**ALKBH8 promotes bladder cancer growth and progression through regulating the
expression of survivin**

Ikumi Ohshio*, Ryoji Kawakami*, Yohei Tsukada, Kazuhiro Nakajima, Kaori Kitae,
Tomoki Shimano, Yasuka Saigo, Hiroaki Hase, Yuko Ueda, Kentaro Jingushi,
Kazutake Tsujikawa

Laboratory of Molecular and Cellular Physiology, Graduate School of Pharmaceutical
Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka, 565-0871, Japan

Corresponding author: Kentaro Jingushi

Tel: +81-6-6879-8192; Fax: +81-6-6879-8190; E-mail: jingushi-kk@phs.osaka-u.ac.jp

*These authors contributed equally to this work.

Abstract

Human AlkB homolog 8 (ALKBH8) is highly expressed in high-grade, superficially and deeply invasive bladder cancer. Moreover, ALKBH8 knockdown induces apoptosis in bladder cancer cells. However, the underlying anti-apoptotic mechanism of ALKBH8 in bladder cancer cells has thus far remained unclear. Moreover, there is no direct evidence that highly expressed ALKBH8 is involved in tumor progression *in vivo*. We here show that ALKBH8 knockdown induced apoptosis via downregulating the protein expression of survivin, an anti-apoptotic factor also exhibiting increased levels in bladder cancer. We also clarify that *ALKBH8* transgenic mice showed an accelerated rate of bladder tumor mass and invasiveness in an N-butyl-N-(4-hydroxybutyl)-nitrosamine-induced bladder cancer model. These findings suggest that the high expression of ALKBH8 is critical for the growth and progression of bladder cancer.

Key words: ALKBH8, bladder cancer, anti-apoptotic factor, tumor progression

34 **Abbreviations:** ALKBH, AlkB homolog; BBN,
35 N-butyl-N-(4-hydroxybutyl)-nitrosamine; IAP, inhibitor of apoptosis protein; NOX1,
36 NADPH oxidase 1; ROS, reactive oxygen species; siRNA, small interfering RNA; TG,
37 transgenic; XIAP, X-linked inhibitor of apoptosis protein

1. Introduction

Bladder cancer is one of the most common cancers worldwide. Over 76,000 patients, representing 4.5% of all cancer patients, are diagnosed with bladder cancer annually in the USA [1]. One of the most outstanding characteristics of bladder cancer is the high recurrence rate after local surgical resection. Approximately 70% of postoperative superficial bladder cancer patients show recurrence, and 30% of such cases progress to invasive bladder cancer [2,3]. Therefore, identification of a prominent target molecule for bladder cancer treatment is desired.

Escherichia coli AlkB is a 2-oxoglutarate and Fe(II)-dependent dioxygenase that repairs alkylated DNA/RNA nucleotides by catalyzing oxidative demethylation [4–6]. This oxidative demethylation role of AlkB is considered to be the third genome restoration system in addition to the base-excision and nucleotide-excision repair systems that contribute to maintaining the stability of the *E. coli* genome. In humans, nine AlkB homolog (ALKBH) family members have been reported [7,8]. The ALKBH family recognizes various kinds of substrates more specifically than AlkB, including single- or double-stranded DNA or RNA [9]. In recent years, some reports have shown that ALKBH family members are highly expressed in various human cancers, including bladder cancer, and contribute to cancer progression [10–12]. For example, ALKBH2

regulates the cell cycle and epithelial-to-mesenchymal transition of bladder cancer via regulating the oncogene *MUC1* [13]. ALKBH3 also has a crucial role in urothelial carcinoma by regulating reactive oxygen species (ROS) generation via Tweak/Fn14/VEGF signaling and subsequent angiogenesis [14].

