Role of CTGF in Sensitivity to Hyperthermia in Ovarian and Uterine Cancers

Graphical Abstract

Highlights
- Ovarian and uterine cancers have variable hyperthermia sensitivities
- CTGF promotes hyperthermia resistance
- Targeting CTGF can sensitize cancers to hyperthermic therapy
- In vivo hyperthermia by copper sulfide nanoparticle-based technology

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In Brief
The predictors of sensitivity of tumors to hyperthermia remain poorly understood. Hatakeyama et al. discovered variable hyperthermia sensitivity of ovarian cancers, and they identified CTGF as a key regulator of hyperthermia resistance. Silencing of CTGF sensitized ovarian tumors to hyperthermia in orthotopic models.

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Role of CTGF in Sensitivity to Hyperthermia in Ovarian and Uterine Cancers

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SUMMARY

Even though hyperthermia is a promising treatment for cancer, the relationship between specific temperatures and clinical benefits and predictors of sensitivity of cancer to hyperthermia is poorly understood. Ovarian and uterine tumors have diverse hyperthermia sensitivities. Integrative analyses of the specific gene signatures and the differences in response to hyperthermia between hyperthermia-sensitive and -resistant cancer cells identified CTGF as a key regulator of sensitivity. CTGF silencing sensitized resistant cells to hyperthermia. CTGF small interfering RNA (siRNA) treatment also sensitized resistant cancers to localized hyperthermia induced by copper sulfide nanoparticles and near-infrared laser in orthotopic ovarian cancer models. CTGF silencing aggravated energy stress induced by hyperthermia and enhanced apoptosis of hyperthermia-resistant cancers.

INTRODUCTION

Therapeutic hyperthermia is based on the premise of raising the temperature of tumor tissue to 40°C–43°C. It has been used for treatment of ovarian and other cancers. The rationale for this therapy is based on the direct-killing effects of temperatures above 41°C–42°C (Wust et al., 2002). Hyperthermia also is applied as an adjunctive therapy with various established cancer treatments, such as radiotherapy and chemotherapy (Moyer and Delman, 2008; Nagata et al., 1997; Palazzi et al., 2010; Lim et al., 2015). Some studies have suggested that hyperthermia activates the immune system against tumor cells, by increasing the release of heat shock proteins (HSPs) associated with tumor-specific antigens from heat-stressed or dying tumor cells that are phagocytized by antigen-presenting cells (APCs) (Binder et al., 2000).

As interest in hyperthermic treatment of cancer has increased, significant progress has been made in developing strategies to heat tumors via local, regional, and whole-body hyperthermia (van der Zee, 2002). Different types of energy have been used, including microwaves and radio waves (Gazelle et al., 2000; Seki et al., 1999), magnetic heating (Lee et al., 2011; Rodriguez-Luccioni et al., 2011), and ultrasound (Jolesz and Hynynen, 2002). Regional hyperthermia (e.g., hyperthermic intraperitoneal chemotherapy [HIPEC] heated to 42°C) theoretically enables direct contact between tumor cells and the chemotherapeutic agent to control residual microscopic disease (Wademan et al., 2012).

Even though hyperthermia is a promising therapeutic approach, multiple obstacles remain to be cleared. One of the major issues is that the tumor temperatures that must be reached for obtaining clinical efficacy are largely undefined (Wust et al., 2002). In the present study, we carried out a series of experiments to determine the molecular mechanisms of tumor response to hyperthermia. Moreover, we utilized nanoparticle (NP)-based approaches to silence key pathways to enhance tumor response to hyperthermia.
RESULTS

