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ARTICLE

A methylation functional detection hepatic cell system validates correlation between DNA methylation and drug-induced liver injury

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Abstract

Drug-induced liver injury (DILI) is a life-threatening, adverse reaction to certain drugs. The onset and extent of DILI can vary drastically in different patients using identical drugs. Association studies suggested that subtle differences in DNA methylation may help explain the individual differences in DILI. However, there are very few experimental methods to confirm such associations. In this study, we established a novel DNA methylation functional detection system in human hepatocytes, using CRISPR/dCas9 for targeted modification of DNA methylation, and set four parameters to indicate the liver injury by cell model. Using this system, we validated the association of hypermethylation of *CYP2D6* and *CYP2E1* with rifampin-induced DILI. Our results revealed that, following treatment of HepaRG cells with rifampin, the methylation levels of *CYP2D6* and *CYP2E1* were inversely proportional to cell viability and glutathione content, and directly proportional to caspase 3/7 activity. We expect that our methylation detection system will serve as a useful tool in validating correlations between DNA methylation and DILI in other in vitro systems. Our results establish a foundation for future investigations to better understand the mechanisms underlying DILI and may aid in advancing personalized DILI medicine.

Introduction

Drug-induced liver injury (DILI) describes liver damage caused directly by a drug or indirectly by its metabolic byproducts [1]. It is the most common and severe adverse drug reaction observed in clinical treatments. In fact, DILI is thought to be responsible for the majority of acute liver failure cases around the world [2]. The incidence of DILI is 14 per 100,000 persons annually [3]. More concerning is that 6% of DILI patients progress to liver-induced death or require liver transplantation [4]. Although DILI is known to have high morbidity and mortality [5], it is challenging to limit the use of medicines that present risk of DILI. Currently, there are over 1000 frequently used drugs that have the potential to cause hepatotoxicity, but which are necessary and efficacious in treating or curing their target diseases, such as anti-tuberculosis and antineoplastic drugs [6]. For years, scientists have sought to develop novel drugs and treatments to minimize the damage caused by DILI. However, effective diagnostic or preventive methods to predict DILI remain unavailable. One reason why it has been difficult to predict DILI is that patients possess unique genetic and epigenetic features, such as genomic variants and differences in DNA methylation [7–9].

DNA methylation, which is known to affect DILI, is a form of epigenetic modification that is crucial for many physiological and pathological processes such as development, cell differentiation, embryonic development, tumorigenesis, and genome imprinting [10, 11]. Aberrant DNA methylation is reported to be associated with several diseases and disorders including DILI [11–14]. A recent study demonstrated an association between the hypermethylation

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of specific CpG-associated promoters and DILI based on a case-control study that included the genes *CYP2D6* and *CYP2E1*, whose hypermethylation was found to be associated with liver injury induced by an anti-tuberculosis drug (ATLI) [15].

At present, research connecting DNA methylation and DILI has been primarily based on association analyses, with limited experimental validation. Therefore, there is an urgent need to establish a human hepatic model to detect DNA methylation and predict DILI in clinical drug treatment.

A suitable cell line is the basis for building a functional methylation model. HepaRG cells, which are derived from hepatocellular carcinoma, exhibit multiple liver-specific functions and maintain a high level of liver-specific enzymatic activity (i.e., major cytochrome P450, phase II enzyme, nuclear receptor, and transporter activity). These cells have been shown to be the most suitable in vitro model for drug metabolism and toxicity studies [16–19]. Therefore, we chose HepaRG as the in vitro model cell line to study the underlying mechanism of DILI. We also used a reprogrammable CRISPR/dCas9-based system to modify site-specific DNA methylation [20], which provided an efficient method to precisely modify DNA methylation.

In this study, we employed CRISPR/dCas9-mediated modification of DNA methylation in a hepatic cell model to establish a functional detection system. We demonstrate the successful use of this system to verify the correlation between DNA methylation and DILI, and to confirm the association between hypermethylation of *CYP2D6* and *CYP2E1* and rifampin-induced DILI.