In various types of cancer, apoptosis signal regulation plays a crucial role in tumor progression and resistance to chemotherapy. The inhibitor of apoptosis protein (IAP) family consists of 8 members, which are anti-apoptotic factors considered to be core factors that suppress apoptosis and regulate the cell cycle of different tumors and at different stages of growth [15,16]. Among the IAP family molecules, X-linked IAP (XIAP) and survivin show particularly strong resistance to apoptosis by orienting apoptosis effectors such as those of the caspase family toward proteasome-dependent degradation. Moreover, they are highly expressed in a malignancy-dependent manner in various cancers, including bladder cancer [17,18].

Recently, Shimada et al. [19] showed that ALKBH8 is highly expressed in bladder cancer tissues and correlated with higher grades and stages. They further showed that ALKBH8 knockdown induced apoptosis in bladder cancer cell lines, through downregulation of ROS via suppression of NADPH oxidase 1 (*NOX1*) mRNA expression. Furthermore, Fu et al. [20] showed that constitutive knockdown of

ALKBH8 increased the genotoxic stress sensitivity induced by methylmethanesulfonate and bleomycin in HeLa cells. These results strongly suggest that ALKBH8 is a molecule related to cell survival signals and the cellular stress response in human cancer cells. However, the underlying apoptotic-related molecular mechanism regulated by ALKBH8 has not been fully elucidated.

In this study, we provide the first demonstration of the impact of ALKBH8 expression on bladder cancer growth and progression *in vivo* using the N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN)-induced bladder cancer mouse model. We also show that ALKBH8 promotes cell proliferation via upregulation of the anti-apoptotic factors XIAP and survivin in bladder cancer cells.

2. Materials and Methods

2.1. Cell culture

The human urinary bladder cancer cell lines UM-UC-2, EJ-1, KU7, and T24 were cultured in Dulbecco's modified Eagle medium (Wako), RT4 and 5637 cells were cultured in RPMI-1640 (Wako), and J82 cells were cultured in Eagle's minimum essential medium (Wako) under a 5% CO₂ atmosphere. SV-HUC-1 human ureteral transitional cells transformed by Simian vacuolating virus were cultured in Ham's F-12 (Wako). All media used for cell culture were supplemented with 10% fetal bovine

serum and 100 mg/L kanamycin at 37°C.

2.2. Antibodies and chemical reagents

Anti-ALKBH8 polyclonal antibody was purchased from Abcam. Anti-XIAP and anti-lamin A/C monoclonal antibodies were obtained from Becton Dickinson (BD). Polyclonal anti-survivin and monoclonal anti-Bcl-xL antibodies were obtained from Merck Millipore. Monoclonal anti-survivin antibody were obtained from Cell Signaling Technology (CST). Polyclonal anti-cIAP1 and cIAP2 antibodies were from Novus Biological. Monoclonal anti- β -actin and anti- β -tubulin antibodies were from Sigma-Aldrich. BBN was purchased from Tokyo Chemical Industry.

2.3. Western blotting analysis

The whole cell lysates were resolved on 7.5–15% sodium dodecyl sulfate-polyacrylamide gels and transferred onto a polyvinylidene difluoride (Merck Millipore) membrane using a semi-dry transfer system (Bio-Rad) for 1 h at 12 V followed by blocking in 5% skim milk at room temperature for 1 h. The membranes were incubated with the indicated primary antibodies overnight and then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (CST).

Immunoreactive proteins were detected by treatment with a detection reagent (ECL prime western blotting detection reagent, GE Healthcare). The ImageQuant LAS4000 mini system (GE Healthcare) was used for chemiluminescence detection.

2.4. Small interfering RNA (siRNA) transfection

siRNA duplexes used to knockdown *ALKBH8* mRNA (*ALKBH8* siRNA; UGGAAAAAGUGCAGUGGAATT) and the negative control siRNA duplex (Control siRNA; AUCCGCGCGAUAGUACGUATT) were purchased from B-Bridge. siRNAs of *XIAP* (ACACUGGCACGAGCAGGGUUUCUUU) and survivin (CACCGCAUCUCUACAUAAGAACU) were purchased from Thermo Fisher Scientific. All siRNAs were transfected at a concentration of 10 nM using Lipofectamine RNAiMAX (Thermo Fisher Scientific) in accordance with the manufacturer's protocol.

