

Deactivation of glycogen synthase kinase-3 β by heat shock-inducible tumor small protein attenuates hyperthermia-induced pro-migratory activity in colorectal cancer cells

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Abstract. Hyperthermia is a promising approach for improving cancer treatment in combination with chemotherapy, radiotherapy and/or immunotherapy; however, its molecular mechanisms remain unclear. Although heat shock proteins (HSPs) are involved in hyperthermia via antigen presentation and immune activation, major HSPs including HSP90 are associated with cancer progression via tumor cell migration and metastasis. The present study showed that heat shock-inducible tumor small protein (HITS) could counteract the pro-migratory effects of HSPs in colorectal cancer (CRC) cells, which represents a novel function. Western blotting analysis revealed that overexpression of HITS increased the protein level of glycogen synthase kinase-3 β (GSK3 β) phosphorylated (p) at the serine 9 (pGSK3 β ^{S9}; inactive form) in HCT 116, RKO and SW480 CRC cells. GSK3 β ^{S9} phosphorylation was reported to suppress migration in some cancer types; therefore, by using the wound healing assay, the present study revealed that HITS overexpression decreased the migration activity of CRC cells. Induction of HITS transcription was observed at 12 and 18 h after heat shock (HS) by using semi-quantitative reverse transcription-PCR analysis, followed by increased levels of pGSK3 β ^{S9} protein at 24 and 30 h in CRC cells in western blotting. Thus, HS

induced not only HSPs to promote cell migration, but also HITS to counteract the migratory activity of these HSPs in CRC cells. HITS knockdown in CRC cells subject to HS showed increased cell migration in wound healing assay, which was decreased by the GSK3 β inhibitor AR-A014418, confirming the anti-migratory effect of HITS via the deactivation of GSK3 β . The present findings indicated that the deactivation of GSK3 β sufficiently offset the pro-migratory effect of hyperthermia via major HSPs in CRC.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer type and the second leading cause of cancer-related deaths worldwide (1,2). Patients with CRC develop metastatic disease in >50% of cases, especially in the liver, which results in the death of >2/3 of the patients (3-5). Advances in chemotherapy, radiotherapy and immunotherapy have improved the survival of patients with metastatic CRC (6,7); however, resistance to anticancer drugs, radiation and immune therapies remains a major therapeutic challenge (7-9).

Increasing evidence has demonstrated that the combination of hyperthermia with the aforementioned therapies can overcome tumor resistance to antitumor treatments and improve their efficacy (10-12). In addition, heat shock (HS) proteins (HSPs) are involved in enhancing the cytotoxic activity of natural killer (NK) cells (11,13), inducing maturation and antigen presentation of dendritic cells and activating T-cells (11,14). By contrast, previous studies have indicated that HSPs including HSP90 promote cancer cell proliferation, invasion and metastasis, as well as tumor angiogenesis, which can negatively affect the hyperthermia efficacy (15,16). These major HSPs are highly expressed in some malignant tumor types and are inversely correlated with prognosis, leading to these HSPs being used as therapeutic targets (15,16). Nonetheless, the overall involvement of HSPs in hyperthermia remains unclear.

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Heat shock-inducible tumor small protein (HITS) is an 18 kDa protein that was originally identified as a molecule upregulated upon HS treatment in Jurkat cells (17). The induction of HITS protein as well as HSP90 protein has been confirmed in THP-1 cells *in vitro* and transplanted rat walker 256 sarcoma cells *in vivo* (17). HITS is highly homologous to downregulated in renal cell carcinoma 1 (DRR1), a putative tumor suppressor that plays important roles in actin and microtubule cytoskeleton organization, and is downregulated in renal cell carcinoma (17-19). Our previous studies indicate that HITS expression can be observed in various cancer cells and is downregulated during tumor progression in colon cancer as well as cervix, thyroid and breast cancers compared with the corresponding healthy tissues (17,20). Unlike the major HSPs, overexpression of HITS shows tumor suppressive phenotype in the mouse cervical cancer xenograft model (20).

The present study showed that HITS overexpression increased the levels of glycogen synthase kinase-3 β (GSK3 β) phosphorylated (p) at serine (S) 9 (pGSK3 β ^{S9}), resulting in its deactivation in CRC cells (21-23). To the best of our knowledge, while GSK3 β deactivation has been reported to prevent β -catenin degeneration (23), no previous studies have shown a direct association of cellular GSK3 β deactivation with cancer development or progression (22). GSK3 β does not participate in the canonical β -catenin destruction complex in the majority of CRCs due to the mutations in either adenomatous polyposis coli (APC; <90% of cases), catenin β -1 (CTNNB1; ~5% of cases) or axis inhibition protein 1 (AXIN1) (22). By contrast, previous studies have demonstrated that pharmacological inhibition of GSK3 β activity suppresses cancer progression by attenuating tumor cell migration and invasion in several cancer types, including colorectal, breast and pancreatic cancers, in addition to glioblastoma (21-29). The present study showed that HITS upregulation induced by HS exerted an anti-migratory effect via GSK3 β deactivation (S9 phosphorylation) in human CRCs, thereby counteracting the pro-migratory effects of HSPs. This novel anti-migratory mechanism may play an important role in reducing the risk of cancer metastasis during hyperthermia.

Materials and methods

Cell culture. HCT 116, RKO and SW480 cells were purchased from American Type Culture Collection and maintained in Dulbecco's Modified Eagle's Medium-high glucose (cat. no. 043-30085; Fujifilm Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum (FBS) (cat. no. F7524; Sigma-Aldrich; Merck KGaA) in a humidified incubator with 5% CO₂ at 37°C. Cells were tested for mycoplasma contamination.

