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Construction of klhdc7b Gene Knockout Human Normal Hepatocyte L-02 Stable Strain by CRISPR/Cas9 Technology and its Preliminary Biological Function Analysis

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Abstract

To knock out the klhdc7b gene in L-02 cells using CRISPR/Cas9 genome engineering technology, and screen klhdc7b knockout L-02 stable strains for analysis of its biological functions. Using the klhdc7b gene sequence retrieved from NCBI, three CRISPR sequences were designed for construction of three eukaryotic recombinant expression plasmids which could simultaneously express sgRNA, Cas9 and puromycin screening markers.After the activity of the transformants were verified by RT-qPCR, using respectively the three recombinant plasmids and transfection reagent infect L-02 cells in an optimal ratio. The stable klhdc7b knockout strains were screened through the stress of puromycin, and the knockout effect was detected by Western blotting.MTT assay and flow cytometry were used to detect the effects of klhdc7b on cell proliferation and apoptosis in normal L-02 cells and L-02 cells under tunicamycin-induced endoplasmic reticulum stress.Verified by RT-qPCR, the most effective guide RNA was sgRNA1, and Western Blot detection showed that the expression of klhdc7b protein in the monoclonal cells was significantly lower than that in the empty group and the normal group ($p<0.001$).MTT assay showed that klhdc7b had no significant effect on the proliferation of L-02 cells when they were in a normal state; when L-02 cells were in a state of endoplasmic reticulum stress, klhdc7b played a role in inhibiting cell proliferation. The results of flow cytometry showed that when L-02 cells were in a normal state, klhdc7b had no significant effect on cell apoptosis; when L-02 cells were in a state of endoplasmic reticulum stress, klhdc7b played a role in promoting cell apoptosis. The L-02 klhdc7b knockout stable strain was successfully constructed, and our preliminary experiments demonstrated that klhdc7b gene can inhibit proliferation and promote apoptosis in L-02 cells under ER stress.

Keywords

L-02; Gene Knockout; klhdc7b; CRISPR / Cas9; Proliferation; Apoptosis.

1. Introduction

With the change of people's lifestyle, viral hepatitis, fatty liver, alcoholic liver disease, liver failure and other liver diseases caused by liver damage caused by staying up late, alcohol abuse, accelerated pace of life, and increased work pressure are becoming more and more serious. It affects human life and health [1]. The previous research of our group found that in the model of cell injury induced by tunicamycin (TM)-induced L-02 endoplasmic reticulum stress (ERS) [2], shRNA (small hairpin RNA) silenced the endoplasmic reticulum stress marker factor C/Ebp-homologous protein (C/EBPho-mologousprotein, CHOP), and performed RNA-seq (RNA-sequencing).The klhdc7b (Kelch domain containing 7B) gene has a very significant differential expression, suggesting that there may be a close relationship between klhdc7b and CHOP, and plays an important regulatory role in endoplasmic

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reticulum stress, but there is no relevant report yet. klhdc7b encodes a protein of unknown function that contains a Kelch repeat β-helix that is known to bind other proteins in various ways [3]. It has been reported in the literature that klhdc7b is a potential epigenetic mark. Neven Papic et al. have shown that the expression of klhdc7b is up-regulated in cells infected with acute hepatitis C virus (HCV).The study of JeongG, BaeH et al. showed that klhdc7b is hypermethylated at the promoter, and the methylation levels of 14 CpG sites in the promoter region of this gene in 50 pairs of breast cancer tissue and adjacent normal tissue samples were found in cancerous tissues medium (72-93%) higher than normal tissue (31-83%). At the same time, it was found that its expression was up-regulated in breast cancer cell lines MCF-7 and MDA-MB-231 [4]. When klhdc7b is up-regulated, MCF7 apoptosis is reduced and proliferation is increased; while when klhdc7b is down-regulated, MCF7 apoptosis is reversed, indicating that it has oncogenic properties [5]. However, the encoded protein and its role in these cancers remain largely unknown. Kinnosuke Yahiro et al. found that klhdc7b is located in the cytoplasm and nucleus and is regulated by the PERK/ATF4/CEBPB/CHOP pathway. klhdc7b controls the pro-apoptotic protein Harakiri (HRK) belonging to the Bcl-2 family and is involved in apoptosis induced by Subtilase cytotoxin (SubAB) [6]. In addition to SubAB, interferon, TNF-α and IL-4 can also induce the expression of klhdc7b. Furthermore, siRNA silencing of klhdc7b inhibited HCV RNA replication and infectious virus production in cell culture. In addition, klhdc7b can also be used as a protective gene with alternative splicing function, which may be involved in the occurrence and development of cervical squamous cell carcinoma (CSCC), and can be used as a biomarker for the diagnosis and prognosis of CSCC [7].However, so far, there is no literature on the function of klhdc7b in human normal hepatocytes L-02, so this study will study the possible role of klhdc7b in L-02 cells. CRISPR (Clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) 9 system, that is, a pair of single-stranded guide RNA (single guide-RNA, sgRNA) targets the klhdc7b gene and recruits Cas9 nuclease to cut it [8], thereby knocking out the klhdc7b gene , to construct a stable Klhdc7b knockout cell line in L-02 cells and conduct preliminary cell biological function studies.

