

# The DR1-CSE/H<sub>2</sub>S system inhibits renal fibrosis by downregulating the ERK1/2 signaling pathway in diabetic mice

HONGZHU LI<sup>1,2\*</sup>, FENGQI SUN<sup>2\*</sup>, SHUZHAI BAI<sup>2\*</sup>, GUIQUAN CHANG<sup>2</sup>, REN WU<sup>2</sup>,  
YAXIN WEI<sup>2</sup>, XIN WEN<sup>2</sup>, YUXIN XI<sup>2</sup>, JINGHUI HAO<sup>2</sup> and ALTAANY ZAID<sup>3</sup>

<sup>1</sup>Department of Pathophysiology, School of Medicine, Xiamen University, Xiamen, Fujian 361100;

<sup>2</sup>Department of Pathophysiology, Harbin Medical University, Harbin, Heilongjiang 150081, P.R. China;

<sup>3</sup>Faculty of Medicine, Yarmouk University, Irbid 21163, Jordan

Received September 7, 2021; Accepted November 1, 2021

DOI: 10.3892/ijmm.2021.5062

**Abstract.** Glomerular mesangial cell (MC) proliferation and extracellular matrix deposition are the main pathological changes in diabetic nephropathy. Hydrogen sulfide (H<sub>2</sub>S) inhibits the proliferation of MCs. Dopamine 1 receptors (DR1) are expressed in MCs and serve important physiological roles. However, it is unclear whether DR1 activation inhibits MC proliferation by increasing endogenous H<sub>2</sub>S. The present study found that the production of H<sub>2</sub>S and the expression of DR1 and cystathionine-γ-lyase (CSE) were decreased in the renal tissues of diabetic mice and high glucose (HG)-induced MCs. SKF38393 (a DR1 agonist) increased the production of H<sub>2</sub>S and the expression of DR1 and CSE and NaHS (an exogenous H<sub>2</sub>S donor) only increased H<sub>2</sub>S production and CSE expression but not DR1 expression. HG increased the thickness of the glomerular basement membrane, cell viability and proliferation, the expression of cyclin D1, PCNA, collagen 1 and α-smooth muscle actin and the activity of phosphorylated ERK1/2 and decreased the expression of P21 and MMP9. SKF38393 and NaHS reversed the effects of HG. PPG (a CSE inhibitor) abolished the beneficial effects of SKF38393. The beneficial effects of SKF38393 were similar to those of PD98059 (an ERK1/2 inhibitor). Taken together, the findings suggested that the DR1-CSE/H<sub>2</sub>S pathway activation attenuated diabetic MC proliferation and extracellular matrix deposition by downregulating the ERK1/2 signaling pathway.

## Introduction

Diabetic nephropathy (DN) is a common and debilitating complication of diabetes (1). According to the United States renal data system, DN is the leading cause of end-stage renal disease and a major contributor to morbidity and mortality in patients with diabetes worldwide (2). Glomerular mesangial cell (MC) proliferation and extracellular matrix deposition are prominent features of DN (3).

Under physiological conditions, MCs are located between glomerular capillary loops and secrete extracellular matrix, produce cytokines, support the glomerular capillary plexus, phagocytize and clear macromolecular substances and contract (4). In the presence of hyperglycemia and other stimulating factors, MCs proliferate abnormally and secrete a large amount of extracellular matrix, which is deposited in the mesangial area (5). MCs can migrate to the gap between glomerular capillary endothelial cells and the basement membrane and even protrude into the surrounding capillary cavity, resulting in capillary occlusion and ultimately leading to glomerulosclerosis (6). MC proliferation serves a key role in the process of DN, but the mechanism of MC proliferation in diabetic patients is complex. It is generally believed that the kidneys of diabetic patients produce too much reactive oxygen species (ROS) due to stimulation of the mitochondrial respiratory chain by long-term hyperglycemia (7). Low levels of ROS serve roles in signal transduction and proliferation, which may be one of the important mechanisms of the abnormal proliferation of MCs. In addition, transforming growth factor β, connective tissue growth factor, angiotensin II and other cellular growth factors are also involved in the abnormal proliferation of MCs (8,9). Studies have also shown that a decrease in the level of hydrogen sulfide (H<sub>2</sub>S) serves an important role in the proliferation of MCs (10).

H<sub>2</sub>S has been traditionally viewed as a toxic gas. However, few individuals realize that H<sub>2</sub>S is also a biological gas that is endogenously produced by the cytosolic enzymes cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE), which use homocysteine and L-cysteine as substrates (11). H<sub>2</sub>S can not only regulate inflammation, oxygen sensing, cell growth and ageing but also protect a variety of organs from ischemia-reperfusion injury, serving an important

*Correspondence to:* Professor Hongzhu Li, Department of Pathophysiology, School of Medicine, Xiamen University, 4221 Xiang'an South Road, Xiang'an, Xiamen, Fujian 361100, P.R. China  
E-mail: lhz2020@xmu.edu.cn

\*Contributed equally

**Key words:** dopamine 1 receptors, hydrogen sulfide, glomerular mesangial cells, proliferation, diabetic nephropathy



biological role *in vivo* (12). There is abundant endogenous H<sub>2</sub>S in kidney tissues (13). H<sub>2</sub>S can increase the glomerular filtration rate, inhibit the reabsorption of sodium by the renal tubules and inhibit the proliferation of glomerular MCs (10,14).

Dopamine receptors (DRs) have seven transmembrane domains and belong to the G protein coupled receptor superfamily (15). According to their different effects on adenylate cyclase, DRs are divided into two subfamilies: DR1 (D1, D5) and DR2 (D2, D3, D4) (16). DR1 binds to Gs protein to activate adenylate cyclase and stimulate phospholipase C, resulting in an increase in the intracellular free calcium concentration [ $\text{Ca}^{2+}$ ]<sub>i</sub>; DR2 inhibits adenylate cyclase and calcium channels (17). DRs are mainly distributed in the central nervous system and in the cardiovascular system, kidneys and adrenal glands. DR1 is also expressed and serves an important role in glomerular MCs (18).

It has been reported that H<sub>2</sub>S can inhibit the proliferation of MCs (10). Yang *et al* (19) found that CSE is physiologically activated by calcium-calmodulin, which increases the production of endogenous H<sub>2</sub>S in vascular endothelial cells. Our research group reported that DR1 activation increases the  $\text{Ca}^{2+}$ <sub>i</sub> in myocardial ischemia-reperfusion injury (20). However, it is not clear whether DR1 activation inhibits MC proliferation by increasing endogenous H<sub>2</sub>S. To examine this possibility, the present study performed animal and cellular experiments and measured changes in related factors and signaling pathways.

## Materials and methods

**Materials and drugs.** Mouse renal MC lines (SV40-MES13) were purchased from Shanghai Cell Bank of Chinese Academy of Science. SKF38393 (DR1 agonist), sodium hydrogen sulfide (NaHS), PPG (a CSE inhibitor), PD98059 (an ERK1/2 inhibitor), streptozocin (STZ) and 7-Azido-4-Methylcoumarin (H<sub>2</sub>S probe) were purchased from Sigma-Aldrich (Merck KGaA). The primary antibodies for anti-CSE, cyclin D1, PCNA, P21, COL1,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and MMP9 were from ProteinTech Group, Inc. DR1 antibody was purchased from GeneTex, Inc. The total (t)-ERK1/2 and phosphorylated (p)-ERK1/2 antibodies were obtained from Cell Signaling Technology, Inc. The Cell Counting Kit-8 (CCK-8) kit and the primary antibody for  $\beta$ -actin were obtained from Wuhan Boster Biological Technology, Ltd. The EdU kit was obtained from Guangzhou RiboBio Co., Ltd. The BCA Protein Assay kit and Enhanced Chemiluminescence (ECL) reagent were purchased from Beyotime Institute of Biotechnology.

**Animal use and experimental design.** At total of 48 C57BL/6J (male:female 50:50; 6-weeks-old; 20-22 g) mice were provided by the Experimental Animal Center of Second Affiliated Hospital in Harbin Medical University. All experiments were approved by the Animal Care Committee of Harbin Medical University for the use of experimental animals (approval no. SCXK2013-001). All animals were given free access to normal chow and water and were housed in cages at room temperature with 40-70% humidity and a fixed 12-h light/dark cycle.