Western blotting. Cells were lysed with 2X sample buffer containing 4% SDS (cat. no. 08933-34; Nacalai Tesque, Inc.), 20% glycerol (cat. no. 17018-25; Nacalai Tesque, Inc.), 0.001% bromophenol blue (cat. no. 05808-61; Nacalai Tesque, Inc.), 0.125 M Tris HCl (cat. no. T1503, Sigma-Aldrich; Merck KGaA) and 10% 2-mercaptoethanol (cat. no. 21418-42; Nacalai Tesque, Inc.) and boiled for 5 min. The protein concentration was measured using BCA Protein Assay Kit (cat. no. 23225; Thermo Fisher Scientific, Inc.). Lysates (20 μ g/lane) were

separated by SDS-PAGE on an 8.5% gel and transferred onto PVDF membrane (cat. no. IPVH00010; MilliporeSigma). To calculate relative intensity, cell lysates to compare were loaded in the same gel. Membranes were blocked with phosphate-buffered saline [PBT; 0.01 M Na₂HPO₄; cat. no. 31801-05 and KH₂PO₄; cat. no. 28721-55; pH 7.4 with 0.15 M NaCl; cat. no. 31320-05; and 0.1% Tween-20 (cat. no. 35624-02; Nacalai Tesque, Inc.)] containing 5% non-fat dry milk or 5% bovine serum albumin (cat. no. 019-21272; Fujifilm Wako Pure Chemical Corporation) for 1 h at room temperature. After washing with PBT, the membranes were incubated with the primary antibodies against GSK3 β (1:20,000 or 40,000; cat. no. 9315; Cell Signaling Technology, Inc.), pGSK3 β ^{S9} (1:1,000 or 2,000; cat. no. 9336; Cell Signaling Technology, Inc.) and β -tubulin (1:120,000 or 40,000; cat. no. 017-25031; Fujifilm Wako Pure Chemical Corporation) overnight at 4°C. This was followed by incubation with anti-rabbit (1:4,000 dilution; cat. no. 5220-0336; SeraCare Life Sciences, Inc.) and anti-mouse (1:4,000 dilution; cat. no. 5220-0341; SeraCare Life Sciences, Inc.) horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The protein bands were developed using the ECL Prime Western Blotting Detection Reagent (cat. no. RPN2232; Cytiva) and the chemiluminescence was detected using a ChemiDoc Touch MP Imaging System with Image Touch 2.4 software (Bio-Rad Laboratories, Inc.) or C-Digit Blot scanner (LI-COR Biosciences).

RNA extraction and semi-quantitative reverse transcription (RT)-PCR. Total RNA was extracted from cells using TRIzol™ (cat. no. 15596026; Thermo Fisher Scientific, Inc.) and reverse-transcribed to complementary DNA (cDNA) using ReverTra Ace™ (cat. no. FSQ-201; Toyobo Life Science) with Random Primer (cat. no. FSK-301; Toyobo Life Science) according to the manufacturer's instructions. Semi-quantitative RT-PCR was performed using a LifeECO thermal cycler (Yakukensha Co., Ltd.). The reaction mixture comprised 0.25 μ l Blend Taq plus (2.5 U/ μ l; cat. no. BTQ-201; Toyobo Life Science), 2.5 μ l 10X buffer, 2.5 μ l dNTP (2 mM; cat. no. NTP-501; Toyobo), 0.25 μ l primers (10 μ M; cat. no. FSK-301; Toyobo) and 1-3 μ l of synthesized cDNA. The following primer pairs were used for semi-quantitative RT-PCR: HITS forward, 5'-CCACCTGAGGATATTGACCATAA-3' and reverse, 5'-TTCTGTGCTTCTTCTCCTTCTG-3'; matrix metalloproteinase-3 (MMP-3), forward, 5'-CTCAGGAAGCTTGAACCTGAAT-3' and reverse, 5'-CAGCTCGTACCTCATTTCCTCT-3'; MMP-13, forward, 5'-TTACCAGTCTCCGAGGAGAAAC-3' and reverse, 5'-TTTTGGAAGACCCAGTTCAGAT-3'; and TATA-box binding protein (TBP) forward, 5'-AGAAAGTGAACATCATGGATCAGA-3' and reverse, 5'-GTTTACAACCAAGATTCAGTGTGG-3'. TBP was amplified as an internal control to normalize the expression of the target gene. The PCR reaction was performed under the following conditions: Initial denaturation at 94°C for 1 min; followed by cycling at 94°C for 20 sec, 55°C (HITS, MMP-13 and TBP) or 53°C (MMP-3) for 20 sec and 72°C for 20 sec; and final extension at 72°C for 1 min. The numbers of cycles were 29 and 30 for HITS, TBP; 36 for MMP-3, 36 for MMP-13 and 33 for TBP. The DNA products were run on a 1.2% agarose gel containing ethidium bromide (cat. no. 312-01193; Nippon Gene Co., Ltd.) and imaged using a ChemiDoc MP Imaging

System (Bio-Rad Laboratories, Inc.). The intensity of each band was quantified using ImageJ software, ver. 1.50 (National Institutes of Health).

Plasmid construction and overexpression. The amino acid sequence of the mouse HITS open reading frame (ORF) is 98.47% homologous to the human HITS; therefore, mouse HITS ORF was cloned for transfection (17). The HITS ORF fragment with EcoRV and BamHI ends was amplified from HeLa-Tet-HITS (16) using PCR (initial denaturation at 94°C for 1 min; 25 cycles of 94°C for 20 sec, 55°C for 20 sec and 72°C for 20 sec; and a final extension at 72°C for 1 min) and the following primers: forward, 5'-GATATCATGGCTGAGCCA GACTACATAGAAG-3', and reverse, 5'-GGATCCCTAGGA CTCCTGGGCCTGAGCCACC-3'. The amplified DNA was cloned into PCR2.1 vector using the TOPO™ TAT™ Cloning Kit (cat. no. K450002; Thermo Fisher Scientific, Inc.) and subcloned into the EcoRV/BamHI sites of pCAG-IRES-EGFP, which was kindly supplied by Dr T. Kawauchi (30). The generated plasmid (0.5 µg/well), namely pCAG-HITS-IRES-EGFP, and pEGFP (30) were mixed with transfection reagents and kept for 10 min then transfected into HCT 116, RKO and SW480 cells, using Lipofectamine 3000™ (cat. no. L3000001; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The cells were incubated for 36 h and obtained cell lysates for western blotting, wound healing assay or MTT assay as described later. Transfection efficiency was tested using semi-quantitative RT-PCR as described in the previous section (Fig. S1).

RNA interference (RNAi). The sequences of small interfering RNAs (siRNAs) targeting HITS were as follows: HITS-siRNA#1, 5'-GGAUUAUUGACCAUAAGGACUC AUAU-3'; and HITS-siRNA#2, 5'-GCCUCAGAAACU GAUCAAUCCUGUA-3' (Thermo Fisher Scientific, Inc.). Lipofectamine™ RNAiMAX (cat. no. 13778030; Thermo Fisher Scientific, Inc.) was used to transfect HITS siRNAs and control siRNA (cat. no. 12935300; Thermo Fisher Scientific, Inc.). A total of 10 pmol/well siRNAs were mixed with transfection reagents and kept for 5 min according to the manufacturer's instructions. The cells were incubated for 36 h and obtained cell lysates for western blotting or wound healing assay as described later.

