Inhibition of Histone Demethylase JMJD1A Improves Anti-Angiogenic Therapy and Reduces Tumor-Associated Macrophages

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Abstract

Antiangiogenic strategies can be effective for cancer therapy, but like all therapies resistance poses a major clinical challenge. Hypoxia and nutrient starvation select for aggressive qualities that may render tumors resistant to antiangiogenic attack. Here, we show that hypoxia and nutrient starvation cooperate to drive tumor aggressiveness through epigenetic regulation of the histone demethylase JMJD1A (JHDM2A; KDM3A). In cancer cells rendered resistant to long-term hypoxia and nutrient starvation, we documented a stimulation of AKT phosphorylation, cell morphologic changes, cell migration, invasion, and anchorage-independent growth in culture. These qualities associated in vivo with increased angiogenesis and infiltration of macrophages into tumor tissues. Through expression microarray analysis, we identified a cluster of functional drivers such as VEGF, FGF18, and JMJD1A, the latter which was upregulated in vitro under conditions of hypoxia and nutrient starvation and in vivo before activation of the angiogenic switch or the prerefractory phase of antiangiogenic therapy. JMJD1A inhibition suppressed tumor growth by downregulating angiogenesis and macrophage infiltration, by suppressing expression of FGF2, HGF, and ANG2. Notably, JMJD1A inhibition enhanced the antitumor effects of the anti-VEGF compound bevacizumab and the VEGFR/KDR inhibitor sunitinib. Our results form the foundation of a strategy to attack hypoxia- and nutrient starvation–resistant cancer cells as an approach to leverage antiangiogenic treatments and limit resistance to them. Cancer Res; 73(10); 3019–28. ©2013 AACR.

Introduction

Angiogenesis is essential for tumor growth and metastasis (1, 2). VEGFs and their receptors (VEGFR) are key regulatory factors of angiogenesis (3, 4). Therefore, targeting the VEGF/VEGFR pathways by antiangiogenesis is a clinically validated anticancer treatment (5). Antiangiogenic therapies are, however, limited to certain types of cancer and may elicit malignant progression and metastasis (6, 7). Thus, the molecular mechanism of eliminating resistant and refractory cancer cells by using such antiangiogenic treatments needs to be elucidated. Under such treatments tumor cells may be exposed to both hypoxia and nutrient starvation. We previously reported that hypoxic and nutrient-starved tumor microenvironment may play a critical role in tumor aggression and the refractoriness to the antiangiogenic treatments (8) and that affected host cells resulted in secondary leukemia in murine cancer models (9). In addition, we found that an epigenetic regulator, histone demethylase JHDM1D, suppressed tumor growth by regulating angiogenesis under hypoxia and nutrient starvation (10). Epigenetic regulation of angiogenesis under hypoxia and nutrient starvation can be important for tumor progression and aggression, but its mechanism has not yet been elucidated. The present study was conducted to investigate the mechanism of tumor aggressiveness, and our findings show that histone demethylase JMJD1A plays an important role in tumor progression under hypoxia and nutrient starvation.

Materials and Methods

Cell culture, hypoxia, and nutrient starvation deprivation

Human cervix HeLa, hepatic HepG2, epidermoid A431 carcinomas, glioblastoma T98G, rhabdomyosarcoma A673, and murine melanoma B16 cell lines were purchased from American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 1× penicillin/streptomycin, and 1× glutamine. For hypoxic conditions, cells were cultured in a humidified incubator with 5% CO2 at 37°C, and the atmosphere was replaced with nitrogen-gas before the addition of culture medium. For nutrient starvation, cells were cultured in a media with only serum-free RPMI 1640 media. Cells were maintained under hypoxic and nutrient starvation conditions in a separate incubator before the addition of culture medium. Cells were then incubated for 48 h before experiments. For hypoxia and nutrient starvation, cells were cultured in a humidified incubator with 5% CO2 at 37°C, and the atmosphere was replaced with nitrogen-gas before the addition of culture medium. Cells were then incubated for 48 h before experiments. For hypoxia and nutrient starvation, cells were cultured in a humidified incubator with 5% CO2 at 37°C, and the atmosphere was replaced with nitrogen-gas before the addition of culture medium. Cells were then incubated for 48 h before experiments.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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the American Type Culture Collection. The murine uterine cancer cell line, HSML, was kindly provided by Dr. Kudoh (Hirosaki University, Hirosaki, Japan). Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Nacalai Tesque) supplemented with 10% FBS at 37°C in a 5% CO₂ atmosphere, except HSML cells, which were maintained in RPMI-1640 medium (Nacalai Tesque). Nutrient deprivation was conducted as previously described under hypoxia (Fig. IA; refs. 8, 9).

