# Enhancements of hyperthermia sensitivity by novel molecular targets of heat shock transcription factor 1-related proteins in human cancer cells

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Contents

## 1. Summary

[Background and Aims]

Hyperthermia (HT) has been considered a promising approach in cancer therapy and the anticancer effects have been verified by clinical trials for various types of cancer. However, the cytoprotective effects caused, at least in part, by an increase in heat shock proteins (HSPs) in some cancer cells have rendered HT less effective. The induction of HSPs is mainly mediated by the activation of heat shock transcription factor 1 (HSF1). In this study, we targeted HSF1-related proteins such as Bcl-2 associated athanogene 3 (BAG3) and polo-like kinase 1 (PLK1) in order to attenuate the thermoresistance of HT. BAG3, a co-chaperone of the Hsp70, is a stress-inducible protein and confers cytoprotection against various stresses, including heat stress. PLK1 plays a role in activation of HSF1 and acts as an important regulator of mitosis in several cancer cells. Therefore, we examined the inhibitory effects of these HSF1-related proteins on sensitivity to HT in human cancer cells.

[Methods]

We examined the effects of BAG3-knockdown by small interfering RNA (siRNA) on the sensitivity to HT (44 °C, 60 or 90 min) in human oral squamous cell carcinoma HSC-3 cells and human retinoblastoma Y79 cells, and examined the effects of PLK1-knockdown by siRNA or by the inhibition

of PLK1 activity with PLK1 inhibitor (BI-2536) on the sensitivity to HT (44 °C, 60 min) in human retinoblastoma Y79 and WERI-Rb-1 cells. Induction of apoptosis and inhibition of cell proliferation were used for the evaluation of hyperthermic effects. Apoptosis was monitored by chromatin condensation analysis, the sub-G1 phase of the cell cycle, and detection of cleavage of caspase-3. Cell proliferation was monitored by WST-8 assay and cell cycle analysis. Furthermore, we examined the role of the c-Jun N-terminal kinase (JNK) pathway and nuclear factor kappa B (NF-κB) activity in BAG3 silencing sensitized HT-induced apoptosis. We also investigated the effects of activating HSF1 through a combination of PLK1-knockdown and HT.

[Results]

1) Although the expression level of BAG3 was low under the non-stress conditions, it significantly increased in HSC-3 and Y79 cells treated with HT.

2) BAG3-knockdown enhanced HT-induced apoptosis in HSC-3 and Y79 cells.

3) The combination of BAG3-knockdown with inhibition of the JNK pathway further enhanced HT-induced apoptosis in HSC-3 cells.

4) BAG3-knockdown maintained HT-induced NF-κB inactivity through IKKγ degradation and the subsequent inhibition of the phosphorylation of IκB and p65 in Y79 cells.

5) PLK1-knockdown or PLK1 inhibitor (BI-2536) enhanced HT-induced apoptosis in the two retinoblastoma cell lines.

6) PLK1-knockdown increased the percentages of G2/M and sub-G1 phases

significantly, under both the control and HT conditions in the two retinoblastoma cell lines. In the cell proliferation assay, the inhibition of viable cell proliferation was enhanced by the combination of PLK1-knockdown and HT.

7) PLK1-knockdown inhibited HT-induced activation of HSF1, and the inactivation of HSF1 reduced the expression of HSPs and BAG3 in the two retinoblastoma cell lines.

[Conclusions]

As shown results of 1)-4), the silencing BAG3 sensitized HT in HSC-3 and Y79 cells. As shown results of 5)-7), the combination of PLK1 inhibition and HT enhanced apoptosis and inhibited cell proliferation through the inactivation of HSF1 concomitant with reductions in HSPs and BAG3 in human retinoblastoma cells. These findings indicated that the disruption of functions of HSF1-related proteins such as BAG3 and PLK1 may serve as a potential therapeutic strategy for HT therapy in patients with cancer tumors.

## 2. Introduction

### 2.1 Hyperthemia (HT) and Heat shock proteins (HSPs)

Local hyperthermia (HT) has been used to treat patients with various cancers [Wust et al., 2002; Huilgol et al., 2010].The combination of HT with chemotherapy, radiotherapy, or both has been clinically used for patients with cancer in various organs, and the anti-tumor effects of these combinations have been verified by clinical trials [Van et al., 2000; Harima et al., 2001; Xia et al., 2006; Aktas et al., 2007; Van et al., 2010]. However, the cytoprotective effects caused, at least in part, by an increase in heat shock proteins (HSPs) in some cancer cells have rendered HT less effective [Parsell et al., 1993; Ohtsuka et al., 2000]. HSPs are highly conserved proteins whose expression is induced by various stresses, especially heat [Garrido et al., 2006]. HSPs have cytoprotective functions against various stresses and work as molecular chaperones. There are several families of HSPs, and the members of one of them, the Hsp70 family, play a central role as molecular chaperones.

### 2.2 Retinoblastoma and HT

Retinoblastoma is the most frequent intraocular malignancy of childhood [MacCarthy et al., 2006], and is an inheritable cancer caused by mutations or deletions in the *Rb1* gene [Classon et al., 2002]. Several therapeutic modalities have been employed to successfully manage most cases of retinoblastoma [Lin et al., 2009]. Chemotherapy coupled with

adjuvant focal treatments such as photocoagulation, cryopexy, and thermotherapy, forms the standard therapeutic regimen for the treatment retinoblastoma [Shields et al., 1996; Shields et al., 1997]. In addition, transpupillary thermotherapy by using an 810 nm diode laser is the most commonly prescribed focal treatment.

Several *in vitro* studies have demonstrated the efficacy of HT in the treatment of retinoblastoma [Inomata et al., 2002; Choi et al., 2003]. Clinically, transpupillary thermotherapy can successfully treat over 90% of small (less than 3.0 mm in diameter) tumors [Abramson et al., 2004; Shields et al., 1999]. However, tumors that are large in height or basal diameter are more difficult to control with HT therapy, and in such cases there is a high risk of HT complications, such as focal iris atrophy, peripheral focal lens opacity, retinal traction, retinal vascular obstruction, and transient localized serous retinal detachment [Shields et al., 1999]. Furthermore, it is considered that tumors with well-differentiated characteristics do not adequately respond to HT in retinoblastoma [Francis et al., 2013].

### 2.3 BAG3 (Bcl-2 associated athanogene 3)

BAG3 (Bcl-2 associated athanogene 3) is a family of co-chaperones that interact with the ATPase domain of Hsp70 through the BAG domain (110-124 amino acids) [Takayama et al., 1999; Rosati et al., 2007]. The induction of BAG3 is at least partly mediated by the activation of heat shock transcription factor 1 (HSF1) as in the cases of HSPs [Franceschelli et al., 2008]. Although the expression level of the BAG3 protein is low in

normal human cells, the level is increased in response to a wide variety of stresses, including oxidative stress [Bonelli et al., 2006], heavy metals [Pagliuca et al., 2003], heat stress [Liao et al., 2001], pulsed ultrasound [Tabuchi et al., 2007], retinal light damage [Chen et al., 2004] and HIV infection [Rosati et al., 2009]. BAG3 has been reported to be expressed in several tumors, such as pancreatic cancer [Liao et al., 2001], thyroid carcinoma [Chiappetta et al., 2007], prostate carcinoma [Staibano et al., 2010], kidney cancer [Wang et al., 2009], glioblastoma [Festa et al., 2011] and ovary cancer [Suzuki et al., 2011]. It has been demonstrated that over expression of BAG3 plays a role in survival, whereas inhibition of BAG3 expression improves the apoptotic response to drugs in cancer cells [Liu et al., 2009; Du et al., 2008; Wang et al., 2007; Zhu et al., 2012]. Previously, Liao et al. reported that BAG3 is more strongly induced after heat stress than after treatment with tumor necrosis factor-alpha in pancreatic cancer cells [Liao et al., 2001]. However, to our knowledge, there has been no report that BAG3 is involved in the HT sensitivity. In this study, we examined the effects of down-regulation of BAG3 on the sensitivity to HT in human oral squamous cell carcinoma (OSCC) cells and human retinoblastoma cells.

### 2.4 HT and c-Jun N-terminal kinase (JNK) pathway

Past reports have shown that HT-induced apoptosis was related to activation of the JNK pathway [Alcala et al., 2010; Hayashi et al., 2011; Gaitanaki et al., 2008; Gabai et al., 2000] and was suppressed by the JNK

inhibitor [Chung et al., 2003]. In contrast, several investigators have suggested that JNK pathway enhancement was associated with anti-apoptotic effects [Wang et al., 2009; Sloss et al., 2008]. We therefore considered that it would be of interest to examine the role of the JNK pathway in BAG3 silencing sensitized HT-induced apoptosis. We also investigated the effects of JNK inhibition in combination with BAG3-knockdown on HT in HSC-3 cells.

### 2.5 BAG3 and nuclear factor kappa B (NF-κB) activity

The major signaling pathways that govern cell proliferation and anti-apoptosis involve nuclear factor kappa B (NF-κB) protein in cancer cells [Chuma et al., 2014; Bassères et al., 2006; Wang et al., 1996]. BAG3 is known to sustain NF-κB activation by inhibiting the delivery of inhibitor of kappa B kinase γ (IKKγ) to the proteasome [Ammirante et al., 2010; Rosati et al., 2012]. NF-κB is crucial for cell viability and cell cycle progression in human retinoblastomas [Poulaki et al., 2002; Qu et al., 2011]. Previous studies demonstrate that HT induces proteasome inhibition, and thus inhibits the activity of NF-κB [Aravindan et al., 2009; Kokura et al., 2003; Mattson et al., 2004; Pajonk et al., 2005]. However, to our knowledge, the role of BAG3 in the HT sensitivity in retinoblastoma is unknown. In this study, we examined the effect of BAG3 on the HT sensitivity and investigated whether the NF-κB pathway is involved in the HT-induced expression of BAG3 in human retinoblastoma cells.

## 2.6 Polo-like kinase 1 (PLK1) and heat shock transcription factor 1 (HSF1)

The induction of HSPs is mainly mediated by the activation of HSF1, which binds to conserved regulatory sequences called heat shock elements (HSE) that are located in the promoter regions of HSP genes [Morimoto et al., 1998]. HSF1 largely localizes to the cytoplasm as an inactive monomer. Under stresses such as heat shock, HSF1 forms an active trimer and translocates from the cytoplasm to nucleus. This active HSF1 binds to the HSE of DNA, thereby activating the transcription of HSPs [Morimoto et al., 1998]. It has been reported that phosphorylation of HSF1 and HSF1 nuclear translocation are regulated by polo-like kinase 1 (PLK1) [Kim SA et al., 2005; Lee et al., 2008]. PLK1 is an important regulator of mitosis and plays a role in G2/M phase progression by regulating CDK1, cyclin B1, and cdc25C [Roshak et al., 2000; Schmit et al., 2009]. Overexpression of PLK1 has been reported in many cancer cells [He et al., 2009; Wang et al., 2012; Ito et al., 2004], and several studies have shown that the depletion of PLK1 using RNA interference inhibits cancer cell proliferation and induces apoptosis [Guan et al., 2005; Nogawa et al., 2005]. It has been demonstrated that PLK1 depletion using RNA interference or PLK1 inhibitor enhances the effects of chemotherapy and radiotherapy without affecting normal cells [Liu et al., 2006; Maire et al., 2013; Gerster et al., 2010]. In addition, a PLK1 inhibitor (BI-2536) has been used in a multi-center, multi-tumor phase II trial [Schöffski et al., 2010]. However, to our knowledge, there has been no report that PLK1 is involved in HT sensitivity, even though PLK1

plays a role in the activation of HSF1. In this study, we examined the effects of PLK1-knockdown (PLK1-targeted small interfering RNA) and PLK1 inhibitor (BI-2536) on sensitivity to HT in human retinoblastoma cells.