Wound healing assay. HCT 116 cells were seeded at a density of 5×10^5 cells/well in a 24-well plate and transfected with HITS the following day. At 10 h after transfection, the cells were trypsinized, re-seeded at a density of 8×10^5 cells/well, and incubated for 18 h (the confluency of the cells reached 80-90%). The cells were then scratched with a 200-µl pipette tip and the medium was replaced to medium containing 1% FBS to prevent the loose cells from settling back down. Images of the cells were captured at 0 and 24 h under a phase-contrast and fluorescence microscope (BZ-X700; Keyence Corp.). Multiple images were captured with the microscope to cover an entire scratched area and merged into one image to compare the same wound gap positions between 0 and 24 h. The wound healing rate was calculated as follows: Wound healing rate (%) = (wound area at 0 h - wound area at 24 h) / (initial wound at 0 h) $\times 100$. For the assay performed on HS-induced cells (HS at 42°C for 1 h), 8.5×10^5 cells/well were plated. For RNAi followed by HS and

inhibitors treatment, the CytoSelect 24-well Wound Healing Assay Kit (Cell Biolabs, Inc.) was used. Plastic inserts provided with the kit were placed onto the wells to create wound gaps where cells were plated at a density of 8.5×10^5 cells/well. Some groups of cells were treated with the HSP90 inhibitor 17-AAG (cat. no. CS-0161; Funakoshi Co., Ltd.) or the GSK3β inhibitor AR-A014418 (cat. no. A3230; Sigma-Aldrich; Merck KGaA), both of which were dissolved in dimethyl sulfoxide (DMSO) to prepare 10 mM stock solutions. DMSO was added to control and 17-AAG samples to adjust the DMSO concentration to 0.2%. The concentration of 17-AAG was optimized by testing different concentrations, which showed suppression of cell migration but no effect against cell survival (data not shown). The concentration of AR-A014418 was determined based on our previous studies (29).

MTT assay. HCT 116 cells were seeded at a density of 1×10^4 cells/well in a 96-well plate. The expression vectors pCAG-HITS-IRES-EGFP and pEGFP were transfected into the cells the following day, as previously described. The MTT assay (cat. no. 11465007001; Sigma-Aldrich; Merck KGaA) was performed 2 days after transfection, according to the manufacturer's instructions. Methanol was used to dissolve formazan. Colorimetric detection was done at a wavelength of 550 nm and reference wavelength was 690 nm.

Statistical analysis. Data are expressed as the mean $\pm$ standard error. Statistical analysis was performed using the unpaired Student's t-test to compare two groups of data and one-way analysis of variance (ANOVA) followed by Dunnett's test or two-way ANOVA followed by Tukey's test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

HITS promotes the phosphorylation of GSK3β S9 in CRC cells. The effects of HITS on GSK3β, which plays important roles in tumorigenesis (21,22), were investigated to study the role of HITS in cancer progression. Overexpression of HITS in HCT 116, RKO and SW480 cells were followed by a significant increase in pGSK3β^{S9} level, while no significant changes were observed in the expression of GSK3β (Fig. 1A-C) nor in the levels of GSK3β phosphorylation at tyrosine (Y)216 (pGSK3β^{Y216}; active form; data not shown).

HCT 116 is one of the human CRC cell lines that were established to extrapolate clinical CRC (31,32). Our previous studies showed the distinct and common tumor-promoting roles of active GSK3β (lower and higher phosphorylation of its S9 and Y216 residues, respectively) in multiple human CRC cell lines including HCT 116 cells (29,33-35). In addition, no biological association between the pathological (tumor-promoting) property of deregulated GSK3β and the pathways mediated by activated β-catenin and phosphoinositide 3-kinase (PI3K)/Akt in human CRC cell lines including HCT 116 cells as well as in clinical CRC tumors (29). Therefore, the present study used HCT 116 cells for the subsequent analyses.

To confirm the effects of HITS on the expression and phosphorylation of GSK3β, HITS RNAi was performed on CRC cells. Semi-quantitative RT-PCR indicated that transfection with

