ORIGINAL PAPER

Matrine has pro-apoptotic effects on liver cancer by triggering mitochondrial fission and activating Mst1-JNK signalling pathways

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Abstract

Mitochondrial homeostasis is closely associated with liver cancer progression via multiple mechanisms and is also a potential tumour-suppressive target in clinical practice. However, the role of mitochondrial fission in liver cancer cell viability has not been adequately investigated. Matrine, a type of alkaloid isolated from *Sophora flavescens*, has been widely used to treat various types of cancer. However, the molecular effect of matrine on mitochondrial homeostasis is unclear. Therefore, the aim of the current study was to determine the role of mitochondrial fission in cell apoptosis, viability, migration and proliferation of HepG2 cells in vitro. The effect of matrine on mitochondrial fission and its mechanism were also explored. The results of our study showed that HepG2 cells treated with matrine had reduced viability, an increased apoptotic rate, a blunted migratory response, and impaired proliferation capacity. At the molecular level, matrine treatment activated mitochondrial fission, which promoted mitochondrial dysfunction, caused cellular oxidative stress, disrupted cellular energy metabolism and initiated cell apoptotic pathways. However, blockade of mitochondrial fission abolished the deleterious effects of matrine on HepG2 cells. Further, we demonstrated that the Mst1-JNK signalling axis was required for matrine-modulated mitochondrial fission. Matrine-mediated mitochondrial dysfunction was reversed by inhibiting Mst1-JNK pathways. Together, our results demonstrated that mitochondrial fission could be a potential upstream tumour-suppressive signal for liver cancer by modifying mitochondrial function and cell death. By contrast, matrine exerted an anticancer function in liver cancer by activating mitochondrial fission mediated by Mst1-JNK pathways.

Keywords Matrine · Mitochondrial fission · Mst1-JNK pathways · Liver cancer · Apoptosis

Introduction

Matrine, a type of alkaloid, is isolated from *Sophora flavescens*. Several studies confirmed the tumour-suppressive effects of matrine. For example, matrine induces apoptosis in human oesophageal squamous cancer [1], inhibits neuroblastoma cell proliferation and migration [2], represses

the progression of prostate cancer [3], regulates pancreatic cell epithelial-mesenchymal transition and invasion [4], and suppresses metastasis in human cervical cancer [5]. These data confirmed that matrine has inhibitory effects on cancer development and progression. However, the exact mechanisms by which matrine mediates liver cancer cell death and the key cellular parameters that matrine affects remain to be elucidated.

Mitochondrial homeostasis is a potential target to reduce cancer cell viability and thus slow tumour progression [6]. Recently, mitochondrial fission has been acknowledged as the initial signal for mitochondrial damage. In cardiac ischaemia–reperfusion injury, excessive mitochondrial fission promotes cardiomyocyte death, and the deletion of genes related to mitochondrial fission attenuates the damage exerted by reperfusion injury [7, 8]. Additionally, in liver fatty disease, abnormal mitochondrial fission interrupts hepatocyte energy metabolism, and pharmacological inhibition of mitochondrial fission reverses hepatic steatosis in a

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mouse model of fatty liver disease [9]. However, the functional role of mitochondrial fission in liver cancer cell death remains to be elucidated. Moreover, whether mitochondrial fission is involved in matrine-mediated liver cancer cell apoptosis remains an interesting question.

Previous studies proposed several upstream regulatory pathways for mitochondrial fission. In a model of endothelial oxidative stress, the BI1/Syk/COX-1 signalling pathway was identified as the key mediator for mitochondrial fission activation [10, 11]. In colorectal cancer, the JNK-Drp1-HtrA2/Omi axis was confirmed to be the stimulus for mitochondrial fission [12, 13]. Furthermore, in endometriosis, mitochondrial fission is primarily regulated by the Mst1/Drp1 cascade [14, 15]. Interestingly, in liver cancer, there is strong evidence supporting the role of the Mst1 and JNK pathways in managing mitochondrial homeostasis, including mitochondrial oxidative stress, mitochondrial calcium overload and mitophagy [16–19]. Based on this information, we examine whether the Mst1-JNK axis is involved in mitochondrial fission in liver cancer in the presence of matrine. The aim of our study was to explore whether the anti-tumour action of matrine on hepatocellular carcinoma (HCC) is mediated through the modulation of mitochondrial fission and modification of the Mst1-JNK pathway.

Materials and methods

Liver cancer cell lines

HepG2 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) and the Huh7 liver cancer cell line (Cell Bank of the Chinese Academy of Sciences) were used to explore the role of matrine in the liver cancer phenotype in vitro. Analytically pure matrine, purchased from Sigma-Aldrich (Cat. No.M5319, St Louis, MO, USA), was incubated with HepG2 cells for 12 h at different doses (0–20 nM). To activate mitochondrial fission in HepG2 cells, cells were pre-treated with Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (5 μM) for approximately 30 min. To inhibit mitochondrial fission, cells were treated with mitochondrial division inhibitor-1 (Mdivi-1, 10 mM; Sigma-Aldrich; Merck KGaA) for 2 h [20].

Cellular proliferation evaluation and LDH release assay

Cellular proliferation was evaluated via EdU assay. Cells were seeded onto a 6-well plate, and the Cell-Light™ EdU Apollo®567 In Vitro Imaging Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA; Catalogue No. A10044) was used to observe the EdU-positive cells according to the manufacturer's instructions [21]. Cellular lactate production in the medium was measured via a lactate assay kit (#K607-100; BioVision, Milpitas, CA, USA) according to a previous study [22].

Quantitation of cellular ATP levels and Transwell assay

Cellular ATP content was measured according to a previous report via ELISA assay. Cells were washed with PBS and then collected at room temperature. Subsequently, a luciferase-based ATP assay kit (Celltiter-Glo Luminescent Cell Viability assay; Promega, Madison, WI, USA; Catalogue No. A22066) was used according to the instructions [23]. For Transwell migration assays, the upper chambers of 24-well Transwell assay plates were seeded with 2×10^3 HepG2 cells in 200 μl serum-free medium per well. The lower chambers were filled with 600 μl medium containing 0.5% fetal bovine serum (FBS) [24]. After a 24-h incubation in a humidified incubator at 37 °C, 5% CO_2, cells that had migrated to the underside of the membranes were fixed and stained with 0.1% crystal violet. After washing with distilled water, pictures of each chamber were randomly taken using a $200\times$ microscope field, and these images were used to quantify the total number of migrated cells [25].

