Mitochondrial Respiratory Dysfunction Induces Claudin-1 Expression via Reactive Oxygen Species-mediated Heat Shock Factor 1 Activation Leading to Hepatoma Cell Invasiveness

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Background: Increased claudin-1 (Cln-1) expression in human hepatoma cells with mitochondrial defects leads to high tumor cell invasiveness.

Results: This Cln-1-mediated hepatoma cell invasiveness occurs via heat shock factor 1 (HSF1) activation.

Conclusion: The mitochondrial defect-ROS-HSF1 axis controls hepatoma cell invasiveness.

Significance: The HSF1 is a key mitochondrial retrograde responsive transcription factor to control hepatoma cell invasiveness.

ABSTRACT

Although mitochondrial dysfunction has been implicated in tumor metastasis, it is unclear how it regulates tumor cell aggressiveness. Previously, we reported that human hepatoma cells harboring mitochondrial defects have high tumor cell invasion activity via increased claudin-1 (Cln-1) expression. In this study, we demonstrated that mitochondrial respiratory defects induced Cln-1 transcription via reactive oxygen species (ROS)-mediated heat shock factor 1 (HSF1) activation, which contributed to hepatoma invasiveness. We first confirmed the inverse relationship between mitochondrial defects and Cln-1 induction in SNU hepatoma cells and hepatocellular carcinoma (HCC) tissues. We then examined five different respiratory complex inhibitors, and complex 1 inhibition by rotenone most effectively induced Cln-1 at the transcriptional level. Rotenone increased both mitochondrial and cytosolic ROS. In addition, the rotenone-induced Cln-1 expression was attenuated by N-acetyl cysteine, an antioxidant, and exogenous H2O2 treatment was enough to increase Cln-1 transcription, implying the involvement of ROS. Next, we found that ROS-mediated HSF1 activation via hyperphosphorylation was the key event for Cln-1 transcription. Moreover, the Cln-1 promoter region (from -529 to +53) possesses several HSF1 binding elements and this region showed increased promoter activity and HSF1 binding affinity in response to rotenone treatment. Finally, we demonstrated that the invasion activity of SNU449 cells, which harbor mitochondrial defects, was blocked by siRNA-mediated HSF1 knockdown. Taken together, these results indicate mitochondrial respiratory defects enhance Cln-1-mediated hepatoma cell invasiveness via mitochondrial ROS-mediated HSF1 activation, presenting a potential role for HSF1 as a novel mitochondrial retrograde...
Mitochondria-mediated HSF1 activation induces Cln-1 signal-responsive transcription factor to control hepatoma cell invasiveness.

Mitochondrial respiratory defects are distinct metabolic features of cancer cells (1, 2). In the early stages of cancer development, actively proliferating tumor cells cannot avoid hypoxia due to their rapid nodular mass formation. Therefore, this respiratory impairment has long been recognized as an epiphenomenon of tumors. However, mitochondrial defects and the accompanying activated glycolysis persist in metastatic malignant cancers, even after a normoxic environment is restored by activated angiogenesis. Similar observations were made with cultivated tumor cells under normoxia (3), indicating that cancerous respiratory defects are not just a transient consequence of environmental constraints in tumor tissue, but may be the result of genetic changes or oncogenic modulations. Indeed, the mitochondrial defects found in cancer cells are mostly associated with mitochondrial DNA (mtDNA) damage, such as deletions and/or point mutations, as described in many types of cancers (4-7). Mitochondrial dysfunction is also induced by oncogenic Myc-mediated pyruvate dehydrogenase (PDH) inactivation (8) or by oncogenic Ras-mediated mitophagic degradation (9). Interestingly, mtDNA damages or mitochondrial defects may contribute to cancer promotion, metastasis, and chemoresistance in addition to primary tumor growth (6, 10-12). These observations suggest that mitochondrial defects have causative roles in tumor progression. However, the underlying molecular mechanisms of how mitochondrial dysfunction regulates tumor progression are not fully understood.

Increased levels of reactive oxygen species (ROS) and the resultant oxidative stress are also key features of tumor cells. Cancer cells with a high metastasis capacity have more ROS accumulation than those with a low tendency for metastasis (13-15). In addition to ROS-induced oxidative damages to cellular proteins, lipids, and nucleotides, ROS also function as second messengers to regulate various metastasis-related signaling pathways via reversible oxidative posttranslational modifications (16), subsequently contributing to tumor angiogenesis, cancer cell invasiveness, and metastasis. Interestingly, increased ROS in cancer cells is primarily emanated from defective mitochondria as a result of an incomplete and inefficient electron transfer process in the defective respiratory chains (17-19), supporting the mechanistic link between tumor progression and mitochondrial defects. Inhibition of complex I activity by stable suppression of GRIM-19 or NDUF53 decreased complex I-enhanced cell migration, invasion, and spheroid formation through ROS production (10). The contribution of mitochondrial dysfunction-mediated ROS to cell migration activity was also demonstrated in SC-M1 human gastric cancer cells (20). These observations emphasize the key role of ROS in mitochondrial dysfunction-mediated tumor progression.

Claudins are a family of tight junction components which form paracellular barriers, thereby controlling the flow of molecules in the intercellular space of the epithelium. However, aberrant up-regulation of claudins has often been reported in human cancers, including hepatocellular carcinoma (HCC) and primary colon carcinoma (21, 22). Among claudins, claudin-1 (Cln-1) is known to play a key role in the invasive behavior of human liver cells via the c-Abl-PKC signaling pathway (23). Overexpression of Cln-1 augments the invasiveness of tumor cells through increased secretion and activation of metalloproteinases (24, 25), which supports the tumor-promoting activity of Cln-1. Recently, we reported that hepatoma cells harboring mitochondrial dysfunctions control cell invasion activity through augmenting Cln-1 expression. However, the molecular mechanism of how mitochondrial dysfunction controls Cln-1 expression remains unclear. In the present study, we demonstrate that mitochondrial respiratory defects augment Cln-1 expression through ROS-mediated mediated heat shock factor 1 (HSF1) activation, contributing to hepatoma cell invasiveness.