Materials and methods

Plasmid construction and sgRNA design

Plasmids pdCas9-Tet1-CD (Addgene #83340), pcDNA3.1-MS2-Tet1-CD (Addgene #83341), and TetO-dcas9-D3A (Addgene #78254) were purchased from Addgene (http://www.addgene.org/). The anti-puromycin element was inserted into TetO-dcas9-D3A to replace the original cassette of mCherry using ClonExpress® II One Step Cloning Kit (Vazyme, China). This new plasmid was named pTetO-dcas9-D3A. Target sequences of the aberrant methylation region in *CYP2E1* and *CYP2D6* that associated with DILI [15] were uploaded and analyzed using the MIT sgRNA design tool (http://crispr.mit.edu) to obtain the target sites of the CRISPR/dCas9 modification system. sgRNAs with high scores and low homology (high specificity) in the whole genome were primarily selected. According to a previous finding that the strongest DNA methylation induction was expected to occur when two adjacent sgRNAs were 50 bp apart from each other [20], the sgRNA sets were selected to meet this criterion, respectively. The designed sgRNAs were synthesized as oligos, annealed, and cloned into the pbluescript-U6-gRNA (PUG, a kind gift from Prof. Daru Lu, FDU, China) vector at the B*bs* I digestion site. A PUG variant carrying a scrambled sequence was designed and used as a control.

Cell culture and transfection

HepaRG (Shanghai Guandao Biological Engineering CO. Ltd., Shanghai, China) cells were cultured in RPMI 1640 Medium (Gibco^{TM}, USA) supplemented with 10% fetal bovine serum (Gibco^{TM}, USA) and 1% penicillin/streptomycin (Sangon Biotech, China). The cells were incubated in a humidified 5% CO_2 air incubator at 37 °C. In preparation for transfection, 5×10^5 HepaRG cells were seeded per well in six-well culture plates, and after incubation for 24 h, the cells were transfected using jetPEI reagent (Polyplus transfection, France). The jetPEI: plasmid ratio was 6 µl:4 µg.

In order to identify cell lines that stably carried the demethylation system., pcDNA3.1-MS2-Tet1-CD was first transfected into HepaRG cells. Both pcDNA3.1-MS2-Tet1-CD and jetPEI were diluted with 150 mM NaCl. jetPEI solution was then added to the plasmid solution. Next, the mixed plasmid solution was vortexed for 15 s and incubated for 15 min at room temperature. The solution was then added to HepaRG cells. Thirty-six hours post transfection, the cells were washed and re-plated in fresh medium containing 75 µg/ml hygromycin (Sangon Biotech, China). The medium was changed every 3 days until we failed to observed cell death. Plasmid pdCas9-Tet1-CD was then transfected into pcDNA3.1-MS2-Tet1-CD-expressing cells using the same method. Medium containing hygromycin (75 µg/ml) and puromycin (1 µg/ml, Sangon Biotech, China) was changed every 3 days to obtain cells that stably expressed both plasmids (pcDNA3.1-MS2-Tet1-CD and pdCas9-Tet1-CD).

In order to identify cell lines that stably carried the methylation system, pTetO-dcas9-D3A was transfected into HepaRG cells following the procedure described above. Cell lines that stably expressed pTetO-dcas9-D3A were selected using puromycin (1 µg/ml, with medium changes every 3 days).

Methylation-sensitive high resolution melting (MS-HRM) analysis

Genomic DNA was extracted from cells using a cell genome extraction kit (Tiangen, China). Genomic DNA was bisulfite-converted using the EZ Research EZ DNA methylation kit (Zymo research, USA). Primers were designed by EptiDesigner^{TM} (www.epidesigner.com). Bisulfite-converted template DNA was first amplified using

a PCR Accessory Set (SEQUENOM, USA). The cycling protocol consisted of pre-denaturation at 95 °C for 240 s, followed by 45 cycles at 95 °C for 20 s, 56 °C for 30 s, 72 °C for 60 s, and a terminal extension at 72 °C for 180 s. The product was mixed with 2 × HRM Analysis Premix (with EvaGreen, Tiangen, China) for the MS-HRM assay. The parameters of the melting curve analysis were as follows: 95 °C for 1 min, 40 °C for 1 min, then the melting curve data were collected from 72 °C to 90 °C, at an increase of 0.02 °C/s. Fluorescence data were collected 25 times per 1 °C. We then used the LightCycler 480 (Roche, Switzerland) gene scanning program to obtain the methylation pattern.

