ORIGINAL ARTICLE
Metformin alters DNA methylation genome-wide via the H19/SAHH axis

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The molecular mechanisms underlying the antineoplastic properties of metformin, a first-line drug for type 2 diabetes, remain elusive. Here we report that metformin induces genome-wide alterations in DNA methylation by modulating the activity of S-adenosylhomocysteine hydrolase (SAHH). Exposing cancer cells to metformin leads to hypermethylation of tumor-promoting pathway genes and concomitant inhibition of cell proliferation. Metformin acts by upregulating microRNA let-7 through AMPK activation, leading to degradation of H19 long noncoding RNA, which normally binds to and inactivates SAHH. H19 knockdown activates SAHH, enabling DNA methyltransferase 3B to methylate a subset of genes. This metformin-induced H19 repression and alteration of gene methylation are recapitulated in endometrial cancer tissue samples obtained from patients treated with anti-diabetic doses of metformin. Our findings unveil a novel mechanism of action for the drug metformin with implications for the molecular basis of epigenetic dysregulation in cancer. This novel mechanism of action also may be occurring in normal cells.

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INTRODUCTION
Metformin, a biguanide compound, is among the most commonly used drugs worldwide for the treatment of type 2 diabetes owing to its high efficacy and minimal side effects. In recent years, there has been significant interest in metformin as a potential cancer chemopreventive and/or therapeutic agent (reviewed in Pollak1 and Pryor and Cabreiro2). Despite extensive research and ongoing multicentered randomized clinical trials on the efficacy of metformin as an anticancer agent, the antineoplastic mechanisms of action of this drug remain enigmatic. Both indirect (systemic) and direct mechanisms have been proposed (reviewed in Pollak, Pryor and Cabreiro, Foretz et al., and Pavlova and Thompson). By altering the endocrine-metabolic milieu of the host that is, reducing systemic levels of glucose and insulin, metformin may decrease cancer cell proliferation (reviewed in Pavlova and Thompson). However, the magnitude of these systemic effects may not be sufficient to impact cancer cell growth directly, especially in patients with normal baseline glucose and insulin levels or in cancers that are insensitive to insulin. Alternative systemic effects of metformin have, therefore, been proposed, including inhibition of pro-inflammatory cytokines and augmentation of host immune response to cancer cells. In contrast, dozens of other studies using in vitro and/or rodent models suggest that metformin may directly interact with cancer cells to elicit its antineoplastic effect. Favored mechanisms, to date, include induction of ‘energy stress’ (through inhibition of the mitochondrial respiratory chain complex I) and activation of the key energy sensor AMPK and subsequent mTOR inhibition, thereby leading to tumor suppression. While alterations in cancer cell metabolism through both AMPK-dependent and AMPK-independent pathways contribute to the direct cellular effects of metformin, the precise mechanisms leading to these metabolic changes have yet to be defined. In addition, it remains to be determined whether the effects of metformin observed in vitro and in animal models actually occur in human tissues and in particular, whether conventional anti-diabetic doses of metformin are adequate to achieve these effects, in part, because most preclinical studies to date have used drug levels significantly higher than those used in patients with diabetes. Adequately addressing these questions will be key to the success of ‘repurposing’ metformin for its potential use as a cancer chemopreventive or therapeutic agent.

We previously reported that metformin reduces the motility and invasiveness of both endometrial and ovarian cancer cells in part by decreasing the production of H19. H19 is a long noncoding RNA that is highly expressed during fetal development but strongly downregulated in most adult tissues. Interestingly, however, H19 is aberrantly expressed in almost all cancer types tested, where it has been shown to play an important role in tumor biology (reviewed in Matouk et al. and Raveh et al.7). We recently found in muscle cells (murine) that H19 alters DNA methylation genome-wide through its interaction with S-adenosylhomocysteine hydrolase (SAHH), the only eukaryotic enzyme capable of hydrolyzing S-adenosylhomocysteine (SAH), a strong feedback inhibitor of SAM-dependent methyltransferases including DNA methyltransferases (DNMTs). H19 binds to SAHH and...
inhibits its hydrolytic activity.\textsuperscript{8} We showed that in mouse skeletal muscle cells decreasing H19 by siRNA activates SAHH and facilitates the removal of SAH, which in turn relieves the inhibition of DNMT3B by SAH and enables it to methylate a subset of genes.\textsuperscript{8} Exposing human endometrial or ovarian cancer cells to metformin decreases H19 levels (although how metformin decreases H19 remains to be investigated), with a concomitant increase in methylation within the promoter region of H19.\textsuperscript{5} Whether this methylation change is mechanistically linked to metformin’s action in cancer cells or is just coincidental remains to be investigated. These findings raise the intriguing possibility that metformin may function to alter gene methylation in malignant cells.

