

## Apoptosis-Related Gene Transcription in Human A549 Lung Cancer Cells via A<sub>3</sub> Adenosine Receptor

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### Key Words

A<sub>3</sub> adenosine receptor • A549 lung cancer cell • Apoptosis • Transcription • Bcl-2 family

### Abstract

**Background/Aims:** Extracellular adenosine induces apoptosis in a variety of cancer cells via diverse signaling pathways. The present study investigated the mechanism underlying adenosine-induced apoptosis in A549 human lung cancer cells. **Methods:** MTT assay, TUNEL staining, flow cytometry using propidium iodide and annexin V-FITC, real-time RT-PCR, Western blotting, monitoring of mitochondrial membrane potentials, and assay of caspase-3, -8, and -9 activities were carried out in A549 cells, and the siRNA to silence the A<sub>3</sub> adenosine receptor-targeted gene was constructed. **Results:** Extracellular adenosine induces A549 cell apoptosis in a concentration (0.01-10 mM)-dependent manner, and the effect was inhibited by the A<sub>3</sub> adenosine receptor inhibitor MRS1191 or knocking-down A<sub>3</sub> adenosine receptor. Like adenosine, the A<sub>3</sub> adenosine receptor agonist 2-Cl-IB-MECA also induced A549 cell apoptosis. Adenosine increased expression of mRNAs for Puma, Bax, and Bad, disrupted mitochondrial

membrane potentials, and activated caspase-3 and -9 in A549 cells, and those adenosine effects were also suppressed by knocking-down A<sub>3</sub> adenosine receptor. **Conclusion:** Adenosine induces A549 cell apoptosis by upregulating expression of Bax, Bad, and Puma, to disrupt mitochondrial membrane potentials and to activate caspase-9 followed by the effector caspase-3, via A<sub>3</sub> adenosine receptor.

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### Introduction

Accumulating evidence has shown that extracellularly applied adenosine is capable of inducing apoptosis in a variety of cancer cells via intrinsic and extrinsic pathways. For the intrinsic pathway, extracellular adenosine is taken up into cells by adenosine transporters and converted to AMP by adenosine kinase, which serves as apoptosis-initiating signals to downregulate expression of c-Fas-associated death domain protein (FADD)-like interleukin-1 $\beta$ -converting enzyme inhibitory protein (c-FLIP), that neutralizes caspase-8 inhibition due to c-FLIP, thereby activating caspase-8 and the effector caspase-3, or to upregulate DIABLO expression and downregulate

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inhibitor of apoptosis protein (IAP) expression, thereby activating caspase-3 following neutralization of caspase-3 inhibition due to IAP, responsible for adenosine-induced apoptosis in HuH-7 human hepatoma cells [1, 2]. Moreover, intracellularly transported adenosine induces apoptosis in HepG2 human hepatoma cells by tuning apoptosis-mediator gene transcription [3] or in HuH-7 cells by upregulating expression of apoptosis-inducing factor-homologous mitochondrion-associated inducer of death (AMID) [4]. Converted AMP from adenosine in cells, alternatively, activates AMP-activated protein kinase (AMPK) to induce apoptosis in GT3-TKB human lung cancer cells and HuH-7 cells [5, 6].

For the extrinsic pathway, adenosine receptors ( $P_1$  purinoceptors) mediate adenosine-induced apoptotic cell death. Adenosine receptors include  $A_1$  receptor linked to  $G_i$  protein,  $A_{2a}$  and  $A_{2b}$  receptors linked to  $G_s$  protein, and  $A_3$  receptor linked to  $G_i$  and  $G_q$  protein.  $A_1$  receptor mediates apoptosis in CW2 human colonic cancer cells and RCR-1 astrocytoma cells [7, 8].  $A_2$  adenosine receptor mediates apoptosis in glioma cells, myeloid leukemia cells, breast cancer cells, and colonic cancer cells [9-11]. Emerging evidence has pointed to  $A_3$  adenosine receptor as a new target for cancer therapy. Chloro- $N^6$ -(3-iodobenzyl)-adenosine-5'- $N$ -methyl-uronamide (Cl-IB-MECA), an agonist of  $A_3$  adenosine receptor, induces cell cycle arrest at the  $G_0/G_1$  phase and apoptosis in lung cancer cells by downregulating cyclin D1, c-myc, and CDK4, activating caspase-3 and -9, cleaving poly(ADP-ribose) polymerase, and inhibiting Akt [12] or induces anti-proliferation and apoptosis in bladder cancer cells via an extracellular signal-regulated kinase (ERK)/c-Jun N-terminal kinase (JNK) pathway [13]. Cl-IB-MECA enhances TRAIL-induced apoptosis in thyroid cancer cells by modulating an NF- $\kappa$ B signaling pathway [14] or induces apoptosis in leukemia cells by upregulating TRAIL receptors [15, 16]. IB-MECA also induces  $G_1$  cell cycle arrest through a p53/Cdk4/cyclinD1 pathway or apoptosis in prostate cancer cells by downregulating Bcl-2, disrupting mitochondrial membrane potentials, and activating caspase-3 [17]. CF102, an agonist of  $A_3$  adenosine receptor, induces apoptosis in hepatocellular carcinoma cells by de-regulating Wnt/NF- $\kappa$ B signal transduction pathways [18]. Overexpression of  $A_3$  adenosine receptors suppresses proliferation and induces apoptosis in malignant mesothelioma cells by inhibiting an Akt/NF- $\kappa$ B signaling pathway [19].  $A_3$  adenosine receptor, thus, regulates proliferation and apoptosis in a variety of cancer cells via diverse signaling pathways.

The present study investigated adenosine-induced apoptosis in A549 human lung cancer cells. We show here that adenosine induces A549 cell apoptosis by upregulating expression of Bax, Bad, and Puma via  $A_3$  adenosine receptor.

## Materials and Methods

### Cell culture

A549 cells were purchased from Health Science Research Resources Bank (Osaka, Japan). Cells were grown in minimum essential medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 0.1 mM non-essential amino acids, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5%  $CO_2$  and 95% air at 37 °C.

