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**Research Article**

# Molecular-Targeted Hyperthermia Therapy for Breast Cancer Cells

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## Abstract

Hyperthermia is a minimally invasive cancer therapy that sensitizes cancer cells to chemotherapy and radiotherapy by improving the tumor microenvironment such as blood flow and acidic conditions. Because stress-related and anti-apoptotic proteins exert a cytoprotective effect and are often up-regulated in many types of cancer, molecular-targeted therapy may be useful in combination with hyperthermia. In the present study, we found that hyperthermia induced myeloid-cell leukemia 1 (MCL-1) anti-apoptotic protein expression among several apoptosis-related molecules. MCL-1 knockdown promoted the anti-tumor effect of hyperthermia by increasing apoptosis. miR-29b-3p, which downregulated MCL-1 protein expression, also promoted the anti-tumor effect of hyperthermia. Moreover, we showed that hyperthermia facilitated the translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) stress-related protein to the nucleus and expression of the downstream target heme oxygenase 1 (HO-1) antioxidant gene. The knockdown of Nrf2 promoted the anti-tumor effect of hyperthermia by suppressing expression of stress-related genes such as HO-1 and NAD (P) H quinone dehydrogenase 1 and by increasing reactive oxygen species production. Our results suggest the potential usefulness of molecular-targeted hyperthermia therapy using nucleic acid medicine.

**Keywords:** Molecular-targeted hyperthermia; Nucleic acid medicine; Stress-related proteins; Anti-apoptotic proteins; Breast cancer

**Abbreviations:** HSPs: Heat Shock Proteins; ROS: Reactive Oxygen Species; Nrf2: Nuclear Factor Erythroid 2-related factor 2; Keap1: Kelch-like ECH-associated protein 1; Maf: Musculo-aponeurotic Fibrosarcoma Oncogene Homolog; ARE/EpRE: Antioxidant/Electrophile responsive element; HO-1: Heme Oxygenase-1; NQO1: NAD (P) H Quinone Dehydrogenase 1; MCL-1: Myeloid-Cell Leukemia 1; BCL-2: B-cell lymphoma 2; sCA: Super Carbonate Apatite; HSF1: Heat Shock Factor 1; BAG3: Bcl-2-Associated Athanogene Domain 3.

## Introduction

Hyperthermia is minimally invasive cancer therapy, using a treatment temperature of 39°C-45°C in the clinic. A lower temperature of around 42°C is considered mild hyperthermia. Mild hyperthermia does not have a direct cytotoxic effect but increases tumor blood flow and vascular permeability [1,2], which sensitizes cancer cells to chemotherapy and radiotherapy. The higher temperature (>42°C) induces cell death mainly because of protein denaturation in the nucleus, cytoplasm, and plasma membrane.

It is generally considered that monotherapy with hyperthermia is insufficient as a strategy in cancer treatment, and many groups have reported that combination therapy with hyperthermia and chemotherapy or radiotherapy is effective [3-5]. One problem in hyperthermia-based cancer therapy is thermal tolerance, the protective cellular response caused by stress response proteins, largely heat shock proteins (HSPs). A molecular-targeted therapy may overcome this cytoprotective mechanism of tumor cells, but reports are lacking that show a clinical benefit of small molecule inhibitors or specific antibodies in combination with hyperthermia.

The anti-apoptotic machinery is also a cytoprotective system for cancer cells. Induction of apoptosis is determined by the balance of anti-apoptotic proteins such as myeloid-cell leukemia (MCL)-1, B-cell lymphoma (BCL)-2, and BCL-xL and proapoptotic proteins such as Bax and Bak. For this reason, therapeutics targeting anti-apoptotic proteins have been developed and tested in clinical trials [6,7]. Among them, MCL-1 localizes at the outer mitochondrial membrane to regulate apoptosis via modulation of mitochondrial function. MCL-1 is upregulated in many types of cancer, including breast, colon, pancreas, and lung cancers, and is related to anti-tumor drug resistance through suppression of apoptosis [8]. Although anti-apoptotic molecules may function as cytoprotective factors under hyperthermia, little is known about the behavior of apoptosis-related proteins.

Hyperthermia induces many cellular responses, including the intracellular accumulation of reactive oxygen species (ROS) [9-12]. It increases ROS production through suppressing mitochondrial membrane potential [13]. Accumulated ROS in the cytoplasm induces cell death by promoting protein denaturation and DNA damage. Here, we focused on nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that regulates antioxidant genes and suppresses ROS production and a key molecule in the cellular response by hyperthermia [14].

Under normal conditions, Nrf2 is constantly ubiquitinated and degraded by interacting with the Kelch-like ECH-associated protein 1 (Keap1) and Cul3 protein in the cytoplasm [15,16]. Under oxidative stress, Nrf2 is released from Keap1 and translocates to the nucleus, where it forms a heterodimer with small musculoaponeurotic fibrosarcoma oncogene homolog (Maf). The Nrf2/Maf heterodimer binds to antioxidant/electrophile responsive element (ARE/EpRE) and activates transcription of downstream genes such as heme oxygenase-1 (HO-1) and NAD (P) H quinone dehydrogenase 1 (NQO1), which have a role in antioxidation and detoxification to inhibit ROS production [17]. Nrf2 is activated in many types of cancers including breast, gastric, and lung cancers [18]. The excess nuclear accumulation of Nrf2 reduces ROS cytotoxicity and contributes to chemoresistance and radiotherapy resistance [19]. Although recent papers showed that hyperthermia affected the localization of Nrf2 protein and the combination treatment of Nrf2 knockdown and hyperthermia further decreased cell viability and increased apoptosis in esophageal cancer, lung cancer and mouse hippocampus cell lines [20-22], the relationship between hyperthermia and Nrf2 in the refractory breast cancer remains unknown.

In this study, we found that hyperthermia induced expression of the anti-apoptotic protein MCL-1 among several apoptosis-related proteins in the triple-negative breast cancer cell line MDA-MB231. Moreover, we found that hyperthermia facilitated the translocation of Nrf2 to the nucleus and induced expression of its downstream antioxidant gene *HO-1*. Our findings propose the potential usefulness of molecular-targeted hyperthermia against cytoprotective molecules using siRNA/microRNA.

## Materials and Methods

### Cell culture

Human colorectal cancer cell line HT29 and DLD-1 were cultured in RPMI 1640 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Human breast cancer cell line MDA-MB231, MCF7, and human colorectal cancer cell line HCT116 cells were cultured in DMEM (Thermo Fisher Scientific, Inc.). Both RPMI 1640 and DMEM contained 10% fetal bovine serum (Biowest, Nuaillé, France), Penicillin-Streptomycin Mixed Solution (NACALAI TESQUE, INC., Kyoto, Japan). All cells were cultured at 37°C in 5% $CO_2$. All cells were obtained from American Type Culture Collection (ATCC).

