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High glucose regulates ERp29 in hepatocellular carcinoma by LncRNA MEG3-miRNA 483-3p pathway

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ABSTRACT

Aims: Blood glucose dysregulation is an adverse factor in the prognosis of hepatocellular carcinoma (HCC). Endoplasmic reticulum (ER) is thought to be crucial component in the development of cancer and diabetes. This study aimed to investigate the mechanisms of poor outcomes in HCC patients with diabetes.

Main methods: ER protein 29 (ERp29) was predicted by proteomics, immunohistochemistry, Western blot, Cell Counting Kit-8 (CCK-8) and cell scratch test were used to identify the expression and biological effects of ERp29 under high glucose (HG) in HCC cells. Bioinformatics found a competing endogenous RNAs (ceRNAs) regulatory network between microRNA-483-3p (miR-483-3p) and Long noncoding RNA (LncRNA MEG3), the above methods also were used to identify their expression, biological effects and their roles of HG on regulation of REp29 in HCC cells, Dual-luciferase reporter assay was carried out to study the interaction of ERp29 with miR-483-3p and miR-483-3p with MEG3.

Key findings: HG upregulated miR-483-3p expression in HCC cells and miR-483-3p overexpression suppressed ERp29 expression and also increased HCC cell proliferation and migration. Furthermore, we found that MEG3 was decreased in HCC cells incubated in medium with high glucose and knockdown of MEG3 downregulated ERp29 expression. Bioinformatics analysis found that MEG3 mediated its protective effects via binding to miR-483-3p.

Significance: Overall, our study established a novel regulatory network of LncRNA MEG3/miR483-3p/ERp29 in HCC which may be helpful in better understanding the effect of high glucose on poor prognosis of HCC and in exploring new diagnostic and therapeutic tools for managing HCC in patients with diabetes.

1. Introduction

The prevalence of diabetes mellitus (DM) is a public health concern worldwide [1]. The estimated overall prevalence of DM in adults in China is 10.9% [2]. DM is a disorder characterized by a chronically raised blood glucose and emerging evidence indicates that DM is an independent risk factor for development of cancer such as pancreatic and liver cancer [3–5]. Hepatocellular carcinoma (HCC) is the 5th most common malignancy worldwide. Effective treatments for HCC include liver resection, liver transplant and local radiotherapy however, post-operative survival rate of HCC is 5-year [6–9]. Existing reports show that the incidence of HCC is two to three times higher in patients suffering from DM and the prognosis and survival rate of these patients is significantly decreased [10–12]. Dysregulation of blood glucose is an adverse factor affecting progression and prognosis of HCC but the underlying mechanism of this association are not fully understood.

Recently, it is been shown that Endoplasmic reticulum (ER) is a crucial component in the development of cancer and DM. On the one hand, ER dysfunction in hepatocytes may aggravate insulin resistance, lipid deposition, and inflammation in DM. On the other hand, there is a correlation between ER stress and apoptosis of tumor cells [11–14]. Taken together, it is particularly important to explore the role of ER in these two diseases. By proteomic and bioinformatic analysis, we identified ERp29, a protein that has been detected in liver and has been demonstrated to play a role in cancer [15,16]. Thus, we explored the role of ERp29 in HCC in the setting of high blood glucose.

Noncoding RNAs (ncRNAs) are RNA transcripts that do not encode any protein and which have been regarded as a vital components in

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tumor biology [17]. NcRNAs are generally categorized into three groups: long noncoding RNAs (lncRNAs), circular RNAs (cirRNAs) and microRNAs (miRNAs). MiRNAs reduce the expression of target protein by binding to the 3′ untranslated region (3′UTR). MiRNAs have been widely studied and their function in the development of HCC has also been demonstrated [18,19]. LncRNAs are ncRNAs > 200 nucleotides which may act as competing endogenous RNA (ceRNA) to negatively regulate the expression of miRNA suggesting that a ceRNAs regulatory network occurs between lncRNAs and miRNAs [20,21].

Taken together, discovery of ERp29 act as a bridge between DM and HCC and understanding the role of high glucose on the expression of this protein via a preliminary regulatory pathway involving ncRNAs would uncover the underlying mechanisms of poor prognosis of HCC patients suffering from DM.

2. Materials and methods

2.1. Cells culture

The HCC cell lines HepG2 and Human Embryonic Kidney (HEK) 293T cells were kindly provided by Endocrinology Institute of Metabolic Disease Hospital of Tianjin Medical University. HCC cells were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum at 37 °C in 5% CO_2. Cells were divided into Control Group (DMEM with low glucose (LG), 5.6 mmol/L) and High Glucose Group (DMEM with high glucose(HG), 25 mmol/L).

2.2. Patient samples

The liver tissues and the paraffin-embedded sections from HCC patients ($n = 7$) with diabetes and HCC patients without diabetes ($n = 14$) were collected from the Tumor Biological Sample Bank of the Cancer Hospital of Tianjin Medical University (Tianjin, China). All patients met the following inclusion criteria: Resected samples were identified as HCC by pathological examination; no radiotherapy or chemotherapy was carried out before surgery. All patients signed informed consent for sample collection and the study protocol was approved by Institutional Research Ethics Committee.

2.3. ER extraction, RNA isolation and quantitative real-time PCR analysis

The isolation and ER from HCC cells was carried out using differential ultracentrifugation and density gradient centrifugation. ER preparations were analyzed by using a Label-free protein quantification method, a method in mass spectrometry that aims to determine the relative amount of proteins in two cell samples. ER-associated candidate protein was identified by bioinformatics. Total RNA was extracted from HepG2 using the RNAiso Plus according to the manufacturer's instruction. The purity and concentration of RNA was measured by Nanodrop2000 (Mrestro). The cDNA was synthesized from RNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa). Quantitative real-time PCR (qRT-PCR) was performed on the LightCycler96 system (Roche). The primer sequences are shown in Table 1. Fold change difference in gene expression was calculated with 2-ΔΔCt method using β-actin as a housekeeping gene.