RNA isolation and qPCR

TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to isolate total RNA from cells. Subsequently, the Reverse Transcription kit (Kaneka Eurogentec S.A., Sering, Belgium) was applied to transcribe RNA (1 μg in each group) into cDNA at room temperature (~25 °C) for 30 min. The qPCR was performed with primers using SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, Inc. Cat. No. 4309155) [26]. The following were primers used in the present study: cadherin, F: 5′-CTAGTGTCGAGCTTCGAA ATCT-3′, R: 5′-CTGTGGTACTGTTGGACCA-3′; vimentin, F: 5′-TAGTGGTTCTTGGATATTCACT-3′, R: 5′-AGAGTT GTCATTGAATTCGG-3′; EGFR, F: 5′-GCTACCTTTGAT GTTAGT-3′, R: 5′-AGAGATACCTGATAGAGTCGT-3′; BRAF: F: 5′-TCAATGACTCCTGGAAGAA-3′, R: 5′-GTG ATTGATCTAATGCCTAT-3′; and GAPDH, F: 5′-AAGTTG TGFATTAGTCA-3′, R: 5′-AGAATAGTCCTATAATCA-3′.

Western blotting

Protein expression was analysed via western blotting. Primary antibodies against the following proteins were used in the present study: caspase-9 (1:1000, Cell Signaling Technology, #9504), pro-caspase-3 (1:1000, Abcam, #ab13847), cleaved caspase-3 (1:1000, Abcam, #ab49822), PARP (1:1000, Abcam, #ab32064), Bcl2 (1:1000, Cell Signaling Technology, #3498), Bad (1:1000, Abcam, #ab90435),

c-IAP (1:1000, Cell Signaling Technology, #4952), Bax (1:1000, Cell Signaling Technology, #2772), Mst1 (1:1000, Cell Signaling Technology, #3682), cyclin D1 (1:1000, Abcam, #ab16663), cyclin E (1:1000, Abcam, #ab171535), cyt-c (1:1,000; Abcam; #ab90529), JNK (1:1000; Cell Signaling Technology, #4672), p-JNK (1:1000; Cell Signaling Technology, #9251), cadherin (1:1000, Abcam, #ab1416), vimentin (1:1000, Abcam, #ab8978) and CDK4 (1:1000, Abcam, #ab137675). After being washed with TBST and further incubated with horseradish peroxidase (HRP)-coupled secondary antibodies (1:2000, Cell Signaling Technology, #7074 and #7076) at 37 °C for 60 min, the blots were developed with an enhanced chemiluminescence (ECL) reagent [27]. The intensity of immunoblot bands was normalized to that of GAPDH (1:1000, Cell Signaling Technology, #5174) and/or β-actin (1:1000, Cell Signaling Technology, #4970).

Immunofluorescence assay

Samples were observed using a Leica DM IL LED inverted fluorescence microscope (magnification, ×400; Leica Microsystems, Inc.). Primary antibodies against the following proteins were used in the present study: cyt-c (1:1000; Abcam; #ab90529), Mst1 (1:1000, Cell Signaling Technology, #3682), p-JNK (1:1000; Cell Signaling Technology, #9251) and Tom20 (mitochondrial marker, 1:1000, Abcam, #ab186735). Mitochondria were stained using Tom20. Mitochondrial length was measured to quantify mitochondrial fission under a laser confocal microscope (TcS SP5; Leica Microsystems, Inc., BuffaloGrove, IL, USA) according to the previous study [28].

Mitochondrial function evaluation

Mitochondrial membrane potential was observed using a JC-1 kit. HepG2 cells were incubated with the JC-1 probe for 30 min at 37 °C in the dark [29]. Subsequently, cells were washed with PBS to remove the free JC-1 probe. Then, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and the mitochondrial potential was assessed under an Olympus IX81 microscope using FV10-ASW 1.7 software. ImageJ software was used to analyse the mitochondrial potential as described previously [30]. In the mPTP opening assay, cells were cultured and then incubated with calcein-AM/$CoCl_2$ staining for 25 min at 37 °C in the dark. Subsequently, the cells were washed with PBS 3 times to remove the free calcein-AM/CoCl2. The change in fluorescence intensity was measured by a fluorescence microscope according to a previous study [31]. Then, the mPTP opening was measured.

ROS measurement via flow cytometry

Cellular ROS measurements were performed using the dihydroethidium (DHE) probe. HepG2 cells were incubated with 5 µM DHE for 30 min at 37 °C in the dark. Then, cells were washed with PBS to remove free ROS probe. Subsequently, cellular ROS was observed under an Olympus IX81 microscope and quantified by fluorescence activated cell sorting (FACS) [32].

Transfection

Transfection with siRNA was used to inhibit the expression of Mst1 in matrine-treated HepG2 cells. Transfection with siRNA was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol [33]. The negative control group was transfected with negative control siRNA. Transfection was performed for approximately 48 h. Then, western blotting was used to observe the knockdown efficiency after harvesting the transfected cells.

Cell apoptosis assay and antioxidant factor measurement

In the TUNEL assays, cells (HepG2 cells and Huh7 cells) were fixed in paraformaldehyde at room temperature for 15 min. Then, cells were washed 3 times in cold PBS and treated with 0.05% Triton X-100 for 15 min on ice [34]. Subsequently, a TUNEL kit (Roche Apoptosis Detection Kit, Roche, Mannheim, Germany) was used to stain cells, as described in a previous study [35]. Additionally, caspase-3 and caspase-9 activity was determined using commercial kits (Beyotime Institute of Biotechnology). The levels of antioxidant factors, including GPX, SOD and GSH, were measured with ELISA kits, which were purchased from Beyotime Institute of Biotechnology [36].

Statistical analysis

Data are presented as the mean ± S.E.M. and were representative of at least three independent experiments. Statistical analyses were performed by one-way analysis of variance (ANOVA). P values less than 0.05 were considered statistically significant.

Results

Matrine induces liver cancer cell death in a dose-dependent manner

To determine whether matrine promotes liver cancer damage, different concentrations of matrine were added into the medium of HepG2 cells and Huh7 cells for 12 h. Then, cellular viability was measured using an LDH release assay. As shown in Fig. 1a, b, matrine significantly reduced the viability of HepG2 cells and Huh7 cells in a dose-dependent manner. These data indicated that matrine treatment induced liver cancer cell death in a dose-dependent manner. Notably, we found no significant differences

between the control group and the 1 nM matrine group. Moreover, the minimum lethal dose of matrine for cell death was 5 nM, and thus, 5 nM matrine was used in the following experiments.

