Cellular stress induces cancer stem-like cells through expression of DNAJB8 by activation of heat shock factor 1

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In a previous study, we found that DNAJB8, a heat shock protein (HSP) 40 family member is expressed in kidney cancer stem-like cells (CSC)/cancer-initiating cells (CIC) and that it has a role in the maintenance of kidney CSC/CIC. Heat shock factor (HSF) 1 is a key transcription factor for responses to stress including heat shock, and it induces HSP family expression through activation by phosphorylation. In the present study, we therefore examined whether heat shock (HS) induces CSC/CIC. We treated the human kidney cancer cell line ACHN with HS, and found that HS increased side population (SP) cells. Western blot analysis and qRT-PCR showed that HS increased the expression of DNAJB8 and SOX2. Gene knockdown experiments using siRNAs showed that the increase in SOX2 expression and SP cell ratio depends on DNAJB8 and that the increase in DNAJB8 and SOX2 depend on HSF1. Furthermore, treatment with a mammalian target of rapamycin (mTOR) inhibitor, temsirolimus, decreased the expression of DNAJB8 and SOX2 and the ratio of SP cells. Taken together, the results indicate that heat shock induces DNAJB8 by activation of HSF1 and induces cancer stem-like cells.

KEYWORDS
cancer stem-like cell, cellular stress, DNAJB8, HSF1, kidney cancer

1 INTRODUCTION

Renal cell carcinoma is a common malignancy the causes about 100 000 deaths annually worldwide.1 RCC in the early stages can be cured by surgical treatment; however, RCC in advanced stages is highly mortal because the disease is resistant to standard chemotherapies.2 CSC/CIC have emerged as a distinctive subpopulation of cancer cells that are resistant to standard treatments including chemotherapy and molecular targeted therapy.3 CSC/CIC are defined by their high tumor-initiating ability, self-renewal ability and differentiation ability.4 CSC/CIC are resistant to chemotherapy and radiotherapy and to molecular target therapy by various mechanisms, and efficient treatment for CSC/CIC is thus essential to improve current RCC therapy.
CSC/CIC can be isolated from RCC samples as CD105-positive cells, SP cells and sphere-forming cells.\textsuperscript{5–9} Previously, we successfully isolated RCC CSC/CIC as SP cells from human and mouse RCC cell lines, and we identified DNAJB8, a member of the HSP 40 family, as a protein expressed in RCC CSC/CIC.\textsuperscript{8} HSP family proteins work as molecular chaperones and play essential roles in the protection of cells under stress conditions.\textsuperscript{10} Transcription of proteins work as molecular chaperones and play essential roles in the protection of cells under stress conditions.\textsuperscript{10} We previously showed that HSF1 is phosphorylated at serine 326 residue in ovarian CSC/CIC even under non-stress conditions.\textsuperscript{12,13} A previous study showed that HSF1 has a critical role in carcinogenesis, indicating that HSF1 is related to tumor initiation.\textsuperscript{14} However, there has been no study on the relationship between a stress condition and CSC/CIC. In the present study, we therefore analyzed the relationship between cellular stress and CSC/CIC using RCC cells.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Mice were maintained and experimented on in accordance with the guidelines of and after approval by the Committee of Sapporo Medical University School of Medicine, Animal Experimentation Center under permit number 12-069. Any animal found unhealthy or sick was promptly killed.

2.2 | Cell lines, heat shock stress and oxidative stress

ACHN, a RCC cell line, was obtained from ATCC (Manassas, VA, USA) and was maintained in RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FBS. Heat shock was given by incubating the cells at 45°C for 1 hour in a prewarmed incubator. Oxidative stress was carried out as described previously.\textsuperscript{15} Briefly, the cells were treated by H2O2 at a concentration of 100μmol/L for 1 hour and SP analysis was carried out 1 day later.

2.3 | Side population analysis

Side population analysis was carried out as described previously.\textsuperscript{16} Briefly, the cells were labeled with Hoechst 33342 (Lanza, Walkersville, MD, USA) dye for 90 min at a concentration of 2.5μg/mL with or without Verapamil (Sigma-Aldrich), which is an inhibitor of ABC transporters, at concentrations of 100μmol/L. The cells were counterstained with 1μg/mL PI (Sigma-Aldrich) for labeling dead cells. Analyses and sorting were carried out with a FACS Aria II cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA).