EXPERIMENTAL PROCEDURES

Cell cultures and tumor samples. Human hepatoma cells (SNU-354, SNU-387, SNU-423, and SNU-449) were purchased from Korean Cell Line Bank (Seoul, Korea) and were
cultured in GIBCO® RPMI1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% GIBCO® fetal bovine serum (FBS) (Invitrogen) and GIBCO® antibiotics (Invitrogen) at 37°C in a humidified incubator with 5% CO2. Chang cell was obtained from American Tissue Culture Collections (ATCC, Rockville, MD) and Chang cell clones were isolated by single cell dilution and expansion of Chang cell (ATCC, Rockville, MD), and a Chang clone with strong hepatic characteristics (Ch-L) validated by confirming liver specific expressions of albumin and carbamoyl-phosphate synthase-1 was used for this study (26). Ch-L clones were cultured in GIBCO® Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% FBS.

HCC tumor samples and surrounding control tissues were obtained from 28 patients with HCC during the period of 2003 to 2005 at Ajou University Hospital after surgical resection with informed consent through Ajou Institutional Review Board. No patient in the current study received chemotherapy or radiation therapy before the surgery.

Measurement of oxygen consumption rate (OCR).
OCR was also measured in situ with cultured cells using XF-24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA) according to the protocol provided. Briefly, cells were seeded on XF24 cell culture microplates (Seahorse Bioscience) at a density of 10,000 cells per well and preincubated with XF assay medium (Seahorse Bioscience) containing 1 mM pyruvate and 5 mM glucose. Its mitochondrial specificity was confirmed by adding 5 mM KCN.

Immunocytochemistry
Cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% triton X-100 for 10 min, and incubated in blocking solution (2% bovine serum albumin in TBS containing 0.1% Tween 20) for 2 h. After incubated overnight with primary antibody for Cln-1 (717800, Invitrogen, Carlsbad, CA) at 4°C, cells were washed three times and probed with cy3-conjugated anti-rabbit antibody (Jackson Immuno Research Laboratories, West Grove, PA) for 1 h. After washing and mounting with mounting solution, cells were visualized by confocal microscope (LSM710, Carl Zeiss, Oberkochen, Germany).

Estimation of intracellular and mitochondrial ROS level.
To determine intracellular and mitochondrial ROS levels, dichlorofluorescin diacetate (DCFH-DA) (Molecular probe, Eugene, OR) and mitochondrial specific MitoSOX® (Invitrogen) fluorogenic probes were used, respectively (27). Briefly, cells were incubated in media containing DCFH-DA (20 μM) and MitoSOX® (25 μM) for 20 min at 37°C. Stained cells were washed and resuspended in PBS, and analyzed by flow cytometry (FACS Vantage, Becton Dickinson Corp.). Mean values of arbitrary fluorescence units of 10,000 cells were used and expressed as percentage of negative control.

Subcellular fractionation
The nuclear and cytoplasmic fractions were obtained from 90% confluently grown cells on 100 mm dishes as described previously with slight modification (28). Briefly, cells were harvested by trypsinization and resuspended in medium A (250 mM sucrose, 0.1 mM EDTA, 2 mM HEPES, pH 7.4). The cell slurry was homogenized in a Dounce homogenizer (StedFast™ Stirrer, Fisher Scientific, Pittsburgh, PA) and spin at 500 rcf for 10 min to precipitate nuclei. The nuclei pellets were washed three times with buffer A (0.1 mM EDTA, 10 mM KCl, 10 mM HEPES, pH 7.9) containing 1% NP-40 and the final pellets were collected for nuclei fraction. The supernatant cytoplasmic fractions were separately collected. Nuclei and cytoplasmic fractions were subjected to lysis in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% Sodium dodecyl sulfate, and 50mM Tris, pH 8.0) for Western blot analysis.

Construction of HSF1 cDNA plasmids and transfection of cDNA plasmids and siRNAs.
To generate a cDNA plasmid, pcDNA-HSF1-HA, conventional cloning procedures were applied. Briefly, pcDNA-HSF1-HA plasmid was constructed by conventional TA cloning using pGEMT-easy (Promega, Fitchburg, WI) and HSF1 cDNA fragment amplified by PCR using
total cDNAs of Ch-L clone and the primer set, 5'-AGAATTCCATGGATCTGCCC and 5'-TGAGCTCGGAGACAGTGGG. The HSF1 cDNA was subcloned into EcoRI and XhoI sites of the pcDNA3-HA vector constructed previously (29). Cln-1 overexpression plasmid, pcDNA-Cln-1, was constructed previously (26).

To introduce plasmids and small interfering RNAs (siRNAs) into cells, cells were transfected with the plasmids and siRNA duplexes using FuGENE HD (Promega) and Oligofectamine Reagent (Invitrogen), respectively, according to the manufacturer’s instructions. HSF1 siRNAs (#1, 5'-ACUGUAGAUUGCUUCUGUA; #2, 5'-GAACUAAAGCCAAGGGUAU) and negative control siRNAs (5'-CCUACGCCACCAAUUUCGU) were obtained from Bioneer (Seoul, Korea).

Construction of promoter-luciferase reporter plasmid and promoter assay.

Human Cln-1 promoter region of 767 bps (-529 to +238, NG_021418) was cloned by targeted PCR against total genomic DNA of Ch-L using a primer set, 5'-GCTCGAGCCAATCTGTAGAGTGT and 5'-TATAGATCTTCGCTCGGGCGC. Amplified Cln-1 promoter region was inserted between Bgl II and Xho I sites of pGL3-basic vector (Promega). After construction, the inserted promoter was confirmed by DNA sequencing.