### 2.7 Purpose in this study

One of the problems with HT therapy is that cells acquire thermoresistance. In order to attenuate the thermoresistance of HT, we examined the inhibitory effects of HSF1-related proteins such as BAG3 and PLK1 on sensitivity to HT in human cancer cells.

## 3. Materials and Methods

### 3.1 Cell culture and heat treatment

A human OSCC HSC-3 cell line was obtained from the Human Science Research Bank, Japan Health Sciences Foundation (Tokyo, Japan) and human retinoblastoma Y79 and WERI-Rb-1 cell lines were obtained from the Riken Bioresource Center (Tsukuba, Japan). The cells were cultured in E-MEM medium or RPMI-1640 medium (Wako Pure Chemical Industrials, Ltd., Osaka, Japan) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) at 37˚C in humidified air with 5% $CO_2$. When the cells were treated with HT, the cell culture dishes were sealed by wrapping them in parafilm and then soaked in a water bath at 42˚C, 43˚C, or 44˚C for 60 or 90 min. The temperature was monitored with a digital thermometer during heating. After HT, the cells were incubated for 1-24 h at 37˚C.

### 3.2 Silencing of BAG3 using small interfering RNA (siRNA) for HSC-3 cells

Based on the human BAG3 nucleotide database, three siRNAs, designated as BAG3-1, BAG3-2, and BAG3-3, were synthesized (Table 1). Luciferase siRNA (CGUACGCGGAAUACUUCGA) was used as a negative control siRNA. Cells were incubated in Opti-MEM® I Reduced Serum Medium (Life Technologies Japan Ltd., Tokyo, Japan) containing 20 nM siRNA and Lipofectamine™ RNAiMAX (Life Technologies Japan Ltd.) at 37˚C. Six hours after the transfection, the medium was exchanged for

E-MEM medium supplemented with 10% FBS, and the cells were maintained at 37˚C for 42 h.

Table 1. Nucleotide sequences of siRNAs for BAG3.

| Name | Sequence-TT | Position | GC% |
|---|---|---|---|
| siBAG3-1 | AGAGGUGGAUUCUAAACCU | 1338 | 42 |
| siBAG3-2 | UGCAGAAGAUCCCCACACA | 1929 | 53 |
| siBAG3-3 | GGUGGAUUCUAAACCUGUU | 1341 | 42 |

Human BAG3 nucleotide database : GenBank accession number, NM_004281

### 3.3 Silencing of BAG3 or PLK1 using siRNA for Y79 and WERI-Rb-1 cells

The three BAG3 siRNAs and four PLK1 siRNAs used for PLK1-knockdown were designed by Nippon EGT Co., Ltd (Toyama, Japan). The sequences of the siRNAs are listed in Table1, 2. A lusiferase siRNA (CGUACGCGGAAUACUUCGA) was used as a negative control siRNA. Cells were incubated in Opti-MEM® I Reduced Serum Medium (Life Technologies Japan Ltd., Tokyo, Japan) containing 50 nM siRNA and Lipofectamine™ RNAiMAX (Life Technologies Japan Ltd.) at 37˚C. Twenty-four hours after transfection, the medium was exchanged for RPMI-1640 medium supplemented with 10% FBS, and the cells were maintained at 37˚C for 24 h.

Table 2. Nucleotide sequences of siRNAs for PLK1.

| Name | Sequence-TT | Position | GC% |
|---|---|---|---|
| siPLK1-1 | AGAUCACCCUCCUUAAAUA | 1477 | 37 |
| siPLK1-2 | UCACCCUCCUUAAAUAUUU | 1480 | 32 |
| siPLK1-3 | CACGCCUCAUCCUCUACAA | 1372 | 53 |
| siPLK1-4 | ACCGAGUUAUUCAUCGAGA | 562 | 42 |

Human PLK1 nucleotide database : GeneBank accession number, NM_005030

### 3.4 Compound treatment

A JNK inhibitor SP600125 (R&D Systems, Inc., Minneapolis, MN, USA) and PLK1 inhibitor BI-2536 (Selleck Chemicals, Houston, TX, USA) were dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium. After 1 h of compound treatment, cells were exposed to HT.

### 3.5 Western blotting

The cells were dissolved in a lysis buffer (150 mM NaCl, 1% Nonidet P-40 and 50 mM Tris-HCl, pH 8.0) containing protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). After electrophoresis on SDS-polyacrylamide gel, the proteins were transferred electrophoretically onto polyvinylidene fluoride membranes. The following primary antibodies were used: rabbit monoclonal anti-BAG3 (Funakoshi Co., Tokyo, Japan);

mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and PLK1 (Millipore Co., Temecula, CA, USA); rabbit polyclonal anti-cleaved caspase-3, caspase-3, HSF1, cdc25c, CDK1, c-Jun, JNK, and phospho-JNK (Cell signaling Technology Inc., Beverly, MA, USA); mouse monoclonal anti-HSP70 and HSP40 (MBL Co., Ltd. Nagoya, Japan); rabbit polyclonal anti-HSP27 antibody (MBL Co., Ltd.); rabbit polyclonal anti-IκBα and IKKγ (Cell signaling Technology Inc., Bevery, MA, USA); and rabbit monoclonal anti-phospho-IκBα, cyclinB1, and phospho-c-Jun (Cell signaling Technology Inc.); rabbit monoclonal anti-phospho-HSF1 (Gene Tex Inc., Irvine, CA, USA); mouse monoclonal anti-Histone H1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The immunoreactive proteins were visualized by a luminescent image analyzer (LAS 4000mini, GE Healthcare Co., Tokyo, Japan) using an enhanced chemiluminescence detection system. GAPDH served as the loading control. The nuclei and cytoplasm were separated by using a Nuclear / Cytosol Fractionation Kit (BioVision, Inc., Mountain View, CA, USA) according to the manufacturer's protocol [Mani et al., 2006]. The proteins in the nuclear and cytoplasmic fractions were used for western blotting.

### 3.6 RNA isolation

Total RNA was extracted from the cells using an RNeasy Total RNA Extraction kit (Qiagen K.K., Tokyo, Japan) and was treated with on-column DNase I (RNase-free DNase kit, Qiagen K.K., Tokyo, Japan ) [Tabuchi et al., 2013].

### 3.7 Real-time quantitative polymerase chain reaction (qPCR) assay

Real-time qPCR was performed on a Real-time PCR system Mx3000P (Agilent Technologies, Inc., Santa Clara, CA, USA) using a SYBR PreMix ExTaq kit (Takara Bio Inc., Shiga, Japan). The relevant primer sequences are listed in Table 3. The mRNA expression level of the proteins was normalized to that of GAPDH [Tabuchi et al., 2013].

Table 3. Nucleotide sequences of primers and a probe for target genes

| Genes | Orientation | Nucleotide sequence (5'→3') | GeneBank accession no. |
|---|---|---|---|
| PLK1 | Sense<br>Antisense | CGAGGACAACGACTTCGTGT<br>GGTTGCCAGTCCAAAATCCC | NM_005030 |
| HSF1 | Sense<br>Antisense | GGGAACAGCTTCCACGTGTT<br>TGGAACTCCGTGTCGTCTCT | NM_005526 |
| BAG3 | Sense<br>Antisense | CGACCAGGCTACATTCCCAT<br>TCTGGCTGAGTGGTTTCTGG | NM_004281 |
| IKKγ | Sense<br>Antisense | GTCCCCTCTTTTGGGGTAGA<br>CAAGTGGTTCGAGCAGACAG | NM_001099856 |
| GAPDH | Sense<br>Antisense | AAGGACTCATGACCACAGTCCAT<br>CCATCACGCCACAGTTTCC | NM_002046 |

### 3.8 Cell cycle analysis

Cells were exposed to HT, and then cultured at 37˚C. After 24 h of culturing, the cells were fixed in 70% ice cold ethanol for at least 24 h at -20˚C and subsequently treated with 0.25 mg/ml RNase A. After the staining with propidium iodide (PI) at a concentration of 0.05 mg/ml, the samples were finally run on an Epics XL flow cytometer (Beckman Counter,

Fullerton, CA, USA) [Furusawa et al., 2012].

### 3.9 Analysis of cell death

Cells were exposed to HT, and then cultured at 37˚C. After 12 h of culturing, the cells were harvested to examine cell death. We performed chromatin condensation analysis using a Nuclear-ID Green Chromatin Condensation Kit (Enzo Life Sciences Inc., Farmingdale, NY, USA) according to the manufacturer's protocol [Park et al., 2011]. The samples were run on an Epics XL flow cytometer.

### 3.10 Cell viability and cell proliferation analysis by WST-8 assay

Cells were treated with HT and then incubated for 24 h at 37 °C. After incubation, the cells were seeded with a volume of 100μL onto 96-well-plates. The Cell Count Reagent SF (Nacalai Tesque, Inc., Kyoto, Japan), a water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]-based assay was used to analyze cell proliferation. In brief, 10 μL of the reagent solution was added to each well, followed by incubation at 37°C for 3 h. The concentration of the formazan dye produced was determined from the absorbance at 450 nm by using a microplate reader (Bio-Rad, Hercules, CA, USA).

### 3.11 Immunocytochemistry

Cells were washed with PBS and fixed in methanol for 10 min at room

temperature. Then, the cells were seeded in an eight-well chamber slide, and incubated with Blocking One Histo™ (Nacalai Tesque, Inc.) for 1 h at room temperature. Next, cells were incubated with the first antibody against HSF1 (Cell signaling Technology Inc.) for 24 h at 4˚C and treated with the Chromeo™ 488-labeled secondary antibody (Active Motif, Carlsbad, CA, USA) for 1 h at 25˚C. Finally, cells were stained with 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Invitrogen™, Eugene, OR, USA) for 5 min for nuclear visualization and thoroughly washed before observation under a fluorescent microscope (Olympus BX-61 microscopic image analyzer; Olympus Co., Ltd., Tokyo, Japan).

### 3.12 Statistical analysis

Data are represented as mean ± S.D. Student's *t*-test was used for statistical analysis and $p < 0.05$ was considered to be statistically significant.

# 4. Results

## 4.1 Effects of HT on apoptosis in human OSCC HSC-3 cells

At first, we examined the effects of HT on apoptosis in HSC-3 cells using chromatin condensation, a marker for apoptosis. When HSC-3 cells were incubated at different temperatures for 90 min, apoptosis was not observed at 41 and 42˚C, but significantly increased at 43 and 44˚C. The percentage of apoptosis at 44˚C (mean ± SD; 14.5 ± 0.4%) was greater than that at 43˚C (6.4 ± 1.8%) (Figure 1). Therefore, we selected the HT at 44˚C for 90 min for use in the following experiments.

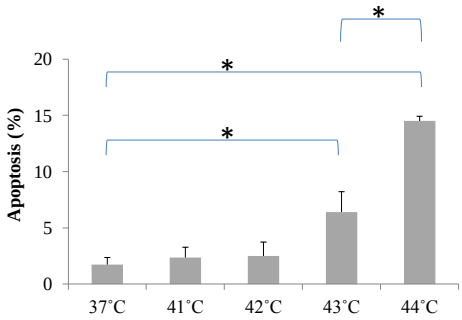


**Figure 1.** Effects of HT on apoptosis in human OSCC HSC-3 cells. The cells were incubated at different temperatures for 90 min, and then cultured at 37˚C for 12 h. The apoptosis was monitored using chromatin condensation. Data are presented as means ± SDs (n = 4). *: $p<0.05$ (Student's t-test).