Multiparametric assay

Owing to the diversity in DILI-related mechanisms, multiple distinct parameters were required to accurately predict cell injury which represent DILI in HepaRG cells. Four parameters, including cell viability, leakage of lactate dehydrogenase (LDH), caspase 3/7 activity, and glutathione content, were estimated using a recently reported multiparametric assay [17]. Cell viability and intracellular reduced glutathione (GSH) were assayed after 12 h rifampin (Selleck, USA) treatment at IC_{30} (i.e., 300 μg/ml) using a Cell Counting Kit-8 (Dojindo Laboratories, Japan) and a GSH Assay Kit (Nanjing Jiancheng Bioengineering Institute, China), respectively. The leakage of LDH into the culture medium, along with caspase 3/7 activity, were assayed after 24 h rifampin treatment at IC_{50} (i.e., 500 μg/ml) using a Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories, Japan) and a Caspase 3/7 Activity Apoptosis Assay Kit *Green Fluorescence (Sangon Biotech, China), respectively. All assays were performed according each manufacturer instruction and for three independent replicates.

Statistical analysis

The statistical significance of comparisons between two groups was analyzed with Student's *t* test. Differences were considered to be statistically significant when two-tailed *P* values were <0.05.

Results

Construction of a functional DNA methylation detection hepatic cell system

To explore the relationship between DNA methylation and DILI, target DNA methylation regions were modified in HepaRG cells using the CRISPR-dCas9 system. We first constructed the hepatic cell lines that carries demethylated and hypermethylated system, respectively. HepaRG cells were successively transfected with plasmids pcDNA3.1-MS2-Tet1-CD and pdCas9-Tet1-CD. After screening, we obtained a demethylation cell line that stably expressed the demethylase (HepaRG-D). We also constructed a methyltransferase-involved cell line (HepaRG-M) with plasmid pTetO-dcas9-D3A. The sgRNA set, which was designed for each target DNA region, was then transfected into these two cell lines separately. After 48 h, the extent of methylation at each target region was detected by MS-HRM assay. These cell lines would be treated with specific drugs known to induce liver injury. After exposure to these drugs, which were added to the culture medium, a multiparametric assay would be carried out to measure cell viability, LDH leakage, caspase 3/7 activity, and GSH content. These results would indicate the degree of cell injury in the hepatic cell lines and be later used to predict DILI. The overall workflow of this model system is shown in Fig. 1.

Validation of correlation between DNA methylation and DILI

The correlation between DILI triggered by anti-tuberculosis drugs and hypermethylation of *CYP2D6* and *CYP2E1* has been demonstrated previously based on a case-control study [15]. To verify this result, we made the use of our functional

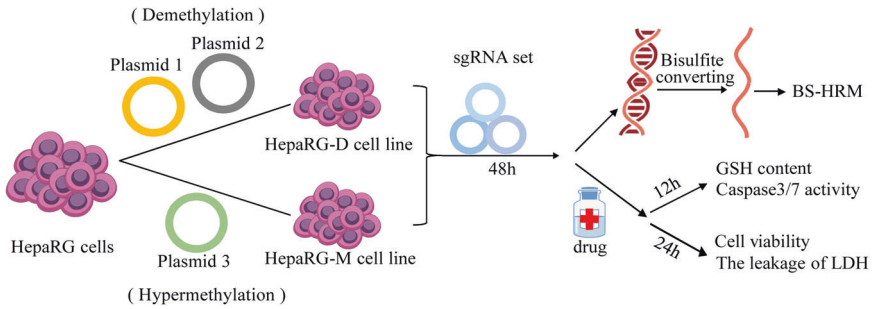

Fig. 1 Flow chart of the construction of the functional DNA methylation detection system in human hepatic cells. Plasmids 1, 2, and 3 represent pcDNA3.1-MS2-Tet1-CD, pdCas9-Tet1-CD, and plasmid pTetO-dcas9-D3A, respectively. Caspase 3/7 activity and GSH content were measured 12 h after drug exposure, while cell viability and LDH leakage were measured 24 h after drug exposure.

methylation detection system. Three and two sgRNAs (defined as sgRNA set 1 and sgRNA set 2, Fig. 2) were designed to target the promoter CpG island regions of *CYP2D6* and *CYP2E1*, respectively. Both HepaRG-D cells and HepaRG-M cells were transfected with sgRNA set 1 and sgRNA set 2. Two days after transfection, we analyzed the methylation status, which showed that the DNA methylation levels of both genes were distinct from control cells (Fig. 3), which indicated that the target DNA regions had been successfully modified. We also detected the CYP2D6 and CYP2E1 expression after hypermethylation or demethylation in each cell line, the results were shown in Supplementary Fig. S1.