Diversity of Hyperthermia Sensitivity in Ovarian and Uterine Cancer Cell Lines

We first monitored the temperature transition in tumors during HIPEC in high-grade serous ovarian cancer patients (Figure S1; Table S1). Even though the perfusion temperature at the entrance was maintained at 42.5°C, the temperature in most of the tumors was about 40°C and the potential clinical benefit of these lower temperatures is unclear. We sought to determine the temperatures at which various cancer cells lose viability and to identify potential molecular regulators of sensitivity to hyperthermia. To address these questions, we tested hyperthermia sensitivity in a range of ovarian (A2780, A2780CP20, SKOV3, ES2, HeyA8, and PEO4) and uterine (Hec-1A, SKUT-2, KLE, and ISHIKAWA) cancer cells. We treated each cell line with hyperthermia for 1 hr, equivalent to the duration of HIPEC, using an incubator at the indicated temperatures, and we assessed cell viability (Figure 1A). We defined the temperature that produced 50% cell death as the median lethal temperature 50 (LT50) using curve fitting.

Based on the calculation of LT50s, the difference between the lowest and highest LT50 was about 4°C, suggesting that the cells showed substantial variability in sensitivity to hyperthermia. The median LT50 in the tested cells was 46.5°C. We classified cells with LT50s above the median, SKOV3, HeyA8, ES2, PEO4, and KLE, as hyperthermia-resistant (HTR) cells. We classified cells with LT50s below the median, A2780, A2780CP20, Hec-1A, ISHIKAWA, and SKUT-2, as hyperthermia-sensitive (HTS) cells (Figure 1B). Since hyperthermia can increase blood flow, we also examined whether endothelial cells are affected by hyperthermia. For these experiments, we used RF24 endothelial cells (Pecot et al., 2013). The LT50 of RF24 cells was ~45.5°C; thus, these cells were classified as HTS cells.

Figure 1. Variability in Hyperthermia Sensitivity of Ovarian and Uterine Cancer Cell Lines

(A) Ovarian and uterine cancer cells were treated with hyperthermia for 1 hr at the indicated temperatures and then incubated at 37°C. Cell viability was determined using an MTT assay at 24 hr. The value in each graph represents the LT50 that produced 50% cell death. Average, ± SEM; n = 3–6.

(B) Difference in each LT50 from the median LT50 (46.5°C) in the tested cells. The cells having LT50s above or below the median LT50 were classified as HTR or HTS, respectively.

See also Figures S1 and S2.
Hyperthermia treatment also enhanced sensitivity of HTS A2780 and HTR HeyA8 cells to cisplatin (Figure S2B).

Specific Gene Signatures in HTR Cells as Determined Using Comprehensive Gene Expression Analysis

To identify potential molecular regulators of sensitivity of cancer cells to hyperthermia, we compared the gene expression profiles for HTS and HTR cells using the Cancer Cell Line Encyclopedia (CCLE) database. Specifically, we compared the gene expression profiles of HTS (A2780, ISHIKAWA, and Hec-1A) and HTR (SKOV3, HeyA8, ES2, and KLE) cell lines. We identified differentially expressed genes between HTS and HTR lines setting the cutoff at p value < 0.05 and fold change > 2. This identified 327 upregulated genes and 92 downregulated genes in HTR cells (Figure 2A; Table S2). Heatmaps of the expression of the top 30 upregulated and downregulated genes in HTR cells are shown in Figure 2B. Enriched gene ontology (GO) annotations for biological processes for these top 30 genes were related to wound healing, cell motion, and cell adhesion (Figure S3A). We then examined these 30 genes using ingenuity pathway analysis (IPA) to identify molecules that can possibly interact with each other. The baseline expression of these genes was generally correlated with increased gene expression in HTR cells in the CCLE database. Among the 15 genes, the expression of FN1, CXCL1, and IL8 was highly increased in HTR compared with HTS cells.

Determination of the Difference in Response of Ovarian Cancer Cells to Hyperthermia Using Quantitative Proteomics

To examine the mechanisms by which HTR cells survive under hyperthermia, we first compared global changes in gene expression between HTS A2780 and HTR SKOV3 cells after hyperthermia using gene microarrays. Most genes that were significantly upregulated after hyperthermia were common between A2780 and SKOV3 cells (Figures S3C and S3D; Table S3). These results suggested that hyperthermia sensitivity was either not dependent on changes in gene expression or that there are changes in protein abundance in response to hyperthermia.