## 4.2 HT-induced apoptosis in retinoblastoma cells under various temperature conditions

Next, we examined the effects of HT on apoptosis in Y79 and WERI-Rb-1 cells in order to determine the appropriate HT conditions for the induction of apoptosis. When we monitored apoptosis using chromatin condensation as an apoptotic marker, the percentages of apoptotic cells were 4.72 ± 1.1% in the control Y79 cells and 4.98 ± 1.1% in the control WERI-Rb-1 cells. When Y79 and WERI-Rb-1 cells were incubated at 42˚C for 60 min, apoptosis did not increase significantly during the recovery periods after HT, but it did increase significantly during the recovery following 60-min incubation at 43˚C or 44˚C (Figure 2). The percentages of apoptotic cells at 43˚C in the Y79 cells were 6.2 ± 1.6% at 6 h after HT, 7.7 ± 0.8% at 12 h after HT ($p<0.05$), and 7.1 ± 0.9% at 24 h after HT ($p<0.05$); in the WERI-Rb-1 cells, they were 6.4 ± 0.7% at 6 h after HT, 7.6 ± 0.5% at 12 h after HT ($p<0.05$), and 7.6 ± 0.8% at 24 h after HT ($p<0.05$). The percentages of apoptotic cells at 44˚C in the Y79 cells were 8.2 ± 1.1% at 6 h after HT ($p<0.05$), 11.3 ± 0.9% at 12 h after HT ($p<0.05$), and 10.1 ± 2.2% at 24 h after HT ($p<0.05$); in the WERI-Rb-1 cells, they were 8.3 ± 1.2% at 6 h after HT ($p<0.05$), 13.0 ± 1.3% at 12 h after HT ($p<0.05$), and 11.5 ± 0.9% at 24 h after HT ($p<0.05$). The percentages of apoptosis at 44˚C were thus greater than those at 43˚C. Furthermore, the increase in apoptosis at 44˚C reached a plateau at 12 h after HT. Therefore, we concluded that the conditions most appropriate for the evaluation of HT-induced apoptosis in Y79 and WERI-Rb-1 cells were the observation at 12 h after HT at 44˚C for 60 min.

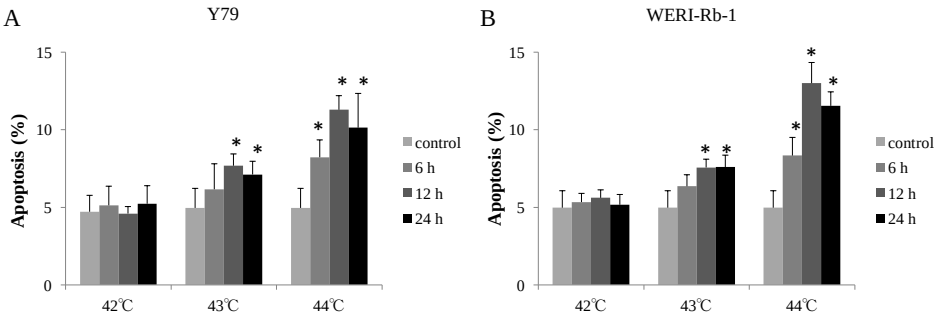


**Figure 2.** Effects of HT on apoptosis in human retinoblastoma Y79 (A) and WERI-Rb-1 (B) cells. The cells were incubated at different temperatures for 60 min and then cultured at 37˚C for 6-24 h. The apoptosis was monitored using chromatin condensation. Data are presented as means ± SDs (n = 4). *: $p<0.05$ vs. control (Student's *t*-test).

## 4.3 Induction of BAG3 by HT and BAG3-knockdown using siRNA in HSC-3 cells

We performed western blotting to monitor the expression level of BAG3 in HSC-3 cells. Although the expression level of BAG3 was low under the non-stress conditions, it significantly increased in HSC-3 cells treated with HT at 44˚C for 90 min (Figure 3A). The treatment of the cells with siRNA for BAG3 almost completely suppressed the expression of BAG3 under either the non-HT- or HT-induced condition. Since the efficacy of silencing of this protein by each of three BAG3 siRNAs (siBAG3-1, -2 and -3) was similar, we chose the siBAG3-3 siRNA for use in the subsequent experiments. In

addition, the expression level of BAG3 was time-dependently increased, with the peak expression being observed from 6 to 12 h after the HT treatment. In contrast, the expression of BAG3 was very low during the course of recovery time periods after HT in HSC-3 cells transfected with the siBAG3-3 siRNA (Figure 3B).

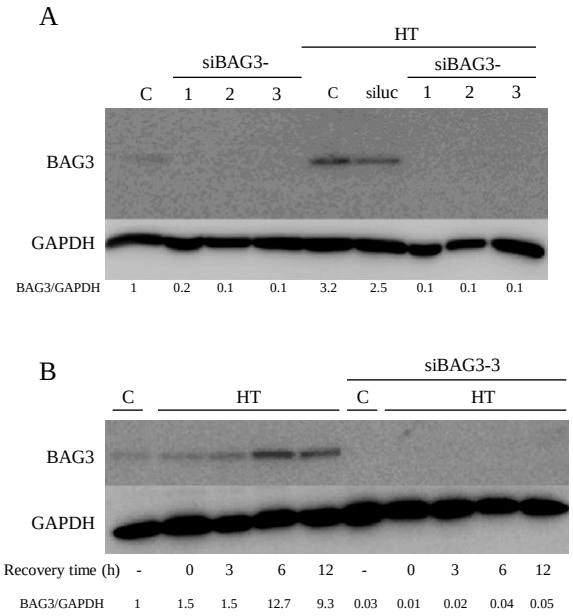


**Figure 3.** Induction of BAG3 by HT and knockdown of BAG3 using siRNAs in HSC-3 cells**.** The cells transfected with each siRNA (siBAG3-1 to -3; 20 nM) were maintained at 37˚C. Forty-eight hours after transfection, cells were treated with HT at 44˚C for 90 min. (A) Inhibition of BAG3 by BAG3 siRNAs. Six hours after HT, cells were harvested. (B) Time-course of change in the expression of BAG3 in the cells treated with a siBAG3-3. After HT exposure, the cells were cultured at 37˚C for 0, 3, 6 and 12 h. Western blotting was performed using a specific primary antibody for BAG3 or GAPDH. GAPDH served as a loading control. Bands were quantified densitometrically and normalized to GAPDH. C, control (non-treatment). siluc, siRNA (20 nM) for luciferase.

### 4.4 HT-induced BAG3 expression and BAG3-knockdown using siRNA in Y79 cells

We examined the expression of BAG3 in the human retinoblastoma Y79 cells by western blotting. Although the expression level of BAG3 was very low under normal conditions, it was induced significantly by HT at 44°C for 60 min. The transfection of the cells with each of the 3 BAG3 siRNAs (siBAG3-1, siBAG3-2, or siBAG3-3) almost completely suppressed the HT-induced BAG3 expression at both 50 nM and 100 nM siRNA concentrations. We selected the siBAG3-3 siRNA for transfection at a concentration of 50 nM in the subsequent experiments (Figure 4A). Additionally, effective knockdown of the mRNA level of BAG3 by siBAG3-3 was confirmed using real-time qPCR (Figure 4B). Next, the time-dependent protein expression of BAG3 and HSPs such as HSP70 and HSP40 was analyzed after HT. The expression of BAG3 was found to increase gradually with time for up to 12 h after HT. Similarly, the expression of HSP70 and HSP40 increased gradually after HT treatment, regardless of the BAG3-knockdown (Figure 4C).

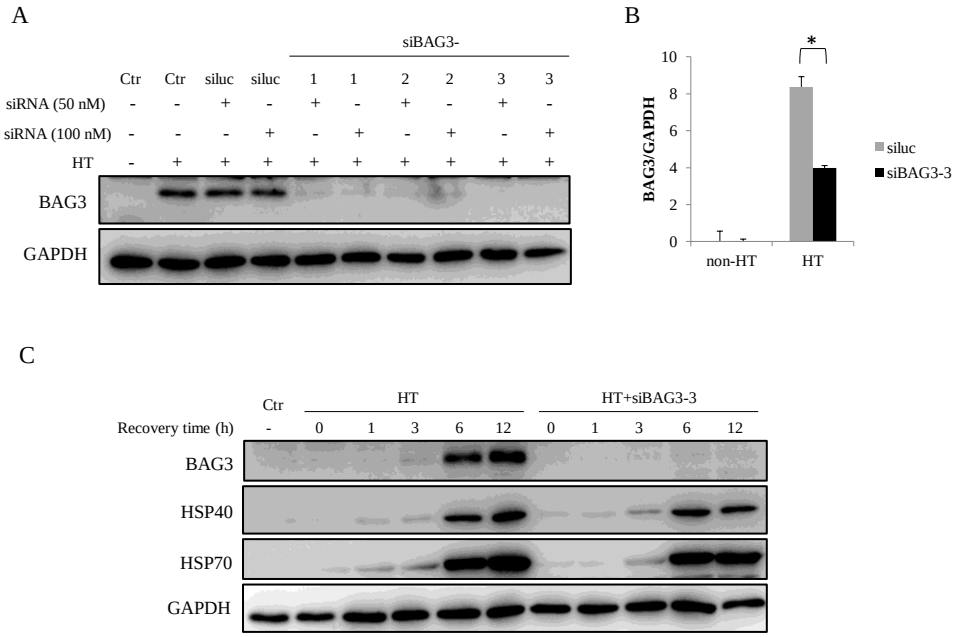


**Figure 4. BAG3-knockdown using siRNA and HT-induced BAG3 expression in Y79 cells.** Y79 cells were transfected with 50 or 100 nM siRNA (siBAG3-1, siBAG3-2, or siBAG3-3) and cultured at 37°C for 48 h. After HT treatment (44°C, 60 min), the cells were incubated at 37°C. Six hours after HT, the cells were harvested for (A) western blotting and (B) real-time quantitative PCR. The cells were transfected with 50 nM siBAG3-3. The mRNA expression level of BAG3 was normalized to that of GAPDH. Date are presented as mean ± S.D. (n=4) * $p<0.05$ (Student's *t*-test) (C) Time-course changes in the protein expression of BAG3, HSP70, and HSP40. Y79 cells were transfected with 50 nM siBAG3-3 and harvested at 0, 1, 3, 6, and 12 h after HT. Western blotting was performed and GAPDH was used as the loading control. Ctr: control (non-treatment). siluc: siRNA for luciferase.

## 4.5 HT-induced cell death and its enhancement by the silencing of BAG3 in HSC-3 cells

When we monitored the cell death using PI staining, the percentage of cell death was very low, i.e., 1.7 ± 0.6% (mean ± SD), in the control cells transfected with the siRNA for luciferase. On the other hand, HT at 44˚C for 90 min significantly elevated the cell death to 17.6 ± 3.9% (Figure 5A). Although silencing of BAG3 did not affect cell death under the non-stress

conditions, a significant increase in cell death (37.7 ± 6.5%) was detected in the cells treated with HT (Figure 5A).