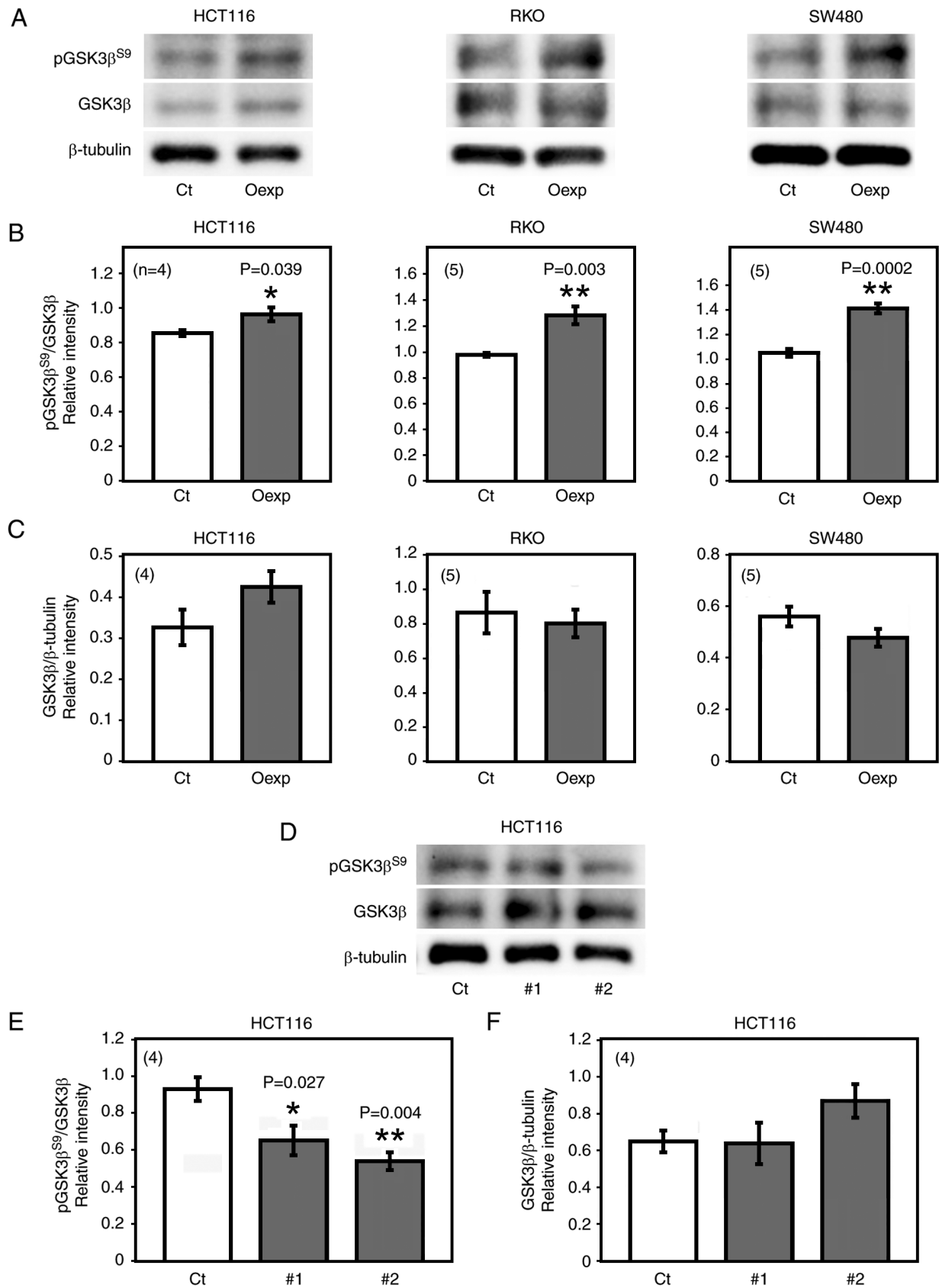


Figure 1. Analysis of HITS overexpression or knockdown effects on GSK3 β . (A-C) Western blotting analysis of HCT 116, RKO and SW480 colon cancer cells transfected with pEGFP (Ct) or pCAG-HITS-IRES-EGFP (Oexp) vector. (A) Expression of β -tubulin was monitored as the loading control. (B) Level of pGSK3 β ^{S9} was normalized to the amount of GSK3 β . (C) Relative level of GSK3 β expression was normalized to the β -tubulin expression in the same cells. (D) Western blotting analysis of the levels of pGSK3 β ^{S9} and GSK3 β expression in HCT 116 cells transfected with control stealth siRNA (Ct), HITS-siRNA#1 (#1) or HITS-siRNA#2 (#2). Expression of β -tubulin was monitored as the loading control. The levels of (E) pGSK3 β ^{S9}/GSK3 β and (F) pGSK3 β / β -tubulin were normalized. The number of replicates is indicated in parenthesis in each panel. * P <0.05 and ** P <0.01 compared with Ct group. HITS, heat shock-inducible tumor small protein; GSK3 β , glycogen synthase kinase-3 β ; p, phosphorylated; EGFP, enhanced green fluorescent protein; S, serine; siRNA, short interfering RNA; Oexp, overexpression.

