

行政院國家科學委員會專題研究計畫 期中進度報告

抑制粒線體蛋白 HAX-1 對病毒感染的影響(1/2)

計畫類別：個別型計畫

計畫編號：NSC93-2314-B-002-134-

執行期間：93年08月01日至94年07月31日

執行單位：國立臺灣大學醫學院小兒科

計畫主持人：呂俊毅

共同主持人：黃立民

計畫參與人員：楊子璿，楊欣穎

報告類型：精簡報告

處理方式：本計畫可公開查詢

中 華 民 國 94 年 5 月 27 日

行政院國家科學委員會補助專題研究計畫
期中進度報告

計畫名稱：抑制粒線體蛋白 HAX-1 對病毒感染的影響(1/2)

計畫類別： 個別型計畫 整合型計畫

計畫編號：NSC - 93 - 2314 - B - 002 - 134

執行期間： 2004 年 8 月 1 日至 2005 年 7 月 31 日

計畫主持人：呂俊毅

共同主持人：黃立民

計畫參與人員：楊子璿、楊欣穎

成果報告類型(依經費核定清單規定繳交)： 精簡報告 完整報告

本成果報告包括以下應繳交之附件：

赴國外出差或研習心得報告一份

赴大陸地區出差或研習心得報告一份

出席國際學術會議心得報告及發表之論文各一份

國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、
列管計畫及下列情形者外，得立即公開查詢

涉及專利或其他智慧財產權， 一年 二年後可公開查詢

執行單位：國立臺灣大學醫學院小兒科

中 華 民 國 94 年 5 月 27 日

Introduction

The purpose of this project was to determine the role of a cellular protein HAX-1 in various virus infections. SiRNA was used to knockdown HAX-1 expression in cells and these cells were used to test the effects of virus infections.

The HAX-1 was first identified as a protein that associates with hemopoietic specific protein (HS1), which is thought to play a role in B cell receptor signal transduction and involved in B cell apoptosis (1, 2). In addition to HS1, HAX-1 has been shown to interact with several structurally distinct proteins including PKD2 (polycystic kidney disease protein), cortactin (cytoskeleton protein), IL-1 NTP (interleukin 1 α N terminus), but the biological functions of these interactions remain to be elucidated (1, 4, 5). Protein domain and motif analysis of HAX-1 shows it contains a PEST site at residues 104 to 117 and a putative hydrophobic transmembrane domain at residues 261 to 273, and shows similarity to the apoptosis-related proteins Bcl-2 and Nip3 (1, 6). Nip3 was shown to interact with some anti-apoptotic cellular and viral proteins, Bcl-2 and BHRF-1 (EBV Bcl-2 homolog) (1, 8). Recent studies demonstrated that HAX-1 is a potent inhibitor of Bax-induced apoptosis (6). Our previous results provided evidence that over-expressed HAX-1 in HeLa cells blocks the nuclear transport of Vpr protein and reverts apoptosis induced by HIV-1 vpr. In addition to HIV-1, nuclear antigen leader protein (EBNA-LP) of Epstein-Barr virus and K15 protein of human herpesvirus 8 also interact with HAX-1 (3, 6, 7). Taking these data together, we strongly believe that HAX-1 is involved in the process of cellular apoptosis induced by various viral infections.

In summary, the fact that a single cellular protein (HAX-1) is targeted by various viral proteins suggests that HAX-1 plays an important role in a variety of viral pathogenesis. We thus hypothesize that HAX-1 is (one of) the cellular factor(s) counteracting with apoptotic effect of various viral proteins. In this project, we are first going to confirm the anti-apoptosis function of HAX-1 by using the siRNA technology. Then, we will test the hypothesis that HAX-1 is a cellular factor involved in apoptosis induced by infections of various viruses, mainly EBV and enterovirus 71. The final goal of this project will be unraveling of the function of HAX-1 and design of possible intervention strategies for clinical viral infections.

Up to now, the project is going smoothly. We have successfully created a HAX-1-silenced cell line by using siRNA technology. In this mid-term report, we are going to present the evidence of effective silencing of HAX-1 in our cell lines. The growth rate of the HAX-1-silenced cells decreased markedly. The HAX-1-silenced cells also showed a strong tendency to die through apoptosis, either under toxic drug stress or pro-apoptotic genes transfection.

