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# Glucose starvation induces resistance to metformin through the elevation of mitochondrial multidrug resistance protein 1

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Metformin, a drug for type 2 diabetes mellitus, has shown therapeutic effects for various cancers. However, it had no beneficial effects on the survival rate of human malignant mesothelioma (HMM) patients. The present study was performed to elucidate the underlying mechanism of metformin resistance in HMM cells. Glucose-starved HMM cells had enhanced resistance to metformin, demonstrated by decreased apoptosis and autophagy and increased cell survival. These cells showed abnormalities in mitochondria, such as decreased ATP synthesis, morphological elongation, altered mitochondrial permeability transition pore and hyperpolarization of mitochondrial membrane potential (MMP). Intriguingly, Mdr1 was significantly upregulated in mitochondria but not in cell membrane. The upregulated mitochondrial Mdr1 was reversed by treatment with carbonyl cyanide m-chlorophenyl hydrazone, an MMP depolarization inducer. Furthermore, apoptosis and autophagy were increased in multidrug resistance protein 1 knockout HMM cells cultured under glucose starvation with metformin treatment. The data suggest that mitochondrial Mdr1 plays a critical role in the chemoresistance to metformin in HMM cells, which could be a potential target for improving its therapeutic efficacy.

**KEYWORDS**
drug resistance, glucose starvation, metformin, mitochondria, multidrug resistance protein 1

## 1 | INTRODUCTION

A tumor microenvironment of glucose starvation, hypoxia, and acidic conditions is a typical feature for tumor masses and is generated by the abnormal tumor vasculature causing malignancy.[1-4] Adapted cancer cells to the tumor microenvironment show metabolism alteration that leads to their survival and enhances chemoresistance, which is the main obstacle to cancer therapy.[5-10] Several mechanisms have been suggested for chemoresistance, such as protection against DNA damage, anti–apoptosis, the overexpression of drug transporters, and the existence of cancer stem cells.[9,11] In particular, multidrug resistance protein 1 (MDR1), an ABC transporter, effluxes xenobiotics from cells and is highly expressed in cancer cells that are resistant to chemotherapy.[12,13] The regulation of the MDR1 expression level is an important strategy for decreasing the cancer cells resistance to anticancer drugs.

**Abbreviations:** ABC transporter, ATP-binding cassette transporter; ABCG2, ATP-binding cassette sub-family G member 2; AMPK, AMP-activated protein kinase; ATP, adenosine triphosphate; CCCP, carbonyl cyanide m-chlorophenyl hydrazine; CRISPR, clustered regularly interspaced short palindromic repeats; HMM, human malignant mesothelioma; LC3, 1A/1B-light chain 3; MDR1, multidrug resistance protein 1; MMP, mitochondrial membrane potential; mPTP, mitochondrial permeability transition pore; mTOR, mammalian target of rapamycin; PINK1, PTEN-induced putative kinase 1; TEM, transmission electron microscopy.

The effects and mechanism of glucose starvation in the context of chemoresistance of cancer cells are largely unknown. Glucose is metabolized mainly in mitochondria, which is essential for generating energy for cell proliferation. Glucose starvation disturbs mitochondrial function and changes the cellular phenotype.[6,14-16] Under glucose-deficient conditions, autophagy provides energy to sustain cellular metabolism and considered a hindrance to cancer therapy that disturbs cellular physiology.[17,18] In contrast, excessive autophagy contributes to cell death, which is considered to be a target for effective cancer therapy.[19] HMM is an aggressive tumor that is closely associated with exposure to asbestos fibers approximately 20-40 years prior to tumorigenesis. HMM patients present poor therapeutic response due to traditional anticancer drug resistance, such as cisplatin and pemetrexed.[20] Metformin, which is commonly used for the management of type II diabetes mellitus, has been suggested as an alternative anticancer drug.[21,22] Recent studies have shown the potential therapeutic value of metformin in HMM cells, which is exerted through the inhibition of mTOR and cell cycle arrest.[21] However, metformin treatment causes resistance in human breast cancer cells, although the precise mechanism has not been elucidated.[23] Metformin treatment has shown no evidence to relieve HMM patients, which may be due to the tumor microenvironment.[24] Expanding our understanding of the mechanism of drug resistance is urgently needed to improve the prognosis in cancer patients and to develop effective therapeutic strategies. The present study was performed to determine the impact of glucose deficiency on the development of resistance to metformin and the underlying mechanism in HMM cells. The results of the present study suggest that glucose starvation enhances drug resistance in HMM cells via mitochondrial Mdr1 elevation. Identification of the mechanism associated with enhanced resistance to metformin will be valuable for improving therapeutic efficacy in cancer patients.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell lines and culture conditions

The HMM cell lines MS1 and NIH-513 (H513) were kindly provided by Dr Jablons (University of California San Francisco, San Francisco, CA, USA) and Dr R. Kratzke (University of Minnesota, Minnesota, MN, USA), respectively. Met-5A benign transformed mesothelial cells were purchased from the ATCC (Manassas, VA, USA). These mesothelial cell lines were cultured as described previously,[25] and various concentrations of glucose (0, 1, 5 and 10 mmol/L) were added to the medium.

### 2.2 | Cell proliferation and viability assay

Cell viability was evaluated by using an MTT assay (Sigma-Aldrich, St. Louis, MO, USA). Briefly, MTT solution (MTT dissolved in PBS, 5 mg/mL) was added to each well and incubated for 1 hour at 37°C. Subsequently, each well was treated with 100 mL of a solution which contained DMSO (Sigma-Aldrich) and 2-propanol (Millipore, Billerica, MA, USA) at a 9:1 ratio and absorbance was measured at a wavelength of 570 nm using a microplate reader (BioTek Epoch, Izasa, Barcelona, Spain). For the cell proliferation assay, $5 \times 10^4$ cells were seeded in 6-well plates and incubated in conditioned medium with glucose concentrations ranging from 0 to 10 mmol/L for 3 days. The surviving cells on each day were counted manually under a light microscope.

### 2.3 | Apoptosis assay

The apoptosis assay was performed using an EzWay Annexin V-FITC Apoptosis Detection Kit (Koma Biotech, Seoul, Korea). Annexin V reagent was added to harvested cells and incubated for 15 minutes in the dark. After washing, the cells were incubated in 1× binding buffer containing propidium iodide (PI). The stained cells were immediately analyzed by flow cytometry (Becton Dickinson, Mountain View, CA, USA). Total apoptosis included late (positive for Annexin and PI) and early (positive for Annexin and negative for PI) apoptotic cells.