2.4 | Xenograft transplantation in NOD/SCID mice

ACHN cells or HS-treated ACHN cells were resuspended at concentrations of $1 \times 10^4$ cells in PBS and Matrigel (BD Biosciences, San Jose, CA, USA) mixture (1:1), and were injected s.c. into the right and left mid back areas of anesthetized NOD/SCID female mice (Charles River Laboratory Japan, Yokohama, Japan) at the age of 4-6 weeks. Tumor growth was monitored weekly, and tumor volume was calculated by $XY^{2}/2$ ($X =$ long axis, $Y =$ short axis).

2.5 | Apoptosis assay

Apoptotic activity of SP and MP cells was measured using an Annexin V binding assay. After staining with Hoechst 33342, cells were stained with the Annexin V-Fluos Staining Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s instructions. In brief, cells were incubated with a staining solution containing Annexin V-fluorescein and PI for 15 minutes at room temperature. Apoptotic cells were visualized by a FACS Aria II cell sorter.

2.6 | Quantitative real-time RT-PCR analysis (qRT-PCR) and RT-PCR

Quantitative real-time PCR was carried out using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. DNAJB8 (Hs00542087_s1), SOX2 (Hs01053049_s1), and HSF1 (Hs00232134_m1) primers and probes were designed by the manufacturer (TaqMan Gene expression assays; Applied Biosystems). Thermal cycling was done using 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Each experiment was done in triplicate, and the results were normalized to the GAPDH gene as an internal control.

Expressions of DNAJB8, SOX2, POU5F1, SNAI1, SNAI2 and TWIST1 were evaluated by RT-PCR as described previously.\textsuperscript{8}

2.7 | Western blotting

Western blotting was carried out as described previously.\textsuperscript{17} Cell lysate with SDS sample buffer was separated by denaturing SDS-PAGE. Separated proteins were transferred onto nitrocellulose membranes and probed with each of the following antibodies. Anti-DNAJB8 antibody (clone #EMR-DNAJB8.214-8) was used at 200-times dilution.\textsuperscript{8} Anti-HSF1 rabbit monoclonal antibody (Abcam, Cambridge, UK) and anti-phosphoHSF1 (pSer326) rabbit polyclonal antibody (Abcam) were used at 2000-times dilution. Anti-HSP72 mouse monoclonal antibody (Enzo Life Sciences, Farmingdale, NY, USA) and anti-β-Actin mouse monoclonal antibody (Sigma-Aldrich) were used at 2000-times dilution. Anti-mouse IgG and anti-rabbit IgG second antibodies (KPL) were used at 5000-times dilution. The membrane was visualized with Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA, USA) according to the manufacturer’s instructions. In brief, the cells were incubated with a staining solution containing Annexin V-fluorescein and PI for 15 minutes at room temperature. Apoptotic cells were visualized by a FACS Aria II cell sorter.
2.8 | siRNA-mediated knockdown

DNAJB8 siRNAs (HSS136480, HSS136482 and HSS176060) were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and HSF-1 siRNAs (Hs_HSF1_7735(i); Hs_HSF1_7746(iii)) were purchased from Sigma-Aldrich. The siRNAs were transfected using Lipofectamine RNAi MAX reagent (Thermo Fisher Scientific) according to the protocol of the manufacturer. Cells were transfected with siRNA 72 hours before analysis. Non-targeting siRNA (Stealth RNAi Negative Control; Invitrogen, Carlsbad, CA, USA) was used as a negative control. DNAJB8 and HSF-1 gene knockdown was confirmed by RT-PCR.

2.9 | DNAJB8 and HSF1 overexpression

Transduction of genes into cells was carried out by a retrovirus-mediated method as described previously.18 PLAT-A cells, amphototropic packaging cells, were transiently transduced with a pMXs-puro (kind gift from Dr T. Kitamura, Tokyo, Japan) retroviral vector expressing FLAG-tagged DNAJB8 using Lipofectamine 2000 (Thermo Fisher Scientific). Retroviral supernatants were harvested 48 hours after transfection. The supernatant was used for infection of ACHN cells in the presence of 8 mg/mL polybrene (Sigma-Aldrich) overnight. For the generation of a stable transfectant, the infected cells were selected with 1 mg/mL puromycin. DNAJB8 expression was confirmed by western blot analysis.

HSF1-encoding plasmid was transfected using Lipofectamine 2000, and then the cells were selected with 1 mg/mL puromycin to establish a stable transfectant as described previously.13

2.10 | Statistical analysis

Statistical analysis was done with Stat Mate III (ATMS Co., Ltd). Data were shown as means ± SD of at least 3 independent experiments. Student’s t test was used to assess statistically significant differences (P < .05).

