

# Knockdown of SMYD3 by RNA interference inhibits cervical carcinoma cell growth and invasion *in vitro*

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**Elevated expression of SMYD3 is a frequent genetic abnormality in several malignancies. Few studies knocking down SMYD3 expression in cervical carcinoma cells have been performed to date. In this paper, we established an inducible short hairpin RNA expression system to examine its role in maintaining the malignant phenotype of HeLa cells. After being induced by doxycycline, SMYD3 mRNA and protein expression were both reduced, and significant reductions in cell proliferation, colony formation and migration/invasion activity were observed in the SMYD3-silenced HeLa cells. The percentage of cells in sub-G1 was elevated and DNA ladder formation could be detected, indicating potent induction of apoptosis by SMYD3 knockdown. These findings imply that SMYD3 plays crucial roles in HeLa cell proliferation and migration/invasion, and that it may be a useful therapeutic target in human cervical carcinomas. [BMB reports 2008; 41(4): 294-299]**

## INTRODUCTION

The importance of epigenetic changes in cancer has been acknowledged for about two decades (1, 2). The potential reversibility of epigenetic states offers exciting opportunities for novel cancer drugs (3). DNA methyltransferases and histone deacetylases are the two major drug targets for epigenetic inhibition used to date (4), and histone methyltransferases are expected to be added in the near future (5, 6). Since many of the reported DNA methyltransferase and histone deacetylase inhibitors have pleiotropic or toxic effects, we used an inducible RNA interference (RNAi) technology in the present work to repress the recently identified SET and MYND domain-containing histone methyltransferase, SMYD3.

RNAi mediated by short hairpin RNAs (shRNAs) is a powerful tool for efficiently suppressing target genes (7-10). However, constitutive gene silencing cannot be used when the target

genes are essential for cell survival, cell cycle regulation and cell development. Thus conditional gene silencing induced by administration or withdrawal of a small inducer molecule has been developed (11). Several trials have explored the oncogenic activity of SMYD3 and have shown that elevated expression is frequently observed in several malignancies, including colorectal carcinoma, hepatocellular carcinoma and breast cancer (12, 13). However, there have been few studies knocking down SMYD3 expression in carcinoma cells (14-17).

In our previous publication, we reported that overexpression of SMYD3 could affect cell viability, adhesion and migration (18). To clarify its role in the malignant phenotype, we established a doxycycline (Dox)-inducible shRNAs expression system in HeLa cells. Using this system, we demonstrated that down-regulation of SMYD3 expression resulted in inhibition of cell growth, colony formation, migration/invasion activity and induction of apoptosis. This suggested that SMYD3 expression is essential for the proliferation and migration of HeLa cells and that SMYD3 might serve as a new therapeutic target in human cervical carcinomas.

## RESULTS

### Development of the inducible shRNAs expression system

An inducible shRNAs expression system that permits control of the RNAi effect in mammalian cells facilitates the application of RNAi (19, 20). Our inducible system consists of three components, *i.e.*, a doxycycline-controlled transcription repressor TetR, an inducible RNA polymerase III H1 promoter containing a Tet operator sequence, and the inducing agent Dox. Transcription of the shRNA driven by RNA polymerase III H1 promoter is blocked in cells expressing TetR. Addition of Dox to the medium inhibits the binding of TetR, which derepresses transcription.

Construction of the inducible shRNAs expression system in HeLa cells is described in Materials and Methods. The positive cell lines were designated as Scr-HeLa, S920-HeLa, S879-HeLa, S761-HeLa and S581-HeLa.

### Inducible knockdown of SMYD3 mRNA and protein expression

After induction with 2 µg/ml Dox for 48h, RT-PCR and Western

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blot analysis indicated that S920-HeLa, S879-HeLa, S761-HeLa and S581-HeLa all led to sharp reductions of SMYD3 expression compared to HeLa TR, while no significant change was observed with Scr-HeLa and HeLa TR. Three independent experiments showed that the mRNA level inhibition ratios for Scr-HeLa, S920-HeLa, S879-HeLa, S761-HeLa and S581-HeLa were  $8.55 \pm 2.25\%$ ,  $86.05 \pm 3.31\%$ ,  $69.96 \pm 2.28\%$ ,  $72.99 \pm 0.82\%$  and  $57.95 \pm 1.41\%$ , while the protein level inhibition ratios were  $3.38 \pm 1.25\%$ ,  $53.18 \pm 3.66\%$ ,  $27.71 \pm 4.18\%$ ,  $44.07 \pm 1.44\%$  and  $13.15 \pm 2.12\%$ , respectively (Fig. 1).