Material and Methods

Constructions of DNA plasmids expressing HAX-1 and viral proteins and confirmation of expression of specific proteins

HIV-vpr, EV71 2A, and HAX-1 have already been cloned into vectors with CMV promoter. A tag of HA or FLAG has been added. Western blotting were used to confirm the correct expression of HAX-1 and various viral proteins. Cell extracts is prepared by washing with cold PBS and scraped into the lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA and 0.5% NP-40). Cell extracts is then mixed with an equal volume of 2X sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.2% bromophenol blue), separated by SDS-PAGE and transferred onto nitrocellulose membranes. Blots are incubated with specific antibodies. Antibodies against the fused tags will be used if specific proteins are not available. Blots are then incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Sigma), the secondary antibody. Proteins were detected using the Enhanced Chemiluminescence Western Blotting kit (Amersham).

Construction of DNA plasmids expressing siRNAs

The vector pSilencer 1.0-U6(13) from Ambion company will be used. Briefly, at least three appropriate ~55 bp insert sequence targeting 5'-end, middle portion, and 3' end of HAX-1 will be designed and synthesized. The double-stranded DNA insert should include 4 nucleotide overhangs complementary to the Apa I and EcoR I restriction sites, as well as the sense and antisense sequences of the desired siRNA separated by a small loop sequence. This double-stranded DNA insert is then ligated into the linearized (Apa I and EcoR I) vector and introduced into E. coli cells. The resulting plasmid is produced in E. coli, purified and then transfected into cells.

Establishment of HAX-1-silenced cell lines

African green monkey kidney cell Vero E6 (CRL-1587) was purchased from the American Type Culture Collection (VA, USA). All cell lines were maintained in minimal essential medium with Earle's balance salts (EMEM, JRH Bioscience, Kansas, USA) with 10% fetal bovine serum (FBS, Biological industries, Israel). Vero-E6 cells (3 x 10⁵ cells/well) were seeded into 6-well-plates in EMEM with 10% fetal calf serum one day before transfection to get a 70% confluence at transfection. PSilencer-ACE2 plasmids were transfected by using lipofectamine (Invitrogen, CA, USA) with a ratio of 4 ug DNA mix to 10 uL lipotectamine per well according to manufacturer's instructions. Transfection of the same plasmids was repeated once 48 hours later. The cells were subcultured 24 hours after the transfections and then every 2-3 days in complete medium containing 2 mg/ml G418 (Sigma, MO, USA). The concentration of G418 was reduced to 1 mg/ml 14 days later. The cells were then diluted and single colonies were picked for further experiments.

Validation of effectiveness of siRNA By Western blotting and Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Western blotting and qRT-PCR were used to validate the effectiveness of HAX-1 silencing. Cells transfected with various pSilencer-HAX-1 vectors with different sequences targeting HAX-1 were checked. Western blotting will be done on cell lysates with anti-HAX-1 antibody. The construct with most significant degree of suppression of HAX-1 will be used for further experiments. Supernatants of cell cultures infected with SARS CoV were used for RNA extraction according to the mini spin protocol of the QIAamp virus RNA mini kit (Qiagen, Hilden, Germany). The extracted RNA was eluted in 50 µl RNase free water, treated with deoxyribonuclease I (Life Technologies, California, USA) to digest genomic DNA, and stored at -80°C before use. Quantitative RT-PCR for SARS CoV RNA was done by using the RealArt HPA-coronavirus LC RT PCR Reagent (Artus Biotech, Hamburg, Germany) on Light Cycler instrument from Roche diagnostics (Mannheim, Germany).

Real time RT-PCR was also done to quantify the amount of HAX-1 mRNA. A primer pair (1528-AAAGTGGTGGGAGATGAAGC and 1572-GTTTCATCATGGGGCACA) which extends over two exons of ACE2 was used to amplify an amplicon of 63 nucleotides. A fluorophore and quencher dual-labelled probe (Human #77 from Exiqon, Denmark) was used on Roche diagnostics (Mannheim, Germany). GADPH mRNA was also amplified as internal control with the primer pair: CTCATGACCACAGTCCATGC (left) and CCCTGTTGCTGTAGCCAAAT (right).

Cell culture and DNA transfection

HeLa cell and 293T cells will be used first. These cells will be maintained in DMEM with 10% FBS and incubated in 5% CO₂ incubator. Transfection of DNA will be performed by Lipofectamine method according to the manufacturer's instructions (GIBCO). Electroporation might be needed if chemically transfection is proved to be ineffective in certain cell lines or primary cell cultures.