### Assay of cell viability

Cell viability was assayed by the method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as described previously [1].

### Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining

TUNEL staining was performed to detect *in situ* DNA fragmentation as a marker of apoptosis using an In Situ Apoptosis Detection Kit (Takara Bio, Otsu, Japan). Briefly, fixed and permeabilized A549 cells were reacted with terminal deoxynucleotidyl transferase and fluorescein isothiocyanate (FITC)-deoxyuridine triphosphate for 90 min at 37 °C. FITC signals were visualized with a confocal scanning laser microscope (LSM 510, Carl Zeiss Co., Ltd., Oberkochen, Germany).

### Apoptosis assay

Cells were stained with both propidium iodide (PI) and annexin V-FITC, and loaded on a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA) available for FL1 (annexin V) and FL2 (PI) bivariate analysis. Data from 10,000 cells/sample were collected, and the quadrants were set according to the population of viable, unstained cells in untreated samples. CellQuest analysis of the data was used to calculate the percentage of the cells in the respective quadrants.

### Construction and transfection of siRNA

The siRNA to silence human  $A_3$  adenosine receptor-targeted gene ( $A_3$ R siRNA) and the negative control siRNA (NC siRNA) were obtained from Ambion (Austin, TX, USA). The sequences of  $A_3$ R siRNA was: 5'-GGG AGU GAA UUG AAU UUA ATT-3' and 5'-UUA AAU UCA AUU CAC UCC CTG-3'.

The NC siRNA or the  $A_3$ R siRNA was reverse-transfected into A549 cells using a Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). Cells were used for experiments 48 h after transfection.

### Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs of A549 cells were purified by an acid/guanidine/thiocyanate/chloroform extraction method using the Sepasol-RNA I Super kit (Nacalai, Kyoto, Japan). After purification, total RNAs were treated with RNase-free DNase I (2 units) at 37 °C for 30 min to remove genomic DNAs, and 10 µg of RNAs was resuspended in water. Then, random primers, dNTP, 10x RT buffer, and Multiscribe Reverse Transcriptase were added to an RNA solution and incubated at 25 °C for 10 min followed by 37 °C for 120 min to synthesize the first-strand cDNA. Real-time RT-PCR was performed using a SYBR Green Realtime PCR Master Mix (Takara Bio) and the Applied Biosystems 7900 real-time PCR detection system (ABI, Foster City, CA, USA). Thermal cycling conditions were as follows: first step, 94 °C for 4 min; the ensuing 40 cycles, 94 °C for 1 s, 65 °C for 15 s, and 72 °C for 30 s. The expression level of each mRNA was normalized by that of GAPDH mRNA. Primers used for real-time RT-PCR are shown in Table 1.

### Western blotting

Cells were lysed with 1% (w/v) sodium dodecyl sulfate (SDS). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a TGX gel (BioRad, Hercules, CA, USA) and then transferred to polyvinylidene difluoride membranes. Blotting membranes were blocked with TBS-T [150 mM NaCl, 0.1% (v/v) Tween20 and 20 mM Tris, pH 7.5] containing 5% (w/v) bovine serum albumin and subsequently incubated with an anti- $A_1$  receptor antibody (Oncogene, Cambridge, MA, USA), an anti- $A_{2a}$  receptor antibody (Oncogene), an anti- $A_{2b}$  receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), an anti- $A_3$  receptor antibody (Santa Cruz Biotechnology), or an anti- $\beta$ -actin antibody (Sigma, St Louis, MO, USA). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibody. Immunoreactivity was detected with an ECL kit (GE Healthcare, Piscataway, NJ, USA) and visualized using a chemiluminescence detection system (GE Healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Pierce, Rockford, IL, USA).

### Assay of mitochondrial membrane potentials

Mitochondrial membrane potentials were measured using a DePsipher™ kit. A549 cells untreated and treated with adenosine were incubated in a DePsipher™ solution at 37 °C for 20 min. Then, cells were washed with 1 ml of a reaction buffer containing a stabilizer solution. The fluorescent signals were observed with a laser scanning microscopes (LSM 510) equipped with an epifluorescence device using a fluorescein long-pass filter (fluorescein and rhodamine) at an absorbance of 590 nm for red aggregations and at an absorbance of 530 nm for green aggregations.

### Enzymatic assay of caspase-3, -8, and -9 activities

Caspase activity was measured using a caspase fluorometric assay kit (Ac-Asp-Glu-Val-Asp-MCA for a caspase-3 substrate peptide; Ac-Ile-Glu-Thr-Asp-MCA for a

PCR primers	Oligonucleotide sequence
p53	Sense: 5'-GCCATCTACAAGCAGTCACAGCACAT-3' Anti-sense: 5'-GGCACAAACACGCACCTCAAAGC-3'
Noxa	Sense: 5'-GCAGAGCTGGAAGTCGAGTG-3' Anti-sense: 5'-GAGCAGAAGAGTTTGGATATCAG -3'
Puma	Sense: 5'-GACGACCTCAACGCACAGTA-3' Anti-sense: 5'-AGGAGTCCCATGATGAGATTGT -3'
Bax	Sense: 5'-CGGACCCGGCGAGAGGC-3' Anti-sense: 5'-TCAGCTTCTTGGTGGACGCATCC-3'
Bad	Sense: 5'-CTGGGGCTGTGGAGATCCGGAGTCGCC-3' Anti-sense: 5'-TCACTGGGAGGGGGCGGAGCTTCCCC-3'
AIF	Sense: 5'-TCACAAAGACACTGCGATTCAAACAGT-3' Anti-sense: 5'-GTTGCTGAGGTATTTCGGGGAGGAT-3'
GAPDH	Sense: 5'-GACTTCAACAGCGACACCCACTCC-3' Anti-sense: 5'-AGGTCCACCACCCTGTTGCTGTAG-3'

**Table 1.** Primers used for real-time RT-PCR.

caspase-8 substrate peptide; and Ac-Leu-Glu-His-Asp-MCA for a caspase-9 substrate peptide) as previously described [11]. Briefly, A549 cells were harvested before and after treatment with adenosine, and then centrifuged at 3,000 rpm for 5 min at 4 °C. The pellet was incubated on ice in cell lysis buffer for 10 min, and reacted with the fluorescently labeled tetrapeptide at 37 °C for 2 h. The fluorescence was measured at an excitation of wavelength of 400 nm and an emission wavelength of 505 nm with a fluorometer (Fluorescence Spectrometer, F-4500, HITACHI, Japan).