**Cell proliferation assay**

Cell proliferation was measured using the sulforhodamine B (SRB) assay as previously described (8).

**Phase-contrast microscopy**

Cells were plated on Petri dishes and images were captured using the ×10 to ×20 objective lenses of an Olympus inverted microscope. Phase-contrast images were acquired using KinetiC Imaging software connected to a Hamamatsu CCD camera (Hamamatsu Photonics K.K).

**Wound-healing assay**

Cells were seeded onto 12-well plates at a density of 1 × 10⁶ cells. Following attachment, 3 to 6 hours after seeding, scratches were made within the monolayer by using a sterile pipette tip. The same spots were photographed under phase-contrast microscopy at 12 hours post-“wounding.”

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**Figure 1.** Hypoxia and nutrient starvation double-deprivation increases Akt phosphorylation, cellular migration and invasion, and anchorage-independent tumor cell growth in vitro. A, schematic diagram of a hypoxia and nutrient starvation DDS cycle. Human cancer cells were cultured under hypoxia (pO₂, 1%) and nutrient starvation (1:100 ratio) for approximately 3 days and recovered by culturing under normoxia in 30% nutrition for 1 day and then 100% nutrition for more than a day until 80% to 90% confluence was achieved. This hypoxia and nutrient starvation cycle was conducted for 10 or more cycles. B, ten cycles of DDS (DDS10) in HeLa and T98G cells induced a cell morphologic change from epithelial cobblestone-like cells to mesenchymal-like spindle cells. C, DDS stimulated Akt phosphorylation in HeLa and T98G cells. D, DDS increased the cellular migration, which was examined using a wound-healing assay. E, DDS increased cellular invasion into Matrigel migration chambers. F, DDS increased anchorage-independent tumor cell growth in HeLa and T98G cells. G, HeLa-DDS10 and T98G-DDS10 cells become less sensitive to pacritaxel. Cells were treated with 10 μmol/L pacritaxel for 3 days. *, P < 0.05; **, P < 0.01, determined by the Student t test.
migrated distance was assessed using 9 measurements at time point. Wound area was calculated using the following equation: Wounded filled (%) = ([wound width at 12 hours – wound width at 0 hours]/wound width at 0 hours) × 100. The experiments were carried out in triplicate. SEM was calculated and analyzed using the unpaired, 2-sided Student t test.

### Invasion assay

Twenty microliters of Matrigel (BD Biosciences) was added to a Boyden chamber insert containing 8-µm pores (BD Falcon) and solidified for 2 hours in 37°C. Then, 200 µL of DMEM supplemented with 0.5% FBS containing 5 × 10⁴ cells were plated on the upper chamber, and 300 µL DMEM supplemented with 10% FBS was added in the lower chamber. Cells were cultured for 8 to 48 hours depending on the cell types, and the number of invasive cells was counted following staining with crystal violet.

### Colony formation assay

Cells were suspended in DMEM containing 0.6% methylcellulose (Wako) supplemented with 10% FBS and seeded into 6-well plates at a density of 1 × 10⁵ cells per well in triplicate. After 7 to 10 days, anchorage-independent colonies were counted using a microscope.

### Western blotting

Cell lysates were applied to a 7.5% to 15% PAGE and transferred to a polyvinylidene difluoride membrane (Invitrogen). The membrane was incubated with rabbit polyclonal anti-JHDMA2 antibody, anti-histone H3K9me1, anti-histone H3K9me3, anti-histone H3, anti-Akt, anti-phosphorylated Akt, or mouse monoclonal anti-histone H3K9me2 and anti-β-actin antibody (1:1,000; Millipore), followed by incubation with horseradish peroxidase–conjugated secondary antibodies (GE Healthcare). Signals were detected using enhanced chemiluminescence detection reagents (GE Healthcare) and images were acquired using a luminescent image analyzer (LAS3000, FujiFilm).