Table 1
The primer sequences for qRT-PCR.

miR-483-3p	Forword	5′-gcgcgTCACTCCTCTCCTCC-3′
	Reverse	5′- ATCCAGTGCAGGGTCCGAGG-3′
miR-26b-5p	Forword	5′- accgcgcgcgTTCAAGTAATTCAGG-3′
	Reverse	5′- ATCCAGTGCAGGGTCCGAGG-3′
miR-29b-3p	Forword	5′- cgcgcgcgTAGCACCATTTGAAATC-3′
	Reverse	5′- ATCCAGTGCAGGGTCCGAGG-3′
miR-135a-5p	Forword	5′-gcgcgcgcgTATGGCTTTTTATTCCT-3′
	Reverse	5′- ATCCAGTGCAGGGTCCGAGG-3′
miR-376a-3p	Forword	5′- cgcgcgcgcgATCATAGAGGAAAAT-3′
	Reverse	5′- ATCCAGTGCAGGGTCCGAGG-3′

Table 2
The primer sequences for cell transfection.

ERp29 siRNA	5′-GUGAGUCCCUUGUGGAAUATT-3′
	5′-UAUUCCACAAGGGACUCACTT-3′
miR-483-3p mimics	5′-UCACUCCUCUCCUCCCGUCUU-3′
	5′-GACGGGAGGAGAGGAGUGAUU-3′
miR-483-3p mimics NC	5′-UUCUCCGAACGUGUCACGUTT-3′
	5′-ACGUGACACGUUCGGAGAATT-3′
miR-483-3p inhibitor	5′-AAGACGGGAGGAGAGGAGUGA-3′
miR-483-3p inhibitor NC	5′-CAGUACUUUUGUGUAGUACAA-3′

2.4. Cell transfection

ERp29 (pEGFP-ERp29-C1) plasmid and control plasmid (pcDNA3.1), siRNAs targeting ERp29, MEG3 (EX-Y0475-M03) plasmid and controls (pcDNA3.1) were generated. The siRNAs sequences are shown in Table 2. We also synthesized MiR-483-3p mimics and controls, miR-483-3p inhibitor and controls (Table 2). HCC cells were transfected using Lipofectamine 2000 (Thermo Fisher Scienfic) according to the manufacturer's protocol.

2.5. Western blotting analysis

Western blotting was performed according to standard procedures using primary antibodies, anti-calreticulin diluted at 1:1000, anti-COSIV diluted at 1:1000, anti-58 k Golgi diluted at 1:1000, anti-β-actin diluted at 1:3000, anti-E-cadherin diluted at 1:1000, anti-Vimentin diluted at 1:1000, anti-N-cadherin diluted at 1:1000, anti-ERp29 diluted at 1:1000. Protein bands were visualized by Immobilon Western Chemiluminescent HRP Substrate.

2.6. Cell proliferation and migration

Cells were seeded in 96-well plates and cell proliferation was measured by Cell Counting Kit-8(CCK-8). The optical density (OD) at 450 nm was determined using a microplate reader. The migratory ability of HCC cells was detected by cell scratch assay. On the backs of 6-well plates a line was drawn every 1.0 cm with a marker pen perpendicular to the horizontal axis. Cells were grown to 70–90% confluency and a scratch was made along the marked line at the head perpendicular to the surface of plate. Cells were then incubated at 37 °C for various periods. Cells were photographed using an inverted microscope at 0, 24, and 48 h. ImageJ software was used to calculate average scratch width.

2.7. Dual luciferase reporter assay

The wild-type ERp29 UTR and a mutant ERp29 UTR containing the putative miR-483-3p binding sites; wild-type MEG3 UTR and a mutant MEG3 UTR containing the putative miR-483-3p binding sites were cloned into the pMIR-REPORT luciferase reporter plasmid. The luciferase vectors were transfected with the miR-483-3p mimics or negative control into HEK-293T cells using Lipofectamine 2000. Forty eight hours after transfection, cells were collected and lysed. The Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA) was used to detect the relative luciferase activity.

2.8. Immunohistochemistry

HCC paraffin sections were baked at 60 °C for 2 h, deparaffinized and rehydrated in xylene and graded ethanol respectively. Sections were submerged into citrate buffer (pH 6.0) and boiled for antigenic retrieval. Endogenous peroxidase inhibitor was utilized to quench

peroxidase activity. Rabbit anti-ERp29 was incubated with slides overnight at 4 °C. After washing in phosphate-buffered saline (PBS), slides were treated with enzyme labeled goat anti-mouse/rabbit lgG polymer secondary antibody, stained by 3,3′-diaminobenzidine (DAB) system and counterstained with hematoxylin, dehydrated and desiccated in ethanol and xylenes. Then the samples were observed under microscope after sealing with neutral balsam. The expression status of immunostaining was scored by two pathologists based on positive cells proportion and staining intensity. Scoring for percentage was as follows (A): 0% (0), < 10% (1), 10–50% (2), 50–80% (3), and > 80% (4). Scoring for intensity (B): negative (0), weakly positive (1), positive (2), strongly positive (3). Immunohistochemical score (IHS) = A × B.

2.9. Statistical analysis

Data analysis was carried out by SPSS version 20.0. Data were primarily tested for normal distribution and homogeneity test of variance. Independent *t*-test was used for comparison between two groups; One-way ANOVA was carried out for multiple comparisons. The data is represented by means ± SD, $p < 0.05$ was considered statistically significant. All the experiments were repeated three times independently.

3. Results

3.1. ERp29 identified by proteomics

We isolated ER from HCC cells using differential ultracentrifugation and density gradient centrifugation and confirmed the purity of ER by western blot (Fig. 1A). The mass spectrometric analyses of ER isolated from HCC cells grown in low glucose (LG) and high glucose (HG) were performed. According to mass spectrometric analysis results and Uniprot protein database, a total of 1362 proteins were identified of which 28 proteins were excluded owing to the lack of fluorescence intensity. 1334 proteins were included in subcellular location study to screen ER-associated proteins through analysis of Prolocate and Compartments databases. And then, 40 candidate proteins were identified by comparative study on the detection of fluorescence intensity of HG and LG. A screening standard for these proteins was carried out after NCBI database search according to the following criteria: a. the present proteomics results were identical to previous publications; b. there were no > 50 articles discussing the candidate proteins; c. there was significant difference of fluorescence intensity between HG and LG. Finally, ERp29 was identified as the candidate protein for subsequent analysis (Fig. 1B).