### Inhibition of HeLa cells growth by suppression of SMYD3

To investigate the role of SMYD3 in cell growth, cell growth curves and a colony formation assays were performed. The cell growth curves showed that proliferation of S920-HeLa, S879-HeLa, S761-HeLa and S581-HeLa were markedly inhibited, and the highest inhibitory rates were  $74.03 \pm 0.9\%$ ,  $62.89 \pm 1.8\%$ ,  $65.61 \pm 2.1\%$ ,  $46.45 \pm 1.4\%$  ( $P < 0.05$ ) on day 5, respectively. However, there was no significant difference in cell proliferation between Scr-HeLa and HeLa TR ( $P > 0.05$ ; Fig. 2A).

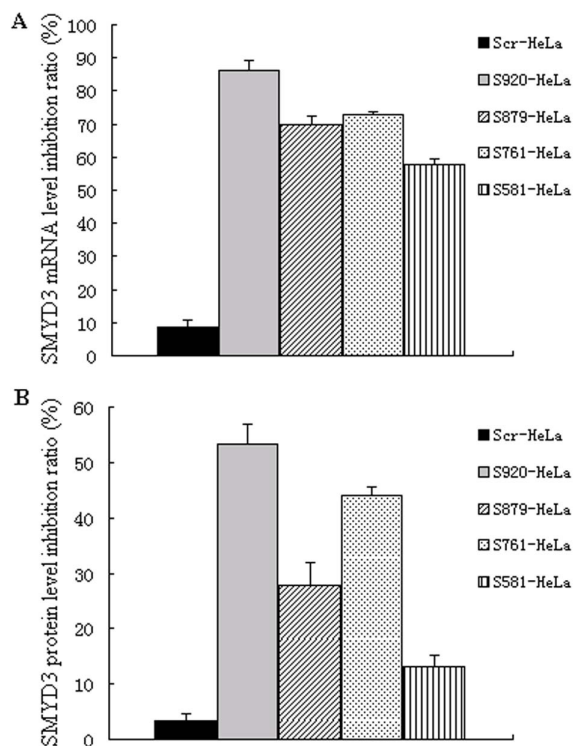
The colony formation assay showed that S920-HeLa, S879-HeLa, S761-HeLa and S581-HeLa cells formed markedly less

colonies compared with HeLa TR cells (Fig. 2B). Furthermore, the colonies formed by the S920-HeLa, S879-HeLa, S761-HeLa and S581-HeLa cells were significantly smaller than those formed by the HeLa TR and Scr-HeLa cells (data not shown).

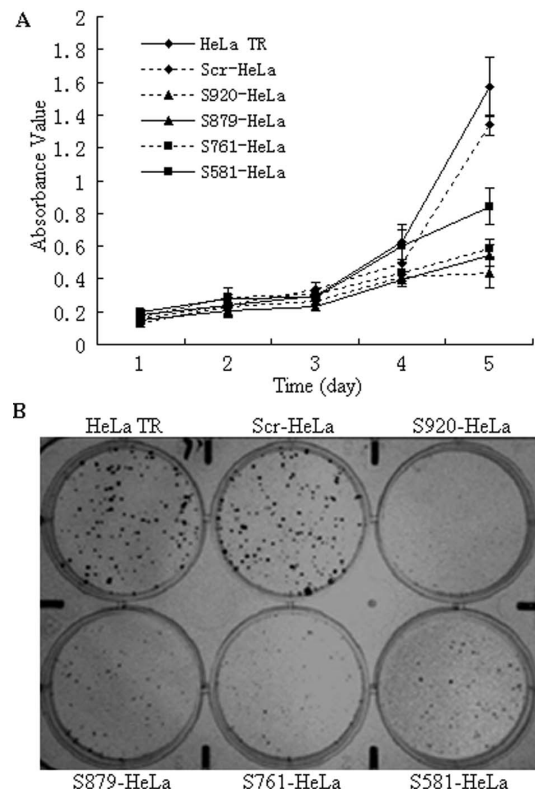
### Inhibition of HeLa migration/invasion by downregulation of SMYD3 expression

To analyze a possible effect of SMYD3 knockdown on the migratory behavior of HeLa cells, wound-healing scratching assays were carried out. The results showed that knockdown of SMYD3 delayed wound closure. As shown in Fig. 3A, the migration of SMYD3 knockdown cells was decreased by 22-38%.