### Gene expression analysis using real-time PCR

Total RNA was extracted from cells using the Isogen reagent (Nippon Gene), converted to cDNA by using the Prime Script reverse transcriptase (Takara) as per the manufacturer’s instructions, and used for quantitative real-time PCR amplification using SYBR Green (Takara; Supplementary Table S1).

### Gene expression profiling and statistical analysis

Total RNA (5 µg) was converted to aminoallyl-modified cDNA by random 6-mer and oligo(dt)-primed polymerization using PrimeScript reverse transcriptase (Takara), coupled to X-hydroxysuccinimidyl esters of Cy3 (Agilent), and then hybridized to a human Oligo Microarray slide (human G4110F, Agilent) as per the manufacturer’s instructions. Arrays were scanned using a G2565BA Microarray Scanner System and analyzed using the GeneSpring GX software (Tomy Digital Biology).

### Animal studies and tumor xenografts

Murine B16 or human HeLa or A673 cells (1 × 10⁷) were subcutaneously injected into C57BL/6J or C.B17/Icr-scid/scid mice, respectively. Mice were treated with i.v. injection of 1 to 10 mg/kg/wk intraperitoneally (i.p.) of either human IgG or anti-VEGF antibody (Chugai-Roche) every 7 days starting on day 2. VEGFR inhibitor (sunitinib) was solubilized in methylcellulose and administrated orally at the concentration of 40 mg/kg/d. To visualize the hypoxic region in the tumor tissue, 60 mg/kg of pimonidazole was administrated (i.p./i.v.) at 30 to 45 minutes before perfusion fixation. Tumor volume was measured, and the data were analyzed using the Student t test. All animal care procedures were in accordance with institutional guidelines approved by the University of Tokyo and Tokyo Medical and Dental University.

### Histological analysis and immunohistochemistry

Tumor tissues were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and subjected to hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) staining.

### Immunofluorescence staining

Freshly frozen or 4% PFA-fixed tumor tissues were cut 14-µm thick by using a Cryostat (Leica) and stained with hamster anti-CD31 (BD Biosciences), rat anti-CD11b (BD Biosciences), rat anti-F4/80 (AbD Serotec), mouse monoclonal anti-αSMA1 (Sigma), and mouse monoclonal anti-pimonidazole (Cosmo Bio). The sections were incubated with the appropriate secondary antibody and the nuclear-staining dye To-Pro-3 (Invitrogen Life Technologies) and then analyzed using a confocal microscope (Radiance 2000; Bio-Rad). We quantified the number of CD31⁺/αSMA1⁺ blood vessels/pericytes in the total area of the tumor. We analyzed 5 to 10 fields per sample (n = 5) for quantification of blood vessel density (k pixels). We also counted the number of CD11b⁺ and F4/80⁻ cells in the total area of the tumor in 5 to 10 fields for each sample (n = 5).

### siRNA

siRNAs were designed against human JMJD1A and obtained commercially (Invitrogen Life Technologies). In this study, 2 sequences of siRNAs were used against hJMJD1A, designated as siRNA1 (AGAGAAUUCAAGAGAUCCAGGAG) and siRNA2 (AACACAUCCAGGUCUUAUGU). Cells were transfected with JMJD1A siRNAs or negative (scramble) control siRNA by using the Lipofectamine RNAiMAX transfection reagent according to the manufacturer’s instructions (Invitrogen Life Technologies). The ability of the siRNA to inhibit JMJD1A expression was assessed at 48 hours after transfection.