### 2.4 | Western blotting assay

The cells were lysed using EzRIPA buffer (ATTO, Tokyo, Japan). The lysate protein concentration was quantified by a Bradford assay (BioRad, Hercules, CA, USA) and measured using a BioTek Epoch Microplate Reader. Twenty micrograms of protein were subjected to 10%-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham, GE Healthcare, Barcelona, Spain) using the electrophoretic method. The membrane was blocked by a PBS-T solution which contained 5% skim milk for 60 minutes at room temperature. The primary antibodies, PINK1 (Novus Biologicals, Littleton, CO USA, #NB100-493), phosphorylated AMPK (Cell Signaling, Boston, MA, USA, #2531), MDR1 (Santa Cruz biotechnology, CA, USA, #Sc-55510) and β-actin (Cell Signaling, Boston, MA, USA, #4967) were diluted 1:1000 in blocking solution (PBS-T with 4% BSA) and incubated overnight at 4°C. The secondary HRP-conjugated anti–rabbit (Santa Cruz Biotechnology) and anti–mouse (Santa Cruz Biotechnology) antibodies were used at a dilution of 1:1000 in blocking solution for 2 hours. Protein expression was detected by a chemiluminescence imaging system (ATTO) after spreading the Luminata Forte Western HRP Substrate (Millipore).

### 2.5 | Subcellular fractionation assay

Subcellular fractionation of the cytoplasm, mitochondria and nucleus was achieved using a fractionation kit (Abcam, Burlingame, CA, USA) according to the manufacturer's recommendations. Alpha-tubulin (Santa Cruz Biotechnology, #sc-8035), COX IV (Abcam, #ab14744) and Lamin A (Santa Cruz Biotechnology, #sc-6214) were used as endogenous control markers for the cytoplasm, mitochondria and nucleus, respectively.

### 2.6 | Quantitative real-time PCR

Total RNA was extracted using TRIzol LS Reagent (Ambion, Austin, TX, USA) and quantitation was performed using a BioTek Epoch Microplate Spectrophotometer (Izasa, Barcelona, Spain). A total of 1000 ng of RNA was subjected to cDNA synthesis using a QuantiTect Reverse Transcription Kit (Enzynomics, Seoul, South Korea). An SYBR Green RT-PCR Kit (Enzynomics) was used for gene expression analysis. The primer sequences are presented in Table S1. The relative changes in gene expression levels were normalized to 18S rRNA and calculated using the $\Delta\Delta C_t$ method.[26]

### 2.7 | Autophagy detection

The autophagy activity was assessed using a Cyto-ID Autophagy detection kit (Enzo Life Sciences, Farmingdale, NY, USA). Briefly, the Cyto-ID Autophagy Detection Reagent was added to the cell pellet, and incubated for 30 minutes at 37°C protected from light and analyzed using flow cytometry (Becton Dickinson).

### 2.8 | Immunofluorescence assay

Human malignant mesothelioma cells were seeded in 8-well chamber slides (SPL Life Sciences, Pocheon, Korea) and incubated with MitoTracker Deep Red (Molecular Probes, Eugene, OR, USA) for 30 minutes in the dark. Fixation, permeabilization and blocking were carried out using 4% paraformaldehyde (Millipore), 0.1% Triton X-100 (Amresco, Solon, OH, USA) and blocking solution (BSA 3% in PBS with 0.1% Tween-20 [PBST]) for 15, 10 and 30 minutes, respectively. After washing with PBS, Mdr1 antibody was added in blocking solution and incubated overnight at 4°C. Subsequently, the Alexa Fluor 488-conjugated anti–mouse secondary antibody (Molecular Probes) was added in blocking solution and incubated for 2 hours in the dark. In addition, nuclear was stained using DAPI (Molecular Probes). Fluorescence images were captured using an LSM710 confocal laser scanning microscope (CLSM; Carl Zeiss, Göttingen, Germany) and analyzed using LAS AF Lite software (Leica, Wetzlar, Germany).

### 2.9 | Transmission electron microscopy

Cell pellets were immersed in Karnovsky's solution (2% glutaraldehyde, 0.05 mol/L cacodylate, 2% paraformaldehyde and distilled water) and incubated overnight.[27] After washing with 0.05 mol/L sodium cacodylate buffer, the cells were subjected to post–fixation using 2% osmium tetroxide for 2 hours, followed by washing in distilled water. For fixation, 0.5% uranyl acetate was added, and the cells were then washed with ethanol. Propylene oxide was added to the pellet for transition. For infiltration, the cells were incubated in propylene oxide and Spurr's resin mixed at a 1:1 ratio for 2 hours at room temperature. For solidification, the solution was replaced with fresh Spurr's resin and incubated at 70°C overnight. After thin sectioning using an ultramicrotome (MT-X; RMC, Tucson, AZ, USA), the intracellular organelles morphology was examined using a JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan).

### 2.10 | Assessment of mitochondrial function

The cellular level of ATP was measured using the ATP Colorimetric/Fluorometric Assay Kit (BioVision, Milpitas, CA, USA), according to the manufacturer's recommendations. Briefly, a mixture of ATP assay buffer, probe, converter and developer was added to the cell lysate obtained from $1 \times 10^6$ cells. In addition, the resulting absorbance was measured at a wavelength of 570 nm using a microplate reader (BioTek Epoch) and calculated using a standard curve.

Mitochondrial membrane potential was evaluated using 5,5′,6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolylcarbocyanine iodide; JC-1, Molecular Probes). HMM cells were treated with 2.5 μmol/L JC-1 solution and incubated at 37°C for 30 minutes in the dark. Subsequently, MMP was analyzed by flow cytometry (Becton Dickinson), and compartmentalized as green and red in a dot plot. As depolarization control, 50 μmol/L carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was added to the cells prior to JC-1 treatment. Using the depolarization baseline with red/green ratio decreased by CCCP treatment, the MMP data were normalized.

### 2.11 | Production of knockout cells using the clustered regulated interspaced short palindromic repeats/Cas9 technique

Human malignant mesothelioma cells were transfected with 2 μg of MDR1 CRISPR/Cas9 KO plasmids containing a GFP-coding region and either control or MDR1 (Table S1; Santa Cruz Biotechnology) using the HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) following the manufacturer's recommendations. GFP-positive cells were selectively collected by using a BD Aria III cell sorter (BD Biosciences Clontech, Palo Alto, CA, USA) 3 days post–transfection. The knockout efficiency for the target gene was verified by real-time RT-PCR for MDR1.

### 2.12 | Statistical analysis

The experiments described above were performed independently at least 3 times. Data were expressed as the mean ± SD. GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) was used for all graphs and statistical analysis. Tukey's pairwise comparison and one-way ANOVA were applied for comparisons between groups. Statistical significance was accepted at $P < 0.05$.

