Original Research

Nuclear factor E2-related factor 2 knockdown enhances glucose uptake and alters glucose metabolism in AML12 hepatocytes

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Impact statement

Increasing evidence supports the complexity of Nrf2 functions beyond the antioxidant and detoxification response. Previous *in vivo* studies employing either Nrf2-knockout or Nrf2-activated mice have achieved a similar endpoint: protection against an obese and insulin-resistant phenotype that includes impaired lipogenesis and gluconeogenesis in the liver. These apparently paradoxical observations led us to evaluate the impact of Nrf2 in liver cells in the absence of any influence from the systemic environment, including changes in the secretion of adipokines and proinflammatory cytokines by adipose tissues. In the present study, Nrf2 knockdown was sufficient to induce fundamental changes in the glucose metabolism of AML12 hepatocytes in addition to its classical cytoprotective functions. We also discuss similarities and differences between our *in vitro* study and previous *in vivo* studies, which may be helpful to dissect and better understand *in vivo* data that represents the culmination of both local and systemic alterations.

Abstract

Nuclear factor E2-related factor 2 (Nrf2) is a transcription factor known to induce the expression of a variety of antioxidant and detoxification genes. Recently, increasing evidence has revealed roles for Nrf2 in glucose, lipid, and energy metabolism; however, the exact functions of Nrf2 in hepatocyte biology are largely unclear. In the current study, the transient knockdown of Nrf2 via siRNA transfection enhanced the glucose uptake of fasting AML12 hepatocytes to $325.3 \pm 11.1\%$ ($P < 0.05$) of that of untransfected control cells. The impacts of Nrf2 knockdown (NK) on the antioxidant system, inflammatory response, and glucose metabolism were then examined in AML12 cells under both high-glucose (33 mmol/L) and low-glucose (4.5 mmol/L) conditions. NK lowered the gene and protein expression of the anti-oxidases heme oxygenase-1 and NAD(P)H: quinone oxidoreductase 1 and increased *p*-eukaryotic initiation factor-2α^{S51}, p-nuclear factor-κB p65^{S276}, and its downstream proinflammatory factors, including interleukin-1 beta, tumor necrosis factor-α, matrix metalloproteinase 2, and matrix metalloproteinase 9, at the protein level. NK also altered the protein expression of fibroblast growth factor 21, glucose transporter type 4, insulin-like growth factor 1, forkhead box protein O1, p-AKT^{S473}, and p-GSK3$\alpha/\beta^{Y279/Y216}$, which are involved in glucose uptake, glycogenesis, and gluconeogenesis in AML12 cells. Our results provide a comprehensive understanding of the central role of Nrf2 in the regulation of glucose metabolism in AML12 hepatocytes, in addition to its classical roles in the regulation of redox signaling, endoplasmic reticulum stress and proinflammatory responses, and support the potential of Nrf2 as a therapeutic target for the prevention and treatment of obesity and other associated metabolic syndromes.

Keywords: Nuclear factor E2-related factor 2, AML12 hepatocytes, glucose uptake, hyperglycemia, glycogenesis, gluconeogenesis

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Introduction

Nuclear factor E2-related factor 2 (Nrf2) is a basic leucine zipper (bZIP) transcription factor that regulates the expression of many antioxidant and phase II detoxifying enzymes.[1,2] Under static conditions, Nrf2 is retained in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) and undergoes constant ubiquitination and proteasomal degradation. Upon oxidative or electrophilic stress, the Nrf2 protein is released from Keap1, translocates to the nucleus, forms heterodimers with other bZIP proteins, binds to the antioxidant response element in the upstream promoter regions of target genes, and induces the expression of many cytoprotective proteins, including heme oxygenase (HO-1), glutathione-*S*-transferases (GST), NAD(P)H: quinone oxidoreductase 1 (NQO1), and others.[1–4]

Extensive evidence has indicated Nrf2 functions are not limited to exerting antioxidant and detoxification effects but are also implicated in many other molecular processes and diseases, including inflammatory responses, cancers, metabolic diseases, cell proliferation, senescence, and survival.[5–9] The emerging role of Nrf2 in glucose, lipid and energy metabolism has been increasingly recognized following numerous reports of the effects of loss of Nrf2 function (Nrf2 deletion) on the prevention of high-fat diet-induced obesity in mice.[10–13] The majority of these studies have converged on the same endpoint: protection against an obese and insulin-resistant phenotype that includes impaired adipogenesis and adipose functions as well as impaired lipogenesis and gluconeogenesis in the liver.[10–13] Nrf2 in particular has been identified as a novel regulator of fibroblast growth factor 21 (FGF21) activity in the crosstalk between cellular stress defenses and metabolism regulation.[10,12] Paradoxically, the genetic activation of Nrf2 via the hypomorphic knockdown of Keap1[14,15] or the oral administration of Nrf2 inducers[14,16,17] has also been reported to repress gluconeogenesis and lipogenesis in murine livers and prevent the onset of diabetes mellitus in diabetic *db/db* mice.