After animal adaptation to the environment for 1 week, male and female C57BL/6J (6-weeks-old, 20-22g) mice

were made diabetic by a single injection of streptozotocin (STZ; 150 mg/kg, intraperitoneally). The STZ was dissolved in 100  $\mu$ M citrate buffer (citric acid: sodium citrate=1:1.32). After 3 days, the mice with glucose levels >16.67 mmol/l were considered hyperglycemic (diabetic) (21). The daily water intake and food intake of these diabetic mice were simultaneously recorded and the weekly weight change monitored. At the same time, blood glucose was monitored weekly to eliminate mice whose blood glucose had returned to <16.67 mmol/l.

Briefly, the experimental groups were as follows (n=8): i) Control group (Control); the mice were injected with the same volume of physiological saline solution daily. ii) Diabetes group (T1D); the diabetic mice were injected with the same volume of physiological saline solution daily. iii) Diabetes + SKF38393 group (T1D + SKF38393); the diabetic mice were injected with SKF38393 (50  $\mu$ g/kg) daily. iv) Diabetes + NaHS group (T1D + NaHS); the diabetic mice were injected with NaHS (100  $\mu$ M/kg) daily. According to the results of pre-experiment and published papers, a dose of 100  $\mu$ M/kg/day NaHS was used in present study (11,22). All drugs were dissolved in physiological saline solution and all the above mice were injected intraperitoneally. After the treatment reached 4, 8 and 12 weeks, the mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (60 mg/kg) and then the chest was opened and the kidneys, hearts, livers and arteries of mice were removed for related experimental research.

**Renal morphology assessment.** Kidneys were dehydrated in a series of alcohols and then embedded. Tissue sections from paraffin-embedded kidneys with a 4  $\mu$ m thickness were stained with hematoxylin and eosin and Masson's trichrome at room temperature for 3 min following deparaffinization. Staining was conducted to assess renal morphology and collagen accumulation to estimate the progression of diabetic nephropathy. Images of random fields under a microscope (Olympus Corporation) were captured by an individual blinded to the grouping.

**Transmission electron microscopy.** Renal tissues (1 mm<sup>3</sup>) were immersed immediately in fixative (2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate, pH 7.2). Following 2-3 days of storage at 4°C, specimens were rinsed in PBS, postfixed in cacodylate-buffered 1% osmium tetroxide, dehydrated in an ethanol series, and embedded in polybed 812. Ultra-thin (90 nm) sections were made with microtome, and ultrastructural changes of glomerular basement membrane were observed under an electron microscope. The thickness of glomerular basement membrane was quantified using ImageJ software (National Institutes of Health; version 1.48).

**Measurement of H<sub>2</sub>S levels in the kidney.** H<sub>2</sub>S production rate was measured as described previously (23). In brief, following different treatments, the kidney tissues were collected and homogenized in 50 mM ice-cold potassium phosphate buffer (pH 6.8). The flasks containing the reaction mixture (100 mM potassium phosphate buffer, 10 mM l-cysteine, 2 mM pyridoxal 5-phosphate and 10% cell homogenates) and center wells containing 0.5 ml 1% zinc acetate and a piece of filter paper (2x2.5 cm) were flushed with N<sub>2</sub> gas and incubated at



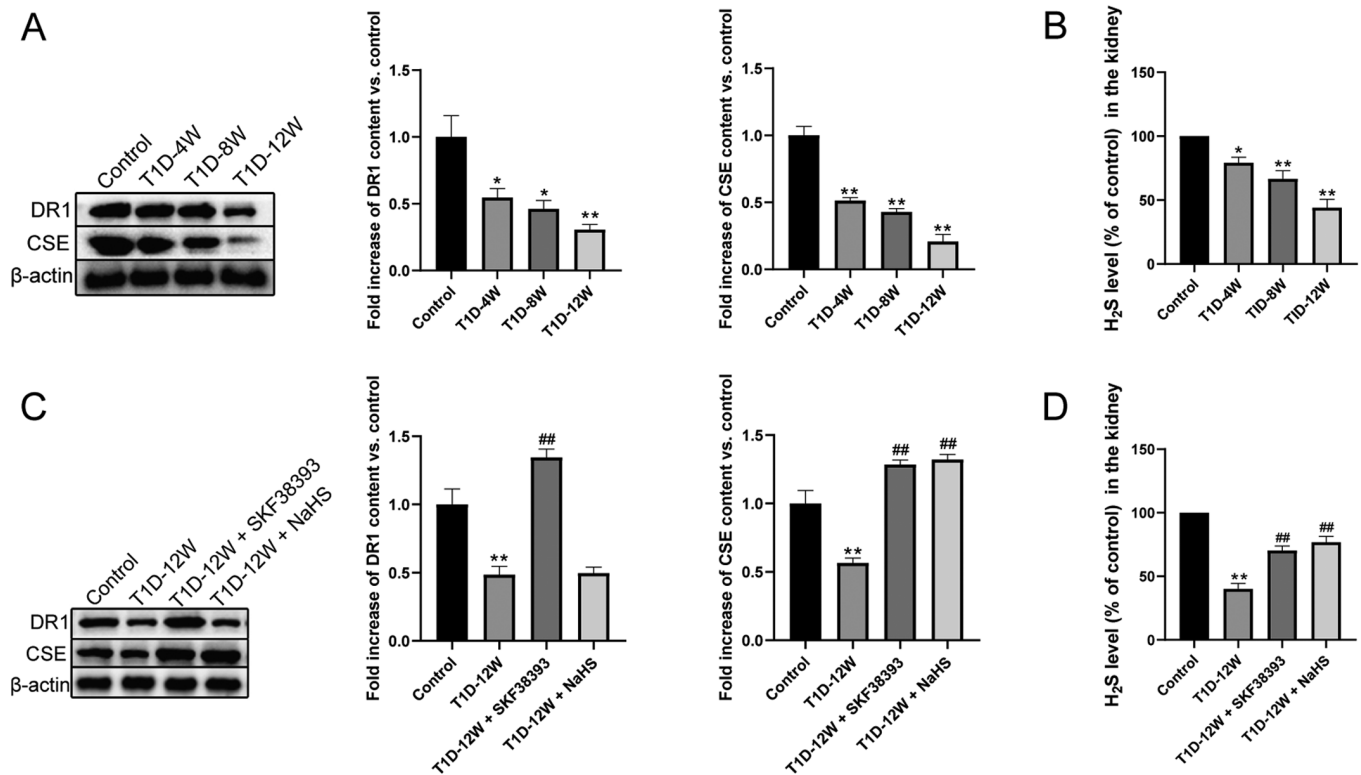


Figure 1. Activation of DR1 upregulates the CSE/H<sub>2</sub>S pathway in the kidneys of T1D mice. (A and C) The expression of DR1 and CSE in the kidneys was determined by western blotting (n=3). The intensity of each band was quantified by densitometry and data was normalized to the  $\beta$ -actin signal. (B and D) H<sub>2</sub>S levels were detected by ultraviolet spectrophotometer in the kidneys (n=6). The results were expressed as the mean  $\pm$  standard error of the mean. \*P<0.05, \*\*P<0.01 vs. control group; ##P<0.01 vs. T1D-12 W group. DR1, dopamine 1 receptors; CSE, cystathionine- $\gamma$ -lyase; T1D, diabetes group; W, week.

37°C for 90 min. The reaction was stopped by adding 0.5 ml of 50% trichloroacetic acid and the flasks were incubated at 37°C for another 60 min. The contents of the center wells were transferred to test tubes, each containing 3.5 ml of water. Then 0.5 ml of 20 mM N, N-dimethyl-p-phenylenediamine sulfate in 7.2 M HCl and 0.5 ml 30 mM FeCl<sub>3</sub> in 1.2 M HCl were added. The absorbance of the resulting solution at 670 nm was measured 20 min later with an ultraviolet spectrophotometer.