Then, the TUNEL assay was performed to quantify cellular death. As shown in Fig. 1c–e, matrine treatment increased the proportion of TUNEL-positive cells in a concentration-dependent manner. To provide additional support for the pro-apoptotic action of matrine on liver cancer cells, caspase-3-related apoptosis signalling was measured because caspase-3 is the executor of cellular apoptosis. As shown in Fig. 1f–k, a western blotting assay demonstrated that caspase-3 expression was increased in response to matrine treatment compared to the control treatment. Along with caspase-3 activation, PARP expression was correspondingly
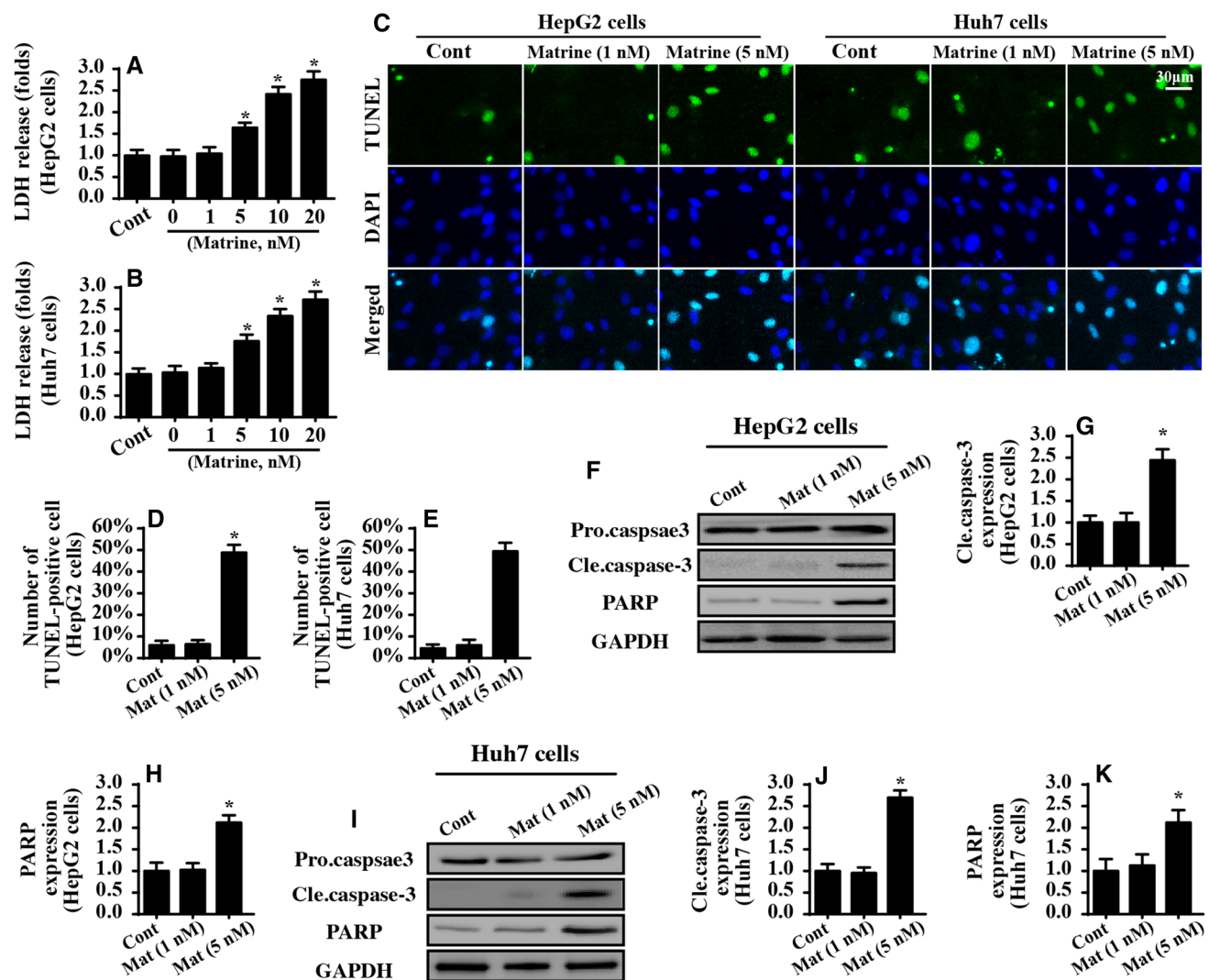

Fig. 1 Matrine induces HepG2 and Huh7 cell death. **a**, **b** An LDH release assay was performed to analyse cell death. Different doses of matrine were added into the medium of HepG2 cells and Huh7 cells. **c–e** Cell death was quantified via TUNEL assays. The number of TUNEL-positive cells was monitored. **f–k** Western blotting was used to analyse the expression of caspase-3 and PARP. Experiments were repeated 3 times ($n=3$), and data are shown as the mean ± SEM. *$P < 0.05$ vs the control group. *Cont* control, *Mat* matrine

elevated in matrine-treated cells. Together, these data identified matrine as a tumour suppressor that acted by promoting liver cancer cell apoptosis. Notably, because no phenotypic differences were noted between HepG2 cells and Huh7 cells, HepG2 cells were used in the following experiments.

Matrine impairs HepG2 cell migration and proliferation

In addition to cellular apoptosis, we further observed the regulatory role of matrine in liver cancer cell migration and proliferation. In a Transwell assay, matrine-treated HepG2 cells migrated approximately 2 times less than the control cells (Fig. 2a, b), suggesting that matrine repressed liver cancer cell mobilization. Additionally, qPCR analysis demonstrated that matrine treatment significantly inhibited the transcription of metastasis genes, such as EGFR and BRAF (Fig. 2c, d), compared to the control treatment. Moreover, the expression of migration-related factors, including cadherin and vimentin, was also downregulated in response to matrine treatment (Fig. 2e–g).

Regarding cancer cell proliferation, cyclin proteins were measured after matrine treatment using western blotting. The results shown in Fig. 2h–j demonstrate that matrine drastically reduced the expression of cyclin D and CDK4. Given that the cancer cell cycle transition is primarily regulated by the cyclin D/CDK4 complex, we questioned whether matrine treatment promoted HepG2 cell S-phase arrest. To explore this question, an EdU assay was used to observe the replicated cells. As shown in Fig. 2k, l, matrine treatment reduced the proportion of EdU-positive cells, indicating that matrine treatment inhibited cell DNA replication. Together, the above data confirmed that matrine strongly repressed HepG2 cell growth and migratory responses.

Matrine mediates HepG2 cell death via the caspase-9-dependent apoptotic pathway

Next, experiments were performed to determine the molecular mechanism by which matrine mediated liver cancer cell death. Previous studies reported that mitochondria are the potential target for evoking cancer cell death [17, 37]; therefore, the mitochondria-dependent apoptotic pathway was evaluated in the current study. First, mitochondrial membrane potential was observed to reflect mitochondrial function. As shown in Fig. 3a, b, mitochondria in the control group exhibited more red fluorescence, whereas matrine-treated cells exhibited more green fluorescence. This result indicated that matrine caused a decrease in mitochondrial membrane potential. In response to the mitochondrial potential reduction, excess electrons cannot be captured by mitochondria, causing cellular oxidative stress. To observe this phenomenon, an ROS probe was used, and the results

showed that matrine-treated cells produced more ROS compared to the control cells (Fig. 3c, d). Due to extensive ROS production, the concentrations of antioxidants, such as GSH, SOD and GPX, were significantly reduced by matrine (Fig. 3e–g). Moreover, excessive ROS may also attack the mitochondrial membrane, resulting in the opening of the mitochondrial permeability transition pore (mPTP) [38]. As shown in Fig. 3h, compared to the control treatment, matrine treatment extended the time of mPTP opening. As a consequence of mPTP opening, mitochondrial pro-apoptotic factors, such as cyt-c, are liberated into the cytoplasm and/or nucleus [39]. The immunofluorescence assay for cyt-c demonstrated that matrine stress caused greater cyt-c translocation from the mitochondria into the nucleus (Fig. 3i–j). These results were further validated by western blotting, which confirmed that matrine induced a decrease in mitochondrial cyt c (mito-cyt c) expression and a parallel increase in the expression of cytoplasmic cyt c (cyto-cyt c) (Fig. 3k–m). Therefore, these data established a central role for matrine in activating mitochondrial apoptosis in HepG2 cells.