## 3 | RESULTS

### 3.1 | Survived human malignant mesothelioma cells under glucose-starved conditions desensitized against to metformin treatment

To assess the impact of glucose concentration on cell proliferation, the MS1, H513 and Met-5A cell lines were cultured in conditioned

medium containing 0, 1, 5 and 10 mmol/L glucose. The proliferation of HMM cells was decreased in medium with 0 and 1 mmol/L glucose (Figure 1A), while the number of cells increased in medium with 5 and 10 mmol/L glucose. Met-5A cells cultured under glucose-deficient conditions did not survive after 3 days. In contrast, the HMM cell lines, MS1 and H513, survived under glucose-deficient conditions. These results demonstrated that glucose is essential for cell proliferation and that HMM cells have a distinct capacity for survival under glucose starved conditions compared to benign mesothelial cells.

Human malignant mesothelioma cells were precultured in medium with 0, 1, 5 and 10 mmol/L glucose for 2 days, followed by treatment with 5 mmol/L metformin for 1 day. The viability of HMM cells cultured with 5 and 10 mmol/L glucose was significantly decreased by metformin treatment, but this feature was minimally affected in HMM cells precultured under glucose-deficient conditions (Figure 1B,C). To determine glucose starvation-enhanced metformin resistance, HMM cells were precultured in medium containing different concentrations of glucose for 2 days, replated with identical numbers of cells and treated with metformin for 1 day. HMM cells precultured in medium with 5 or 10 mmol/L glucose exhibited a significant reduction of cell viability compared to 0 or 1 mmol/L glucose (Figure 1D,E). These data revealed that adapted HMM cells to glucose-deficient conditions acquired greater resistance to metformin than did cells cultured under glucose abundant conditions.

### 3.2 | Treatment of human malignant mesothelioma cells cultured under glucose starvation with metformin suppressed apoptosis and autophagy

Total apoptosis was suppressed by metformin treatment in HMM cells cultured under glucose-deficient conditions. In contrast, glucose starvation or metformin treatment increased the total apoptosis ratio (Figure 2A). Furthermore, either metformin treatment or glucose starvation increased the expression of cleaved Parp-1 (Figure 2B). However, the treatment of glucose-starved HMM cells with metformin significantly reduced the expression of cleaved Parp-1 compared to those cells cultured in complete medium. In line with that, autophagic activity was increased by glucose starvation or metformin treatment (Figure 2C). In contrast, the treatment of glucose-starved HMM cells with metformin significantly suppressed autophagic activity compared to those cells cultured in complete medium.

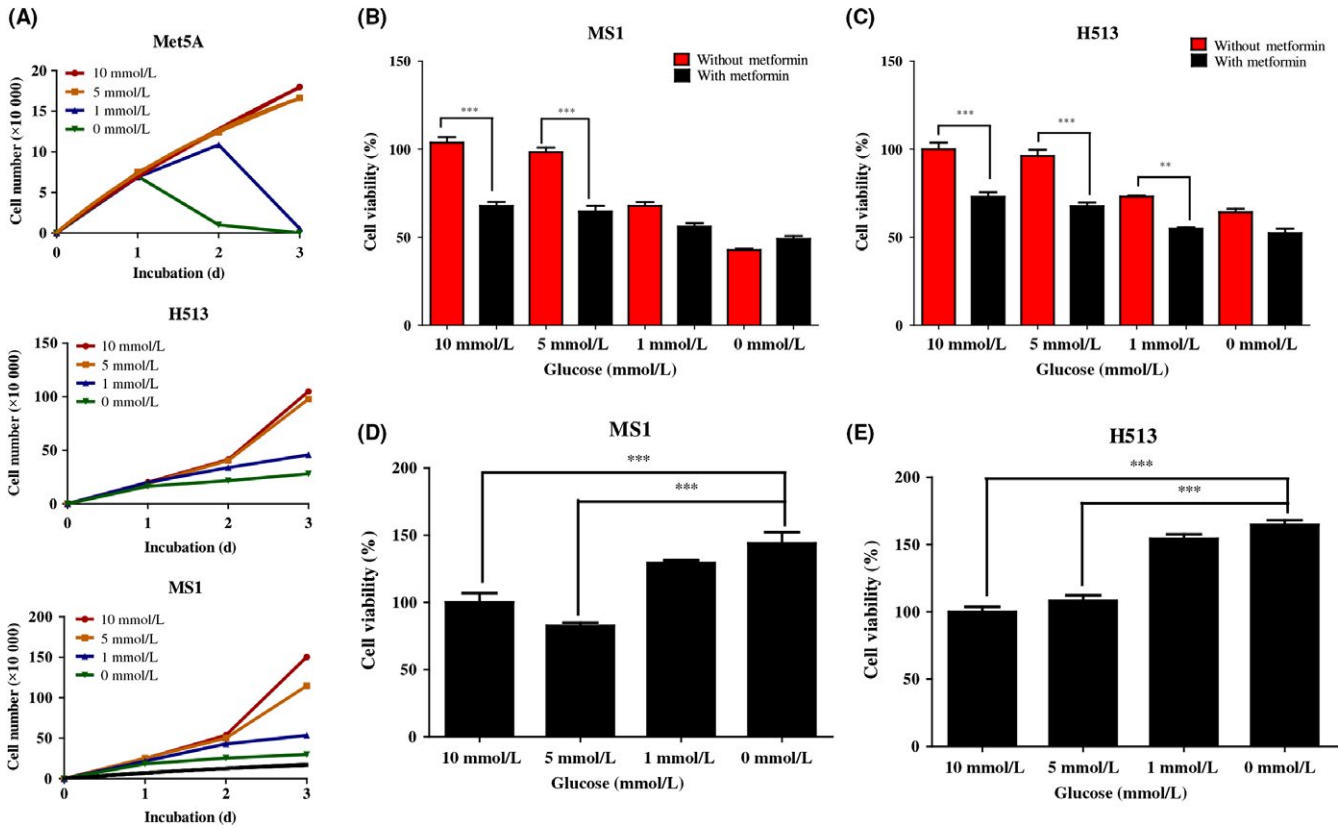



**FIGURE 1** Assessment of glucose concentration effect in cell proliferation and metformin resistance. A, Proliferation of human malignant mesothelioma cells under each glucose concentration contained medium was measured for 3 d by macroscopic analysis. B, C, MS1 and H513 cells were cultured in medium containing the various concentrations of glucose for 2 d and subsequently treated with 5 mmol/L of metformin for 1 d. Red column: metformin nontreatment. Black column: 5 mmol/L metformin treatment. D, E, MS1 and H513 cells incubated in the various concentrations of glucose for 2 d were replated same number and treated 5 mmol/L metformin for 1 d. All error bars indicate the SEM with n = 8. Significant differences are indicated by $^{**}P < .01$, $^{***}P < .001$