To test this hypothesis, we subjected A2780 and SKOV3 cells treated with hyperthermia to isobaric tandem mass tags (TMT) labeling and offline two-dimensional liquid chromatography-tandem mass spectrometry (2D-LC-MS/MS) to comprehensively assess changes in protein expression induced by hyperthermia. We treated the cells with hyperthermia at 46°C for 1 hr and harvested protein after incubation at 37°C for 4 hr. We identified 53 upregulated and 159 downregulated proteins in A2780 cells, and...
Figure 3. Determination of the Difference in Response to Hyperthermia in A2780 and SKOV3 Cells Using Quantitative Proteomics

(A) Volcano plots of differentially expressed proteins in HTS A2780 (left) and HTR SKOV3 (right) cells after hyperthermia identified using 2D-LC-MS/MS with TMT. Upregulated and downregulated proteins (fold change, > 0.5; p < 0.2 for A2780 and p < 0.05 for SKOV3) are highlighted in red and green, respectively.

(B) Graphs show the top ten enriched GO annotations for biological processes for differentially expressed proteins in A2780 (upper) and SKOV3 (lower) cells.

(C) Graph shows the top ten canonical pathways in SKOV3 cells in response to hyperthermia as determined using IPA.

(D) Left: schematic of the glycolysis pathway. Enzymes highlighted in green represent downregulated proteins in SKOV3 cells after hyperthermia. Right: heatmap shows differential expression patterns for proteins involved in the glycolysis pathway according to proteomic analysis.

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we identified 309 upregulated and 536 downregulated proteins in SKOV3 cells (Figure 3A; Table S4). We analyzed the GO terms for biological processes for the differentially expressed proteins in A2780 and SKOV3 cells using the Database for Annotation, Visualization and Integrated Discovery (DAVID), and we found that processes involved in glucose metabolism were specifically enriched in SKOV3 cells after hyperthermia (Figure 3B). We also found that glycolysis was the primary pathway affected by hyperthermia in SKOV3 cells according to canonical pathway analysis (Figure 3C). Expression of most of the proteins involved in the glycolysis pathway was downregulated in SKOV3 cells, suggesting that the glycolysis pathway was impaired after hyperthermia treatment (Figure 3D). Upon examining the interactions between the 15 molecules that are differentially expressed between HTS and HTR cells and the glycolysis-related molecules identified in the proteomics described here, we identified FN1, SGK1, CTGF, and CXCL1 to be the potential players regulating the glycolysis pathway in HTR cells (Figure 3E).

Genes Responsible for Hyperthermia Resistance in Ovarian Cancer Cells
According to network mapping, FN1, SGK1, CTGF, and CXCL1 were likely linked with glycolysis (Figure 3E). Because CXCL1 was likely downstream of the glycolysis pathway, we focused our effort on FN1, SGK1, and CTGF. We measured FN1, SGK1, and CTGF protein expression in ten cell lines via western blotting (Figures 4A and S4A). The expression was increased in HTR cells, which was generally consistent with the FN1, SGK1, and CTGF gene expression profiles of the ten cell lines (Figure S3B).

We then examined the potential effects of silencing CTGF, FN1, or SGK1 on response to hyperthermia in HTR (SKOV3, HeyA8, ES2, and KLE) and HTS (A2780) cells using small interfering RNA (siRNA) (Figure 4B). Silencing of FN1 and SGK1 did not sensitize HTR or HTS cells to hyperthermia (Figures S4B–S4E). On the other hand, silencing of CTGF sensitized the HTR cells to hyperthermia with a decrease in the LT50 by 1.0°C–2.5°C (Figure 4C). Downregulation of CTGF expression had no effect on sensitization to hyperthermia in A2780 cells. No sensitization was observed when HTS RF24 endothelial cells were treated with siCTGF (Figures S2C and S2D). Transfection of a CTGF-expressing vector enhanced CTGF mRNA levels in HeyA8 cells, which made the cells tolerant of hyperthermia with about a 2°C increase in the LT50 (Figures 4D and 4E). Because CTGF is a secreted molecule, we treated HTR cells with recombinant human CTGF (rCTGF) in a medium to examine its functional effect. HTR HeyA8 and ES2 cells treated with CTGF siRNA (siCTGF) exhibited tolerance of hyperthermia with LT50 increases of 2.3°C and 1.0°C following the addition of rCTGF (Figure 4F). Conversely, anti-CTGF antibody treatment sensitized SKOV3 cells to hyperthermia (Figure S2E).

