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# Induction of apoptosis in p16$^{INK4A}$ mutant cell lines by adenovirus-mediated overexpression of p16$^{INK4A}$ protein

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## Abstract

**The tumor suppressor gene p16$^{INK4A}$ is a cyclin-dependent kinase inhibitor (CDKI) and an important cell cycle regulator. We have previously constructed a recombinant adenovirus which expresses p16 (Adp16) and shown that infection in a variety of human tumor cell lines with this recombinant virus results in high levels of p16$^{INK4A}$ protein expression resulting in cell cycle arrest and loss of cyclin-cdk activity. Furthermore, adenoviral-mediated overexpression of wild-type p16$^{INK4A}$ is more toxic in cancer cells which express mutant forms of p16$^{INK4A}$ compared to cancer cell lines containing endogenous wild-type p16. TUNEL assay and DAPI staining following infection of MDA-MB 231 breast cancer cells with Adp16 indicate that p16$^{INK4A}$-mediated cytotoxicity was associated with apoptosis. This is supported by studies demonstrating a decrease in cpp32 and cyclinB1 protein levels and induction of poly (ADP-ribose) polymerase (PARP) cleavage following infection of MDA-MB-231 cells with Adp16. These results suggest that gene therapy using Adp16 may be a promising treatment option for human cancers containing alterations in p16 expression.** *Cell Death and Differentiation* (2000) **7,** 706–711.

**Keywords:** adenovirus; p16$^{INK4A}$; apoptosis; gene therapy

**Abbreviations:** CDKI, cyclin-dependent kinase inhibitor; DAPI, 4,6-Diamidino-2-phenylindole; PARP, poly (ADP-ribose) polymerase; TUNEL, TdT-mediated dUTP-biotin nick end labeling

## Introduction

Cell proliferation is regulated by cell cycle arrest and programmed cell death (or apoptosis). The tumor suppressor gene p53, one of the most commonly mutated genes in human tumors, controls both cell cycle arrest and apoptosis in response to DNA damage.[1] Other tumor suppressor genes, including p27$^{Kip1}$, p21$^{Waf1}$, and p16$^{INK4A}$ also regulate the cell cycle by inhibiting the coalition of cyclins with cyclin-dependent kinases. p16$^{INK4A}$ maps to chromosome region 9p21[2,3] and is commonly mutated and/or deleted in many primary human tumors and human tumor cell lines. In addition to mutations in p16$^{INK4A}$, other mechanisms of decreased p16$^{INK4A}$ gene expression in human tumor cells have been proposed, including hypermethylation of the p16$^{INK4A}$ gene and chromatin condensation.[4–6] p16$^{INK4A}$-mediated cell cycle arrest involves inhibition of cyclin D-Cdk4/6 complex formation via direct binding of p16$^{INK4A}$ with Cdk4/6. This binding to Cdk4/6 inhibits pRb phosphorylation resulting in G1 arrest.[7]

Previously we reported the construction of a recombinant adenoviral vector expressing wild-type p16$^{INK4A}$. Adenoviral-mediated overexpression of p16$^{INK4A}$ in cancer cells resulted in G1 arrest, an effect that was related to p16$^{INK4A}$ and Rb status of the tumor cell lines.[8] Most tumor cells containing mutated p16$^{INK4A}$ are pRb positive and our previous studies indicated that human tumor cell lines expressing mutant p16$^{INK4A}$ and wild-type RB protein were more sensitive to the cytotoxic effects of Adp16 infection compared to tumor cell lines expressing wild-type p16$^{INK4A}$. In the present studies, we have explored the mechanism of Adp16-mediated cytotoxicity and the role of p16$^{INK4A}$ overexpression in the induction of apoptosis in human tumor cells.