To monitor the Cln-1 promoter activity, cells were transfected with total 1 μg DNA (700 ng of pcDNA3 or pcDNA3-HSF1-HA, 250 ng of the cloned reporter plasmid and 50 ng of thymidine kinase promoter-driven Renilla luciferase plasmid as an internal control) using FuGENE HD reagent. After 2 days, luciferase activity of cell lysate was measured by Synergy 2 Multi-Mode Reader (BioTek Instruments, Inc., Winooski, VT) according to the protocol provided with the Dual-Luciferase Reporter Assay System (Promega). The Cln-1 promoter-mediated luciferase activities of the reporter plasmid were normalized by the Renilla luciferase activity.

Chromatin immunoprecipitation (ChIP) assay.

ChIP assay was performed according to the ChIP Assay kit protocol (Upstate Biotechnology Inc., Lake Placid, NY) with slight modification. Briefly, cells were treated with 1% formaldehyde to cross-link stably the DNA-interacting proteins to genomic DNA. After lysis, the lysates were briefly sonicated to shear genomic DNA, and centrifuged at 13,000 rpm for 10 min. An aliquot was saved for input control and the other aliquots were subjected to ChIP using HSF1 antibody (4356S, Cell Signaling) and protein-G agarose bead (Millipore corp, Billerica, MA). The eluted DNA was purified by using DNA extraction kit (Inclone Biotech, Seoul, Korea) and the specific Cln-1 promoter region (-490 to +53, 543 bp) was amplified by PCR with the primer sets, 5'-GAGACAAGTGATGGGAACGC and 5'-CTGGAGTCTGGAGACTAGAAGC (Bioneer, Seoul, Korea) and sequenced for validation.

Cell invasion assay.

Cell invasion assays were basically performed with Transwell® Permeable Supports (Corning, Acton, MA) according to the manufacturer's instructions. Briefly, cells (2×10⁴) pre-starved with serum-free RPMI for 16 h were placed into the upper chamber with 0.2 ml of serum-free RPMI. RPMI (0.8 ml) supplemented with 10% fetal bovine serum was placed in the lower chamber as a chemo-attractant. Uncoated porous filters (8-μm pore size) were pre-coated with 7% Growth Factor Reduced BD Matrigel™ Matrix (Becton Dickinson Labware, Franklin Lakes, NJ) for the assay. The cells invaded into the lower surface were fixed by 100% methanol for 1 min, stained with Hematoxylin Solution (Sigma-Aldrich, Saint Louis, MO) and Eosin Y Solution (Sigma-Aldrich), and counted. All experiments were performed in independent triplicate experiments.

Western blot analysis.

Western blotting was performed using standard procedures. Cln-1 antibody (717800) was purchased from Invitrogen (Carlsbad, CA) and HSF1 antibody (4356S) was from Cell Signaling (Danvers, MA). Antibody for phospho-HSF1 (phospho S326, ab115702) was obtained from Abcam (Cambridge, UK). Antibodies for β-actin (sc-1616) and α-tubulin (sc-5286) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against for NDUFA9 of complex I
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(A21344) and MTCOII of complex IV (A6404) were from Molecular Probes (Eugene, OR).

Reverse transcription-polymerase chain reaction (RT-PCR).

Total RNA was isolated using Trizol (Invitrogen) and cDNA was prepared using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI). PCR was performed with 25-30 cycles of the reaction involving 95°C for 30 seconds, 53-58°C for 30 seconds, and 72°C for 70-90 seconds. The PCR primer sets for Cln-1 (5’-GAGCGAGTCATGGCCAACGC and 5’-GCCTCTGTTCACACAGTAGT), NDUFA3 (5’-CAGTGCTGGTCGTGCCTTC and 5’-GGCATGTCCCATCAGC), UQCRB (5’-GCCTTTCTGTTCGCCATG and 5’-ATCCAGGCACCTTGCTGATG), ATP5G3 (5’-TCTCGACCAAGGCTATGG and 5’-TTGCAGCACCTGCAACATA), and ß-actin (5’-CCTTCCTGGGCATGGAGTCCTGT and 5’-GCACTCTTGGCATGGAGTCTG) were produced by Bioneer (Seoul, Korea). Quantitative real-time RT-PCR (qRT-PCR) was performed by using THUNDERBIRD™ SYBR™ qPCR Mix (Toyobo Co., Ltd, Osaka, Japan) and Bio-Rad CFX96™ Real Time System (Hercules, CA).

RESULTS

Increased expression of Cln-1 is associated with mitochondrial dysfunction of hepatoma cells.

First, we examined the relationship between mitochondrial dysfunction and Cln-1 expression in hepatoma cells using four different SNU hepatoma cells (SNU-354, SNU-387, SNU-423, and SNU449). A previously characterized Ch-L clone that possesses liver-specific genes (30) was used as a control with active mitochondria. The SNU-354, SNU-423, and SNU449 cells with defective mitochondrial respiration showed increased Cln-1 expression at both the mRNA and protein levels; whereas, the cells with active mitochondria (SNU387 and Ch-L clone) had low Cln-1 expression (Fig. 1A-1B). Increased Cln-1 expression was mostly localized in the cell-to-cell contact region of plasma membrane (Fig. 1C), implying its usual function as a tight junction component. Next, we monitored the expression levels of Cln-1 and a core subunit of mitochondrial respiratory complex IV subunit in 28 HCC tumor samples and compared the levels with surrounding tissues (Fig. 1D and 1E). There was no clear one on one correlation between decreased complex IV subunit and increased Cln-1 expressions in human hepatoma tissues. However, 20 tumor samples had decreased expression (below 0.2 fold) of the complex IV subunit, implying mitochondrial defects. Among 20 samples with mitochondrial defects, 14 (63.6%) had increased expression (over 2.0 fold induction) of Cln-1 (Fig. 1E). These results suggest that up-regulation of Cln-1 expression may be associated with mitochondrial dysfunction in some hepatoma cells.