3.2. ERp29 expression in HCC cells and in HCC liver tissues

HCC cells were grown in LG or HG for 24 h, 48 h and 72 h. Western Blot analysis showed that the expression of ERp29 in HCC cells grown in HG was decreased in a time-dependent manner when compared to that in cells grown in LG (Fig. 2A). Immunohistochemistry showed that the expression of ERp29 in liver tissues of HCC patients with diabetes was remarkably lower than that in liver tissues of HCC patients without diabetes (Fig. 2B). The IHS further supported these observations (Fig. 2C). These results were further confirmed by Western blot which showed that expression level of ERp29 in liver tissues of HCC patients with diabetes was lower than that in liver tissues of HCC patients without diabetes (Fig. 2D). In addition, qPCR analysis showed that ERp29 mRNA expression was decreased in HCC patients with diabetes when compared to that in HCC patients without diabetes (Fig. 2E).

To further explore the clinical significance of ERp29 expression in HCC patients with or without diabetes, we divided all the patients into two groups: the ERp29 high expression group (above the median ERp29 expression, $n = 13$) and the ERp29 low expression group (below the median ERp29 expression, $n = 8$). The correlation between ERp29 expression and clinical features is summarized in Table 3. There was no significant correlation between ERp29 expression and patient's age, gender, HCV antigen, serum AFP, alcohol intake, tumor nodule number, vascular invasion and TNM in 21 HCC cases. However, we observed that the ERp29 was negatively associated with differentiation ($P = 0.006$), tumor size ($P = 0.007$) and T2DM (0.002) in HCC patients.

3.3. Effect of ERp29 on proliferation and migration of HCC cells

To evaluate the biological effect of ERp29 on HCC cells, ERp29 (pEGFP-ERp29-C1) plasmid and empty vector (pcDNA3.1) as well as siRNAs targeting ERp29 were constructed and transfected into HCC cells in HG (Fig. 3A). A CCK-8 test was performed and the results showed that the overexpression of ERp29 suppressed the proliferative capacities of HCC cells in comparison to the cells transfected with empty vector. Conversely, knocking down ERp29 with siRNA enhanced the proliferation of HCC cells (Fig. 3B). To determine whether ERp29 regulated HCC cell migration, a cell scratch assay was performed. We found that HCC cells overexpressing ERp29 showed a slower closing of scratch wounds when compared to the HCC cells transfected with empty vector. Reverse effects were observed in cells where ERp29 expression was silenced (Fig. 3C). Overall, these results demonstrated that ERp29 inhibited HCC cell proliferation and migration in HG.

3.4. ERp29 regulates epithelial-mesenchymal transition in HCC cells

Cancer cells acquire the ability to invade through the Epithelial-Mesenchymal Transition (EMT). A regulatory role of ERp29 on EMT has been found in breast cancer cells. In order to determine whether ERp29 could modulate EMT process in HCC cells, we analyzed the expression of EMT-associated markers (Vimentin, N-cadherin and E-cadherin) by Western blot. We found that that overexpression of ERp29 in cells grown in HG led to a decreased expression of epithelial biomarkers (E-cadherin) and an increase in the expression of mesenchymal markers (Vimentin, N-cadherin) (Fig. 3D). Converse was observed in the cells with a knockdown of ERp29. These findings suggested that ERp29 suppressed the EMT process in HCC cells which explains the increased migratory effects in HCC with lower expression of ERp29.

3.5. MiR-483- negatively regulates ERp29 in HCC cells

Five potential ERp29-associated miRNA (miR-483-3p, miR-29b-3p, miR-26b-5, miR-135a-5p, miR-376a-3p) were predicted and identified by the bioinformatics (TargetScan, Tarbase, Starbase). QPCR showed that among these 5 miRNAs, expression of miR-483-3p was significantly increased in HCC cells grown in HG compared to the cells grown in LG (Fig. 4A). To assess the biological effect of miR-483-3p on HCC cells, mimics and inhibitor of miR-483-3p were transfected into HCC cells (Fig. 4B). The binding sites of miR-483-3p to ERp29 3'UTR are illustrated in Fig. 4C. To investigate whether ERp29 was a functional target of miR-483-3p, dual-luciferase gene reporter assays were carried out. We found that the luciferase activity in ERp29-3′UTR-WT + miR-483-3p mimic group was significantly decreased when compared to the ERp29-3′UTR-WT + miR-483-3p-NC group. No significant difference in luciferase activity was observed between the ERP29-3′UTR-MUT + miR-483-3p mimic group and the ERp29-3′UTR-MUT + miR-483-3p-NC group (Fig. 4D). This finding revealed that MiR-483-3 targeted and negatively regulated ERp29. We also analyzed the effects of miR-483-3p on ERp29 expression by Western blot and found that miR-483-3p overexpression inhibited ERp29 expression in cells grown in HG compared to the control. Conversely, down-regulation of miR-483-3p caused an increase in the expression of ERp29 (Fig. 4E). To further confirm that the regulatory effect of HG on ERp29 is mediated by miR-483-3p, we first suppressed miR-483-3p expression in HCC cells followed by culturing these in medium with HG. Western blot analysis