We next studied whether BAG3-knockdown induced apoptosis in HSC-3 cells under the control and HT conditions. The results are shown in Fig. 5B-F. When apoptosis was monitored by chromatin condensation, the percentage of apoptosis was 1.4 ± 0.6% in the control cells. Treatment of the cells with HT increased the percentage of apoptosis to 14.5 ± 0.4%. Under HT-treated cells, knockdown of BAG3 by siBAG3-3 effectively enhanced the HT-induced apoptosis (46.7 ± 4.8%) (Figure 5B). The raw data for the chromatin condensation are shown in Figure 5C. A similar enhancement of apoptosis was detected when HSC-3 cells were transfected with siRNA3-1 or siRNA3-2 (Figure 5D).

The sub-G1 phase of the cell cycle, a marker for apoptosis, was studied using a flow cytometer. In control cells, the percentage of the sub-G1 phase was 1.8 ± 0.5%, whereas it was 6.4 ± 1.4% in the cells treated with HT. Under HT-treated cells, BAG3 silencing significantly increased the percentage of cells in the sub-G1 phase (12.4 ± 2.9%) (Figure 5E). We further performed western blotting to confirm the induction of apoptosis detected by cleavage of caspase-3. Caspase-3 is a critical executioner of apoptosis, and proteolytic processing is required to generate the 17- and 19-kDa activated cleavages from its inactive 35-kDa full-length precursor during apoptosis [Nicholson et al., 1996]. Although the expression levels of the 17- and 19-kDa activated fragments of caspase-3 were very low in the control cells, significant elevations of these fragments were observed in HT-exposed cells, and they

were further enhanced by knockdown of BAG3 (Figure 5F).

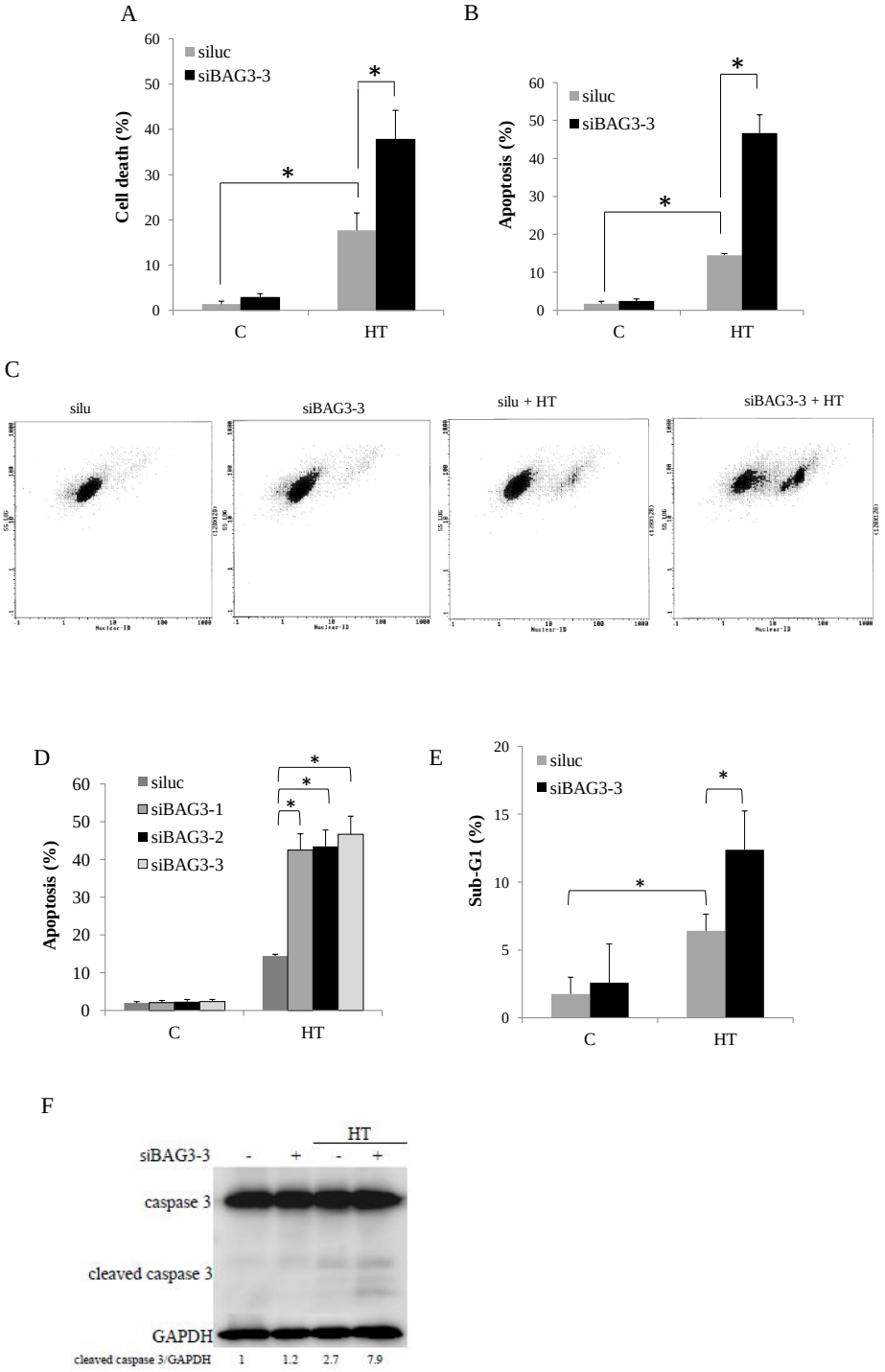


**Figure 5.** HT-induced cell death and its enhancement by the silencing of BAG3 in HSC-3 cells. The cells transfected with siBAG3-3 (20 nM) were maintained at 37°C. Forty-eight hours after transfection, cells were treated with HT at

44˚C for 90 min, and then cultured at 37˚C for 12 h. The cell death was monitored using various indexes, such as PI staining (A), chromatin condensation (B), the raw data for the chromatin condensation (C), similar apoptotic effects of knockdown of BAG3 using siRNAs (siBAG3-1 to -3; 20 nM) (D), and the sub-G1 phase in the cell cycle (E). Analyses were performed using flow cytometry. Data are presented as means ± SDs (n = 4-8). C, control (non-treatment). siluc, siRNA for luciferase (20 nM). *: $p<0.05$ (Student's *t*-test). (F) Western blotting was performed using a specific primary antibody for caspase-3 or GAPDH. GAPDH served as a loading control. Bands were quantified densitometrically and normalized to GAPDH.

## 4.6 HT-induced apoptosis and its enhancement by BAG3-knockdown in Y79 cells

Next, we examined whether BAG3-knockdown induced apoptosis in the Y79 cells under non-HT and HT conditions. The Nuclear-ID Green Chromatin Condensation Kit was used to monitor chromatin condensation as an indicator of apoptosis. As shown in Figure 6A, under non-HT conditions, the percentage of apoptotic cells treated with siRNA for luciferase or BAG3 was 5.3% ± 0.6% or 6.0% ± 0.5%, respectively, indicating that the BAG3-knockdown did not affect apoptosis (n=5, p=0.09). On the other hand, under HT conditions, BAG3-knockdown significantly enhanced the HT-induced apoptosis; the percentage of apoptotic cells treated with siRNA for luciferase or BAG3 was 12.7% ± 1.0% or 22.6% ± 2.4%, respectively (n=5, p=0.0003). We further examined the activation of caspase-3, an indicator of apoptosis, using an anti-cleaved caspase-3 antibody by western blotting and found that caspase-3 is cleaved under conditions of HT and is enhanced by BAG3-knockdown (Figure 6B).

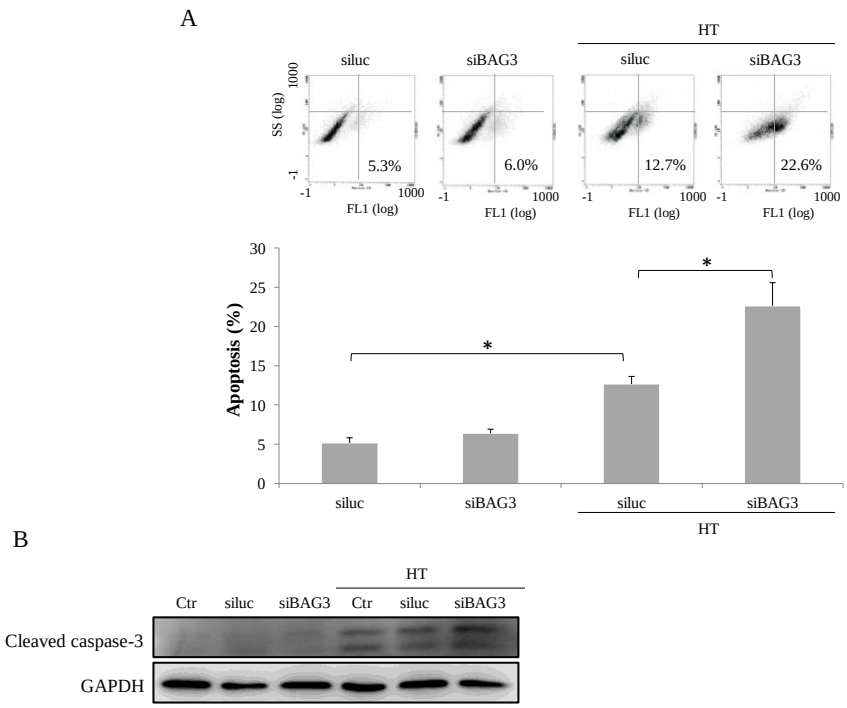


**Figure 6. HT-induced apoptosis is enhanced by BAG3-knockdown in Y79 cells.** Y79 cells transfected with 50 nM siBAG3 were subjected to HT at 44°C for 60 min, and then incubated at 37°C for 12 h. (A) Apoptosis was analyzed by flow cytometry. Date are presented as mean ± S.D. (n=5) *$p$<0.05 (Student's $t$-test) (B) Activation of caspase-3 was monitored by western blotting using an anti-cleaved-caspase-3 antibody. GAPDH was used as the loading control. Ctr: control (non-treatment). siluc: siRNA for luciferase.

## 4.7 The effect of BAG3-knockdown on NF-κB activity in HT-exposed Y79 cells

To examine the effect of BAG3-knockdown on the activity of NF-κB in HT-exposed cells, western blotting was performed. We first examined the phosphorylation level of p65, a subunit of NF-κB, and IκBα. As shown in Figure 7A and B, under normal conditions, p65 and IκBα were highly phosphorylated in Y79 cells, whereas the phosphorylation levels were decreased immediately after HT treatment. This attenuation of the phosphorylation of p65 and IκBα gradually recovered in a time-dependent

manner 12 h after HT. BAG3-knockdown inhibited the recovery of phosphorylation of p65 and IκBα after HT. Next, we evaluated the expression level of IKKγ protein in the cells treated with siBAG3 and HT. BAG3-knockdown reduced the expression of IKKγ. However, when the cells were pre-treated with MG132, a proteasome inhibitor, the expression of IKKγ remained unchanged in the cells treated with siBAG3 and HT (Figure 7C). In this experiment, we chose a concentration of 10 μM of MG132 based on previous reports that demonstrated effective inhibition of IKKγ degradation by this compound [Chuma et al., 2014; Ammirante et al., 2010]. On the other hand, BAG3-knockdown did not change the mRNA levels of IKKγ (data not shown).