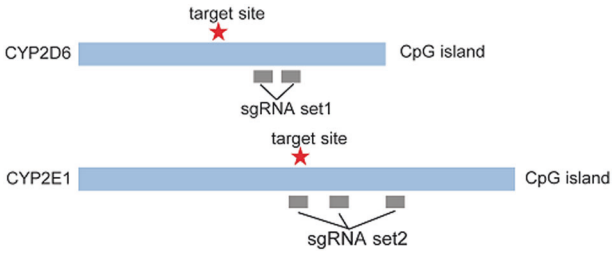

Fig. 2 Diagrams of the positions of sgRNA sets and target methylation sites in the CpG island promoter regions of *CYP2D6* and *CYP2E1*.

We then compared the phenotypes of cell injury when the methylation levels of *CYP2D6* and *CYP2E1* were reduced. As shown in Fig. 4, after the treatment of rifampin (a widely used anti-tuberculosis drug reported to induce DILI), HepaRG-D cells transfected with either sgRNA set 1 or sgRNA set 2 showed significantly increased cell viability ($P < 0.05$, Fig. 4a) and GSH content ($P < 0.05$ and $P < 0.01$, respectively, Fig. 4d) compared with the control. In addition, HepaRG-D cells transfected with either sgRNA set 1 or sgRNA set 2 exhibited significantly decreased caspase 3/7 activity ($P < 0.001$ and $P < 0.01$, respectively, Fig. 4c). On the other hand, the leakage of LDH did not show a significant difference between cells transfected with sgRNAs and control plasmid (Fig. 4b). The increase in cell viability and GSH content, together with the decrease in caspase 3/7 activity, in HepaRG-D cells transfected with sgRNAs suggested that decreasing the methylation levels of *CYP2D6* and *CYP2E1* lowered the level of injury to hepatic cells.

To further verify the correlation between DNA methylation and DILI, we made use of our hypermethylation system. As shown in Fig. 5, after the treatment of rifampin