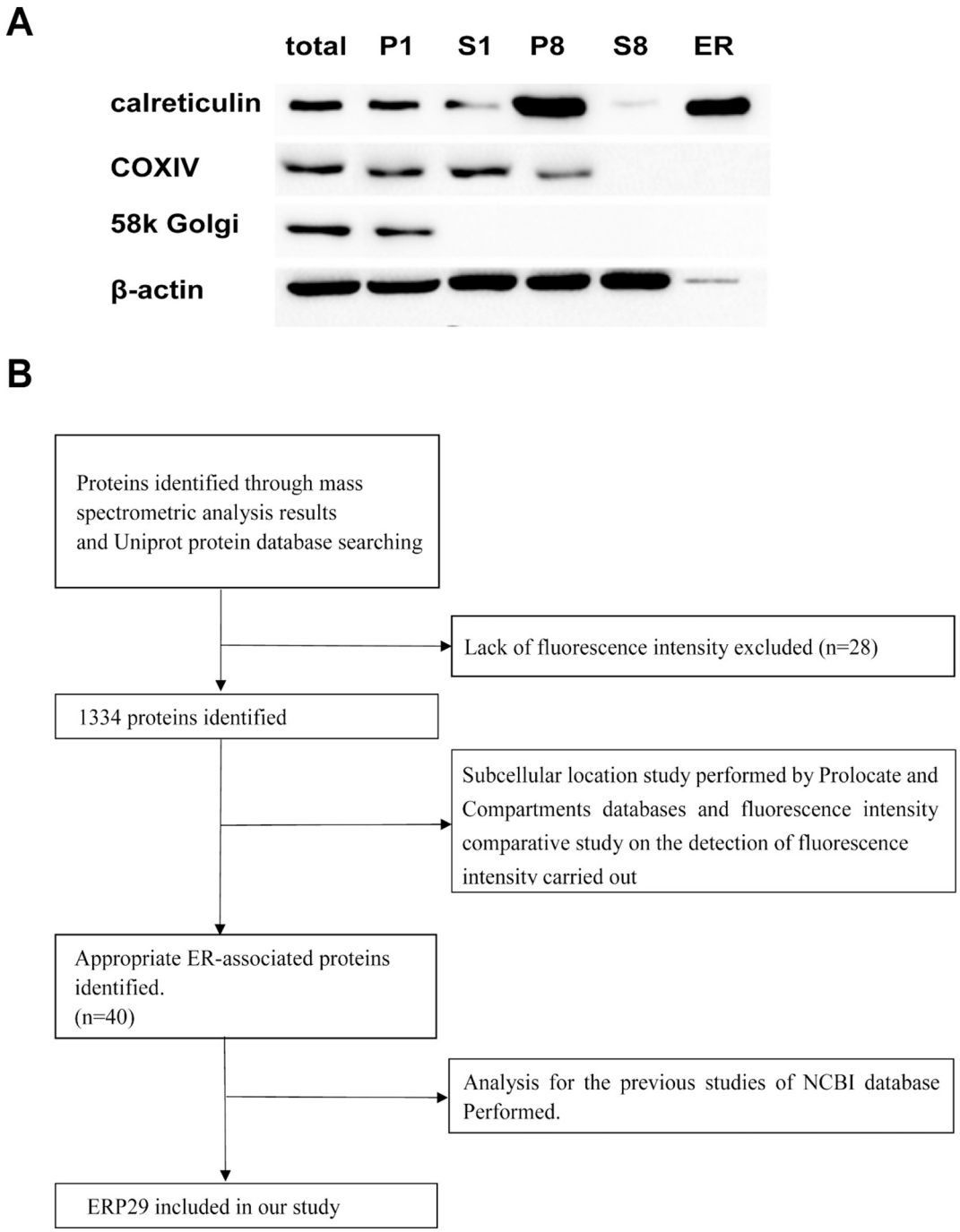

Fig. 1. ERp29 was identified by proteomics. A. Detection of organelle specific markers (total, total cell protein; P1, 1000 centrifuge after precipitation; S1, 1000 centrifuge after supernatant; P8, 8000 centrifuge after precipitation; S8, 8000 centrifuge after supernatant). B. Identification of ERP29 by proteomics.

indicated that there was no significant different between the ERp29 expression in these HCC cells when compared to the cells grown in LG (Fig. 4F). These findings suggest that HG suppresses ERp29 expression in a miR-483-3p-dependent manner. Furthermore, CCK-8 assay demonstrated that miR-483-3p overexpression promoted the proliferation of HCC cells (Fig. 4G). The scratch test indicated that HCC cells overexpressing miR-483-3p underwent a quicker closing of scratch wounds which suggested that miR-483-3p could promote HCC cells migration (Fig. 4H).

3.6. LncRNA MEG3 is downregulated in HCC cells in HG and promotes ERp29 expression by regulating MiR-483-3p

Three potential ceRNAs (LncRNA MEG3, LncRNA H19 and LncRNA SNHG14) of miR483-3p were predicted and identified by the bioinformatics (Lncbase v., Starbase v2.0). QPCR results suggested that the expression level of LncRNA MEG3 was significantly decreased in HCC cells grown in HG when compared to the cells grown in LG (Fig. 5A, B). In order to explore the effects of MEG3 on miR-483-3p and ERp29, plasmid expressing MEG3 or empty plasmid was transfected into HCC cells. The qPCR results showed that over-expression of MEG3 downregulated miR-483-3p expression when compared to the empty vector-transfected cells (Fig. 5C). Western blot revealed that the over-