3 | RESULTS

3.1 | Induction of DNAJB8 by heat shock stress

Several methods for isolation of CSC/CIC have been described. In our previous study, we showed that human renal cell carcinoma stem cells can be isolated as SP cells from human kidney cancer cell line ACHN.8 DNAJB8, a member of the HSP 40 family, has a role in the maintenance of ACHN SP cells. As DNAJB8 is a HSP, we hypothesized that HS may induce SP cells through expression of DNAJB8. We thus treated ACHN cells at 45°C for 60 minutes and analyzed them (Figure 1A). Ratios of SP cells were 0.82% ± 0.10% in untreated cells and 1.77% ± 0.48% in HS-treated cells. SP cell increase was also observed in another kidney cancer cell line, Caki-1 (Figure S1). mRNA expression and protein expression of DNAJB8 and a stem cell-related marker SOX2 were examined by qRT-PCR and western blot analysis. Both DNAJB8 and SOX2 were increased at the mRNA level and protein level by HS (Figure 1B,C). HS increased the expressions of another stem cell-related marker POU5F1, and EMT markers SNAI1 and TWIST1 (Figure S2).

As CSC/CIC are defined by higher tumorigenicity, we addressed the tumorigenicity of HS-treated ACHN cells. HS-treated ACHN showed significantly higher tumorigenicity compared with the tumorigenicity of wild-type ACHN cells indicating that CSC/CIC are increased in HS-treated ACHN cells (Figure S3).

3.2 | Heat shock induces cancer stem cells from a non-stem cell population

HS increased the ratio of SP cells in ACHN cells. CSC/CIC are resistant to cellular stress,12 and there are 2 hypotheses to explain the increase of CSC/CIC by HS. One hypothesis is that non-CSC/CIC are more sensitive to HS and that only HS-resistant CSC/CIC remain after HS (hypothesis #1). The other hypothesis is that HS induces CSC/CIC from a non-CSC/CIC population (hypothesis #2) (Figure 2A). To explore the 2 hypotheses, we investigated the cell death status under the condition of HS. ACHN cells and HS-treated ACHN cells were stained with PI and Annexin-V. HS at 45°C slightly increased the ratio of necrotic cells but did not increase the ratio of apoptotic cells. HS at 50°C increased only the ratio of necrotic cells (Figure 2B).

We then examined the ratios of cells undergoing apoptotic cell death in MP cells and SP cells to address hypothesis #1. The ratios of cells undergoing apoptotic cell death in untreated SP cells and MP cells were 1.3% and 1.8%, respectively, and the ratios of cells undergoing apoptotic cell death in HS-treated SP cells and MP cells were only 1.5% and 1.5%, respectively, indicating that SP cells and MP cells subjected to HS treatment do not die by apoptosis (Figure 2C).

To address hypothesis #2, we isolated MP cells and cultured the cells for 2 weeks for them to propagate and then treated the cells with HS. The ratio of SP cells in MP cells was SP 0.22% ± 0.23%, which is comparable to the ratio of SP cells in Verapamil-treated negative control cells, indicating that there are almost no SP cells in MP cells. In contrast, the ratio of SP cells in HS-treated MP cells was SP 1.52% ± 0.38%, indicating that SP cells were induced from MP cells by HS treatment (Figure 2D). The results support hypothesis #2. There are several types of cellular stress, and we addressed whether other types of stress may induce SP cells. We treated ACHN cells with H2O2 to induce oxidative stress and observed an increase in SP cell rate (Figure S4).

3.3 | DNAJB8 has a role in the induction of SP cells under the condition of HS treatment

We previously found that DNAJB8, a member of the HSP 40 family, was expressed preferentially in CSC/CIC and that DNAJB8 has a role in the maintenance of cancer stem cells.8 We thus analyzed...
FIGURE 1  Induction of cancer stem cells by heat shock. A, Cancer-initiating cell/cancer stem-like cell (CSC/CIC) induction by heat shock. The human renal cell carcinoma (RCC) cell line ACHN was treated at 45°C for 60 min and then side population (SP) cell analysis was carried out (n = 3). Percentages indicate SP cell ratios. Verapamil was used as a negative control for SP analysis. Right panel: SP cell ratios are shown in a graph. Data are shown as means ± SD. Asterisk indicates statistically significant differences. B, mRNA expression of DNAJB8 and SOX2. mRNA expression of DNAJB8 and SOX2 was examined by qRT-PCR. ACHN cells were treated at 45°C for 60 min and then cultured for 0, 24, 48 and 72 h. Non-treated cells were used as negative control cells. Data are shown as means ± SD. Asterisks indicate statistically significant differences. C, Protein expression of DNAJB8 and SOX2. Protein expression of DNAJB8 and SOX2 was examined by western blot analysis. ACHN cells were treated at 45°C for 60 min and then cultured for 0, 24, 48 and 72 h. Non-treated cells were used as negative control cells. Numerical data indicate relative intensity of the bands determined by ImageJ software.