2. Materials and Methods

2.1 Experimental Materials

2.1.1 Plasmids, Strains and Cells

Plasmid pYSY-CMV-Cas9-U6-klhdc7b-sgRNA-EFla-puro and competent bacteria DH5α were purchased from Nanjing Yaoshunyu Biotechnology Co., Ltd. L-02 cells were preserved in the laboratory of the Scientific Research Center of the Basic Medical College of Henan University of Science and Technology.

2.1.2 Enzymes and Main Reagents

RPMI1640 culture medium was purchased from Beijing Soleibo Technology Co., Ltd.; 2xTaq MasterMix was purchased from Nanjing Novizan Biotechnology Co., Ltd.; cellular genomic DNA extraction kit was purchased from Beijing Soleibo Technology Co., Ltd.; PCR primers were purchased from Shanghai Sangon Biotechnology Co., Ltd. Engineering Co., Ltd.; BCA protein detection kit was purchased from Beijing Soleibao Technology Co., Ltd.; Lipofectamine 3000 was purchased from Invitrogen Company of the United States; Opti-MEM medium was purchased from Gibco Company; reverse transcription kit was purchased from Nanjing Novizan Biotechnology Co., Ltd.; qPCR reaction kit was purchased from Nanjing Novizan Biotechnology Co., Ltd.; Trizol was purchased from Beijing Soleibao Technology Co., Ltd.; Boaosen Biotechnology Co., Ltd.

2.2 Method

2.2.1 Design of sgRNA Oligo Sequences

Use the https://www.ncbi.nlm.nih.gov/ website to determine the gene sequence of the CDs region of klhdc7b, and the sgRNA sequence design reference http://crispr.mit.edu/ website [9]. The design principles are as follows: (1) The first exon is preferred for sequence analysis and target design. If

ISSN: 2414-1895 DOI: 10.6919/ICJE.202206_8(6).0007

exon1<100 bp, an exon of appropriate size will be selected in extension[8]. (2) According to the results of website analysis feedback, select a pair of sequences with higher scores [10], and remove the bases of the PAM sequence. (3) If the first base at the 5′ end of sgRNA is not G, it needs to be supplemented with G in front. (4) Using the sgRNA sequence as a template, design its complementary strand, and add enzyme cleavage sites at both ends. That is, CACC is added to the 5' end of the F chain of each sgRNA sequence, and AAAC[11] is added to the 5' end of the R chain, which is complementary to the sticky end formed after the pX330 plasmid is digested with BbsI (Table 1). The three pX330 recombinant plasmids are hereinafter referred to as pX330-klhdc7b-1, pX330-klhdc7b-2 and pX330-klhdc7b-3, respectively.