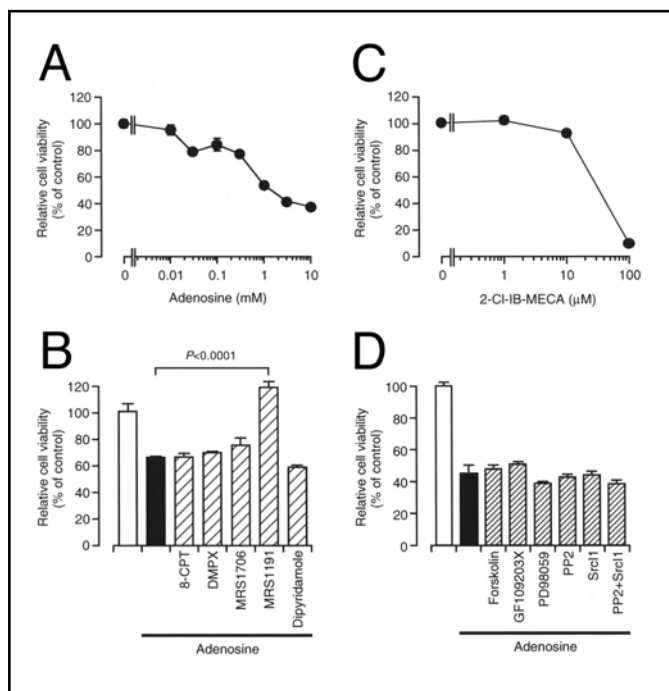
### Statistical analysis

Statistical analysis was carried out using unpaired *t*-test and Dunnett's test.

## Results

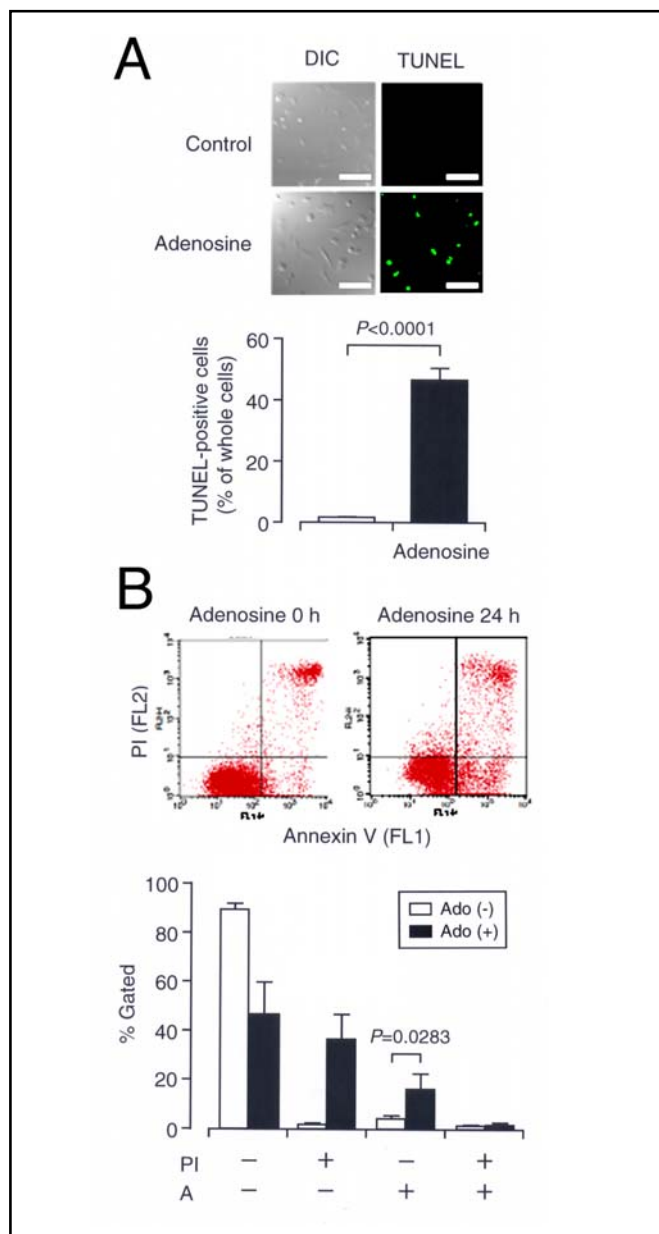
### Adenosine induces A549 cell apoptosis via $A_3$ adenosine receptor

In the MTT assay, 24-h treatment with adenosine reduced A549 cell viability in a concentration (10 µM-10 mM)-dependent manner (Fig. 1A). For cells treated with adenosine (3 mM) for 24 h approximately 45% of total cells was positive to TUNEL, while for cells untreated with adenosine TUNEL-positive cells were within 3% of total cells (Fig. 2A). In the flow cytometry using PI and annexin V-FITC, PI is a marker of dead cells and annexin V, detecting externalized phosphatidylserine residues, is a marker of apoptotic cells. Treatment with adenosine (3 mM) for 24 h significantly increased the population of PI-negative and annexin V-positive cells, which corresponds to early apoptosis (Fig. 2B). Taken together, these results indicate that adenosine induces apoptosis in A549 cells.