Functional assays of apoptosis

Cell growth and viability

Cell viability will be determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, using an assay kit from Boehringer Mannheim as instructed by the manufacturer. Percentage viability will be determined by comparing the number of viable cells in treated cultures to the number of cells in a parallel control culture (treated with ethanol for equivalent time).

Caspase assays

Induce cells to undergo apoptosis by transfection of HIV-1 vpr or appropriate controls. Remove media, wash cells, and scrape cells into PBS. Pellet cells gently and resuspend in cell lysis buffer (10 mM HEPES, 2 mM EDTA, 0.1 % CHAPS / NP40, 5 mM DTT, 1 mM PMSF, 10 mg/ml pepstatin A, 20 mg/ml leupeptin, 10 mg/ml aprotinin). Incubate on ice for 10-15 minutes. Centrifuge at 10,000g for 1 minute at 4 degrees C. Transfer supernatant to a new tube and keep on ice. Measure protein content of the supernatant. In a microplate reader add: 178 µl reaction buffer (100 mM HEPES, 20% v/v glycerol, 0.5 mM EDTA, 5 mM DTT added fresh), up to 20 µl

cell lysate (containing 100-200 mg total protein) and 2 ml caspase substrate. Ac- Ile- Glu- Thr- Asp- MCA (Peptide Institute, 3195-v) will be used as substrate for caspase 8, Ac- Leu- Glu- His- Asp- MCA (Peptide Institute, 3198-v) will be used as substrate for caspase 9. Adjust volume to 200 µl with cell lysis buffer if required. Incubate at 37 degrees C and measure the absorbance at 405 nm every 30 minutes.

Determine the effects of HAX-1 knockdown on viral infections

SiRNA technology will be used to knockdown the endogenous HAX-1 expression in RD cells. Cells with and without HAX-1 knockdown will be infected by enterovirus 71 inoculation. Compare the efficiencies of enterovirus 71 infection and proliferation.

Results

Successful knockdown of HAX-1 expressions by siRNA

By using a DNA vector with a pol III promoter, U6, shRNAs targeting HAX-1 were introduced into HeLa cells and successfully silenced HAX-1 expression both in protein (Figure 1) and mRNA levels (Figure 2).

HAX-1-silenced cells grow slower and are prone to apoptosis induced by various drugs and EV71 2A protease

Grossly, the HAX-1-silenced cells showed normal morphology. The HAX-1-silenced cells showed decreased growth rate in comparison to original HeLa cells (Figure 3). When various toxic drugs were added to the cells, the HAX-1-silenced cells showed higher susceptibility to the drugs, especially H₂O₂ and actinomycin-D.

When the HAX-1 silenced cells were transfected with EV71 2A protease, more cell death was noted in HAX-1-silenced cells (figure 4). The cells died via apoptosis evidenced by the fact that cleavage of PARP was shown in cells with EV71 2A transfection (Figure 5). By caspase-3 assay, the magnitude of apoptosis was determined to be significantly higher in HAX-1 silenced cells (Figure 6). When the EV71 2A or an empty vector was transfected with pMito-YFP, which expresses YFP in mitochondria, destruction of mitochondria was clearly shown in HAX-1-silenced cells but not normal HeLa cells (Figure 7).

Conclusions and Discussions

In the first 10 months of this 2-year project, we have successfully knockdown HAX-1 expressions by siRNA technology in HeLa cells. Stable clones of HAX-1-silenced cells were created. The effective knockdown of HAX-1 has been proved by using real-time RT-PCR and Western blotting at the mRNA and protein levels, respectively.

We have also shown the HAX-1-knockdown cells grow slower in spite of having normal morphology. Apoptosis can be easily induced in these cells. This provided evidence that HAX-1 is an anti-apoptosis protein. When our original hypothesis that HAX-1 is an anti-apoptosis factor

in various virus infections were then tested, we found some exciting results. EV71 2A protease induces cellular apoptosis. This phenomenon was significantly enhanced in cells lacking HAX-1. We already have preliminary results to support our hypothesis.

In summary, this project has proceeded smoothly in the past 10 months. We are confident here and now that this project will be done as proposed. The results are very promising that we have found an important protein involved in the apoptosis process induced by various virus infections. After the project is completed, we will be able to have 1 or 2 high quality publications about this.