Complex I inhibition by rotenone effectively induces Cln-1 expression at the transcriptional level.

Next, we investigated the relationship between Cln-1 expression and mitochondrial defects. Overexpression of Cln-1, regardless of whether transient or stable, in Ch-L clones did not affect the OCR (Fig. 2A and 2B). However, when Ch-L clones were treated with the respiratory inhibitors rotenone (complex I inhibitor), thenoyltrifluoroacetone (TTFA, complex II inhibitor), antimycin A (complex III inhibitor), and oligomycin (complex V inhibitor), Cln-1 expression was significantly increased at the transcription level without altering respiratory subunit expression without any clear alterations in respiratory subunit expressions (data not shown); while, KCN (complex IV inhibitor) did not lead to increased Cln-1 expression (Fig. 2C and 2D). Among the five respiratory complex inhibitors, complex I inhibition by rotenone showed the most effective Cln-1 induction. This induction started with 1 μM rotenone and progressively increased 3 h after treatment at the transcription level (Fig. 2E and 2F). We further demonstrated that mitochondrial defect by siRNA-mediated knockdown of NDUFA9, a complex I subunit, induced Cln-1 expression (Fig. 2G). These results indicate that mitochondrial complex I defect is involved in Cln-1 expression and also suggest that
Mitochondria-mediated HSF1 activation induces Cln-1 expression can be used as a proper model to elucidate the molecular link between mitochondrial defects and Cln-1 expression.

**Mitochondrial ROS generated by rotenone is a key regulator of Cln-1 expression.**

Next, we asked how complex I inhibition by rotenone regulated Cln-1 transcription. To address this, we monitored intracellular and mitochondrial ROS levels in the hepatoma cells and compared with that of the Ch-L clones. The hepatoma cells with mitochondrial defects had increased levels of both mitochondrial and intracellular ROS (Fig. 3A). When the Ch-L clone was treated with rotenone, both mitochondrial and intracellular ROS increased in a dose-dependent manner, accompanying Cln-1 mRNA induction and showing an inverse relationship with respiratory inhibition (Fig. 3B). Pretreatment with N-acetyl cysteine (NAC), an antioxidant, effectively blocked the rotenone-induced Cln-1 transcription (Fig. 3C). When Ch-L clone was exposed to subcytotoxic dose of hydrogen peroxide (H2O2), intracellular ROS increased in a biphasic manner and mitochondrial ROS increased progressively (Fig. 3D, a and 3F, a), implying that the second peak of intracellular ROS may be linked with mitochondrial ROS which was probably triggered by H2O2–induced mitochondrial defect (Fig. 3F, b). This biphasic ROS generation by subcytotoxic dose of H2O2 corresponds well with the previous report (31). Interestingly, in this condition Cln-1 mRNA expressions also showed a biphasic profile, suggesting that certain level of ROS may be required for Cln-1 induction (Fig. 3D and 3E). These results indicate that mitochondrial ROS generated by complex I inhibition is critically involved in Cln-1 transcription.

**ROS-mediated HSF1 phosphorylation is a key regulatory event for Cln-1 expression.**

To investigate how mitochondrial ROS modulates Cln-1 expression, we analyzed potential transcription factor binding sites in the Cln-1 promoter region (-2729 to +271 bps, NG_021418 from NCBI) using the TFSEARCH program. The most abundant transcription binding site in the promoter region was for HSF1, which is known as a stress responsive transcription factor (data not shown) that is regulated by oxidative signaling (32), suggesting its potential involvement in mitochondrial ROS-mediated Cln-1 transcription. Upon exposure to rotenone, the mobility of the HSF1 protein band was delayed on SDS-PAGE (Fig. 4A) and this band was hyper-phosphorylated, as shown by complete dephosphorylation using λ phosphatase (Fig. 4B). This finding implied it was activated, as shown previously (33). The delayed band was phosphorylated on serine (Ser) 326 (Fig. 4A), the activating phosphorylation site (34). Rotenone-induced HSF1 activation was further demonstrated by augmented transcription of HSP70, a known major target of HSF1, (Fig. 4C) and nuclear targeting of HSF1 (Fig. 4D). The HSP70 transcription was faster than Cln-1 transcription (Fig. 2F, b), implying differential regulation by HSF1. The delayed HSF1 mobility was recovered by NAC pretreatment and induced by exogenous H2O2 treatment, corresponding with the Cln-1 expression pattern (Fig. 4E and 4F). We then further investigated whether rotenone-induced Cln-1 expression was truly mediated through HSF1. Induction of Cln-1 by rotenone was effectively suppressed by siRNA-mediated HSF1 knockdown and HSF1 overexpression was sufficient to induce Cln-1 in the Ch-L clone (Fig. 4G and 4H). These results support that complex I inhibition by rotenone induced Cln-1 transcription through ROS-mediated HSF1 activation.