FIGURE 2  Cancer-initiating cells/cancer stem-like cells (CSC/CIC) are induced from non-CSC/CIC by heat shock. A, Two hypotheses that are related to induction of CSC/CIC. Hypothesis #1: Main population (MP) cells may be sensitive to heat shock (HS) and side population (SP) cells tend to survive HS. Hypothesis #2: SP cells may be induced from differentiated MP cells by HS. B, Detection of dead cells by FACS. Heat shock-treated ACHN cells were stained with Annexin-V and propidium iodide (PI) and then analyzed by FACS. Annexin-V-positive, PI-negative cells are apoptotic cells. PI-positive cells are necrotic cells. C, Detection of apoptotic cells in SP cells and MP cells under an untreated condition and an HS condition. ACHN cells were stained with Hoechst 33342, Annexin-V and PI. SP cells and MP cells were separated by FACS and then the apoptotic cell ratio was examined. Upper, untreated cells; lower, HS-treated cells. D, Induction of SP cells by HS from isolated MP cells. MP cells were isolated and the cells were cultured for 2 weeks, and then MP cells were treated by HS and SP analysis was carried out (n = 3). Wild-type ACHN cells were used as control cells. Right panel, SP cell ratios are shown in a graph. Data are shown as means ± SD. Asterisk indicates statistically significant differences.
the function of DNAJB8 in HS-induced SP cells by gene knockdown using siRNA. DNAJB8 mRNA was significantly decreased by siRNA transfection (Figure 3A). DNAJB8 knockdown decreased the expression of SOX2 in both untreated cells and HS-treated cells (Figure 3B). The ratio of SP cells was increased in control siRNA-transfected ACHN cells by HS treatment (from 1.4% to 3.4%), but the increase in the ratio of SP cells was smaller in DNAJB8 siRNA-transfected ACHN cells (from 1.1% to 1.5%) (Figure 3C), indicating that DNAJB8 has a role in the increase of SP cells under HS treatment.

3.4 Activated HSF1 induced SP cells

The transcription factor HSF1 is a key molecule for stress responses, and recent studies have shown that HSF1 is related to carcinogenesis.19 The expression level of HSF1 mRNA was slightly increased by HS treatment (Figure 4A). Phosphorylation status at serine 326 residue was increased by HS treatment, whereas total HSF1 protein expression level was not changed by HS treatment (Figure 4B). To address the functions of HSF1 in induction of DNAJB8 and SOX2 under the conditions of HS treatment, we knocked down HSF1 by
siRNAs (Figure 4C). Knockdown of HSF1 decreased the expression of DNAJB8 under HS treatment, whereas knockdown of HSF1 did not change the expression of DNAJB8 in untreated cells (Figure 4D). Knockdown of HSF1 decreased the expression of SOX2 in both untreated cells and HS-treated cells (Figure 4E).

To further confirm the function of HSF1 in HS-induced SP cells, we treated HSF1-overexpressed cells with HS. Overexpression of HSF1 and phosphorylation status under the condition of HS treatment were confirmed by western blot analysis (Figure 4F). The ratio of SP cells was increased by overexpression of HSF1 (from 0.2% to 1.1%). The ratios of SP cells were increased by HS treatment (Figure 4G), indicating that HSF1 has a role in induction of DNAJB8 and SOX2 and increase in SP cells under the condition of HS treatment.

### 3.5 mTOR activates HSF1 and induces SP cells

It is known that phosphorylation of HSF1 at serine 326 residue requires mTOR protein kinase. We thus used an mTOR inhibitor,
temsirolimus, to investigate the functions of mTOR in induction of DNAJB8 and SP cells. Treatment with temsirolimus decreased phosphorylated HSF1 at serine 326 residue (Figure 5A). Treatment with temsirolimus significantly decreased the expression levels of DNAJB8 and SOX2 (Figure 5B). Furthermore, temsirolimus treatment decreased the ratio of SP cells (Figure 5C). These results indicate that phosphorylation of HSF1 at serine 326 residue has a role in the expression of DNAJB8 and SOX2 and induction of SP cells under the condition of HS treatment.