References:

1. Suzuli, Y., C. Demoliere, D. Kitamura, H. Takeshita, U. Deuschle, and T. Watanabe. HAX-1, a novel intracellular protein, localized on mitochondria, directly associates with HS1, a substrate of src family tyrosine kinase. *J. Immunol.* 1997; 158: 2376-2744.
2. Yamanashi, Y., T. Fukuda, H. Nishizumi, T. Inazu, K. Higashi, D. Kitamura, T. Ishida, H. Yamaura, and T. Yamamoto. Role of tyrosine phosphorylation of HS1 in B cell antigen receptor-mediated apoptosis. *J. Exp. Med.* 1997; 185:1387-1392.
3. Martin D., Maria O., and Lars R. Epstein-Barr virus nuclear antigen 5 interacts with HAX-1, a possible component of the B-cell receptor signalling pathway. *J Gen Virol.* 2001; 82: 1581-1587.
4. Gallagher, A. R., A. Cedzich, N. Gretz, S. Somlo, and R. Witzgall. The polycystic kidney disease protein PKD2 interacts with Hax-1, a protein associated with the actin cytoskeleton. *Proc. Natl. Acad. USA* 2000; 97:4017-4022.
5. Yiu, H., H. Morioka, C. A. Towle, M. Vidal, T. Watanabe, and L. Weissbach. Evidence that HAX-1 is an interleukin-1 alpha n-terminal binding protein. *Cytokine* 2001;15:122-137.
6. Tyson V. S, Hsei-Wei W, Andrew K, Daniel H, Yoshio E, Hongtao Y, Ming-Qing D, Chris B. K15 protein of Kaposi's sarcoma-associated herpesvirus is latently expressed and binds to HAX-1, a protein with antiapoptosis function. *J Virol* 2002; 76:802-816.
7. Yasushi K, Kaori N, Mie I, Tomoko M, Michiko T, Mikiko et al. Interaction of Epstein-Barr virus nuclear antigen leader protein with HS1-associated protein X-1: implication of cytoplasmic function of EBNA-LP. *J Virol* 2000; 74: 10104-10111.
8. Boyd, J. M., S. Malstrom, T. Subramanian, L. K. Venkatesh, U. Schaeper, B. Elangovan, C. D'Sa-Eipper, and G. Clinnadurai. Adenovirus E1B 19 kDa and Bcl-2 protein interact with a common set of cellular proteins. *Cell.* 1994; 79: 341.
9. Choi, J. K., B. S. Lee, S. N. Shim, M. Li, and J. U. Jung. Identification of novel K15 protein gene at the rightmost end of Kaposi's sarcoma-associated herpesvirus genome. *J. Virol.* 2000. 74:436-446.
10. Glenn, M., L. Rainbow, F. Aurad, A. Davison, and T. F. Schulz. Identification of a spliced gene from Kaposi's sarcoma-associated herpesvirus encoding a protein with similarities to latent membrane protein 1 and 2A of Epstein-Barr virus. *J. Virol.* 1999; 73:6953-6963.
11. Kuo RL, Kung SH, Hsu YY, Liu WT. Infection with enterovirus 71 or expression of its 2A protease induces apoptotic cell death. *J Gen Virol.* 2002 Jun;83(Pt 6):1367-76.

12. Li ML, Hsu TA, Chen TC, Chang SC, Lee JC, Chen CC, Stollar V, Shih SR. The 3C protease activity of enterovirus 71 induces human neural cell apoptosis. *Virology* 2002;293(2):386-95.
13. <http://www.ambion.com/techlib/tn/94/941.html>
14. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hygiene* 1938;27:493-7.

Figure 1. Expression of HAX-1 was shown by Western blots in various clones of HeLa cells transfected with *pSilencer-HAX-1*. Clone 13, 20, and 21 showed lowest amount of HAX-1 expressions and were used for further experiments.