### Results

**Hypoxia and nutrient starvation accelerate tumor aggressiveness and resistance to chemotherapy**

To clarify whether the hypoxia and nutrient starvation double-deprivation stress (DDS) stimulates tumor aggressiveness in human cancer cells, we used the well-known human cancer cell lines HeLa (human cervical carcinoma) and T98G (human glioblastoma). As previously reported, we developed a cell culture system for maintaining these cells under DDS...
Hypoxia and nutrient starvation double-deprivation stimulates tumorigenicity associated with increased angiogenesis and macrophage infiltration in vivo. A, anti-VEGF treatment decreases tumor growth after 20 days. Tumor (HeLa cells-bearing C.B17/SCID scid/scid mice were treated with 10 mg/kg/wk of control immunoglobulin G or anti-VEGF antibody (n = 5; P < 0.05). B, antiangiogenic treatment expands the hypoxic and nutrient-starved areas in the tumor tissue. Tumor (HeLa xenograft) tissues were stained with a modified PAS (P, nutrient-rich area in pink) and pimonidazole/hypoxyprobe (H, hypoxic area in brown). V, blood vessels indicated by red arrows; left. Schematic diagram of hypoxia and nutrient starvation in the tumor tissue (right). C, representative micrographs of immunohistochemical staining indicate tumor xenograft of HeLa and HeLa-DDS cells in mice with CD31 (red), CD11b (green), and To-Pro-3 nuclear staining (blue) in tumor tissues. Scale bars, 150 μm. D, DDS increases tumor growth, E, tumor angiogenesis (CD31+ area), F, infiltration of CD11b+ macrophages in vivo. HeLa and HeLa-DDS10 cells were subcutaneously inoculated into C.B17/SCID scid/scid mice (n = 5) and the experiment was carried out on day 10 after tumor injection (P < 0.05). G, representative micrographs of immunohistochemical staining indicate transplantation of B16 and B16-DDS cells in mice with CD31+ (red), CD11b+ (green), and To-Pro-3 nuclear staining (blue) in tumor tissues. Scale bars, 150 μm. H, DDS increases tumor growth, I, tumor angiogenesis (CD31+ area). J, infiltration of CD11b+ macrophages in vivo. B16 and B16-DDS10 cells were subcutaneously inoculated into C57BL/6J mice (n = 5) and experiment was carried out on day 10 after tumor injection (P < 0.05).

(Fig. 1A; refs. 8, 9). HeLa and T98G cells were continuously exposed to 10 cycles of DDS (HeLa-DDS10 and T98G-DDS10 cells). HeLa-DDS10 and T98G-DDS10 cells showed cell morphologic changes (Fig. 1B), increased Akt phosphorylation (Fig. 1C), cellular migration (Fig. 1D), invasion (Fig. 1E), and anchorage-independent cell growth (Fig. 1F) in vitro. To investigate whether increased invasion is due, in part, to the high proliferation rate of DDS cells, we compared the cell proliferation rates of untreated HeLa and T98G cells to the proliferation rates of HeLa- and T98G-DDS10 cells. We found no significant difference in the rate of cell proliferation in HeLa and T98G cells (Supplementary Fig. S1), suggesting that cell proliferation rate was not responsible for the increased migration and invasiveness of DDS cells (Fig. 1D and E). Notably, HeLa-DDS10 and T98G-DDS10 cells were less sensitive to conventional chemotherapeutic agents such as paclitaxel (Fig. 1G, Supplementary Fig. S2; ref. 8). This finding suggests that hypoxia- and nutrient starvation–resistant human cancer cells develop tumor aggressiveness and resistance to chemotherapeutic agents.
Cancer cells resistant to hypoxia and nutrient starvation increased tumor growth and tumor angiogenesis in vivo

The extent of hypoxia and nutrient starvation in tumor tissues following treatment with antiangiogenic agents was examined by treating tumor-bearing immunodeficient mice with VEGF-neutralizing antibody (bevacizumab; 0–10 mg/kg/wk i.p.), and the hypoxic- and nutrient-rich areas were visualized using anti-pimonidazol and a modified PAS, respectively. While tumor growth was significantly suppressed after anti-VEGF treatment (Fig. 2A, Supplementary Fig. S3A), hypoxic (pimonidazol) and nutrient-starved (PAS) areas were increased in tumor tissues in HeLa and A673 cells (Fig. 2B, Supplementary Fig. S3B). In conclusion, hypoxia alone does not affect cell proliferation, but hypoxia and nutrient starvation cooperatively suppressed the cell proliferation of cancer cells in vitro (Supplementary Fig. S4).

To investigate whether cancer cells resistant to hypoxia and nutrient starvation stimulate tumorigenicity, we subcutaneously inoculated 1 × 10⁶ of original HeLa and HeLa-DDS10 cells in scid/scid mice. HeLa-DDS10 cells showed increased initial tumor growth (Fig. 2C and D), increased CD31vascular formation (Fig. 2E), and infiltration of CD11b/monocytes (Fig. 2F) and F4/80macrophages (Supplementary Fig. S5), suggesting a contribution of tumor-associated monocytes/macrophages (TAM; refs. (11, 12). In addition, orthotopic transplantation of B16-DDS10 cells in immunocompetent mice showed a significant increase in the initial tumor growth (Fig. 2G and H), angiogenesis (Fig. 2I), and monocyte (Fig. 2J) and macrophage infiltration (Supplementary Fig. S5) compared with implantation with original B16 cells (11, 13). Notably, blood vessel functionality and coverage of pericytes (α-SMA+) on CD31blood vessels were not significantly different between control and DDS cells in vivo (Supplementary Fig. S6). In addition, HeLa-DDS10 cells were still sensitive to treatment with anti-VEGF antibody (bevacizumab; 5 mg/kg/wk i.p.) in vivo (Supplementary Fig. S7). Thus, cancer cells survived under the DDS-stimulated tumorigenicity by regulation of tumor angiogenesis.