Here, we show that metformin induces let-7-mediated H19 degradation through activation of AMPK. Exposing endometrial cancer cells to metformin leads to global DNA methylation changes mediated through the H19/SAHH axis. Impressively, these same metformin-induced H19 repression and gene methylation changes are also observed in human tumor samples derived from endometrial cancer patients treated with metformin. These studies provide further support for the notion that the dose range of metformin commonly used for the treatment of diabetes has the potential to directly impact cancer cell biology through an AMPK-dependent mechanism.

\textbf{RESULTS}

Metformin increases H19 methylation through the H19/SAHH axis

We previously observed that treatment of the endometrial cancer-derived cell line ARK2 with the biguanide antidiabetes drug metformin results in a decrease in H19 levels and an increase in methylation within the promoter region of H19.\textsuperscript{5} Whether these changes were mediated by the H19/SAHH axis, however, as previously had been reported in normal mouse muscle cells\textsuperscript{8} was unclear. To address this possibility, we first asked whether downregulation of H19 following metformin exposure would activate SAHH. ARK2 cells were incubated with metformin followed by measurement of H19 levels and SAHH activity 24 h later. Metformin treatment reduced H19 levels by ~ 4-fold (Figure 1a), and increased SAHH activity by ~ 20% (Figure 1b) without affecting its protein level (Figure 1c). Importantly, knockdown of H19 expression using siH19\textsuperscript{5} to levels comparable to those induced by metformin (Figure 1a, compared with Figure 1a) also increased SAHH activity by ~ 20% (Figure 1e) without altering the level of this protein (Figure 1c). Moreover, the physical interaction between SAHH and H19, which previously had been observed in mouse muscle cells,\textsuperscript{8} also was observed in ARK2 cells by RNA immunoprecipitation. Using an SAHH-specific antibody (Anti-SAHH), we observed an ~ 8-fold enrichment of H19 in SAHH-containing ribonucleoprotein complexes (RNPs) relative to control immunoglobulin G (IgG) immunoprecipitates (Figure 1g, left column), whereas a GAPDH mRNA (control) was not enriched.

\begin{figure}[h]
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\caption{Metformin-induced H19 reduction activates SAHH leading to DNMT3B-dependent hypermethylation of H19 promoter. (a–c) ARK2 cells were treated with (+) or without (−) metformin (metf) at a final concentration of 2 mM for 48 h. H19 RNA levels (a), \textit{in vivo} SAHH activity (b) and SAHH protein levels (c) were assessed by reverse transcriptase (RT)–quantitative PCR (qPCR), SAHH activity assay and western blot analysis, respectively. In (c), representative gel images of three independent western blot experiments are shown, with quantification on the right. (d–f) ARK2 cells were transfected with control siRNA (siCon) or H19-specific siRNA (siH19). H19 RNA levels (d), \textit{in vivo} SAHH activity (e) and SAHH protein levels (f) were assessed. (g) RNA immunoprecipitation (RIP) with mouse monoclonal anti-SAHH antibody or preimmune IgGs from ARK2 cells. RNA levels in immunoprecipitates were determined using RT-qPCR. Levels of H19 and GAPDH mRNA are presented as fold enrichment in anti-SAHH relative to IgG immunoprecipitates. (h) ARK2 cells were transfected with siCon or Dnmt3b-specific siRNA (siDnmt3b). Twenty-four hours later, cells were treated with or without metformin, followed by QMSP analysis of H19 promoter methylation 24 h later. (i) Confirmation of Dnmt3b knockdown by RT-qPCR (left panel) and western blot analysis (right panel). Numbers are mean ± s.d. (n = 3). **P < 0.01. NS, no statistical difference.}
\end{figure}
under these circumstances (right column). Together, these results support the hypothesis that metformin-induced downregulation of H19 activates SAHH in human endometrial cancer cells.

Changes in SAHH activity change could potentially affect all DNMTs, including DNMT1, DNMT3A, and DNMT3B. Here we focused initial studies on DNMT3B, because it previously has been shown to be associated with the H19/SAHH pathway.\(^8\) To determine whether DNMT3B may contribute to the observed metformin-induced H19 promoter methylation, DNMT3B was downregulated in ARK2 cells using siDNmt3b,\(^9\) and the effects on H19 promoter methylation were evaluated using quantitative methylation-specific PCR (QMSP).\(^6\) While metformin increased H19 promoter methylation (Figure 1h, compare second bar with first bar on left) as previously reported,\(^5\) the combination of this drug with Dnmt3b knockdown (Figure 1i) restored the level of H19 promoter methylation to that of the control (Figure 1h, third and fourth bars on right). These results suggest that DNMT3B may contribute to metformin-induced H19 hypermethylation, although possible contributions from other DNMTs cannot be excluded at this time.