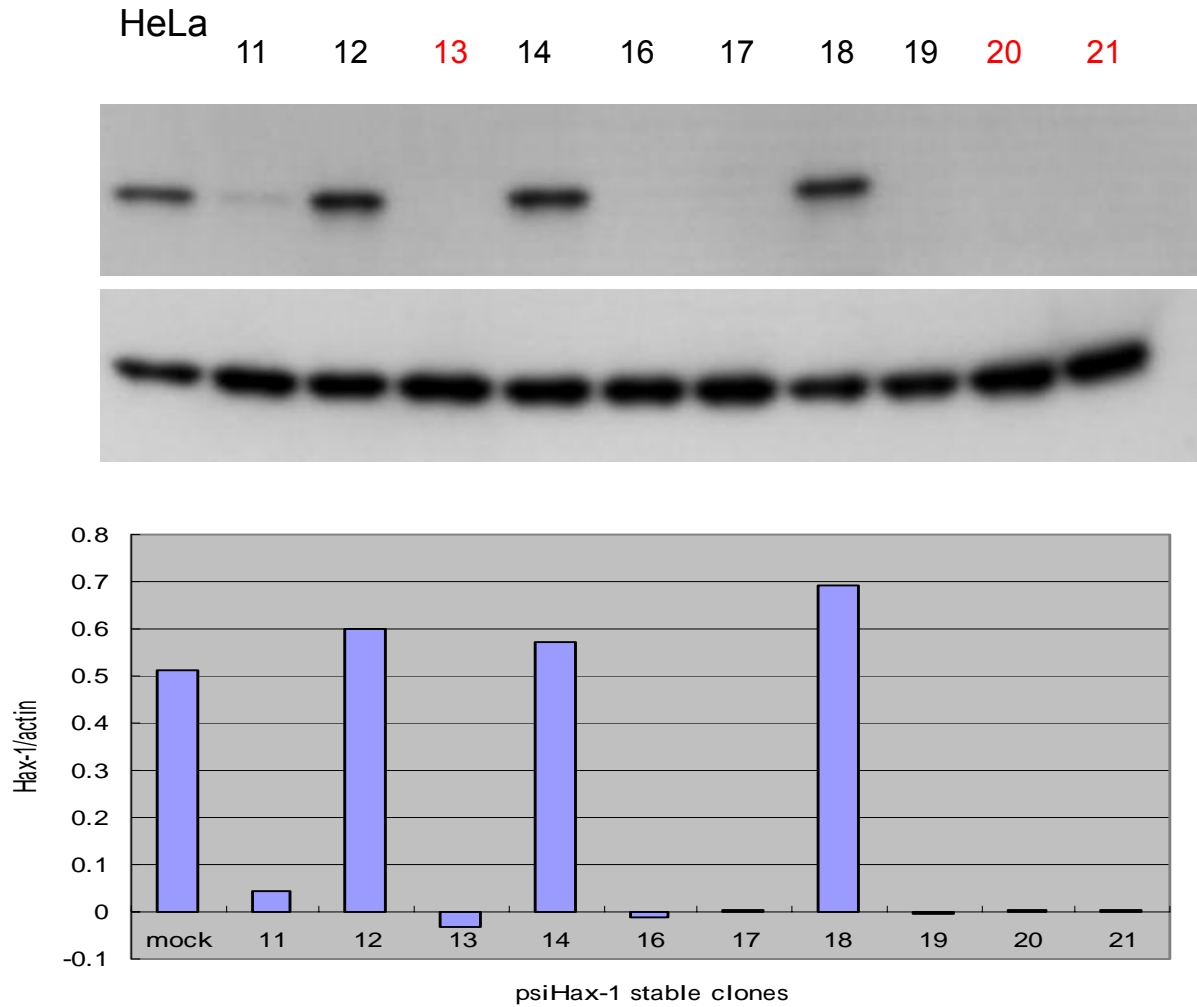


Figure 2. Amounts of HAX-1 mRNA normalized by amount of GAPDH mRNA determined by quantitative RT-PCR. The amount of HAX-1 mRNA was significantly reduced in various cell lines with shRNA targeting HAX-1.