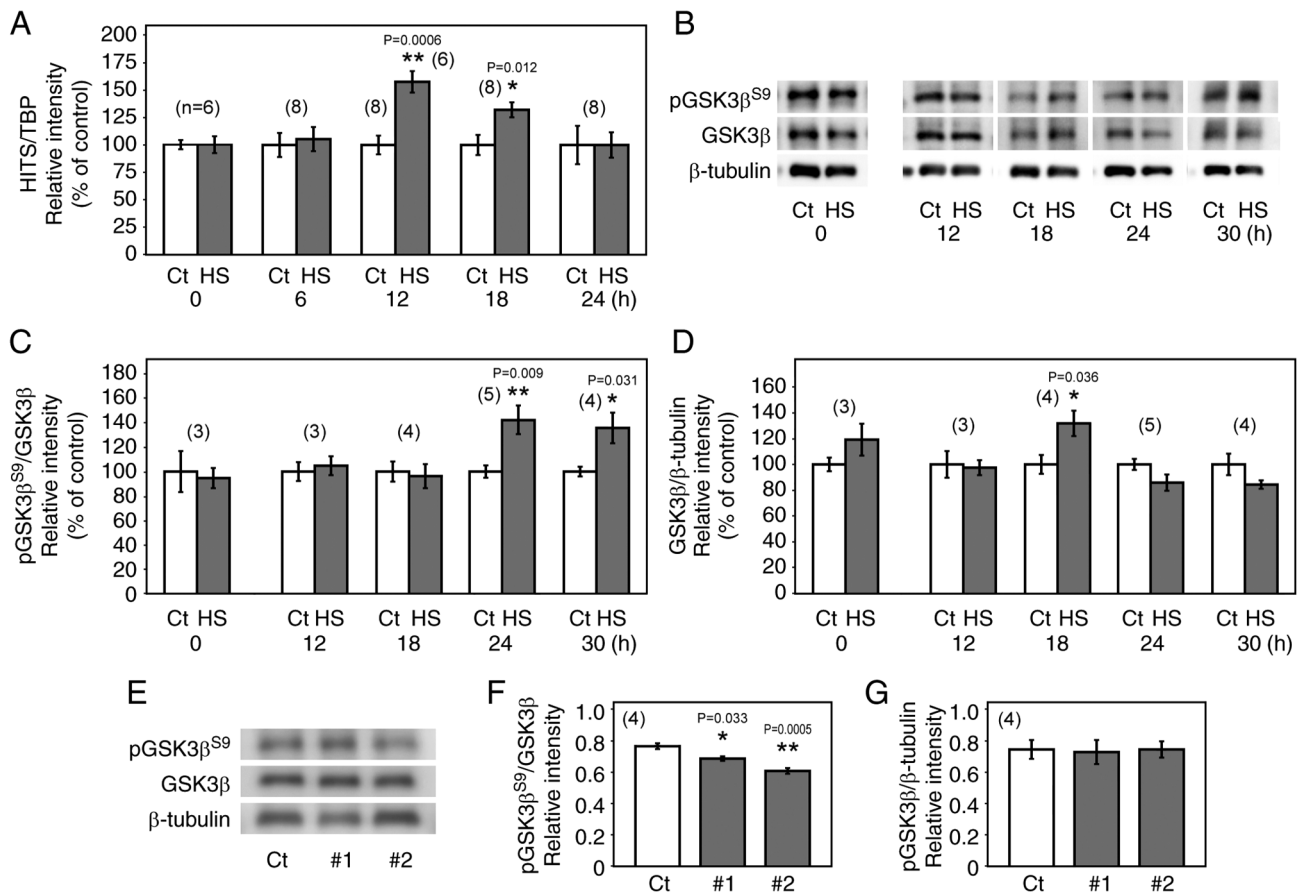


Figure 2. HS effects on HITS transcription and GSK3 β protein in HCT 116 cells. (A) Relative levels of HITS mRNA at several time points following treatment with HS compared with those in the untreated Ct cells. (B) Western blotting and subsequent statistical analysis of the protein levels of (C) pGSK3 β ^{S9}/GSK3 β and (D) GSK3 β / β -tubulin at several time points following treatment with HS compared with those in the untreated Ct cells. (E) Western blotting and subsequent statistical analysis of the protein levels of (F) pGSK3 β ^{S9}/GSK3 β and (G) GSK3 β / β -tubulin in cells transfected with control siRNA (Ct), HITS-siRNA#1 (#1) or HITS-siRNA#2 (#2), followed by HS and measured after 24 h. (B and E) Expression of β -tubulin was monitored as the loading control. Relative levels of (C and F) pGSK3 β ^{S9} and (D and G) GSK3 β expression in each sample were normalized to the amounts of GSK3 β and β -tubulin, respectively. The number of replicates is indicated in parenthesis in each panel. * $P < 0.05$ and ** $P < 0.01$ compared against respective Ct groups. HITS, heat shock-inducible tumor small protein; GSK3 β , glycogen synthase kinase- β ; HS, heat shock; Ct, control; p, phosphorylated; siRNA, short interfering RNA.

either HITS siRNA#1 or HITS siRNA#2 significantly reduced HITS mRNA expression by >90% compared with the that in cells transfected with control siRNA (Fig. S2). Transfection of these HITS siRNAs in HCT 116 cells significantly decreased the level of pGSK3 β ^{S9} compared with that in cells transfected with control siRNA, but did not affect the GSK3 β expression (Fig. 1D-F). The present data demonstrated that HITS promoted the phosphorylation of GSK3 β at S9 in CRC cells.

HS induces GSK3 β S9 phosphorylation via HITS upregulation. Our previous study showed that HS induced HITS protein expression both *in vitro* and *in vivo* (17). To study the transcriptional response to HS in detail, the levels of HITS mRNA were monitored in HCT 116 cells in response to HS (42°C, 1 h). Semi-quantitative RT-PCR analysis showed that the level of HITS mRNA increased relatively slowly following HS, with its expression peaking at 12 h after HS (Fig. 2A). The level of pGSK3 β ^{S9} in the cells, measured using western blotting, increased significantly at 24 and 30 h after HS compared with their respective controls (Fig. 2B and C). This was likely caused by HITS upregulation in response to HS, since HITS knockdown in the heat-shocked cells significantly decreased

the level of pGSK3 β ^{S9} at 24 h after HS compared with the control group (Fig. 2E and F). Significantly increased expression of GSK3 β was observed 18 h after HS compared with the control (Fig. 2B and D); however, HITS knockdown induced no significant change in the GSK3 β expression (Fig. 2E and G).

HITS suppresses cell migration but not proliferation. It is hypothesized that HITS is involved in suppressing tumor progression via phosphorylation-dependent deactivation of GSK3 β . To investigate the effects of HITS on the migration of CRC cells, a wound healing assay was performed on HCT 116 cells transfected with a pEGFP control vector expressing GFP or pCAG-HITS-IRES-EGFP vector expressing both HITS and GFP. Compared with that in the control cells, cells overexpressing HITS showed a significant suppression of cell migration (Fig. 3A and B). This was not due to a cell proliferation suppression caused by HITS because no significant changes were observed in cell proliferation upon HITS overexpression (Fig. 3C).

MMPs are key proteases involved in cancer cell migration, invasion and metastasis by degrading the extracellular matrix (ECM) (36). MMP-3 and MMP-13 have been reported