## Results

### Overexpression of p16$^{INK4A}$ induces apoptosis

We have previously reported that infection with Adp16 is cytotoxic to human cancer cell lines and that this cytotoxicity is related to the p16 and Rb status of the cancer cell lines.[8] To determine the possible contribution of apoptosis to Adp16-mediated cytotoxicity, TUNEL analyses were performed by flow cytometry on MDA-MB-231 cell, a breast cancer line which has a homozygous deletion of p16. MDA-MB-231 cells were infected with 100 p.f.u./cell of Adp16 or AdControl for 1, 3 and 5 days, and TUNEL assays were performed as described in the Materials and Methods. In this assay, induction of apoptosis is represented by an increase in DNA fragments that are labeled with biotin-dUTP and detected by binding to avidin-FITC. As shown in Figure 1A, infection of MDA-MB-231 cells with Adp16 resulted in a rightward shift of

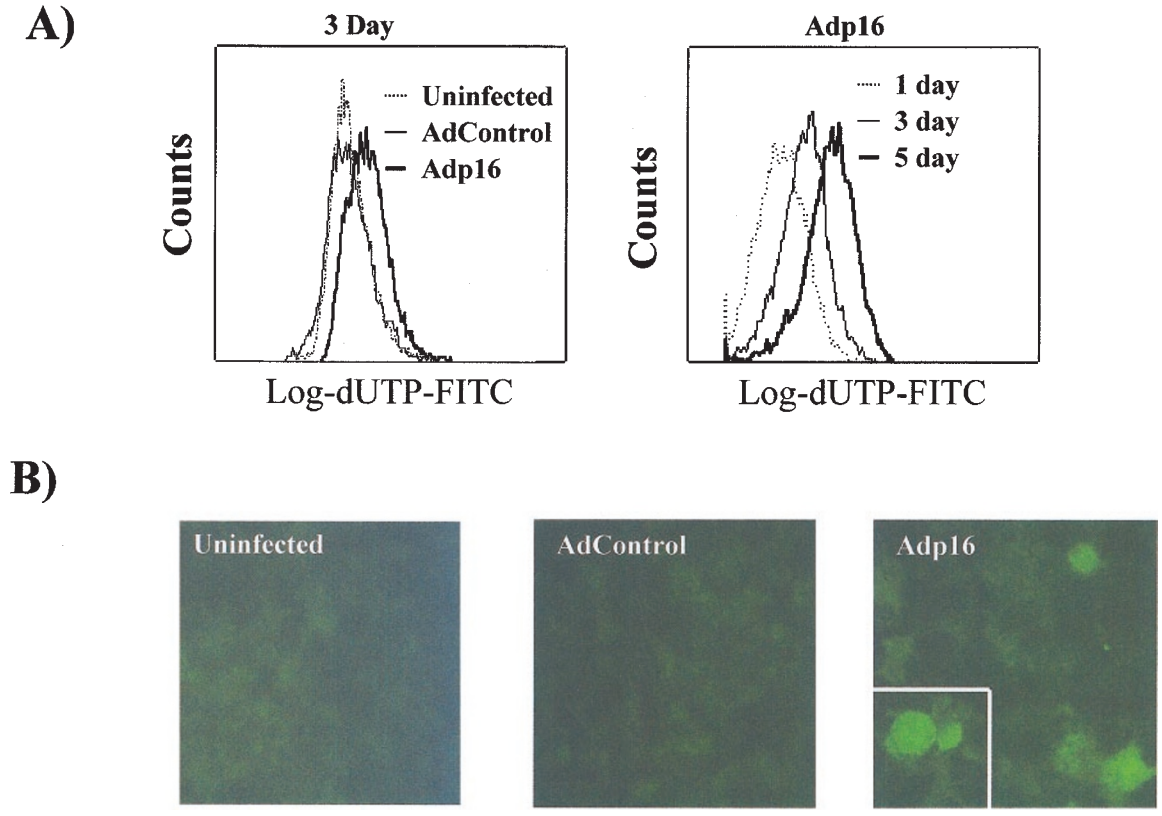


**Figure 1** (**A**) Flow cytometric TUNEL analyses for apoptosis on MDA-MB-231 cells. Cells ($2 \times 10^6$), uninfected (- - - -) and infected with 100 p.f.u./cell of Adp16 (bold line) or AdControl (thin line) for 3 days were harvested and analyzed (left panel). Cells infected with 100 p.f.u./cell of Adp16 for 1, 3 and 5 days were analyzed (right panel). (**B**) TUNEL nuclear staining on MDA-MB-231 cells. Cells were infected with 100 p.f.u./cell of AdControl or Adp16 and incubated for 3 days. Whole cells were collected, mounted on slides, subjected to TUNEL nuclear staining, and viewed by fluorescence microscopy. Inset of Adp16 panel is one apoptotic cell at a higher magnification

the mean fluorescence intensity when compared to the baseline fluorescence following infection with AdControl. Although a shift in fluorescence intensity was observed 3 days after infection, this shift was maximal following 5 days after infection with Adp16 (Figure 1A). This can also be detected by an increase in the nuclear staining seen by fluorescence microscopy after *in situ* TUNEL (Figure 1B).