Mitochondrial fission is required for matrine-triggered mitochondrial apoptosis

Mitochondrial fission is acknowledged as an early event during mitochondrial apoptosis [7, 8]. Accordingly, we questioned whether mitochondrial fission is implicated in matrine-mediated mitochondrial apoptosis. To address this question, an immunofluorescence assay for mitochondria was performed. As shown in Fig. 4a, several spindle-shaped mitochondria were observed in control cells, whereas abundant small, round mitochondria appeared in matrine-treated cells, indicating that matrine treatment triggered mitochondrial fission in HepG2 cells. Subsequently, the average length of mitochondria was measured. Cells in the control group contained a population of highly elongated mitochondria with a length of ~9.2 μm (Fig. 4b). However, the average length of mitochondria decreased to ~3.5 μm after treatment with matrine. These data confirmed the supportive role of matrine in mitochondrial fission.

To determine the contribution of mitochondrial fission to matrine-mediated activation of the mitochondrial apoptosis pathway, we performed loss- and gain-of-function assays for mitochondrial fission. Mdivi-1, an inhibitor of mitochondrial fission, was added to matrine-treated cells to block mitochondrial fission. Additionally, FCCP, an agonist of mitochondrial fission, was applied to control cells, which were used as the positive control group. Then, the mitochondrial apoptotic pathway was measured using western blotting. Matrine treatment upregulated the expression of pro-apoptotic proteins, including caspase-9, Bax and Bad (Fig. 4c–h), and this effect was similar to the
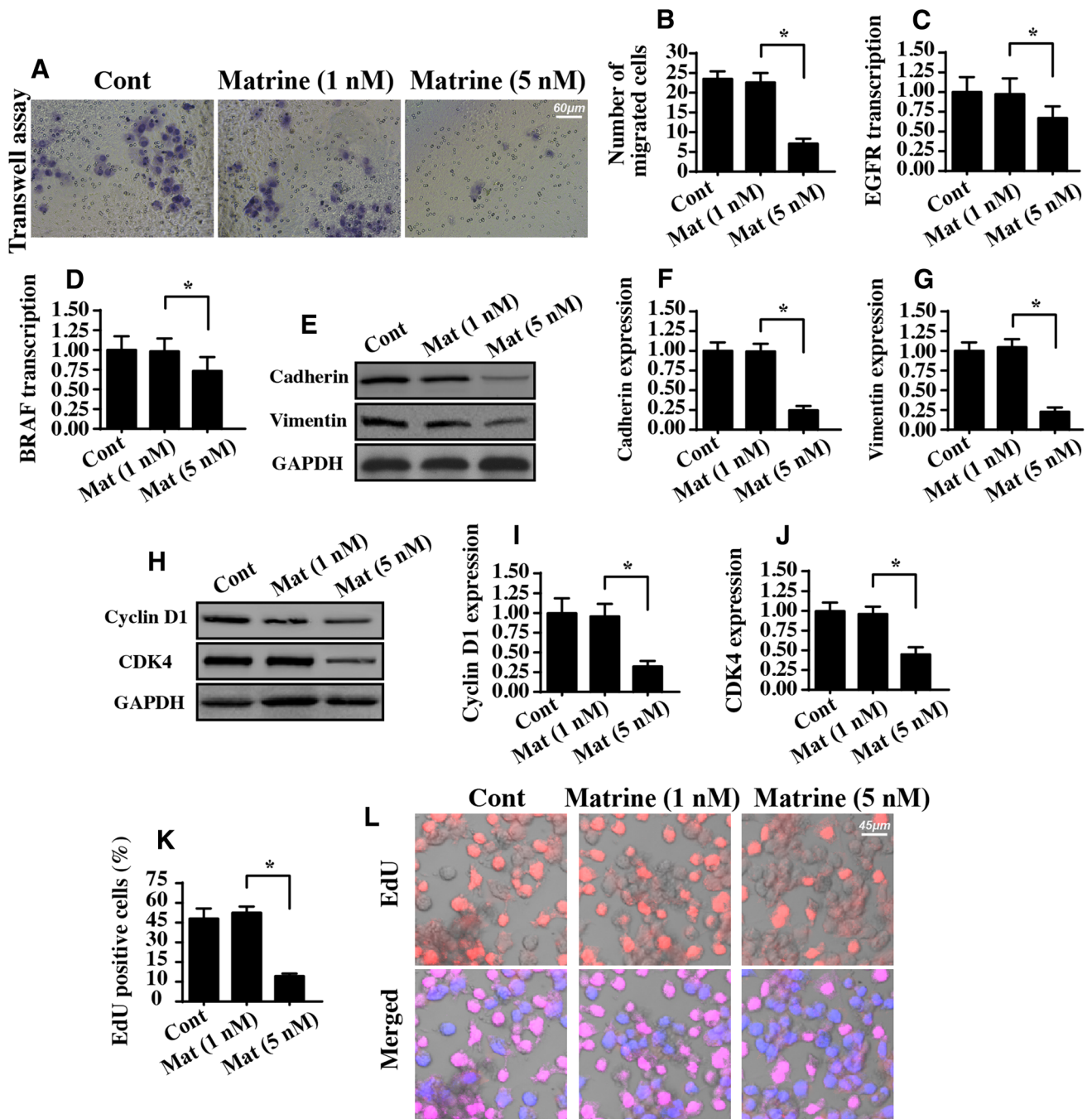
Fig. 2 Matrine regulates HepG2 cell migration and proliferation. **a**, **b**. A Transwell assay was used to observe the migratory response of HepG2 cells in the presence of matrine. **c**, **d** The alteration of metastasis gene expression was analysed via qPCR assays. **e–g** Western blotting was used to analyse the protein expression of cadherin and vimentin. **h–j** The expression of cyclin was assessed via western blotting. **k**, **L** An EdU assay was performed to analyse cellular proliferation. The number of EdU-positive cells was recorded. Experiments were repeated 3 times ($n=3$), and data are shown as the mean $\pm$ SEM. *$P < 0.05$ vs the control group. *Cont* control, *Mat* matrine