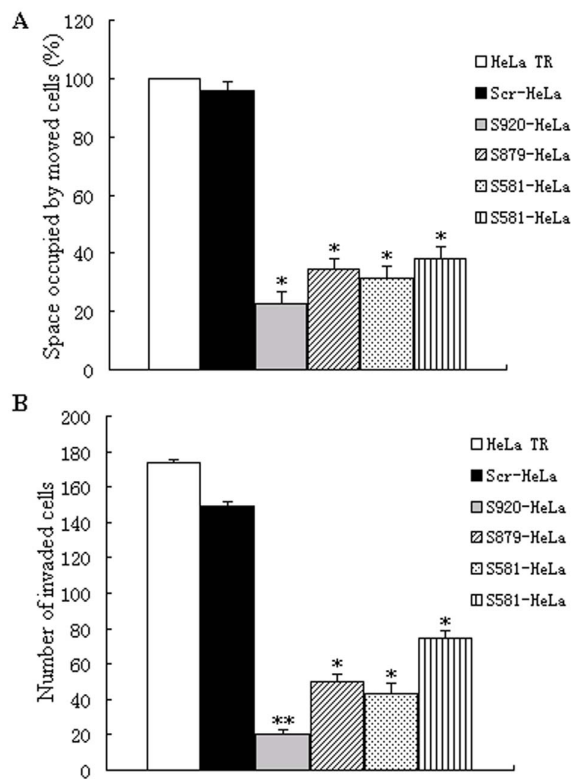
For invasion assays, cells were incubated in transwell plates with 2  $\mu\text{g/ml}$  Dox for 48 h. As shown in Fig. 3B, the number of invading SMYD3 knockdown cells was significantly less than that of HeLa TR cells. These results show that repression of SMYD3 inhibits the proliferation and migration/invasion of HeLa cells.



**Fig. 1.** Inducible silencing of SMYD3 mRNA and protein expression. (A) SMYD3 suppression at the mRNA level, as determined by RT-PCR analysis. (B) Western blot analysis of the inhibition of SMYD3 at the protein level. All experiments were performed in triplicate.



**Fig. 2.** Effects of SMYD3 knockdown on the growth of HeLa cells. (A) SMYD3 knockdown leads to reduced cell growth rate, as detected by the MTT assay. (B) Knockdown of SMYD3 reduces the colony formation ability of HeLa cells. Representative wells demonstrating the total number of colonies formed after 2 weeks of incubation.



**Fig. 3.** Downregulation of SMYD3 expression inhibits the migration/invasion ability of HeLa cells. (A) Cell migration activity is presented. The percent area relative to area at 0h (n=3) was calculated. (B) The cells that invaded through the Thincert inserts were stained and counted under a light microscope at 100×magnification. All experiments were performed in triplicate. Significant differences are indicated by asterisks: \**P* < 0.05 and \*\**P* < 0.01 compared with HeLa TR cells.

### Induction of apoptosis by knockdown of SMYD3 gene expression

To determine whether the growth and migration inhibition resulting from inhibition of SMYD3 was caused by induction of apoptosis, two assays were performed. Firstly, flow cytometry demonstrated that knockdown of SMYD3 gene expression increased the number of sub-G1 cells. With 2 µg/ml Dox in the culture medium for 48 h, in 0.73% and 4.21%, respectively of the HeLa TR and Scr-HeLa were apoptotic compared with apoptosis rates in the S920-HeLa, S879-HeLa, S761-HeLa and S581-HeLa cells of 45.27%, 29.08%, 32.35% and 18.81%, respectively (Fig. 4A).

Secondly, we examined DNA fragmentation after 96h in the presence of Dox. DNA ladder formation was detected after 72 h and 96 h of Dox induction (Fig. 4B), whereas there was no detectable ladder formation after 24 h, 48 h of Dox induction (data not shown) or in the control. These results indicate that apoptosis was induced as consequence of SMYD3 inhibition.

## DISCUSSION

A variety of systems allowing controllable gene knockdown have been developed (21-23). Dox-controlled gene-knockdown is one of the most popular (11, 24, 25).