The liver is a major site of glucose and lipid metabolism in addition to its detoxification functions. Hepatocytes play important roles in maintaining plasma glucose homeostasis by adjusting the balance between hepatic glucose production and utilization via the gluconeogenic and glycolytic pathways. Prolonged hyperglycemia has been widely recognized as a state of chronic oxidative stress and inflammation, caused by enhanced levels of reactive oxygen species (ROS) that are functionally linked to the increased formation of advanced glycation end-products (AGEs) and the expression of their cognate receptor for advanced glycation end-products (RAGEs).[18,19] In the liver, chronic hyperglycemia is a major cause underlying the development of the early stage of hepatic simple steatosis and nonalcoholic fatty liver disease (NAFLD),[20,21] accompanied with significantly elevated nuclear Nrf2 in liver cells.[20] In this regard, Nrf2 plays a central role in the crosstalk dictating the regulation of glucose and lipid metabolism, antioxidant defenses, and inflammatory responses in the liver.

In Nrf2-knockout[10–13] or Nrf2-activated mice,[14–17] alterations in Nrf2 expression affect the liver in two different ways: either through direct effects exerted by Nrf2 on liver cells or indirect effects due to alterations in the environment, particularly altered adipose tissues that participate in crosstalk with the liver via the secretion of a variety of hormones and proinflammatory cytokines, e.g. FGF21.[22–24] The controversial phenotypic changes of the liver observed in Nrf2-knockout or Nrf2-activated mice represent the cumulative direct and indirect effects of Nrf2 alterations; however, the direct effects of Nrf2 on the liver remain largely uncharacterized.

In the current *in vitro* study, we knocked down Nrf2 expression in AML12 hepatocytes to assess the impact of Nrf2 knockdown on liver cells and to better understand the causes of the controversial phenotypes observed in previous *in vivo* studies.

Materials and methods

Cell culture

AML12 hepatocytes were cultured as monolayers in Dulbecco's modified Eagle's medium/F12 (GIBCO, Waltham, MA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 40 ng/mL dexamethasone and insulin–transferring–selenium (ITS) (containing 5 mg/L insulin, 5 mg/L transferrin and 5 μg/L selenium). The cells were grown in flasks and kept at 37°C in a humidified atmosphere consisting of air (i.e. approximately 21% O_2) and 5% CO_2. The cells were grown to 70–80% confluence and subjected to the following treatments.

Knockdown of Nrf2 expression by siRNA

The small-interfering RNA (siRNA) utilized in this study targeted the following sequences of the mouse Nrf2 gene: sense, 5′-GAAUUACAGUGUCUUAAUATT-3′, and antisense, 5′-UAUUAAGACACUGUAAUUCTT-3′. All siRNA duplexes were synthesized by Stealth RNAi (Invitrogen, Eugene, OR). AML12 hepatocytes were transfected with 100 nmol of each siRNA duplex using Lipofectamine 2000 and Opti-MEM for 24 h according to the manufacturer's instructions. Control experiments were performed using equivalent amounts of the Stealth™ RNAi Negative Control, Med GC (Invitrogen).

Glucose uptake cell-based assay

A glucose uptake cell-based assay kit was purchased from Cayman Chemical Co. (Cat.#: 600470, Ann Arbor, MI). The assay was performed according to the manufacturer's instructions with slight modifications. Briefly, AML12 hepatocytes were seeded at 3×10^4 cells/well in a 96-well plate and incubated overnight. The next day, the cells were incubated in 100 μL of glucose-free culture medium containing 10% fetal bovine serum, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 40 ng/mL dexamethasone and ITS for 24 h before 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) was added into the medium to a final concentration of 200 μg/mL. Then, 8 h after 2-NBDG treatment, the plate was centrifuged for 5 min at 400 *g* at room temperature, and the supernatants were aspirated. Next, 200 μL of cell-based assay buffer was carefully added into each well without disturbing the cell layer and allowed to incubate for 10 min before the plate was centrifuged for 5 min at 400 *g* at room temperature. The supernatants were aspirated, 100 μL of cell-based assay buffer was added to each well, and then the samples were analyzed immediately with fluorescent filters (excitation/emission = 485/535 nm).

Quantitative real-time polymerase chain reaction analysis

Eight hours after siRNA transfection, AML12 hepatocytes were incubated in either high-glucose (HG, 33 mmol/L) or low-glucose (LG, 4.5 mmol/L) Dulbecco's modified eagle

Table 1 Primer sequences used for qPCR

Gene	Accession #	Primer (F: forward 5′–3′ R: reverse 3′–5′)
Nrf2	NM_090424	(F) CTACAGTCCCAGCAGGA CATGGATTTG
		(R) GTTTTCGGTATTAAGACAC TGTAATTCGGGAATGG
HO-1	NM_010442	(F) CCCCACCAAGTTCAAACAGC
		(R) AGCTCCTCAAACAGCTCAATGT
NQO1	NM_008706	(F) AGGATGGGAGGTACTCGAATC
		(R) TGCTAGAGATGACTCGGAAGG
FGF21	NM_020013	(F) CCGCAGTCCAGAAAGTCTCC
		(R) CTGCAGGCCTCAGGATCAAA
Sirt1	NM_001159589	(F) CATTTATCAGAGTTGCCACCAA
		(R) ACCAACAGCCTTAAAATCTGGA
PGC-1α	NM_008904	(F) CCGAGAATTCATGGAGCAAT
		(R) GTGTGAGGAGGGTCATCGTT
UCP1	NM_009463	(F) AACTGTACAGCGGTCTGCCT
		(R) TAAGCCGGCTGAGATCTTGT
Gapdh	NM_001289726	(F) AATGGTGAAGGTCGGTGT
		(R) GTGGAGTCATACTGGAACATGTAG