data obtained with the supplementation of FCCP in control cells (Fig. 4c–h). Conversely, anti-apoptotic factors were downregulated in response to matrine treatment or FCCP administration (Fig. 4c–h). Interestingly, the pro-apoptotic

action of matrine was mostly inhibited by Mdivi-1, suggesting that mitochondrial fission was activated by matrine and contributed to HepG2 cell death through the mitochondrial apoptosis pathway.
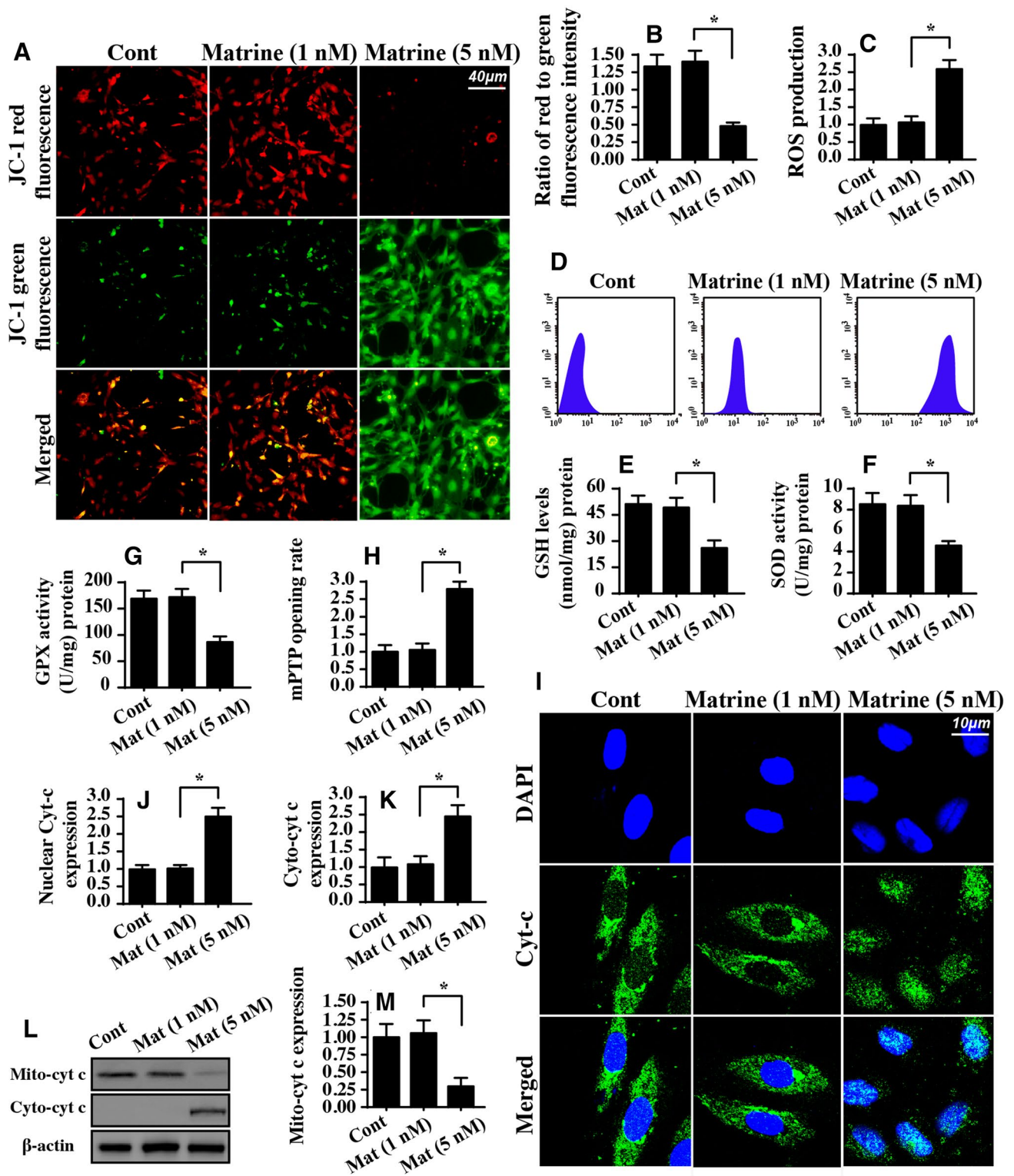
Fig. 3 Matrine modulates mitochondrial function in HepG2 cells. **a**, **b**. Mitochondrial membrane potential was observed using JC-1 staining. The ratio of *red* to *green* fluorescence intensity was measured. **c**, **d** ROS production was monitored with flow cytometry. **e–g** The concentration of antioxidants was measured with ELISA. **h** The ratio of mPTP opening with or without matrine treatment was recorded. **i**, **j**. The immunofluorescence assay for cyt-c was conducted in HepG2 cells. DAPI was used to stain the nuclei. The relative expression of nuclear cyt-c was recorded. **k–m** Western blotting was performed to analyse the expression of cyt-c. Experiments were repeated 3 times ($n = 3$), and data are shown as the mean ± SEM. *$P < 0.05$ vs the control group. *Cont* control, *Mat* matrine
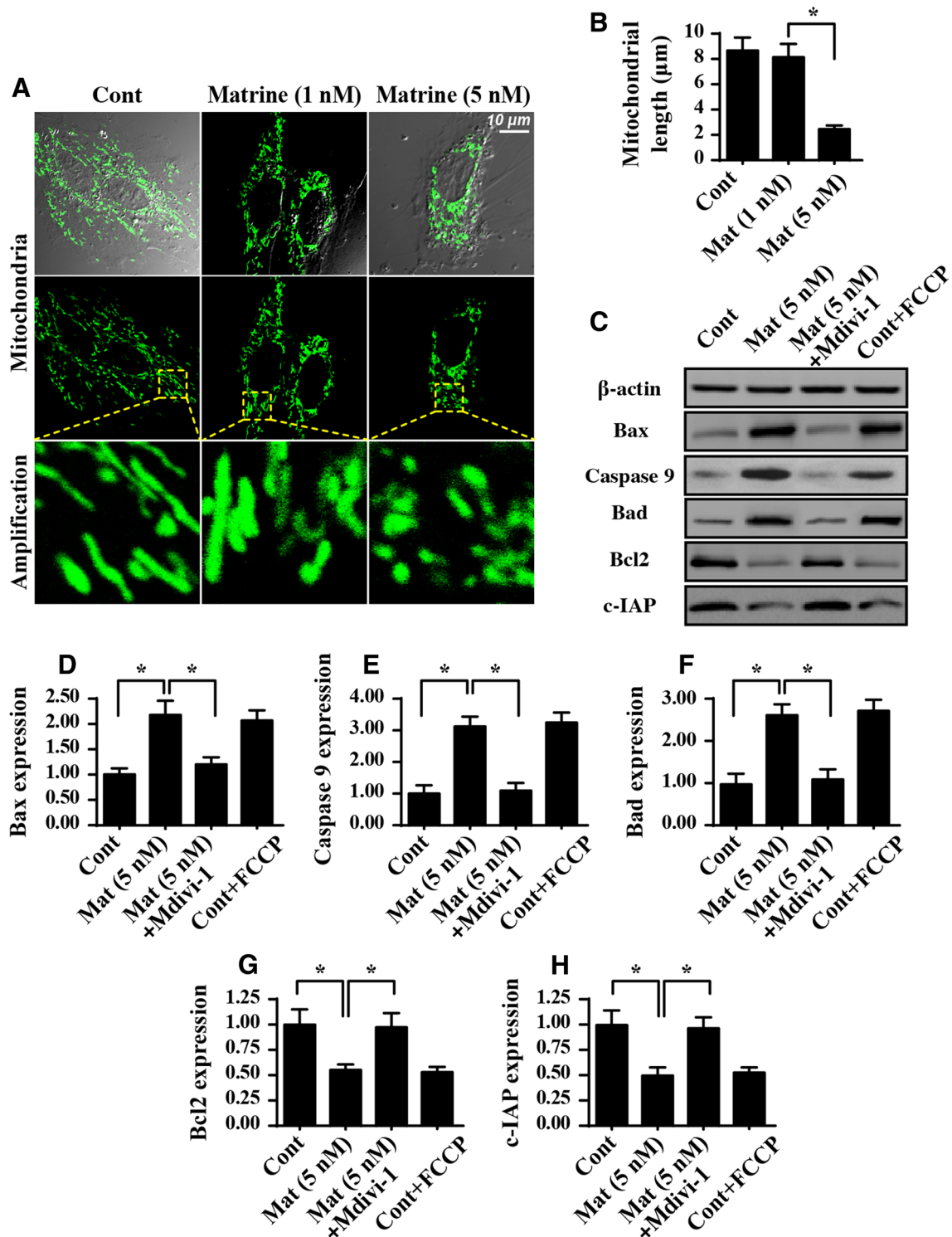
Fig. 4 Matrine activates mitochondrial fission, leading to mitochondrial apoptosis. **a**, **b** Mitochondrial fission was detected using an immunofluorescence assay. The average length of mitochondria was recorded to quantify mitochondrial fission. Mdivi-1, an inhibitor of mitochondrial fission, was used in matrine-treated cells. Additionally, FCCP, an activator of mitochondrial fission, was added to the control group, which was used as the positive control group. **c–h** Western blotting was conducted to determine the activation of mitochondrial apoptosis. The levels of pro-apoptotic proteins, such as Bax, Bad and caspase-9, were measured. The levels of anti-apoptotic factors, such as Bcl2 and c-IAP, were monitored. Experiments were repeated 3 times ($n = 3$), and data are shown as the mean $\pm$ SEM. *$P < 0.05$ vs the control group. *Cont* control, *Mat* matrine