**Let-7 mediates metformin-induced H19 destabilization**

Our previous studies from mouse muscle cells revealed a double negative-feedback loop between H19 and let-7 in which H19 blocks let-7 function by sequestering let-7, while binding of let-7 to H19 promoter methylation could activate SAHH in human endometrial cancer cells. Together, these results suggest that metformin upregulates let-7 in endometrial cancer cells, resulting in H19 degradation.

**Figure 2.** Metformin increases let-7 production, which targets H19 for destabilization. (a, b) ARK2 cells were treated with metformin, followed by RNA extraction and reverse transcriptase (RT)–quantitative PCR (qPCR) to determine RNA levels of let-7 (a) and H19 (b). (c, d) ARK2 cells were incubated with metformin or water control (Con) for 2 h. Transcription inhibitor actinomycin D was added at a final concentration of 5 \(\mu\)g/ml, followed by RNA extraction at the indicated time points. mRNA levels of H19 (c) and RPL22 (d, as a negative control) were measured by RT–qPCR. (e, f) ARK2 cells were transfected with let-7 mimic (Let-7) or negative control microRNA (miCon) in the presence of actinomycin D. H19 (e) and RPL22 (f) mRNA levels were determined by RT–qPCR. Numbers are mean ± s.d. \((n = 3)\). **\(P < 0.01\). *\(P < 0.05\).
KSRP at both the mRNA (Figure 3d) and protein (Figure 3e) levels. In control siRNA-transfected cells, metformin increased let-7 (Figure 3f, left two columns) and decreased H19 RNA levels (Figure 3g, left two columns). These effects were lost when KSRP was downregulated (Figures 3f and g, right two columns), suggesting that KSRP is required for metformin-induced let-7 upregulation and subsequent H19 destabilization.

The H19/SAHH/DNMT3B pathway mediates metformin effects in other cancer cells

To address the possibility that the H19/SAHH/DNMT3B pathway mediates metformin effects in other cancer cells, we tested the effects of metformin on the human breast cancer-derived cell line MCF-7. As mentioned above, previous studies have shown that metformin treatment results in an increase in let-7 levels in breast cancer-derived MCF-7 cells. Here we confirmed and extended these results by demonstrating that metformin (or AICAR) treatment leads to an increase in let-7 (Figure 4a) and a decrease in H19 (Figure 4b) and an increase in SAHH activity (Figure 4c) in MCF-7 cells. Metformin treatment also results in increased H19 promoter methylation in MCF-7 cells (Figure 4d, left two columns), and this increase could be abrogated (Figure 4d, right two columns) by repression of DNMT3B expression using siDnmt3b (Figure 4e). Similar results were observed in human liver cancer cell lines (data not shown). Together, these results suggest that the H19/SAHH/DNMT3B axis is active in multiple cancer types, where it can mediate metformin-induced hypermethylation of H19.

Metformin alters DNA methylation genome-wide

Given that siRNA-mediated H19 knockdown alters DNA methylation genome-wide in mouse muscle cells, and that metformin downregulates H19 (Figure 1a), we sought to determine whether exposure of endometrial cancer cells to metformin would induce changes in global DNA methylation. Thus, ARK2 cells were incubated with metformin for 48 h, followed by genome-scale DNA methylation profiling using previously described methods. Metformin treatment led to extensive methylation changes genome-wide compared with control-treated cells (Supplementary Figure S2 and Supplementary Data S1, and Gene Expression Omnibus (GEO) accession number GSE85974). Some genes became hypermethylated, others became hypomethylated and a third group showed no significant change in methylation. These observations were reminiscent of our previous findings studying siRNA-mediated H19 downregulation in mouse muscle cells. The differential effects of metformin on gene methylation were not surprising since these changes likely reflect a composite of both the direct and indirect effects of metformin. For example, activation of SAHH as a result of H19 repression by metformin would facilitate not only DNA methylation by DNMTs (direct effects) but also methylation of proteins that regulate chromatin structures (and DNMT accessibility), which in turn could influence DNA methylation (indirect effects). Indeed, the methylation status of a given gene is determined not only by activation of DNMTs but also by modifications of chromatin-bound proteins.