Hypoxia and nutrient starvation cooperatively induce the epigenetic regulator JMJD1A

To determine the molecular targets against oxygen and nutrition DDS-resistant cancer cells, we conducted a microarray analysis of 4 different human cancer cells (HeLa, HepG2, A431, and T98G) that were subjected to hypoxia, nutrition starvation, and DDS. We found that a cluster of DDS-responsive genes such as VEGF-A, PGF18, and jumonji domain-containing protein 1A (JMJD1A) was upregulated in cancer cells (Fig. 3A–C, Supplementary Table S2 and Supplementary Fig. S8). Histone demethylase JMJD1A was upregulated under DDS in both murine and human cancer cells (Fig. 3D, Supplementary Table S3) and the upregulation prominently increased following long-term exposure to DDS (48–72 hours; Fig. 3E). In addition, JMJD1A expression was normalized by returning the

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**Figure 3.** Hypoxia and nutrient starvation DDS induces DDS-responsive gene clusters including JMJD1A. A, cluster of DDS-inducible genes following DDS in human cancer cells (HeLa, HepG2, A431, and T98G) under hypoxia, nutrient starvation, and DDS. B, expression of JMJD1A mRNA was cooperatively upregulated under hypoxia and nutrient starvation (C, control; H, hypoxia, NS, nutrient starvation; DDS, hypoxia and nutrient starvation double stress). *P < 0.05. C, JMJD1A expression was upregulated under DDS treatment at the protein level. D, Venn diagram of DDS-responsive genes common to human and mouse cancer cells. E, JMJD1A mRNA expression was especially increased in human and murine cancer cells in response to long-term exposure to DDS.

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Inhibition of JMJD1A Improves Antiangiogenesis
Inhibition of JMJD1A suppresses tumor growth and angiogenesis, sensitizing cancer cells to VEGF and VEGFR inhibitors

To investigate the role of JMJD1A in tumor progression, we silenced JMJD1A in HeLa and A673 cells by using siRNAs (Fig. 5A). We chose these cell lines because previous work has shown that these cells can grow as xenografts in scid/scid mice (14) whereas T98G cells cannot (9). In addition, because A673 cells have been shown to be sensitive to anti-VEGF antibody treatment (15), we examined the tumorigenicity of HeLa and A673 cells following treatment with antiangiogenic agents. JMJD1A inhibition showed a minor effect on cell proliferation under hypoxia, nutrient starvation, and DDS (Supplementary Fig. S11), as well as anchorage-independent tumor cell growth in HeLa and A673 cells in vitro (Supplementary Fig. S12). JMJD1A inhibition slightly decreased cell migration and invasion in vitro (Supplementary Figs. S13 and S14).

To investigate the role of JMJD1A in tumor growth in vivo, we subcutaneously inoculated $1 \times 10^7$ of JMJD1A-silenced HeLa and A673 cells in scid/scid mice ($n = 5$). JMJD1A inhibition significantly suppressed HeLa and A673 cell tumorigenicity in vivo (Fig. 5B). The siRNAs against JMJD1A effectively suppressed $JMJD1A$ expression for 8 days in HeLa and A673 cells (Supplementary Fig. S15), resulting in an overall reduction of tumor growth in vivo for longer than 8 days (Fig. 5B). In contrast, overexpression of JMJD1A significantly promoted initial tumor growth in vivo (Supplementary Fig. S16).

We next investigated whether DDS cells are sensitive to siRNA-mediated knockdown of JMJD1A in vivo. Because HeLa-DDS10 cells were more tumorigenic than the original HeLa cells (Fig. 2D), HeLa-DDS10 cells were more sensitive to siRNA against JMJD1A than the original HeLa cells (Supplementary Fig. S17).