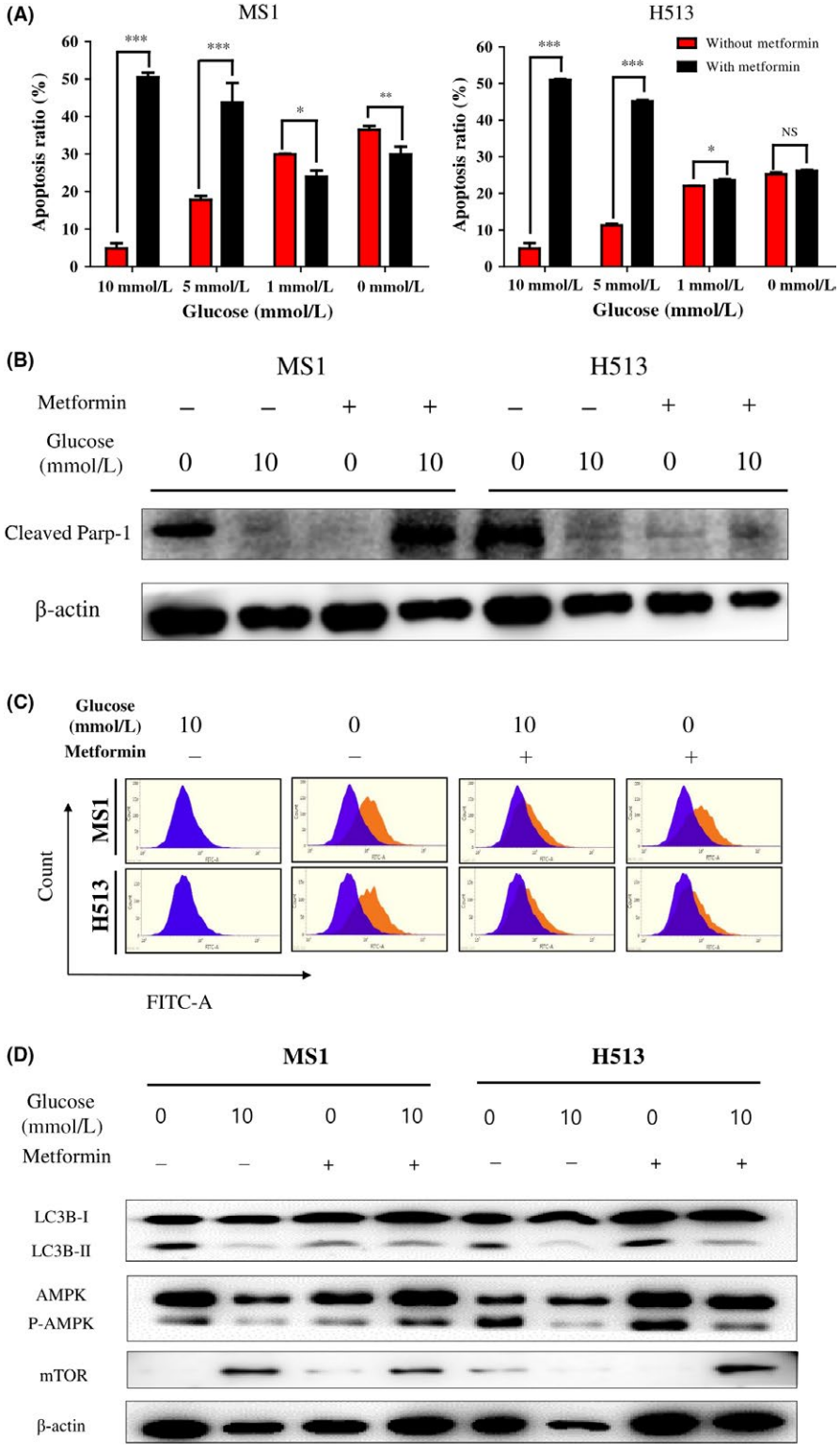



**FIGURE 2** Glucose starvation and/or metformin treatment regulated apoptosis and autophagy. A, Total apoptosis in human malignant mesothelioma cells cultured under each glucose concentration containing medium and/or 5 mmol/L metformin treatment are presented. All error bars represent SEM with n = 3. Significant differences are indicated by $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. B, Western blot analyses of the expression of cleaved Parp-1 and β actin. C, Autophagy activity was assessed by flow cytometry. Blue: 10 mmol/L glucose. Orange: each glucose concentration and/or 5 mmol/L metformin treatment. D, Autophagy indicated molecules, LC3B, AMPK, p-AMPK, mTOR and β-actin; expression was confirmed by western blot assay

The expression of LC3B, p-AMPK and AMPK was increased by either glucose starvation or metformin treatment in HMM cells (Figure 2D). However, that was decreased in the treatment of glucose-starved HMM cells with metformin. In addition, mTOR expression was decreased by either glucose starvation or metformin treatment in MS1.

### 3.3 | MDR1 is significantly elevated in mitochondria of glucose-starved HMM cells by treatment with metformin

The treatment of glucose-starved HMM cells with metformin increased the mRNA expression of MDR1 and ABCG2 (Figure 3A,B). In particular, MDR1 mRNA expression in glucose-starved cells with metformin