**Cell culture and treatment.** The SV40-MES13 were cultured in DMEM/F-12 medium (3:1) (cat. no. PM150323; Procell Life Science & Technology Co., Ltd., and cat. no. 31600; Beijing Solarbio Science & Technology Co., Ltd.) supplemented with 14 mM HEPES, 5% fetal bovine serum, 1% penicillin and streptomycin in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. For the following experiments, the SV40-MES13 were incubated with serum-free DMEM/F-12 for 12-16 h (the cells reached 70-80% confluence) and then the cells were randomly divided into six groups: i) normal-glucose control group (Control); incubated in DMEM/F-12 medium containing 5.6 mM glucose. ii) high glucose group (HG); incubated in DMEM/F-12 medium containing 30 mM glucose for 48 h. iii) SKF38393 treated group (HG + SKF38393); 10  $\mu$ M SKF38393 was added to the medium 1 h before HG induction. iv) NaHS treated group (HG + NaHS); 100  $\mu$ M NaHS was added to the medium 1 h before HG condition. v) PPG and SKF38393 treatment group (HG + PPG + SKF38393); 2 mM PPG and 10  $\mu$ M SKF38393 were added to the medium 1 h before HG condition. vi) PD98059 treatment group (HG + PD98059); 10  $\mu$ M

PD98059 (an ERK1/2 inhibitor) was added to the medium 1 h before HG condition.

**Cell viability assay.** Cell viability was measured by Cell Counting Kit-8. Cells were seeded in 96-well plates at a concentration of  $1 \times 10^3$  cells/well. Prior to different treatments, the cells were serum starved for 12 h. Subsequently, they were incubated in 10  $\mu$ l of CCK-8 reagent at 37°C for 1 h. A microplate reader was used to determine the optical density at 450 nm.

**Measurement of cell proliferation.** 5-Ethynyl-20-Deoxyuridine (EdU) incorporation assay was performed with the Cell-Light EdU *In Vitro* Imaging kit to detect the proliferation rates of SV40-MES13 (21). Briefly, cells subjected to different treatment were incubated with 20 mM EdU in complete growth medium at 37°C for 2 h before fixation in 4% paraformaldehyde at room temperature for 30 min. After EdU staining, cell nuclei were stained with Hoechst 33342 at room temperature for 30 min in a darkroom and visualized under fluorescence microscopy (magnification, x200). The percentage of EdU-positive cells was calculated from six random fields in three wells.

**Detection of H<sub>2</sub>S by 7-Azido-4-Methylcoumarin.** The fluorescence response of H<sub>2</sub>S in MCs was detected using 7-Azido-4-Methylcoumarin (C-7Az; Sigma-Aldrich; Merck KGaA), as described previously (24,25). MCs were incubated with 1 ml PBS (containing 12  $\mu$ l C-7Az) for 30 min at room temperature and then the cells were washed with PBS.



The fluorescence activation response of C-7Az to H<sub>2</sub>S in MCs were visualized using a microscope (Olympus Corporation) and six fields (magnification, x200) were randomly selected for statistical analysis.

**Western blotting analysis.** Western blotting assay was conducted as described previously (12). In brief, cells were lysed and extracted total protein with RIPA lysis buffer pre-mixed with 1 mM PMSF. The protein concentration in each sample was determined using the BCA Protein Assay kit. In each western blot analysis, the same amount of total protein (25–50  $\mu$ g) from each group was separated by 8–12% SDS-PAGE and electro-transferred to PVDF membranes. The membranes were blocked for 1.5 h at room temperature in TBST (Tris-buffered saline with 0.05% Tween-20, pH 7.4) plus 5% non-fat milk and incubated overnight at 4°C with diluted primary antibodies against DR1 (cat. no. GTX100354, 1:1,000; Gene Tex, Inc.), CSE (cat. no. 12217-1-AP, 1:1,000; ProteinTech Group, Inc.), cyclin D1 (cat. no. 60186-1-Ig, 1:5,000; ProteinTech Group, Inc.), PCNA (cat. no. 10205-2-AP, 1:5,000; ProteinTech Group, Inc.), P21 (cat. no. 27296-1-AP, 1:500; ProteinTech Group, Inc.),  $\alpha$ -SMA (cat. no. 14395-1-AP, 1:1,000; ProteinTech Group, Inc.), COL1 (cat. no. 14695-1-AP, 1:1,000; ProteinTech Group, Inc.), MMP9 (cat. no. 10375-2-AP, 1:1,000; ProteinTech Group, Inc.), total (t)-ERK1/2 (cat. no. 4695, 1:5,000; Cell Signaling Technology, Inc.), phosphorylated (p)-ERK1/2 (cat. no. 4370, 1:5,000; Cell Signaling Technology, Inc.), or  $\beta$ -actin (cat. no. BM0627, 1:1,000; Boster Biological Technology, Ltd.). Secondary goat anti-rabbit antibody and goat anti-mouse antibody were used respectively (diluted 1:5,000, cat. no. BA1054 and cat. no. BA1050; Boster Biological Technology, Ltd.). The secondary antibodies were incubated for 1.5 h at room temperature (25°C). The signals were detected by an ECL kit and a multiplex fluorescent imaging system (ProteinSimple). The integrated optical density of the analyzed bands on the film was quantified using ImageJ software (National Institutes of Health; version 1.48). The levels of analyzed proteins were normalized to the internal control ( $\beta$ -actin).

**Statistical analysis.** All data are expressed as the mean  $\pm$  standard error of the mean and represent at least three independent experiments. Statistical comparisons were made using one-way ANOVA followed by a post hoc analysis (Tukey test) where applicable.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**DR1 activation upregulates the CSE/H<sub>2</sub>S pathway in the kidneys of T1D mice.** As shown in Fig. 1, hyperglycemia down-regulated DR1 and CSE expression and H<sub>2</sub>S production in the T1D-4 W, T1D-8 W and T1D-12 W groups compared with the control group. Compared with that in the control group, this effect was most evident in the T1D-12 W group. Compared with the T1D-12 W group, SKF38393 (DR1 agonist) treatment significantly increased the expression of DR1 and CSE and the production of H<sub>2</sub>S. NaHS (an exogenous H<sub>2</sub>S donor) treatment only increased the expression of CSE and the production of H<sub>2</sub>S but had no significant effect on the expression of DR1.