Table 1. The sequences of klhdc7b sgRNA oligo

Primer name	oligo DNA(5′→3′)
oligo1	oligo1-F: CACCGGTTAGAAGCCACCCCTTCCCC
	oligo1-R: AAACGGGGAAGGGGTGGCTTCTAACC
oligo2	oligo2-F: CACCGGCAGGGCCACCAGCTTGAGCT
	oligo2-R: AAACAGCTCAAGCTGGTGGCCCTGCC
oligo3	oligo3-F: CACCGGCCAGCTCAAGCTGGTGGCCC
	oligo3-R: AAACGGGCCACCAGCTTGAGCTGGGG

2.2.2 Construction and Identification of Recombinant Eukaryotic Expression Plasmids pX330-klhdc7b-1, pX330-klhdc7b-2 and pX330-klhdc7b-3

Firstly, the synthesized primers were gradient annealed with T4 PNK to form dimers, and the annealed oligo1, oligo2, and oligo3 were inserted into the pX330 vector digested by BbsI, and then the ligation product system was transformed into competent bacteria DH5α, and coated with On the ampicillin-resistant plate, single clones were picked the next day for sequencing and identification [12].

2.2.3 Culture and Transfection of L-02 Cells

L-02 cells were cultured in RPMI1640 medium, and transfection required cells to grow to approximately 80% confluence. Recombinant plasmids pX330-klhdc7b-1, pX330-klhdc7b-2 and pX330-klhdc7b-3 were extracted from the clones with correct sequencing and mixed with Lipofectamine 3 000 in an appropriate ratio according to the instructions of the transfection reagent, and incubated at room temperature for 15 minutes and then transferred into Cells were routinely cultured in a 37°C, 5% CO2 incubator.

2.2.4 Determination of Optimal Puromycin Concentration

(1) Microscopic observation method First, inoculate L-02 cells in a 96-well plate at 1×104 cells/well, and then culture them in a cell incubator at 37°C and 5% CO2. After the cells were completely attached, the original culture medium was discarded, and different concentrations of puromycin culture medium were added. The set concentration gradient was 0 μg/ml, 0.5 μg/ml, 0.75μg/ml, 1.5 μg/ml, 2 μg/ml, 3 μg/ml, 4 μg/ml, 6 μg/ml, 8 μg/ml, and change the cell culture medium with the same puromycin concentration every two days. The optimal puromycin concentration is the lowest concentration of puromycin that kills all cells within one week of continuous screening [13]. (2) MTT method L-02 cells were inoculated in 96-well plates at 1×104 cells/well, and culture medium containing different concentrations of puromycin was added to the administration group, and the concentration gradient was set as above, and cultured for 48 h. 4 h before the end of the culture, MTT was added, the absorbance A was detected, and the survival rate was calculated.

ISSN: 2414-1895 DOI: 10.6919/ICJE.202206_8(6).0007

2.2.5 Acquisition of klhdc7b Knockout Monoclonal Cells

The three recombinant plasmids were transferred into cells respectively, and were replaced with normal culture medium 8h after transfection. After 48h, 1μg/ml of puromycin was added for screening, and the screening medium was changed every two days. After 7 days of continuous screening, the respective monoclonal cell lines were obtained by limiting dilution method.

2.2.6 Validation of sgRNA1, sgRNA2 and sgRNA3 Activity by RT-qPCR

Collect the L-02 cells and the cells constructed with the three recombinant plasmids, isolate and extract total RNA from the cell samples using TRizol reagent [11], dissolve the extracted RNA in RNase-free water, and reverse it using a reverse transcription kit. Transcribed into complementary DNA (cDNA) [14]. The qPCR primers for amplifying the klhdc7b gene were designed. Using L-02 cells as a control and β-actin as an internal reference, qPCR was used to detect the expression levels of klhdc7b mRNA in the three cells respectively. The primer sequences are shown in Table 2.

Table 2. RT-qPCR primer

Primer name	Primer sequence (5′→3′)
klhdc7b	F: ACGGCCCCTCTTCTTCAGTG
	R: ATCCGTTGGTCTCGGAGCTT

2.2.7 Western Blot Detection of klhdc7b Protein Expression

Collect sgRNA-active cells transfected with pX330-klhdc7b-1 (knockout group), add 100 μl of lysis buffer (PMSF:RIPA=1:100), lyse on ice for 30 min, and use every 5 min Vortex once. The supernatant was collected by centrifugation, and the sample concentration was determined by the BCA protein concentration kit. At the same time, the protein extracted from L-02 cells (normal group) and the cells transfected with empty plasmid (pX330-NC) (empty group) were As a control, SDS-PAGE electrophoresis was performed. The samples were loaded according to the grouping order, electrophoresed first, then electrotransferred to PVDF membrane, blocked with 5% nonfat milk powder for 1 h, incubated with primary antibody (1:1500) and secondary antibody (1:3000), and finally exposed. Using β-actin as an internal reference, the expression levels of klhdc7b protein in the normal group, empty group and knockout group were compared by gray value analysis.