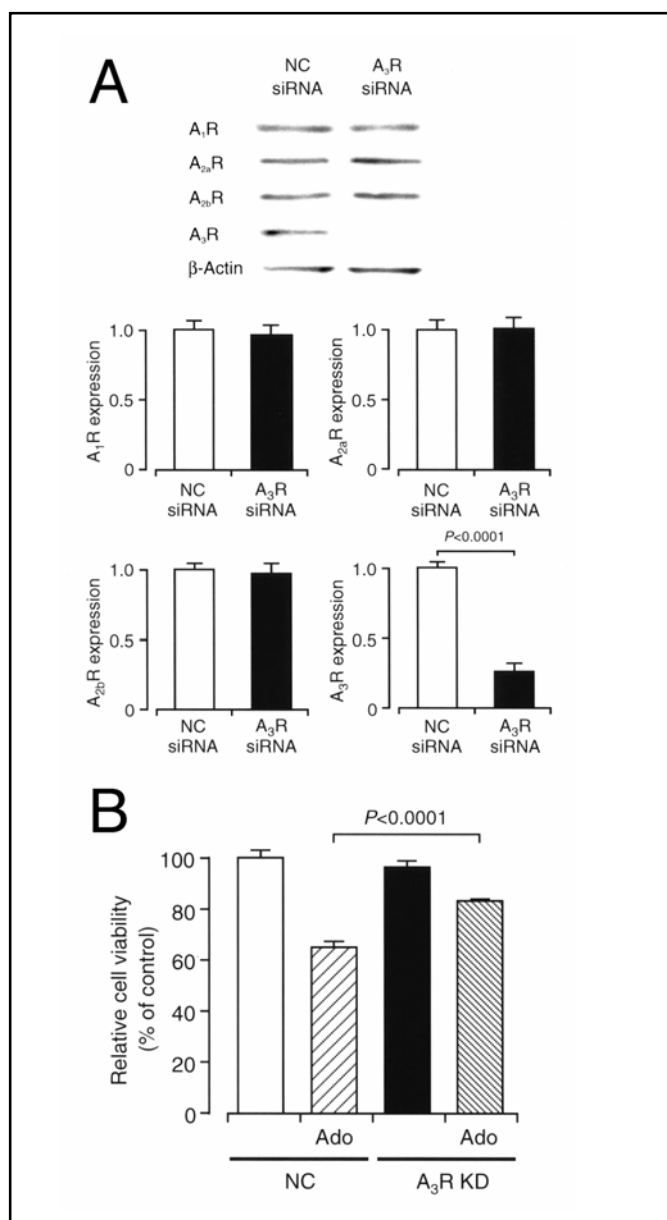
**Fig. 1.** Adenosine-induced A549 cell death. (A) Cells were treated with adenosine at concentrations as indicated for 24 h and MTT assay was carried out. In the graph, each point represents the mean ( $\pm$  SEM) percentage of basal cell viabilities (MTT intensities before adenosine treatment)( $n=4$  independent experiments). (B) MTT assay was carried out in cells treated with adenosine (3 mM) for 24 h in the presence and absence of 8-CPT (10  $\mu$ M), DMPX (10  $\mu$ M), MRS1706 (50 nM), MRS1191 (10  $\mu$ M), or dipyridamole (10  $\mu$ M). In the graph, each column represents the mean ( $\pm$  SEM) percentage of basal cell viabilities (MTT intensities before adenosine treatment in the absence of inhibitors)( $n=4$  independent experiments).  $P$  value, Dunnett's test. (C) Cells were treated with 2-Cl-IB-MECA at concentrations as indicated for 24 h and MTT assay was carried out. In the graph, each point represents the mean ( $\pm$  SEM) percentage of basal cell viabilities (MTT intensities before 2-Cl-IB-MECA treatment)( $n=4$  independent experiments). (D) MTT assay was carried out in cells treated with adenosine (3 mM) for 24 h in the presence and absence of forskolin (10  $\mu$ M), GF109203X (100 nM), PD98059 (50  $\mu$ M), PP2 (100 nM), or Src11 (200 nM). In the graph, each column represents the mean ( $\pm$  SEM) percentage of basal cell viabilities (MTT intensities before adenosine treatment in the absence of inhibitors)( $n=4$  independent experiments).

Adenosine-induced A549 cell death was reversed by MRS1191 (10  $\mu$ M), an antagonist of  $A_3$  adenosine receptor, but otherwise the death was not affected by 8-cyclopentyltheophylline (8-CPT)(10  $\mu$ M), an antagonist of  $A_1$  adenosine receptor, 3,7-dimethyl-1-propargylxanthine (DMPX)(10  $\mu$ M), an antagonist of  $A_{2a}$  adenosine receptor, MRS1706 (50 nM), an antagonist of



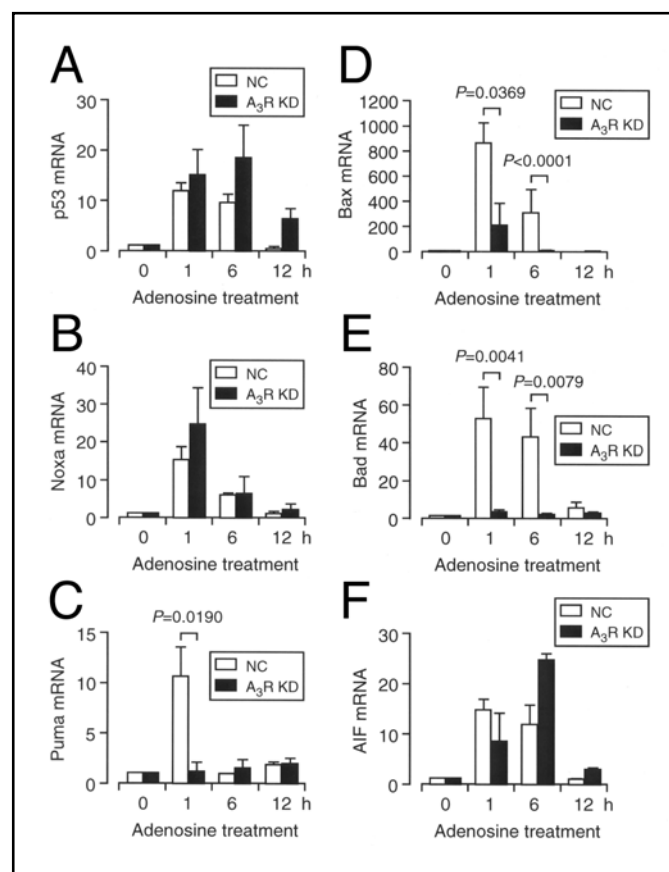
**Fig. 2.** Adenosine-induced A549 cell apoptosis. (A) Cells were untreated and treated with adenosine (3 mM) for 24 h, followed by TUNEL staining. TUNEL-positive cells were counted in the area (0.4 mm x 0.4 mm) selected at random. DIC, differential interference contrast. Bars in the pictures, 100  $\mu$ m. In the graph, each column represents the mean ( $\pm$  SEM) TUNEL-positive cell percentage of whole cells ( $n=4$  independent experiments).  $P$  values, Dunnett's test. (B) Cells were untreated and treated with adenosine (3 mM) for 24 h, and flow cytometry using PI and annexin V-FITC (A) was carried out. Typical profiles are shown in the upper column. In the graph, each column represents the mean ( $\pm$  SEM) percentage of cells in 4 fractions against total cells ( $n=4$  independent experiments).  $P$  value, Dunnett's test.