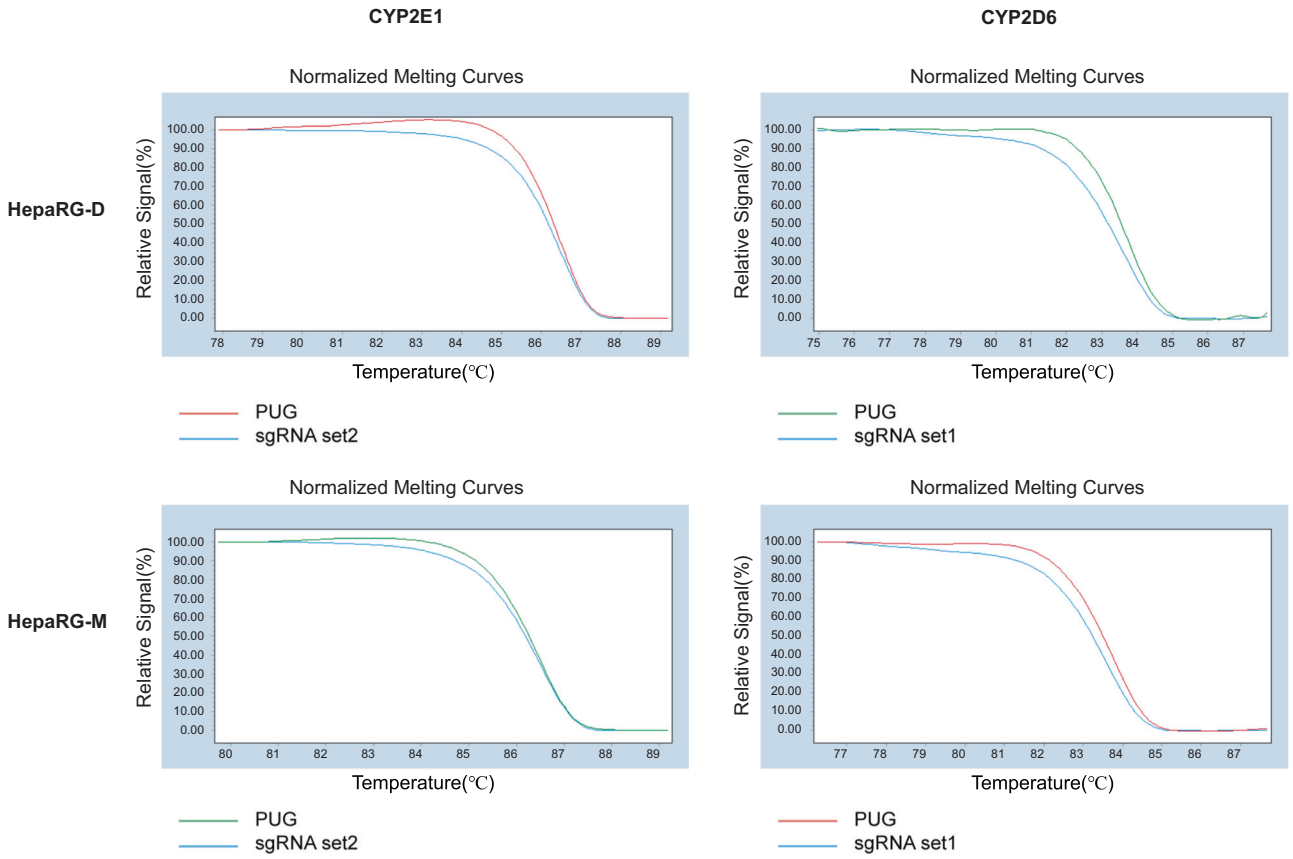

Fig. 3 MS-HRM analysis of modified HepaRG cells. The curves of sgRNA sets 1 and 2 represent the melting curve of bisulfite-converted genomic DNA extracted from cells following transfection with sgRNA set 1 or 2, while the curve of PUG represents the melting curve of bisulfite-converted genomic DNA extracted from cells following transfection the control PUG plasmid, which lacked sgRNA.