2.5. Cell proliferation assay

For the anchorage-dependent cell proliferation assay, 4.0×10^2 cells were seeded on a polystyrene-coated 96-well plate. At 24, 48, 72, 96, and 120 h after transfection, water-soluble tetrazolium salt-1 (WST-1) reagent (Dojindo) was added to the culture

medium and incubated at 37°C, 5% CO₂ for 2 h. Optical density was read at a wavelength of 450/630 nm (measurement/reference) using a microplate reader (Bio-Rad).

2.6. *Soft agar colony formation assay*

A soft agar colony formation assay was used to examine anchorage-independent cell proliferation. Briefly, 1.0×10^3 cells/well were suspended into the medium containing 0.3% agarose and seeded on a 6-well plate coated with medium containing 0.4% agarose as the bottom agar. Two weeks later, formed colonies were stained with a 0.05% crystal violet/50% methanol solution and the colony numbers were counted.

2.7. *Apoptosis analysis*

UM-UC-2 cells transfected with *ALKBH8* siRNA, or with *XIAP* or survivin siRNAs were cultured for 96 h or 72 h, respectively, and stained with propidium iodide (PI) and fluorescein isothiocyanate-conjugated Annexin V (AnnexinV-FITC apoptosis detection kit, BioVision) according to the manufacturer's protocol. Flow cytometric analysis was conducted using a FACS Calibur system (BD).

2.8. Caspases 3, 8, and 9 activity assay

Caspase activity was analyzed by APOPCYTO Caspases 3, 8 and 9 Colorimetric Assay Kit (MBL) in accordance with the manufacturer's protocol. Cells transfected with *ALKBH8* siRNA for 72 h were resuspended in 80 μ L cell lysis buffer and incubated on ice for 10 min. Caspase substrates were added to the cell lysate and the absorbance at 405 nm was measured using a microplate reader (Bio-Rad).

2.9. Establishment of cell lines with stable *ALKBH8* overexpression

Complete alignment of *ALKBH8* cDNA was amplified from a human normal pancreas cDNA library, followed by cloning into the pEBMulti-Neo vector (Wako). The primer sequences for gene amplification were as follows: forward 5'-CACCATGGACAGCAACCATCAAAG-3' and reverse 5'-ATCAGGCCTTTTGAAGAATCAC-3'. An *ALKBH8*-expressing vector (pEBMulti-*ALKBH8*) was transfected into UM-UC-2 (5.0×10^4 cells/well) cells seeded on a 12-well plate using Lipofectamine 2000 (Thermo Fisher Scientific). After 24 h incubation, the culture medium was changed to a medium containing 800 μ g/mL G418 (Wako) for selection.

165 2.10. BBN-induced bladder cancer model in *ALKBH8* transgenic (TG) mice

166 For establishment of the *ALKBH8* TG mice, *ALKBH8* cDNA was amplified from
167 *ALKBH8* full-length cDNA as described previously [21] as a template using the
168 following primers: forward
169 5'-CAATTGCACCATGGACAGCAACCATCAAAGTAATTACAACTCAG-3' and
170 reverse 5'-CAATTGTCAGGCCTTTTGAAGAATCACACACCAGTTTCC-3'. The
171 polymerase chain reaction (PCR) product was cloned into pT7Blue vector (Merck
172 Millipore) and the sequence was confirmed. Then, the PCR product was digested by
173 *Mfe*I and inserted into a pCAGGS vector (supplied from Genome Information Research
174 Center, Research Institute for Microbial Disease, Osaka University, Osaka, Japan) and
175 digested by *Eco*RI with correct direction. The constructed pCAGGS vector containing
176 *ALKBH8* cDNA was microinjected into a fertilized ovum at the Genome Information
177 Research Center, Osaka University. The genotyping of the offspring was analyzed by
178 PCR using DNA extracted from the mouse tails as a template with the following
179 primers: forward 5'-ATCGTTTCTCTCAGTTTGGG-3' and reverse
180 5'-GCTTCTTTATCACTCTCTGG-3'.