Thus, it is reasonable to predict that a subset of the genes that become hypermethylated following metformin treatment may result from the direct effect of metformin. To test this hypothesis we randomly selected four genes from the top 2000 gene list showing strong hypermethylation in the promoter region to test as proof of principle (in addition to H19) following metformin treatment (Supplementary Data S1). Among these genes, DMRTA2 encodes a transcription factor involved in human female germ cell development. KCNG2 encodes a potassium channel subunit whose altered expression has been associated with malignant glial...
Constitutive activation of this gene contributes to the development of tumors; and PSMD10 encodes a regulatory subunit of the 26S proteasome. Constitutive activation of this gene contributes to hepatocarcinogenesis. TRA2A encodes an RNA binding protein involved in the regulation of pre-mRNA splicing. Its increased expression has been found in hepatocellular carcinoma. ARK2 encodes an RNA binding protein involved in the regulation of pre-mRNA splicing. Its increased expression has been found in hepatocellular carcinoma. ARK2 encodes an RNA binding protein involved in the regulation of pre-mRNA splicing. Its increased expression has been found in hepatocellular carcinoma. ARK2 encodes an RNA binding protein involved in the regulation of pre-mRNA splicing. Its increased expression has been found in hepatocellular carcinoma. ARK2 encodes an RNA binding protein involved in the regulation of pre-mRNA splicing. Its increased expression has been found in hepatocellular carcinoma. ARK2 encodes an RNA binding protein involved in the regulation of pre-mRNA splicing. Its increased expression has been found in hepatocellular carcinoma. ARK2 encodes an RNA binding protein involved in the regulation of pre-mRNA splicing. Its increased expression has been found in hepatocellular carcinoma. ARK2 encodes an RNA binding protein involved in the regulation of pre-mRNA splicing. Its increased expression has been found in hepatocellular carcinoma. ARK2 encodes an RNA binding protein involved in the regulation of pre-mRNA splicing. Its increased expression has been found in hepatocellular carcinoma. ARK2 encodes an RNA binding protein involved in the regulation of pre-mRNA splicing. Its increased expression has been found in hepatocellular carcinoma. ARK2 encodes an RNA binding protein involved in the regulation of pre-mRNA splicing. Its increased expression has been found in hepatocellular carcinoma. ARK2 encodes an RNA binding protein involved in the regulation of pre-mRNA splicing. Its increased expression has been found in hepatocellular carcinoma. ARK2 encodes an RNA binding protein involved in the regulation of pre-mRNA splicing. Its increased expression has been found in hepatocellular carcinoma.
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**Figure 5.** SAHH and DNMT3B are required for metformin-induced methylation changes in the DMRs of the indicated genes. (a) ARK2 cells were treated with control (Con), metformin (Metf) or metformin plus inhibitor (DEA). DNAs were isolated 48 h later and analyzed by QMSP using primers specific for the individual DMR. (b) ARK2 cells were transfected with siCon or siDnmt3b. Twenty-four hours later, cells were treated with or without metformin, followed by DNA extraction and QMSP analysis of the indicated DMRs 24 h later. Numbers are mean ± s.d. (n = 3). **P < 0.01. *P < 0.05. NS, no statistical difference.

methylation were measured in tumor tissue samples from both pre- and postoperative samples. As previously reported, preoperative metformin treatment resulted in an increase in AMPK phosphorylation (Figure 6a) as well as decreased tumor cell proliferation (Figure 6b). Strikingly, metformin treatment also resulted in decreased H19 levels (Figure 6c) and increased methylation in each of the five genes (Figure 6d) previously tested in vitro (see Figure 5a).

**DISCUSSION**

Using human cancer cell lines as a model system, we show that metformin functions to alter DNA methylation in a genome-wide fashion and that this action is at least in part mediated by the H19/SAHH/DNMT3B axis. As illustrated in Figure 6e, exposing cells to metformin activates AMPK leading to increased levels of let-7, which in turn targets H19 for degradation. This frees SAHH from sequestration by H19, which would otherwise prevent it from hydrolyzing SAH, a feedback inhibitor of DNMT3B, with an end point of increased DNA methylation. The biological significance of these results is highlighted by the observation of similar findings in parallel studies using human endometrial cancer tissue samples from patients treated with doses of metformin commonly used for the treatment of diabetes. Together, these studies provide strong support for a direct role of metformin in regulation of those gene expression events that play a vital role in aspects of cancer cell biology. Given that this regulatory pathway also appears to function in other cancer cell types (Figure 4 and data not shown), and that diverse types of cancer cells express H19 (reviewed in Matouk et al9 and Raveh et al4), our findings support the conclusion which have proposed that metformin warrants further clinical investigation as both a chemopreventative agent and as a potential therapeutic agent in certain cancer patients.

Moreover, our studies establish an important mechanistic link between metformin and H19 promoter hypermethylation. It is intriguing to speculate that H19 degradation as a result of AMPK activation and let-7 upregulation induced by metformin may serve as an initial trigger that ultimately leads to chronic inhibition of H19 transcription due to promoter hypermethylation. Such a feedforward mechanism would help to reinforce metformin’s action on H19 repression in cancer cells.