**Complex I inhibition increases Cln-1 promoter activity through HSF1 binding on the promoter.**

We then examined how HSF1 regulated Cln-1 transcription. We constructed a reporter plasmid containing the Cln-1 promoter region including the transcription start, from -529 to +238. This promoter region contains more than 15 potential HSF1 binding sites (Fig. 5A). The cloned promoter region was sufficient for activation by rotenone treatment and the increased promoter activity was diminished by NAC pretreatment (Fig. 5B and 5C). Overexpression of HSF1 itself increased the promoter activity and additional rotenone treatment further enhanced the activity, but led to only a minor increase (Fig. 5D). We further demonstrated that complex I inhibition truly increased HSF1 binding to the Cln-1
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**HSF1 activation regulates hepatoma cell invasiveness.**

Finally, we investigated whether HSF1 activation induced by mitochondrial ROS is truly involved in hepatoma cell invasiveness. SNU hepatoma cells (SNU354, SNU423, and SNU449) harboring high Cln-1 expression and mitochondrial ROS had high cell invasion activity and this invasiveness was significantly blocked by Cln-1 suppression, but not completely (Fig. 6A and 6B). Cln-1 overexpression and NDUFA9 knockdown-mediated Cln-1 induction in Ch-L clone also enhanced cell invasion activity (Fig. 6C), implying the involvement of mitochondrial defect-mediated Cln-1 expression in hepatoma cell invasiveness. Furthermore, those cell lines harboring high Cln-1 expression showed the delayed HSF1 mobility on SDS-PAGE (Fig. 6A). However, SNU354 cells had low expression of both HSF1 and Cln-1 without activating phosphorylation of Ser326; whereas, SNU423 and SNU449 cells had high HSF1 phosphorylation and Cln-1 expression (Fig. 6A). Unexpectedly, the invasion activity of SNU423 was not altered by HSF1 knockdown (data not shown), suggesting possible involvement of additional inhibitory mechanisms on HSF1 activity in this cell. Therefore, we employed SNU449 cells to evaluate the role of HSF1 in hepatoma cell invasiveness. The high Cln-1 expression in SNU449 cells was obviously diminished by NAC treatment and siRNA-mediated HSF1 knockdown (Fig. 6D and 6E, a). Eventually, siRNA-mediated HSF1 knockdown significantly decreased the invasion activity of SNU 449 cells (Fig. 6E, b). Taken together, these results indicate that ROS-mediated HSF1 phosphorylation (activation) is involved in certain hepatoma cell invasiveness via Cln-1 expression.

**DISCUSSION**

Mitochondrial respiratory defects that are accompanied by mitochondrial gene mutations are common in most cancers (35, 36); thus, they are considered a metabolic hallmark of cancer. Although the contribution of mitochondrial defects to cancer development, such as tumor angiogenesis, metastasis, and chemoresistance, has often been reported (6, 10, 11, 37-39), it is unclear how mitochondrial impairment regulates tumor progression. One plausible explanation is that an altered mitochondrial metabolism communicates with the nucleus through “mitochondrial retrograde signaling”. The retrograde signaling begins with the release of second messengers, ROS and Ca++, from defective mitochondria (40). These messengers then activate several cytosolic signaling transducers through redox modification or post-translational modification. In turn, certain transcription factors or cofactors are activated and/or trans-localize into the nucleus, switching on gene transcriptions de novo (40, 41). Therefore, the key molecular link between mitochondrial defects and tumor progression is the activation of certain transcription factors.

Several key transcription factors, including CREB and PGC-1, were identified to regulate mitochondrial defect-responsive transcriptional reprogramming (40). In this study, we demonstrated that HSF1 is another mitochondrial retrograde responsive transcription factor regulated by mitochondrial ROS. This is the first report to show a direct link between the mitochondrial defect-ROS-HSF1 axis and cancer development. The transcription factor HSF1 is multifaceted and controls protein homeostasis in response to diverse proteotoxic stress (42). Upon stress, HSF1 trimerizes and is then transported into the nucleus where it is hyperphosphorylated and activates several heat shock proteins such as HSP70 by binding to the consensus heat shock element (HSE). The HSF1 activity is attenuated by a negative feedback loop with binding of the produced HSP70 to its transactivation domain (43). Recently, enhanced activation or expression of HSF1 was associated with a poor prognosis in several types of cancer (42, 44, 45). The HSF1 promotes HCC cell migration and invasion by activating expression of HSP27 (46). In addition, HSF1 is activated by redox regulation (47, 48). Therefore, it is reasonable to hypothesize that HSF1 may play an important role in transcriptional reprogramming that occurs in response to cellular stress by mitochondrial ROS.
Indeed, upon exposure to complex I inhibition, HSF1 was activated by hyperphosphorylation, especially at Ser326. The HSP70 transcription was rapidly induced and slowly attenuated, implying its negative feedback regulation. However, Cln-1 transcription followed HSP70 expression and corresponded with the HSF1 hyperphosphorylation pattern. These results suggest that transcriptional regulation by HSF1 may vary depending on the target gene.

The HSF1 binds specifically to HSE, which has a consensus-sequence that is a tandem array of three oppositely oriented ‘NGAAN’ motifs or degenerate versions of them, that bind with trimerized HSF1 (49). However, the Cln-1 promoter sequence did not have the consensus sequence for HSE, but had several dispersed direct or inversed repeats. Nevertheless, HSF1 binding to the Cln-1 promoter was confirmed by ChIP assay. Some explanations for this unexpected finding are that the HSF1 trimer may bind three dispersed HSE sequences that are placed closely by the looping DNA strand or that binding of hyperphosphorylated HSF1 to single HSE may activate the Cln-1 promoter with the help of other factors activated by mitochondrial defect-mediated retrograde signaling. In this way, HSF1 may regulate the transcriptions of various target genes such as Cln-1, in addition to HSPs. However, if any inhibitory factors exist in some cellular context, HSF1 activity on Cln-1 promoter may be invalid as the case of SNU423. These results contribute to a better understanding of the novel regulatory mechanism of HSF1.