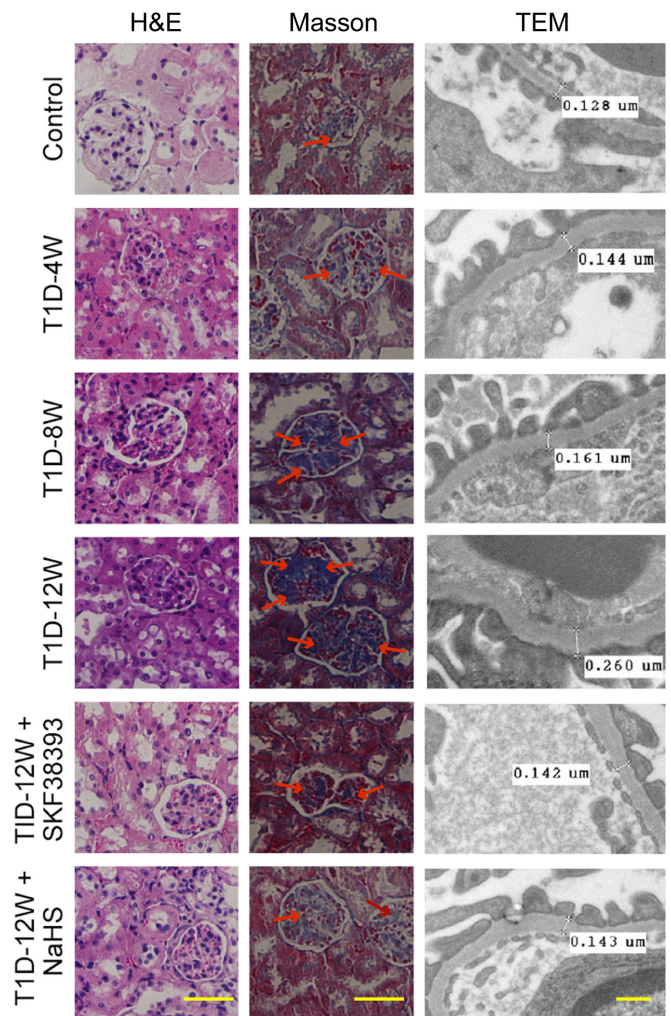


Figure 2. DR1 activation inhibits cell proliferation and collagen deposition by upregulates the CSE/H<sub>2</sub>S pathway in the kidneys of T1D mice. Morphology of kidney by hematoxylin and eosin staining (magnification, x400; scale bars, 100  $\mu$ m), collagen deposition by Masson staining (magnification, x400; scale bars, 100  $\mu$ m, the blue stain by the arrow indicated is collagens) and thickness of renal basement membrane by transmission electron microscope (magnification, x20,000; scale bars, 2  $\mu$ m). Renal injury was aggravated, including glomerular hypertrophy, collagen deposition in interstitial kidney tissue and the thickness of the glomerular basement membrane were increased in the T1D-4 W, T1D-8 W and T1D-12 W groups. These changes were most significant in the T1D-12 W group. Pretreatment with SKF38393 or NaHS significantly reduced these pathological changes in the T1D-12 W group. All data were from at least 4 independent experiments. DR1, dopamine 1 receptors; CSE, cystathionine- $\gamma$ -lyase; T1D, diabetes group; W, week.

**DR1 activation inhibits cell proliferation and collagen deposition by upregulating the CSE/H<sub>2</sub>S pathway in the kidneys of T1D mice.** The data showed that compared with those in the control group, renal injury was aggravated, including glomerular hypertrophy, collagen deposition in interstitial kidney tissue and the thickness of the glomerular basement membrane were increased in the T1D-4 W, T1D-8 W and T1D-12 W groups. These changes were most significant in the T1D-12 W group. Pretreatment with SKF38393 or NaHS significantly reduced these pathological changes in the T1D-12 W group (Fig. 2).

**DR1 activation increases the CSE/H<sub>2</sub>S pathway in HG-induced MCs.** As shown in Fig. 3, the levels of DR1, CSE and H<sub>2</sub>S



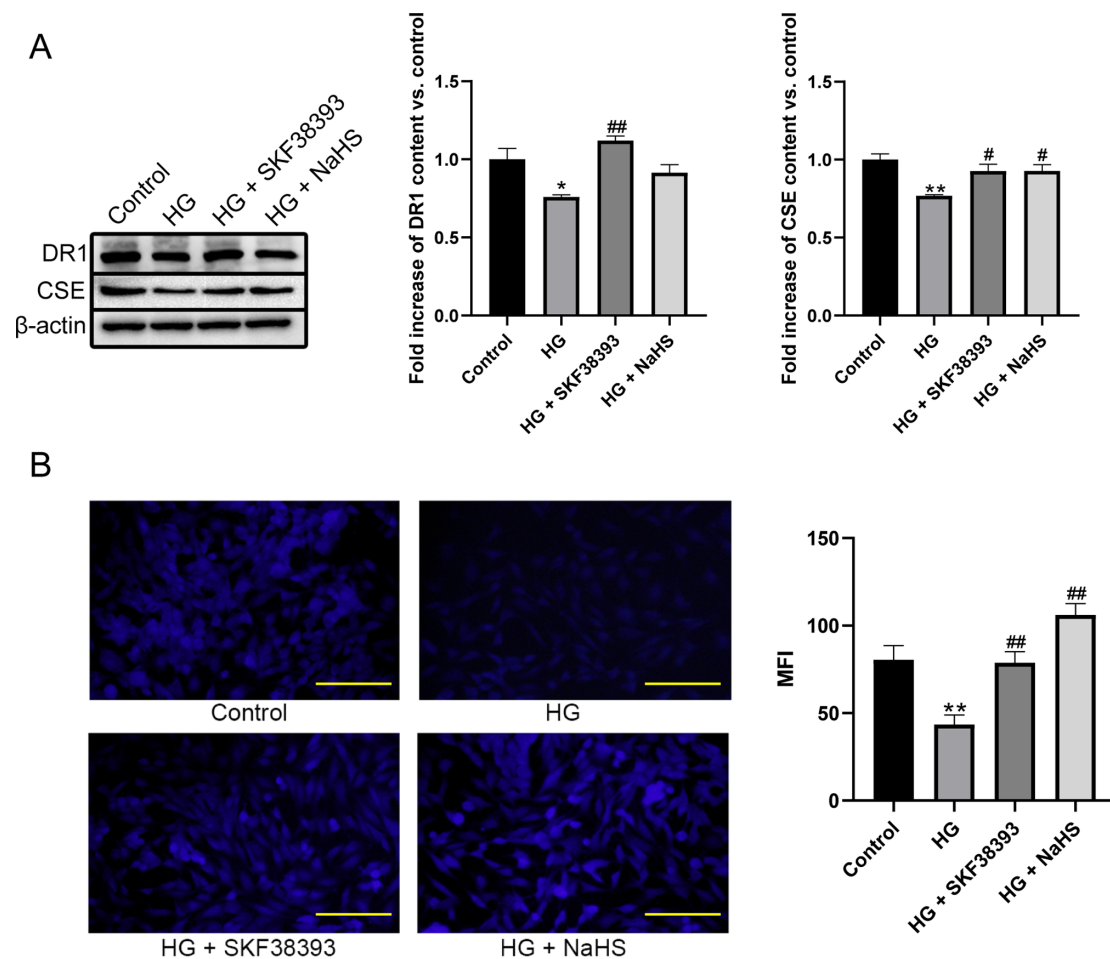


Figure 3. DR1 activation increases the CSE/H<sub>2</sub>S pathway in the HG-induced MCs. (A) The expression of DR1 and CSE in the HG-induced MCs was determined by western blotting (n=3). The intensity of each band was quantified by densitometry and data was normalized to the  $\beta$ -actin signal. (B) H<sub>2</sub>S levels were detected by fluorescence microscopy in the HG-induced MCs (n=6, magnification, x200; scale bars, 200  $\mu$ m). The results were expressed as the mean  $\pm$  standard error of the mean. \*P<0.05, \*\*P<0.01 vs. control group; #P<0.05, ##P<0.01 vs. HG group. DR1, dopamine 1 receptors; CSE, cystathionine- $\gamma$ -lyase; HG, high glucose; MC, mesangial cell.

were lower in the HG group compared with the control group. Compared with those in the HG group, the levels of DR1, CSE and H<sub>2</sub>S were significantly increased in cells treated with HG + SKF38393. Treatment with HG + NaHS only increased the levels of CSE and H<sub>2</sub>S but had no significant effect on DR1 levels. These results were consistent with the results of the animal experiments.

**Activation of the DR1-CSE/H<sub>2</sub>S pathway attenuates HG-induced MC proliferation.** Cell viability, the proliferation rate and the expression of cyclin D1 and PCNA were increased and the expression of P21 was decreased in the HG group compared with the control group. Compared with the HG group, HG + SKF38393 and HG + NaHS reduced cell viability, the proliferation rate and the expression of cyclin D1 and PCNA and increased the expression of P21. PPG (an inhibitor of CSE) abrogated the inhibitory effect of SKF38393 on MC proliferation. In addition, the effect of HG + SKF38393 on these indicators was similar to that of HG + PD98059 (an ERK1/2 inhibitor; Fig. 4).

**Activation of the DR1-CSE/H<sub>2</sub>S pathway inhibits HG-induced collagen deposition in MCs.**  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and collagen 1 (COL1) expression was significantly upregulated

and MMP9 expression was downregulated in the HG group compared with the control group. Compared with that in the HG group, the expression of  $\alpha$ -SMA and COL1 was markedly decreased and the expression of MMP9 was significantly increased in the HG + SKF38393 and HG + NaHS groups. These beneficial effects of SKF38393 were blocked by PPG. The effect of SKF38393 on collagen deposition was similar to that of PD98059 (Fig. 5).