Morphological changes in response to Adp16 infection of MDA-MB-231 cells were examined by fluorescence microscopy after staining with DAPI. As shown in Figure 2A, brightly stained condensed nuclei appeared in approximately 50% of cells. Pyknotic nuclei and condensed ball of chromatin were apparent on day 3 and 5 days after Adp16 infection. These results provide further evidence that Adp16 infection of MDA-MB-231 breast cancer cells produces apoptosis.

## Adp16-mediated apoptosis is associated with PARP cleavage and CPP32 cleavage

The process of apoptosis involves activation of a cascade of proteolytic enzymes belonging to the caspase family resulting in cleavage of several key cellular components including poly (ADP-ribose) polymerase (PARP) and CPP32.[9,10] Thus, detection of cleavage of these proteins following AdP16 infection is further evidence for apoptosis. As shown in Figure 2B, MDA-MB-231 cells infected with Adp16 (1, 10, and 100 p.f.u./cell) resulted in marked overexpression of p16$^{INK4A}$ protein. Adp16 infection of MDA-MB-231 cells was also associated with the appearance of a specific $M_r$ 85 000 cleavage product of PARP. This specific $M_r$ 85 000 band was not observed in the uninfected MDA-MB-231 cells or in cells infected with a control adenovirus (AdControl).

Degradation of CPP32 and cyclin $B_1$ has also been reported to be associated with apoptosis.[9,11] We therefore examined levels of CPP32 and cyclin $B_1$ by Western blot analysis in MDA-MB-231 cells that were either uninfected or infected with 1, 10, and 100 p.f.u./cell of AdControl or Adp16. As shown in Figure 2B, CPP32 and cyclin $B_1$ level decreased following infection with increasing doses of Adp16 but not with AdControl. The results in Figure 2 provide additional evidence that overexpression of p16$^{INK4A}$ following Adp16 infection results in apoptosis in MDA-MB-231 breast cancer cells.

## Induction of apoptosis by overexpressed p16$^{INK4A}$ is related to the p16 status of the cell

We previously reported that the cytotoxicity of Adp16 was related to the p16$^{INK4A}$ status of the cell lines.[8] We therefore