181 The 8-week-old *ALKBH8* TG mice and their wild type (WT) littermates were
182 given drinking water with 0.05% BBN (Tokyo Chemical Industry) for 12 weeks, and

were subsequently provided with natural water. When these mice reached 40 weeks old, they were sacrificed and the bladder and proximal organs were harvested. Bladder weight (mg) was measured on an electronic scale. A whole bladder with tumor was fixed in 10% neutral buffered formalin (Wako) overnight for pathological examination. The bladder tissues were embedded in paraffin and cut into 3- μ m sections. Hematoxylin and eosin staining was performed and the pathological findings were recorded by a certified pathologist (Applied Medical Research). All procedures were performed under a protocol approved by the Animal Experimentation Committee at Osaka University.

2.11. Statistical analysis

The results are presented as the mean $\pm$ standard deviation. The data were statistically analyzed by the Student's t-test or Welch's t-test after examination of the dispersion difference by the F-test. One-way analysis of variance with Bonferroni post-hoc tests was applied for multiple comparisons. A P-value <0.05 was considered statistically significant.

3. Results

3.1. ALKBH8 knockdown induced apoptosis in UM-UC-2 cells

To investigate the function of ALKBH8 in bladder cancer cells, we first analyzed the expression levels of ALKBH8 in bladder cancer cell lines and a normal urothelial cell line, SV-HUC-1. Among the seven cancer cell lines tested (KU7, RT4, UM-UC-2, T24, EJ-1, 5637, and J82 cells), marked expression of ALKBH8 was observed in all cancer cell lines except J82 (Supplementary Fig. S1A). No expression of ALKBH8 was detected in SV-HUC-1 cells. Therefore, we selected UM-UC-2 cells for the following experiments because of its high transfection efficiency. We then performed knockdown experiments using *ALKBH8* siRNA in UM-UC-2 cells. *ALKBH8* knockdown significantly suppressed the UM-UC-2 cell proliferation (Fig. 1A). Increases in the Annexin V (+) PI (-) population (Fig. 1B) and caspases 3, 8, and 9 activities (Fig. 1C) were detected, indicating a relationship between the suppression of UM-UC-2 cell proliferation and apoptosis induction. Suppressed cell proliferation, increased Annexin V (+) PI (-) population, and enhanced caspases 3, 8, and 8 activities were also observed in EJ-1 cells transfected with *ALKBH8* siRNA (Supplementary Fig. S2).

To examine the potential molecular mechanism of apoptosis induction by *ALKBH8* siRNA, western blot analyses for anti-apoptotic molecules was carried out. As shown in Fig. 1D, *ALKBH8* knockdown obviously inhibited the protein expression of XIAP and survivin. Interestingly, no effect of *ALKBH8* siRNA on the expression of *XIAP* and

survivin mRNA was found (Supplementary Fig. S3A).

3.2. *ALKBH8* overexpression upregulated the expression of *XIAP* and *survivin*

ALKBH8 cDNA was stably transfected in UM-UC-2 cells, and the expression of *XIAP* and *survivin* was determined by western blot analysis. As shown in Fig. 2A, overexpression of *ALKBH8* upregulated the expression of *XIAP* and *survivin*. Again, *ALKBH8* overexpression had no effect on the mRNA levels of *XIAP* and *survivin* (Supplementary Fig. S3B), suggesting that *ALKBH8* might regulate the expression of *XIAP* and *survivin* at a post-transcriptional level in UM-UC-2 cells. Neither anchorage-dependent nor anchorage-independent cell proliferation was observed in *ALKBH8*-overexpressing UM-UC-2 cells (Fig. 2B, C).