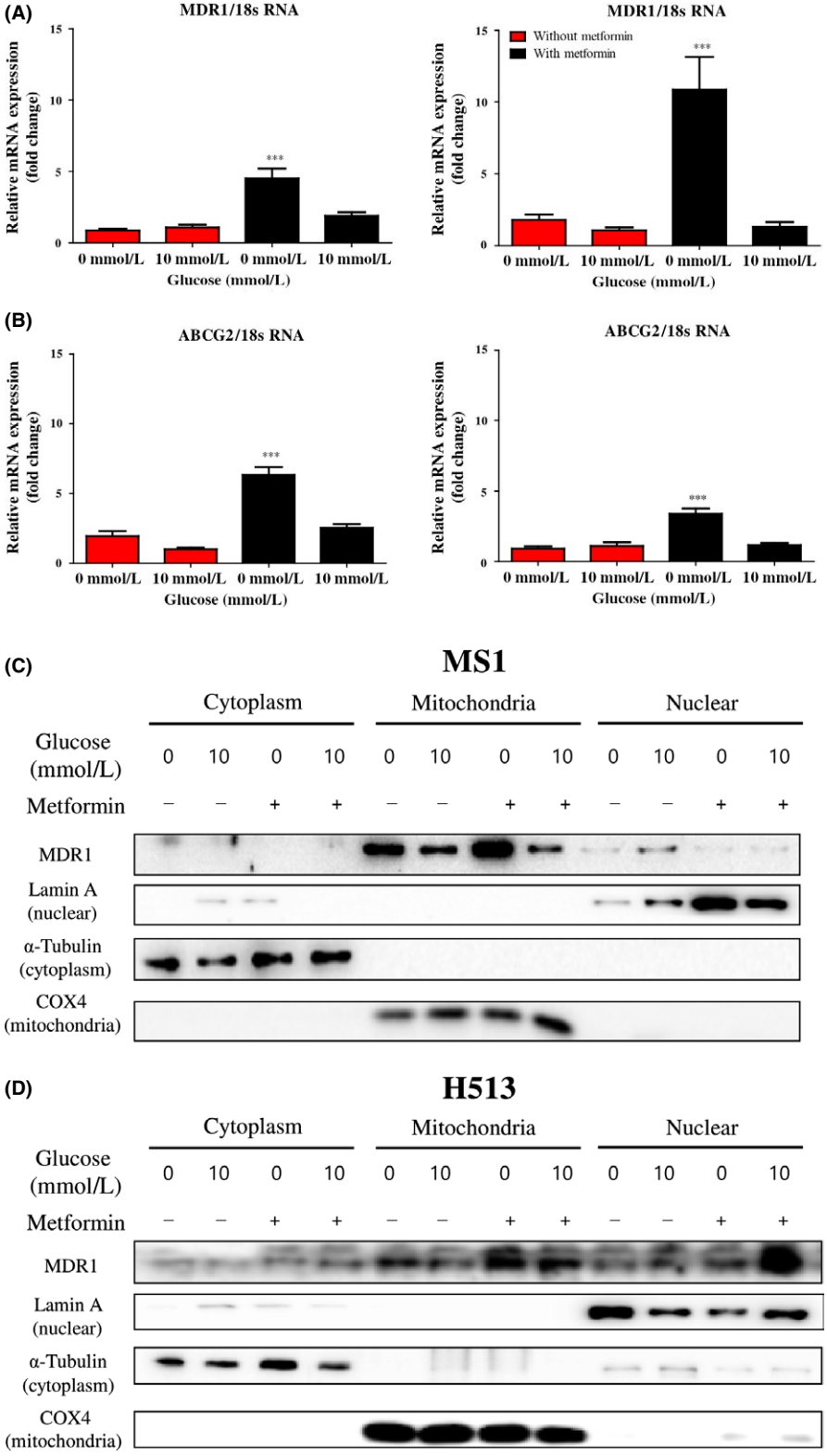



**FIGURE 3** Regulation of drug-related molecule expression by glucose concentration and/or metformin treatment. A, B, The mRNA expression of multidrug resistance protein 1 (MDR1) and ATP-binding cassette sub-family G member 2 expression was measured in MS1 and H513 cells. Significance: **P* < .05, ***P* < .01, ****P* < .001. Each data represents the mean ± SEM (n = 4). C, D, Western blot analysis of Mdr1 expression from mitochondria, cytoplasmic and nuclear fractions. The α-tubulin, COXIV and Lamin A were used for normalization of cytoplasmic, mitochondria and nuclear fractions. E, Immunofluorescence of MDR1 and mitotracker in MS1 was exhibited by confocal laser scanning microscope. The Mdr1 and mitotracker expressed green and red fluorescence

treatment was approximately 10-fold higher than those cells cultured in complete medium. To determine Mdr1 expression in subcellular fractions, the cytoplasm, nucleus and mitochondria were isolated. The expression level of membrane Mdr1 was not significantly altered by glucose content or metformin treatment (Figure S1). In MS1 cells, the majority of Mdr1 expression was found in the mitochondria and minimal expression was found in the nuclear and cytoplasmic fractions. In addition, mitochondrial Mdr1 expression was significantly increased in the treatment of glucose-starved conditions with metformin (Figure 3C,D). These results suggested that the treatment of glucose-starved HMM cells with metformin elevated Mdr1 expression exclusively in fractionated mitochondria. In H513 cells, however, Mdr1 was found in all subcellular fractions,

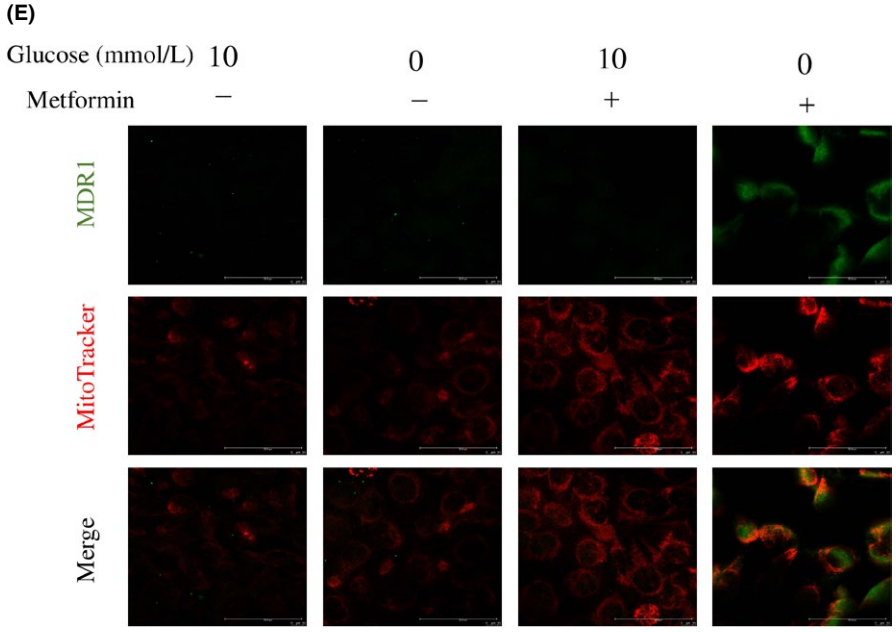



**FIGURE 3** (Continued)

although substantial enrichment in the mitochondrial and nuclear fractions was observed under glucose-starved conditions.

Previous research reported that MDR1 is increased in hepatocellular carcinoma cells with mitochondrial defects.[28] Moreover, dependent on glucose content decreasing and metformin treatment, the mRNA expression of MDR1 and PINK1 showed positive correlation (Figure S2). The Mdr1 fluorescence in glucose-starved MS1 cells with metformin treatment was significantly increased and exhibited co-localization with MitoTracker (Figure 3E). However, the Mdr1 expression in HMM cells cultured in complete medium was unchanged by metformin treatment. In addition, mitotracker expression increasing which was a mitochondria damage indicator was detected in HMM cells cultured under glucose starvation and metformin treatment compared to those HMM cells cultured in complete medium (Figure S3).