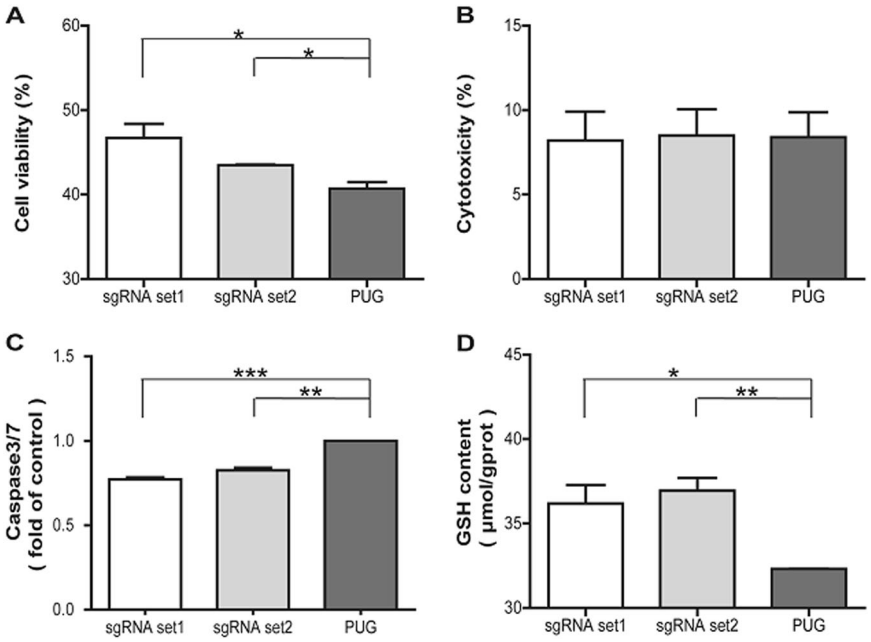

Fig. 4 Representative data from the mu0ltiparametric assay in which HepaRG-D cells were treated with rifampin. Cytotoxicity rates were inferred from leakage of LDH. Cell viability and LDH leakage into the culture medium were measured 24 h after drug exposure at a concentration of 500 μg/ml. Caspase 3/7 activity and GSH content were measured 12 h after drug exposure at a concentration of 300 μg/ml. Caspase 3/7 activity is expressed as a percentage of the control. Data are presented as the mean of three independent replicates. Error bars are defined as S.E.M. The individual results are shown in Supplementary Fig. S2.

HepaRG-M cells transfected with either sgRNA set 1 or sgRNA set 2 displayed significantly reduced cell viability ($P < 0.01$ and $P < 0.05$, respectively, Fig. 5a) and GSH content ($P < 0.001$ and $P < 0.01$, respectively, Fig. 5d) compared with the control. In contrast, caspase 3/7 activity increased in cells transfected with sgRNAs compared with that in the control ($P < 0.001$ and $P < 0.01$, respectively, Fig. 5c). Similar to the results from the previous experiment, LDH leakage did not seem to show any difference (Fig. 5b). Collectively, these results confirmed the correlation between hypermethylation of *CYP2D6* and *CYP2E1* and DILI, and demonstrate the utility of our functional methylation detection system in hepatic cells.

Discussion

The correlation between DILI and the hypermethylation of genes such as *CYP2D6*, *CYP2E1*, *GSTP1*, and *CYP1A1* has been discovered through previous case-control studies [15, 21]. However, such association analyses demanded enormous amounts of time and expense to collect sufficient samples. Animal models have also been used for such analysis and have led to the identification of associated genes such as *CYP1A1* and *CYP1B1* [22, 23]. However, animal models have extensive laboratory cycles and are not suitable for high-throughput screening. In addition, the use of animals in toxicology studies is controversial for ethical and scientific reasons [16].

In this study, we designed a DNA methylation functional detection system in human hepatic cells to verify the correlation between hypermethylation of specific genes and DILI. We chose *CYP2D6* and *CYP2E1* as examples, and our results were consistent with the findings of the association study [15]. This demonstrated that our experimental platform is an effective tool for discovering and verifying changes in DNA methylation that are correlated with DILI in vitro. Using our cell system, researchers can perform multisite high-throughput verification for a large number of drugs that have potential hepatotoxicity, thus increasing the efficiency and breadth of drug development and clinical studies. This system can be also utilized to better understand the molecular mechanisms behind the correlation between DNA methylation and DILI.

DILI is a complex phenomenon that involves many mechanisms such as bioactivation, apoptosis, and oxidative stress [24]. Based on previous studies [17], we chose four parameters—cell viability, LDH leakage, caspase 3/7 activity, and GSH content—to predict DILI. Cellular redox state and cell apoptosis, which are regarded as early to mid-stage indicators of liver cell injury, were assessed by examining the GSH content and caspase 3/7 activity, respectively, after 12 h of drug treatment at the IC_{30} dose (i.e., 300 μg/ml). Membrane disruption and the overall vitality of cells, which served as indicators of the mid-late stages of liver cell injury, were estimated by examining the LDH leakage and cell viability, respectively, after 24 h of drug treatment at the IC_{50} dose (i.e., 500 μg/ml).

For a long time, specific liver-related enzymes have been used to predict DILI, such as alanine aminotransferase, alkaline phosphatase, and aspartate aminotransferase [5]. Recently, microRNA-122 in serum has been proposed as a candidate biomarker of DILI [14, 25]. However, these enzymes and microRNA-122 are usually detected in blood