**DISCUSSION**

Cancer stem-like cells/cancer-initiating cells comprise a small subpopulation of cancer cells that are defined by their high tumor-initiating ability. The highly tumorigenic subpopulation can be isolated by several methods including SP analysis, ALDEFLUOR assay, use of cell surface markers and sphere-forming culture.²¹,²² CSC/CIC make a hierarchical differentiation model like normal stem cells;²³ however, recent studies have shown that CSC/CIC can be produced from non-CSC/CIC by several types of stimulation including tumor microenvironment and oxidative stress.¹⁵,²⁴-²⁷ HSF1 is a key transcription factor for stress response,¹¹ and recent studies have shown that HSF1 has a critical role in tumor initiation in several models.¹⁴,¹⁹ Interestingly, the transcriptional pattern was shown to be different in non-cancer cells under a stress condition and cancer cells.²⁸

Several markers have been used for isolation of kidney cancer stem cells.²⁹ In the present study, we showed that cell stress induces SP cells. Furthermore, HS increased tumorigenicity of ACHN cells. In this study, we did not address other CSC/CIC markers; however, expression of other stem cell-related markers including CD44, CD73, CD105 and CD133 might be increased by HS, because the stem cell-related marker-positive subpopulation shows higher tumorigenicity as do the cells treated by HS. Interestingly, kidney cancer stem cells defined by the expression of CD44 are induced by TNF-α that is secreted from TAM.³⁰ Likewise, DAMP are known to induce acute inflammation, and inflammatory cytokines are related to induce CSC/CIC. Thus, the tumor microenvironment, including TAM and
inflammation, might also be an important factor for the induction of CSC/CIC.

In our previous study, we showed that HSF1 is phosphorylated at serine 303, 320 and 326 residues in ALDH high CSC/CIC isolated from gynecological cancers even in a non-stress condition.\(^\text{13}\) Furthermore, HSP27, a member of the small HSP family, is expressed in CSC/CIC and has a role in the maintenance of CSC/CIC. Phosphorylation of HSF1 at serine 326 residue is related to the expression of HSP27 and the maintenance of CSC/CIC. As HSF1 is phosphorylated by cellular stresses including HS, we hypothesized that HS may induce CSC/CIC. In the present study, we showed that HS induces SP cells of ACHN human RCC cells. Interestingly, HS can induce SP cells from isolated MP cells, indicating that CSC/CIC can be induced from non-CSC/CIC by cellular stress. CSC/CIC could be induced by oxidative stress in ACHN cells (data not shown), indicating that several cellular stresses that can activate HSF1 may be involved in the de-differentiation of non-CSC/CIC. CSC/CIC have been shown to be resistant to cellular stresses including chemotherapy, radiotherapy and molecular targeted therapy.\(^\text{31}\) However, our observations indicate that cellular stress is also responsible for inducing highly tumorigenic CSC/CIC from non-CSC/CIC. An anti-de-differentiation approach may emerge as a new aspect of CSC/CIC-targeting therapy.

A previous study showed that the kinase complex mTOR has a role in phosphorylation of HSF1 at serine 326 residue under the condition of HS.\(^\text{20}\) However, we previously showed that HSF1 serine 326 residue is phosphorylated in CSC/CIC under a non-stress condition.\(^\text{12,13}\) Similarly, several HSP have been reported to be related to CSC/CIC under a non-stress condition.\(^\text{8,13,32-34}\) Our results showed that cellular stress induces CSC/CIC-related DNAJB8 by activation of HSF1. Therefore, both constitutively activated HSF1 and HSF1 activated by cellular stress can induce CSC/CIC. As the molecular functions of DNAJB8 are still elusive, our results indicated that DNAJB8 might induce expression of the stem cell-related transcription factor SOX2 (Figure 6). Therefore, the HSF1/DNAJB8/SOX2 axis might be important for induction of CSC/CIC by cellular stress (Figure 6). As HSF1 and its downstream HSPs comprise a common anti-stress machinery, it is difficult to target HSF1/HSP. However, DNAJB8 is a CSC/CIC-specific HSP and can be targeted. CTL-based cancer immunotherapy is a promising approach for targeting DNAJB8.\(^\text{35}\) DNAJB8-targeted cancer immunotherapy might be effective for inducing CSC/CIC by cellular stress. A recent review article pointed out that niche signals should be targeted in CSC/CIC induced by niche signals.\(^\text{36}\) In this stress-induced CSC/CIC, HSF1 signal should be targeted. In this regard, DNAJB8 is a reasonable target for CSC/CIC-targeting therapy.

In summary, we found that cellular stress induces CSC/CIC from non-CSC/CIC. HSF1 is a key transcription factor for inducing the expression of DNAJB8 and SOX2. The kinase complex mTOR has a role in activation of HSF1 and induction of CSC/CIC (Figure 6). Our observations show a novel aspect of CSC/CIC, and a stress response signal might emerge as a novel signal for CSC/CIC-targeting therapy.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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