Sensitization of Tumors to Hyperthermia by CTGF Silencing In Vivo
Next, we evaluated the effect of CTGF silencing on sensitization to hyperthermia and tumor growth in orthotopic models of ovarian cancer. First, in the HeyA8 model, we administered siCTGF into the peritoneal cavity twice weekly using the 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) nanoliposomal delivery platform (Figure 5A). This resulted in decreased CTGF expression in tumors (Figures SS4A). The day before near-infrared (NIR) laser treatment, we intravenously injected CuS NPs modified with poly(ethylene glycol) (PEG-CuS NPs) into tumor-bearing mice (day 18). PEG-CuS NPs about 10 nm in diameter (Figures SSD and SSE) circulate in the blood for long periods after systemic administration, and they passively accumulate in tumors via an enhanced permeability and retention (EPR) effect (Zhou et al., 2010; Hatakeyama et al., 2011). We then treated tumors with local hyperthermia using an NIR laser at 980 nm for 5 min on day 19, because CuS NPs can convert the optical energy of an NIR laser to thermal energy (Figure S5F) (Zhou et al., 2015). As temperatures of 46°C–60°C are associated with reversible cellular damage in proportion to the exposure time (Vanagas et al., 2010; Wood et al., 2002), we maintained the tumor temperature at about 50°C (Figure 5B).

We harvested HeyA8 tumors on day 30, and we found that local hyperthermia decreased tumor weights and nodule numbers in control siRNA (siCont)- and siCTGF-treated mice (Figures 5C–5F). Furthermore, tumor growth and metastasis were markedly suppressed by the combination of CTGF silencing and local hyperthermia, with no changes in body weight (Figures 5 and S5B). A significant increase in cleaved caspase-3-positive cells was observed after hyperthermic treatment in HeyA8 tumors treated with siCTGF (Figure 5G). No obvious decrease in the number of Ki67-positive tumor cells was observed in the siCTGF plus hyperthermia treatment group compared to the siCont group (Figure S5C). Next, we used the HTR SKOV3 tumors surgically implanted into the ovary of nude mice (n = 9–10 per group). The tumor weight and metastasis were significantly decreased by the combination of CTGF silencing and local hyperthermia (Figures 5H–5K). These results demonstrated that CTGF silencing in vivo sensitized ovarian tumors to local hyperthermia treatment.

Identification of the Mechanism by which CTGF Causes Hyperthermia Resistance
To further examine how CTGF silencing sensitized HTR cells to hyperthermia, we examined CTGF levels in HeyA8 and SKOV3 cells longitudinally. Since the expression of some housekeeping genes might be affected by hyperthermia, we confirmed that β-actin and 18S could be used as reference genes in a hyperthermia experiment (Figures S6A–S6C). CTGF expression increased after hyperthermic treatment, and the increase of

(E) Network map of 15 upregulated and downregulated genes in HTR cells that were identified in the CCLE database and glycolysis-involved proteins that were identified in SKOV3 cells using 2D-LC-MS/MS with TMT. Red and green indicate upregulation and downregulation, respectively, with darker shades indicating greater changes in expression. The lines indicate binding of two gene products, and lines terminating with arrows indicate one gene product acting on another gene product.

See also Figure S3.
CTGF after hyperthermia was suppressed by siCTGF-based treatment (Figure 6A). CTGF protein expression also increased after hyperthermic treatment in HeyA8 cells, but the increase was blocked by siCTGF-based treatment over 24 hr (Figure 6B). Silencing of CTGF increased the population of sub-G0/G1 (apoptotic) cells at 24 hr after hyperthermia (Figure 6C), and it induced cell death at 24 hr after hyperthermia treatment (Figure 6D).