These findings also identify a new mechanism of epigenetic dysregulation in cancer cells. Many mechanisms have been proposed to explain H19’s role in tumor initiation and progression, including inhibition of the tumor suppressor retinoblastoma Rb through H19-encoded miR-675, alteration of gene transcription via interaction with Polycomb repressive complex 2 components, and sequestration and inhibition of the tumor suppressor microRNA let-7. Our studies suggest that another mechanism of H19-mediated carcinogenesis may also be at play: genome-wide dysregulation of gene methylation. Since the initial discovery of methylation changes in specific genes in primary human tumors, epigenetic dysregulation has been increasingly recognized as a hallmark of cancer (reviewed in Sandoval and Esteller). Three types of alterations in DNA methylation have been documented in cancers: hypermethylation, hypomethylation and loss of imprinting. Epigenetic alterations in tumors also include histone modifications and H19-induced SAHH activity changes which itself could alter histone modification. We propose here that H19, in conjunction with SAHH, may contribute to alterations in the epigenetic landscape in cancer cells. Similar to other hallmarks of cancer, H19 may therefore also serve as a useful biomarker for such clinical applications as predicting cancer recurrence, metastasis, or even as a potential marker predicting therapeutic response, since DNA methylation-based biomarkers have found increasing utility in the clinic in recent years (reviewed in Sandoval and Esteller).

While the proposal that metformin induces hypermethylation of certain tumor-promoting pathway genes (Supplementary Table S2) is consistent with metformin’s proposed anticancer mechanism of action, we acknowledge that this interpretation is based on the assumption that hypermethylation will result in inhibition of gene expression. In fact, the relationship between methylation and gene expression is far more complex; not only is it genomically context-dependent, it is also influenced (positively and negatively) by factors such as distance between CpGs relative to
transcription start-sites and whether these sites are localized in promoters, enhancers or gene bodies. Adding an additional layer of complexity, the patterns and types of modifications to chromatin-bound proteins also can affect gene expression. We further acknowledge that the changes we have observed in SAHH activity could potentially affect many SAM-dependent methyltransferases that methylate other cellular components (for example, methylated proteins). We, therefore, propose here that it is ultimately the net affect of metformin's influence on gene expression, mediated at least in part via DNA methylation, that results in its anticancer potential.

Based on these results, we propose that metformin warrants further study as a potential anticancer agent, including the mechanisms, such as regulation of gene expression, that may contribute to this anticancer activity. In this regard, metformin has been widely prescribed not only for the treatment of diabetes (type 2) but also for the prevention of diabetes in patients with insulin resistance. The patients included in our study were not diabetic but did have insulin resistance/glucose intolerance. There is, therefore, precedent for the safe use of metformin in endometrial cancer patients (for the prevention of diabetes), as well as an extensive history of using this drug in humans (for diabetes), suggesting that the risk profile associated with this drug, as part of future testing as an anticancer agent, should be acceptable. Therefore, metformin is potentially useful in the prevention of diabetes in endometrial cancer patients.

In conclusion, our studies extend the known mechanisms by which metformin can exert its effects on gene expression in cancer cells, and demonstrate for the first time that metformin is able to directly impact cancer cell proliferation by altering DNA methylation via regulation of the H19/SAHH axis. These studies further suggest that this mode of action may contribute to the observed and predicted anticancer activities of metformin in vivo. Future studies aimed at developing a better understanding of the molecular interplay between various epigenetic factors will be required to more fully appreciate the specific role of metformin-induced epigenetic modifications in specific cancers, such as endometrial and breast cancer, and such studies should be complemented through the conduct of ongoing clinical trials of metformin as a potential chemopreventive agent in endometrial cancer—particularly in high-risk populations (https://clinicaltrials.gov/ct2/show/NCT01697566). It remains to be determined whether this newly identified metformin mechanism also exists in non-cancerous cells.

MATERIALS AND METHODS

Antibodies, siRNAs, miRNAs, activators and inhibitors

Antibodies for SAHH (for RNA immunoprecipitation, Santa Cruz, Dallas, TX, USA; sc-271389; for western blot, Proteintech Group, Chicago, IL, USA; 10757-2-P), DNMT3B (Novus, Littleton, CO, USA; NB300-516), KSRP (Cell Signaling, Boston, MA, USA; 13398), Phospho-AMPK-alpha (Thr172) (Cell Signaling; 2535), AMPK alpha (Cell Signaling; 2532), β-tubulin (Abcam, Cambridge, MA, USA; ab6046), β-actin (Abcam; ab8226) and mouse preimmune IgGs (Chemicon, Billerica, MA, USA; PP54) were purchased. Control siRNA (siCon; Ambion, Grand Island, NY, USA; AM4636), siRNAs specific for human H19 (siH19; Ambion; 4390771/n272452), KSRP (siKsrp; Ambion; 4390824/s16322) and DNMT3B (siDnmt3b; Santa Cruz; SC-37759), and let-7a mimics (Let-7; Ambion; AM17100/PM10050), Pre-miR negative control (miCon; Ambion; AM17110), Let-7 inhibitor (iLet-7; Ambion; 4392431) and miRNA control (Ambion; AM17010) were purchased. Metformin (ALX-270-432-G005), AICAR (123040) and D-Eritadenine (sc-207632) were from ENZO Life Sciences International Inc. (Uniondale, NY, USA) Calbiochem (Billerica, MA, USA), and Santa Cruz, respectively. Metformin and AICAR were used at final concentrations of 2 and 0.5 mM, respectively.