### 3.4 | Dysfunctional mitochondria induced by glucose starvation and/or metformin treatment

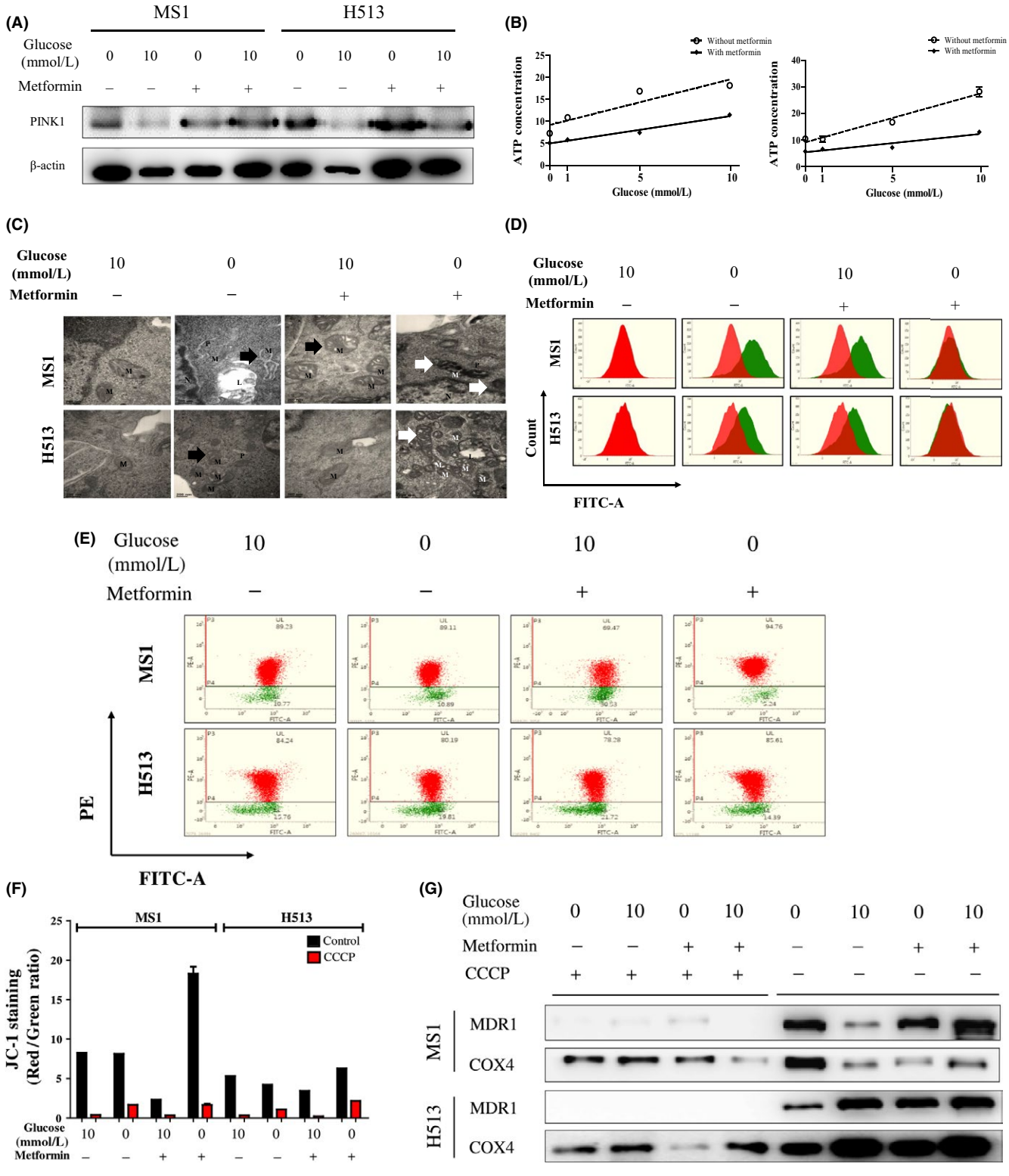
To further examine the mitochondria integrity, the expression of Pink1, a mitochondrial damage marker, was measured. The expression of Pink1 was increased by glucose starvation and/or metformin treatment (Figure 4A). Moreover, ATP synthesis was assessed in HMM cells cultured under glucose starvation and/or metformin treatment. The ATP level was decreased by either glucose starvation or metformin treatment in HMM cells (Figure 4B). The ATP level in the treatment of glucose-starved HMM cells with metformin was lower than 4-fold compared to those HMM cells cultured in complete medium. To evaluate the mitochondrial ultrastructure, TEM was performed. Glucose starvation or metformin treatment induced mitochondrial swelling and abnormal morphology and this effect was more severe in MS1 cells (Figure S4). Intriguingly, elongated mitochondria with condensed cristae were observed in glucose-starved HMM cells with metformin treatment (Figure 4C). In the mPTP assay, the FITC intensity was significantly increased in HMM cells cultured under glucose-starved conditions or metformin treatment, but not in glucose-starved HMM cells with metformin treatment (Figure 4D). Metformin treatment induced the depolarization of the MMP, but glucose starvation alone did not affect the MMP. In contrast, the treatment of glucose-starved HMM cells with metformin induced MMP hyperpolarization, especially in MS1 cells (Figure 4E,F).

**FIGURE 4** Mitochondrial dysfunction and morphological alteration in glucose-starved human malignant mesothelioma (HMM) cells in cases treated or not treated with metformin. A, The expression of Pink1 and β-actin expression was assessed by western blot assay. B, Adenosine triphosphate (ATP) generation was measured in MS1 and H513 cells. C, Cellular morphological alteration was detected by transmission electron microscopy assay which is usually performed to classify the intracellular organelle morphology. L, lysosome; M, mitochondria; N, nuclear; P, phagophore. D, The mitochondrial permeability transition pore assay was performed in HMM cells cultured under glucose-starved condition and/or 5 mmol/L metformin treatment by flow cytometry. Red: 10 mmol/L glucose. Green: each glucose concentration contained medium and/or 5 mmol/L metformin treatment. E, Analysis of the mitochondrial membrane potential (MMP) by flow cytometry in HMM cells cultured under glucose starvation and/or 5 mmol/L metformin treatment. Data were normalized with the red/green ratio result of MMP depolarization induced by 50-μm carbonyl cyanide m-chlorophenyl hydrazine (CCCP) treatment used as positive control. The green part increasing compared to the red portion indicates hyperpolarization. F, The ratio of red to green is presented. The red and black columns show incubation with or without 5 mmol/L CCCP for 3 d. All error bars represent SEM with (n = 3). G, The expression of Mdr1 in mitochondria with or without 5 mmol/L CCCP was measured by a western blotting assay in HMM cells cultured with glucose starvation and/or 5 mmol/L metformin treatment

Mitochondrial membrane potential regulated protein import in mitochondria.[29] Therefore, to determine the MMP hyperpolarization in the treatment of glucose-starved HMM cells with metformin-regulated mitochondrial Mdr1 expression, 5 μmol/L CCCP was treated for 3 days. CCCP treatment suppressed the increased MMP and Mdr1 expression in mitochondria of HMM cells cultured under glucose starvation with metformin (Figure 4F,G).