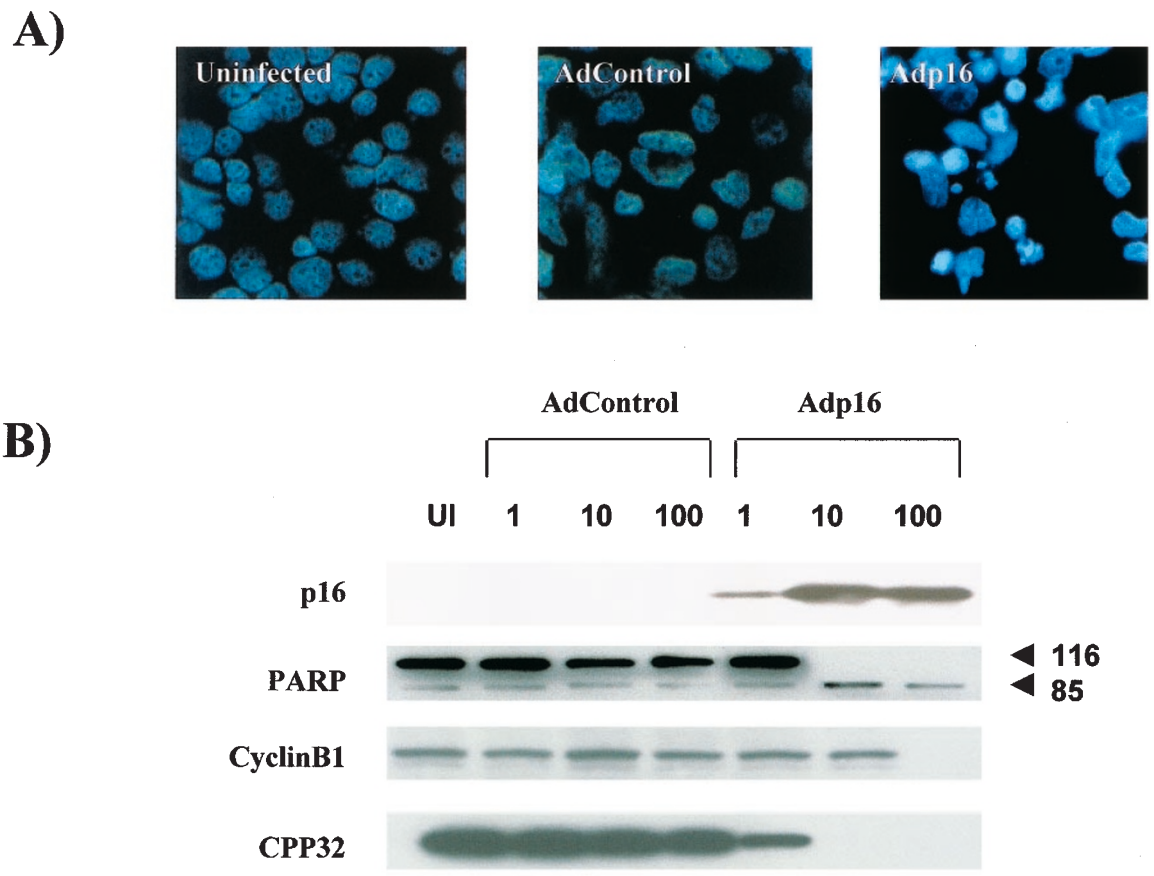


**Figure 2** (**A**) DAPI staining on MDA-MB-231. Cells were infected with 100 p.f.u./cell of AdControl or Adp16 and incubated for 3 days. Whole cells were collected and stained by DAPI and viewed under the fluorescence microscopy. (**B**) Western blot analyses of p16$^{INK4A}$, PARP, cyclin B1, CPP32 and actin in MDA-MB-231 cells. Cells ($5 \times 10^6$) were infected with 1, 10, and 100 p.f.u./cell of AdControl or Adp16. After 3 days of incubation, whole cells were collected and subjected to Western blot analysis

examined the induction of apoptosis following infection of several human cell lines by Adp16 and compared this to their endogenous p16$^{INK4A}$ status. Figure 3 shows the results of TUNEL assays following infections of H358 lung cancer cells, as well as U2OS, and Saos-2 osteosarcoma cells. MDA-MB-231, U2-OS and H358 cell lines, each of which express wild-type pRb but do not express functional p16$^{INK4A}$ protein, resulted in marked apoptosis as determined by TUNEL assay following Adp16 infection. In contrast, Saos-2, which expresses wild-type p16$^{INK4A}$ but does not express functional pRb did not show evidence of apoptosis by TUNEL assay. We also examined *in situ* TUNEL microscopy and Western blot analyses for PARP and CPP32 cleavage (Figure 4) following infection of Saos-2 cells with Adp16. These experiments indicated that overexpression of p16$^{INK4A}$ in Saos-2 cells, in contrast to the results obtained in MDA-MB-231 cells, did not result in apoptotic cell death.