**Activation of the DR1-CSE/H<sub>2</sub>S pathway downregulates the ERK1/2 pathway in HG-induced MCs.** The activity of p-ERK1/2 was significantly higher in the HG group than in the control group. HG + SKF38393 or HG + NaHS significantly decreased p-ERK1/2 activity and similar suppression was observed in the HG + PD98059 group. The inhibitory effect of SKF38393 on p-ERK1/2 activity was abolished by PPG. The activity of t-ERK1/2 remained unchanged in different treatments (Fig. 6).

## Discussion

Diabetic nephropathy is one of the leading causes of mortality in individuals with diabetes and is characterized by diffuse or nodular glomerulosclerosis (26). MC proliferation and



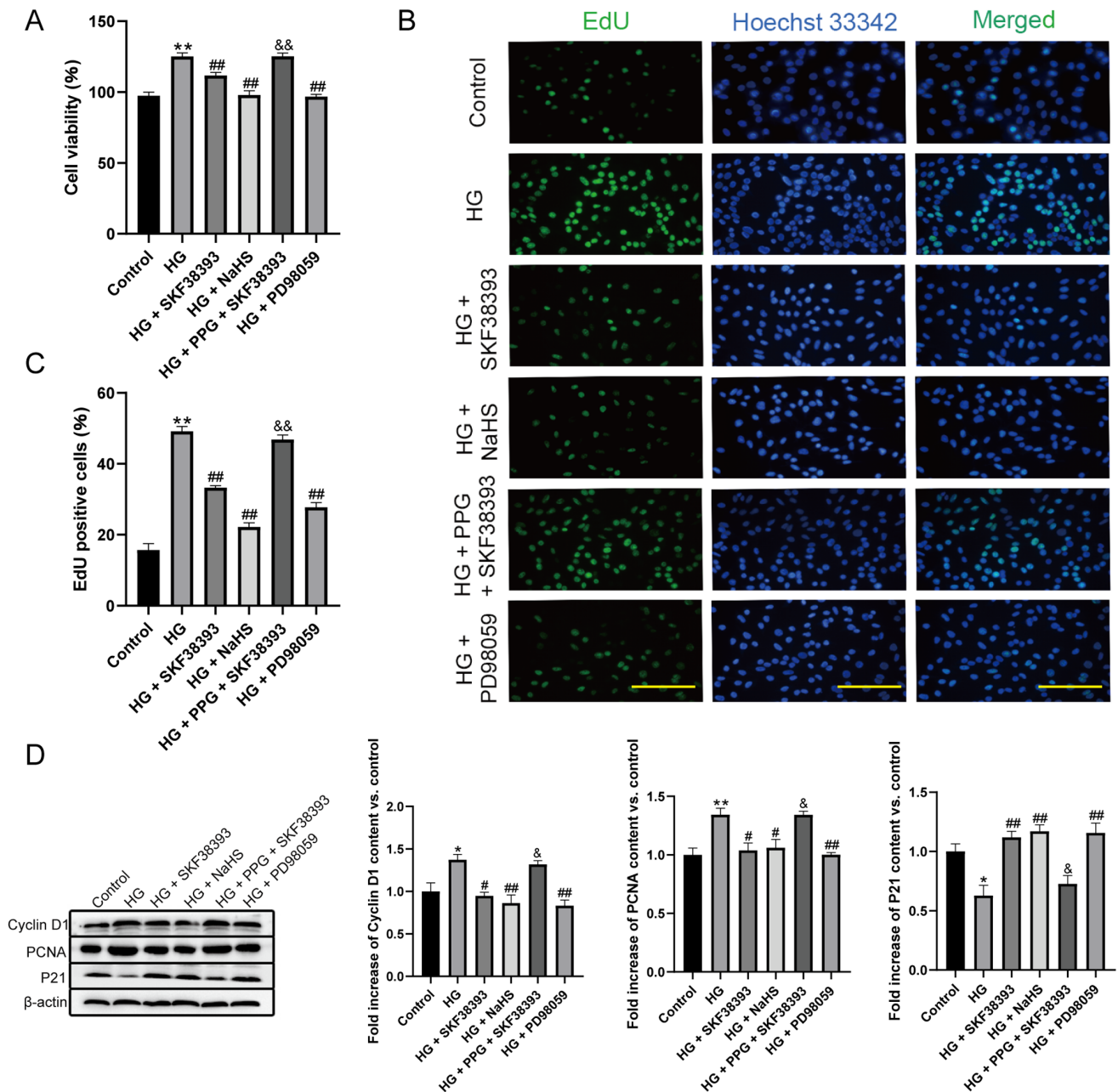


Figure 4. Activation of the DR1-CSE/H<sub>2</sub>S pathway attenuates HG-stimulated MC proliferation. (A) Cell viability was measured by CCK-8 assay (n=8). (B) Cell proliferation rate was measured by EdU assay (n=6, magnification, x200; scale bars, 200  $\mu$ m). (C) Quantify the results of (B) with a histogram (n=6). (D) The expression of cyclin D1, PCNA and P21 in the HG-induced MCs was determined by western blotting (n=3). The results were expressed as the mean  $\pm$  standard error of the mean. \*P<0.05, \*\*P<0.01 vs. control group; #P<0.05, ##P<0.01 vs. HG group; &P<0.05, &&P<0.01 vs. HG + SKF38393 group. DR1, dopamine 1 receptors; CSE, cystathionine- $\gamma$ -lyase; HG, high glucose; MC, mesangial cell.

extracellular matrix deposition are the main causes of glomerulosclerosis (3). Proliferating MCs secrete a large amount of extracellular matrix, resulting in glomerular basement membrane thickening and mesangial expansion (27). Inhibiting the abnormal proliferation of MCs is critical for diabetic patients. The results of the present study showed that the level of H<sub>2</sub>S and the expression of DR1 and CSE were decreased *in vivo* and *in vitro* (Figs. 1 and 3). This finding suggests that the proliferation of MCs is associated with a decrease in DR1 expression and the downregulation of the CSE/H<sub>2</sub>S pathway. To test this hypothesis and examine the relationship between these factors, SKF38393 (a DR1 agonist) and NaHS (an exogenous H<sub>2</sub>S donor) were administered. Our

previous research showed that SKF38393 could increase the expression and activity of DR1 (20). It has been reported that 120  $\mu$ M exogenous H<sub>2</sub>S increased the transcription and expression of CSE, while at concentrations >160  $\mu$ M, the transcription and expression of CSE were completely inhibited, suggesting that higher levels of H<sub>2</sub>S may be toxic (28,29). The experimental results of the present study showed that SKF38393 increased the expression of DR1 and CSE/H<sub>2</sub>S pathway factors, but NaHS only increased CSE/H<sub>2</sub>S pathway factors and did not affect the expression of DR1. In addition, it was also found that PPG (a CSE inhibitor) abolished SKF38393-mediated enhancement of the CSE/H<sub>2</sub>S pathway in the pre-experiment (data not shown).