$A_{2b}$  adenosine receptor, or dipyridamole (10  $\mu$ M), an inhibitor of adenosine transporter (Fig. 1B). This suggests



**Fig. 3.** A<sub>3</sub> adenosine receptor-dependent A549 cell apoptosis. (A) Cells were transfected with the NC siRNA or the A<sub>3</sub>R siRNA, and Western blotting was carried out using antibodies against A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub> adenosine receptors 48 h after transfection. Signal intensities for adenosine receptor proteins were normalized by β-actin signal intensities. In the graph, each column represents the mean (± SEM) protein intensity (n=4 independent experiments). *P* values, unpaired *t*-test. (B) Cells transfected with the NC siRNA (NC) or the A<sub>3</sub>R siRNA (A<sub>3</sub>R KD) were untreated and treated with adenosine (Ado)(3 mM) for 24 h, and then, MTT assay was carried out. In the graph, each column represents the mean (± SEM) percentage of basal cell viabilities (MTT intensities before adenosine treatment)(n=4 independent experiments). *P* value, Dunnett's test.

A<sub>3</sub> adenosine receptor-dependent A549 cell apoptosis. Like adenosine, 24-h treatment with 2-Cl-IB-MECA, an



**Fig. 4.** Real-time RT-PCR analysis in A549 cells. Cells transfected with the NC siRNA (NC) or the A<sub>3</sub>R siRNA (A<sub>3</sub>R KD) were treated with adenosine (3 mM) for 1-12 h, and then real-time RT-PCR for each gene as indicated was carried out. The mRNA quantity for each gene was calculated from the standard curve made by amplifying different amount of the GAPDH mRNA, and normalized by regarding the average of independent basal mRNA quantity at 0 h as 1. In the graphs, each column represents the mean (± SEM) ratio (n=4 independent experiments). *P* values, Dunnett's test. (A) p53, (B) Noxa, (C) Puma, (D) Bax, (E) Bad, and (F) AIF.

agonist of A<sub>3</sub> adenosine receptor, reduced A549 cell viability in a concentration (1-100 μM)-dependent manner (Fig. 1C), supporting the note that adenosine induces A549 cell apoptosis via A<sub>3</sub> adenosine receptor.

To obtain further evidence for this, we knocked-down A<sub>3</sub> adenosine receptor using the A<sub>3</sub>R siRNA. For A549 cells transfected with the A<sub>3</sub>R siRNA, expression of A<sub>3</sub> adenosine receptor protein, but not A<sub>1</sub>, A<sub>2a</sub>, or A<sub>2b</sub> adenosine receptor protein, was apparently suppressed as compared with the expression for cells transfected with the NC siRNA (Fig. 3A), confirming A<sub>3</sub> adenosine receptor knock-down. Adenosine-induced reduction in A549 cell viability was significantly inhibited by knocking-

down  $A_3$  adenosine receptor (Fig. 3B). Collectively, these results provide evidence that adenosine induces A549 cell apoptosis by activating  $A_3$  adenosine receptor.

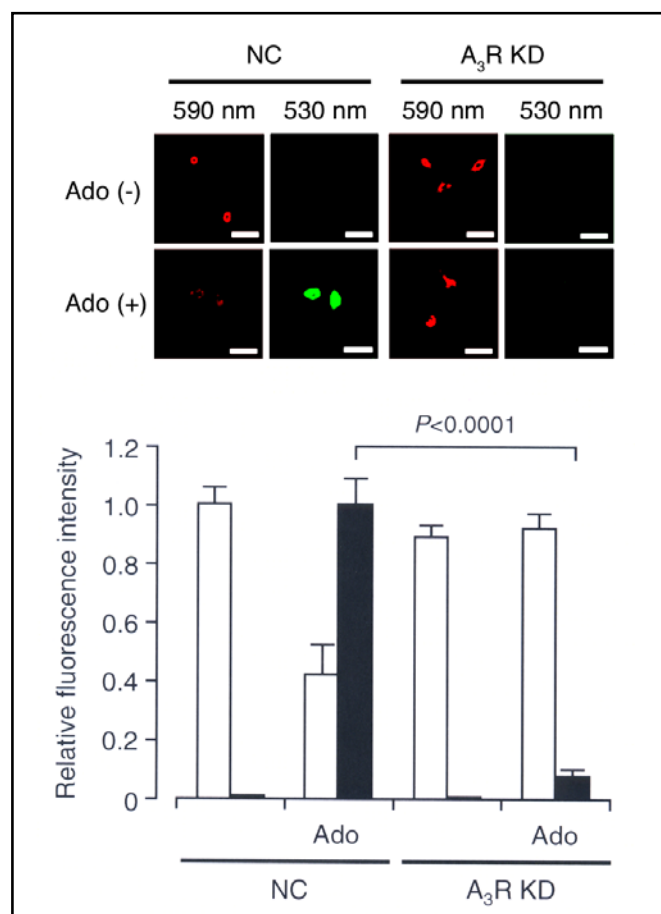
$A_3$  adenosine receptor is linked to  $G_i$  protein bearing adenylate cyclase inhibition responsible for reduced cAMP production/reduced activity of protein kinase A (PKA) and to  $G_q$  protein bearing phospholipase C activation responsible for protein kinase C (PKC) activation [20]. Adenosine-induced A549 cell death was not affected by forskolin (10  $\mu$ M), an activator of adenylate cyclase, GF109203X (100 nM), an inhibitor of protein kinase C (PKC), PD98059 (50  $\mu$ M), an inhibitor of mitogen-activated protein (MAP) kinase kinase (MEK), or the Src family inhibitors PP2 (100 nM) and/or Src11 (200 nM)(Fig. 1D). This implies that adenosine induces A549 cell apoptosis via  $A_3$  adenosine receptor, but independently of PKA inhibition, PKC activation, MAP kinase activation, or Src activation.