Cell culture and siRNA/let-7 transfection

The ARK2 and MCF-7 cells were authenticated and were free from mycoplasma contamination. The cells were cultured in RPMI1640 (Gibco, Grand Island, NY, USA; 11965-092) supplemented with 10% fetal bovine serum, heat inactivated, 1% amphotericin B, 1% penicillin/streptomycin and 1% L-glutamine. Cells were transfected in a 48-well plate scale. To prepare siRNA transfection solution for each well, 16 pmol of siCon or siH19 was mixed with 50 µl OPTI-MEM by gentle pipetting. In parallel, 0.5 µl Lipofectamine 2000 was mixed with 50 µl OPTI-MEM. Following 5 min of incubation at room temperature, the two were mixed by gentle pipetting and incubated for 20–30 min at room temperature to allow
siRNA/lipid complexes to form. At the end of incubation, the 100 μl transfection solution was used to re-suspend cell pellet (4×10⁷ cells). After incubation at room temperature for 10 min, regular growth medium was added at a ratio of 1:5 (1 volume of transfection solution/5 volumes of growth medium) and the cell suspension was transferred to the culture plate. After 12 h incubation at 37 °C in 5% CO₂, the medium was replaced with fresh growth medium. RNAs and proteins were extracted and analyzed at the indicated time points following transfection. For metformin/iLet-7 experiments, 45 pmol of iLet-7 or control inhibitor were added for each well of cells.

In vivo SAHH activity assay

The experiments were performed in a 96-well scale using the Human Homocysteine (Hcy) ELISA Kit (Mybiosource, San Diego, CA, USA; MBS260128) that allows quantitative measurement of homocysteine concentration in cell extracts, according to the manufacturer’s instructions. The concentration of homocysteine in cell extracts was used as a readout for in vivo SAHH activity, as SAHH hydrolyzes SAH to homocysteine and adenosine. Briefly, ARK2 cells were washed with cold phosphate-buffered saline and lysed on plate in 200 μl of lysis buffer (40 mM hexadecyltrimethylammonium bromide, 75 mM Tris- HCl, pH 8.0, 1 mM NaCl, 15 mM EDTA). The lysate was cleared of insoluble materials by centrifugation at 15 000 g at 4 °C for 15 min. Immediately following the centrifugation, 100 μl of the supernatant was collected and used for SAHH activity measurement. The absorbance of the samples was determined using a FilterMax F3&S5 Multi-Mode Microplate Reader (Molecular Devices).

RNA extraction and reverse transcriptase–quantitative PCR

Total RNAs were extracted from cells using PureLink RNA Mini Kit (Ambion; catalog number 12183025). cDNA was synthesized using PrimeScript RT Reagent Kit (Takara, Mountain View, CA, USA; RR037A) in a 20 μl reaction containing 100–500 ng of total RNA. Real-time quantitative PCR (qPCR) was performed in a 15 μl reaction containing 200–800 ng of total RNA, PCR was performed by initial denaturation at 95 °C for 5 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Specificity was verified by melting curve analysis and agarose gel electrophoresis. The threshold cycle (Ct) values of each sample were used in the post-PCR data analysis. Gene expression levels were normalized against β-tubulin. Real-time PCR primers are listed in Supplementary Table S2.

Let-7 miRNA quantification

Total RNAs were extracted from ARK2 cells using the PureLink RNA Mini Kit. Levels of mature let-7 were determined by reverse transcriptase–qPCR using miScript reverse transcription kit (catalog number 218161) and miScript SYBR Green PCR kit (catalog number 218073) according to the manufacturers’ instructions. PCR primer sets (miScript primer) specific for let-7a (MS00006482) and snRNA U6 (MS00033740) were purchased from Qiagen (Hilden, Germany). The indicated miRNA levels were normalized against U6.