### 3.5 | MDR1 regulates chemoresistance under glucose-starved conditions

To evaluate whether MDR1 is directly mediated metformin resistance in glucose-starved HMM cells, MDR1 was knocked out using the CRISPR/CAS9 system. The MDR1 gene was significantly decreased close to zero in MDR1 knockout (KO) HMM cells compared with



control cells (Figure 5A). The viability of the MDR1 KO HMM cells was similar to those of the parental cells. In contrast to the parental HMM cells, the treatment of glucose-starved MDR1 KO HMM cells with metformin induced a significant reduction of viability (Figure 5B). Apoptosis and autophagy assays were performed to assess cell death pathways that were regulated by MDR1. Compared to those of the control, increased apoptosis was found in the glucose-starved MDR1 KO HMM cells by metformin treatment (Figure 5C). The autophagic response was increased in metformin treatment against glucose-starved MDR1 KO cells, while this response was decreased in the control cells (Figure 5D). However, a consistent level of autophagy was exhibited in MDR1 KO HMM cells cultured under glucose starvation or metformin treatment compared to those of the parental cells.

## 4 | DISCUSSION

Despite efforts in anticancer drug development, cancer mortality remains high due to the therapeutic resistance of cancer cells. During progression to the malignant stage, cancer cells survive in an adverse microenvironment and develop drug resistance.[8-10] This study was performed to elucidate the mechanism of enhanced metformin resistance in glucose-starved HMM cells. HMM cells used a distinct metabolism to generate energy compared to normal mesothelial cells and glucose starvation promoted more severe metabolic alterations.[30,31] Therefore, energy-deficient cancer cells survived through PKA over activated metabolism alteration.[32] Surviving HMM cells under glucose starvation exhibited metformin