Table 2 Antibodies used for Western blot analysis

Antibody	Company	Reference	Dilution
Nrf2	Santa Cruz	Sc-722	1:1000
HO-1	Abcam	ab52947	1:1000
NQO1	Proteintech	11451-1-AP	1:1000
p-EIF2α^{S51}	Millipore	04-342	1:1000
EIF2α	Proteintech	11233-1-AP	1:1000
IL-1β	Ruiying Biological	RLT2322	1:1000
TNF-α	Ruiying Biological	RLM3477	1:1000
p-NF-κB p65^{S276}	Ruiying Biological	RLP0187	1:1000
MMP2	Ruiying Biological	RLT2798	1:1000
MMP9	Ruiying Biological	RLT1892	1:1000
FGF21	Abcam	ab171941	1:1000
AMPKα	Ruiying Biological	RLT0215	1:1000
Sirt1	Cell signaling	Q96E86	1:1000
PGC-1α	Abcam	ab54481	1:1000
UCP1	Abcam	ab23841	1:1000
Glut-4	Ruiying Biological	RLT1930	1:1000
IGF-1R	Ruiying Biological	RLT2282	1:1000
FOXO1	Ruiying Biological	RLT1757	1:1000
p-AKT^{S473}	Ruiying Biological	RLP0006	1:1000
AKT	Ruiying Biological	RLT0178	1:1000
GSK3α/β	Proteintech	22104-1-AP	1:500
p-GSK3α/β$^{Y279/Y216}$	Signalway	11002	1:500
Gapdh	Santa Cruz	Sc420485	1:1000

medium (DMEM) medium for 24 h before the cells were collected for total RNA extraction and western blot analysis.

Total RNA from each group was isolated using a Hipure Total RNA Mini Kit (Cat.#: R4111-03, Magen, Guangzhou, Guangdong, China). cDNA was synthesized from 2 μg of extracted total RNA using a ReverTra Ace qPCR RT kit (Cat.#: FSQ-101, TOYOBO, Kita-ku, Osaka, JAPAN). Quantitative real-time polymerase chain reaction (qPCR) was carried out using a SYBR Green PCR master mix (Cat.#: QPK-201, TOYOBO) and on a StepOne Quantitative real-time PCR System (Applied Biosystems, StepOne Plus, CA). Each sample was loaded in triplicate. The glyceraldehyde 3-phosphate dehydrogenase (*Gadph*) gene was amplified as an internal reference gene, and the $2^{-\Delta\Delta Ct}$ method was used for data analysis. The relative mRNA expression of each gene is expressed as the mean and standard error of the mean (SEM) of six independent experiments. The primers used in this study are listed in Table 1. The ratios of the mean values of mRNA level in AML12 cells between 2 selected groups are listed in Supplementary Table 1.

Western blot analysis

Protein lysates were resolved by gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was blocked for 1 h in 5% nonfat milk, subsequently probed with specific antibodies and visualized using a ChemiDoc^{TM} MP system (Bio-Rad, Hercules, CA). GAPDH was used as the loading control. Protein band density was quantified using ImageJ software and normalized to that of Gapdh. The antibodies used in this study are listed in Table 2. The ratios of the mean values of protein level in AML12 cells between two selected groups are listed in Supplementary Table 2.

Statistical analysis

The data are presented as the mean ± SEM for the number of replicates indicated. All data were analyzed using SPSS 17.0 and GraphPad Prism 5.0 software. Statistical analysis was carried out by performing an unpaired Student's *t* test between two selected groups and two-way analysis of variance (ANOVA) for the siRNA transfection and glucose treatments as two variables among groups. Significant differences were defined as $P < 0.05$.

Results

Knockdown of Nrf2 resulted in increased ER stress, impaired antioxidant pathways, and increased glucose uptake in AML12 cells

Nrf2 knockdown after 24 h of siRNA transfection was confirmed by qPCR and Western blot analysis. Nrf2 mRNA and protein levels in transfected AML12 cells (NK, Nrf2 knockdown) were reduced to 9.9 ± 2.6% ($P < 0.05$) (Figure 1(a)) and 23.2 ± 6.4% ($P < 0.05$) (Figure 1(b)), respectively, of that of untransfected control cells (Con, control). Additionally, the rate of glucose uptake by fasting AML12 cells in the NK group increased to 325.3 ± 11.1% ($P < 0.05$) of that of Con cells (Figure 1(c)).

The effects of Nrf2 knockdown on Nrf2-dependant antioxidant pathways were examined in AML12 cells 48 h after