## Transfection of siRNA and miRNA

Cells were transfected with small-interfering (si)RNA or micro (mi)RNA by using Lipofectamine RNAiMAX regent (Thermo Fisher Scientific, Inc.) following the manufacture's protocol. All siRNAs and miRNAs used in this study were obtained from GeneDesign, Inc. (Osaka, Japan). The siRNAs and miRNAs used were as follows:

*Nrf2-siRNA #1*, sense (S) :

5'-CCCAUUGAUGUUUCUGAUCUA-3'

antisense (AS) : 5'-UAGAUCAGAAACAUCAAUGGG-3';

*Nrf2-siRNA #2*, S: 5'-AAGGAUUAUUAUGACUGUUAA-3'

AS: 5'-UUAACAGUCAUAAUAAUCCUU-3'

*Nrf2-siRNA #3*, S: 5'-GAGACUACCAUGGUUCCAA-3'

AS: 5'-UUGGAACCAUGGUAGUCUC-3'

*Nrf2-siRNA #4*, S: 5'-GUGAGAACACACCAGAGAA-3'

AS: 5'-UUCUCUGGUGUGUUCUCAC-3'

*MCL-1-siRNA*, S: 5'-GGACUUUUAUACCUGUUAU-3'

AS: 5'-AUAACAGGUAUAAAAGUCC-3'

*miR-29b-3p*, S: 5'- UAGCACCAUUUGAAAUCAGUGUU -3'

AS: 5'- AACACUGAUUUCAAAUGGUGCUA -3'

*negative control RNA*, S: 5'-AUCCGCGCGAUAGUACGUA-3'

AS: 5'-UACGUACUAUCGCGCGGAU-3'

## Hyperthermia

Hyperthermia was performed in the $CO_2$ incubator. Cells were incubated at 43°C in 5% $CO_2$ for the indicated time (0-120 minutes). In the combination treatment with siRNA or miRNA, hyperthermia treatment started 24 hours after transfection.

## Western blotting and protein isolation

Cells were lysed in RIPA buffer (0.05 M Tris-HCl pH 7.6, 0.15 M NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate) with proteinase inhibitor and phosphatase inhibitor. The cell lysate was subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Merck Millipore, Burlington, MA, USA). The primary antibodies used in this study were as follows: anti-Nrf2 rabbit monoclonal antibody [EP1808Y] (abcam, Cambridge, UK); anti-HSP70 mouse monoclonal antibody [W27] (Santa Cruz Biotechnology, Dallas, TX, USA); anti-HSP90 mouse monoclonal antibody [C45G5] (Cell Signaling Technology, Danvers, MA, USA); anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody [0411] (Santa Cruz Biotechnology);

anti-lamin B1 rabbit polyclonal antibody (abcam); anti-BCL-2 rabbit monoclonal antibody [E17] (abcam); anti-BCL-xL rabbit polyclonal antibody (Cell Signaling Technology); anti-Bax rabbit polyclonal antibody (Cell Signaling Technology); anti-Bak rabbit monoclonal antibody [D4E4] (Cell Signaling Technology); anti-MCL-1 rabbit monoclonal antibody [D35A5] (Cell Signaling Technology); and anti-β-actin rabbit monoclonal antibody [13E5] (Cell Signaling Technology).

Nuclear and cytoplasmic fractions were isolated using the Nuclear Extraction Kit (Abcam) following the manufacturer's protocol. Band image was detected by the ImageQuant LAS 4000mini (GE Healthcare, Chicago, IL, USA). The band intensity was calculated using ImageJ software.

## Immunocytochemistry

Cells were cultured in a six-well plate with glass cover slides at a density of $1.0 \times 10^5$ cells/well. Forty hours later, cells were incubated at 43°C in 5% $CO_2$ for 90 minutes. Cells were fixed 6 hours after hyperthermia at room temperature by using the 4% paraformaldehyde for 5 minutes and incubated in 0.25% Triton/phosphate-buffered saline (PBS) for 2 minutes at room temperature, followed by washing with PBS three times. Immunostaining was performed using anti-Nrf2 rabbit monoclonal antibody (EP1808Y, abcam) and VECTASTAIN Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's protocol.

## Immunofluorescence

Cells were cultured in a six-well plate with glass cover slides at a density of $1.0 \times 10^5$ cells/well. Forty hours later, cells were incubated at 43°C in 5% $CO_2$ for 90 minutes. Cells were fixed 6 hours after hyperthermia at room temperature by using the 4% paraformaldehyde for 5 minutes and incubated in 0.25% Triton/ PBS for 2 minutes at room temperature, followed by washing with PBS three times. Blocking was performed by using Blocking One Histo (NACALAI TESQUE, INC) for 15 minutes at room temperature. Cells were incubated with anti-Nrf2 rabbit monoclonal antibody (EP1808Y, abcam) for 1 hour at room temperature, followed by washing with PBS-T (PBS with Tween 20) three times. Then, cells were incubated with FITC-conjugated anti-rabbit IgG antibody (Dako, Denmark) for 1 hour at room temperature, followed by washing with PBS-T three times and PBS one time. Cells were mounted by using ProLong Gold Antifade Reagent with DAPI (Thermo Fisher Scientific, Inc.).

## Quantitative RT-PCR

Cells were lysed in TRIzol regent (Thermo Fisher Scientific, Inc.), and total RNA was extracted by following the standard protocol. Complementary DNA was generated from RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.). qPCR was performed using the LightCycler 480 System II (Roche Diagnostics, Rotkreuz, Switzerland). Relative

expression was quantified using the ΔΔCt method. Each value was normalized to GAPDH expression. Primer sequences were listed below;

GAPDH – Forward (F): 5'-CAACTACATGGTTTACATGTTC-3'

Reverse (R): 5'-GCCAGTGGACTCCACGAC-3'

Nrf2 – F: 5'-ATAGCTGAGCCCAGTATC-3'

R: 5'-CATGCACGTGAGTGCTCT-3'

HO-1 – F: 5'-GCTGCTGACCCATGACACCAAGG-3'

R: 5'-AAGGACCCATCGGAGAAGCGGAG-3'

NQO-1 – F: 5'-GCACTGATCGTACTGGCTCA-3'

R: 5'-CGCAGGGTCCTTCAGTTTAC-3'

**Cell viability**

Cell viability was assessed by cell count. A total of 1.0-2.0×10⁵ cells were plated and treated with hyperthermia and/or siRNA or miRNA. Cells were trypsinized at 48 hours after the treatment and mixed with 0.5% trypan blue staining solution. Viable cell number was calculated using a LUNA cell counter (Logos Biosystems, Gyeonggi-do, South Korea).