2.2.8 Detection of Cell Proliferation by MTT Assay

The cells of the normal group, the empty group and the knockout group were seeded in 96-well plates at 1×104 cells/well, with 3 replicate wells in each group. After the cells were completely adhered and cultured for 44 h, 15 μL of MTT was added to each well. Continue to culture at 37°C for 4 h, aspirate the medium, add 150 μL of dimethyl sulfoxide and shake for 10 min. A microplate reader was used to measure the A value of each well at the wavelength of 592 nm, and the average value of each group was taken to calculate the cell proliferation rate [cell proliferation rate (%) = (A value of the experimental group - A value of the blank group)/(A value of the control group) -Blank group A value) x 100%][15]. At the same time, three additional groups were set up to detect the proliferation rate of cells in the normal group, empty group, and knockout group under endoplasmic reticulum stress. Among them, the ERS model of L-02 cells was constructed using the effective protocol obtained in the previous research of our group, that is, 75 μM tunicamycin (TM) was added for 24 h after the cells were completely adhered to the wall, so the three groups were used. Named as normal+TM group, empty+TM group, knockout+TM group. The rest of the operation steps are the same as above.

2.2.9 Detection of Apoptosis by Flow Cytometry

The same as 2.2.8, the cells of the normal group, empty group, knockout group, normal+TM group, empty+TM group, and knockout+TM group were seeded in 6-well plates at 1×106 cells/well, respectively. Set 3 duplicate holes in each group. After the cells were completely adhered and cultured

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for 48 h, the cells of each group were collected. After washing, they were resuspended in binding buffer, and then Annexin V-FITC and PI were added respectively. After mixing, they were incubated in the dark for 15 min, and the apoptosis rate of cells in each group was detected by flow cytometry [16].

2.2.10 Statistical Method

SPSS 21.0 software was used for statistical analysis, and the experimental results were repeated 3 times independently. The experimental data were expressed in the form of mean±standard deviation (Mean±SD), and ANOVA method was used for difference analysis [17], where $P<0.05$ was considered to be statistically significant.

3. Result

3.1 Construction and Identification of Recombinant Eukaryotic Expression Plasmids pX330-klhdc7b-1, pX330-klhdc7b-2 and pX330-klhdc7b-3

The previously verified positive clones were sent to a sequencing company for sequencing. Through sequence comparison, it could be determined that the target plasmid was successfully constructed [18], see Figure 1 and Figure 2.