RNA immunoprecipitation

To prepare antibodies, 20 μl of protein A Sepharose beads were incubated with 20 μg of monoclonal anti-SAHH antibody or 20 μg of mouse preimmune IgG in 500 μl IP buffer (0.5% Triton X-100, 200 mM NaCl, 10 mM Tris-HCl at pH 7.5 and 10 mM EDTA) at 4 °C overnight. The next day, the beads were washed three times with IP buffer and kept on ice until used. To prepare cell lysates, ARK2 cells (from 1 well of a 6-well plate) were harvested and cell pellets resuspended in 600 μl of freshly prepared lysis buffer (0.5% Triton X-100, 10 mM NaCl, 10 mM Tris-HCl at pH 7.5, 10 mM EDTA, 0.5 mM PMSF, 1 mM DTT, 1 × protease inhibitor cocktail (Calbiochem) and 400 units/ml RNase inhibitor). The suspensions were incubated on ice for 20 min. After removing insoluble materials by centrifugation, lysates were precleared using 10 μl of protein A sepharose (NaCl was added to a final concentration of 200 mM), followed by addition of yeast tRNA (Ambion) to a final concentration of 40 μg/ml. The cleared lysates were transferred to tubes containing antibody or preimmune IgG-coated beads, and IP was carried out by rotating the tubes at 4 °C for 3 h. Following IP, the beads were washed five times with IP buffer by adding 1 ml of the buffer and rotating the tube at 4 °C for 2 min each time. RNA was extracted from the beads using PureLink RNA Mini Kit (Ambion; catalog number 12183018A). Reverse transcription was performed in a 40 μl reaction volume using the Bio-Rad Script cDNA synthesis kit, followed by qPCR.

RNA stability analysis

To evaluate let-7 effects on H19 RNA stability, miRNA transfection (48-well plate scale) combined with actinomycin D time course analysis was performed. To prepare transfection cocktail, 1 μl of control miRNA (miCon) or let-7a mimic was mixed with 50 μl of OPTI-MEM. In parallel, 0.5 μl of Lipofectamine 2000 was mixed with 50 μl of OPTI-MEM. Following 5 min of incubation, the two solutions were mixed and incubated at room temperature for 20 min. The resulting 100 μl of transfection cocktail was added to ARK2 cells pre-washed with OPTI-MEM. Upon adding the transfection cocktail, actinomycin D was also added to each well at a final concentration of 10 μg/ml. Total RNA was extracted at the indicated time points, followed by reverse transcriptase–qPCR analysis. Results are presented after normalization against β-tubulin miRNA levels with 0 time point RNA levels arbitrarily set as 1.

Western blot analysis

Cell pellets were quickly lysed in five volumes of 2× sodium dodecyl sulfate sample buffer heated at 95 °C for 5 min, with occasional vortexing. Five to 10 μl of homogenized samples were loaded onto 10% SDS gel, followed by western blot analysis. The linear dynamic range of each protein of interest was determined by serial dilutions. Bands on western blot gels were quantified using Image J.

Genomic DNA extraction

Genomic DNA was isolated using Quick-gDNA MicroPrep (Zymo, Irvine, CA, USA; D3021) according to the manufacturer’s instructions.

Quantitative methylation-specific PCR (Q MSP)

Genomic DNA was extracted from ARK2 and MCF-7 cells in one well of six-well plates using Quick-gDNA MicroPrep. For bisulfite treatment, 400–500 ng of DNA was used for each column using EZ DNA Methylation-Gold Kit (Zymo; D5006). Two hundred microliters of water was used to elute DNA from each column. Real-time qPCR was performed in a 15 μl reaction containing 1 μl of the eluant using iQSYBRGreen (Bio-Rad, Hercules, CA, USA) in a Bio-Rad iCycler. The PCR primers (Supplementary Table S3) for methylated DNA were used at a final concentration of 0.3 μM each in PCR reaction. PCR was performed by initial denaturation at 95 °C for 5 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Specificity was verified by melting curve analysis and agarose gel electrophoresis. The threshold cycle (Ct) values of each sample were used in the post-qPCR data analysis. Albumin DNA was used as loading controls for all QMSP normalization.

Methyl-MiniSeq library construction

Libraries were prepared from 200 to 500 ng of genomic DNA digested with 60 units of Tagaq and 30 units ofMspI (NEB) sequentially and then extracted with Zymo Research (ZR) DNA Clean & Concentrator-5 kit (Cat #: D4003). Fragments were ligated to preannealed adapters containing 5’-methyl-cytosine instead of cytosine according to Illumina’s specified guidelines (www.illumina.com). Adaptor-ligated fragments of 150–250 and 250–350 bp in size were recovered from a 2.5% NuSieve 1:1 agarose gel (Zymoclear Gel DNA Recovery Kit, ZR Cat#: D4001). The fragments were then bisulfite-treated using the EZ DNA Methylation-Lightning Kit (ZR, Cat#: D5020). Preparative-scale PCR was performed and the resulting products were purified (DNA Clean & Concentrator-ZR, Cat#:D4005) for sequencing on an Illumina HiSeq.