**Cell proliferation**

Cell proliferation was assessed by cell count. A total of 0.8×10⁵ cells were plated and treated with hyperthermia. Cells were trypsinized at 1, 4, 6 days after the treatment and mixed with 0.5% trypan blue staining solution. Viable cell number was calculated using a LUNA cell counter (Logos Biosystems).

**ROS detection**

A total of 1.2-1.5×10⁵ cells were plated and treated with hyperthermia and/or siRNA. Treated cells were suspended and stained with CellROX™ Deep Red Flow Cytometry Assay Kit (Thermo Fisher Scientific, Inc.) by following the manufacturer's protocol. Cells were analyzed by flow cytometry using the Spectral Analyzer SA3800 (SONY, Tokyo, Japan).

**Annexin V assay**

A total of 9.0×10⁵ cells were plated and treated with hyperthermia and/or siRNA. Treated cells were suspended and stained using the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Thermo Fisher Scientific, Inc.), following the manufacture' s protocol. The population of apoptotic cells was assessed by flow cytometry using the Spectral Analyzer SA3800.

**Statistical analysis**

All data are shown as means ± standard deviations. Student's t test was conducted to calculate the statistical significance. A $P<0.05$ was considered as significant.

## Results

### Hyperthermia induces anti-apoptotic protein MCL-1 expression

To determine the condition of hyperthermia treatment, we examined cell viability after hyperthermia treatment in triple-negative breast cancer cell line MDA-MB231. Cell viability decreased with increased hyperthermia treatment. In most of the subsequent experiments, hyperthermia treatment was performed for more than 90 minutes, and cell viability was decreased to less than 70% at 48 hours after treatment (Supplementary figure S1A). We also performed a time course study and found that hyperthermia treatment suppressed cell proliferation in MDA-MB231 cells (Supplementary figure S1B).
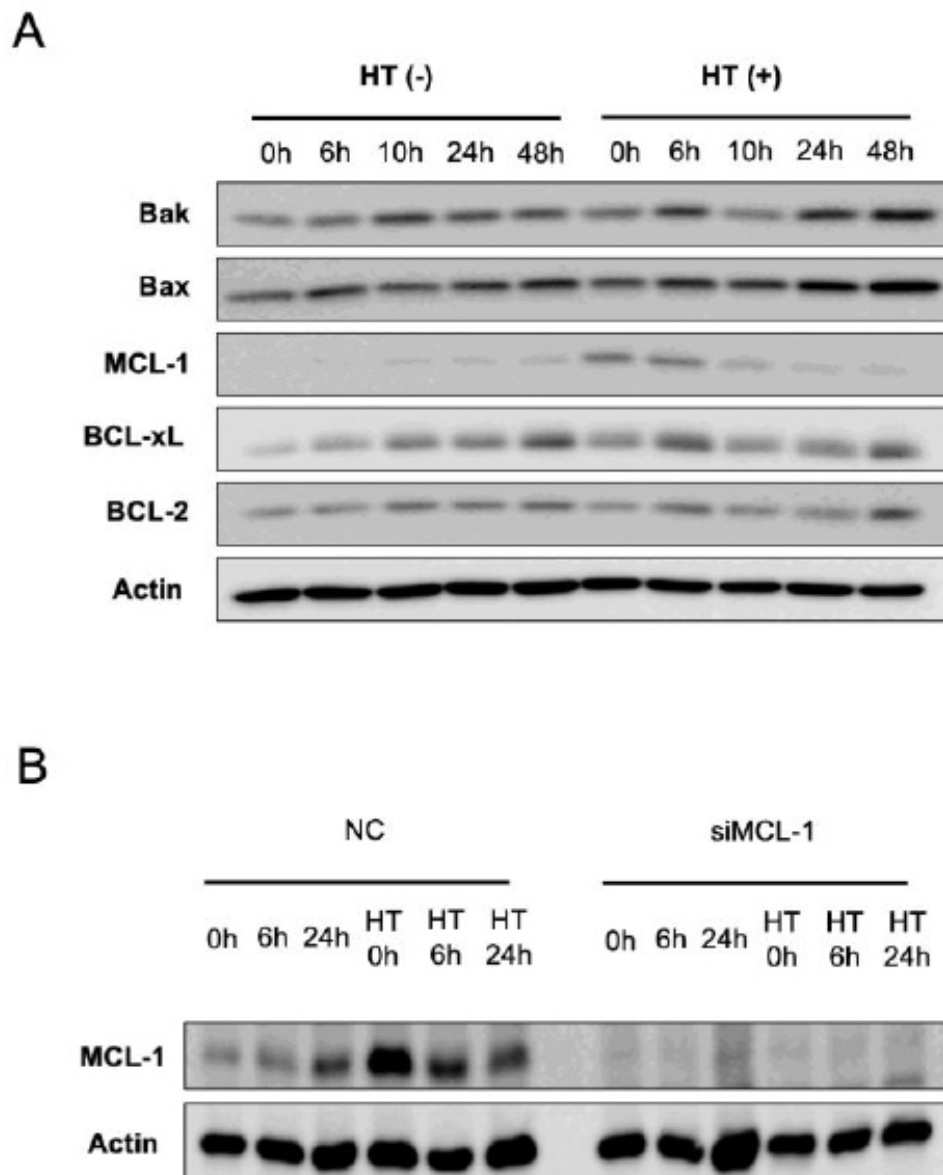
**Figure 1:** MCL-1 expression was induced by hyperthermia; (A) The expression of anti-apoptotic, and proapoptotic proteins in control and hyperthermia-treated cells. Hyperthermia was performed for 100 minutes at 43°C. The whole cell lysates were collected 0–48 hours after hyperthermia treatment. HT: hyperthermia; (B) The knockdown efficiency of siMCL-1. siRNA transfection was performed 24 hours prior to hyperthermia treatment, which was performed for 60 minutes at 43°C. MCL-1 protein expression was examined 0, 6, and 24 hours after hyperthermia treatment. b-actin is a loading control. HT: hyperthermia.

The anti-apoptotic machinery is also a cytoprotective system for cancer cells. For this reason, we hypothesized that hyperthermia might induce certain anti-apoptotic proteins. To confirm this idea, we examined the expression of apoptosis-related proteins. Hyperthermia induced one anti-apoptotic protein: MCL-1 expression levels increased right after hyperthermia, and the effect was maintained for at least 6 hours (Figure 1A). This outcome was reproducible, and siRNA for MCL-1 considerably suppressed MCL-1 protein expression even after hyperthermia treatment (Figure 1B).