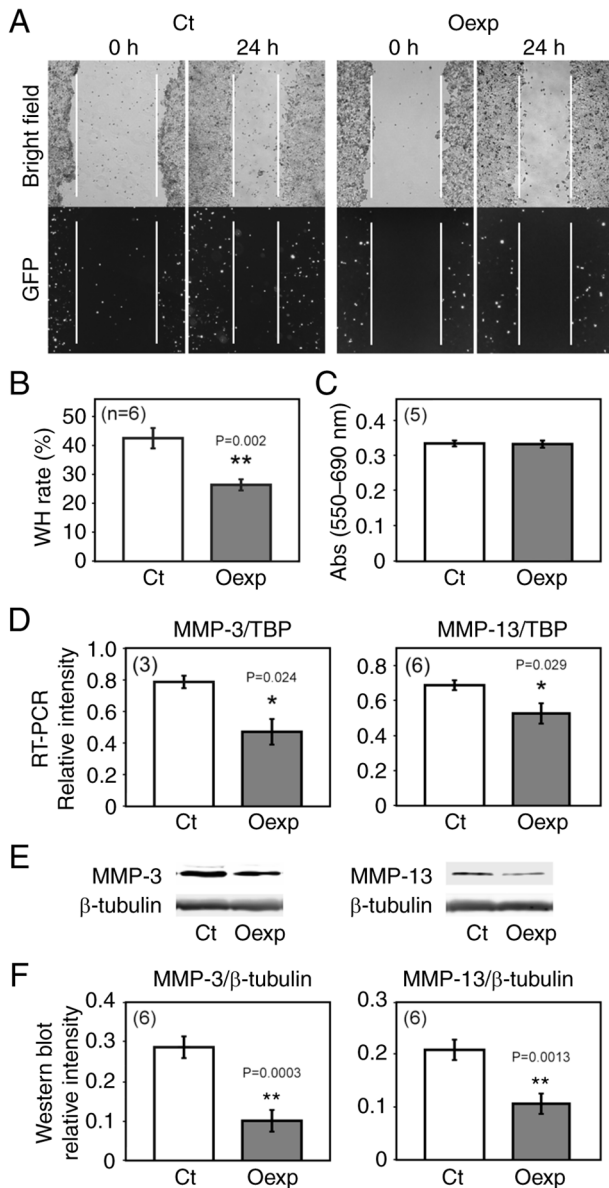


Figure 3. Effects of HITS on the migration and the proliferation of HCT 116 cells. (A and B) Wound healing assay was performed to compare the migration of cells transfected with pEGFP (Ct) or pCAG-HITS-IRES-EGFP (Oexp) vector. (A) Representative of bright field images (upper panels) and fluorescence images (lower panels) showing GFP-positive cells. (B) Comparison of the WH rate between cells transfected with pEGFP (Ct) or pCAG-HITS-IRES-EGFP (Oexp) vector. (C) Comparison of cell survival measured using the MTT assay between cells transfected with pEGFP (Ct) or pCAG-HITS-IRES-EGFP (Oexp) vector. (D) Comparative semi-quantitative RT-PCR analysis of MMP-3 and MMP-13 mRNA expression in HCT 116 cells transfected with pEGFP (Ct) or pCAG-HITS-IRES-EGFP vector (Oexp). Expression of HITS mRNA was normalized by TBP amplified as an internal control. (E and F) Western blotting analysis of MMP-3 and MMP-13 protein levels in cells transfected with pEGFP (Ct) or pCAG-HITS-IRES-EGFP (Oexp) vector. Expression of β -tubulin was monitored as the loading control (E). The number of replicates is indicated in parenthesis in each panel. * $P < 0.05$ and ** $P < 0.01$ compared with Ct group. HITS, heat shock-inducible tumor small protein; EGFP, enhanced green fluorescent protein; WH, wound healing; RT-PCR, reverse transcription PCR; TBP, TATA-box binding protein.

to be regulated by GSK3 β (37); therefore, the present study investigated if HITS caused any changes in the expression of MMPs. Semi-quantitative RT-PCR and western blotting analysis revealed significant downregulation of MMP-3 and

MMP-13 mRNA and protein in the HITS overexpressing cells (Fig. 3D-F), indicating that HITS suppressed cell motility, at least in part, by downregulating MMP-3 and MMP-13.

Anti-migratory effect of the HITS-GSK3 β pathway offsets the pro-migratory effects of HSPs after HS. Previous studies have indicated that, while HS increases HITS expression, HS also induces expression of HSPs, including HSP90 (17,38,39), which in turn promotes the migration of cancer cells (15,16,40-42). Therefore, it remains unclear whether the overall effects of HS on CRC cell migration are promotive or suppressive. Thus, a wound healing assay was performed to compare the migration of cells with and without HS. To cover all the time points where the S9 phosphorylation or the protein expression of GSK3 β was upregulated by HS, HCT 116 cells were scratched and observed at 14 and 38 h (0 and 24 h in the wound healing timeframe, respectively) after HS. No significant change was observed in the migration of these cells (Fig. 4A), suggesting that the anti-migratory effect of the HITS-GSK3 β pathway offset the pro-migratory activities of HSPs after HS. To understand the HITS-GSK3 β effect, HITS siRNAs were transfected into HCT 116 cells under the same HS conditions, and a wound healing assay was performed following the time schedule shown in Fig. 4B. The wound healing rate was significantly increased in cells transfected with HITS siRNAs compared with that in the control, demonstrating that HITS decreased the migration of the cells induced with HS (Fig. 4C and D). Subsequently, the pro-migratory effect of HSP90 was further demonstrated using 17-AAG which is metabolized by NAD(P)H:quinone oxidoreductase 1 (NQO1) and selectively inhibits HSP90 (43). 17-AAG decreased the wound healing rate of HS-treated cells (Fig. 4C and D). Moreover, the migratory effect of the HITS knockdown was not observed in cells treated with 17-AAG, which suggested that the pro-migratory pathway mediated by HSP90 was the target of HITS. The function of HITS was mediated by GSK3 β because the treatment with the specific GSK3 β inhibitor, AR-A014418 (44) counteracted the effects of the HITS knockdown on cell migration (Fig. 4C and D). Taken together, the present findings indicated that HS not only induced the pro-migratory pathways mediated by HSPs, but also induced the anti-migratory pathway involving HITS and GSK3 β , and that HITS-mediated GSK3 β deactivation sufficiently offset the pro-migratory activity in CRC cells.

Discussion

The present study explored the effects of HS on CRC cell migration by focusing on HITS and its putative downstream target GSK3 β . The level of pGSK3 β ^{S9} (GSK3 β inactive form) was significantly increased in CRC cells overexpressing HITS, whereas its knockdown showed an opposite effect. These data demonstrated that HITS expression suppressed GSK3 β activity by increasing the levels of pGSK3 β ^{S9}. Our previous studies have indicated that the deactivation of GSK3 β by S9 phosphorylation suppresses tumor progression in various CRC cells such as RKO, SW480, SW48, SW620 and HT29, as well as HCT 116 *in vitro* (29) and xenograft *in vivo* (35). The wound healing assay showed that overexpression of HITS in the CRC cells significantly suppressed cell migration compared with that of the control cells. This was most likely mediated