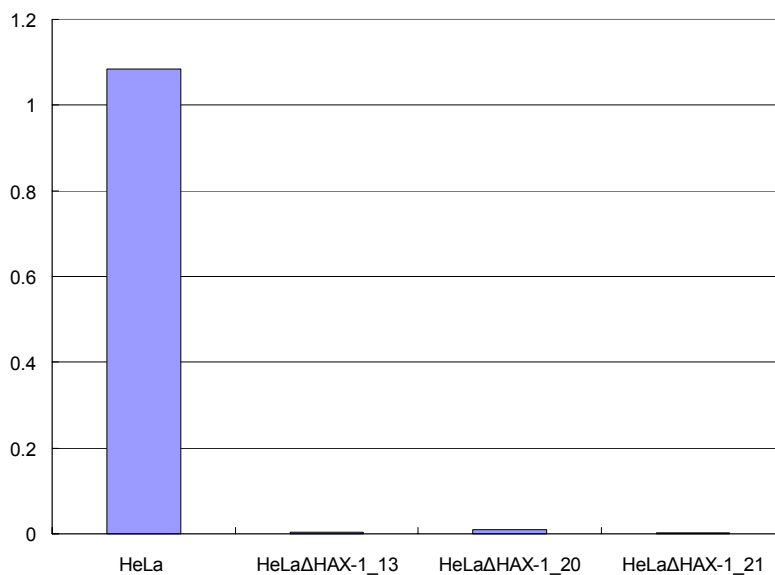


Figure 3. Growth curves of HeLa cell and HAX-1-silenced cell (HAX_N_13). The growth rate of HAX-1-silenced HeLa cells was significantly reduced.

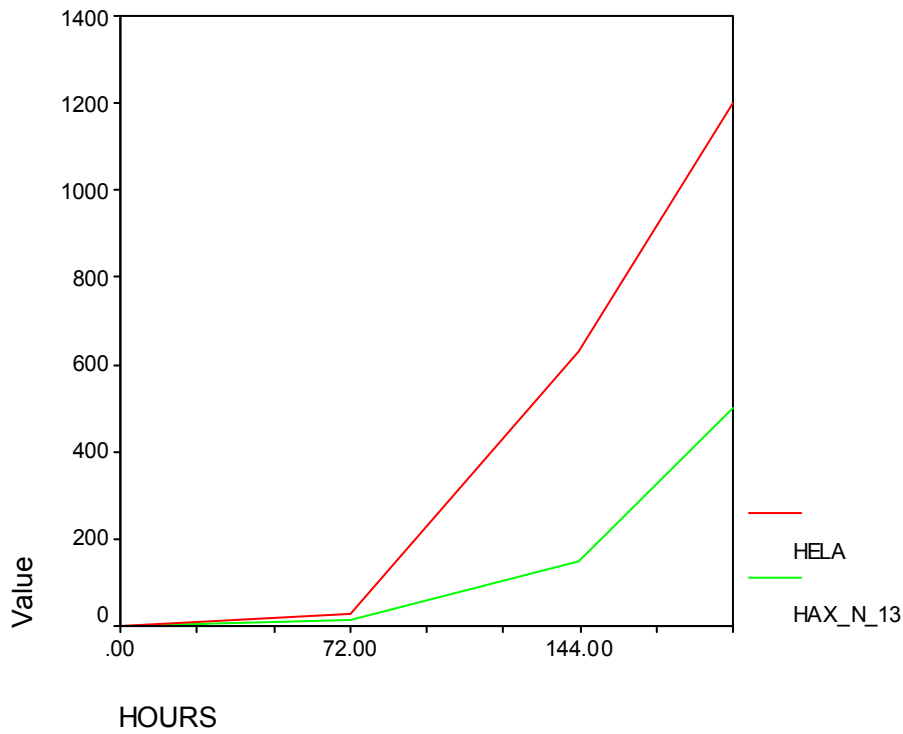


Figure 4. Relative numbers of viable cells determined by MTT assay in HeLa cells with and without HAX-1 silencing after t EV71 2A protease transfection. Silencing of HAX-1 resulted in more cell death after EV71 2A transfection.