As shown in Figure 3D, quantitative proteomic analysis suggested that glycolysis was impaired in the HTR SKOV3 cells after hyperthermia treatment. Inhibition of glycolysis results in energy stress. AMPK-activated protein kinase (AMPK) plays a key role in regulating cellular energy homeostasis under energy stress. AMPK is activated by phosphorylation when energy stress occurs (Hardie et al., 2012). We observed increased phosphorylated AMPK levels induced by hyperthermia, and these levels were enhanced in HeyA8 and SKOV3 cells treated with siCTGF (Figure 6E). The increase was independent of LKB1 activity (Figure S6D). The sensitization to hyperthermia also was observed when SKOV3 cells were treated with metformin (Figure S2F), which can activate AMPK phosphorylation and induce energy stress (Lengyel et al., 2015). It is possible that CTGF regulates glycolytic activity by modulating the expression of glycolysis-related genes after hyperthermia. To confirm that inhibition of the glycolysis pathway affected sensitivity of HTR cells to hyperthermia, we treated HeyA8 and SKOV3 cells with a glycolysis inhibitor, 2-deoxy-D-glucose (2-DG). This treatment sensitized HTR HeyA8 and SKOV3 cells to hyperthermia with a decrease in the LT50 by about 2°C (Figure 6F), and it increased the population of sub-G0/G1 in HeyA8 and SKOV3 cells (Figure 6G).
Figure 5. CTGF Silencing-Induced Sensitization of HeyA8 Tumors to Hyperthermia by Local Thermal Ablation with CuS NPs and an NIR Laser In Vivo

(A) Our animal experiment schedule. Mice were injected intraperitoneally with luciferase-transfected HeyA8 cells or injected into the ovary with luciferase-transfected SKOV3. SiRNA/DOPC liposomes (5 μg siRNA/mouse) were administered via intraperitoneal injection twice weekly beginning on day 7 after cell injection. On day 18, CuS NPs (8 OD/mouse) were injected intravenously into the mice, and, on day 19, tumors were treated with an NIR laser for 5 min. Tumors were collected from the mice on day 30 for the HeyA8 model or day 40 for the SKOV3 model.

(B) Representative transition of the temperature of an HeyA8 tumor. The temperature was monitored during local hyperthermic treatment using a thermal camera (red line) and fiber probe (black line).

(C–E) Average tumor weights (C), numbers of tumor nodules (D), and numbers of distant metastatic nodules (E) of HeyA8 model on day 30 are shown (n = 4–5/group). Average, ± SEM.

(F) Representative pictures show HeyA8 tumor burdens in mice (arrows).

(legend continued on next page)
DISCUSSION

Here we identified molecular determinants of cancer cell sensitivity to hyperthermia. We characterized the role of CTGF as a key target for enhancing response to hyperthermia treatment. CTGF silencing inhibited the recovery of glycolytic activity after hyperthermia, which resulted in energy stress-induced cell death (Figure 7).

The temperatures used in clinical hyperthermia treatment range from 40°C to 45°C; typically, the temperature is 42.5°C in cases of regional hyperthermia, such as during HIPEC (Wademan et al., 2012). However, most of the prior studies lacked any actual assessment of temperature in tumors. Moreover, given the gradient between the temperature of infusate and actual tumor-level temperatures, true hyperthermia is unlikely to have been achieved in such treatments. The clinical benefit of such a low temperature for cancer therapy is unclear. We discovered that cancer cells exhibited substantial diversity in their sensitivity to hyperthermia as determined by the LT50.

We observed little difference in gene expression changes after hyperthermia between HTS versus HTR cells despite substantial difference in protein expression. It is known that heat shock response where heat shock proteins are upregulated rapidly is highly conserved in all organisms for the protection of cells from a wide range of harmful conditions, including heat shock (Jolly and Morimoto, 2000), which is consistent with our observation that enhanced gene expression induced by hyperthermic treatment occurred independently of hyperthermia sensitivity. While RNA molecules are typically only damaged above 85°C–90°C, thermal stability of protein represents a wide range (Bischof and He, 2005). These may account for wider effects on expression of protein than RNA by hyperthermia.