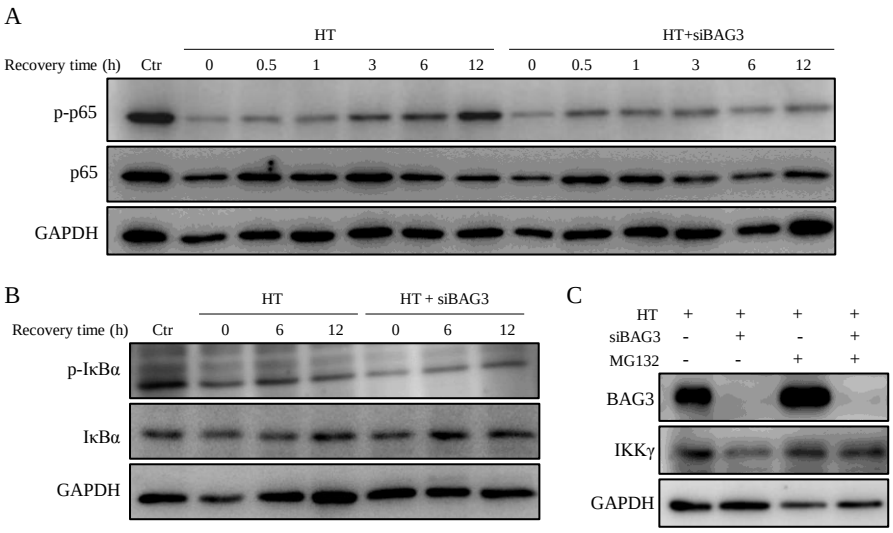


**Figure 7. The effect of BAG3-knockdown on NF-κB activity in HT-exposed Y79 cells.** Y79 cells transfected with 50 nM siBAG3 were treated with HT at 44°C for 60 min, and then incubated at 37°C for 0–12 h. Western blotting was performed. (A, B) Time-course changes in the protein expression of p65, p-p65, IκBα, and p-IκBα. (C) The cells were treated with 10 μM MG132 for 1 h and then subjected to HT. After 12 h, the cells were harvested. GAPDH was used

as the loading control. Ctr: control (non-treatment). siluc: siRNA for luciferase.

## 4.8 The effects of BAG3 silencing on the activation of the JNK pathway due to HT in HSC-3 cells

The effects of BAG3 silencing on the activation of the JNK pathway due to HT were examined using western blotting. The expression levels of JNK and c-Jun proteins were almost constant in any treatment tested. On the other hand, the phosphorylation level of either JNK (p-JNK) or c-Jun (p-c-Jun) was increased in HT-treated cells, and silencing BAG3 further increased each HT-induced elevation of the phosphorylation level (Figure 8A). These HT-activated phosphorylations were inhibited effectively by the treatment of cells with the JNK inhibitor SP600125 at a concentration of 20 μM (Figure 8B). Previous reports have indicated that the phosphorylation level of JNK is markedly inhibited in the cells treated with this compound (20 μM) [Wang et al., 2009; Lu et al., 2010]. Based on these facts, we chose a compound concentration of 20 μM to be used in the present study.

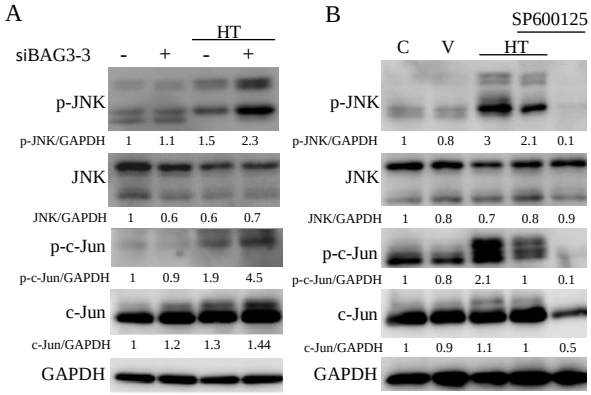


**Figure 8.** Effects of BAG3 silencing on activation of the JNK pathway due to HT in HSC-3 cells. (A and B) The cells transfected with siBAG3-3 (20 nM) were maintained at 37˚C. Forty-eight hours after transfection, cells were treated with HT at 44˚C for 90 min, and then cultured at 37˚C for 12 h. Western blotting was performed using specific primary antibodies, such as JNK, p-JNK, c-Jun, p-c-Jun and GAPDH. (B) The cells were pre-treated with vehicle (V; 1% DMSO) or SP600125 at a concentration of 20 μM for 1 h, and then exposed to HT. GAPDH served as a loading control. Bands were quantified densitometrically and normalized to GAPDH. C, control (non-treatment).

## 4.9 The effects of SP600125 on apoptosis induced by the combination of BAG3 silencing with HT in HSC-3 cells

To examine the effects of SP600125 on BAG3 silencing plus HT-induced apoptosis, a chromatin condensation assay was performed using a flow cytometer. The results are shown in Figure 9A. In both the control and HT-treated cells, the JNK inhibitor SP600125 did not affect apoptosis. In contrast, the inhibitor significantly elevated the level of apoptosis in the cells treated with the combination of BAG3 silencing and HT. The raw data are

also shown in Figure 9B.

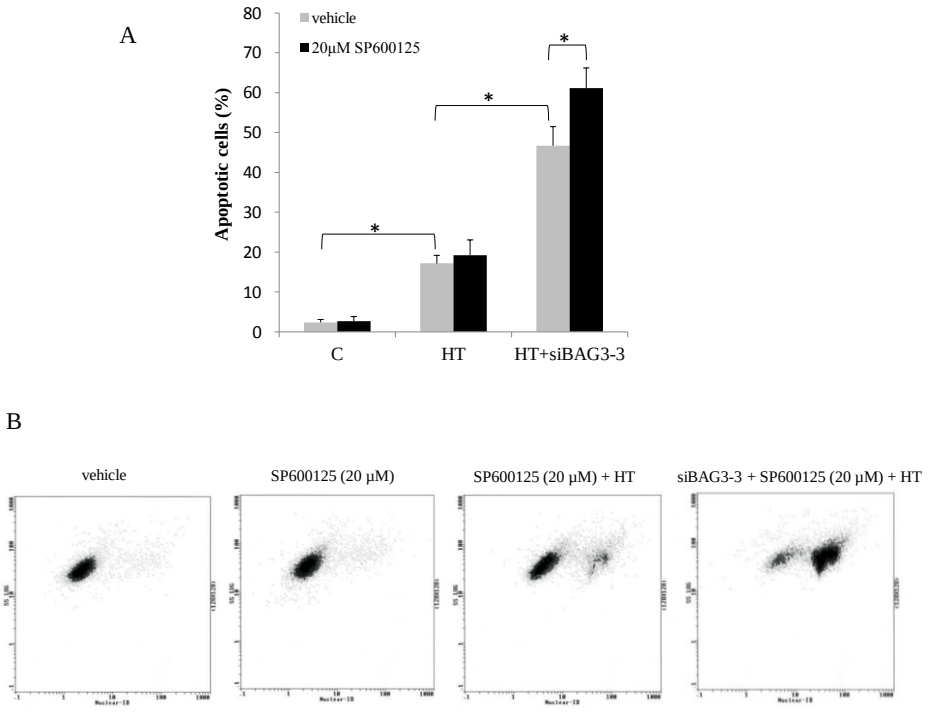


**Figure 9.** Effects of the JNK pathway the on the apoptosis induced by the combination of BAG3 silencing with HT in HSC-3 cells. The cells transfected with siBAG3-3 (20 nM) were maintained at 37˚C. Forty-seven hours after transfection, cells were treated with vehicle (1% DMSO) or SP600125 (20 μM) for 1 h, and then exposed to HT at 44˚C for 90 min. Twelve hours after HT, a chromatin condensation assay was performed using a flow cytometer (A). The raw data for the chromatin condensation (B). Data are presented as means ± SDs (n = 4). C, control (non-treatment). *: $p<0.05$ (Student's *t*-test).

## 4.10 PLK1-knockdown using siRNA in Y79 and WERI-Rb-1 cells

We performed western blotting to select the most effective siRNA for PLK1. Forty-eight hours after siRNA transfection, siPLK1-1 markedly decreased the protein level of PLK1 in Y79 and WERI-Rb-1 cells (Figure 10A and B). On the other hand, the siRNA for luciferase, a negative control, did not affect PLK1 expression. Therefore, we chose the siPLK1-1 siRNA for use in the subsequent experiments. In addition, an effective knockdown of the

PLK1 mRNA level was verified using the real-time qPCR assay using siPLK1-1 (Figure 10C and D). We next examined whether the PLK1-knockdown would affect the expression levels of CDK1, cyclinB1, and cdc25c, which are required for cell cycle progression and are known downstream from PLK1 [Roshak et al., 2000; Schmit et al., 2009]. PLK1-knockdown induced the accumulation of CDK1, cyclinB1, and cdc25c. A similar protein expression pattern was found for the downstream targets of PLK1, such as CDK1, cyclinB1, and cdc25c (Figure 10E and F) .These results suggest that CDK1, cyclinB1, and cdc25c are targets of PLK1 and that PLK1-knockdown affects PLK1 downstream proteins in retinoblastoma cells, as reported previously [Gerster et al., 2010; Grinshtein et al., 2011].

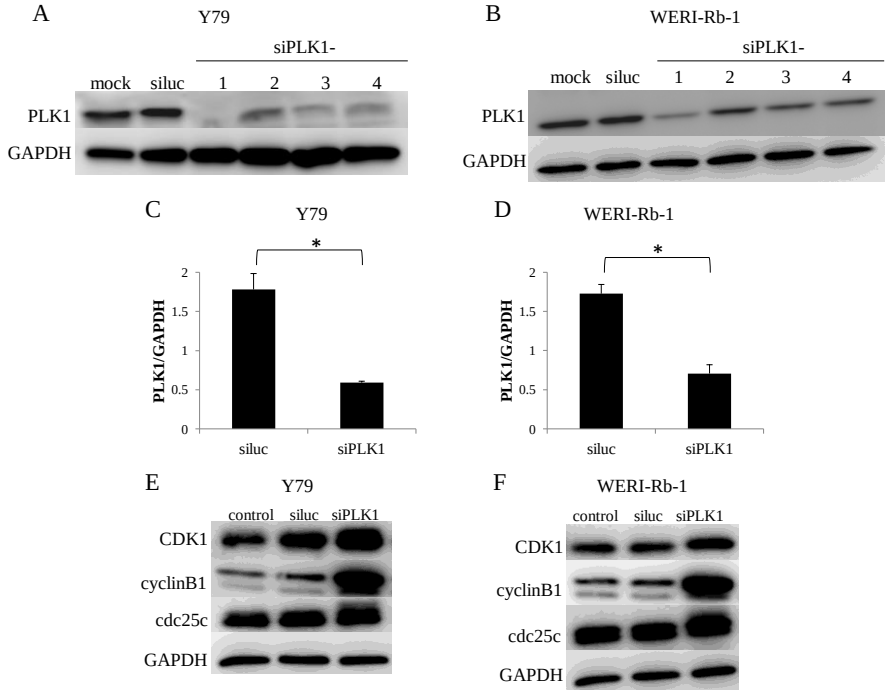


**Figure 10.** PLK1-knockdown using siRNAs in Y79 (A) and WERI-Rb-1 (B) cells**.** Cells transfected with each siRNA (siPLK1-1 to -4; 50 nM) were maintained at 37˚C. Forty-eight hours after transfection, the cells were harvested for western blotting. Western blotting was performed using a specific primary antibody for PLK1 or GAPDH. GAPDH served as a loading control. The sequences of the four siRNAs are listed in Table 2. mock, siRNA transfection

reagent only. siluc, siRNA for luciferase (50 nM). (C and D) The effects of siRNA for PLK1 (siPLK1-1) on the mRNA expression level of PLK1 in Y79 and WERI-Rb-1 cells. Real-time quantitative PCR assay was performed with specific primers for PLK1 or GAPDH. The PLK1 mRNA level was normalized to the GAPDH expression level. Date are presented as mean ± SDs (n = 4). *: $p<0.05$ vs. siluc (control) (Student's *t*-test). siluc, siRNA for luciferase. (E and F) PLK1-knockdown affects the expression of CDK1, cyclinB1, and cdc25c, which are known downstream of PLK1. Western blotting was performed using specific primary antibodies for CDK1, cyclinB1, cdc25c, and GAPDH. GAPDH served as a loading control. siluc, siRNA for luciferase (50 nM).