## Discussion

Cell proliferation is controlled via a network of extracellular and intracellular signaling pathways and involve process both negative and positive signals of cell growth. The ultimate recipients of many of these signals are cyclin dependent kinases (CDKs), a family of enzymes which catalyze events required for cell cycle transitions. CDKs require association with cyclins for activation, and the timing of CDK activation is dependent largely upon the timing of cyclin expression.[1] In mammalian cells, positive growth signals lead to expression of $G_1$ cyclins, which serve as substrates of CDKs 2, 3, 4 and 6 thus facilitating passage through the $G_1$ cell cycle restriction point.[12]

A number of biochemical pathways, referred to as checkpoints,[13] coordinate cell cycle transitions and CDK activities are potential targets of checkpoint function. The activities of CDKs are regulated by their association with various CDK inhibitors (CDKIs). Two distinct families of CDKIs have been defined, based on their relative affinities for various CDKs as well as sequence homology. One CDKI family includes p27$^{Kip1}$, p21$^{Cip1/Waf1}$ and p57$^{Kip2}$, which although universal inhibitors of CDKs, have preferential inhibitory effect on CDK2. Sequence homology in this CDKI family is apparent in the $NH_2$-terminal CDK binding/inhibitory domain. p16$^{INK4A}$ differs somewhat from p21$^{Cip1/Waf1}$ and p27$^{Kip1}$ in that it is a relatively selective inhibitor of kinases which preferentially activate D-type

cyclins (CDK4 and CDK6). The p16$^{INK4A}$ gene maps to human chromosome 9p21, a site that is frequently deleted in melanomas and gliomas[2,3] and the p16$^{INK4A}$ (MTS1) locus is commonly altered in many human tumor cells.

We previously reported that a recombinant adenovirus expressing p16 (Adp16) induces cell cycle arrest and loss of cyclin-cdk activity in p16−/Rb+ cell lines after infection with Adp16.[8] In the present studies, we have found that infection of several human tumor cell lines containing mutant forms of endogenous p16 undergo apoptosis following infection with Adp16. This is associated with Adp16-induced cleavage of PARP and decreased levels of cyclin B1 protein. While the studies in this report have shown that Adp16 induces apoptosis, the mechanism which triggers apoptosis in these cells is unknown. To explore the mechanism by which Adp16 induces apoptosis, we examined several apoptosis-related proteins including Bax, Bcl2 and Fas by Western blot analysis. However, following infection of MDA-MB-231 by Adp16, we could not detect any significant changes of these protein levels (data not shown). Thus the mechanism of p16-mediated apoptosis in MDA-MB-231 cells is still not clear.

Recent studies have indicated that p16$^{INK4A}$-mediated cell cycle arrest can result in apoptosis of transformed cells containing wild-type p53.[14] Similarly, Frizelle reported that p16 expression in mesothelioma cells containing wild-type p53 induced cell cycle arrest and apoptotic cell death.[15] While these studies are in accordance with the studies in this report demonstrating Adp16-mediated apoptosis in human tumor cell lines, it should be noted that Adp16 infection resulted in apoptosis in MDA-MB-231 breast cancer cells, which contain mutant p53, as well as in H358 lung cancer cells, which are null for p53. Thus