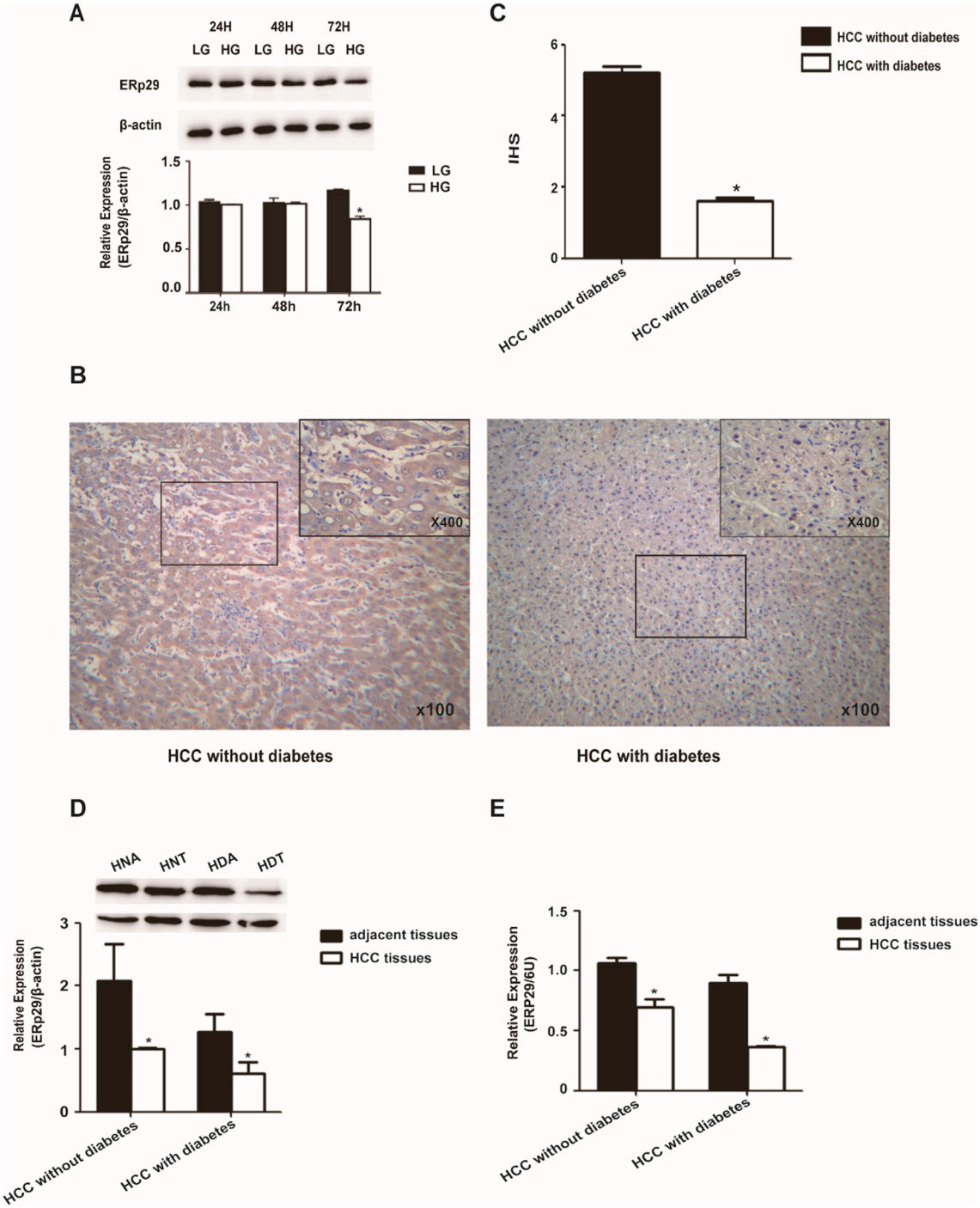

Fig. 2. ERp29 Expression in HCC Cells in HG and in liver tissues of HCC patients with or without diabetes. A. Expression levels of ERp29 under different glucose concentrations as measured by western blotting assay. B. Immunohistochemistry of ERp29 in liver tissues of HCC patients with and without diabetes (magnification, 100×, 400×; arrow, the location of ERp29). C. Immunohistochemical score (IHS) of liver tissues of HCC patients with and without diabetes (*$P < 0.05$ versus HCC patients without diabetes). D. Expression levels of ERp29 in liver tissues of HCC patients with and without diabetes (*$P < 0.05$ versus HCC patients without diabetes). F. Expression levels of ERp29 mRNA in liver tissues of HCC patients with and without diabetes (*$P < 0.05$ versus HCC patients without diabetes).

expression of MEG3 increased the expression of ERp29 when compared to the control cells with empty vector (Fig. 5D). In order to understand whether MEG3 mediates its positive effects on ERp29 via miR-483-3p, we first overexpressed miR-483-3p in HCC cells and then overexpressed MEG3 and analyzed the expression of ERp29 in these cells. We found that overexpression of MEG3 did not regulate the ERp29 expression when miR-483p was overexpressed, suggesting that the regulatory effect of MEG3 on ERp29 are mediated by miR-483-3p (Fig. 5E). The binding sites of miR-483-3p to MEG3 3′UTR are illustrated in Fig. 5F. To investigate whether MEG3 was a functional target of miR-483-3p, dual-luciferase gene reporter assays were carried out. We found that the luciferase activity in MEG3-3′UTR-WT + miR-483-3p mimics group was significantly decreased when compared to the MEG3-3′UTR-WT + miR-483-3p-NC group. No significant difference was observed in luciferase activity between the MEG3-3′UTR-MUT + miR-483-3p mimics group and the MEG3-3′UTR-MUT + miR-483-3p-NC group (Fig. 5G). This finding revealed that MEG3 was a target of miR-483-3p and negatively regulated by miR-483-3p. The CCK-8 assay demonstrated that

Table 3
Correlation between ERp29 expression and clinical parameters of HCC.

Parameter		ERp29		*P*-value
		Low expression	High expression	
All cases		8	13	
Age(years)	≤60	1	4	0.340
	> 60	7	9	
Gender	Male	6	12	0.271
	Female	2	1	
HBs antigen	Negative	7	2	0.001*
	Positive	1	11	
HVC antigen	Negative	7	12	0.716
	Positive	1	1	
Serum AFP(ng/ml)	≤20	6	9	0.776
	> 20	2	4	
Liver cirrhosis	Absence	2	6	0.332
	Presence	6	7	
Differentiation	High	0	2	0.006*
	Median	2	10	
	Low	6	1	
Alcohol intake	Absence	6	10	0.92
	Presence	2	3	
Tumor size(cm)	≤5	3	12	0.007*
	> 5	5	1	
Tumor nodule number	≤2	6	12	0.271
	> 2	2	1	
Vascular invasion	Absence	4	9	0.378
	Presence	4	4	
TNM	I-II	2	8	0.104
	III-IV	6	5	
DM	Absence	2	12	0.001*
	Presence	6	1	

overexpression of MEG3 suppressed the proliferation of HCC cells (Fig. 5H). Moreover, the scratch test showed that HCC cells overexpressing MEG3 underwent a slower closing of scratch wounds than the control HCC cells grown in suggesting that MEG3 suppressed HCC cell migration (Fig. 5I).

4. Discussion

This study investigated the underlying mechanisms of poor outcomes in HCC patients with diabetes. We found that HG upregulated miR-483-3p expression in HCC and that miR-483-3p overexpression suppressed ERp29 expression and increased HCC cell proliferation and migration. Furthermore, LncRNA MEG3 was decreased in HCC cells grown in HG and knockdown of MEG3 downregulated ERp29 expression. Bioinformatics analysis and luciferase reporter assay found MEG3 mediated its effects via binding to miR-483-3p.