Matrine regulates mitochondrial fission via Mst1-JNK pathways

Next, experiments were performed to analyse the molecular mechanism by which matrine triggers mitochondrial fission. Previous studies identified the Mst1-JNK pathway as a novel regulator of mitochondrial homeostasis in metastatic liver cancer [16, 37]. Accordingly, we questioned whether the Mst1-JNK pathway is required for matrine-mediated mitochondrial fission. As shown in Fig. 5a–c, matrine treatment significantly increased the expression of Mst1, which was followed by an increase in phosphorylated JNK levels. Subsequently, an siRNA against Mst1 was used to inhibit matrine-mediated Mst1 upregulation. The transfection efficiency was confirmed by western blotting, as shown in Fig. 5d–e. Interestingly, transfection with Mst1 siRNA clearly inhibited JNK phosphorylation (Fig. 5a–c). Similar results were obtained using immunofluorescence (Fig. 5f–h). JNK phosphorylation and Mst1 expression were drastically elevated by matrine and were repressed by Mst1 siRNA (Fig. 5f–h). These data indicated that the Mst1-JNK pathway was activated by matrine. To validate whether the Mst1-JNK pathway is responsible for matrine-related mitochondrial fission, an immunofluorescence assay for mitochondria was performed. As shown in Fig. 5i–j, matrine treatment caused mitochondria to divide into several fragments that had a shorter diameter, and this effect was reversed by Mst1 siRNA. Collectively, these data indicated that the Mst1-JNK pathway is responsible for matrine-triggered mitochondrial fission and HCC cell apoptosis.

Mst1-JNK pathways are also implicated in matrine-mediated cell migration impairment and cell proliferation arrest

To confirm whether Mst1-JNK pathways are also involved in matrine-modulated HepG2 cell mobilization and growth, a Transwell assay and EdU staining were used. Compared to the control treatment, matrine treatment significantly repressed HepG2 cell migration (Fig. 6a–b), and this effect was abolished by Mst1 siRNA. Moreover, the transcription of metastasis genes, such as cadherin and vimentin, was downregulated in response to matrine treatment, and this change was reversed to near-normal transcript levels with Mst1 siRNA transfection (Fig. 6c–d). These data confirmed that matrine regulates HepG2 cell migration via Mst1-JNK pathways.

Regarding cell proliferation, the EdU assay demonstrated that the number of EdU-positive cells decreased in response to matrine treatment and returned to near-normal levels after Mst1 siRNA administration (Fig. 6e–f). Additionally, the protein expression of cyclin D1/E was negatively modulated by matrine in an Mst1/JNK-dependent manner (Fig. 6g–i).

These data demonstrated that the Mst1-JNK pathway is also required for the anti-proliferative effects of matrine in HepG2 cells.

Discussion

In the United States, there are ~ 17,000 new cases and ~ 15,000 deaths resulting from primary liver cancer per year [40]. More importantly, the incidence and mortality of liver cancer have gradually increased in recent decades. This trend is in contrast to that of several other common cancers, such as colorectal cancer, gastric cancer and non-small cell lung cancer, which show either decreases or no changes in incidence and mortality over the last 10 years [41]. Accordingly, determining effective approaches to control HCC development and progression will bring clinical benefits to patients with liver cancer. In the present study, we tested the impact of matrine on HepG2 cell apoptosis, migration and proliferation. We demonstrated that matrine induced HepG2 cell apoptosis in a dose-dependent manner. Moreover, matrine-induced stress impaired HepG2 cell migration and proliferation. At the molecular level, matrine activated the Mst1-JNK pathway to initiate excessive mitochondrial fission, which induced mitochondrial apoptosis, inhibited cell migration and arrested cell proliferation. To our knowledge, this is the first study to investigate the molecular mechanism underlying matrine-mediated HCC cell apoptosis, with a focus on mitochondrial fission, the Mst1-JNK pathway and mitochondrial apoptosis.