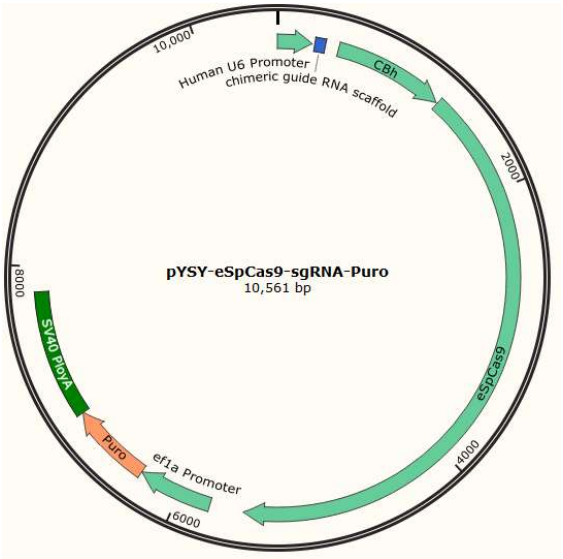
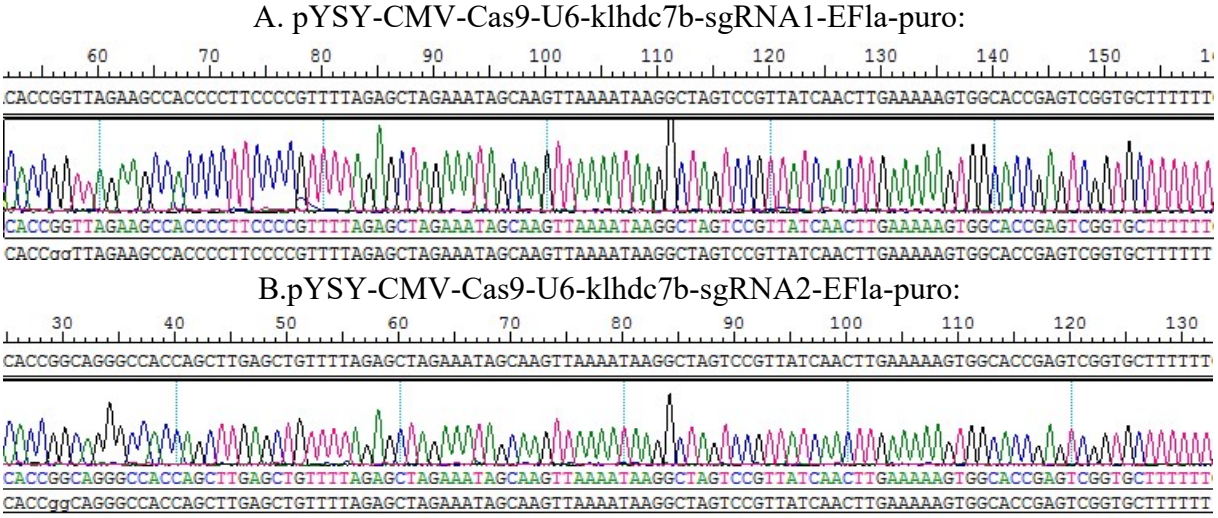


Figure 1. Related plasmid map



ISSN: 2414-1895 DOI: 10.6919/ICJE.202206_8(6).0007

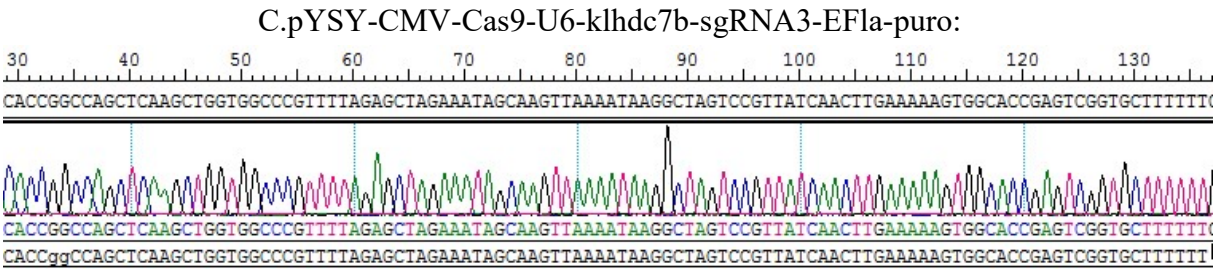


Figure 2. U6 primer sequencing comparison results.Note:A:pX330-klhdc7b-1 sequencing result;B:pX330-klhdc7bklhdc7b-2 sequencing result;C:pX330-klhdc7b-3 sequencing result

3.2 Determine the Optimal Ratio for Transfection

Use a 96-well plate to screen the ratio with higher transfection efficiency. Cells in logarithmic growth phase were seeded in 96-well plates at a density of 1×104 cells/well. When the cell density of each well is 80%, the empty vector plasmid with fluorescent label can be used for transfection. The normal culture medium was replaced 8 h after transfection, and the transfection effect was observed after 48 h [19], as shown in Figure 3. By comparison, the ratio of plasmid with higher transfection efficiency to transfection reagent is 1:1.5.