Major targets of HSF1 are known to be HSPs, which restore the structures of proteins distorted by proteotoxic stress. Therefore, induction of HSPs by HSF1 under our mitochondrial oxidative stress must be required to maintain essential cellular functions for tumor progression. However, it is still questionable why HSF1 should activate Cln-1 transcription. Although recent studies support the involvement of Cln-1 in tumor metastasis, the detailed molecular mechanisms of how Cln-1 regulates tumor activities remain unclear. In general, tight junctions regulate the passage of ions and molecules through the paracellular pathway in epithelial and endothelial cells. However, Cln-1 also has unexpected roles in tumor cells; it recruits and promotes the activation of the matrix metalloprotease-2 and makes tumor cells aggressive (25). Interestingly, Cln-1 alone is sufficient to exert a tight junction-mediated gate function in metastatic tumor cells even in the absence of other tight junction-associated proteins (50). These findings suggest that Cln-1 may be required to overcome the cellular stress triggered by mitochondrial defects by exporting excess ions or stressors out of the cell. Our results from this study demonstrate that Cln-1 is up-regulated in response to mitochondrial ROS in the early stage of HCC invasiveness via HSF1 activation. The detailed molecular mechanisms of how Cln-1 controls HCC invasion activity remain to be elucidated.

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The abbreviations used are: Cln-1, claudin-1; HSF1, heat shock factor 1; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; mtDNA, mitochondrial DNA; PDH, pyruvate dehydrogenase, Ch-L, Chang clone with liver characteristics; OCR, oxygen consumption rate; TTFA, thenoyltrifluoroacetone; NAC, N-acetyl cysteine; ChIP, chromatin immunoprecipitation; HSP, heat shock protein; HSE, heat shock element.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: JHL, YKL, and JJL performed and analyzed most experiments, with assistance by HOB for a few experiments. IP and GHK performed a few experiments added newly to the revised
Mitochondria-mediated HSF1 activation induces Cln-1 version. WGX and HJW provided human hepatoma tissues and analyzed the results shown in Fig. 1D and 1E. GY designed and coordinated the study and wrote the paper.

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FIGURE LEGENDS

Fig. 1. Inverse relationship between Cln-1 expression and mitochondrial respiratory defects in hepatoma cells and tissues. Ch-L clone and SNU hepatoma cell lines (SNU354, SNU387, SNU423l and SNU449) were cultured for 2 days to maintain exponentially growing state. (A) Cln-1 protein levels by Western blot analysis. OCRs of the cells were measured as described in ‘Materials and methods’ and their percentages of control (Ch-L) are shown in the bottom panel. **, p<0.01 vs. Ch-L by student t-test. (B) Cln-1 mRNA levels by RT-PCR. (C) Immunocytochemistry for detecting intracellular localization of Cln-1. Red arrows indicate enhanced expression of Cln-1 in the cell-to-cell contact regions of cytoplasmic membrane. (D) Protein expression ratios of Cln-1 and complex IV subunit in 28 HCC tumor samples and their surrounding tissues from the Western blot analysis of (E). (E) Western blot analysis of 28 HCC tumors and their surrounding specimen. Numbers of tumor samples with down-regulated mitochondrial activity (Mito-down, below 0.2 fold) which were estimated by complex IV subunit expression, with up-regulated Cln-1 expression (Cln-1 up, over 2 fold induction), and with both Mito-down plus Cln-1 up.

Fig. 2. Pharmacological inhibition of respiratory complex I effectively induces Cln-1 expression at transcriptional level. (A) OCR was measured after Cln-1 was overexpressed in Ch-L clone, using pcDNA-Cln-1. (B) Ch-L clones stably expressing Cln-1 were isolated after transfecting cells with pcDNA-Cln-1 plasmid, and then subjected to OCR assay (upper panel) and Western blot analysis (lower panel). C-D) Ch-L clone was challenged with 5 μM Rotenone (Ro), 200 μM TTFA (TT), 5 μM antimycin A (AA), 5 mM KCN (KCN) or 5 μM oligomycin (Oli) for 12h. (C) Representative Western blot image for Cln-1 (a) and quantified results (b) are shown. (D) Cln-1 mRNA levels by RT-PCR. Representative gel
image (a) and quantified results (b) are shown. (E) Ch-L clone was exposed to the indicated concentration of rotenone for 12h. Western blot (a) and RT-PCR (b) results are shown. (F) Ch-L clone was challenged with 5 μM rotenone for the indicated time periods. Western blot (a) and RT-PCR (b) results are shown. (G) Ch-L clone was transfected with siRNA (50 or 100 pmoles) for NDUFA9 for 3 days and subjected to OCR measurement (a) and Western blot analysis (b). **, p<0.01 vs. control by student t-test.

Fig. 3. Complex I inhibition-induced Cln-1 expression is mediated by ROS production. (A) Ch-L clone and SNU hepatoma cell lines (SNU354, SNU387, SNU423l and SNU449) were cultured for 2 days to maintain exponentially growing state. Mitochondrial and cytoplasmic ROS levels were measured by cytofluorometric analysis after staining cells with MitoSOX (□) or DCFH-DA (■) fluogenic dyes, respectively. (B) Ch-L clone was treated with the indicated concentrations of rotenone for 12h. Mitochondrial ROS levels (a), intracellular ROS levels (b), OCR (c), and Cln-1 mRNA levels by RT-PCR were monitored. (C) Ch-L clone was challenged with 5 μM rotenone for 12h with or without pretreatment of NAC for 6h. Cln-1 mRNA levels were examined by RT-PCR. (D) Ch-L clone was exposed to 300 μM H₂O₂ for the indicated time periods. Intracellular ROS levels (a) and mRNA levels (b) are shown. (E) RT-PCR for Cln-1 was performed after Ch-L clone was treated with the indicated concentrations of H₂O₂ for 3 days. (F) Ch-L clone was exposed to 300 μM H₂O₂ for the indicated time periods. Mitochondrial ROS levels (a) and mRNA levels of some respiratory complex subunits by qRT-PCR (b) are shown. *, p<0.05; **, p<0.01 vs. control by student t-test.