Our findings related to the role of CTGF in glycolysis-related genes are supported by its role in development (de Winter et al., 2008). For example, downregulation of glycolysis-related genes, such as Pgml, Pgam1, and Eno1, and the decrease in glycolytic activity and ATP levels have been reported in chondrocytes from Ctgf (Ccn2)-null mice (Maeda-Uematsu et al., 2014; Murase et al., 2016). It is likely that CTGF regulates the expression of glycolysis-related genes after hyperthermia to prevent cancer cells from energy-stressed cell death. However, some studies have reported that energy stress downregulates the expression of CTGF via the inactivation of YAP in the Hippo pathway in HEK293 cells (Mo et al., 2015; Wang et al., 2015). It is possible that such signaling may be context dependent, since the 14-3-3 signaling pathway was downregulated in the HTR SKOV3 cells after hyperthermia (Figures 3C and S3E).

To overcome the challenges with fluid-based hyperthermia, we used local thermal ablation with CuS NPs and an NIR laser. CuS NPs have smaller size than plasmonic gold-based nanostructures, which give them better chances to extravasate into extravascular fluid space. In addition, the absorption properties of CuS NPs are not subject to change upon repetitive laser irradiation. Our approach for gene silencing with a DOPC nanoliposomal delivery system can be readily translated into clinical settings (Rupaimoole et al., 2014; Wu et al., 2014) to sensitize cancers to hyperthermia, and it can be used for other key targets. Such an approach also could be beneficial for cancer cells only exposed to sub-lethal thermal stress by HIPEC.

The tumor endothelial cell is also an important target for hyperthermia. Based on the LT50 of RF24 cells, endothelial cells were sensitive to hyperthermia. Thus, while our study focuses on the effect of hyperthermic treatment on tumor cells, it is possible that the decreases in tumor burden after hyperthermia treatment in vivo were due to the effects of both tumor and endothelial compartments.

In summary, we discovered that cancer cells have diverse hyperthermia sensitivity. CTGF is an important regulator of hyperthermia sensitivity, and it may represent an important target for further development.

EXPERIMENTAL PROCEDURES

Our materials; HIPEC procedure; cell culture; the cell-cycle study; immunoblotting; RNA extraction; qRT-PCR; TMT labeling and 2D-LC-MS/MS-based quantitative proteomics; cDNA microarray analysis, synthesis, and transmission electron microscopy (TEM) of CuS NPs; and immunohistochemistry are described in the Supplemental Experimental Procedures. 12 patients were enrolled in the study following IRB approval.

Hyperthermic Treatment of Cancer Cells In Vitro

The details are described in the Supplemental Experimental Procedures. Briefly, cells were treated with hyperthermia via heating cells in an incubator for 1 hr at the indicated temperatures followed by incubation at 37°C in a regular cell culture incubator. At the indicated time points, viability was determined by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. The LT50 that produced 50% cell death was calculated via curve fitting. Transfection of siRNA or pDNA and treatment procedures are described in the Supplemental Experimental Procedures.

CCLE Database Analysis

The expression data for HTR cells (SKOV3, HeyA8, ES2, and KLE) and HTS cells (A2780, Hec-1A, and ISHIKAWA) were downloaded from the CCLE website (https://portals.broadinstitute.org/ccle/home). The details are available in the Supplemental Experimental Procedures.

DOPC Liposome Preparation

DOPC liposomes encapsulating siRNA were prepared as described previously (Rupaimoole et al., 2014; Wu et al., 2014).

Synthesis of CuS NPs

CuS NPs were synthesized according to previously reported procedures (Zhou et al., 2010, 2015). The details are available in the Supplemental Experimental Procedures.