*Adenosine upregulates expression of the Puma, Bax and Bad mRNAs in A549 cells in an  $A_3$  adenosine receptor-dependent manner*

We probed adenosine-regulated expression of apoptosis-related molecule mRNAs in A549 cells. In the real-time RT-PCR analysis, treatment with adenosine (3 mM) for 1–12 h increased expression of mRNAs for p53, Noxa, Puma, Bax, Bad, and apoptosis inducing factor (AIF), while it did not affect expression of mRNAs for Bcl-2, Bcl-X<sub>L</sub>, Mcl-1, Bid, Hrk, and AIF-homologous mitochondrion-associated inducer of death (AMID)(data not shown). We, therefore, focused upon p53, Noxa, Puma, Bax, Bad, and AIF, and examined whether  $A_3$  adenosine receptor regulates transcription of those genes. Adenosine-induced upregulation of mRNA expression for Puma, Bax, and Bad was drastically prevented by knocking-down  $A_3$  adenosine (Fig. 4C,D,E). In contrast, the effect of adenosine on the mRNA expression for p53, Noxa, and AIF was not inhibited or conversely further enhanced by knocking-down  $A_3$  adenosine (Fig. 4A,B,F). These results raise the possibility that  $A_3$  adenosine receptor mediates A549 cell apoptosis by upregulating expression of Bax, Bad, and Puma.

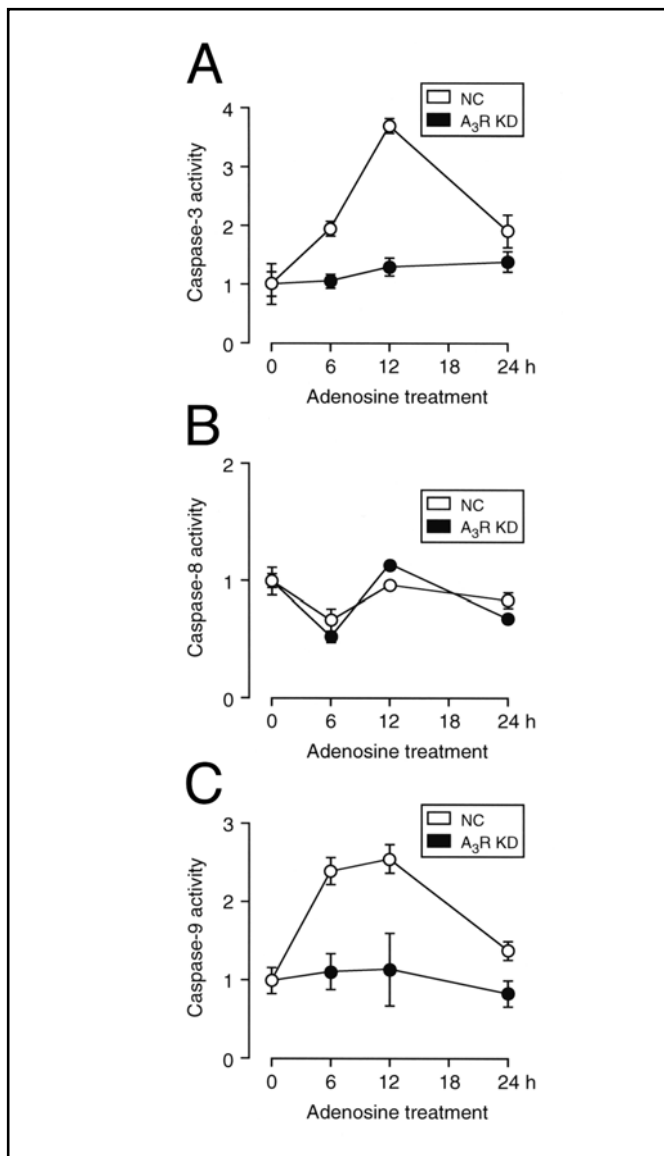
*Adenosine disrupts mitochondrial membrane potentials and activates caspase-9 followed by caspase-3 in A549 cells*

We thought that upregulation of expression for Puma, Bax, and Bad should perturb mitochondrial membrane potentials. To address this question, we monitored mitochondrial membrane potentials using DePsipher™, a



**Fig. 5.** Mitochondrial membrane potentials in A549 cells. For cells transfected with the NC siRNA (NC) or the  $A_3$ R siRNA ( $A_3$ RKD), mitochondrial membrane potentials were monitored before [Ado (-)] and after 24-h treatment with adenosine (3 mM)[Ado (+)]. Typical fluorescent images are shown in the upper panel. DIC, differential interference contrast. Bars, 20  $\mu$ m. Red and green fluorescent intensities in the area (0.4 mm x 0.4 mm) selected at random were measured using ImageJ (Bethesda, MD, USA), and red fluorescent intensities at an absorbance of 590 nm for cells untreated with adenosine or green fluorescent intensities at an absorbance of 530 nm for cells treated with adenosine were regarded as 1. In the graph, each column represents the mean ( $\pm$  SEM) intensity ( $n=4$  independent experiments). Open bars, red fluorescent intensities; closed bars, green fluorescent intensities.  $P$  value, Dunnett's test.

mitochondrial activity marker. DePsipher™ is detected as an orange-red fluorescence at an absorbance of 590 nm for normal mitochondrial membrane potentials, but in case of the disruption the dye turns into green fluorescence at an absorbance of 530 nm. For A549 cells transfected with the NC siRNA, the mitochondria exhibited orange-red fluorescent signals alone without green fluorescent signals before adenosine treatment (Fig. 5). In contrast, 24-h treatment with adenosine (3