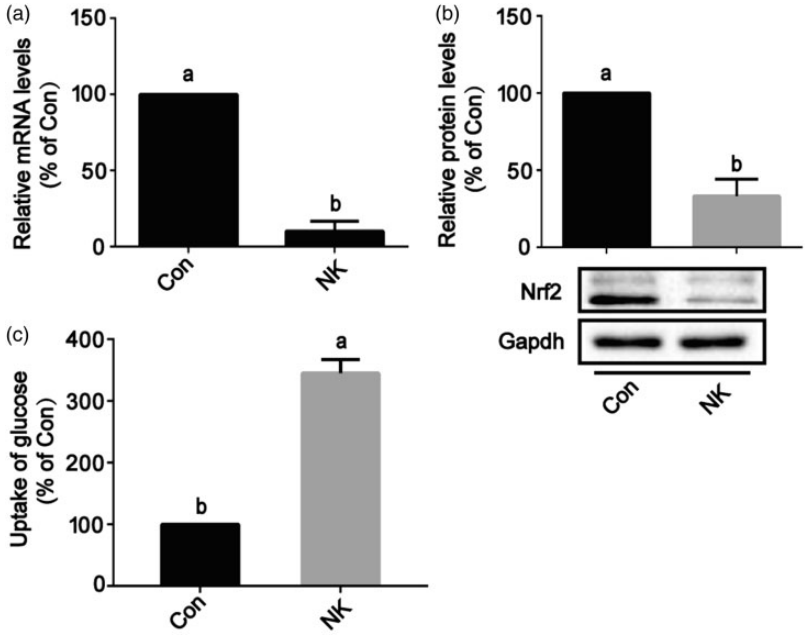

Figure 1 Nrf2 knockdown enhanced the glucose uptake of fasting AML12 hepatocytes. Relative Nrf2 mRNA levels (a), relative Nrf2 protein levels and a representative Nrf2 Western blot (b) are shown for AML12 cells. Glucose uptake (c) was determined using fasting AML12 cells pre-incubated in glucose-free medium for 24 h. The data are expressed as the mean ± SEM, $n = 6$/group. Different letters indicate differences between the treatment groups, $P < 0.05$. Nrf2: nuclear factor E2-related factor 2; NK: Nrf2 knockdown in AML12 cells; Con: untransfected AML12 cells

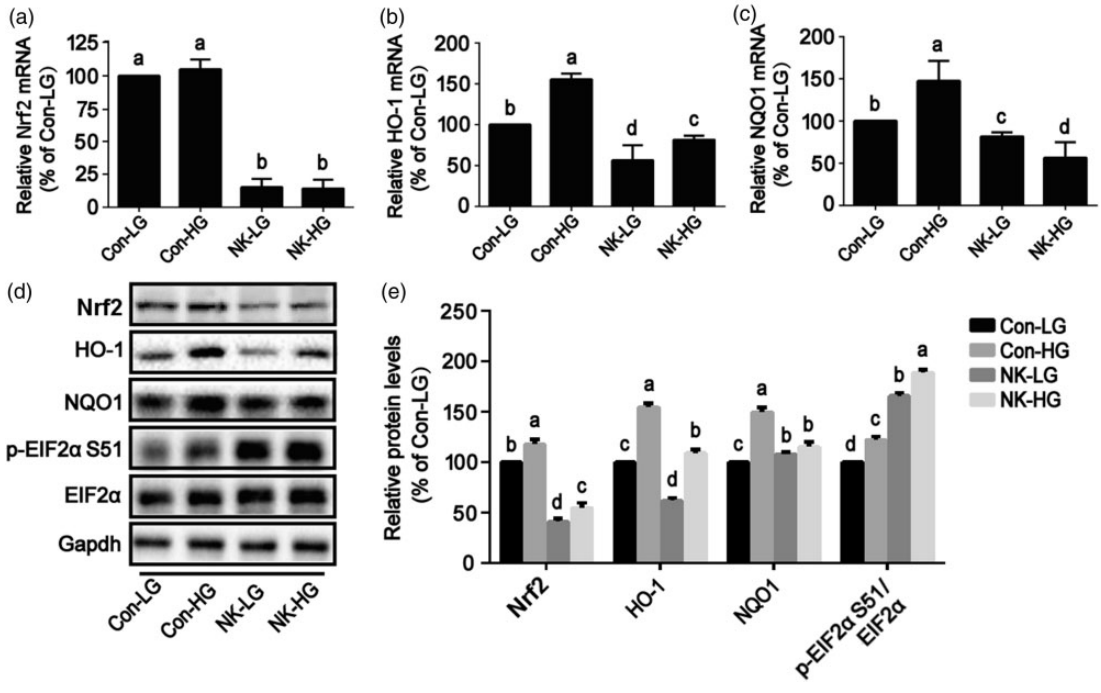

Figure 2 Nrf2 knockdown impaired antioxidant defenses and increased endoplasmic reticulum stress in AML12 hepatocytes. Relative mRNA levels of Nrf2 (a), HO-1 (b) and NQO1 (c), representative Western blots for Nrf2, HO-1, NQO1, EIF2α, and p-EIF2α^{S51} protein expression (d), and relative protein levels (e) in AML12 cells (Con, NK) under both LG (4.5 mmol/L) and HG (33 mmol/L) conditions. The data are expressed as the mean ± SEM, $n = 6$/group. Different letters indicate differences between the treatment groups, $P < 0.05$. HO-1: heme oxygenase; NQO1: quinone oxidoreductase 1; EIF2α: α subunit of eukaryotic initiation factor

Nrf2 transfection in both LG (4.5 mmol/L glucose) and HG (33 mmol/L glucose) cultures. The mRNA level of Nrf2 was remained significantly reduced 48 h after siRNA transfection (Con-LG/HG vs. NK-LG/HG, $P < 0.05$), while was not significantly affected upon HG treatment (Con-LG vs. Con-HG, $P > 0.05$; NK-LG vs. NK-HG, $P > 0.05$) (Figure 2(a)), indicating that the transcription of Nrf2 in AML12 cells was not affected by glucose concentration under the present experimental conditions. The protein level of total Nrf2 was decreased after Nrf2 knockdown (NK-LG vs.