### 4.11 Knockdown of PLK1-induced apoptosis and its enhancement by HT in Y79 and WERI-Rb-1 cells

We next examined whether PLK1-knockdown would induce apoptosis in Y79 and WERI-Rb-1 cells under the control and HT conditions. The results are shown in Figure 11. When apoptosis was monitored by chromatin condensation, the percentages of apoptosis were 4.96 ± 1.26% in the Y79 control cells transfected with the siRNA for luciferase and 5.97 ± 1.08% in the WERI-Rb-1 control cells transfected with the siRNA for luciferase. Treatment of the cells transfected with siPLK1 significantly increased the percentage of apoptosis to 17.20 ± 1.67% in the Y79 cells and to 12.90 ± 0.94% in the WERI-Rb-1 cells. Under HT-treated cells, the percentage of apoptosis was 11.01 ± 1.10% in the Y79 cells and 12.48 ± 1.23% in the WERI-Rb-1 cells, and PLK1-knockdown enhanced HT-induced apoptosis (27.03 ± 2.05% in the Y79 cells and 21.73 ± 1.96% in the WERI-Rb-1 cells) (Figure 11A and B). We further performed western blotting to confirm the induction of apoptosis detected by the cleavage of caspase-3 in the two retinoblastoma cell lines. Although the expression levels of the 17- and 19-kDa activated fragments of caspase-3 were very low in the control cells, significant elevations of these fragments were observed in the knockdown of PLK1 cells. Furthermore, the

combination of PLK1-knockdown and HT enhanced these fragments of caspase-3 (Figure 11C and D).

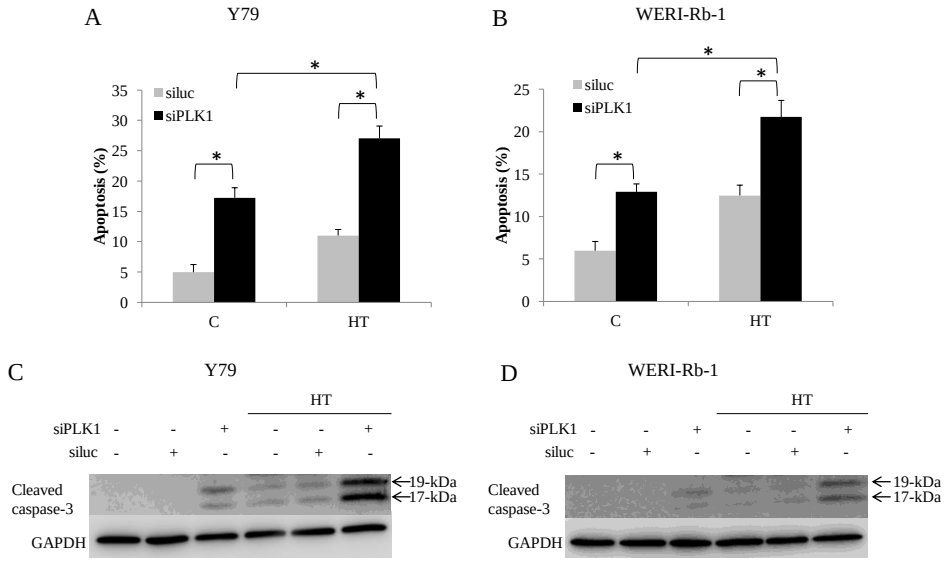


**Figure 11.** PLK1-knockdown induced apoptosis, and this effect was enhanced by HT in retinoblastoma cells. Cells transfected with siPLK1 (50 nM) were maintained at 37˚C. Forty-eight hours after transfection, the cells were treated with HT at 44˚C for 60 min, and then cultured at 37˚C for 12 h. The apoptosis was monitored using chromatin condensation (A and B). Analyses were performed using flow cytometry. Data are presented as means ± SDs (n = 4-6). C, control (non-treatment). siluc, siRNA for luciferase (50 nM). *: $p<0.05$ (Student's *t*-test). (C and D) Western blotting was performed using a specific primary antibody for cleaved caspase-3 or GAPDH. GAPDH served as a loading control.

## 4.12 PLK1 inhibition using BI-2536 decreased cell viability in a dose-dependent manner in Y79 and WERI-Rb-1 cells. BI-2536 induced apoptosis, and its effects were enhanced by HT.

We next analyzed the effects of BI-2536, a PLK1 inhibitor, in the two retinoblastoma cell lines. The cells were treated with BI-2536 at various concentrations, and cell survival was determined using the WST-8 assay.

BI-2536 treatment decreased the viability of Y79 and WERI-Rb-1 cells after 12 h and 24 h of treatment in a dose-dependent manner (Figure 12A and B). Furthermore, when apoptosis was monitored by chromatin condensation, BI-2536 (10 nmol/L)-induced apoptosis was enhanced by HT in the two retinoblastoma cell lines, as was siRNA-mediated PLK1 inhibition (Figure 12C and D).

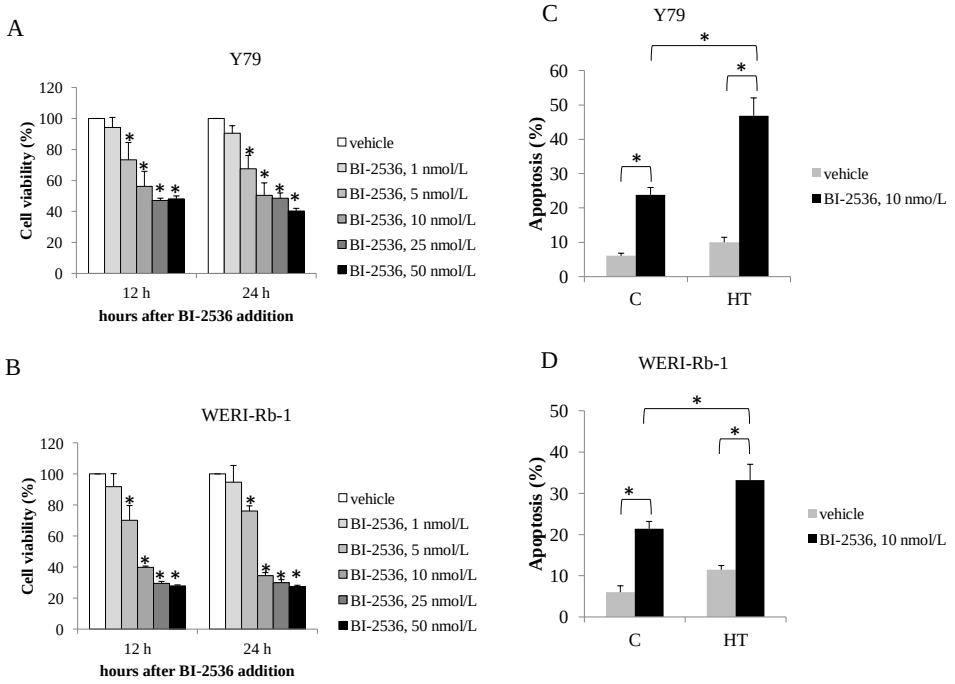


**Figure 12.** PLK1 inhibition using BI-2536 decreased cell viability in retinoblastoma cells. BI-2536 induced apoptosis, and its effect was enhanced by HT. (A and B) For the examination of cell viability, the Y79 and WERI-Rb-1 cells were inoculated at a density of $20 \times 10^4$ cells/ml and treated with various concentrations of a PLK1 inhibitor, BI-2536. After incubation for 12 h or 24 h, cell viability was measured by the WST-8 assay. Data are presented as means ± SDs (n = 4), *: $p<0.05$ vs. vehicle (DMSO only) (Student's *t*-test). (C and D) The Y79 and WERI-Rb-1 cells transfected with siPLK1 (50 nM) were maintained at 37˚C. Forty-eight hours after transfection, cells were treated with vehicle (DMSO only) or BI-2536 (10 nmol/L) for 1 h, and then exposed to HT at 44˚C for 60 min. Twelve hours after HT, a chromatin condensation assay was performed using a flow cytometer. Data are presented as means ± SDs (n = 4). C, control (non-treatment). *: $p<0.05$ (Student's *t*-test).

### 4.13 Effects of PLK1-knockdown on cell cycle analysis and cell proliferation, and its combined effects with HT in Y79 and WERI-Rb-1 cells.

We next examined whether the silencing of PLK1 would affect cell proliferation in Y79 and WERI-Rb-1 cells under the control and HT conditions. The WST-8 assay was used to count the viable cells in the cell proliferation assay. The results are shown in Figure 13A and B. PLK1-knockdown inhibited the viable cell proliferation of Y79 and WERI-Rb-1 cells compared with the control cells. Furthermore, the inhibition of viable cell proliferation was enhanced by the combination of PLK1-knockdown and HT. We next performed cell cycle analysis using flow cytometry. The results are shown in Figure 13C-H. Treatment of the cells transfected with siPLK1 increased the percentages of G2/M and sub-G1 phases significantly, under both the control and HT conditions in the two retinoblastoma cell lines. Although the combination of PLK1-knockdown and HT did not enhance G2/M arrest in comparison with the PLK1-knockdown alone, it did significantly increase the percentage of cells in the sub-G1 phase, a marker for apoptosis in the two retinoblastoma cell lines.