Matrine, a type of extract from the Chinese herb *Sophora flavescens*, was reported to exhibit pro-apoptotic properties, anti-proliferative actions and metastasis-suppressive effects on many types of cancer. Matrine suppresses bladder cancer proliferation [42], triggers endoplasmic reticulum stress in breast cancer [43], promotes necroptosis in cholangiocarcinoma cells [44], inhibits lung cancer growth [45], and blocks the activation and self-renewal of hepatic cancer stem cells [46]. At the molecular level, matrine regulates the cancer cell phenotype via multiple physical processes. Matrine treatment strongly reduces the expression of IL-6 and therefore increases the sensitivity of liver cancer to natural killer cells via JAK/STAT3 signalling pathways [47]. Additionally, matrine application reduces the activity of the PI3 K/Akt pathway, contributing to the augmentation of p21-mediated cell cycle arrest [48]. Moreover, matrine supplementation increases the proteins levels of GRP78, eIF2α and CHOP, activating endoplasmic reticulum stress-mediated cancer cell apoptosis. In the present study, our data provided new evidence to support the pro-apoptotic effects of matrine on liver cancer. We showed that matrine treatment was associated with activation of the Mst1-JNK pathway, which initiated the caspase-9-related cellular apoptotic pathway. Notably,
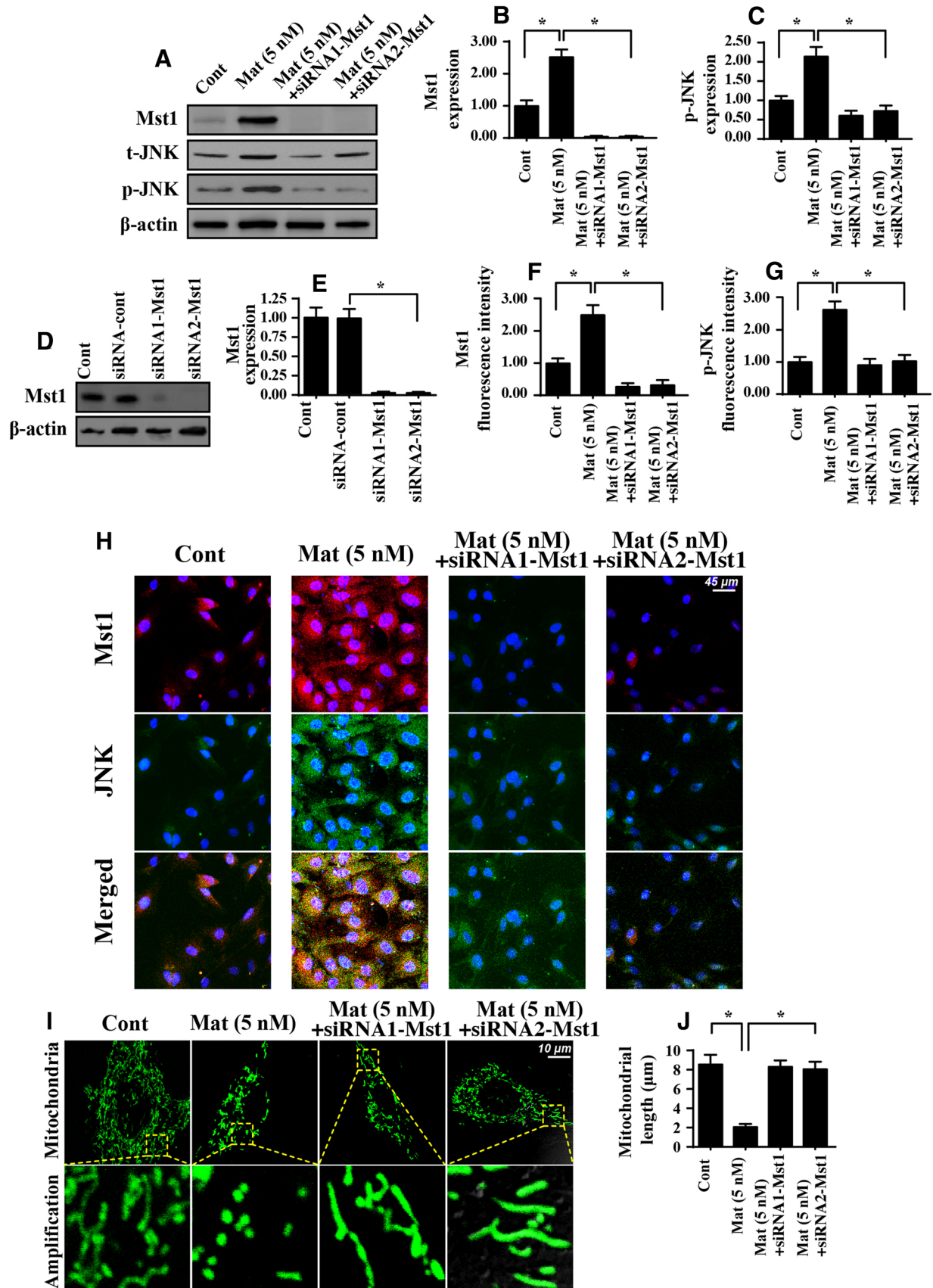
◀**Fig. 5** Matrine regulates mitochondrial fission via the Mst1-JNK pathways. **a**–**c** Western blotting was performed to analyse the expression of Mst1 and JNK phosphorylation in response to matrine. **d**, **e** The siRNA against Mst1 was transfected into HepG2 cells. The knockdown efficiency was verified via western blotting. **f**–**h** The immunofluorescence assay for JNK phosphorylation and Mst1 expression in the presence of matrine. The siRNA was transfected into matrine-treated cells to inhibit the activation of Mst1. **i**, **j** Mitochondrial fission was monitored using an immunofluorescence assay. The average length of mitochondria was recorded. Experiments were repeated 3 times ($n = 3$), and data are shown as the mean ± SEM. *$P < 0.05$ vs the control group. *Cont* control, *Mat* matrine

ample evidence has established the role of the Mst1-JNK pathway in cellular death. In endometriosis, upregulated Mst1 promotes JNK phosphorylation, causing apoptosis through excessive mitochondrial fission. In colorectal cancer, the Mst1-JNK pathway also modulates colorectal cancer cell survival and migration via Bnip3-required mitophagy [37]. In liver cancer, the Mst1/Yap-JNK pathway is reported to be involved in liver cancer cell migration and adhesion via the cofilin/F-actin/lamellipodium axis [16]. These observations, combined with our present data, support the functional importance of the Mst1-JNK pathway in regulating the biological characteristics of HCC.