ERp29 is widely expressed in various tissues [15]. Many studies have demonstrated that ERp29 plays a regulatory role in cancer [16]. For instance, overexpression of ERp29 was found to suppress the proliferation and migration of tumor cells in gastric carcinoma [22,23]. The carcinostatic function of ERp29 has also been found in breast cancer [24]. However, the role of ERp29 in HCC is not clear and the effect of blood glucose dysregulation on expression of ERp29 in HCC has not been reported. In this study, we provide the first evidence that ERp29 expression is downregulated in HCC cells grown in HG and in liver tissues of HCC patients with diabetes. We further showed that overexpression of ERp29 suppressed proliferation and migration of HCC cells whereas knockdown of ERp29 promoted it. These results suggest that ERp29 may act as a tumor suppresser, but HG may weaken the antitumor effects of ERp29 to enhance the proliferation and migration of HCC cells which might result in poor prognosis of HCC. However, the underlying mechanisms of effects of HG on ERp29 need to be

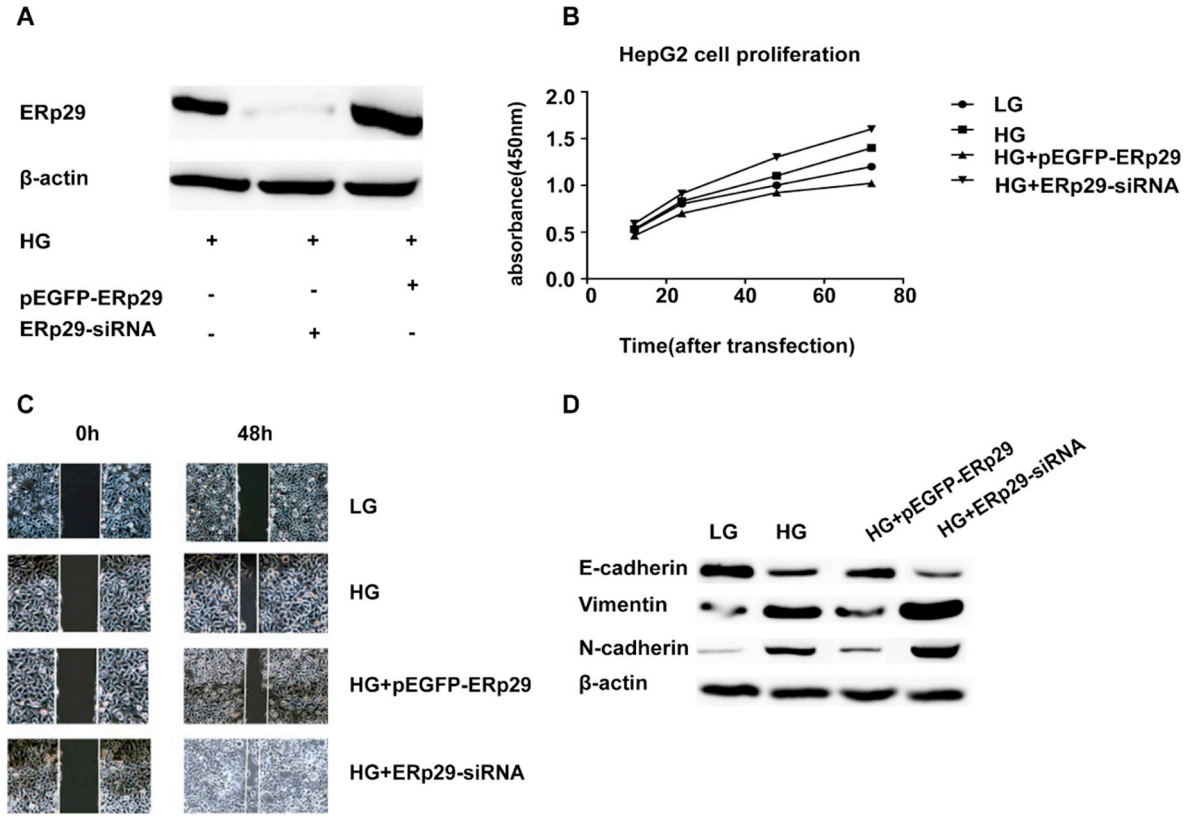

Fig. 3. Biological effect of ERp29 on HCC cells. A. The transfection efficiency of ERp29 was confirmed by Western blot assay. B. CCK-8 was used to evaluate the effect of ERp29 on HCC cell proliferation (magnification, 100 ×). C. Cell scratch assay was used to evaluate the effect of ERp29 on migratory ability of HCC cells. D. Regulation of Epithelial-Mesenchymal Transition process by ERp29 in HCC cells.