**Fig. 6.** Activities of caspase-3, -8, and -9 in A549 cells. Cells transfected with the NC siRNA (NC) or the A<sub>3</sub>R siRNA (A<sub>3</sub>R KD) were treated with adenosine (3 mM) for 6–24 h, and activities of caspase-3, -8 and -9 were enzymatically assayed. In the graphs, each point represents the mean ( $\pm$  SEM) ratio against basal caspase activities (before treatment with adenosine)(n=4 independent experiments).

mM) accumulated green fluorescent signals with weaker orange-red fluorescent signal (Fig. 5). Adenosine-induced accumulation in green fluorescent signals was significantly abrogated by knocking-down A<sub>3</sub> adenosine (Fig. 5). Collectively, the results indicate that adenosine disrupts mitochondrial membrane potentials in A549 cells via A<sub>3</sub> adenosine receptor.

Adenosine-induced perturbation of mitochondrial membrane potentials would allow cytochrome c efflux from the mitochondria into the cytosol, resulting in

activation of caspase-9 and the effector caspase-3. We finally assayed activities of caspase-3, -8, and -9 in A549 cells. Expectedly, adenosine (3 mM) activated caspase-3 and -9, but not caspase-8, and the caspase-3/-9 activation was clearly inhibited by knocking-down A<sub>3</sub> adenosine receptors (Fig. 6A,B,C). Overall, adenosine appears to disrupt mitochondrial membrane potentials, thereby causing activation of caspase-9 followed by caspase-3 via A<sub>3</sub> adenosine receptors, responsible for A549 cell apoptosis.

## Discussion

In the present study, extracellular adenosine or the A<sub>3</sub> adenosine receptor agonist 2-Cl-IB-MECA induced apoptosis in A549 human lung cancer cells. The effect was inhibited by the A<sub>3</sub> adenosine receptor inhibitor MRS1191 or knocking-down A<sub>3</sub> adenosine receptor, but it was not affected by the A<sub>1</sub> adenosine receptor inhibitor 8-CPT, the A<sub>2a</sub> adenosine receptor inhibitor DMPX, the A<sub>2b</sub> adenosine receptor inhibitor MRS1706, or the adenosine transporter inhibitor dipyridamole. This accounts for A<sub>3</sub> adenosine receptor-mediated A549 cell apoptosis.

A<sub>3</sub> adenosine receptor is linked to G<sub>i</sub> protein involving adenylyl cyclase inhibition followed by decreased cAMP production/PKA inhibition and G<sub>q</sub> protein involving phospholipase C activation followed by PKC activation [20]. Adenosine-induced A549 cell death was not prevented by the adenylyl cyclase activator forskolin or the PKC inhibitor GF109203X. This suggests that A<sub>3</sub> adenosine receptor mediates A549 cell apoptosis via a pathway independent of PKA inhibition or PKC activation. Moreover, adenosine-induced A549 cell death was not affected by the MEK inhibitor PD98059 or the Src inhibitors such as PP2 and/or Src11, suggesting no implication of MAP kinase cascades or Src cascades in the adenosine effect.

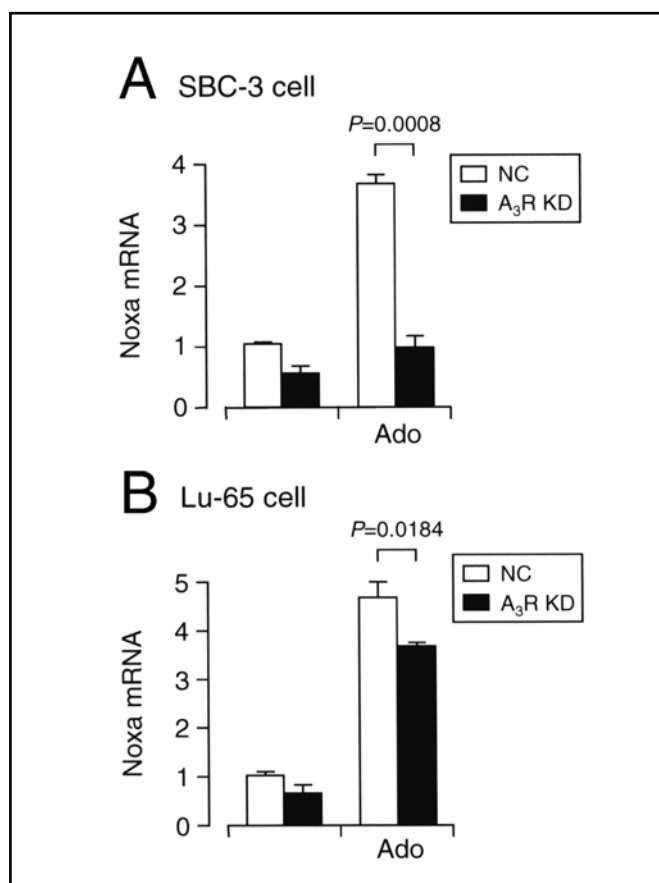
Adenosine increased mRNAs for p53, Noxa, Puma, Bax, Bad and AIF in A549 cells. p53 is well-recognized to serve as a tumor suppressor. DNA damage activates ataxia telangiectasia mutated (ATM), to phosphorylate and activate checkpoint kinase 2 (Chk2). DNA damage, alternatively, activates AT- and Rad3-related (ATR), to phosphorylate and activate Chk1. Activated ATM also phosphorylates p53 at Ser15, dissociating p53 from a complex of p53/Mdm2 or Mdmx, and dissociated p53 is further phosphorylated at Ser20 by Chk2 or Chk1, to activate p53. Activated p53 promotes transcription of the



p21/Waf1 gene, to arrest at the G<sub>1</sub> phase of cell cycling by inhibiting cyclin-dependent kinase, and the p53R2 gene, to repair DNA damage [21, 22]. Phosphorylated p53 at Ser15 and Ser20 is further phosphorylated at Ser46 by Ser46 kinase, which triggers transcription of the p53-regulated apoptosis-inducing protein 1 (p53AIP1) gene followed the Puma or Noxa genes [22]. Adenosine-induced increase in the expression of mRNAs for Puma, Bax, and Bad in A549 cells was suppressed by knocking-down A<sub>3</sub> adenosine, although the increase in the expression of mRNAs for p53, Noxa, and AIF was not inhibited. This indicates that A<sub>3</sub> adenosine receptor upregulates expression of Puma, Bax, and Bad in A549 cells. This also suggests that adenosine here promotes transcription of the Puma gene in a p53-independent manner.