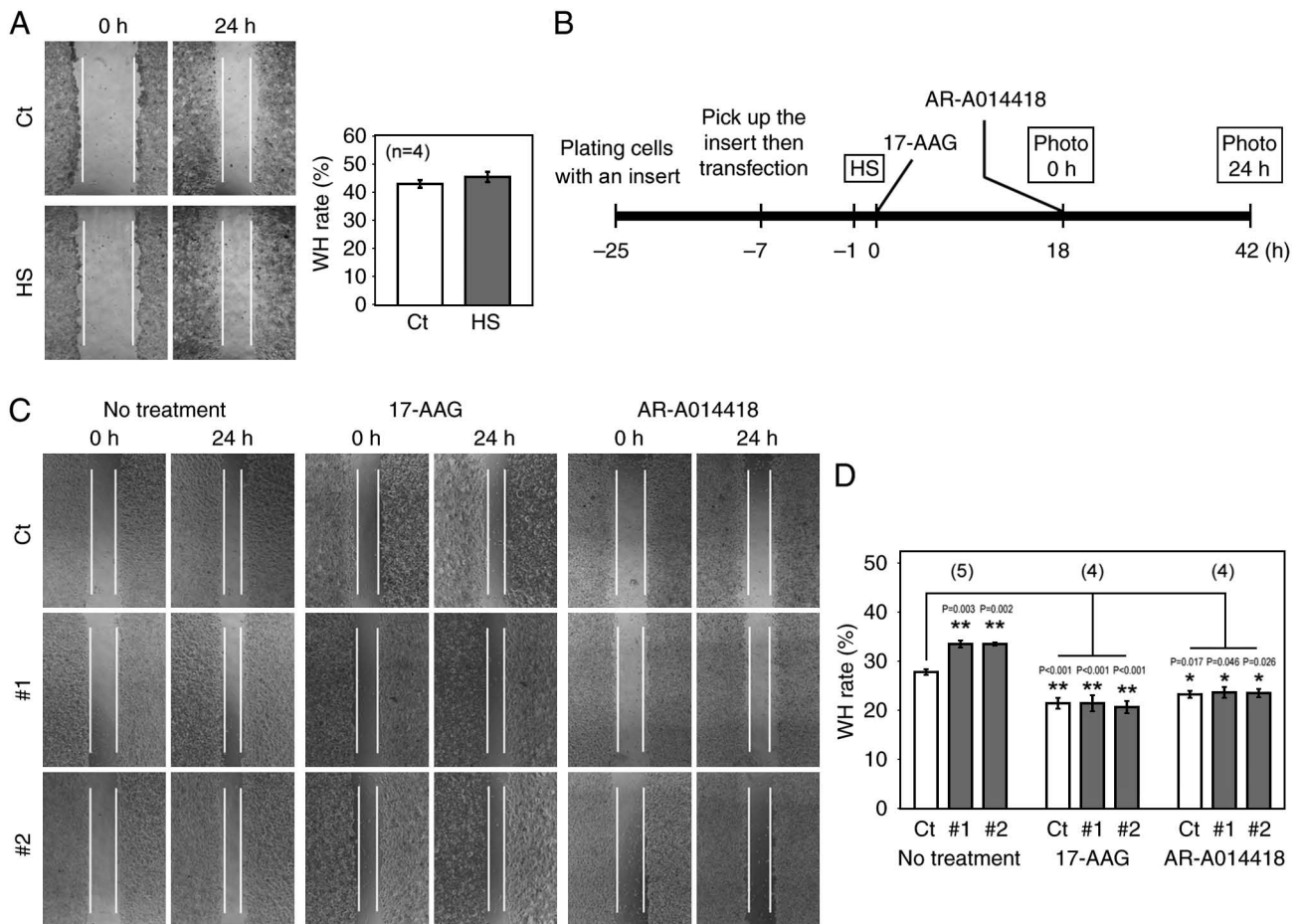


Figure 4. Effect of HITS on the migration of HCT 116 cells after HS. (A) Wound healing assay shows the migration of cells treated with HS compared with that in control cells (without HS; Ct). The left panels show the representative bright field images of the cells. The right panel shows the WH rate. (B-D) Effects of HITS knockdown (HITS-siRNA#1 and HITS-siRNA#2) alone or siRNA treatment in combination with 17-AAG (0.5 μ M) or AR-A014418 (20 μ M) on the migration of cells treated with HS for 1 h. (B) Schematic timeline of the experiment shown in (C) and (D). Each wound gap was made using a plastic insert that was placed on each well 25 h before starting the wound healing assay. (C) Representative bright field images of cells with the respective treatments. (D) Comparison of the WH rate between cells treated as indicated. WH rate of cells treated with inhibitors were compared with that of control cells transfected with control siRNA and were statistically significant as indicated by umbrella lines. The number of replicates is indicated in parenthesis in each panel. * P <0.05 and ** P <0.01 compared with Ct; otherwise statistically insignificant. HS, heat shock; WH, wound healing; siRNA, short interfering RNA; Ct, control; HITS, heat shock-inducible tumor small protein.

by GSK3 β deactivation because the positive effect of HITS knockdown on cell migration was abolished by treatment with the GSK3 β inhibitor AR-A014418.

The molecular mechanisms underlying the anti-migratory and anti-invasive effects of GSK3 β inhibition are of interest. Previous studies have indicated that the inhibition of GSK3 β suppresses F-actin assembly and decreases the formation of lamellipodia and invadopodia via suppression of the phosphorylation-dependent activity of focal adhesion kinase (FAK) (27,28,45), c-Jun N-terminal kinase (JNK) (27) and adenylyl cyclase-associated protein 1 (CAP1) (46,47), the deactivation of guanine nucleotide-exchange factors (GEF) and Ras-related C3 botulinum toxin substrate 1 (RAC1) (27,28,48) and the degradation of nuclear factor of activated T cells (NFAT) (49). Potential involvement of these functional molecules in suppression of CRC cells migration upon the S9 phosphorylation-mediated deactivation of GSK3 β is an aim of our future studies to investigate mechanisms by which HITS regulates tumor cell migration. The deactivation of GEF/RAC1 and JNK pathways reduces the expression of

MMP-2 and membrane type 1 (MT1)-MMP, resulting in the suppression of invadopodia formation in glioblastoma and pancreatic cancer cells (27,28). The present study showed that HITS decreased the expression of MMP-3 and MMP-13 in CRC cells in association with attenuated cell migration (50,51). Therefore, the anti-migratory effect of HITS in cancer cells might be mediated by suppressing these MMPs.

A previous study reported that DRR1, which shares a highly homologous region with HITS but is not induced by HS (18,19,52), can directly bind actin (53). This study elucidated the two actin-binding regions of DRR1, of which the N-terminal region is highly homologous to HITS (70% amino acid identity) and is necessary for suppressing F-actin elongation in HeLa cells. It is possible that HITS also directly binds to actin to suppress F-actin assembly. Guo *et al* (54) indicated that the downregulation of HITS by S100A4, a pro-metastatic protein, increases the migration of gastric cancer MGC803 cells. Considering that S100A4 has been reported to bind F-actin and myosin II heavy chain to promote cytoskeletal formation (55,56), the direct binding of HITS to actin may