By employing this system with HeLa cells, we demonstrated that shRNAs induced by Dox could repress both SMYD3 mRNA and protein expression. However the inhibition ratios at the protein level were lower than at the mRNA level, which is likely to be linked to SMYD3 function as a histone lysine methylase. By methylating H3-K4, SMYD3 regulates the expression of a wide array of genes. There are two potential models for explaining its role in transcriptional activation (26). One possibility is that SMYD3 directly recognizes the SMYD3 binding site and methylates nearby histones with assistance from its associated protein HSP90A, and results in H3-K4 methylation by additional histone lysine methyltransferases. Alternatively, following binding to DNA, SMYD3 may associate with RNA polymerase II and thus produce a wider distribution of H3-K4 methylation across a target gene. In both models, SMYD3 requires protein cofactors for its catalytic activity and this may provide SMYD3 with a longer half-life.

As colorectal carcinoma and hepatocellular carcinoma cells (12, 13), SMYD3-knockdown induced significant reductions in proliferation and colony formation by the HeLa cells. We previously found that SMYD3-transfected NIH3T3 cells displayed enhanced cell migration (18). In the present work, we showed that the migration/invasion activity of SMYD3-knockdown HeLa cells was markedly inhibited, and there was strong induction of apoptosis (17).

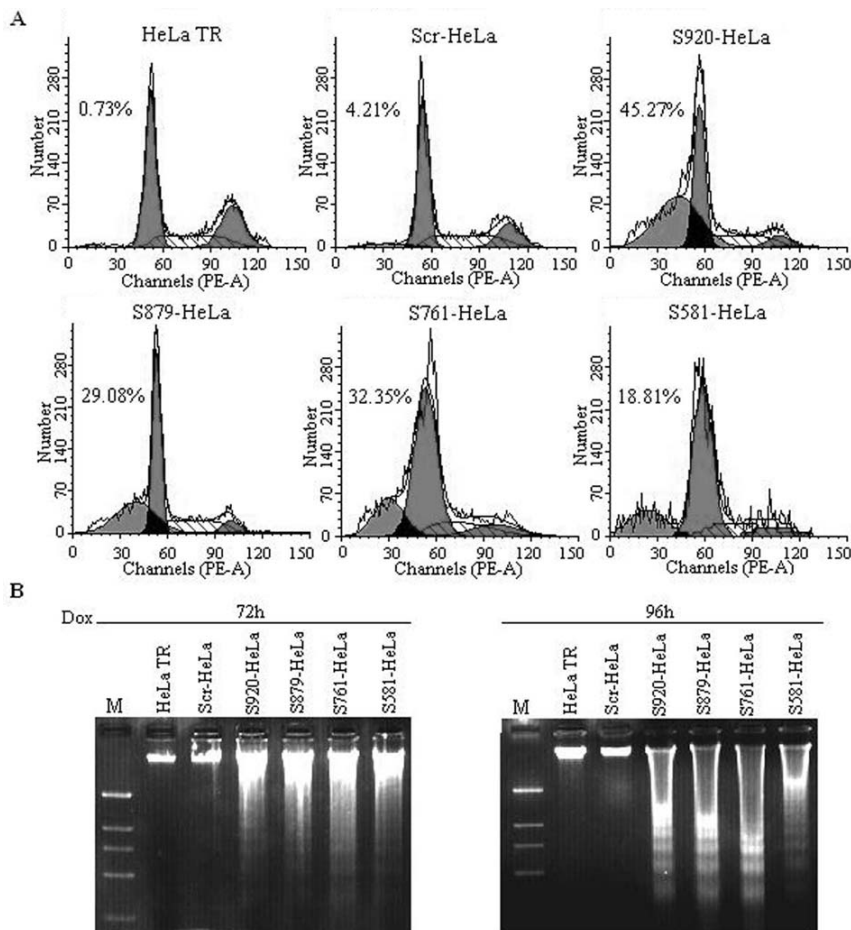
## MATERIALS AND METHODS

### Materials

RPMI 1640, Lipofectamine 2000, blasticidin, zeocin and other reagents for cell culture were purchased from Invitrogen. Doxycycline (Dox) was from Sigma-Aldrich Co. The tetracycline repressor (TetR) expression plasmid pcDNA6/TR and shRNA expression plasmid pTER were from Prof. Hans Clevers (Hubrecht Laboratory, Centre for Biomedical Genetics, Utrecht, Netherlands). SMYD3 polyclonal antibody was prepared in our laboratory.