Furthermore, we determined that mitochondrial fission is required for matrine-mediated liver cancer cell damage. Mitochondrial fission is caused by the Mst1-JNK pathway in the presence of matrine treatment, and excessive mitochondrial fission induced mitochondrial dysfunction as evidenced by increased oxidative stress, reduced mitochondrial potential and activated mitochondrial apoptosis. Similarly, the role of mitochondrial fission in exacerbating mitochondrial dysfunction and promoting cell death is well established. In cardiac ischaemia–reperfusion injury, mitochondrial fission is activated and evokes cardiomyocyte and endothelial cell apoptosis via ROS-mediated cellular oxidative stress and

HK2-induced mPTP opening [8]. Additionally, in chronic fatty liver disease, lipotoxicity-mediated mitochondrial fission disturbs hepatocyte energy metabolism via the NR4A1/DNA-PKcs pathway, contributing to the progression of hepatic stenosis and liver dysfunction [9]. In diabetic mice, hyperglycaemia-evoked mitochondrial fission promotes the senescence of functional cells in an AMPK-dependent manner [49], leading to heart and kidney dysfunction. In the present study, we demonstrated that excessive mitochondrial fission is associated with cell death via mitochondrial apoptosis. Our data and the previous investigations have firmly established a central role of mitochondrial fission activation and subsequent mitochondrial dysfunction that leads to apoptosis in various disease models. Besides, excessive mitochondrial fission may promote matrine-mediated cancer proliferation arrest and migration inhibition. Based on previous studies, excessive mitochondrial fission triggers oxidative stress [49] and F-actin degradation. Redox imbalance and cystoskeleton degradation impair the mobility and proliferation of rectal cancer, liver cancer [16], microvascular endothelial cell [49] and pancreatic carcinoma [50]. Although our study confirmed the inhibitory effects of matrine on HepG2 cell mobilization and proliferation, the exact molecular mechanism has not been fully described. More research is required to validate whether matrine modulates HepG2 cell mobilization and migration via mitochondrial fission.

Together, our report provides new insights into the mechanisms of liver cancer cell death and a novel strategy to promote liver cancer cell death in vitro. Matrine supplementation can induce mitochondrial dysfunction and amplify cell apoptosis via Mst1/JNK-mediated mitochondrial fission. This finding may highlight a new strategy for treating liver cancer by targeting the Mst1-JNK signalling axis and mitochondrial fission.
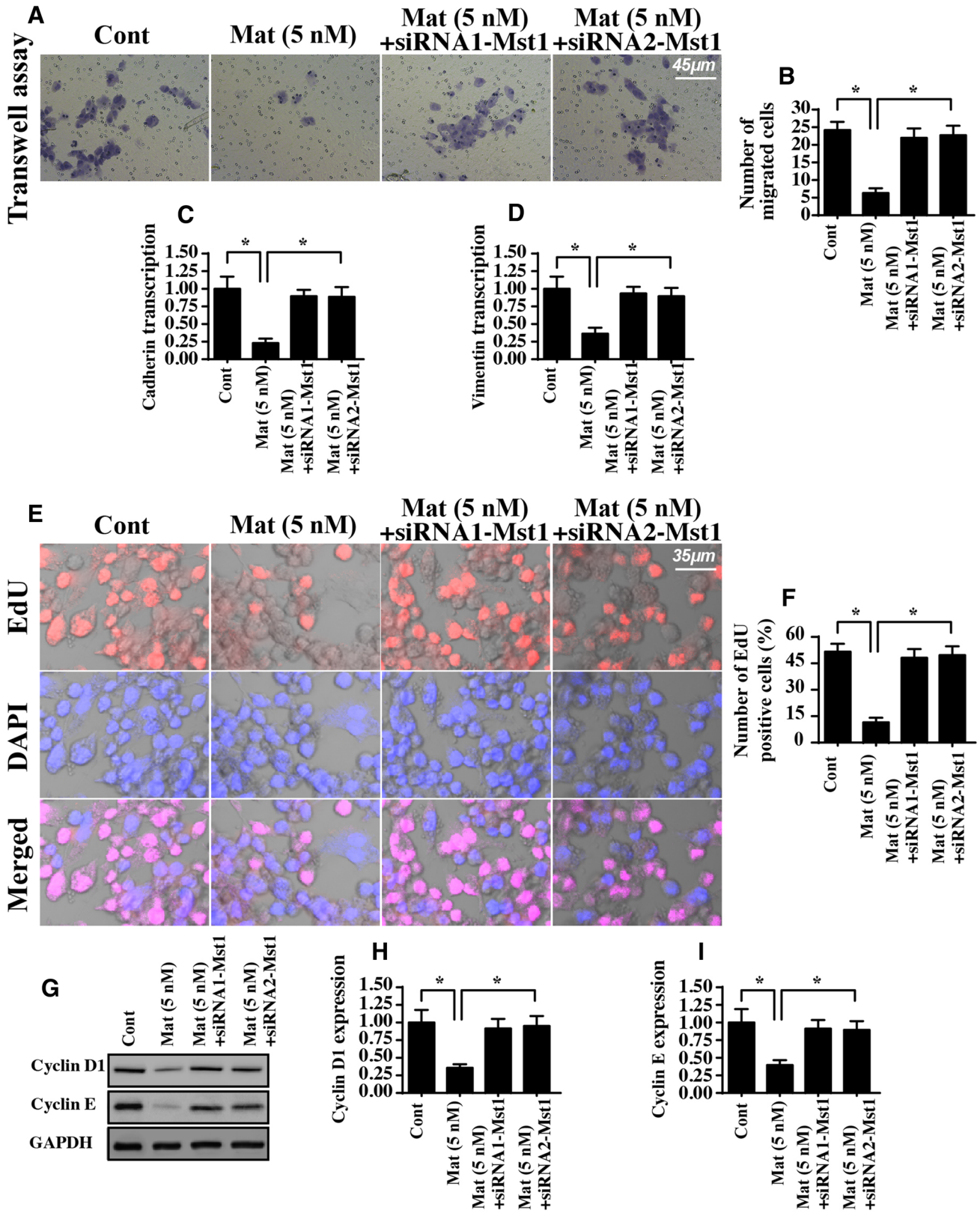
◀**Fig. 6** Mst1-JNK pathways are also involved in matrine-regulated HepG2 cell proliferation and migration. **a**, **b** The HepG2 cell migratory response was observed with Transwell assays. The number of migrated cells was recorded. **c**, **d** Mst1 siRNA was used to inhibit the activity of Mst1-JNK pathways. A qPCR assay was performed to analyse the transcription of metastasis genes, such as cadherin and vimentin. **e**, **f** Cell proliferation was detected using an EdU assay. The number of EdU-positive cells was recorded. **g–i** The expression of cyclins, such as cyclin D1 and cyclin E, was measured using western blotting. Experiments were repeated 3 times ($n = 3$), and data are shown as the mean $\pm$ SEM. *$P < 0.05$ vs the control group. *Cont* control, *Mat* matrine

Compliance with ethical standards

Conflict of interest Not applicable.

Availability of data and materials All data generated or analysed during this study are included in this published article.

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