## MCL-1 knockdown enhances the anti-tumor effect of hyperthermia

We next examined the anti-tumor effect of hyperthermia in combination with MCL-1 knockdown. MCL-1 knockdown enhanced the anti-tumor effect of hyperthermia (Figure 2A). Furthermore, we found that apoptosis was induced in MCL-1 knockdown cells (Figure 2B).
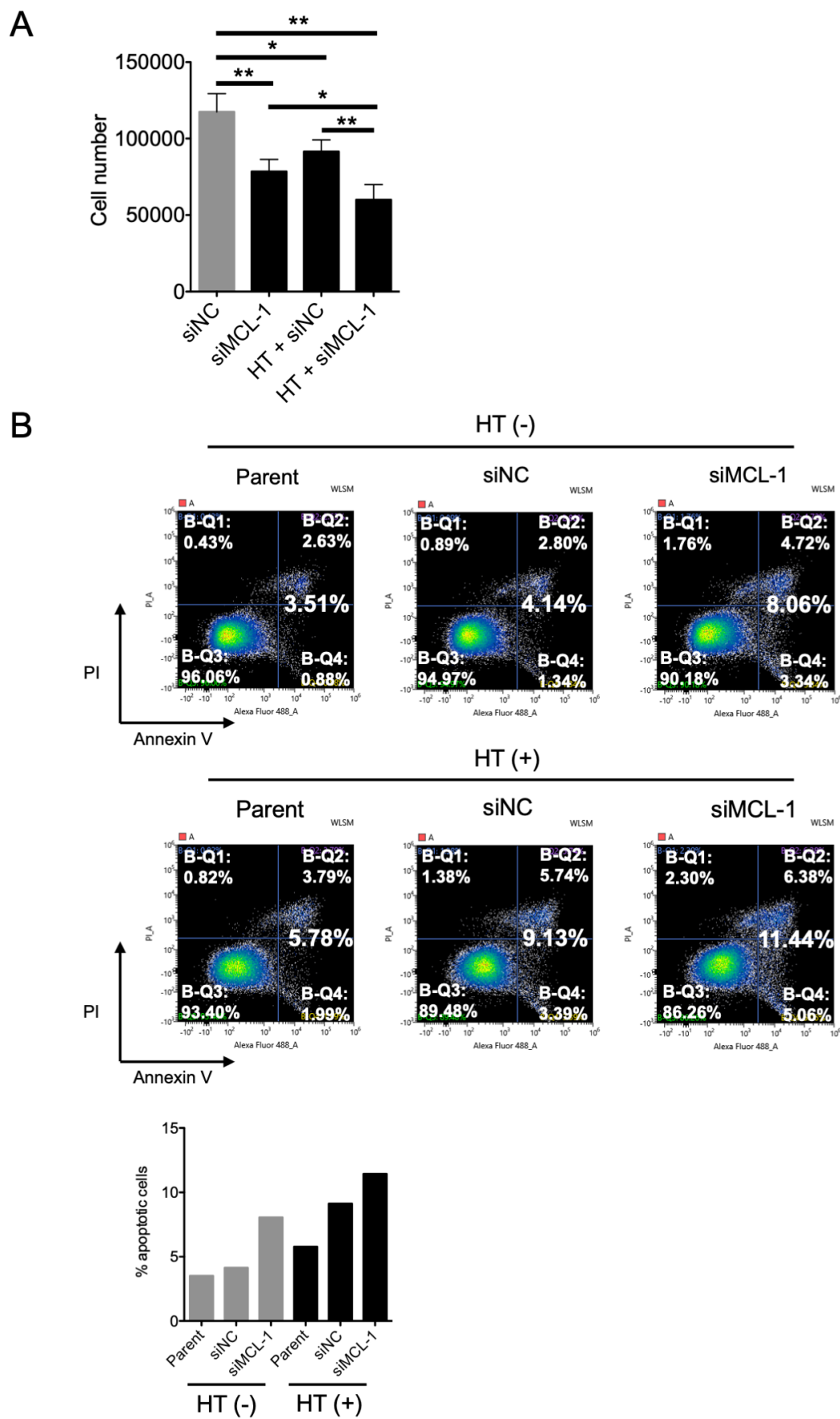


**Figure 2:** MCL-1 knockdown enhanced the anti-tumor effect of hyperthermia; (A) Cell number in si-negative control (NC) or siMCL-1 treated cells with or without hyperthermia treatment. siRNA transfection was performed 24 hours prior to hyperthermia treatment, which was performed for 60 minutes at 43°C. Cell number was counted at 48 hours. *P<0.05; **P<0.01; HT: hyperthermia; (B) Apoptosis detected by Annexin V assay in control and hyperthermia-treated cells with MCL-1 knockdown. siRNA transfection was performed 24 hours before hyperthermia treatment, which was performed for 100 minutes at 43°C. The total percentage of early (B-Q4) and late (B-Q2) apoptotic cells were indicated in the middle right column of each chart and the graph was shown in the bottom. HT: hyperthermia.

**miR-29b-3p promotes the anti-tumor effect of hyperthermia.**

We previously reported that miR-29b-3p suppressed MCL-1 and CDK6 expression and showed an anti-tumor effect in colorectal cancer cell lines [23,24]. As with the colorectal cancer cell lines, miR-29b-3p suppressed hyperthermia-induced MCL-1 expression in a breast cancer cell line (Figure 3A). When we investigated the anti-tumor effect of hyperthermia in combination with miR-29b-3p treatment, a single treatment of miR-29b-3p showed a significant anti-tumor effect ($P<0.01$), and the combination treatment with hyperthermia more strongly suppressed cell growth in a time-dependent manner (Figure 3B).
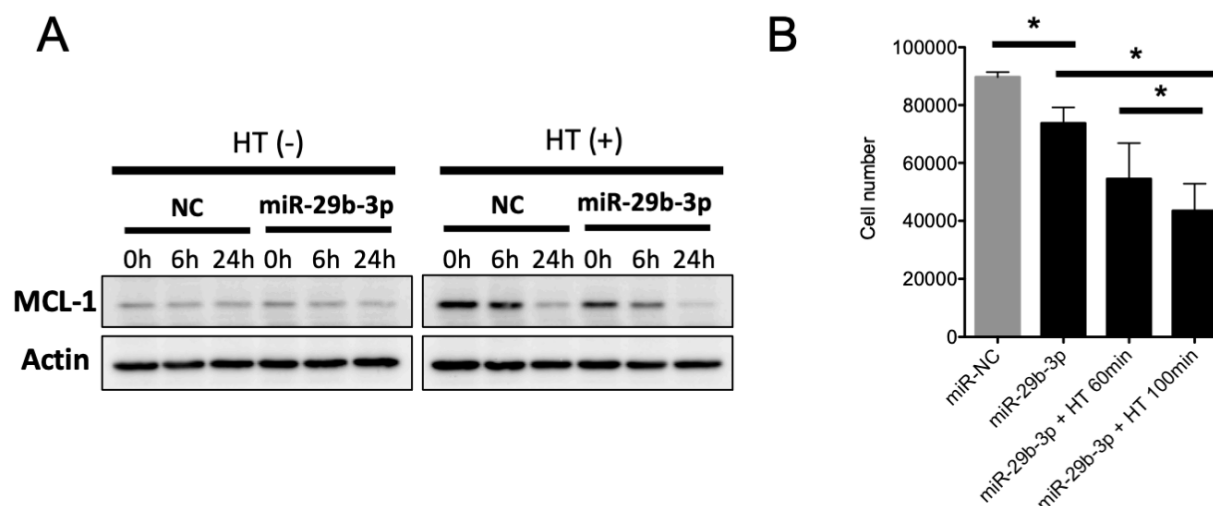