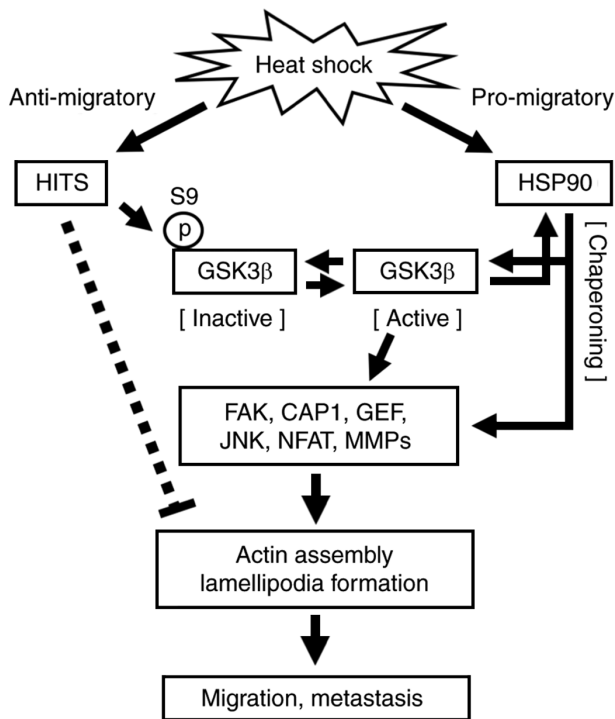


Figure 5. Schematic representation of the hypothetical pro- and anti-migratory pathways activated by heat shock in cancer cells. HITS, heat shock-inducible tumor small protein; HSP, heat shock protein; GSK3 β , glycogen synthase kinase-3 β ; FAK, focal adhesion kinase; CAP1, adenyl cyclase-associated protein 1; GEF, guanine nucleotide-exchange factor; JNK, c-Jun N-terminal kinase; NFAT, nuclear factor of activated T cells.

disturb the interaction between S100A4 and the actin/myosin II heavy chain, resulting in the suppression of cell migration. In conjunction with these previous studies, the present findings suggested that HITS was involved in suppressing actin cytoskeleton formation directly and/or via S9 phosphorylation-mediated deactivation of GSK3 β , which suppresses the formation of lamellipodia and invadopodia to prevent the CRC cell migration that is necessary for invasion (Fig. 5).

A number of previous studies have indicated that HSPs promote the migration, invasion and metastasis of cancer cells (15,16,57-59). Among the HSPs, HSP90 was shown to act as a molecular chaperone able to stabilize and maintain oncoproteins involved in CRC progression such as mutated TP53 (gain-of-function), HER2 and BRAF, especially in stress conditions caused by chemotherapy, radiotherapy and hyperthermia (60,61). GSK3 β has also been shown to be one of the client proteins of HSP90 (62-64), which in turn phosphorylates HSP90 and HSP70 to facilitate cancer progression (65). The upregulation of HSP90 in HCT 116 cells was indicated in a previous report (66). Consistently, the increased expression of GSK3 β observed after HS might be due to the chaperoning function of HSP90 and other HSPs. It has been indicated that HSP90 chaperoning function is required for maintaining the expression and/or function of FAK, NFAT, JNK and MMPs (67-71) that promote the cell migration under the control of GSK3 β , as discussed above. The present study indicated that GSK3 β was involved in the major pro-migratory pathways mediated by HSP90 after HS, and that the inhibition of GSK3 β caused by HITS played a critical role in attenuating

the pro-migratory effect of GSK3 β (Fig. 5). Indeed, HS did not induce significant changes in the migration of HCT 116 cells, although HSP90 was activated and showed pro-migratory activity. This was most likely due to the deactivation of GSK3 β caused by HITS because HITS knockdown, under the same HS condition, showed an increase in wound healing rate, which in turn was decreased by treatment with AR-A014418. Treatment with 17-AAG counteracted the effect of the HITS knockdown, confirming the HSP90 chaperoning effect on GSK3 β and its downstream pathways (Fig. 5). Other studies have also reported the anti-migratory and anti-metastatic effects of hyperthermia *in vitro* or in animal models, although the underlying mechanisms remain unclear (72-74). In these reports, the HITS-GSK3 β pathway might have played a critical role in suppressing cell migration in these reports.

The single or combined use of HSPs inhibitors with other therapies were proposed as attractive strategies for cancer therapy (75,76). Several studies have proposed to use hyperthermia in combination with HSP inhibitors, such as HSP90 inhibitors, which provides successful *in vitro* results (77-79). However, HSPs are involved in the activation of the immune response, including the cytotoxicity of NK cells, maturation and antigen presentation of dendritic cells, and activation of T cells (11,13,14). Previous studies have shown that CRC is a heterogeneous disease consisting of the right-side colon cancer and the left-side colorectal cancer with different molecular, biological and clinicopathological properties (80-82). Consistently, the antitumor immunity activated by HSPs would be beneficial to prevent peritoneal metastasis of the right-sided (proximal) colon cancer with highly immunogenic and intensive lymphocytic infiltration (81,82). Therefore, the combined use of HSPs inhibitors with hyperthermia may reduce the clinical benefits of hyperthermia. The present findings showed that the HITS-GSK3 β pathway prevented hyperthermia-induced cancer cell migration caused by HSP90. One concern of the present study is that, in certain cancer types, HS may not induce HITS considering that the endogenous HITS expression has been reported to be suppressed in various cancer types (17,20,83,84) possibly due to epigenetic modification (84-86). The present data showed that treatment with a GSK3 β inhibitor reinforced the anti-migratory effect via HITS, while other studies also showed the benefit of GSK3 β inhibitors to activate NK cells and dendritic cells and suppress the immune checkpoint protein PD-1 to enhance CD8 $^{+}$ T cells activity (87-90). Grassilli *et al* (91) also indicated another benefit of GSK3 β inhibitors to overcome drug resistance of p53 null colon carcinoma by inducing necroptosis. Accordingly, as well as an investigation of the putative roles of the pathways mediated by HITS and GSK3 β in CRC biology as discussed in the former paragraph, the combination use of GSK3 β inhibitors with hyperthermia would be an aim of our future studies.

Hyperthermia in cancer treatment shows clinical benefits because of its antitumor effects exerted through several processes, such as direct heat-induced cell death, induction of oxidative stress and molecular damage, antitumor immunity boosting, radio- and chemo-sensitivity enhancement, tumor microenvironment modifications, and inhibition of epithelial-to-mesenchymal transition (10-14). While the HSF1-HSPs system, major cellular responses to HS, has long been considered to have a promigratory function that leads

to cancer progression, the present study clarified that HITS could counterbalance the migratory effects caused by major HSPs (15,16), such as HSP90 in CRC cells. Further investigation is required to fully understand the molecular mechanisms underlying the antitumor effects of hyperthermia.

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Availability of data and materials

The datasets used and/or analyzed during of current study are available from the corresponding author on reasonable request.

Authors' contributions

KK conceptualized the study, designed the experiments, acquired data and wrote the primary manuscript. TD acquired data and resources, designed the methodology and critically revised and proofread the manuscript. TM and KS acquired resources, designed methodology and critically revised and proofread the manuscript. HN conceptualized the study, acquired resources, designed methodology and critically revised and proofread the manuscript. All authors read and approved the final version of the manuscript. KK and HN confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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