### shRNAs expression plasmids

We followed reported methods to generate shRNA expression plasmids from pTER (11). The vector was digested with *Bam*HI and *Hind*III, and shRNA oligos (see below) were ligated into this vector. Four resulting RNAi vectors, S920-pTER, S879-pTER, S761-pTER and S581-pTER, were tested for effect on levels of SMYD3 in HeLa cells. S920-pTER, was found to work very efficiently, and was used as a positive control (12). The synthetic DNA sequences for the shRNAs corresponded to GenBank accession number NM\_022743 nucleotides 920-940, 879-897,



**Fig. 4.** Induction of apoptosis by knock-down of SMYD3 gene expression. (A) Flow cytometry analysis of apoptotic cells. The percentages of cells in sub-G1 population are given. Left shaded line, sub-G1; middle shaded line, G1; right shaded line, G2; striped line, S. (B) DNA fragmentation after 2  $\mu$ g/ml Dox induction for 72 h and 96 h. Time is indicated above the open line. M, DL2000.

761-781, and 581-601. Another RNAi vector Scr-pTER was used as negative control; its targeted sequence, 5'-GTAGATGG TCGACCTTCAC- 3', has no significant homology with any known gene. The resulting plasmids were confirmed by sequencing.

#### Cell culture, transfection and selection

The human cervical carcinoma cell line HeLa (ATCC) was grown in RPMI 1640 medium supplemented with 10% NBS, 50 units/ml penicillin, and 50  $\mu$ g/ml streptomycin. Transfection was performed using Lipofectamine 2000. To create a HeLa TR cell line stably expressing TetR, the cells were transfected with pcDNA6/TR and plated with 2  $\mu$ g/ml blasticidin for 2 weeks. Genomic DNA of resistant clones was analyzed by PCR with primers for TetR: TetRF, 5'-CCTTAGCCATTGAGAT GTTAG-3' and TetRR, 5'-TTAGCGACTTGATGCTCTTG-3'. A HeLa TR was then transfected with the shRNAs expression plasmids. Individual clones were isolated and maintained in the presence of 2  $\mu$ g/ml blasticidin and 100  $\mu$ g/ml zeocin. Zeocin resistant clones were analyzed as above with primers

for the zeocin resistance gene: ZeocinF, 5'-ATGGCCAAGTTG ACCAGTG-3' and ZeocinR, 5'-CGAAGCCCAACCTTTCATAG-3'.

#### RT-PCR assay

Total cellular RNA was extracted from cells after induction with 2  $\mu$ g/ml Dox for 48h, using Trizol reagent (Gibco). For each sample, 2  $\mu$ g of total RNA was reverse transcribed using oligo dT 18 primers and MMLV (Promega) following standard protocols. 2  $\mu$ l of the newly synthesized cDNA was used as a template for PCR, which was performed with 1.5 mM MgCl<sub>2</sub>, 2.5U Taq polymerase, and 0.5  $\mu$ M primers. The primer sequences were: for SMYD3, 5'-CCCAGTATCTCTTTGCTCAATC AC-3' and 5'-ACTTCCAGTGTGCCTTCAGTTC-3'; for the GAPDH internal control, 5'-ATTCAACGGCACAGTCAAGG-3' and 5'-GCAGAAGGGGCGGAGATGA-3'. Amplification cycles were 94°C for 5 min, followed by 26 cycles at 94°C for 30 s, 55°C for 45 s, 72°C for 45 s, followed by 72°C for 10 min. Aliquots of PCR products were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining. The DNA bands were analyzed with Quantity

One software and relative mRNA expression levels (REs) were deduced from the ratio of the mean values of SMYD3 to that of GAPDH. HeLa TR was used as blank control. The mRNA inhibition ratio is defined as:

$$IR = \left[ 1 - \frac{RE_{\text{experimental}}}{RE_{\text{control}}} \right] \times 100\%$$

### Western blot analysis

After treatment with 2 µg/ml Dox for 48h, cells were lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholic phenylmethyl-sulfonyl fluoride, 1 µg/ml of aprotinin, and 1 mmol/l of DTT). Twenty microgram aliquots of the lysates were separated on a 12% SDS-polyacrylamide gel and transferred electrophoretically onto a nitrocellulose membrane (Bio-Rad). Blots were blocked for 1 h in blocking buffer (10% non-fat dried milk, 0.5% Tween in TBS) and incubated with anti-human SMYD3 rabbit polyclonal antibody overnight at 4°C, and with anti-human β-actin mouse polyclonal antibody (1:200; Sigma) as a control. The blots were washed, incubated according to standard procedures and developed with the ECL system (Amersham Pharmacia). Protein levels were quantified by densitometric analysis using a GS-700 Densitometer. Relative protein expression levels (REs) were deduced from the ratio of the mean values of SMYD3 to that of β-actin. HeLa TR was used as blank control. Protein inhibition ratios (IR) were obtained using the following formula.