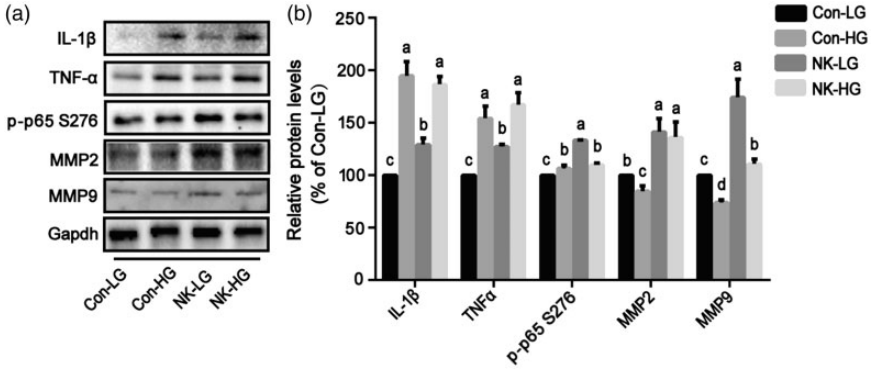

Figure 3 Nrf2 knockdown increased the gross inflammatory status of AML12 hepatocytes. Representative Western blots for IL-1β, TNF-α, p-p65^{S276}, MMP2, and MMP9 protein expression (a) and relative protein levels (b) in AML12 cells (Con, NK) under both LG and HG conditions. The data are expressed as the mean ± SEM, $n = 6$/group. Different letters indicate differences between the treatment groups, $P < 0.05$. IL-1β: interleukin-1 beta; TNF-α: tumor necrosis factor-α; p-p65^{S276}: phosphorylated nuclear factor κB subunit p65; MMP2: matrix metalloproteinase 2; MMP9: matrix metalloproteinase 9

Con-LG, $P < 0.05$; NK-HG vs. Con-HG, $P < 0.05$) and elevated upon HG treatment (Con-LG vs. Con-HG, $P < 0.05$; NK-LG vs. NK-HG, $P < 0.05$) (Figure 2(d), (e)), indicating that HG treatment resulted in higher level of total Nrf2 protein in AML12 cells.

The mRNA expression of Nrf2 downstream antioxidases HO-1 and NQO1 was significantly elevated upon HG treatment (Con-LG vs. Con-HG, $P < 0.05$) and decreased after Nrf2 knockdown (NK-LG vs. Con-LG, $P < 0.05$) (Figure 2(b), (c)). HO-1 and NQO1 mRNA levels in the NK-LG group were decreased to 56.5 ± 10.7% and 76.8 ± 2.9% of that of the Con-LG group ($P < 0.05$), respectively, suggesting that the regulation of NQO1 transcription was less Nrf2-dependent than that of the *HO*-1 gene. HO-1 and NQO1 protein expression was also elevated with HG treatment and decreased after Nrf2 knockdown (Figure 2(d), (e)) ($P < 0.05$), exhibiting similar altered patterns in terms of mRNA expression among different groups, suggesting that HO-1 and NQO1 expression was primarily regulated at the transcriptional level.

Intracellular endoplasmic reticulum (ER) stress levels in AML12 cells were evaluated by measuring the activation of an ER stress reporter, α subunit of eukaryotic initiation factor (EIF2α). EIF2α protein expression remained relatively constant among the different groups, while the p-EIF2α^{S51}/EIF2α ratio was increased upon HG treatment and further elevated after Nrf2 knockdown (Figure 2(d), (e)) ($P < 0.05$), suggesting that both HG treatment and Nrf2 knockdown increased ER stress in AML12 cells.

HG treatment and Nrf2 knockdown elevated the gross inflammatory status of AML12 cells

Next, the impact of Nrf2 knockdown on the gross inflammatory status of AML12 cells was evaluated by examining the protein expression of proinflammatory markers, including interleukin-1 beta (IL-1β), tumor necrosis factor-α (TNF-α), phosphorylated nuclear factor κB subunit p65 (p-p65^{S276}), matrix metalloproteinase 2 (MMP2), and matrix metalloproteinase 2 (MMP9), under both LG and HG conditions.

HG treatment significantly elevated IL-1β, TNF-α (Con-LG vs. Con-HG, $P < 0.05$; NK-LG vs. NK-HG, $P < 0.05$) and p-p65^{S276} protein levels (Con-LG vs. Con-HG, $P < 0.05$) and significantly lowered MMP2 and MMP9 protein levels (Con-LG vs. Con-HG, $P < 0.05$) in AML12 cells (Figure 3). After Nrf2 knockdown, IL-1β, TNF-α, p-p65^{S276}, MMP2, and MMP9 protein levels were all significantly elevated (Con-LG vs. Con-HG, $P < 0.05$) (Figure 3), suggesting that Nrf2 knockdown elevated the gross inflammatory status of AML12 cells.

HG treatment and Nrf2 knockdown altered the FGF21-related metabolic signaling pathway in AML12 cells

We then examined the effects of Nrf2 knockdown on the expression of genes involved in the FGF21-related metabolic signaling pathway, including FGF21, AMP-activated protein kinase α (AMPKα), silent mating type information regulation 2 homolog-1 (Sirt1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and uncoupling protein 1 (UCP1).[25]

FGF21, Sirt1, PGC-1α, and UCP1 mRNA levels were all significantly lowered upon HG treatment (Con-LG vs. Con-HG, $P < 0.05$) and elevated after Nrf2 knockdown (NK-LG vs. Con-LG, $P < 0.05$) (Figure 4(a–d)).