To clarify the tumor-suppressive role following JMJD1A inhibition, we examined the immunohistochemical staining of tumor angiogenesis and the infiltration of TAM lineage cells in the tumor tissues of A673 cell–transplanted mice. CD31$^+$ vascular formation (Fig. 5C and D) and infiltration of CD11b$^+$ macrophage lineage cells (Fig. 5C and E) were significantly decreased in the tumor tissues under JMJD1A inhibition, suggesting that JMJD1A participates in tumor angiogenesis and the infiltration of TAMs.

To further investigate the antiangiogenic effects of JMJD1A inhibition, we specifically examined the mRNA expression of the tumor-secreted (human origin) and host-derived (mouse origin) angiogenic factors in the tumor tissues of JMJD1A-silenced HeLa and A673 cells. The expression of tumor-derived hFGF2, hHGF, and hAng2 and host-derived mAng2 were significantly decreased by JMJD1A inhibition in the tumor tissues of HeLa and A673 cells (Fig. 5F, Supplementary Table S1 and Supplementary Fig. S18). Moreover, JMJD1A inhibition significantly increased the tumor-suppressive effects of another antiangiogenic VEGFR inhibitor (sunitinib) in vivo ($n = 10$; Fig. 6B). We further examined the mechanism of suppression of tumor angiogenesis by inhibition of JMJD1A via microarray and gene ontology analysis following siRNA against JMJD1A and/or anti-VEGF antibody treatment (bevacizumab) in A673 cells. We found that inhibition of JMJD1A specifically decreases expression of genes such as...
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FGF2, CSE, and S100A9 (Supplementary Table S4) related to cell movement, leukocyte and myeloid cell migration, angiogenesis, and lymphangiogenesis (Supplementary Fig. S20). This explains why an inhibition of JMJD1A by siRNAs can prevent the infiltration of immune cells such as monocytes/macrophages into tumor tissue, resulting in decreased tumor angiogenesis (Fig. 5). Introducing epigenetic modifications in hypoxic and nutrient-starved cancer cells, such as by JMJD1A inhibition, is an effective anticancer strategy for improving antiangiogenesis (Fig. 7).

Discussion

Antiangiogenesis is a clinically validated cancer treatment (5). However, the emergence of drug resistance and tumor aggressiveness, partly due to an extreme tumor microenvironment, can be major clinical problems (6–9). In this study, we investigated the mechanism underlying tumor aggressiveness by using a model system of antiangiogenesis (8, 9). Oxygen and nutrition deficiency stimulated cell migration, invasion (Fig. 1), and tumorigenicity associated with increased tumor angiogenesis and infiltration of TAMs (Fig. 2). We have shown that histone demethylase JMJD1A, an epigenetic regulator, is highly expressed in cancer cells in response to hypoxia and nutrient starvation (Fig. 3) and that JMJD1A inhibition suppressed tumor angiogenesis by downregulating fibroblast growth factor (FGF2), hepatocyte growth factor (HGF), and Ang2 and resulted in significant suppression of tumor growth and improvement of anti-VEGF antibody (bevacizumab) and VEGFR inhibitor (sunitinib) treatment (Figs. 5 and 6). Taken together, our results indicate that targeting cancer cell resistance to hypoxia and nutrient starvation can be a strategy for overcoming resistance to antiangiogenic treatments.

Inhibition of angiogenesis suppresses the formation of new tumor vasculature (5) and may normalize the vasculature, resulting in increased efficacy of the combination chemotherapy (16). We found that antiangiogenic treatments expanded the hypoxic and nutrient-starved areas within the tumor tissue (Fig. 2B) and that hypoxia- and nutrient starvation-resistant cancer cells showed tumor aggressiveness such as increased migration, invasion (Fig. 1), tumorigenicity, tumor
angiogenesis, and infiltration of tumor-associated monocytes and macrophage cells, suggesting that DDS10 cells actively recruit monocytes and macrophages (Fig. 2, Supplementary Fig. S5). In addition, hypoxia- and nutrient starvation-resistant cells became less sensitive to conventional chemotherapy (Fig. 1G; ref. 8), suggesting that cancer cell refractoriness following antiangiogenic treatment (6, 7, 17, 18) could be due to resistance to the combinational chemotherapy, in addition to activation of both tumor cells and associated cells (19). Thus, combination chemotherapeutic regimens should be considered to perturb the DDS-resistant cancer cells and tumor-associated cells in combination with antiangiogenic treatment.