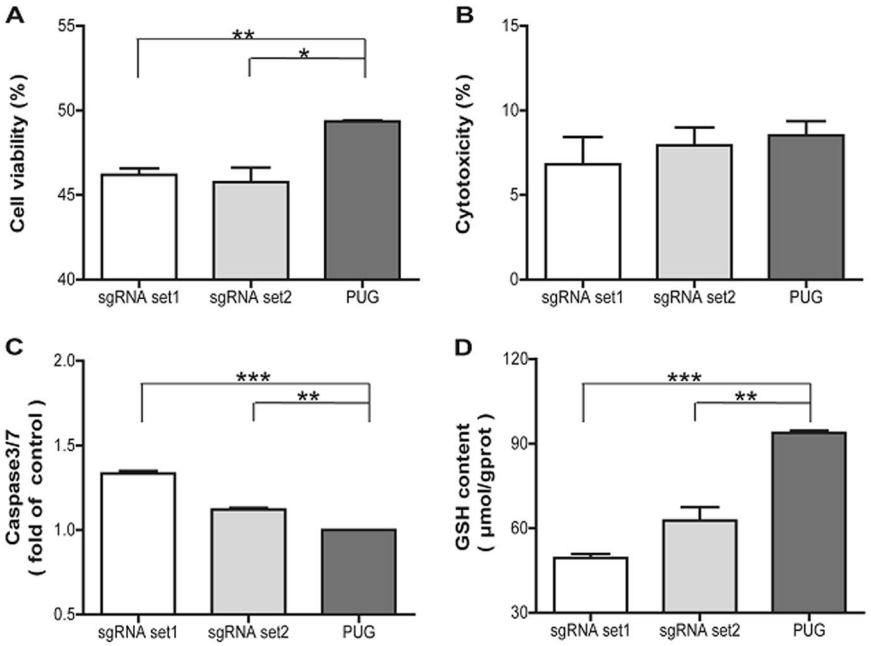

Fig. 5 Representative data from the multiparametric assay in which HepaRG-M cells were treated with rifampin. All data are presented as the mean of three replicate wells. Each parameter was measured on three separate occasions. Error bars are defined as S.E.M. The individual results of which are shown in the Supplementary Fig. S3.

or tissue samples, where the amount of enzyme is much higher than in cells. Therefore, the amount of these enzymes or microRNA secreted into the culture medium is rarely sufficient for accurate detection of hepatocyte damage. Our predictive model of DILI directly detected intracellular mechanisms indicative of DILI, which may be useful for more accurately assessing the degree of hepatic cell damage and better predicting DILI.

In this study, cell viability, caspase 3/7 activity, and GSH content were shown to be significantly altered along with changes in DNA methylation level, indicating these parameters are effective in vitro predictors of DILI. Among them, caspase 3/7 activity was found to be the most sensitive, which is consistent with a previous study [17]. We found that LDH leakage remained unchanged with varying DNA methylation levels, which may be related to the high sensitivity of LDH leakage or the fact that the DNA methylation of *CYP2D6* and *CYP2E1* was not severe enough to cause significant membrane disruption. Another possibility is that different drugs can cause different liver injury patterns, and that rifampin-induced liver injury may not necessarily increase the leakage of LDH.

Our findings verified the association between DNA methylation and rifampin-induced liver injury in vitro, whereas, the mechanism was still need to be further studied. In this study, we did an exploration. We detected the gene expression after methylation modification, and found that the expression of *CYP2D6* and *CYP2E1* were down-regulated after hypermethylation in HepaRG-M, and up-regulated after demethylation in HepaRG-D (Supplementary material, Fig. S1). Possibly, methylation of CpG islands of *CYP2D6* and *CYP2E1* promoter might inhibit the gene expression by regulating and inactivating the gene transcription, and decreased the metabolism capability of rifampin in general, thereby indirectly caused rifampin-induced liver cell injury.

However, there are also some limitations of this system. As the targeted DNA methylation modification is depend on CRISPR/dCas9 system, sgRNAs should be carefully designed to avoid the potential off-target effect. Besides, this detection system is more sensitive to hepatocellular DILI than cholestatic DILI.

In summary, we constructed a functional DNA methylation detection system in hepatic cells and verified the correlation between DILI and hypermethylation of *CYP2D6* and *CYP2E1*. We hope our experimental platform will ultimately help clinicians improve the efficiency of medical treatments for individual patients on the basis of their genetic and epigenetic backgrounds.

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Author contributions YW and CZ conducted the experiments and drafted the paper. YG, WZ, and MW assisted in experiments and data analysis. CL helped to polish the paper. CH and SQ designed the study. All authors reviewed and approved the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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