FGF21, AMPKα, Sirt1, PGC-1α, and UCP1 protein levels were all lowered upon HG treatment (Con-LG vs. Con-HG, $P < 0.05$; NK-LG vs. NK-HG, $P < 0.05$) and elevated after Nrf2 knockdown (NK-LG vs. Con-LG, $P < 0.05$; NK-HG vs. Con-HG, $P < 0.05$) (Figure 4(e), (f)), similar to alterations at the mRNA level.

HG treatment and Nrf2 knockdown altered the expression of proteins involved in glucose homeostasis in AML12 cells

The impacts of HG treatment and Nrf2 knockdown on the expression of proteins involved in glucose metabolism were examined by measuring the protein level of glucose transporter type 4 (Glut4), insulin-like growth factor 1 (IGF-1 R), forkhead box protein O1 (FOXO1), the activation of protein

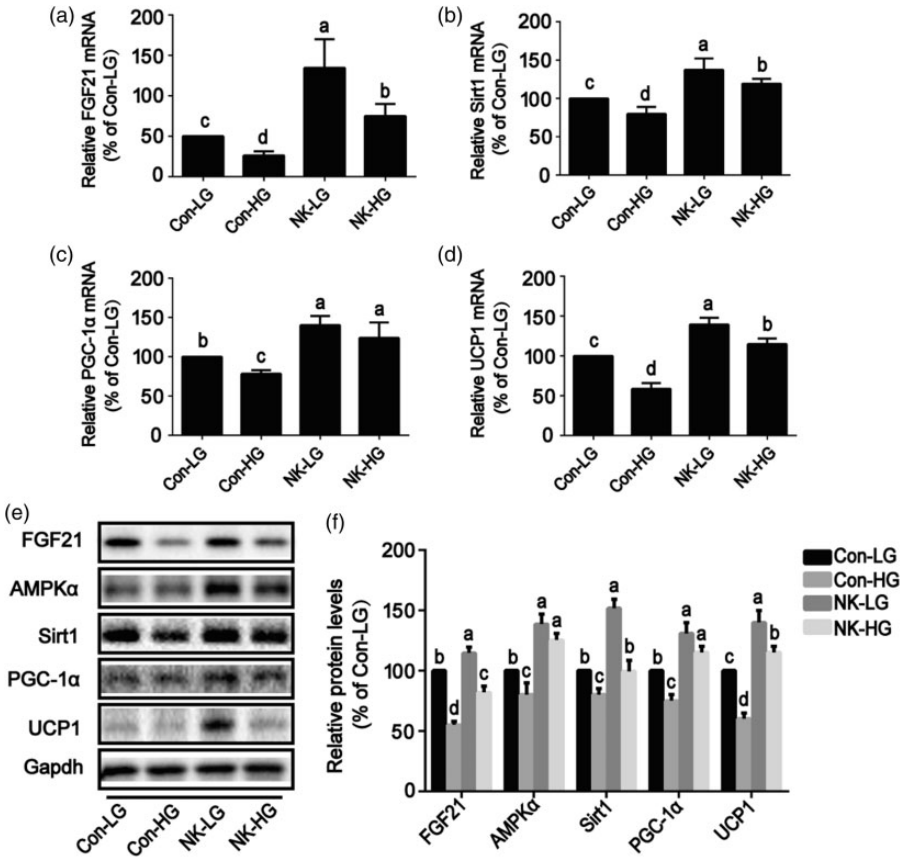

Figure 4 Nrf2 knockdown activated the FGF21 signaling pathway in AML12 hepatocytes. Relative mRNA levels of FGF21 (a), Sirt1 (b), PGC-1α (c), and UCP1 (d); representative Western blots for FGF21, AMPKα, Sirt1, PGC-1α, and UCP1 protein expression (e) and relative protein levels (f) in AML12 cells (Con, NK) under both LG and HG conditions. The data are expressed as the mean ± SEM, $n = 6$/group. Different letters indicate differences between the treatment groups, $P < 0.05$. FGF21: fibroblast growth factor 21; Sirt1: silent mating type information regulation 2 homolog-1; AMPKα: AMP-activated protein kinase α; PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha; UCP1: uncoupling protein 1

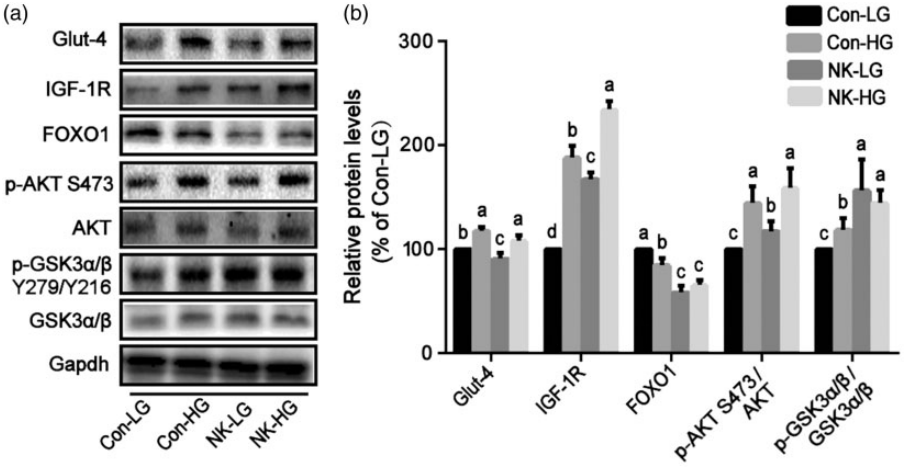

Figure 5 Nrf2 knockdown altered glucose metabolism in AML12 hepatocytes. Representative Western blots for Glut-4, IGF-1 R, FOXO1, p-AKT^{S473}, AKT, p-GSK3α/β$^{Y279/Y216}$, and GSK3α/β protein expression (a) and relative protein levels (b) in AML12 cells (Con, NK) under both LG and HG conditions. The data are expressed as the mean ± SEM, $n = 6$/group. Different letters indicate differences between the treatment groups, $P < 0.05$. Glut-4: glucose transporter type 4; IGF-1 R: insulin-like growth factor 1; FOXO1: forkhead box protein O1; AKT: protein kinase B; GSK3α/β: glycogen synthase kinase 3α and 3β

