sup>.

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