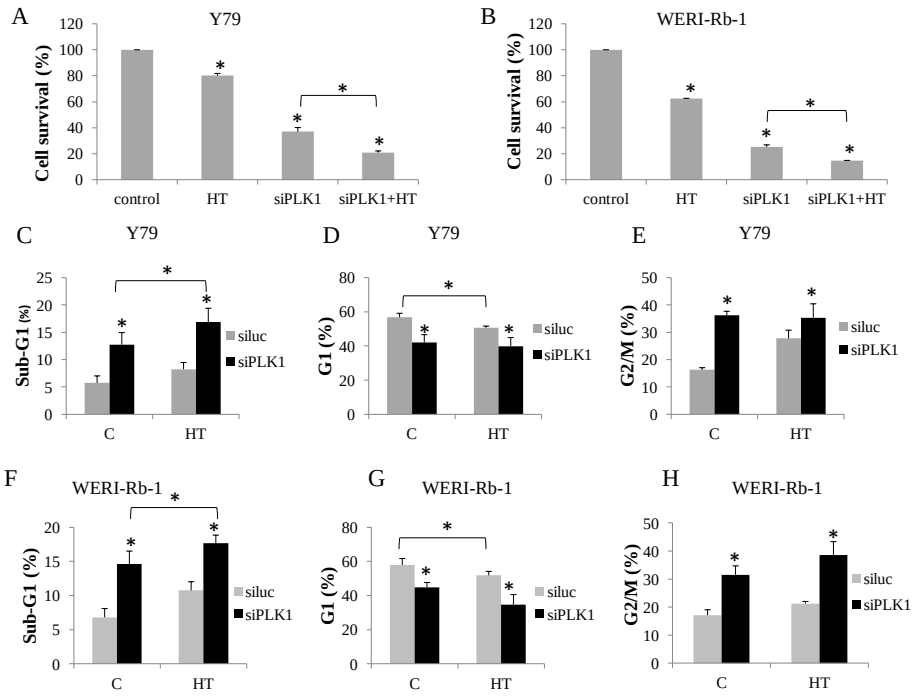


**Figure 13.** Effects of PLK1-knockdown on cell proliferation and cell cycle analysis, and the combined effects of PLK1 knockdown and HT in retinoblastoma cells. The Y79 and WERI-Rb-1 cells were transfected with siRNA for PLK1 (50 nM). Forty-eight hours after transfection, cells were treated with HT at 44˚C for 60 min, and then cultured at 37˚C for 24 h. (A and B) The WST-8 assay was used to count for the viable cells in the cell proliferation assay. Data are presented as the mean ± SD (n=6). *: $p<0.05$ (Student's *t*-test). (C-H) Y79 and WERI-Rb-1 cells were harvested and fixed after 24 h of HT treatment. The percentages of cells in the different cell cycle phases were detected using flow cytometry. C, control (non-treatment). siluc, siRNA for luciferase (50 nM). Date are presented as mean ± SD (n=4-6). *: $p<0.05$ (Student's *t*-test)

## 4.14 PLK1-knockdown inhibits the HT-induced activity of HSF1 in Y79 and WERI-Rb-1 cells.

We examined whether the silencing of PLK1 would affect the expression of HSF1 using real-time qPCR. There were no significant differences in the HSF1 mRNA expression levels after siRNA transfection for PLK1 under the control and HT conditions in Y79 and WERI-Rb-1 cells (Figure 14A and B). Next, we performed western blotting to monitor the activity of HSF1 in Y79

cells. Although the expression level of phosphorylated HSF1 (p-HSF1) was low under the non-stress conditions, it significantly increased in Y79 cells treated with HT at 44˚C for 60 min. The peak expression of p-HSF1 was observed from 1 h to 3 h after the HT treatment. On the other hand, the treatment of the cells with siRNA for PLK1 strongly suppressed the phosphorylation of HSF1 under either the non-HT- or HT-induced condition (Figure 14C). We next investigated the role of PLK1 in HSF1 nuclear translocation in Y79 cells. Under the non-stress condition, expression of HSF1 was observed predominantly in the cytosolic fraction with or without knockdown of PLK1. Under the HT-induced condition, the expression of HSF1 showed an increased localization in the nucleus, but HSF1 nuclear translocation was decreased by the silencing of PLK1 (Figure 14D). To confirm the effect of PLK1-knockdown on the activity of HSF1, we performed immunocytochemistry in Y79 cells. The cells were transfected with siRNA for PLK1, HT was performed at 44˚C for 60 min, and the cells were cultured at 37˚C for 1 h. Under the non-stress condition, almost no formation of nuclear HSF1 granules was observed. On the other hand, under the HT-induced condition, the changes of HSF1 granules in the nucleus increased. However, the HT-induced nuclear granules of HSF1 were decreased by PLK1-knockdown (Figure 14E).

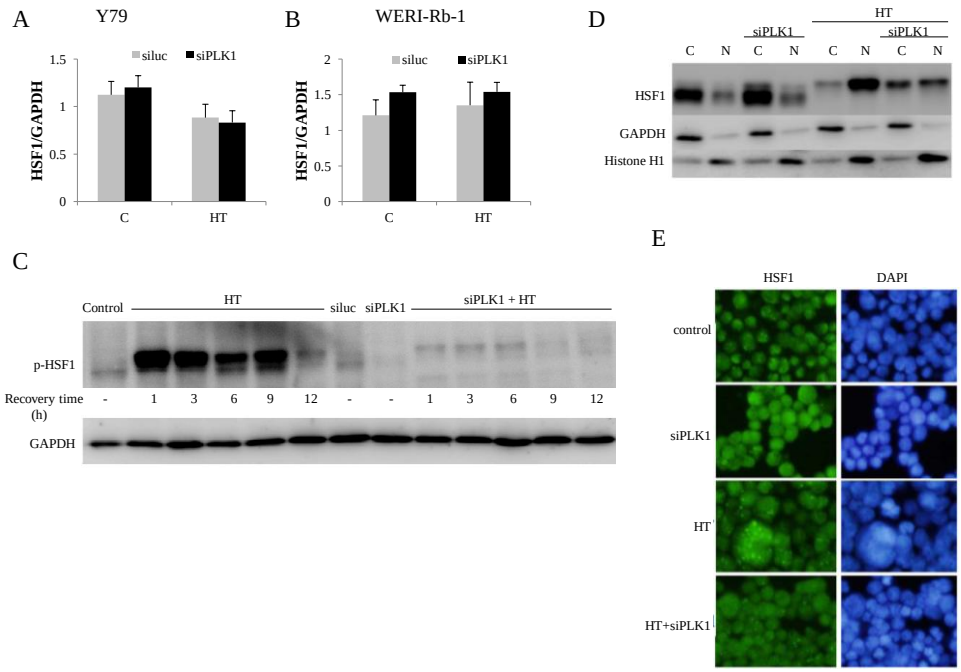


**Figure 14.** PLK1-knockdown inhibits the HT-induced activity of HSF1 in retinoblastoma cells. Cells were transfected with siRNA for PLK1 (50 nM) and then HT was performed at 44˚C for 60 min and then cultured at 37˚C. (A and B) Total RNA was extracted from cells at 3 h after HT treatment. The effects of siRNA for PLK1 on the HSF1 mRNA expression level were investigated under the control and HT conditions in Y79 and WERI-Rb-1 cells. Real-time quantitative PCR assay was performed with specific primers for HSF1 or GAPDH. The HSF1 mRNA level was normalized to GAPDH expression level. Date are presented as mean ± SDs (n = 4). siluc, siRNA for luciferase. (C) After HT treatment, the Y79 cells were cultured at 37˚C for 1, 3, 6, 9 or 12 h. Then, the time-course of changes in the expression of phosphorylated HSF1 (p-HSF1) was examined by western blotting. Western blotting was performed using a specific primary antibody for p-HSF1 or GAPDH. GAPDH served as a loading control. siluc, siRNA for luciferase (50 nM). (D) The nuclei and cytoplasm were separated by using a Nuclear / Cytosol Fractionation Kit at 1 h after HT treatment in Y79 cells. The localization of HSF1 was analyzed by western blotting. Western blotting was performed using specific primary antibodies for HSF1, GAPDH, and Histone H1. GAPDH was used as a loading control of cytoplasm and Histone H1 was used as a loading control of nuclei. C, cytosolic fractions. N, nuclear fractions. (E) Immunocytochemistry was performed at 1 h after HT treatment in order to confirm the activity of HSF1 in Y79 cells. A specific primary antibody for HSF1 was used, and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI).

## 4.15 PLK1-knockdown inhibits HT-induced proteins such as HSPs and BAG3 in Y79 and WERI-Rb-1 cells.

We next investigated whether the HT-induced expression of HSPs and BAG3 would be affected by PLK1-knockdown in Y79 cells and WERI-Rb-1 cells (Figure 15). The cells were transfected with siRNA for PLK1, HT was performed at 44˚C for 60 min, and the cells were cultured at 37˚C for 6 h. Although HT induced the expression of HSP70, HSP40, and BAG3 in control cells and cells transfected with siRNA for luciferase, PLK1-knockdown remarkably inhibited the HT-induced expression of those HSPs and BAG3 in the two retinoblastoma cell lines. These results suggest that PLK1 plays an important role in the induction of HSPs and BAG3. However, PLK1-knockdown did not affect the expression of HSP27.

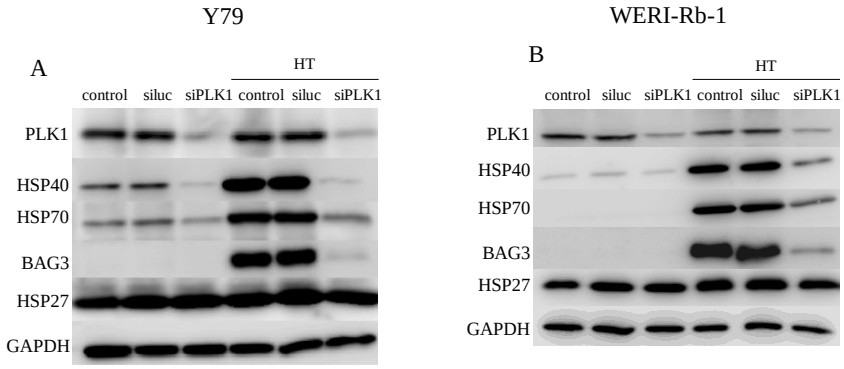


**Figure 15.** PLK1-knockdown inhibits HT-induced proteins such as HSPs and BAG3 in Y79 (A) and WERI-Rb-1 (B) cells. Cells were transfected with siRNA for PLK1 (50 nM) and subjected to HT at 44˚C for 60 min. Then, the cells

were cultured at 37˚C for 6 h and harvested for western blotting. Western blotting was performed using specific primary antibodies for PLK1, HSP70, HSP40, HSP27, BAG3, and GAPDH. GAPDH served as a loading control. siluc, siRNA for luciferase (50 nM).

# 5. Discussion

## 5.1 The combination of silencing BAG3 and inhibition of the JNK pathway enhances HT sensitivity in human oral squamous cells.

HT has been considered a promising approach in cancer therapy and the effectiveness of HT combined with chemotherapy or radiotherapy against various tumors has been shown [Van et al., 2000; Harima et al., 2001; Xia et al., 2006; Aktas et al., 2007; Van et al., 2010]. However, these HT strategies are often insufficient. Previous studies have indicated that, in some cancers, the cytoprotective function to HT therapy is caused at least in part by the elevation of HSPs in the cancer cells [Parsell et al., 1993; Ohtsuka et al., 2000]. BAG3, a co-chaperone of the Hsp70, is a stress-inducible protein and confers cytoprotection against various stresses, including heat stress [Bonelli et al., 2006; Pagliuca et al., 2003; Liao et al., 2001; Tabuchi et al., 2007; Chen et al., 2004; Rosati et al., 2009]. In the present work, the silencing of BAG3 sensitized HT in human OSCC HSC-3 cells. To the best of our knowledge, this is the first report on the enhancement of HT sensitivity through the targeting of BAG3. Moreover, the combination of silencing BAG3 with inhibition of the JNK pathway further enhanced the sensitivity to HT in the HSC-3 cells.

In keeping with the previous reports, we observed a significant

induction of BAG3 expression in HSC-3 cells treated with HT at 44˚C for 90 min [Tabuchi et al., 2012]. Under this condition, HT increased various apoptotic indexes, such as chromatin condensation, the cell population in the sub-G1 phase in the cell cycle and cleavage of caspase 3, suggesting that the type of cell death occurring in HSC-3 cells exposed to HT is apoptosis. It has been demonstrated that BAG3 exerts cytoprotective functions against various stresses in cooperation with Hsp70 [Bonelli et al., 2006; Pagliuca et al., 2003; Liao et al., 2001; Tabuchi et al., 2007; Chen et al., 2004; Rosati et al., 2009]. Furthermore, several kinds of apoptosis induced by the proteasome inhibitor [Liu et al., 2009; Du et al., 2008; Wang et al., 2007], cisplatin [Festa et al., 2011], etoposide [Ammirante et al., 2010], a tumor necrosis factor-related apoptosis-induced ligand [Chiappetta et al., 2007], were reported to be enhanced by the down-regulation of BAG3. Therefore, we assumed that BAG3 silencing enhanced the sensitivity to HT of HSC-3 cells. As expected, BAG3-knockdown markedly enhanced HT-induced apoptosis, indicating that this protein is involved in the HT sensitivity in HSC-3 cells.