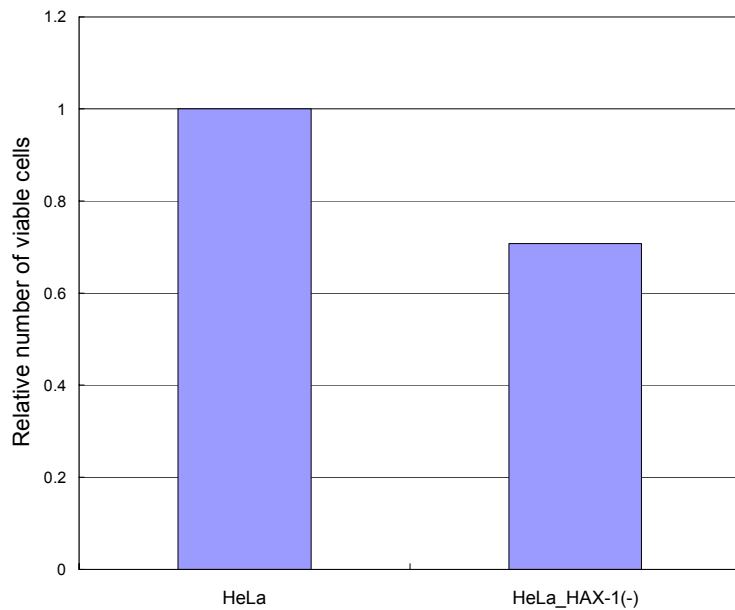


Figure 5. Cleavage of PARP was shown in HAX-1-silenced cells transfected with EV71 2A protease or treated with actinomycine D (ACD).

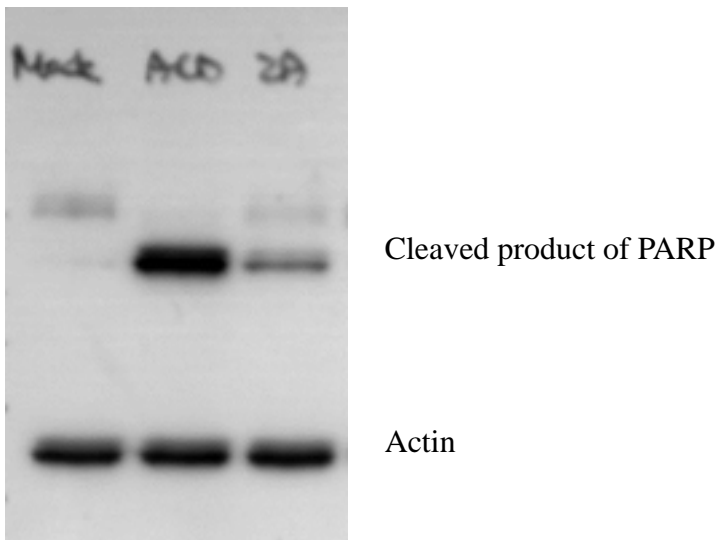


Figure 6. Caspase-3 activities in HeLa cells with or without HAX-1-silenced were treated with actinomycin-D or transfected with EV71 2A. HeLa cells with HAX-1 silenced showed more prominent apoptosis after actinomycin-D treatment or 2A transfection.

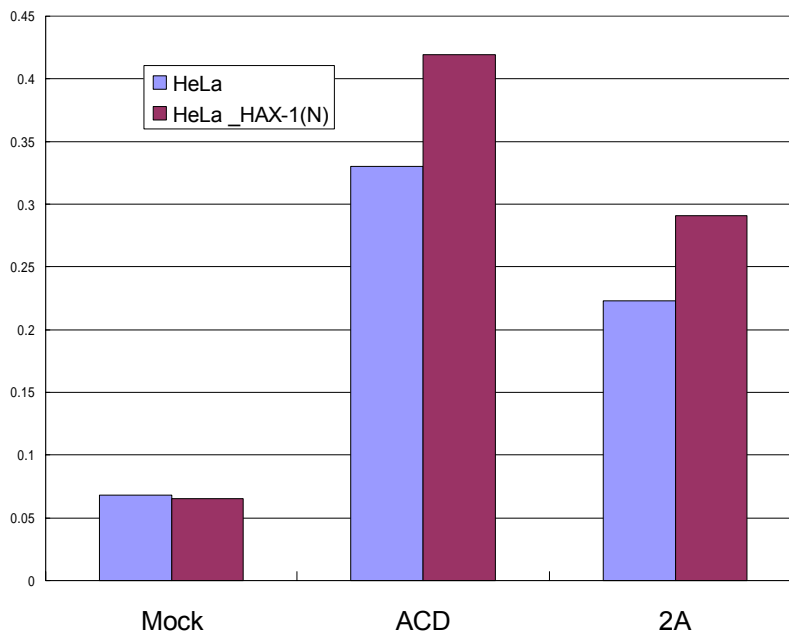


Figure 7. Pictures of HeLa cells with or without HAX-1 silencing. Vectors expressing EV71 2A or empty vector alone was co-transfected with pMito-YFP, which expresses a fluorescence staining mitochondria. HeLa cells whose HAX-1 was silenced showed significantly higher susceptibility to apoptosis induced by EV71 2A transfection.