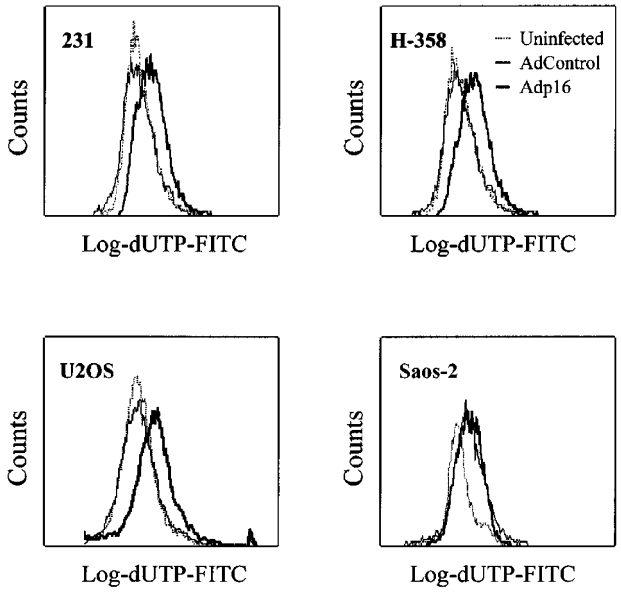


**Figure 3** Flow cytometric TUNEL analysis for apoptosis on different cancer cell lines. Cells ($2 \times 10^6$) were infected with 100 p.f.u./cell of Adp16 or AdControl for 3 days were harvested and analyzed by flow cytometry. DNA fragments were labeled with biotin-dUTP and detected by binding to avidin-FITC. MDA-MB-231, H358 and U2OS cells contain mutant p16$^{INK4A}$, Saos-2 cells contain wild-type p16$^{INK4A}$

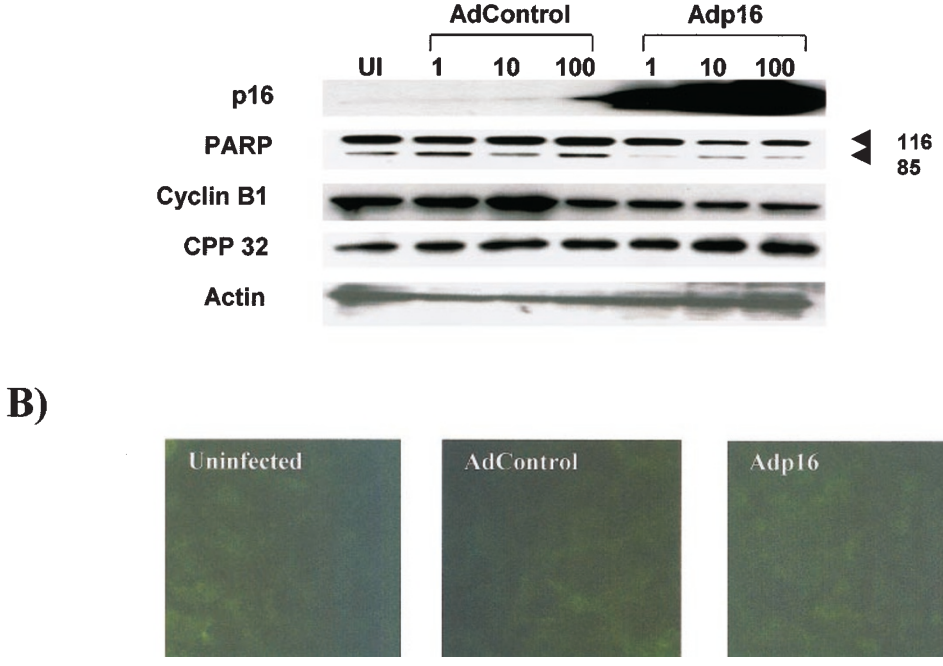


**Figure 4** Western blot analyses of p16$^{INK4A}$, PARP, cyclin B1, CPP32 and actin in Saos-2 cells. Cells ($5 \times 10^6$) were infected with 1, 10 and 100 p.f.u./cell of AdControl or Adp16. After 3 days of incubation, whole cells were collected and subjected to Western blot analyses as described in the Materials and Methods

adenoviral p16-mediated apoptosis is not dependent on the p53 status on human tumor cells.