3.3 *Survivin* knockdown induced apoptosis in UM-UC-2 cells

Since the protein expression of *XIAP* and *survivin* was downregulated and upregulated by *ALKBH8* knockdown and overexpression, respectively, we analyzed the effect of knockdown of each gene on apoptosis in UM-UC-2 cells. Knockdown of *survivin* but not *XIAP* significantly suppressed the proliferation and induced the subsequent apoptosis in UM-UC-2 cells (Fig. 3A–C). These results indicate that the

apoptosis induction by ALKBH8 knockdown was mainly due to downregulation of survivin.

3.4. ALKBH8 TG mice had accelerated tumor growth and progression in a BBN-induced bladder cancer model

To clarify the relationship between the high expression of ALKBH8 and bladder cancer, we generated TG mice that express human ALKBH8 (*ALKBH8* TG) under pCAGGS promoter regulation (Fig. 4A). *ALKBH8* TG mice were born normally and showed neither obvious phenotypes nor tumor formation (data not shown). Then, we orally administered BBN, which is known as an inducer of bladder cancer [22], to WT and *ALKBH8* TG mice. BBN administration for 12 weeks spontaneously induced bladder cancer in both WT and *ALKBH8* TG mice. Interestingly, *ALKBH8* TG mice showed accelerated growth of bladder tumors and increased bladder weights compared to WT mice (Fig. 4B). Then, we performed histopathological analysis of the bladder tumors from both types of mice. In WT mice, the bladder tumor existed in the epithelium tissue and submucosa, and the frequency of muscle invasion tumors was less than 40%. By contrast, over 80% of the tumors in *ALKBH8* TG mice showed invasion to the muscle layer (Fig. 4C, D). These data clearly indicate that ALKBH8 promotes the growth and

invasion of bladder cancer *in vivo*.

4. Discussion

We show here for the first time that ALKBH8 is a critical molecule for bladder cancer growth and progression *in vivo*. An important regulation mechanism of ALKBH8 is upregulation of XIAP and survivin in bladder cancer. These findings strongly support the expression changes of ALKBH8 from noninvasive to invasive bladder cancer in clinical specimens [19].

The regulation mechanism of tumor growth and progression by ALKBH8 has thus far remained elusive. Shimada et al. [19] reported that ALKBH8 knockdown reduced ROS production partly via down-regulation of NOX-1 in UM-UC-2 cells. In contrast, our experiments showed that ALKBH8 knockdown increased ROS generation in the same cell line (data not shown). The reason for the discrepancy between these studies is not clear, but there are other reports showing that NOX-1 induces ROS generation, leading to subsequent apoptosis [23,24]. Moreover, our finding that the expression of XIAP and survivin was downregulated and upregulated by ALKBH8 knockdown and overexpression, respectively, is noteworthy. Survivin is known to be highly expressed in bladder cancer [17,18]. Importantly, the expression regulation of XIAP and survivin

by ALKBH8 was only observed at the translational and not at the transcriptional levels. ALKBH8 protein is unique among the ALKBH family, since it harbors a 2-oxoglutarate and Fe(II)-dependent oxygenase domain as well as an RNA recognition motif and methyl transferase domain. ALKBH8 regulates translation via the methylation and hydroxylation of uridine of an anti-codon loop in four types of tRNA, tRNA^{Arg(UCU)}, tRNA^{Gly(UCC)}, tRNA^{Glu(UUC)}, and tRNA^{Sec}, through these domains [20,25,26]. Using immunofluorescent staining and western blot analysis with anti-ALKBH8 antibody, we confirmed that endogenous ALKBH8 localizes in the cytoplasm of bladder cancer cells (Supplementary Fig. S4). These localization data are consistent with previous reports that the main ALKBH8 substrate is the uridine of tRNAs.

Recently, it was reported that the amount of tRNA likely regulates gene expression in several cancers [27,28], and that the component ratio of tRNAs is significantly different in breast cancer cells compared to that of normal mammary glandular cells [29]. Therefore, dysregulation in the amount and modification of tRNA might be the key mechanism for the effects of ALKBH8 on tumor growth and progression, suggesting ALKBH8 as the molecule responsible for tRNA modification during gene translation in bladder cancer.