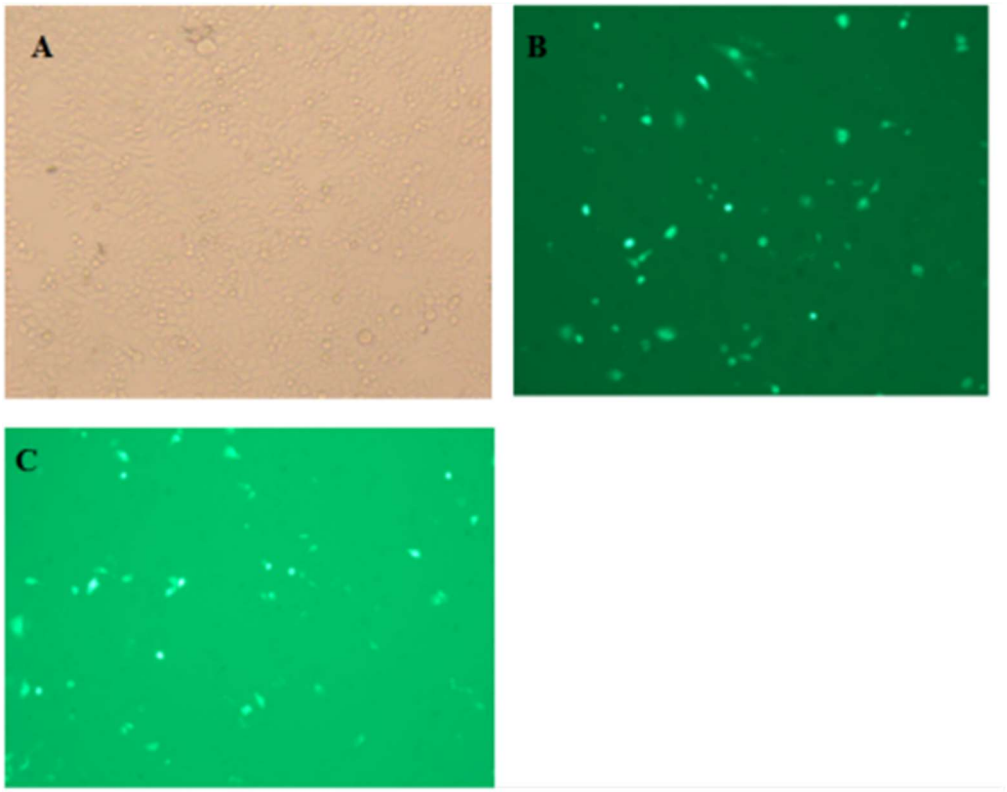


Figure 3. transfection efficiency(×200)

Note: A: untransfected L-02 cells; B: The ratio of plasmid to transfection reagent was 1:1.5; C: The ratio of plasmid to transfection reagent was 1:3

3.3 Determination of Optimal Puromycin Concentration

Through microscope observation, it was concluded that all cells in the wells died within one week when the concentration of puromycin c≥1 μg/ml. At the same time, combined with the results measured by the MTT method, as shown in Figure 4, it can be seen that the puromycin concentration of 1 μg/ml is the lowest puromycin concentration that causes all cells to die [13].

ISSN: 2414-1895 DOI: 10.6919/ICJE.202206_8(6).0007

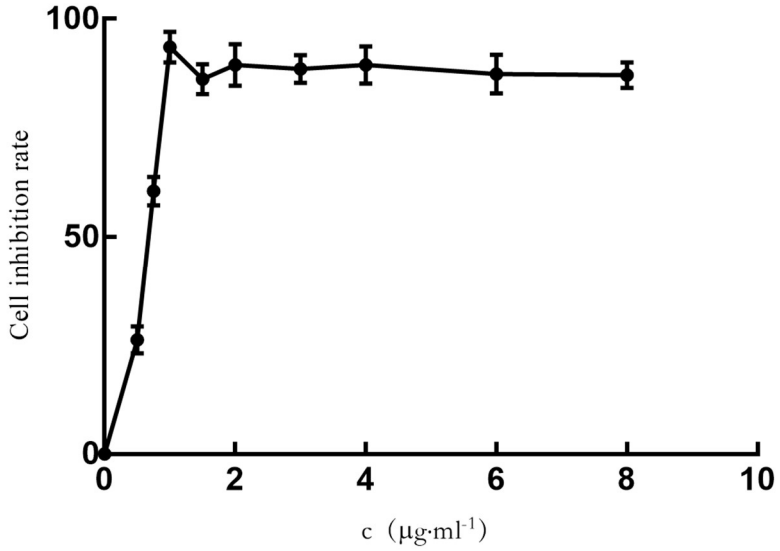


Figure 4. Effect of puromycin on L-02 activity of human normal hepatocytes(x±s, n=6)