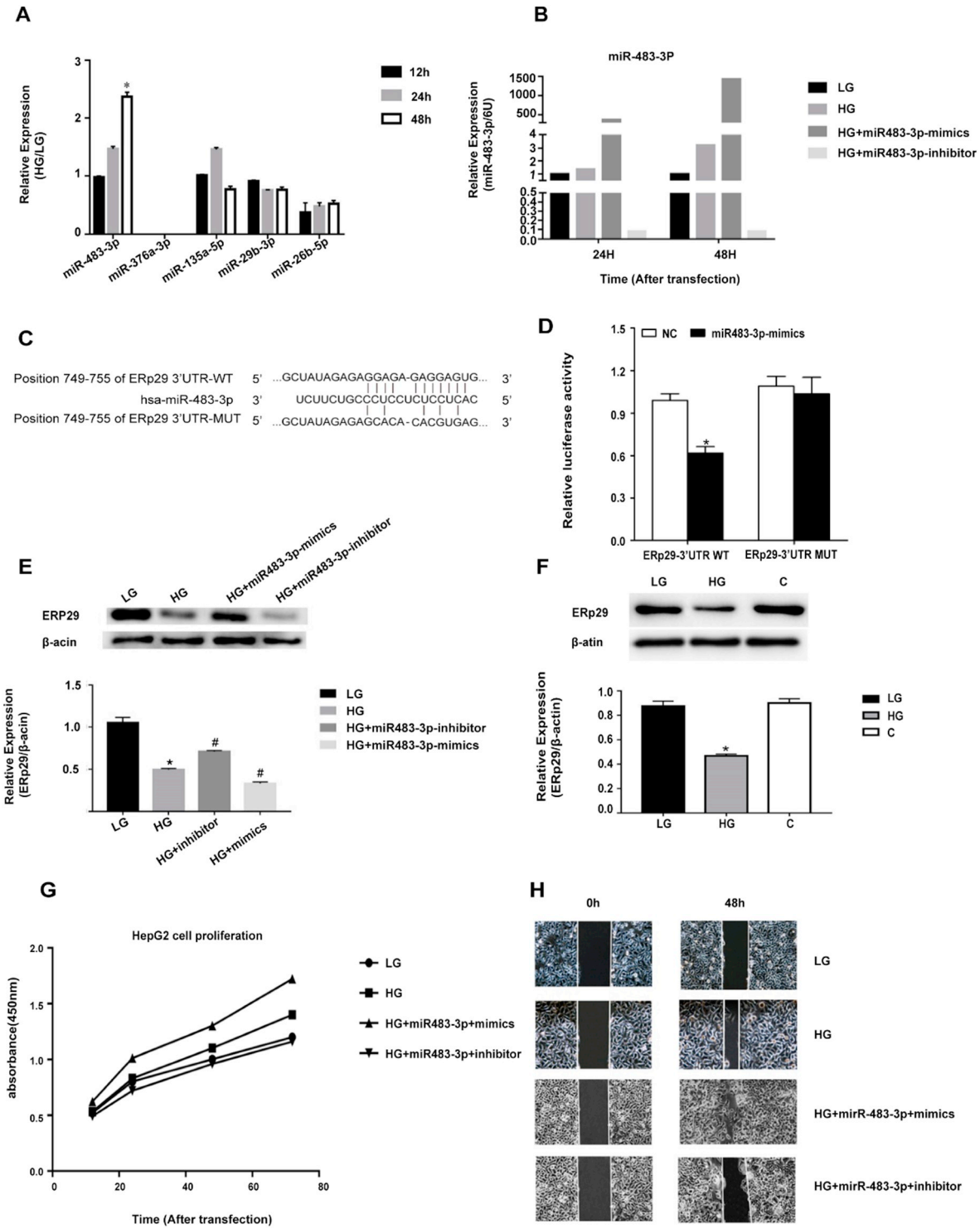

Fig. 4. Effects of MiR-483-3p on ERP29 in HCC cells. A. Expression levels of potential miRNAs in cells grown in LG or HG were measured by qPCR. B. The efficiency of miRNA483-3p transfection was confirmed with qPCR. C. The predicted miR-483-3p binding sites in ERP29-3′UTR (ERP29-3′UTR-WT) and the designed mutant sequence (ERP29-3′UTR-MUT) are indicated. D. Luciferase reporter assay of HCC cells co-transfected with ERP29-3′UTR-WT or ERP29-3′UTR-MUT and miR-483-3p mimics or NC (*$P < 0.05$ versus ERP29-WT + NC group). E. Western blot analysis for miRNA483-3p regulating the expression of ERp29 (*P < 0.05 versus LG, #P < 0.05 versus HG). F. Western blot analysis for the two-step transfection was adopted to assess the association between ERp29 and miR-483-3p, C group represents that suppressing miR-483-3p expression of HCC cells firstly and then culturing these HCC cells at high glucose (*P < 0.05 versus LG). G. CCK-8 was used to evaluate the proliferative capacity of miRNA483-3p on HCC cells (magnification, 100×). H. Cell scratch assay was used to evaluate the migratory ability of miRNA483-3p on HCC cells.

investigated.

MiR-483-3p was first identified in the second intron of the insulin-like growth factor 2 gene (IGF2) and since then, has been shown to play a role in various human malignancies. For instance, MiR-483-3p was found to a promote colorectal cancer [25]. In contrast, tumor suppressor function of miR-483-3p in breast cancer via its targeting of the