We and others have previously reported that adenoviral-mediated overexpression of wild-type p53 resulted in apoptotic cell death in cell lines containing mutant p53. While p53 mediated apoptosis occurs within 24–48 h,[16] Adp16-mediated apoptosis was maximal at 5 days. This suggests that the apoptotic mechanism induced by Adp16 differs from that induced by Adp53. It is possible that prolonged G1 arrest of cells following Adp16 infection ultimately induces apoptosis. However, prolonged G1 arrest by itself is not sufficient to come apoptosis as adenoviral mediated overexpression of p21$^{Cip1/Waf1}$, which also induces G1 arrest, actually renders cells resistant to apoptosis.[17] The reason for this difference in sensitivity of cells to undergo apoptosis following Adp21 *vs* Adp16 infection is unclear.

Mutations that inactivate the CDK inhibitory function of the p16 gene are associated with familial melanoma and occur at high frequencies in esophageal cancers (~30%) and cancers of the biliary tract (~50%).[18,19] Furthermore, homozygous deletions of the p16 locus commonly occur in gliomas (~55%), mesotheliomas (~55%), nasopharyngeal carcinomas (~40%), and acute lymphocytic leukemias (30%). Many other malignant tumors have been reported to have p16 mutations and/or deletions including sarcomas, non-small-cell lung carcinomas, cancers of the bladder, ovary, pancreas, and head and neck.[19] The studies presented in this report indicate that tumor cell lines containing mutations in p16 are sensitive to Adp16-mediated apoptosis and suggests that Adp16 may have therapeutic potential in human tumors containing alterations in the expression of the endogenous p16 gene.

## Materials and Methods

### Tissue culture

MDA-MB-231 (ATCC HTB26) breast cancer cells, U2OS (ATCC HTB96) and Saos-2 (ATCC HTB85) osteosarcoma, and H-358 lung cancer cells (kindly provided by J Minna, University of Texas, Dallas, TX, USA) were cultured in Improved Minimal Essential Medium (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum as described previously.[8]

### Construction of adenoviral vectors and infection procedure

Adp16, a replication-deficient recombinant adenovirus that expresses p16$^{INK4A}$, was constructed in our laboratory as described previously.[8] AdControl is a control recombinant adenovirus that does not carry any transgene but has the same adenoviral backbone as Adp16. Infection with Adp16 and AdControl was accomplished by incubating cells with different doses of the viruses (p.f.u./cell) in serum-free medium for 2 h followed by addition of medium containing 10% serum and further incubation for the required time at 37°C.

### Assays for apoptosis

For detection of apoptosis by the TUNEL (TdT mediated dUTP-biotin nick end labeling) method, cells were infected with the adenoviral vectors, collected by incubation with trypsin-EDTA, and washed two times with PBS. TUNEL was performed using MEBSTAIN Apoptosis Kit (Medical and Biological Laboratories Co., Ltd, Japan) according to the manufacturer's directions. FACS analyses were performed on a FACS Calibur instrument (Becton Dickinson, Mansfield, MA, USA). Cells were also collected by cytospin, mounted on glass slides, and viewed under the fluorescence microscope.

For detection of apoptosis by DNA staining with DAPI, cultured and infected cells were washed with phosphate buffered saline (PBS) and harvested with trypsin-EDTA. Cells (50 000) were resuspended in 50 $\mu$l of PBS, and 100 $\mu$l of 22% bovine serum albumin (BSA) was added to the sample. This suspension was added into the bottom of a cytofunnel mounted with a microscope slide into the cyto-centrifuge and spun at 500 r.p.m. for 5 min. Slides were air dried for 30 min at room temperature and washed with PBS. DAPI (200 $\mu$l solution containing 2.5 $\mu$g/ml in PBS) was applied for 30 min at room temperature. After washing with PBS, samples were stored in the dark at 4°C and viewed under the fluorescence microscope.

### Western blot analysis

Cells ($2 \times 10^6$) were infected by adenoviral vectors, scraped and the lysates subjected to Western blot analysis as previously described.[16] Blots were probed with anti-p16 (PharMingen, San Diego, CA, USA), anti-actin, anti-cyclinB1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-PARP (Ab-2, Oncogene Research Products, Cambridge, MA, USA), anti-CPP32 (Ab-1, Oncogene Research Products, Cambridge, MA, USA).

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