The adaptation of cancer cells to tumor microenvironments involving oxygen or nutrient deficiencies, acidosis, and reactive oxygen species (ROS) is required for tumor progression and metastasis (20, 21). The oxygen or nutrient deficiency and acidosis can be adapted by metabolic alterations to glycolysis (Warburg effect), gluconeogenesis, and other pathways (22–24). Although prokaryotic cells use a "stringent control" under nutrient deficiencies by terminating cell proliferation while synthesizing defective nutrient components (25, 26), the metabolic alteration can be achieved in mammalian cells via transcriptional and epigenetic regulation (27). Epigenetic regulation of metastasis-related genes can also lead to resistance to ROS (28). The "stringent control" of mammalian cells may play important roles during nutrient starvation, but to date there are no studies showing this. We previously reported that histone demethylase JHDM1D is an essential epigenetic regulator in tumor progression under nutrient starvation by angiogenesis downregulation (10). Interestingly, JMJD1A acts as a pro-angiogenic regulator that promotes initiation of tumor growth, whereas JHDM1D suppresses tumor growth, possibly playing a role during the adaptation to both hypoxia and nutrient starvation. Thus, epigenetic regulation under hypoxia and nutrient starvation could also be essential for both tumor aggressiveness and the shift in metabolism, but the control mechanism underlying this has not yet been identified.

We identified that the histone demethylase JMJD1A is an essential epigenetic regulator of tumor progression under hypoxia and nutrient starvation. JMJD1A is involved in spermatogenic development (29), metabolism in obesity (30), and hypoxic cancer cells under HIF1α regulation (31). Expression of JMJD1A can be a poor prognostic maker in patients with cancer (32). Loss of JMJD1A resulted in reduced proliferation and invasiveness of human colon cancer cells in vitro, which may lead to decreased tumor growth in vivo (32). In this study, while JMJD1A significantly decreased tumor growth in vivo (Fig. 5), inhibition of JMJD1A showed only a minor effect on proliferation and invasion of HeLa and A673 cells in vitro (Supplementary Figs. S11 and S12). Therefore, we suggest that JMJD1A alters the tumor microenvironment by affecting angiogenesis and TAMs.

However, the exact role of JMJD1A in tumor progression and aggression has not been elucidated to date. JMJD1A inhibition significantly decreased tumor angiogenesis and macrophage infiltration into tumor tissues (Fig. 5C–E). Although Ang1 and Ang2 conduct antagonistic as well as agonistic functions on the regulation of angiogenesis (33), expression of several
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pro-angiogenic factors such as angiopoietins, FGFs, and HGF was downregulated by JMJD1A inhibition within the tumor tissues of HeLa and A673 cells (Fig. 5F, Supplementary Table S4 and Supplementary Fig. S18). Moreover, JMJD1A was significantly upregulated at the angiogenic switch and the pre-fracture phase of antiangiogenesis (Fig. 4, Supplementary Fig. S10), suggesting the importance of JMJD1A at these stages. Strikingly, JMJD1A inhibition delayed tumor growth in combination with anti-VEGF (bevacizumab) and anti-VEGFR treatment (sunitinib); possibly, by affecting the refractory phase of the tumor against antiangiogenesis (Fig. 6, Supplementary Fig. S19), suggesting that modification of epigenetic regulators can influence both cancer cells and tumor-associated cells.

Recently, small-molecule inhibitors targeting epigenetic modulators have garnered much attention in the development of cancer chemotherapy drugs. For example, DNA demethylase inhibitors, 5-azacytidine and 5-aza-2-deoxycytidine, commonly used in clinical cancer therapy (34). Many inhibitors of histone modifiers such as EzH2 are now in clinical trials. Thus, it is possible that epigenetic modification through inhibition of JMJD1A can be a novel way to overcome the refractoriness of tumors to antiangiogenic therapy. On the basis of our results, therapies targeting cancer cells that are resistant to hypoxic and nutrient-starved tumor microenvironments can comprise an anticancer strategy for sensitizing cells anti-angiogenic treatments and preventing tumor recurrence and aggressiveness.

Disclosure of Potential Conflict of Interest
M. Shibuya has Honoraria from Speakers Bureau as the symposium speaker and is on the Consultant/Advisory Board as a research advisor. No potential conflicts of interest were disclosed by the other authors.

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