The BH3-only Bcl-2 family Puma or Noxa dissociates Bax/Bak from a complex with Bcl-2, Bcl-X<sub>L</sub>, or Mcl-1, and in turn, dissociated Bax forms a Bax/Bax dimer, to damage the mitochondria responsible for mitochondrial apoptosis [23, 24]. Puma or Noxa still induces mitochondrial apoptosis by neutralizing function of Bcl-2, Bcl-X<sub>L</sub>, or Mcl-1 through its direct binding [25]. Bad also participates in mitochondrial apoptosis by dissociating Bax from a complex with Bcl-2 or Bcl-X<sub>L</sub>. Adenosine-induced upregulation of expression for Puma, Bax, and Bad, accordingly, should perturb mitochondrial membrane potentials. Adenosine, indeed, disrupted mitochondrial membrane potentials in A549 cells, and the effect was reversed by knocking-down A<sub>3</sub> adenosine receptor. Damaged mitochondria releases cytochrome c into the cytosol, forming an oligomeric complex with dATP or Apaf-1, to activate caspase-9 and the effector caspase-3 [26-28]. Adenosine here activated caspase-3 and -9, but not caspase-8, in A549 cells, and the activation of caspase-3 and -9 was neutralized by knocking-down A<sub>3</sub> adenosine receptor. Taken together, these results indicate that adenosine induces A549 cell apoptosis by upregulating expression of Puma, Bax, and Bad, thereby disrupting mitochondrial membrane potentials, to activate caspase-9 followed by caspase-3, as mediated via A<sub>3</sub> adenosine receptor. How A<sub>3</sub> adenosine receptor regulates the gene transcription for Puma, Bax, and Bad in A549 cells, however, is presently unknown. To answer this question, we are currently attempting further experiments.

We have examined adenosine-induced apoptosis in other human lung cancer cell lines SBC-3 and Lu-65 cells. For both the cell types, adenosine induced apoptosis in a concentration (0.01-10 mM) and treatment time (24-72 h)-dependent manner, and a similar effect was obtained



**Fig. 7.** Upregulation of Noxa mRNA expression in an A<sub>3</sub> adenosine receptor-dependent manner. SBC-3 (A) and Lu-65 cells (B) transfected with the NC siRNA (NC) or the A<sub>3</sub>R siRNA (A<sub>3</sub>R KD) were treated with adenosine (Ado)(3 mM) for 12 h, and then real-time RT-PCR for Noxa was carried out. The Noxa mRNA quantity was calculated from the standard curve made by amplifying different amount of the GAPDH mRNA, and normalized by regarding the average of basal Noxa mRNA quantity at 0 h as 1. In the graphs, each column represents the mean ( $\pm$  SEM) ratio ( $n=4$  independent experiments). *P* values, Dunnett's test.

with the A<sub>3</sub> adenosine receptor agonist 2-Cl-IB-MECA. Adenosine-induced apoptosis in SBC-3 and Lu-65 cells was commonly inhibited by the A<sub>3</sub> adenosine receptor inhibitor MRS1191, or knocking-down A<sub>3</sub> adenosine receptor. This indicates the implication of A<sub>3</sub> adenosine receptor in adenosine-induced apoptosis in SBC-3 and Lu-65 cells as well as A549 cells. For SBC-3 cells, adenosine upregulated expression of mRNAs for p53, Noxa, Puma, Bcl-2, AIF, and AMID, but expression of mRNAs for Bcl-X<sub>L</sub>, Mcl-1, Bax, Bad, Bid, and Hrk was not affected (unpublished data). Amazingly, adenosine-induced apoptosis in SBC-3 cells was prevented by knocking-down AMID (unpublished data), indicating caspase-independent apoptosis in SBC-3 cells via A<sub>3</sub>



adenosine receptor. This also suggests that A<sub>3</sub> adenosine receptor mediates apoptosis in SBC-3 cells, but that the signaling cascades downstream A<sub>3</sub> adenosine receptor differ between A549 and SBC-3 cells. For Lu-65 cells, adenosine upregulated expression of mRNAs for p53 and Noxa, but expression of mRNAs for Bcl-2, Bcl-X<sub>L</sub>, Bax, Bad, Bid, Hrk, Puma, AIF, and AMID was downregulated and expression of the Mcl-1 mRNA was not affected (unpublished data). In addition, adenosine induced apoptosis in Lu-65 cells in a p53-dependent manner via A<sub>3</sub> adenosine receptor (unpublished data). This suggests again that signaling cascades downstream A<sub>3</sub> adenosine receptor to induce Lu-65 cell apoptosis is distinct from those for A549 or Lu-65 cells. Notably, adenosine-induced upregulation of Noxa mRNA expression in SBC-3 and Lu65 cells, but not in A549 cells, was significantly

suppressed by knocking-down A<sub>3</sub> adenosine receptor, with more efficient suppression for SBC-3 cells (Fig. 7). This, in the light of the fact that Noxa is an apoptosis-inducing protein, suggests that Noxa also participates in A<sub>3</sub> adenosine receptor-dependent apoptosis for SBC-3 and Lu-65 cells, not but for A549 cells.

In conclusion, the results of the present study show that extracellular adenosine upregulates expression of Puma, Bax, and Bad, thereby disrupting mitochondrial membrane potentials, to activate caspase-9 and the effector caspase-3 and to induce apoptosis in A549 human lung cancer cells, in an A<sub>3</sub> adenosine receptor-dependent manner. This may represent further insight into A<sub>3</sub> adenosine receptor signaling pathways relevant to apoptosis-related gene transcription.

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