$$IR = \left[ 1 - \frac{RE_{\text{experimental}}}{RE_{\text{control}}} \right] \times 100\%$$

### MTT assay

For cell proliferation assays, the cells were seeded at a density of  $3 \times 10^3$  cells/well in 96-well plates and grown for 5 days. During this period, three wells were selected from each group of cells every day for the methyl thiazolyl tetrazolium (MTT) (50 µg/well) assay. After the cells had been incubated at 37°C for 4 h, the reaction was stopped by adding 150 µl/well of DMSO, and incubating for 10 min. The color reaction was quantified using an automatic plate reader (Bio Rad) at 570 nm with a reference filter of 620nm. The absorbance value (A) was directly proportional to the number of viable cells. All assays were performed in triplicate.

### Colony formation assay

Cells (300/well) were seeded in six-well plates and maintained in culture medium supplemented with 2 µg/ml Dox. After 2 weeks the colonies were stained with Giemsa. Cultures were analyzed in triplicate, and colonies larger than 100 µm in diameter were counted.

### Migration assay *in vitro*

To analyze two-dimensional migration, a wound-healing scratching assay was performed as described (27).  $3 \times 10^5$

cells in culture medium supplemented with 2 µg/ml Dox were seeded in six-well plates. After confluent monolayers had formed, wounds were created using a plastic tip. Two parallel wounds were created in each six-well, and their location was marked on the bottom of the plate; 6 wounds were created for each cell clone. The areas newly occupied as a result of cell migration were measured 48 h after scratching, by photographing them under a microscope. Wound closure by the control HeLa TR cells was set to 100%.

### Invasion assay *in vitro*

To determine the effect of SMYD3 knockdown on the invasion potential of HeLa cells, an invasion assay *in vitro* was performed using ThinCert™ cell culture inserts for 24-well plates composed of polycarbonate membranes containing 8 µm pores (Greiner, Frickenhausen, German). In brief, cells were first starved overnight in serum-free RPMI 1640 and then seeded on the upper chamber of the Thincert™ at  $5.0 \times 10^4$  cells in 200 µl serum-free RPMI 1640 with 2 µg/ml Dox. The lower chamber was filled with 600 µl RPMI 1640 containing 10% FBS. After 37°C for 48 h, the cells in the upper chamber were removed with a cotton swab, and the cells that had migrated to the lower side of the membrane were fixed with methanol: acetic acid (3:1) for 10 min and stained with 10% Giemsa. The number of migrated cells was counted in five randomly chosen fields of three independent experiments at 100× magnification.

### Flow cytometry

Flow cytometry analysis was used to measure apoptosis of the cells. Cells were deprived of serum for 24h and induced with 2 µg/ml Dox for 48h. They were harvested, washed once in PBS, fixed with 70% ethanol for 20 min and stored at 4°C overnight, then washed with PBS, and stained with 100 µl of 50 mg/l propidium iodide (BD) at 4°C for 30 min. Apoptotic cells were assessed by flow cytometric detection (BD) of sub-G1 DNA content.

### DNA fragmentation assay

After 24h, 48h, 72h and 96h in the presence of 2 µg/ml Dox, cells were harvested and lysed in 20 µl of a lysis buffer (100 mM Tris-HCl pH 8.0, 20 mM EDTA, and 0.8% (w/v) SDS) and 10 µl RNaseA/T1 (500 U/ml, 20000 U/ml, Ambion Inc) for 90-120 min at 37°C. Then 10 µl of Proteinase K (20 mg/ml) was added. After incubation for 90 min at 50°C, the fragmented DNA was electrophoresed on a 2% agarose gel. The gel was stained with ethidium bromide and the state of the DNA was visualized by UV transillumination.

### Statistical analysis

All statistical analyses were performed using SPSS10.0. Data represent the means ± s.d. of three independent experiments, and significance was assessed at  $P < 0.05$  by Student's *t*-test.

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