Previous reports have shown that HT induced apoptosis via activation of the JNK pathway [Alcala et al., 2010; Hayashi et al., 2011; Gaitanaki et al., 2008; Gabai et al., 2000] and that the apoptosis was suppressed by treatment with a JNK inhibitor [Chung et al., 2003]. In contrast, the JNK pathway functions as anti-apoptotic effects [Wang et al., 2009; Sloss et al., 2008], and phosphorylation of c-Jun by activated JNK pathway is

associated with down-regulation of death signaling [Shaulian et al., 2002]. Thus, it is thought that the JNK pathway has functions of both pro- and anti-apoptotic effects. In this study, although we confirmed the activation of the JNK pathway in HT-treated HSC-3 cells, SP600125, a JNK inhibitor, did not affect HT-induced apoptosis. On the other hand, very interestingly, treatment of HSC-3 cells with SP600125 significantly elevated apoptosis induced by the combination of the suppression of BAG3 function with HT. This finding therefore indicates that the JNK pathway may play a role in the cytoprotection conferred by this combination treatment in HSC-3 cells. This finding was also in agreement with previous studies [Wang et al., 2009; Sloss et al., 2008; Meriin et al., 1998; Kim YH et al., 2005]. That is, Lu et al. recently reported that the JNK inhibitor SP600125 enhances dihydroartemisinin-induced apoptosis by increasing the release of mitochondrial apoptotic factors in human lung adenocarcinoma cells [Lu et al., 2010]. Wang et al. demonstrated that inhibition of the JNK signaling pathway enhances proteasome inhibitor-induced apoptosis by suppression of the BAG3 expression in kidney cancer cells [Wang et al., 2009]. At present, we do not know the detailed molecular interactions between BAG3 and the JNK pathway in HT-induced apoptosis. Further investigations will be needed to clarify this issue.

In conclusion, our present findings suggest that disrupting the functions of both BAG3 and the JNK pathway may become an option for HT therapy in OSCC cells.

## 5.2 BAG3 protects against hyperthermic stress by modulating NF-κB activity in human retinoblastoma Y79 cells.

The anti-apoptotic role of BAG3 in heat stress remains unknown. In this study, we examined the effect of BAG3 on the HT sensitivity of human retinoblastoma Y79 cells. To our knowledge, this is the first report that demonstrates the enhancement of HT sensitivity by BAG3-knockdown in retinoblastoma cells. Furthermore, BAG3-knockdown maintained HT-induced NF-κB inactivity through IKKγ degradation and the subsequent inhibition of the phosphorylation of IκB and p65.

The expression of BAG3 as well as HSP70 and HSP40 is induced by HT at 44°C for 60 min in retinoblastoma cells. The combination of these proteins is thought to have an anti-apoptotic role in heat stress. Previous reports demonstrate that BAG3 stabilizes anti-apoptosis-related proteins by protecting them from proteasome-mediated degradation [Boiani et al., 2013; Zhang et al., 2012]. However, in this study, BAG3-knockdown did not influence the expression of HSP70 and HSP40. It is possible that BAG3 does not interfere with the stabilization of HSP70 and HSP40 in retinoblastoma cells.

NF-κB binds to its inhibitor IκB. Under conditions of stress such as hypoxia and ischemia, IKK leads to the phosphorylation and degradation of IκB [Hayden et al., 2004]. As a result, free NF-κB is strongly phosphorylated and then translocated to the nucleus. In this study, we found that HT inhibits the activation of NF-κB and the inactivity of NF-κB recovers

gradually after HT. This phenomenon concurs with the previous reports, in which heat stress inhibited the activity of NF-κB through the impairment of the proteasome function [Aravindan et al., 2009; Pajonk et al., 2005]. Furthermore, compared to HT alone, the combination of BAG3-knockdown and HT reduced the phosphorylation of IκB and p65 during the recovery period. Ammirante et al. reported that BAG3 binds to IKKγ and regulates the NF-κB activity by increasing the availability of IKKγ [Ammirante et al., 2010]. It is possible that BAG3-knockdown destabilizes IKKγ during the HT recovery period and maintains the HT-induced inactive state of NF-κB. Poulaki et al. showed that the inhibition of NF-κB sensitizes the retinoblastoma cells to doxorubicin through the down-regulation of anti-apoptotic proteins such as Bcl-2, A1, and cIAP-2 [Poulaki et al., 2002]. Silencing BAG3 may induce apoptosis, at least in part, by maintaining the HT-induced inactive state of NF-κB.

In conclusion, BAG3-knockdown attenuates thermal resistance by maintaining HT-induced NF-κB inactivity. Our findings suggest that silencing BAG3 may serve as a potential therapeutic strategy for HT therapy in patients with retinoblastoma.

### 5.3 Inhibition of PLK1 promotes HT sensitivity via inactivation of HSF1 in human retinoblastoma cells.

It has been considered that HSPs play a role in the acquisition of thermoresistance [Parsell et al., 1993; Ohtsuka et al., 2000], and the expression of HSPs is mainly regulated by HSF1 [Morimoto et al., 1998]. In

the current study, we focused on PLK1 because there have been several reports that PLK1 regulates the activity of HSF1 [Kim SA et al., 2005; Lee et al., 2008]. PLK1 also acts as an important regulator of mitosis [Roshak et al., 2000], and several studies have shown that the expression of PLK1 is elevated in various types of cancer cells [He et al., 2009; Wang et al., 2012; Ito et al., 2004]. Further, it has been demonstrated that inhibition of PLK1 promotes the sensitivity of cancer cells to chemotherapy or radiotherapy [Liu et al., 2006; Maire et al., 2013; Gerster et al., 2010], and clinical trials employing PLK1 inhibition have been performed for multiple tumors [Schöffski et al., 2010]. However, there have been no reports of the use of PLK1 to enhance HT sensitivity in retinoblastoma. In this study, we demonstrated for the first time that the combination of inhibition of PLK1 and HT enhanced apoptosis and inhibited cell proliferation via the inactivation of HSF1 in human retinoblastoma cells. In addition, siRNA-mediated PLK1-knockdown and PLK1 inhibitor-mediated PLK1 inhibition promoted HT sensitivity similarly.

Previous reports have shown that overexpression of HSF1 was observed in human cancer cells of various origins [Dudeja et al., 2011; Hoang et al., 2000; Cen et al., 2004]. Furthermore, the HSF1-induced overexpression of HSPs, such as HSP27, HSP70, and HSP90, has been reported in various cancers, and it has been demonstrated that these overexpressions are related to the development of cancer, invasiveness, metastasis, resistance to chemotherapy, and radiotherapy [Tang et al., 2005; Ciocca et al., 2002; Kase et al., 2009]. Several authors have reported that inhibition of HSF1 enhances

the effect of HT treatment concomitant with inhibition of HSPs [Rossi et al., 2006; Tabuchi et al., 2011; Nakamura et al., 2010]. With respect to human retinoblastoma, Kase et al. and Jiang et al. demonstrated that HSP27, HSP70, and HSP90 were all significantly expressed in patients with this cancer, and these HSPs were related to cell proliferation and resistance to chemotherapy [Kase et al., 2009; Jiang et al., 2008]. In addition, several clinical reports demonstrated that HT is effective as a focal treatment, but these effects were limited to small retinoblastoma tumors [Shields et al. 1999; Abramson et al., 2004]. Accordingly, we consider that the targeting of HSF1 and HSPs is a good approach to HT treatment in retinoblastoma. However, in this study we targeted PLK1, not HSF1, in order to enhance HT sensitivity, for the following reasons. First, PLK1 inhibition does not have many influences on normal cells even though it has strong anti-cancer activity [Nogawa et al., 2005; Liu et al., 2006; Maire et al., 2013]. Second, PLK1 inhibitor has been used already in clinical trials [Schöffski et al., 2010]. Third, the potential of PLK1 as a therapeutic target for HT has not been reported.

In the current study, the silencing of PLK1 inhibited HT-induced phosphorylation of HSF1, nuclear translocation of HSF1, and the formation of nuclear HSF1 granules. Previous reports have shown that phosphorylation of HSF1 and HSF1 nuclear translocation are regulated by PLK1 [Kim SA et al., 2005; Lee et al., 2008]. Kim et al. showed that inhibition of PLK1 suppresses the expression level of HSF1 after heat shock [Kim et al., 2010]. Moreover, Holmberg et al. showed that granule formation

of HSF1 is required for some of the molecular mechanisms underlying the inducible phosphorylation and transcriptional activation of HSF1 [Holmberg et al., 2000]. In keeping with the previous reports, we here observed that PLK1 modulated activation of HSF1. Therefore, an inhibition of PLK1 may product a synergistic effect in HT through an inactivation of HSF1. Furthermore, we think that an enhancement of HT sensitivity may be involved in the inhibition of HT-induced HSPs, especially HSP70. Chen et al. reported that PLK1 mediated the phosphorylation of HSP70 during mitosis [Chen et al., 2011]. Inhibition of HSP70, a main player in thermoresistance, may be suppressed effectively by the inhibition of PLK1 and resulting inactivation of HSF1. However, HSP27 was not changed significantly by PLK1-knockdown. Additional work will be needed in regard to HSP27 and other HSPs. In addition, the silencing of PLK1 strongly inhibited the HT-induced expression of BAG3. BAG3 is a family of co-chaperones that interact with the ATPase domain of HSP70 through the BAG domain [Rosati et al., 2007]. The expression level of BAG3 is increased in response to various stresses, including heat stress [Kariya et al., 2013; Liao et al., 2001]. The induction of BAG3 is at least partly mediated by HSF1 [Franceschelli et al., 2008]. We recently reported that the silencing of BAG3 enhanced HT sensitivity in human oral squamous cell carcinoma [Yunoki et al., 2013]. In the present work, the enhancement of HT sensitivity by PLK1-knockdown may have been related to the inhibition of BAG3. At present, the detailed molecular mechanisms underlying the relationship between PLK1 and thermoresistance are not well known. Further investigations will be needed

to clarify this issue. Furthermore, there are several limitations to this study. HT under in vitro experimental conditions ensures a more homogeneous heating than under clinical conditions. Additionally, HSPs are thought to be involved in the protection of retinoblastoma cells against heat damage. On the other hand, HSPs have anti-tumor immunity [Gurbuxani et al., 2001; Frey et al., 2012]. These opposite immunogenic and anti-apoptotic effects of HSPs may affect the therapeutic effects of this combination therapy. Accordingly, additional studies, including in vivo experiments, are needed to clarify the effect of combination therapy with HT and PLK1 inhibition.

In conclusion, the inhibition of PLK1 may attenuate the thermoresistance of HT through an inactivation of HSF1 concomitant with reductions in HSPs and BAG3. The combination of PLK1 inhibition and HT may become an option for HT therapy in patients with retinoblastoma.

## 6. Conclusions

In conclusion, our present findings suggest that the disruption of functions of HSF1-related proteins such as BAG3 and PLK1 may serve as a potential therapeutic strategy for HT therapy in patients with cancer tumors.

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