**Figure 3:** miR-29b-3p promoted the anti-tumor effect of Hyperthermia; (A) Expression of Nrf2 protein in control and hyperthermia/miR-29b-3p–treated cells. miRNA transfection was performed 24 hours before hyperthermia treatment, which was performed for 100 minutes at 43°C. The whole cell lysates were collected 0–24 hours after hyperthermia treatment. b-actin is a loading control. HT: hyperthermia; (B) Cell number in control and miR-29b-3p– and hyperthermia-treated cells. miRNA transfection was performed 24 hours before hyperthermia treatment. Hyperthermia was performed for 60 minutes or 100 minutes at 43°C. Cell number was counted at 48 hours. *$P<0.01$; HT: hyperthermia.

**Hyperthermia induces nuclear translocation of Nrf2 protein and HO-1 gene expression**

The triple-negative breast cancer cell line MDA-MB231 expressed a higher level of Nrf2 protein compared to the other breast cancer cell line MCF7 and colorectal cancer cell lines DLD-1, HCT116, and HT29 (Figure 4A). We investigated the effect of hyperthermia on protein expression of Nrf2. We also investigated the HSPs as an indicator of the effect of hyperthermia because it is reported that the expression and localization of HSPs was affected by hyperthermia in many cancer types [21,25-29]. Expression of the HSP70 protein was increased 3 hours after hyperthermia, but expression of Nrf2 and HSP90 was not increased (Figure 4B). Because the activated Nrf2 was translocated from the cytoplasm to the nucleus, we investigated its expression in the cytoplasm and nucleus separately. Nuclear translocation of the Nrf2 protein was noted just after hyperthermia treatment and was sustained until 10 hours after treatment. HSP70 expression was increased in both the cytoplasm and nucleus, and HSP90 expression was slightly increased in the cytoplasm (Figure 5A). The nuclear translocation of Nrf2 by hyperthermia was confirmed by immunocytochemistry and immunofluorescence (Figure 5B and 5C).