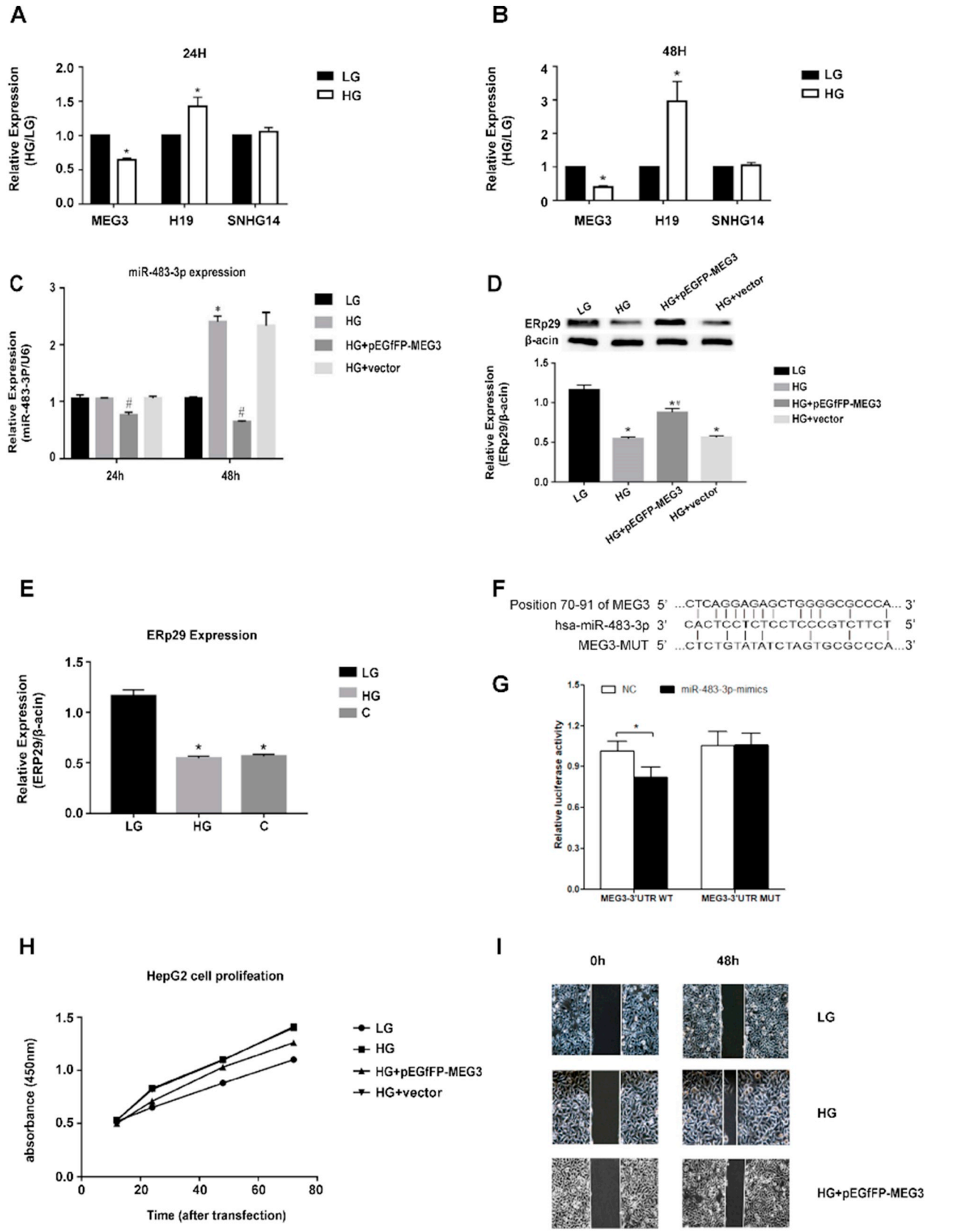

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Fig. 5. Expression of LncRNA MEG3 in HCC cells under HG and mediated by MiR-483-3p. A. Expression levels of potential lncRNAs in cells grown in medium with different glucose levels were measured by qPCR (*$P < 0.05$ versus LG). B. The qPCR analysis of effect of MEG3 on the expression of miRNA483–3p (*$P < 0.05$ versus LG). C. Western blot analysis of effect of MEG3 on the expression of ERp29 (*$P < 0.05$ versus LG, #$P < 0.05$ versus HG). D. Western blot analysis for the two-step transfection was adopted to assess the association between MEG3 and miR-483-3p, C group represents that miR-483-3p expression was upregulating in HCC cells first and then LncRNA MEG3 was overexpressed (*$P < 0.05$ versus LG). E. The predicted miR-483-3p binding sites in MEG3-3′UTR (MEG-3'UTR-WT) and the designed mutant sequence (MEG3-3′UTR-MUT) are indicated. F. Luciferase reporter assay of HCC cells co-transfected with MEG3-3′UTR-WT or MEG3-3′UTR-MUT and miR-483-3p mimics or NC (*$P < 0.05$ versus MEG3-WT + NC group). H. CCK-8 was used to evaluate the effect of MEG3 on proliferative capacity of HCC cells (magnification, 100×). I. Cell scratch assay was used to evaluate the effect of MEG3 on migratory ability of HCC cells.

cyclin E1 gene has also been confirmed [26]. To explore the potential role of miR-483-3p in HCC, we over- or under expressed miR-483-3p in HCC cells and analyzed cell proliferation and migration. As overexpression of miR-483-3p promoted cell proliferation and migration, these results indicate that miR-483-3p acts as an oncogenic factor. We also showed that miR-483-3p overexpression suppressed ERp29 expression and that miR-483-3p binds to ERp29 3′UTR suggesting that ERp29 is a target of miR-483-3p. In addition, we found that HG upregulated miR-483-3p expression in HCC cells. These results indicated HG may suppress ERp29 by upregulating miR-483-3p.

Maternally expressed gene 3(MEG3), a lncRNA, is located in the imprinted DLK1–MEG3 locus on human chromosome 14q32.3 [27]. Published studies have demonstrated that MEG3 is associated with cancer growth, progression, invasion and metastasis, and is decreased or lost in variety of tumors [28]. Some studies reported that overexpressed MEG3 suppressed cell proliferation and promoted apoptosis in HCC partially by the accumulation of p53 [29]. In addition, MEG3 could serve as a potential biomarker for predicting the survival of HCC as it was observed that HCC patients with lower expression of MEG3 had a worse relapse-free survival (RFS) than the HCC patients with higher MEG3 expression [30]. However, the underlying mechanisms of MEG3 in the pathogenesis of HCC remain unclear. It is reported that expression of MEG3 is decreased in human islet cells in diabetes which affects insulin production, secretion and resistance [31–34]. So, we wondered whether HG regulates the effects of MEG3 on the proliferation and metastasis of HCC cells. Our results revealed that the expression of MEG3 was decreased in HCC cells in the presence of HG and that MEG3 overexpression inhibited HCC cell proliferation and migration under HG conditions. LncRNAs could function as molecular miRNA sponges to negatively modulate the expression of miRNAs. For example, LncRNA H19 binds to miR-29a and downregulates the expression of miR-29a in glioma [35]. Similar negative associations are found in other lncRNA/miRNA such as SPRY4-IT1/miR-101-3p [36], MALAT1/miR-200c [37] and MIR31HG/miR-575 [38]. It was reported that MEG3 bound to miR-494 in hemangioma cells [39]. So, we hypothesized that MEG3 may act as a ceRNA which binds to miR-483-3p in HCC in HG. To confirm this notion, bioinformatics method and the dual luciferase reporter assay were conducted to verify the relation between MEG3 and miR-483-3p. The results showed that miR-483-3p bound to MEG3 in a sequence-specific manner. Moreover, we illustrated that overexpression of MEG3 downregulated miR-483-3p and increased the expression level of ERp29 in HG. We also found that MEG3 overexpression did not change the expression of ERp29 when in the presence of miR-483-3p was overexpressed, suggesting that MEG3 regulated ERp29 expression through miR-483-3p. These data revealed that MEG3 positively regulates post-transcriptional expression of ERp29 by sponging miR-483-3p in HCC. These data revealed that MEG3 might be a tumor suppresser. However, HG suppress expression of MEG3 to promote progression of HCC through up-regulating miR-483-3p and then inhibiting expression of ERp29. Through the above mechanisms, we speculated that the high blood glucose caused by DM could lead to poor outcomes of HCC.

There some limitations in this study. First, we did not explore specific functions of ERp29 in the development of HCC. Second, the sample size of the liver tissues and the paraffin sections of HCC with diabetes specimens and HCC without diabetes was small. Third, we lacked animal experiments to verify these results. Further studies are needed to address these issues and to confirm our findings.

5. Conclusion

Our study established a role for regulatory pathway of MEG3/miR-483-3p/ERp29 in HCC under the conditions of high glucose. Overall, our findings suggest that HG negatively regulates the expression of ERp29 by inhibiting the miRNA sponge role of MEG3 on miR-483-3p in HCC cells. However, as LncRNA can function as a ceRNA by binding to various miRNAs, it is important to explore whether other miRNAs are involved in the ceRNAs regulatory network in HCC cells under HG conditions. Furthermore, ERp29 may regulate the biologic functions of HCC cells through EMT process. Overall, these findings may explain the poor prognosis of HCC patients suffering from DM and may shed light on the development of novel therapeutic methods for HCC with DM.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Author contribution

X.L., S.Z., K.Z. and P.Y. originated and designed the study. X.L., T.C. Y·H did the experiments. Y.W., S.Z. and P.Y. drafted the paper. X.L. and P.Y. assured and controlled the paper's quality.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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