Fig. 4. Complex I inhibition induces Cln-1 expression by ROS-mediated HSF1 activation. (A-C) Ch-L clone was treated with 5 μM rotenone for the indicated time periods. A) Western blot analysis. (B) Cell lysates were incubated with lambda (λ) phosphatase for 2h and subjected to Western blot analysis. (C) RT-PCR for mRNA levels. (D) After Ch-L clone was treated with 5 μM rotenone for 12 h, nuclear and cytoplasmic fractions were isolated as described in ‘Materials and methods.’ (E) Ch-L clone was exposed to 5 μM rotenone for 12h with or without pretreatment of NAC for 6h and subjected to Western blot analysis. (F) Ch-L clone was exposed to 300 μM H₂O₂ for the indicated time periods and subjected to Western blot analysis. (G) Ch-L clone was transfected with siRNAs for HSF1 and then exposed to 5 μM rotenone for 12 h. Cell lysates were subjected to Western blot analysis. H) Ch-L clone was transfected with pcDNA-HSF1-HA for 2 days. Cell lysates were subjected to Western blot analysis.

Fig. 5. Complex I inhibition increases Cln-1 promoter activity through binding of HSF1. (A) Design of GRN-pGL3-luciferase reporter plasmid containing promoter region of Cln-1 from -2894 to +230. Putative HSF1 binding sites in the promoter region (-539 to -1) of Cln-1 are shown in the bottom panel. Putative HSF1 binding sequences are underlined with arrows. Bold sequences contain key sequences for HSF1 binding. (B) Ch-L clone was transfected with the GRN-pGL3-luciferase plasmid and treated with 5 μM rotenone for the indicated time periods. Promoter activity was monitored. (C) Promoter activity. Ch-L clone was transfected with the GRN-pGL3-luciferase plasmid and challenged with 5 μM rotenone for 12h with or without pretreatment of NAC for 6h. (D) Promoter activity. Ch-L clone was transfected with the GRN-pGL3-luciferase plasmid and pcDNA-HSF1-HA (HSF1). pcDNA3 was used as mock control for HSF1. (E) Promoter binding activity by ChIP assay using anti-HSF1 antibody. Ch-L clone was treated with 5 μM rotenone for the indicated time periods and subjected to ChIP assay.

Fig. 6. HSF1 regulates hepatoma cell invasion activity through Cln1 expression. (A) Western blot analysis. (B) SNU354, 423, and 449 cells were transfected with siRNA for Cln-1 for 2 days and subjected to cell invasion assay. Representative images of invaded cells are shown in the lower panel. (C) Ch-L clone was transfected with pcDNA-Cln-1, siNDUFA9, and/or siCln-1 for 2 days and then subjected to cell invasion activity (upper panel) and Western blot analysis (lower panel). (D) SNU449 cell was treated with NAC for 6h and subjected to Western blot analysis. (E) SNU449 cell was transfected with siRNAs
Mitochondria-mediated HSF1 activation induces Cln-1 for 2 days and then subjected to Western blot analysis (a) and cell invasion activity (b). **, p<0.01 vs. siNC by student t-test. (F) Schematic diagram for involvement of mitochondrial defect-induced HSF1 activation in Cln-1-mediated hepatoma cell invasiveness.
Fig. 1.

A  Ch-L  354  387  423  449

| OCR (%) | 100 | 39.5 | 85.4 | 32.2 | 20.5 (Ave) |
|---------|-----|------|------|------|-------------|
|         | +18.5 | +19.1 | +32.3 | +25.2 | +10.1 (SD) |

B  Ch-L  354  387  423  449

C

D

Protein expression ratio (tumor/surrounding)

Ch-L  354  387  423  449

β-actin

Cln-1

Comp IV

protein expression ratio (tumor/surrounding)
Fig. 1. - continue

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|----|----|----|
| S | T | S | T | S | T | S | T | S | T | S | T | S | T |
| Comp IV |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Cln 1  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| β-actin|  |  |  |  |  |  |  |  |  |  |  |  |  |

|   | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|----|----|----|----|----|----|----|----|----|----|----|----|
| S | T | S | T | S | T | S | T | S | T | S | T | S | T |
| Comp IV |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Cln 1  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| β-actin|  |  |  |  |  |  |  |  |  |  |  |  |  |

|   | 25 | 26 | 27 | 28 |
|---|----|----|----|----|
| S | T | S | T | S | T |
| Comp IV |  |  |  |  |  |
| Cln-1  |  |  |  |  |  |
| β-actin|  |  |  |  |  |

| Mito-down | 20 / 28 (71.4% of total) |
| Cln-1 up   | 18 / 28 (64.3% of total) |
| Mito-down + Cln-1 up | 14 / 28 (50% of total) |
|            | 14 / 20 (63.6% of Mito down) |
Fig. 2.

A

B

C (a)

D (a)

Cln-1 protein (fold induction)

Cln-1 mRNA (fold induction)

E (a)

F (a)

G (a)
Fig. 3.

A

B (a)

(c)

B (b)

(d)
**Fig. 3. - continue**

**C**

![Image showing Cln-1 and actin expression comparison between control (C), RNAi (R), and RNAi + Ac (R/NAc) groups after 6 hours.](image)

**D(a)**

![Bar graph showing DCF fluorescence intensity (a.u., mean) over time (C 1 3 6 12 24 72 h) for H2O2 concentration (300 μM).](image)

**E**

![Image showing Cln-1 and actin expression comparison between control (C) and H2O2 concentration (100, 200, 300 μM) over 3 days.](image)

**D(b)**

![Composite image showing Cln-1 and actin expression patterns.](image)

**F (a)**

![Graph showing MitoSox fluorescence intensity (a.u., mean) over time (C 1 3 6 12 24 72 h) for H2O2 concentration (300 μM).](image)

**F (b)**

![Graph showing mRNA levels of NDUF3A, UQCRB, and ATP5G3 over time (C 12 24 36 h) for H2O2 concentration (300 μM).](image)
Fig. 4.