Because our results indicated that hyperthermia facilitated nuclear translocation of the Nrf2 protein in breast cancer cells, we examined the expression of downstream genes such as HO-1 and NQO1. Hyperthermia significantly induced HO-1 gene expression, especially 6 hours after treatment, and the expression was gradually decreased at 12 and 24 hours (Figure 5D).
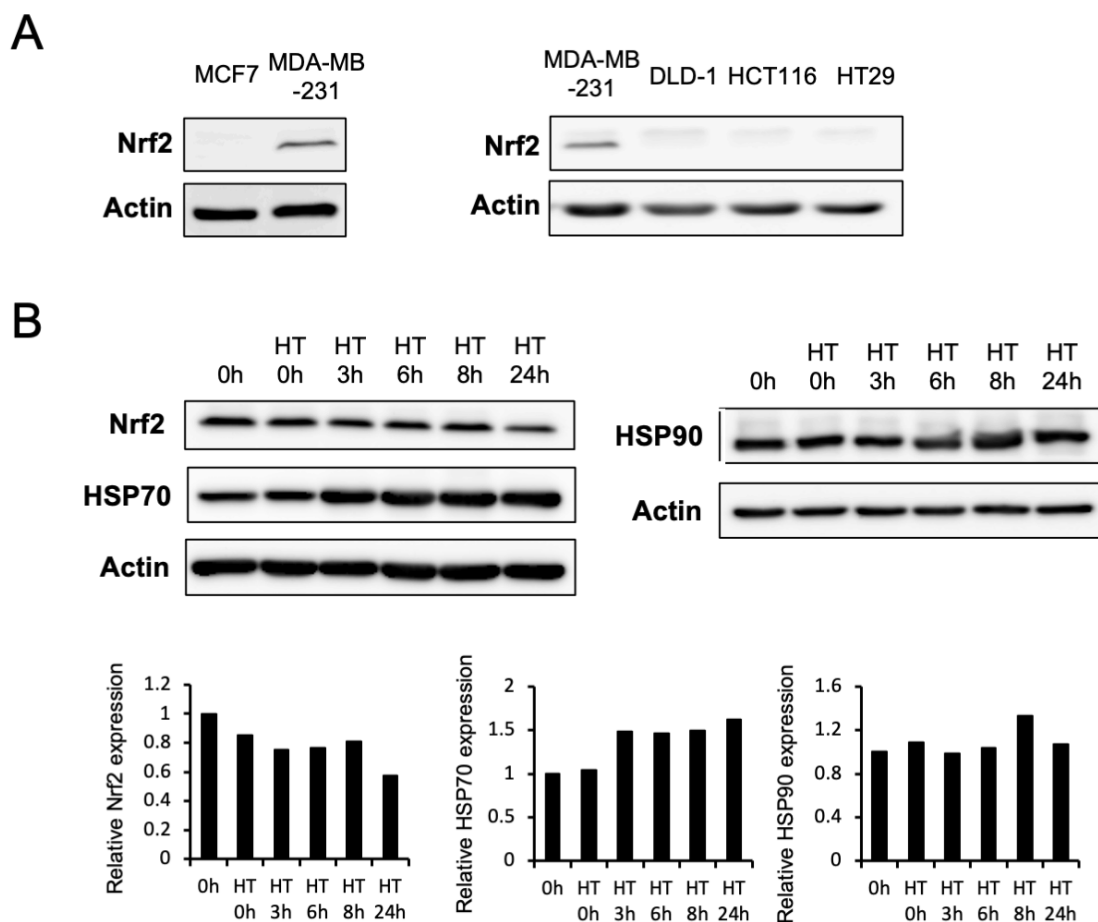
**Figure 4:** Hyperthermia induced HSP70 expression but not Nrf2 and HSP90 expression in whole cell lysate; (A) Nrf2 protein expression in breast and colorectal cancer cell lines; (B) Expression of Nrf2, HSP70, and HSP90 proteins in control and hyperthermia-treated cells in whole cell lysates. Hyperthermia was performed for 90 minutes at 43°C. The whole cell lysates were collected 0-24 hours after hyperthermia treatment. The relative band intensities of Nrf2, HSP70, and HSP90 as compared with the control sample (0 h) are shown. b-actin is a loading control. HT: hyperthermia.
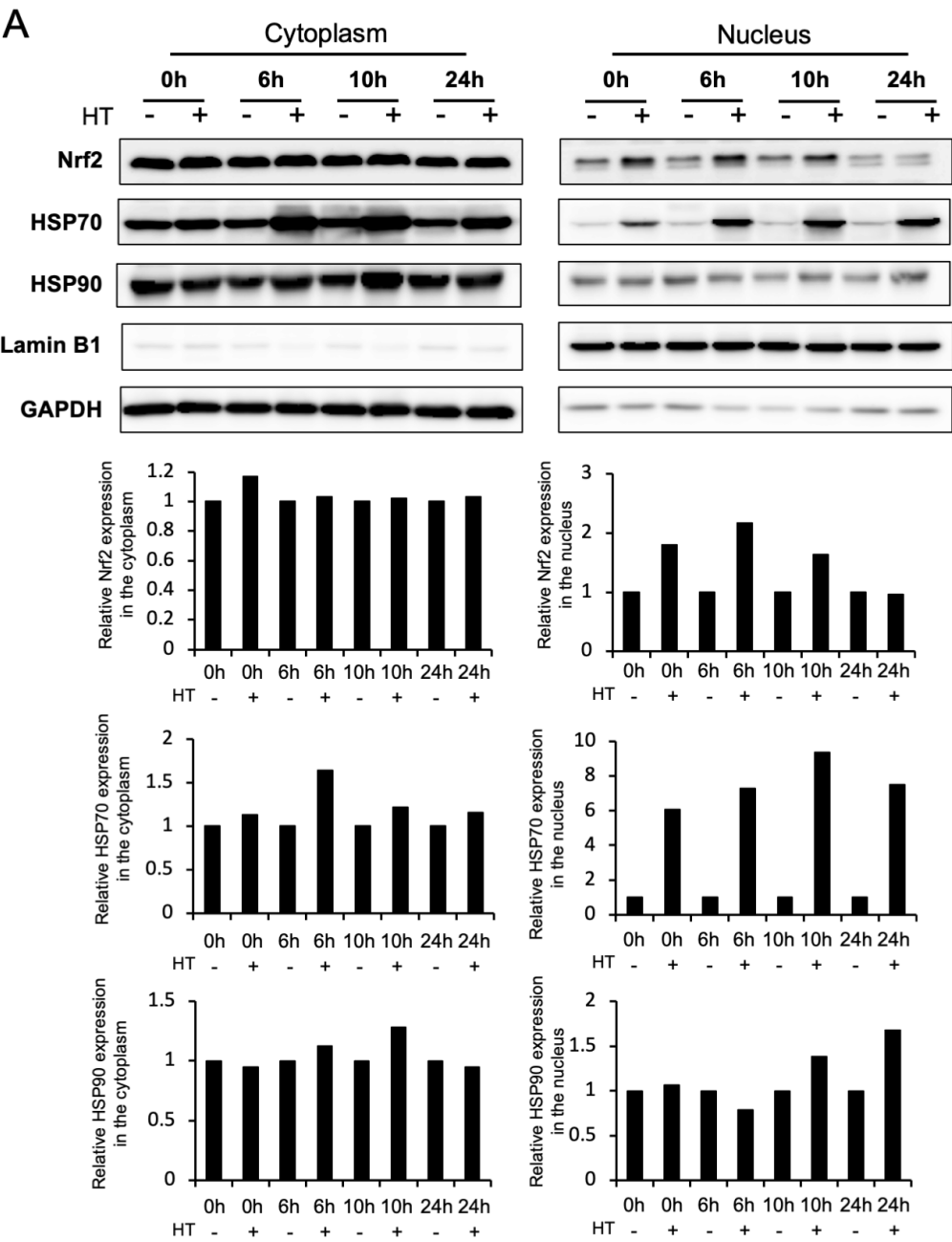
**Figure 5: A)** Hyperthermia induced translocation of the Nrf2 protein to the nucleus; (A) The expression of Nrf2, HSP70, and HSP90 proteins in control and hyperthermia- treated cells in the cytoplasm and nuclear fractions. Each fraction was collected using a Nuclear Extraction Kit. Hyperthermia was performed for 90 minutes at 43°C. GAPDH is a loading control for the fraction of cytoplasm. Lamin B1 is a loading control for the fraction of nucleus. The relative band intensities of Nrf2, HSP70, and HSP90 compared with that of the control sample (0 h, HT-) are shown. HT: hyperthermia.
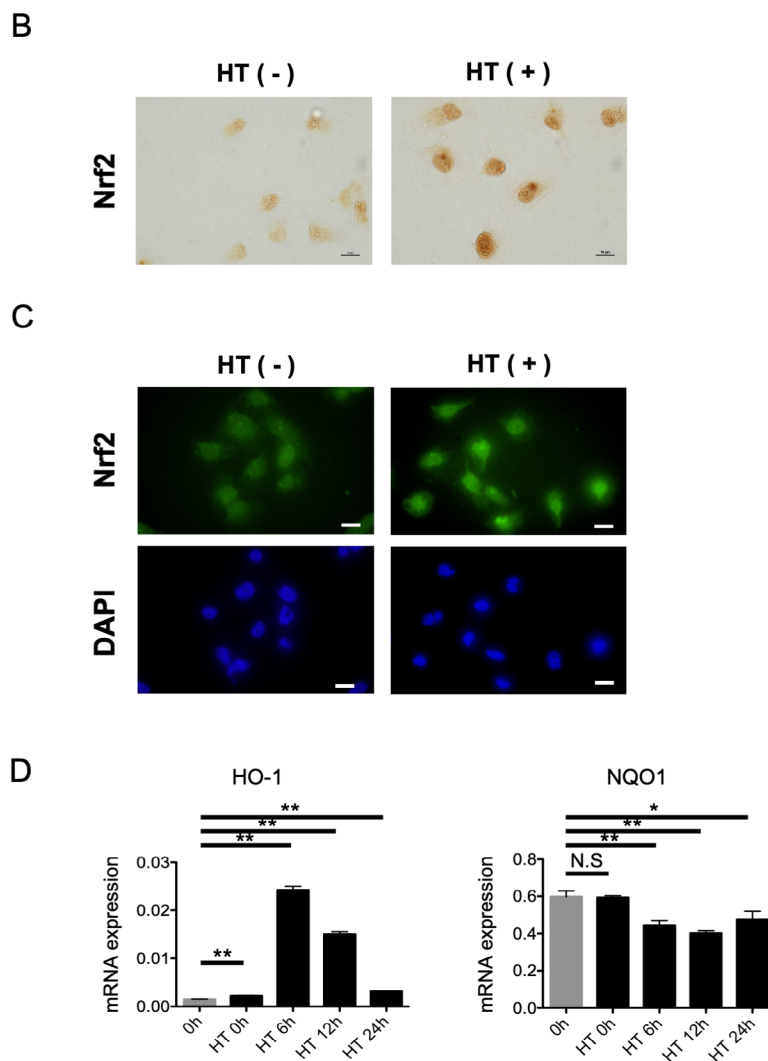
**Figure 5:** B) Immunocytochemistry for the Nrf2 protein. The Nrf2 protein was stained with anti-Nrf2 rabbit monoclonal antibody (EP1808Y, abcam) in control and hyperthermia-treated cells. Hyperthermia was performed for 90 minutes at 43°C. HT: hyperthermia; scale bar: 10 µm; (C) Immunofluorescence for the Nrf2 protein. The Nrf2 protein was stained with anti-Nrf2 rabbit monoclonal antibody (EP1808Y, abcam) in control and hyperthermia-treated cells. Hyperthermia was performed for 90 minutes at 43°C. HT: hyperthermia; scale bar: 10 µm; (D) Expression of HO-1 and NQO1 mRNA in control and hyperthermia-treated cells. Hyperthermia induced HO-1 gene expression. Hyperthermia was performed for 100 minutes at 43°C. Each value was normalized by expression of GAPDH. *$P<0.05$; **$P<0.01$; N.S: not significant ; HT: hyperthermia.