3.4 Validation of sgRNA1, sgRNA2 and sgRNA3 Activity by RT-qPCR

Analysis of RT-qPCR results is shown in Figure 5. Compared with the L-02 cells of the normal group, the relative expression of mRNA in the cell lines transfected with sgRNA1 was significantly different.

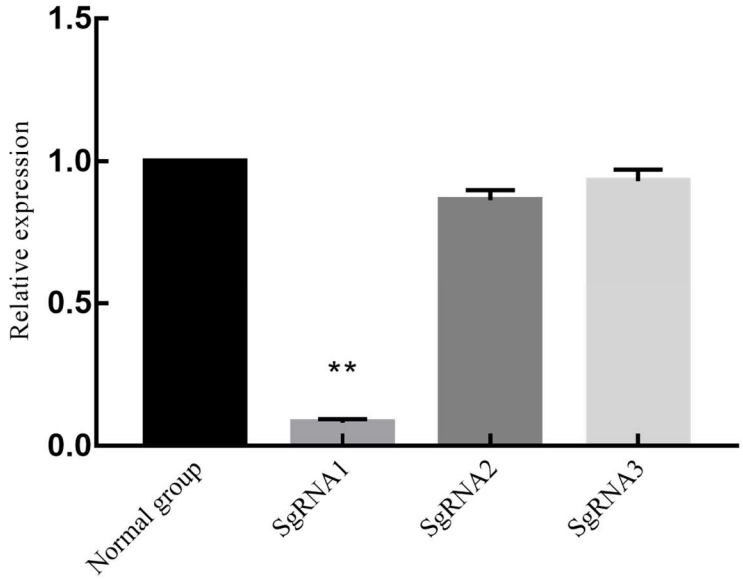


Figure 5. Expression of klhdc7b mRNA in L-02 cells after transfection(x±s,n = 3,compared with normal,* P < 0.05,**P < 0.01)

3.5 Western Blot Detection of klhdc7b Protein Expression

Compared with the normal group and the empty group, the expression of klhdc7b protein in the cells of the knockout group (ie, the cell line transfected with sgRNA1) was significantly decreased (Figure 6).