A) 5μM Rotenone

B) 5 μM Ro

C) 5μM Rotenone

D) 5 μM Ro

E) NAC

F) 300 μM H₂O₂

G) siRNA: siNC #1 #2 Mix

H) pcDNA3 HSF1-HA 3:1 4:1
**Fig. 5.**

A

Cln1 promoter-pGL3

-539 CCAATCTGTA GAGTGAAAAA GTCTCTGAGG CTCCTTGCAAG AGACAAGTGA TGGAAGGACCC TTGACAGAAG
-469 AGAGCAGAGA GAGGAAAGAAG GGGGAGAAGA GCCAAGCAAAA GGGAGAGAAA ATGGTGATG GGGGAGGAG
-399 AGCGGGAGTT GGGAAGAAGA GCTTTTAAAT AAGATATTGG GAAAAGATA TTAAACCTAA AACTGCCAAGCT
-329 CTTGAGGAT CATTTTTTC TCTTTGTGTAG AGGATTCTAC GATACAGTGCA ATAGTAATT TTGGAATAATT
-259 GAGTGAAGT AAAGAAGCG GTGAAAGGCC TTACAGGAGGG GGAAGATCAG ACACAGAGAA GGCGCCAGGG
-179 AGCACGGCTCT GGTCGCTGTT CCTGGCCTGT GGTCCCAACG CGCGCAGCCCG CGCGTCCGCC AACCAGCCGC
-109 TCCCGCGGCC CTCTCGGGTA GCCGCCCTGA ACCGCAAGGG GGGGCCTGGCG CGGCTGCCGG GCTGAGGCCG
-39 GCGGAGCTCTGG CTTTAAATCG CGCGGCCCAAT CGGTCTGGTC ttcagttcc ggacgctgg agcaacgcga
+32 gctctctccag tccagactcag ag

B

Promoter activity (fold induction)

|          | C    | 6    | 12 (h) |
|----------|------|------|--------|
| Rotenone |      |      |        |
| C        | 1.0  | 1.5  | 2.0    |
| 6        | 2.0  | 2.5  | 3.0    |
| 12 (h)   | 3.0  | 2.5  | 2.0    |

C

Promoter activity (fold induction)

|          | NAC | Ro |
|----------|-----|----|
| C        | 1.0 |    |
| 10mM NAC | 1.0 | +  |
| 20mM NAC | 1.0 | +  |

D

Promoter activity (fold induction)

|          | pcDNA3 | HSF1 | pcDNA3 | HSF1 |
|----------|--------|------|--------|------|
| 5μM Rotenone | 1.0    | 1.5  | 1.0    | 1.5  |

E

Input no-Ab α-HSF1 Input no-Ab α-HSF1 Input no-Ab α-HSF1

Promoter binding (fold induction)

|          | C    | 6    | 12 (h) |
|----------|------|------|--------|
| C        | 1.0  | 1.0  | 1.0    |
| 6        | 1.0  | 1.5  | 2.0    |
| 12 (h)   | 1.0  | 1.5  | 2.0    |
Fig. 6.

A

|        | HSF1 | p-HSF1 | Cln-1 | β-actin |
|--------|------|--------|-------|---------|
| Ch-L   | ![Image](114x182 to 215x205) | ![Image](114x130 to 215x152) | ![Image](114x155 to 215x179) | ![Image](114x271 to 215x301) |
| 354    | ![Image](110x243 to 214x269) | ![Image](110x271 to 214x301) | ![Image](110x320 to 214x353) | ![Image](110x389 to 214x410) |
| 387    | ![Image](110x447 to 214x470) | ![Image](110x447 to 214x470) | ![Image](110x447 to 214x470) | ![Image](110x447 to 214x470) |
| 423    | ![Image](290x447 to 366x512) | ![Image](290x447 to 366x512) | ![Image](290x447 to 366x512) | ![Image](290x447 to 366x512) |
| 449    | ![Image](367x447 to 440x570) | ![Image](367x447 to 440x570) | ![Image](367x447 to 440x570) | ![Image](367x447 to 440x570) |

B

|        | Invaded cell # (x 10^2) |
|--------|-------------------------|
| pcDNA3 | ![Image](81x598 to 215x622) |
| pcDNA-Cln-1 | ![Image](81x569 to 215x594) |
| siNC  | ![Image](82x626 to 215x650) |
| siNdufa9 | ![Image](81x652 to 215x692) |
| siNdufa9 + Cln-1 | ![Image](81x698 to 215x722) |

C

|        | Invaded cell # (x 10^2) |
|--------|-------------------------|
| Ch-L   | ![Image](114x389 to 215x410) |
| 354    | ![Image](114x389 to 215x410) |
| 387    | ![Image](114x389 to 215x410) |
| 423    | ![Image](114x389 to 215x410) |
| 449    | ![Image](114x389 to 215x410) |

D

|        | Cln-1 | β-actin |
|--------|-------|---------|
| NAC, 6 h | ![Image](163x224) | ![Image](163x224) |
| 10(mM)  | ![Image](163x224) | ![Image](163x224) |
| 20(mM)  | ![Image](163x224) | ![Image](163x224) |

E (a)

|        | siNC | siHSF1 #1 | siHSF1 #2 |
|--------|------|-----------|-----------|
| HSF1   | ![Image](163x224) | ![Image](163x224) | ![Image](163x224) |
| Cln-1  | ![Image](163x224) | ![Image](163x224) | ![Image](163x224) |
| β-actin| ![Image](163x224) | ![Image](163x224) | ![Image](163x224) |

E (b)

|        | Invaded cell # (x 10^2) |
|--------|-------------------------|
| siNC   | ![Image](163x224) |
| #1     | ![Image](163x224) |
| #2     | ![Image](163x224) |

F

- damaged mitochondria
- ROS → HSF1 Phosphorylation → HSF1 Translocation to Nucleus
- HSF1 Phosphorylation → Cln-1 Transcription
- Cln-1 Phosphorylation → Hepatoma cell invasiveness