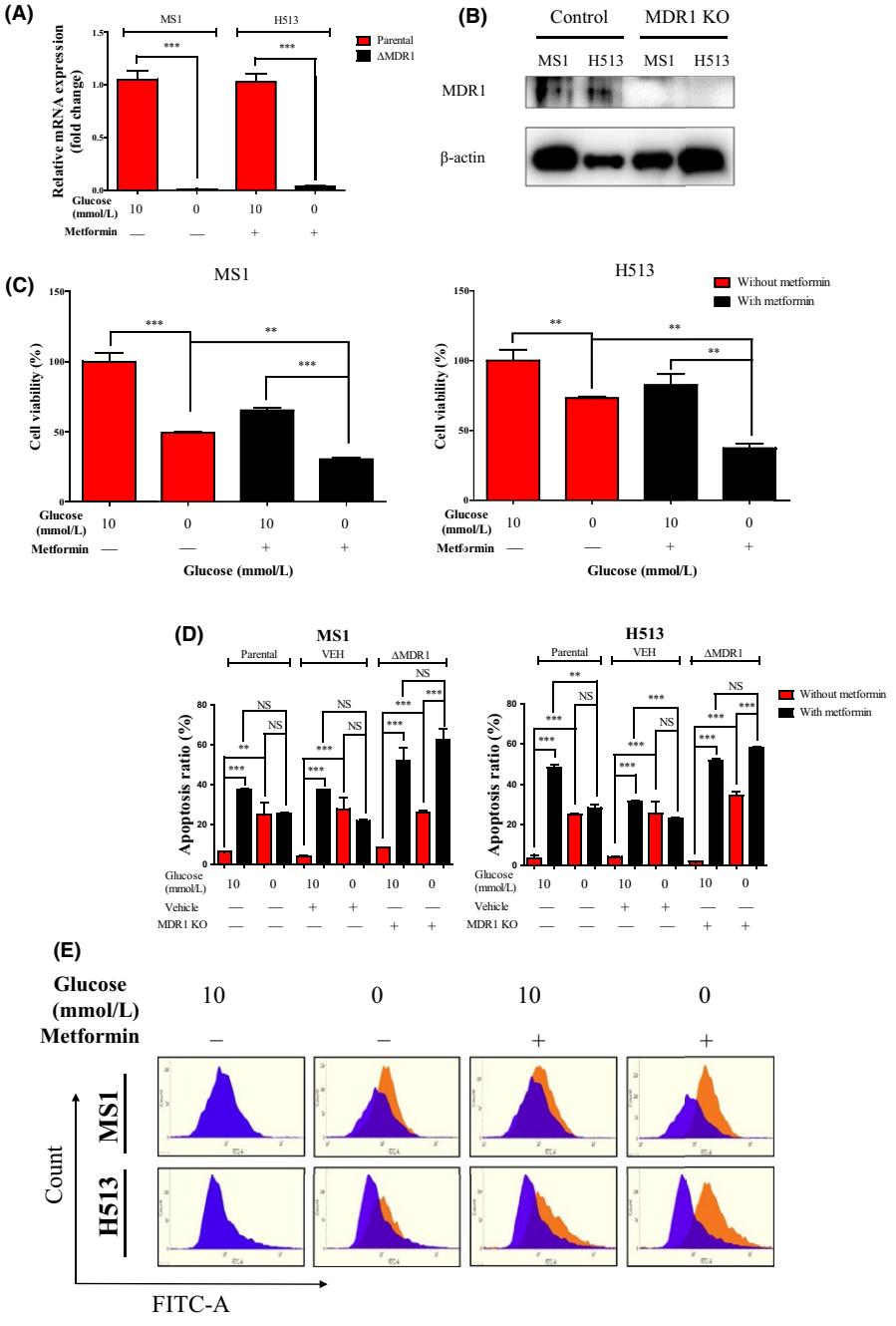



**FIGURE 5** Survival of glucose-starved human malignant mesothelioma (HMM) cells subjected to metformin treatment was controlled by multidrug resistance protein 1 (MDR1). A, The mRNA expression of MDR1 was measured in control and Mdr1-CRISPR/Cas9 plasmid-transfected HMM cells. ΔMDR1: MDR1-CRISPR/Cas9 plasmid-transfected HMM cells. Control: cells transfected with a noncoding CRISPR/Cas9 plasmid. The error bars represent the SEM (n = 4). Significant differences are indicated by $^{**}P < .001$, $^{***}P < .0001$. B, The Mdr1 expression in the parental HMM cells and MDR1 KO HMM cells by western blot assay. MDR1 KO: MDR1-CRISPR/Cas9 plasmid-transfected HMM cells. Control cells were HMM cells transfected with a noncoding CRISPR/Cas9 plasmid. C, The viability of ΔMDR1 HMM cells with glucose starvation and/or metformin treatment. All error bars represent the SEM (n = 8). Significant differences are indicated by $^{**}P < .01$, $^{***}P < .001$. D, Analysis of apoptosis in parental cells, control cells and ΔMDR1 cells. E, Activated autophagy in ΔMDR1 HMM cells cultured under glucose starvation and/or metformin treatment was investigated by flow cytometry (purple: control; red: experimental condition). CRISPR, clustered regulated interspaced short palindromic repeats; KO, knockout

resistance. The viability of HMM cells cultured under glucose starvation with metformin was unchanged and apoptosis was decreased compared to those of cells cultured in complete medium. Excessively activated autophagy induces cell death during cancer therapy.[18] However, the treatment of glucose-starved HMM cells with metformin decreased autophagy activation compared to that of untreated cells. Therefore, during glucose starvation, metabolism-altered HMM cells survived for 3 days and exhibited enhanced resistance to metformin.

Glucose-starved HMM cells were survived by metformin treatment more than those cells cultured in complete medium, although mitochondrial integrity, including ATP generation, morphology, MMP and mPTP, was compromised. The treatment of glucose-starved HMM cells with metformin resulted in a significant ATP synthesis reduction; a major function of mitochondria that supported mitochondrial function was disrupted and could not active mitochondrial-mediated cell death. Moreover, elongated mitochondria and thick cristae were detected in the treatment of glucose-starved HMM cells with metformin. That phenotype was observed during mitochondrial fusion, with inhibited cytochrome C release, leading to anti–apoptotic effects.[33-35] The de-phosphorylation of Ser 656 on dynamin-related protein 1, a mitochondrial fission marker, induced mitochondrial elongation and prompted an anti–apoptotic response.[36] The mPTP penetrated through the mitochondrial inner and outer membranes, which maintain the calcium homeostasis. Excessive calcium was accumulated in damaged mitochondria through mPTP overactivation, which disturbs homeostasis and leads to cell death.[37] In line with this, glucose starvation or metformin treatment induced mPTP overactivation and mitochondrial swelling. However, the treatment of glucose-starved HMM cells with metformin exhibited a similar level of mPTP activation compared to HMM cells cultured under complete medium. Cyclophilin D, which is a component of the mPTP inhibition by cyclosporine A treatment, protects calcium-mediated cell death in cardio myocytes.[38] Furthermore, the treatment of glucose-starved HMM cells with metformin increased MMP. MMP hyperpolarization permitted the hyperaccumulation of anions in the inner membrane, leading to protein import to the intermembrane space.[39-41] Moreover, MMP hyperpolarization is simultaneously observed with mitochondrial p-glycoprotein overexpression under stressful conditions.[42] A comparatively low amount of hyperpolarization was present in H513 cells compared to MS1 cells. The translocation of p53 to the mitochondria alters the MMP; however, H513 cells, which are p53-mutated HMM cells, exhibit disturbances in MMP changes that leading relatively lower mitochondrial Mdr1 expression compared to MS1 cells.[43] Therefore, we proposed that significantly increased MMP in the treatment of glucose-starved HMM cells with metformin protected mitochondrial integrity and promoted cell survival through overexpressed Mdr1 in mitochondria. In contrast, mitochondrial Mdr1 expression was not changed in the treatment of glucose-starved HMM cells with metformin and CCCP. Therefore, MMP hyperpolarization in the treatment of glucose-starved HMM cells with metformin promotes Mdr1 overexpression in compromised mitochondria.

Metformin combined with serum in medium obtains a hydrophobic feature that could be a substrate of MDR1.[44,45]

Multidrug resistance has been attributed to ABC transporters that encode p-glycoprotein, which regulates drug resistance via the efflux of hydrophobic substrates from the intracellular membrane.[46] Furthermore, MDR1 interacts with the membrane as a fluid mosaic model located in lipid bilayer membranes and activate binding with ATP, which contributes to the efflux of the accumulated drug and small molecules to the extracellular space.[47] The MDR1 is expressed not only in the plasma membrane, but also in mitochondria and nucleus.[48] Moreover, p-glycoprotein is highly expressed to protect attenuated mitochondria and promotes ion and chemical homeostasis.[49,50] Mitochondrial Mdr1 has a distinct role as a unidirectional pump that sequesters drug infiltrated mitochondria and is subsequently degraded by lysosome to prevent drug distribution.[48] Overexpressed Mdr1 of mitochondria in glucose-starved HMM cells spill out the accumulated metformin for survival. In contrast to MS1 cells, H513 cells exhibited nonspecific Mdr1 expression in mitochondria. The p53 mutant form induced a high level of Mdr1 expression in colorectal cancer metastases.[51] The role of MDR1 was verified by knockout using the CRISPR/Cas9 system. In contrast to the parental cells, apoptosis and autophagy were increased and viability was decreased in the treatment of glucose-starved MDR1 KO HMM cells with metformin. ATP is required for autophagy activation. However, the ATP amount was insufficient in glucose-starved HMM cells with metformin because that energy was wasted in MDR1 overexpression. Therefore, an inadequate ATP amount could not facilitate autophagy. However, further research is necessary on the relationship between autophagy and MDR1. In addition, the expression level of ABCG2 mRNA was increased in glucose-starved HMM cells with metformin treatment. However, the extent of ABCG2 upregulation was less dramatic than that of MDR1 in our study. The role of ABCG2 in metformin resistance and the underlying mechanism warrants further study.

The present study demonstrated that overexpressed Mdr1 in defected mitochondria rescued glucose-starved HMM cells from metformin treatment. Although MDR1 is important in chemoresistance, the mechanism of its expression and localization are not yet clear in HMM cells. Therefore, tumor recurrence and chemoresistance in malignant mesothelioma patients could be suppressed by Mdr1 repression through MMP reduction, which promote the anticancer efficacy.

## CONFLICTS OF INTEREST

All authors declare no potential of conflicts of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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