kinase B (AKT), and glycogen synthase kinase 3α and 3β (GSK3α/β).

HG treatment significantly elevated the protein levels of Glut4, IGF-1 R, the p-AKT^{S473} /AKT ratio, and the p-GSK3α/β$^{Y279/Y216}$/GSK3α/β ratio (Con-LG vs. Con-HG, $P < 0.05$) and lowered FOXO1 protein levels (Con-LG vs. Con-HG, $P < 0.05$) (Figure 5). After Nrf2 knockdown, IGF-1 R protein levels, the p-AKT^{S473}/AKT ratio, and the

p-GSK3α/β$^{Y279/Y216}$/GSK3α/β ratio were all significantly increased (NK-LG vs. Con-LG, $P < 0.05$), and Glut4 and FOXO1 protein levels were decreased (NK-LG vs. Con-LG, $P < 0.05$) in AML12 cells (Figure 5).

Discussion

Crosstalk between antioxidant and proinflammatory pathways

HG treatment significantly elevated the total Nrf2 protein level, and concurrently elevated the gene and protein expression of its downstream anti-oxidases, HO-1 and BQO1, and activated the ER stress reporter EIF2α via phosphorylation (Figure 2), which is consistent with previous studies that identified hyperglycemia as a state of chronic oxidative stress for cells.[20,21,26] After Nrf2 knockdown, both HO-1 and NQO1 mRNA and protein levels were lowered, accompanied by further phosphorylation of EIF2α, impairment of the Nrf2-dependent antioxidant system and increased ER stress in cells.

Hyperglycemia did not affect transcription of Nrf2 gene, but resulted in higher level of total Nrf2 protein in AML12 cells (Figure 2). Combined with the previous observation of significantly elevated nuclear Nrf2 in liver cells upon HG treatment,[20] it is suggested that HG treatment promoted translocation of Nrf2 to the nuclear of AML12 cells, thus partially prevented cytoplasm Nrf2 from degradation through the Keap1- and ubiquitination-dependent pathway,[1–4] and resulted in higher level of total Nrf2 protein in AML12 cells in the present study.

Nuclear factor-κB (NF-κB) is another redox-sensitive transcription factor that contains two subunits: p65 (RelA), which mediates transcriptional activity, and p50 (NF-κB1), which inhibits p65.[27] The transcription of proinflammatory mediators such as TNF-α, IL-1, MMP2, and MMP9, and others is initiated in response to increased intracellular ROS concentrations.[28–30] The redox activation of Nrf2 and NF-κB has been described as two coordinated and often counter-balanced interactions that share common effectors and regulatory points.[5,27] In the present study, impairment of the Nrf2-dependent antioxidant system was accompanied by an enhanced gross inflammatory state in AML12 cells, suggesting that AML12 cells are predisposed to endure NF-κB-mediated proinflammatory and deleterious processes when their antioxidant defense machinery is compromised.

Nrf2-controlled glucose homeostasis in AML12 cells

Primarily expressed in the liver and adipocytes,[31–33] FGF21 is widely recognized as a key mediator induced by nutrient stress and hypothermia and exerts a number of beneficial effects on glucose and lipid metabolism, as well as energy expenditure.[34–41] In the present study, both FGF21 mRNA and protein levels in AML12 cells were dramatically lowered upon HG treatment; in contrast, elevated serum FGF21 concentrations are observed in diet-induced obese mice[42] and in patients with obesity, type 2 diabetes or hepatic steatosis.[32,43–45] Thus, hyperglycemia alone inhibited FGF21 expression *in vitro* in AML12 hepatocytes, suggesting that the elevated circulating FGF21 levels observed in mice and patients with obesity and metabolic symptoms[32,42–45] do not solely account for the responses of the liver to a hyperglycemic environment; rather, more complex mechanisms may be involved in these processes.

FGF21/AMPK/Sirt1/PGC-1α/UCP1 signaling pathway regulates energy metabolism by enhancing mitochondrial oxidative capacity in adipocytes.[25] In the present study, Nrf2 knockdown increased the protein expression of FGF21, AMPKα, Sirt1, PGC-1α, and UCP1 in AML12 cells (Figure 4(a)), suggesting that Nrf2 could control energy metabolism through the FGF21 pathway in AML12 hepatocytes as in adipocytes. Nrf2 knockdown greatly enhanced glucose uptake in fasting AML12 cells compared to that in untransfected cells (Figure 1(c)). In 3T3-L1 adipocytes, hyperglycemia-enhanced glucose uptake was mediated by FGF21 via modulation of the expression of the glucose transporter Glut1.[31] In the present study, Glut4 expression was enhanced upon HG treatment ($P < 0.05$) but was slightly decreased after Nrf2 knockdown ($P < 0.05$), suggesting that NK-enhanced glucose uptake in AML12 cells occurred via mechanisms other than the regulation of Glut4 expression.