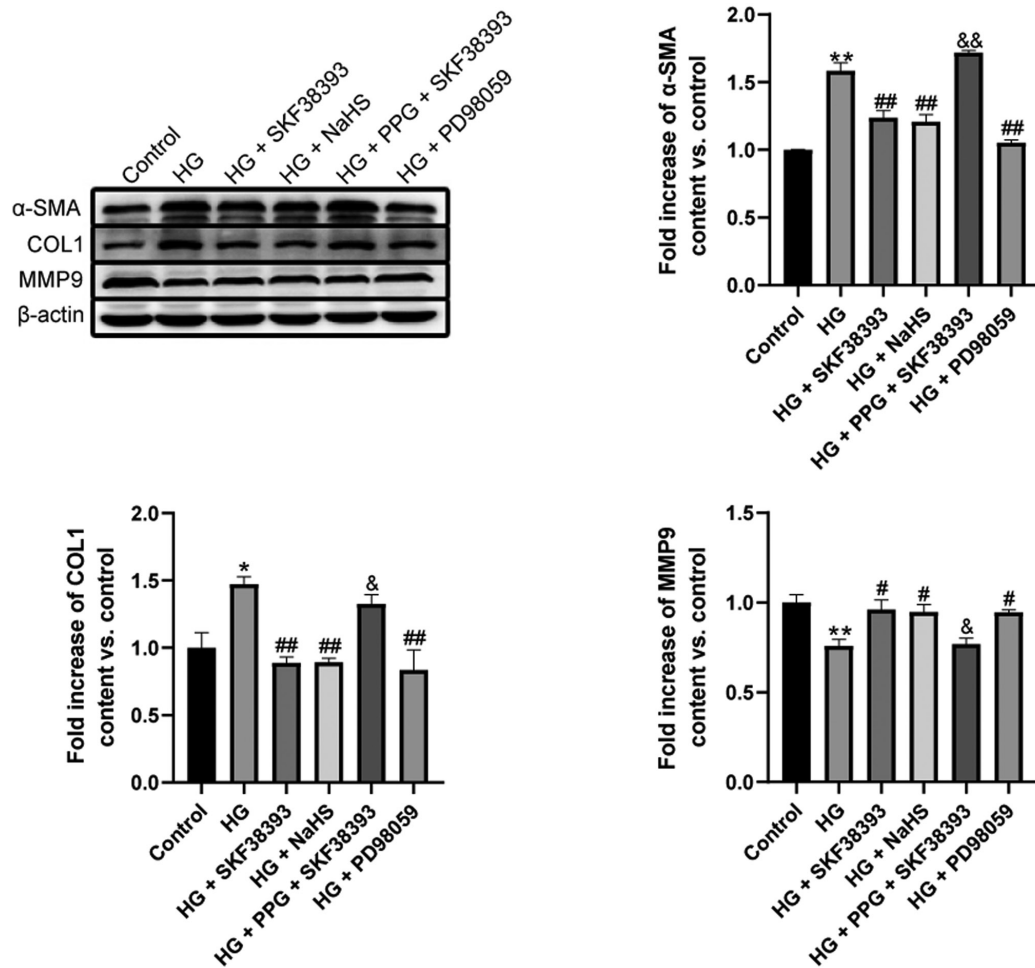


Figure 5. Activation of the DR1-CSE/H<sub>2</sub>S pathway inhibits HG-induced collagen deposition in MCs. The expression of COL1, α-SMA and MMP9 in the HG-induced MCs was determined by western blotting. The experiments were repeated at least three times. The results were expressed as the mean ± standard error of the mean. \*P<0.05, \*\*P<0.01 vs. control group; #P<0.05, ##P<0.01 vs. HG group; &P<0.05, &&P<0.01 vs. HG + SKF38393 group. DR1, dopamine 1 receptors; CSE, cystathionine-γ-lyase; HG, high glucose; MC, mesangial cell; COL1, collagen 1; α-SMA, α-smooth muscle actin.

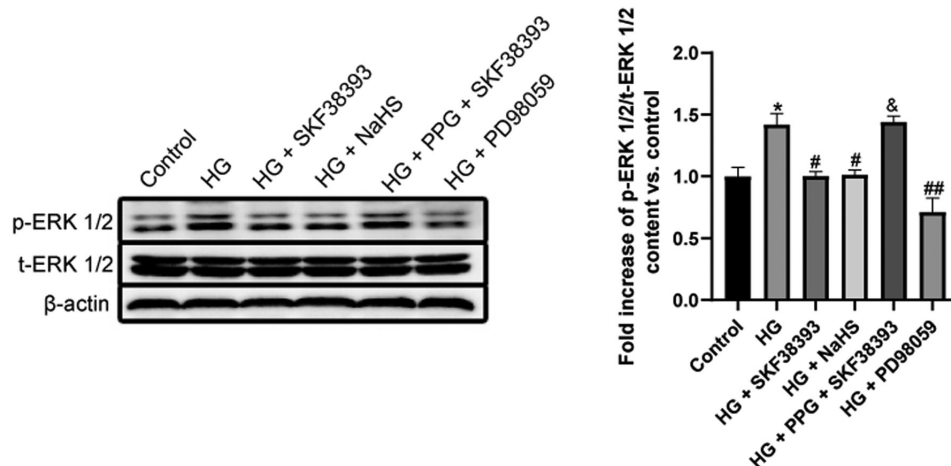


Figure 6. Activation of the DR1-CSE/H<sub>2</sub>S pathway downregulates ERK1/2 pathway in HG-induced MCs. The activity of p-ERK1/2 and t-ERK1/2 was detected through western blotting from different groups in HG-induced MCs. PD98059 is an ERK1/2 inhibitor. The intensity of each phosphorylated band was quantified by densitometry and data was normalized to the corresponding total band signal. The experiments were repeated at least three times. The results were expressed as the mean ± standard error of the mean. \*P<0.05 vs. control group; #P<0.05, ##P<0.01 vs. HG group; &P<0.05 vs. HG + SKF38393 group. DR1, dopamine 1 receptors; CSE, cystathionine-γ-lyase; HG, high glucose; MC, mesangial cell; p-, phosphorylated; t-, total;

These results indicated that DR1 activation inhibited the proliferation of MCs by upregulating the CSE/H<sub>2</sub>S pathway

and that DR1 is an upstream regulatory factor of the CSE/H<sub>2</sub>S pathway (Figs. 1 and 3).



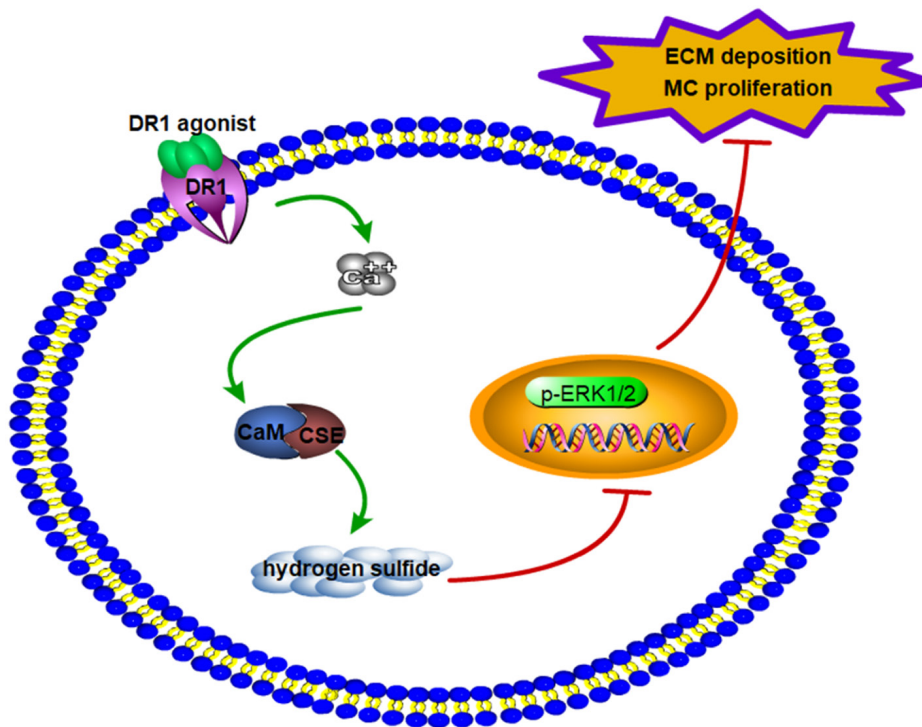


Figure 7. The mechanism of DR1 activation inhibiting ECM deposition and MC proliferation. DR1 activation increases  $(Ca^{2+})_i$  and then enhances interaction of CaM and CSE and promotes generation of endogenous H<sub>2</sub>S, which inhibits MC proliferation and ECM deposition by downregulating ERK1/2 signaling pathway. DR1, dopamine 1 receptors; ECM, extracellular matrix; MC, mesangial cell; CaM, calmodulin; CSE, cystathionine-γ-lyase; p-, phosphorylated.

How can DR1 regulate the CSE/H<sub>2</sub>S pathway to inhibit MC proliferation? DR1, with seven transmembrane domains, belongs to the G protein-coupled receptor superfamily, which can increase  $(Ca^{2+})_i$  (17). A previous study by our research group has also shown that DR1 activation promotes myocardial ischemia-reperfusion injury by increasing  $(Ca^{2+})_i$  (20). It has been reported that an increase in  $(Ca^{2+})_i$  can activate calmodulin (CaM), upregulate the expression of CSE and promote the production of endogenous H<sub>2</sub>S (19). Therefore, DR1 increases endogenous H<sub>2</sub>S production by increasing  $(Ca^{2+})_i$ , which inhibits MC proliferation.