Methyl-MiniSeq sequence alignments and data analysis

Sequence reads from bisulfite-treated EpicQuest libraries were identified using standard Illumina base-calling software and then analyzed using a Zymo Research proprietary analysis pipeline, which is written in Python and used Bismark (http://www.bioinformaticians.ubabraham.ac.uk/projects/bismark/) to perform the alignment. Index files were constructed using the bismark_genome_preparation command and the entire reference genome. The non_directional parameter was applied while running Bismark. All other parameters were set to default. Filled-in nucleotides were trimmed off when doing methylation calling. The methylation level of each sampled CpG was estimated as the number of reads reporting a C, divided by the total number of reads reporting a C or T. Fisher’s exact test or t-test was performed for each CpG site which has at least five reads coverage, and promoter, gene body and CpG island annotations were added for each CpG included in the comparison.
Cell viability and apoptosis

ARK2 and MCF-7 cells were seeded in 96-well plates at a density of 3 × 10^3 well the night before metformin addition. Cell viability and caspase-3/7 activity were measured 48 h post metformin treatment using the CellTiterBlue Cell Viability kit (Promega, Madison, WI, USA) and the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega), respectively, according to the manufacturer’s protocols.

Patients

Study approval was obtained from the Institutional Review Board of Chiba University. Five patients with endometrioid adenocarcinoma were recruited to the study. All patients signed an informed consent form according to the institutional guidelines. Eligibility criteria included an Eastern Cooperative Oncology Group performance status of 0 to 1 and normal renal, liver and cardiac function. Exclusion criteria were as follows: (1) type 2 diabetes requiring medication; (2) history of metformin use; (3) an abnormal blood coagulation profile and/or a history of thromboembolism; and (4) the presence of mental or life-threatening illnesses. All patients were obese with impaired glucose tolerance, but were not diabetic. Detailed characteristics of the patients are shown in Supplementary Table S1.

Metformin (initial dose, 750 mg/day; increased weekly up to 1500 or 2250 mg/day) was administered for 3–12 weeks until the day of scheduled surgery. Patients were not treated with any hormones such as progestin. Tissue specimens were obtained via endometrial curettage at the time of initial diagnosis (before treatment) and hysterectomy (after treatment).

Tissue collection and analyses

For RNA extraction, tissue samples collected at surgery were snap frozen in liquid nitrogen and stored at −80 °C until RNA extraction. RNA was extracted from tissues using Trizol reagent (Life Technologies, Grand Island, NY, USA). cDNA was synthesized from 3 to 5 μg RNA using oligo-deoxythymidine 12–18 (catalog item 18418-012; Invitrogen) and Super-Script II reverse transcriptase (catalog item 18064-014; Invitrogen) in a 20 μl reaction volume according to the manufacturer’s instructions. Real-time qPCR was performed as described above using β-tubulin mRNA as a loading control.

For immunohistochemical staining of Ki-67, 3-μm-thick sections were briefly microwaved in 10 ml of citrate buffer (pH 6.0) and immunostained for Ki-67. The Envision FLEX system (K8000; Dako, Carpinteria, CA, USA) was used to observe the immunostaining using an Autostainer S3400 (Dako). The primary antibody was incubated at room temperature for 60 min at a dilution of 1:100. The secondary antibody (Envision FLEX/HRP; Dako) was incubated at room temperature for 30 min at 1:1000 dilution. Signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Wayne, MI, USA). Lysates (10 mg of protein) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (GE Healthcare Japan, Tokyo, Japan). The primary antibodies were diluted (1:1000 for phospho-AMPKa, AMPKa; and 1:5000 for β-actin) and incubated overnight at 4 °C. The secondary antibody (enhanced chemiluminescence horseradish peroxidase-conjugated anti-rabbit immunoglobulin G and anti-mouse immunoglobulin G; GE Healthcare) was incubated at room temperature for 60 min. Signals were detected using the ECL Select Western Blotting Detection Kit (GE Healthcare). Signal intensity was quantified using a densitometer (CS Analyzer version 3.0 software; ATTO, Tokyo, Japan) and normalized to β-actin levels.

For QMSP analysis, genomic DNA was extracted from frozen tissue samples using the Quick gDNA MicroPrep kit. qMSP was performed as described for ARK2 cells.

Statistical analysis

All data (unless otherwise indicated) are presented as mean ± s.d. All experiments were performed in triplicate and repeated at least three times. Statistical analyses were performed using the Statistical Package for the Social Science (SPSS) computer software version 17.0 (IBM SPSS Statistics, Chicago, IL, USA). The Student’s t-test or the Mann–Whitney U-test, or the Wilcoxon signed-rank test were used to compare differences among quantitative variables when appropriate. The Fisher’s exact test was used when appropriate, for comparing categorical variables (contingency tables). P-values at 0.05 or smaller (two-sided) were considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

YH conceived and directed the project, analyzed the data and wrote the manuscript. TZ, YM, TG and JZ carried out the experiments and analyzed the data. LL and TZ designed primers for QMSP. LL performed Ingenuity Pathway Analysis. AM and MS provided patient tumor tissue samples for QMSP analysis and patient tumor H19 expression data, AMPK western blots data and Ki-67 index data. HST, GGC and NJM provided intellectual insights and critical reading of the manuscript.

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