IGF1 enhances glucose uptake in the liver.[46] In the present study, expression of the IGF1 receptor (IGF1R) was elevated upon HG treatment and was further increased after Nrf2 knockdown in AML12 cells, suggesting that Nrf2 inhibits IGF1R-dependent glucose uptake and contributes to increased glucose uptake in AML12 cells after Nrf2 knockdown.

FOXO1 plays a vital role in maintaining glucose homeostasis by promoting hepatic gluconeogenesis during prolonged fasting or starvation[47,48] and is then inhibited by AKT after feeding.[49] In the present study, HG treatment resulted in decreased FOXO1 protein expression, consistent with decreased hepatic gluconeogenesis under hyperglycemic conditions. NK inhibited FOXO1 expression under both LG and HG conditions (Figure 5), suggesting that NK impaired hepatic gluconeogenesis in AML12 cells. The p-AKT^{S473}/AKT ratio increased upon HG treatment and was further increased after Nrf2 knockdown ($P < 0.05$), suggesting that lowered FOXO1 expression with HG treatment and after Nrf2 knockdown is AKT-dependent and occurs if Nrf2 regulates hepatic gluconeogenesis via the AKT/FOXO1 pathway in AML12 cells.

AKT may also promote glycogenesis by inactivating GSK3 (GSK3α and Gsk3β) via phosphorylation in the liver.[49] In the present study, the p-GSK3α/β$^{Y279/Y216}$/GSK3α/β ratio was increased upon HG treatment, confirming enhanced glycogenesis under hyperglycemic conditions. NK resulted in further phosphorylation of GSK3α/β, suggesting that Nrf2 represses liver glycogenesis via the AKT/GSK3α/β pathway.

Similarities and differences between *in vivo* and *in vitro* studies

Based on previous *in vivo* studies, Nrf2 ablation protects mice against high-fat diet-induced obesity and NAFLD and is accompanied by phenotypes such as enhanced

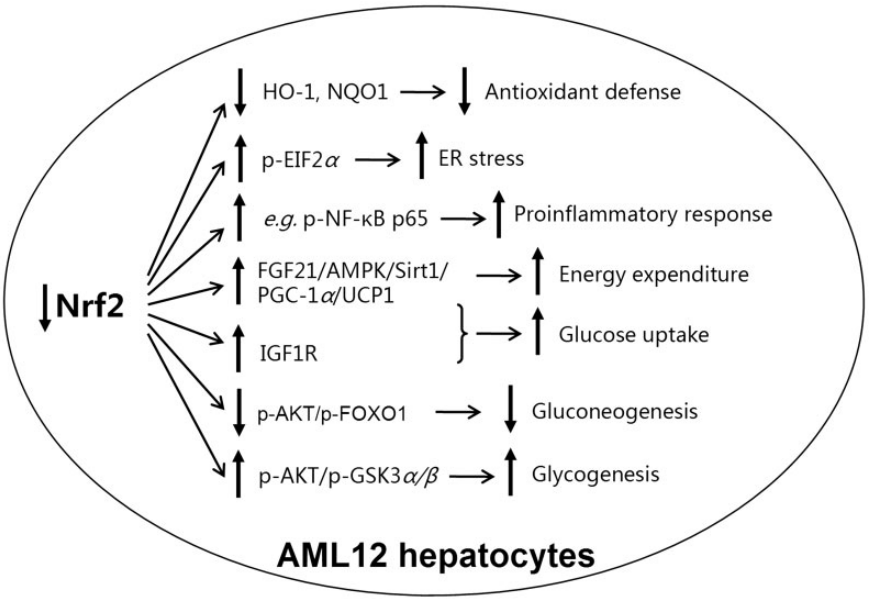

Figure 6 A proposed model of Nrf2 function in AML12 hepatocytes based on the present study

glucose resistance, enhanced energy expenditure, and impaired lipogenesis and gluconeogenesis in the liver.[10–13] The present study is globally consistent with previously reported *in vivo* data[10–13] and provides a comprehensive understanding of the roles of Nrf2 in the regulation of glucose homeostasis in liver cells in the context of a full spectrum of mechanisms, including the regulation of glucose uptake through enhanced IGF-1 R- and FGF21-dependent pathways, enhanced glycogenesis through the AKT/GSK3α/β pathway, and repressed gluconeogenesis through the AKT/FOXO1 pathway (Figure 6).

Notably, HG treatment repressed both the mRNA and protein expression of FGF21 in AML12 cells (Figure 4), in contrast to previous findings that reported increased circulating FGF21 after feeding or under obesity conditions,[38,42,50] suggesting that the latter phenomenon represents the culmination of a systemic response to feeding or obesity; in contrast, liver cells alone were not sufficient to induce increased circulating FGF21 levels under hyperglycemic conditions.

Overall, the present study revealed the central role of Nrf2 in the regulation of the antioxidant response, the proinflammatory system, and glucose homeostasis in liver cells and supports the potential of Nrf2 as a therapeutic target for the prevention and treatment of obesity and other associated metabolic syndromes.

Authors' contributions: All authors participated in study design and interpretation, data analysis, and manuscript review. XY, YH, HH, JW, JY, and LD carried out the experiments; XY, CZ and LZ wrote the manuscript; and LZ takes responsibility for the final content of the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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