(G) Immunohistochemistry assay of cleaved caspase-3 in tumor sections (scale bar, 50 μm). Left: representative images show cleaved caspase-3 staining in HeyA8 tumors. Right: bar graph represents ratio of cleaved caspase-3-positive cells per total cells per hours post fertilization (hpf, n = 13–18 fields). Average, ± SEM.

(H–J) Average tumor weights (H), numbers of tumor nodules (I), and frequency of metastases to distant sites (J) of SKOV3 model on day 40 are shown (n = 9–10/group). Average, ± SEM.

(K) Representative pictures show SKOV3 tumor burdens in mice (arrows). See also Figure S5.
Figure 6. Mechanism by which CTGF Regulates Hyperthermia Resistance

(A) Time profile of CTGF mRNA expression in HeyA8 and SKOV3 cells treated with control siRNA (siCont) or siCTGF, as determined using qRT-PCR. Cells treated with siCont of siCTGF were treated with hyperthermia at 46°C for 1 hr (0–1 hr) and further incubated at 37°C up to the time points. Average, ± SEM; n = 3.

(B) Time profile of CTGF protein expression in HeyA8 cells treated with siCont or siCTGF. Left: immunoblot shows CTGF and β-actin expression. Right: band intensity of CTGF and β-actin was semiquantified using the ImageJ software program (NIH). Average, ± SEM; n = 3.

(C) Sub-G0/G1 (apoptotic) populations of HeyA8 and SKOV3 cells treated with siCont or siCTGF after hyperthermia at the indicated time points are shown. Average, ± SEM; n = 3.

(D) Interference contrast images show HeyA8 and SKOV3 cells treated with siCont or siCTGF after hyperthermia. Scale bars, 100 μm.

(E) Time profiles of phosphorylated AMPK (pAMPK) and AMPK expression in HeyA8 and SKOV3 cells treated with siCont or siCTGF. Left: immunoblot shows pAMPK and total AMPK expression. Right: band intensity of pAMPK and AMPK was semiquantified using the ImageJ software program. Average, ± SEM; n = 3.

(F) The effect of inhibition of glycolytic activity on the sensitivity of HTR HeyA8 and SKOV3 cells to hyperthermia. HeyA8 and SKOV3 cells treated with 2-DG (25 mM) were treated with hyperthermia at the indicated temperatures, and viability was determined at 24 hr after the initiation of hyperthermia. Average, ± SEM; n = 3.

(G) Sub-G0/G1 populations of HeyA8 and SKOV3 cells treated with 2-DG (25 mM) were determined 24 hr after hyperthermic treatment at the indicated temperatures. Average, ± SEM; n = 3.

See also Figures S2 and S6.
Hyperthermia inhibits glycolytic activity. On the other hand, CTGF is upregulated after hyperthermia. Upregulated CTGF may regulate glycolytic activity, which prevents HTR cells from undergoing energy stress and death. Silencing of CTGF presumably inhibits the activation of glycolytic activity in HTR cells after hyperthermia, and it induces energy stress and cell death.

In Vivo Models
Orthotopic mouse models of ovarian cancer were developed as described previously (Rupaimoole et al., 2014; Wu et al., 2014), and the details are available in the Supplemental Experimental Procedures. SiRNA-incorporated neutral DOPC nanoliposomes were injected intraperitoneally at a dose of 5 μg siRNA/mouse twice weekly for about 3 weeks beginning 1 week after cell injection. Photothermal ablation using CuS NPs and an NIR laser was used for in vivo local hyperthermic treatment. The details are described in the Supplemental Experimental Procedures.

Protein Expression Analysis Using TMT Labeling and 2D-LC-MS/MS-Based Quantitative Proteomics
The details of proteome sample preparation, LC-MS/MS analysis, and proteomic data analysis are available in the Supplemental Experimental Procedures. All raw files were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PRIDE: PXD005055 and 10.6019/PXD005055.

ACCESSION NUMBERS
The accession numbers for the raw files reported in this paper are PRIDE: PXD005055 and 10.6019/PXD005055.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.10.020.

AUTHOR CONTRIBUTIONS

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