The cell cycle is precisely controlled by cell cycle regulatory proteins. Cell cycle regulatory proteins can be divided into positive and negative regulatory proteins according to their effects on the cell cycle. Positive regulatory proteins include cyclins and cyclin-dependent kinases; the negative regulatory proteins are mainly cyclin-dependent kinase inhibitors (30). Cyclins bind with CDK to form cyclin/CDK active complexes, which regulate cell cycle initiation and phase transition. CKIs include the INK4 family and CIP/Kip family. The former includes p16<sup>INK4a</sup>, p15<sup>INK4B</sup>, p18<sup>INK4C</sup> and p19<sup>INK4D</sup>, which can inhibit the activity of cyclin/CDK complexes in G1 and S phases; the latter includes p21<sup>WAF1/CIP1</sup>, p27<sup>kip1</sup> and p57<sup>kip2</sup>, which inhibit most cyclin/CDK complexes (31-33). Proliferation-related proteins were measured to explore what caused the inhibition of MC proliferation. The data showed that HG increased the thickness of the glomerular basement membrane, cell viability, proliferation and cyclin D1 and PCNA expression and decreased P21 expression. SKF38393 reduced the thickness of the glomerular basement membrane, cell viability, proliferation and cyclin D1 and PCNA expression and increased P21 expression. This was the same as

the effect of directly using NaHS to supplement exogenous H<sub>2</sub>S in MCs. PPG (a CSE inhibitor) abolished the effect of SKF38393 (Figs. 2 and 4). These results suggested that the DR1-CSE/H<sub>2</sub>S pathway suppresses cell proliferation by affecting the expression of cell proliferation-related proteins (cyclin D1, PCNA and P21).

Renal fibrosis is the common pathway of chronic kidney disease that leads to end-stage renal failure (26). The deposition of extracellular matrix is the main cause of renal fibrosis. Collagen is the most abundant protein in the extracellular matrix and provides structural support to cells (34). α-SMA is an important marker of the phenotypic transformation of MCs. Positive α-SMA expression in diabetic glomeruli is a sign of impaired MC activation (35). Under physiological conditions, MCs have a contractile phenotype, maintaining a balance between the synthesis and degradation of mesangial matrix (36). However, under continuous hyperglycemia, MCs transform to a synthetic phenotype, secrete a large amount of mesangial matrix such as COL1 to resist traumatic stimulation and reduce the expression of MMP9 (37). To examine the protective effect of the DR1-CSE/H<sub>2</sub>S pathway on renal fibrosis, changes in the relevant indicators (COL1, α-SMA and MMP9) of renal fibrosis were measured and it was found that HG increased the expression of COL1 and α-SMA and decreased the expression of MMP9. SKF38393 and NaHS reduced COL1 and α-SMA expression and increased the expression of MMP9. PPG abrogated the effect of SKF38393 (Fig. 5). The results of the present study demonstrated that the DR1-CSE/H<sub>2</sub>S pathway improved renal fibrosis by inhibiting the deposition of collagen and increasing the degradation of extracellular matrix. MMP2 and membrane-type matrix metalloproteinase-1 (MT1-MMP)



are also fibrosis markers of diabetic nephropathy (34-37) and these two indicators will be detected in future studies.

A number of signaling pathways can regulate the proliferation of MCs, such as ERK1/2, TGF- $\beta$ , Wnt/ $\beta$ -Catenin and PI3K-AKT (38-40). ERK1/2 is a major member of the MAPK family that is involved in a number of physiological processes, such as gene expression, mitosis, cell metabolism, survival, apoptosis and differentiation (41). The ERK1/2 pathway activates the cyclin D1 gene, which leads to cell growth, proliferation and differentiation (42). The ERK1/2 pathway becomes activated in MCs cultured under HG conditions (38). The results of the present study showed that HG increased the activity of p-ERK1/2. SKF38393 and that NaHS reduced the activity of p-ERK1/2. PPG reversed the effect of SKF38393 (Fig. 6). The effect of SKF38393 on cell viability, proliferation, proliferation-associated protein expression, fibrosis-associated protein expression and p-ERK1/2 activity was similar to that of PD98059 (Figs. 1-6). This finding suggested that the DR1-CSE/H<sub>2</sub>S pathway inhibited MC dysfunction by inhibiting ERK1/2 activation. However, it remains to be explored whether the DR1-CSE/H<sub>2</sub>S pathway also affects other signaling pathways, such as Wnt/ $\beta$ -Catenin, PI3K-AKT, TGF- $\beta$  and others.

Notably, the roles of SKF38393 (a DR1 agonist), NaHS (an exogenous H<sub>2</sub>S donor) and PD98059 (an ERK1/2 inhibitor) in MC proliferation were similar, suggesting that these factors inhibit MC proliferation by regulating the ERK1/2 pathway. Whether there are other mechanisms involved will be explored in the future.

In conclusion (Fig. 7), the findings of the present study suggested that: i) MC proliferation and extracellular matrix deposition were associated with decreased DR1 expression and the CSE/H<sub>2</sub>S pathway. ii) DR1 activation upregulated the CSE/H<sub>2</sub>S pathway in HG-induced kidney tissues and MCs. iii) The DR1-CSE/H<sub>2</sub>S pathway inhibited MC proliferation and extracellular matrix deposition by downregulating the ERK1/2 signaling pathway. The present study explored the potential protective mechanism of the DR1-CSE/H<sub>2</sub>S pathway in diabetic nephropathy and provided a new therapeutic target for antifibrosis strategies against diabetic nephropathy.

## Acknowledgements

Not applicable.

## Funding

The present study was supported by the National Natural Science Foundation of China (grant nos. 81770486 and 81200160).

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

HL conceived and designed the research. FS conducted cell culture. FS and GC performed animal breeding. RW, YW, JH

and SB provided technical support. YX, XW and FS conducted sample processing and sample extraction. AZ analyzed and interpreted the data. FS and SB wrote and revised the manuscript. HL and FS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

All animal experiments were approved by the Animal Care Committee of Harbin Medical University for the use of experimental animals (approval no. SCXK2013-001).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References

- Adeshara KA, Diwan AG and Tupe RS: Diabetes and complications: Cellular signaling pathways, current understanding and targeted therapies. *Curr Drug Targets* 17: 1309-1328, 2016.
- Yuan CM, Nee R, Ceckowski KA, Knight KR and Abbott KC: Diabetic nephropathy as the cause of end-stage kidney disease reported on the medical evidence form CMS2728 at a single center. *Clin Kidney J* 10: 257-262, 2017.
- Chen B, Li Y, Liu Y and Xu Z: circLRP6 regulates high glucose-induced proliferation, oxidative stress, ECM accumulation, and inflammation in mesangial cells. *J Cell Physiol* 234: 21249-21259, 2019.
- Marciano DK: Mesangial Cells: The tuft guys of glomerular development. *J Am Soc Nephrol* 30: 1551-1553, 2019.
- Abboud HE: Mesangial cell biology. *Exp Cell Res* 318: 979-985, 2012.
- Li J, Zhao Q, Jin X, Li Y and Song J: Silencing of lncRNA PVT1 inhibits the proliferation, migration and fibrosis of high glucose-induced mouse mesangial cells via targeting microRNA-93-5p. *Biosci Rep* 40: 40, 2020.
- Zhang L, Liu J, Zhou F, Wang W and Chen N: PGC-1 $\alpha$  ameliorates kidney fibrosis in mice with diabetic kidney disease through an antioxidative mechanism. *Mol Med Rep* 17: 4490-4498, 2018.
- Zhao JH: Mesangial cells and renal fibrosis. *Adv Exp Med Biol* 1165: 165-194, 2019.
- Buhl EM, Djurdjaj S, Klinkhammer BM, Ermert K, Puelles VG, Lindenmeyer MT, Cohen CD, He C, Borkham-Kamphorst E, Weiskirchen R, *et al*: Dysregulated mesenchymal PDGFR- $\beta$  drives kidney fibrosis. *EMBO Mol Med* 12: e11021, 2020.
- Yuan P, Xue H, Zhou L, Qu L, Li C, Wang Z, Ni J, Yu C, Yao T, Huang Y, *et al*: Rescue of mesangial cells from high glucose-induced over-proliferation and extracellular matrix secretion by hydrogen sulfide. *Nephrol Dial Transplant* 26: 2119-2126, 2011.
- Wang R: Physiological implications of hydrogen sulfide: A whiff exploration that blossomed. *Physiol Rev* 92: 791-896, 2012.
- Liu M, Li Y, Liang B, Li Z, Jiang Z, Chu C and Yang J: Hydrogen sulfide attenuates myocardial fibrosis in diabetic rats through the JAK/STAT signaling pathway. *Int J Mol Med* 41: 1867-1876, 2018.
- Dugbartey GJ: Diabetic nephropathy: A potential savior with 'rotten-egg' smell. *Pharmacol Rep* 69: 331-339, 2017.
- Feliers D, Lee HJ and Kasinath BS: Hydrogen sulfide in renal physiology and disease. *Antioxid Redox Signal* 25: 720-731, 2016.
- Missale C, Nash SR, Robinson SW, Jaber M and Caron MG: Dopamine receptors: From structure to function. *Physiol Rev* 78: 189-225, 1998.
- Beaulieu JM, Espinoza S and Gainetdinov RR: Dopamine receptors - IUPHAR Review 13. *Br J Pharmacol* 172: 1-23, 2015.
- Beaulieu JM and Gainetdinov RR: The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol Rev* 63: 182-217, 2011.