**Nrf2 knockdown suppresses antioxidant gene expression and ROS production promoted by hyperthermia and enhanced the anti-tumor effect of hyperthermia**

To investigate the effect of Nrf2 knockdown in combination with hyperthermia, we selected siRNA #3 because it best suppressed Nrf2 expression at both the protein and mRNA levels (Figure 6A and 6B). Nrf2 knockdown suppressed expression of the downstream antioxidant genes HO-1 and NQO1, even after the hyperthermia treatment (Figure 6C and 6D).

Under the normal condition, the ROS activity of MDA-MB231 cells was scored as 50%-60%. After hyperthermia, ROS activity was increased up to 70%-75%. When *Nrf2* gene expression was knocked down, ROS activity further increased 24 hours after hyperthermia, but it gradually declined in the negative control siRNA-treated cells (Figure 6E). Furthermore, Nrf2 knockdown enhanced the anti-tumor effect of hyperthermia (Figure 6F).
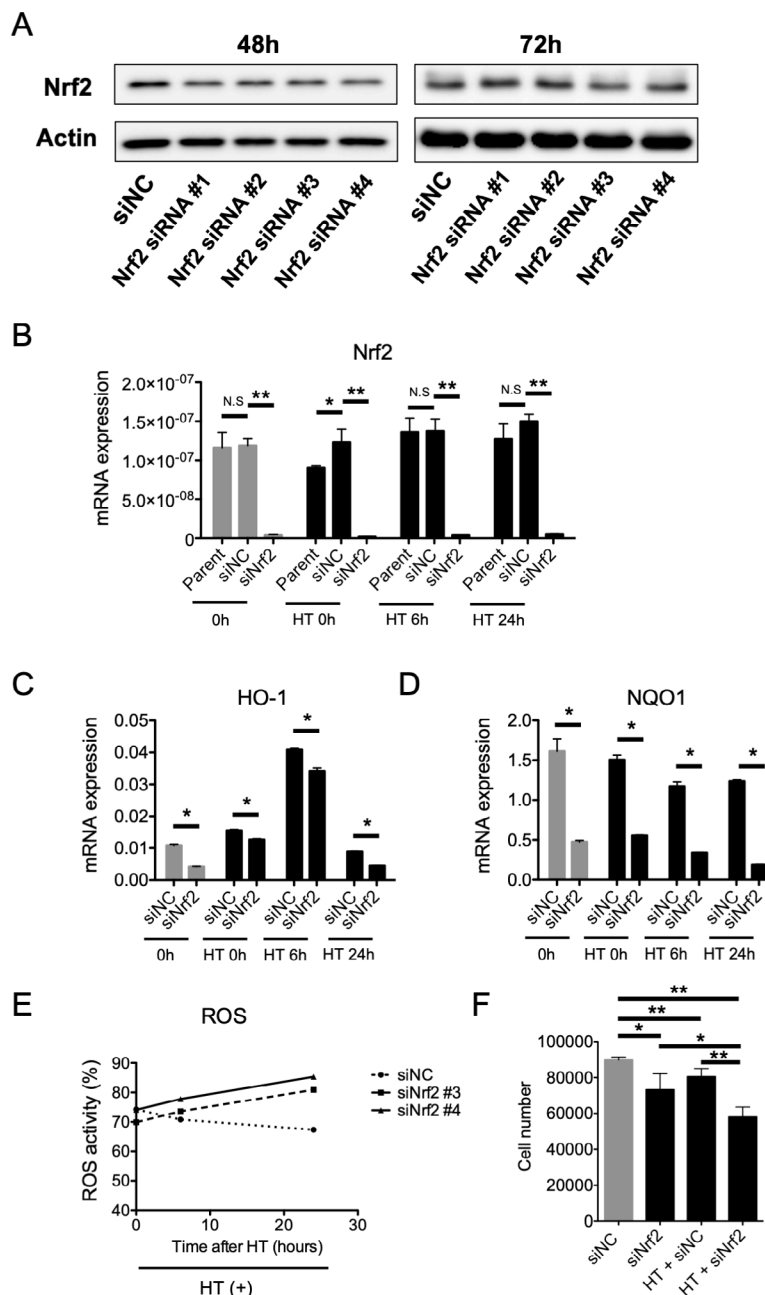
**Figure 6:** Nrf2 knockdown suppressed HO-1 and NQO1 gene expression and enhanced ROS production after hyperthermia; (A) The knockdown efficiency of siNrf2. Nrf2 protein expression was examined 48 and 72 hours after siRNA treatment. b-actin is a loading control; (B) The knockdown efficiency of siNrf2 #3. Nrf2 mRNA expression was examined by quantitative PCR in control and hyperthermia-treated cells. siRNA transfection was performed 24 hours prior to hyperthermia treatment, which was performed for 100 minutes at 43°C. Each value was normalized by the expression of GAPDH. *$P<0.05$; **$P<0.01$; HT: hyperthermia; (C, D) The expression of HO-1 mRNA (C) or NQO1 mRNA (D) in control cells and hyperthermia-treated cells with Nrf2 knockdown. siRNA transfection was performed 24 hours before hyperthermia treatment, which was performed for 100 minutes at 43°C. Each value was normalized by the GAPDH expression. *$P<0.01$; HT: hyperthermia; (E) ROS activity in control and hyperthermia-treated cells with Nrf2 knockdown. siRNA transfection was performed 24 hours prior to hyperthermia treatment, which was performed for 100 minutes at 43°C. HT: hyperthermia; (F) Cell number in si-negative control (NC) or siNrf2 treated cells with or without hyperthermia treatment. siRNA transfection was performed 24 hours prior to hyperthermia treatment, which was performed for 60 minutes at 43°C. Cell number was counted at 48 hours. *$P<0.05$; **$P<0.01$; HT: hyperthermia.