We showed that survivin knockdown significantly suppressed cell growth and

induced apoptosis in UM-UC-2 cells (Fig. 3B and C). Moreover, ALKBH8 knockdown induced the activation of caspases 3, 8, and 9 in the cells (Fig. 1C and Fig. S2C). Since survivin is reported to inhibit caspase 9 activity [30], these results suggest that ALKBH8 might suppress caspase 9 activity by increasing the expression of survivin protein, which highlights an important function of ALKBH8 in bladder cancer cells.

Taken together, our results suggest that ALKBH8 might exert its tumor-promoting function through transcriptional upregulation of anti-apoptotic molecules based on tRNA modification in bladder cancer. By verification *in vitro*, *in vivo*, and in reported clinical samples, ALKBH8 shows potential as a molecular target with a unique regulation mechanism for the treatment of bladder cancer.

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Figure legends

Figure 1. ALKBH8 knockdown induced apoptosis and downregulated XIAP and survivin expression in UM-UC-2 cells.

(A) UM-UC-2 cells transfected with *ALKBH8* siRNA were reseeded on a 96-well plate and WST-1 assay was performed at the indicated times after transfection. UM-UC-2 cells transfected with *ALKBH8* siRNA were stained with propidium iodide and annexin V after 96 h of transfection and analyzed with a flow cytometer (B), and examined for caspase activity after 72 h of transfection (C). (D) Cell lysates of the transfected UM-UC-2 cells were prepared after 72 h of transfection and subjected to western blot analysis with anti-cIAP1, anti-cIAP2, anti-XIAP, anti-survivin, anti-Bcl-xL, anti-ALKBH8, and anti- β -actin antibodies. Values are the mean $\pm$ SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ vs. control siRNA.

Figure 2. ALKBH8 overexpression upregulated the protein expression of XIAP and survivin.

(A) UM-UC-2 cells stably overexpressing ALKBH8 were subjected to western blot analysis with anti-cIAP1, anti-cIAP2, anti-XIAP, anti-survivin, anti-Bcl-xL, anti-ALKBH8, and anti- β -actin antibodies. UM-UC-2 cells stably overexpressing

ALKBH8 or control cells were examined in anchorage-dependent (B) and anchorage-independent (C) cell proliferation assays. Values are the mean $\pm$ SD of three independent experiments.

Figure 3. Survivin but not XIAP knockdown induced apoptosis in UM-UC-2 cells.

(A) Knockdown of XIAP and survivin was confirmed by western blotting in UM-UC-2 cells transfected with *XIAP* and survivin siRNAs for 48 h. (B) UM-UC-2 cells transfected with *XIAP*, survivin were reseeded on a 96-well plate and cell proliferation assay were performed. (C) UM-UC-2 cells were stained with propidium iodide and annexin V after 72 h of transfection and analyzed with a flow cytometer. Values are mean $\pm$ SD of three independent experiments. *, $P < 0.05$; ***, $P < 0.001$ vs. control siRNA.

Figure 4. ALKBH8 TG mice showed accelerated tumor growth and invasion in a BBN-induced bladder tumor model.

(A) Confirmation of human *ALKBH8* expression in *ALKBH8* TG mice by RT-PCR. (B) Representative images (left panel) and weight (right panel) of the bladder from wild type (WT) and *ALKBH8* TG mice orally administered BBN. Scale bar: 1 cm. Values are

shown as the mean $\pm$ SD of 13 (WT) or 15 (*ALKBH8* TG) mice. **, $P < 0.01$ vs. WT mice. (C) The bladders of BBN-administered WT and *ALKBH8* TG mice were stained with hematoxylin and eosin, and images of each representative specimen are shown (C). Scale bars: 1 mm (upper pictures) or 50 μ m (lower pictures). (D) Non-muscle invasive bladder cancer (NMIBC) or muscle invasive bladder cancer (MIBC) were histopathologically examined, and the proportions were calculated from eight (WT) and six (*ALKBH8* TG) specimens.