18. Bryson SE, Drew GM, Hall AS, Ball SG and Balmforth AJ: Characterization of the dopamine receptor expressed by rat glomerular mesangial cells in culture. *Eur J Pharmacol* 225: 1-5, 1992.
19. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, *et al*: H<sub>2</sub>S as a physiologic vaso-relaxant: Hypertension in mice with deletion of cystathionine gamma-lyase. *Science* 322: 587-590, 2008.
20. Li HZ, Han LP, Jiang CM, Li H, Zhao YJ, Gao J, Lin Y, Ma SX, Tian Y, Yang BF, *et al*: Effect of dopamine receptor 1 on apoptosis of cultured neonatal rat cardiomyocytes in simulated ischaemia/reperfusion. *Basic Clin Pharmacol Toxicol* 102: 329-336, 2008.
21. Zhou X, Feng Y, Zhan Z and Chen J: Hydrogen sulfide alleviates diabetic nephropathy in a streptozotocin-induced diabetic rat model. *J Biol Chem* 289: 28827-28834, 2014.
22. Waz S, Heeba GH, Hassanin SO and Abdel-Latif RG: Nephroprotective effect of exogenous hydrogen sulfide donor against cyclophosphamide-induced toxicity is mediated by Nrf2/HO-1/NF-κB signaling pathway. *Life Sci* 264: 118630, 2021.
23. Yang G, Tang G, Zhang L, Wu L and Wang R: The pathogenic role of cystathionine γ-lyase/hydrogen sulfide in streptozotocin-induced diabetes in mice. *Am J Pathol* 179: 869-879, 2011.
24. Chen B, Li W, Lv C, Zhao M, Jin H, Jin H, Du J, Zhang L and Tang X: Fluorescent probe for highly selective and sensitive detection of hydrogen sulfide in living cells and cardiac tissues. *Analyst (Lond)* 138: 946-951, 2013.
25. Olson KR, Gao Y, Arif F, Patel S, Yuan X, Mannam V, Howard S, Batinic-Haberle I, Fukuto J, Minnion M, *et al*: Manganese porphyrin-based SOD mimetics produce polysulfides from hydrogen sulfide. *Antioxidants* 8: 8, 2019.
26. Papadopoulou-Marketou N, Paschou SA, Marketos N, Adamidi S, Adamidis S and Kanaka-Gantenbein C: Diabetic nephropathy in type 1 diabetes. *Minerva Med* 109: 218-228, 2018.
27. Drummond K and Mauer M; International Diabetic Nephropathy Study Group: The early natural history of nephropathy in type 1 diabetes: II. Early renal structural changes in type 1 diabetes. *Diabetes* 51: 1580-1587, 2002.
28. Bithi N, Link C, Henderson YO, Kim S, Yang J, Li L, Wang R, Willard B and Hine C: Dietary restriction transforms the mammalian protein persulfidome in a tissue-specific and cystathionine γ-lyase-dependent manner. *Nat Commun* 12: 1745, 2021.
29. Wang M, Guo Z and Wang S: The effect of certain conditions in the regulation of cystathionine γ-lyase by exogenous hydrogen sulfide in mammalian cells. *Biochem Genet* 51: 503-513, 2013.
30. Shankland SJ: Cell cycle regulatory proteins in glomerular disease. *Kidney Int* 56: 1208-1215, 1999.
31. Lee MH and Yang HY: Negative regulators of cyclin-dependent kinases and their roles in cancers. *Cell Mol Life Sci* 58: 1907-1922, 2001.
32. Thullberg M, Welcker M, Bartkova J, Kjerulff AA, Lukas J, Högberg J and Bartek J: Monoclonal antibody probes for p21WAF1/CIP1 and the INK4 family of cyclin-dependent kinase inhibitors. *Hybridoma* 19: 63-72, 2000.
33. Chen JH, Tseng TH, Ho YC, Lin HH, Lin WL and Wang CJ: Gaseous nitrogen oxides stimulate cell cycle progression by retinoblastoma phosphorylation via activation of cyclins/Cdks [correction]. *Toxicol Sci* 76: 83-90, 2003.
34. Chow MJ, Turcotte R, Lin CP and Zhang Y: Arterial extracellular matrix: A mechanobiological study of the contributions and interactions of elastin and collagen. *Biophys J* 106: 2684-2692, 2014.
35. Johnson RJ, Floege J, Yoshimura A, Iida H, Couser WG and Alpers CE: The activated mesangial cell: A glomerular 'myofibroblast'? *J Am Soc Nephrol* 2 (Suppl 10): S190-S197, 1992.
36. Kreisberg JJ, Venkatachalam M and Troyer D: Contractile properties of cultured glomerular mesangial cells. *Am J Physiol* 249: F457-F463, 1985.
37. Ohtomo S, Nangaku M, Izuhara Y, Yamada N, Dan T, Mori T, Ito S, van Ypersele de Strihou C and Miyata T: The role of megsin, a serine protease inhibitor, in diabetic mesangial matrix accumulation. *Kidney Int* 74: 768-774, 2008.
38. Chen Z, Gao H, Wang L, Ma X, Tian L, Zhao W, Li K, Zhang Y, Ma F, Lu J, *et al*: Farrerol alleviates high glucose-induced renal mesangial cell injury through the ROS/Nox4/ERK1/2 pathway. *Chem Biol Interact* 316: 108921, 2020.
39. Mao Q, Chen C, Liang H, Zhong S, Cheng X and Li L: Astragaloside IV inhibits excessive mesangial cell proliferation and renal fibrosis caused by diabetic nephropathy via modulation of the TGF-β1/Smad/miR-192 signaling pathway. *Exp Ther Med* 18: 3053-3061, 2019.
40. Qian X, He L, Hao M, Li Y, Li X, Liu Y, Jiang H, Xu L, Li C, Wu W, *et al*: YAP mediates the interaction between the Hippo and PI3K/Akt pathways in mesangial cell proliferation in diabetic nephropathy. *Acta Diabetol* 58: 47-62, 2021.
41. Cargnello M and Roux PP: Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev* 75: 50-83, 2011.
42. Bramanti V, Grasso S, Tibullo D, Giallongo C, Raciti G, Viola M and Avola R: Modulation of extracellular signal-related kinase, cyclin D1, glial fibrillary acidic protein, and vimentin expression in estradiol-pretreated astrocyte cultures treated with competence and progression growth factors. *J Neurosci Res* 93: 1378-1387, 2015.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.