## Discussion

Hyperthermia is applicable as a combination therapy with radiation and anti-cancer drugs [5]. It converts the tumor microenvironment from hypoxia and acidic conditions by increasing blood flow and perfusion, which sensitizes cells to radiotherapy. In addition, hyperthermia may augment the cytotoxic effect of drugs, including cisplatin, doxorubicin, and mitomycin C, because of increased intracellular drug uptake and inhibition of DNA damage repair [5]. Molecular-targeted therapy, such as with small molecule inhibitors, antibodies, and nucleic acid medicine, also is anticipated to work in combination with hyperthermia. Although HSPs are the most studied molecules in thermal tolerance, and inhibitors of HSP70 and HSP90 sensitize cancer cells to hyperthermia [30,31], little is known about the molecular dynamics underlying the effects of hyperthermia.

Hyperthermia induces both necrosis and apoptosis. The type of cell death changes from apoptosis to necrosis with the temperature increase [32,33]. In this study, we hypothesized that hyperthermia might activate an anti-apoptotic mechanism and lead to thermal tolerance. We found that the anti-apoptotic protein MCL-1 was upregulated just after the hyperthermia treatment and that MCL-1 knockdown enhanced the anti-tumor effect of hyperthermia and induced apoptosis. These results indicate that the combination of hyperthermia with MCL-1 inhibitors or MCL-1 siRNA may be an option for molecular-targeted hyperthermia.

One possible mechanism for upregulation of MCL-1 by hyperthermia is the heat shock factor 1 (HSF1) and Bcl-2-associated athanogene domain 3 (BAG3) pathway. Under normal conditions, HSF1 is inactivated by interacting with HSP70 or HSP90. Under stress conditions including heat shock, HSF1 dissociates from HSPs and translocates to the nucleus [34], promoting BAG3 transcription [35,36]. BAG3 is reported to prevent MCL-1 degradation by the proteosome [37].

Our earlier studies suggested that miR-29b-3p suppresses MCL-1 expression, so we examined its effect in combination with hyperthermia. The combination treatment indeed largely augmented the anti-tumor effect of hyperthermia. However, although we found downregulation of the MCL-1 protein, it was not prominent, so we hypothesized that other mechanisms might be operating in this combination therapy. In this regard, hyperthermia may enhance efficacy of therapeutic miRNAs, and further study is essential using several potential miRNA or siRNA candidates.

Here, we focused on Nrf2, a major sensor of oxidative stress. It was recently reported that the nuclear translocation of Nrf2 was induced by hyperthermia in a temperature- and time- dependent manner in lung cancer cell lines [20] and another paper showed that increased expression and nuclear translocation of Nrf2 was induced just after hyperthermia treatment in HT22 mouse hippocampal neuronal cell line, but returned after 1 hour [21]. On the other hand, Yang et al. showed that Nrf2 was translocated from the nucleus to the cytoplasm by hyperthermia in esophageal cancer cell line [22].

Our result showed that hyperthermia treatment did not affect the Nrf2 expression itself, but induced Nrf2 nuclear translocation and it was sustained even 10 hours after hyperthermia treatment in MDA-MB231 breast cancer cell line. This difference might be caused by the cell type. Hyperthermia promoted the antioxidant gene expression thought to support cell survival. These results led us to investigate the potential of combination therapy using Nrf2 knockdown and hyperthermia. Nrf2 knockdown enhanced the anti-tumor effect of hyperthermia, which may be attributed to suppression of antioxidant genes such as HO-1 and NQO1 and increased ROS production. This result is consistent with a recent report in lung cancer cell lines [20]. We showed that hyperthermia induced HO-1 expression but not that of NQO1, which is already constitutively expressed in MDA-MB231 cells at a relatively high level. In A549 lung cancer cells, expression of NQO1 is low, and hyperthermia can induce further expression [38]. We infer that the anti-oxidative molecular machinery induced with hyperthermia differs by cancer cell type, possibly because of differences in basal expression levels.

Both MCL-1 and Nrf2 are considered to be a cytoprotective protein. Once the tumors develop, MCL-1 and Nrf2 supports cell survival by inhibiting cell death and inducing cytoprotective gene expression. Several MCL-1 inhibitors such as S64315 and AZD5991 have entered phase I clinical trials for blood cancer and expected as new anti-tumor drugs [39], but further investigation will be required. On the other hand, several Nrf2 inhibitors (ex. halofuginone and brusatol) are also studied as anti-cancer drugs [40] but have not yet reached clinical trials. In this study, we used MCL-1 siRNA, miR-29b-3p and Nrf2 siRNA in combination with hyperthermia. Nucleic acid medicine, including with therapies based on antisense oligonucleotides, siRNAs, and miRNAs, is expected to drive next-generation therapeutics. Clinical challenges with siRNA and miRNA for treatment are instability in the bloodstream and entrapment in liver and kidney. A drug delivery system for stabilizing nucleic acids and effective targeting to the tumor are thus required. Our group developed the super carbonate apatite (sCA) nanoparticle, a pH-sensitive drug delivery system, and showed that it could deliver siRNA and miRNA stably and efficiently to tumors compared to other delivery systems, such as liposome and atelocollagen, without any toxicity [23,41,42]. Although further investigation is needed, combination therapy using hyperthermia with MCL-1 siRNA or miR-29b-3p or Nrf2 siRNA loaded on sCA may be a promising and low-invasive cancer treatment candidate. In addition, it was reported that MCL-1 and Nrf2 were associated with both chemoresistance and radioresistance [19,39]. Therefore, the combination of molecular-

targeted hyperthermia and chemotherapy or radiotherapy might be also the option for cancer treatment. In order to evaluate the potential of these combination therapies, investigation of gene expression and anti-tumor effect under chemotherapy or radiotherapy is essential and this will be our future challenge.

## Conclusion

In conclusion, we showed that hyperthermia induced the anti-apoptotic protein MCL-1 and translocation of Nrf2 to the nucleus, led to downstream antioxidant gene expression, as shown in the scheme depicted in Supplementary figure S2. In this study, we used only one triple-negative breast cancer cell line which is a shortage and further investigation with other refractory cell types is essential at the next stage. Our results showing that knockdown of Nrf2 or MCL-1 or miR-29b-3p treatment enhanced the anti-tumor effect of hyperthermia may offer the first indication of the potential of molecular-targeted hyperthermia.

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