Supplementary Figure 1. Protein expression of ALKBH8 in bladder cancer cell lines.

(A) Cell lysates from cancer cell lines and a normal urothelial cell line SV-HUC-1 were subjected to western blotting with anti-ALKBH8 antibody. A representative result of two independent experiments is shown.

Supplementary Figure 2. ALKBH8 knockdown induced apoptosis in EJ-1 cells.

(A) EJ-1 cells transfected with *ALKBH8* or control siRNAs for 48 h were subjected to western blot analysis with anti-ALKBH8 and anti- β -actin antibodies. The transfected EJ-1 cells were reseeded on a 96-well plate (3.0×10^2 cells/well) and cell proliferation

was examined by a WST-1 assay at the indicated times. After 72 h of transfection, apoptotic analysis (B) and caspases activity assays (C) were performed. Values are mean $\pm$ SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ vs. control siRNA.

Supplementary Figure 3. ALKBH8 knockdown nor overexpression had effect on mRNA expression of XIAP and survivin in UM-UC-2 cells.

mRNA expression of *ALKBH8*, *XIAP*, and survivin in UM-UC-2 cells with ALKBH8 knocked down (A) and overexpressed (B); expression levels. Values are the mean $\pm$ SD of three independent experiments. *, $P < 0.05$; ***, $P < 0.001$ vs. control.

Supplementary Figure 4. ALKBH8 is localized in the cytoplasm.

(A) Immunofluorescence staining was performed with the anti-ALKBH8 antibody and 4',6-diamidino-2-phenylindole (DAPI) in UM-UC-2 cells. Scale bars: 50 μ m. (B) The cytosolic and nuclear proteins of UM-UC-2 cells were fractionated, and the protein samples were subjected to western blot analysis with anti-ALKBH8, anti-lamin A/C (cytosol marker), and anti- β -tubulin (nuclear marker) antibodies.

Supplementary Materials & Methods

1.1. Extraction of total RNAs and real-time RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen) and cDNA was synthesized by Primescript RT reagent Kit (Takara Bio) according to the manufacturer's protocol. Light cycler (Roche) was used for real-time PCR analysis. Thermal cycling conditions included an initial step at 95°C for 30 s and 40 cycles at 95°C for 15 s, 58°C for 30 s (*ALKBH8*, *GAPDH*) or 40 cycles at 95°C for 5 s, 60°C for 20 s (*XIAP* and survivin), and a final step at 72°C for 15 s. The primer sequences used for amplification were as follows: *ALKBH8*, 5'-CCAGTTATGTTGCCTCGTCG-3' (forward) and 5'-TGTTTCGTAGTCCCCTCTTGC-3' (reverse); *GAPDH*, 5'-CCATCACCATCTTCCAGGAG-3' (forward) and 5'-AATGAGCCCCAGCCTTCTCC-3' (reverse); *XIAP*, 5'-CATCCATGGCAGATTATGAAGCA-3' (forward) and 5'-CTTCACTGGGCTTCCAATCAGTTAG-3' (reverse); survivin, 5'-TTCTCAAGGACCACCGCATC-3' (forward) and 5'-GCCAAGTCTGGCTCGTTCTC-3' (reverse).

1.2. Fluorescence microscopy

UM-UC-2 cells were cultured for 48 h on coverslips, and subsequently fixed and permeabilized by 50% methanol/acetone under -20°C for 10 min. After blocking with 5% bovine serum albumin with 0.1% Triton X-100 in phosphate-buffered saline for 1 h, the samples were incubated with the primary antibody at 4°C overnight, followed by Alexa fluor 488-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen) treatment for 1 h at room temperature. Then, the coverslips were mounted on the slide glass using Dapi Fluoromount-G (SouthernBiotech). The fluorescence of stained cells was examined under a fluorescence microscope (Olympus).

Figure 1

A

B

D

C

Fig. 2

Fig. 3

Fig. 4