ISSN: 2414-1895 DOI: 10.6919/ICJE.202206_8(6).0007

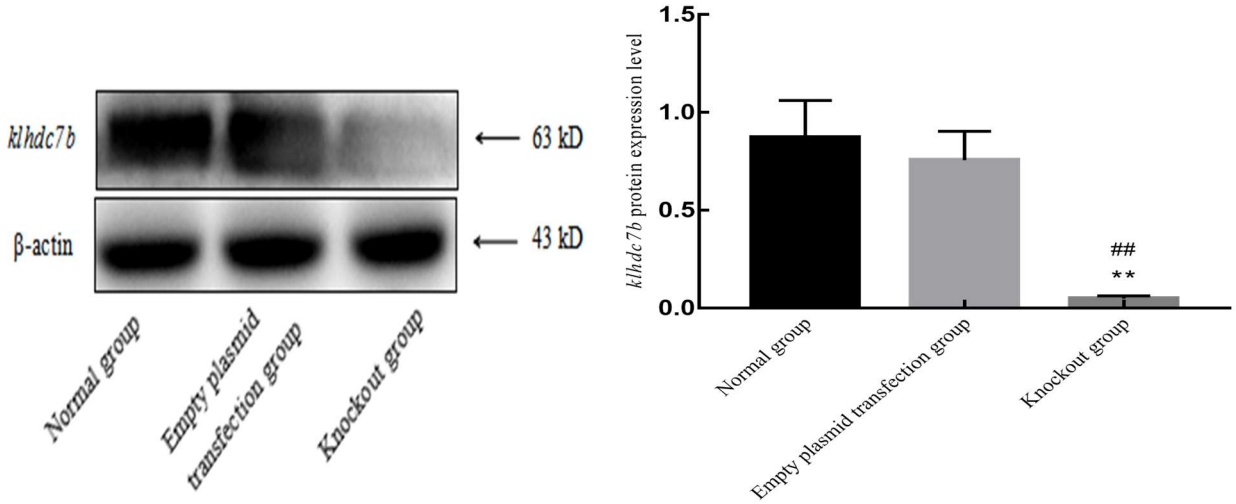


Figure 6. Expression of klhdc7b protein stably transfected by recombinant plasmid pX330-klhdc7b-1(x±s, n=3, *P < 0.05, **P < 0.01 compared with the control group,#p < 0.05, ##p < 0.01 compared with the sgRNA-NC group)

3.6 Detection of Cell Proliferation by MTT Assay

Compared with the normal group and the empty control group, the proliferation rate of the knockout group had no significant difference; the proliferation rate of L-02 cells in the normal + TM group after TM treatment was significantly decreased (P<0.01); Compared with the +TM group, the cell proliferation rate of the knockout +TM group was significantly increased (P<0.01), as shown in Figure 7. The results showed that the stable knockout of the klhdc7b gene significantly promoted the proliferation of L-02 cells induced by TM, and it was speculated that this gene played a role in inhibiting cell proliferation in L-02 cells under endoplasmic reticulum stress.

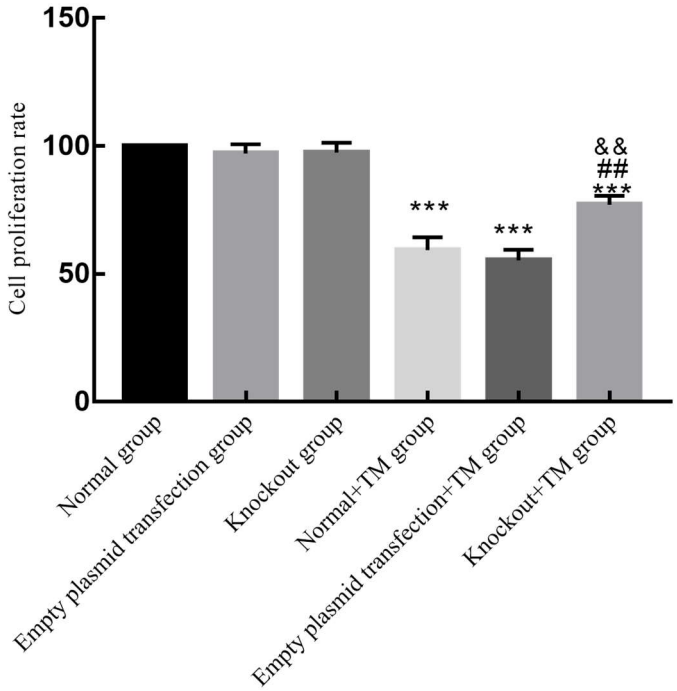


Figure 7. Cell proliferation rate of different groups (x±s, n=3, Compared with the normal group,*P<0.05, **P<0.01,***p<0.001; Compared with the normal + TM group,#P<0.05, ##P<0.01; Compared with no-load + TM group,&P<0.05, &&P<0.01)

ISSN: 2414-1895 DOI: 10.6919/ICJE.202206_8(6).0007

3.7 Detection of Apoptosis by Flow Cytometry

There was no obvious apoptosis of L-02 cells in the normal group. Compared with the normal group, the apoptosis rate of L-02 cells in the normal +TM group after TM treatment was significantly increased (P<0.01); compared with the normal +TM group and the empty +TM group, the apoptosis rate of the knockout group The rate was significantly decreased (P<0.01), see Figure 8. The results showed that the stable knockout of klhdc7b gene had a significant inhibitory effect on TM-induced apoptosis of L-02 cells, and it was speculated that this gene played a role in promoting apoptosis in L-02 cells when ER stress occurred.

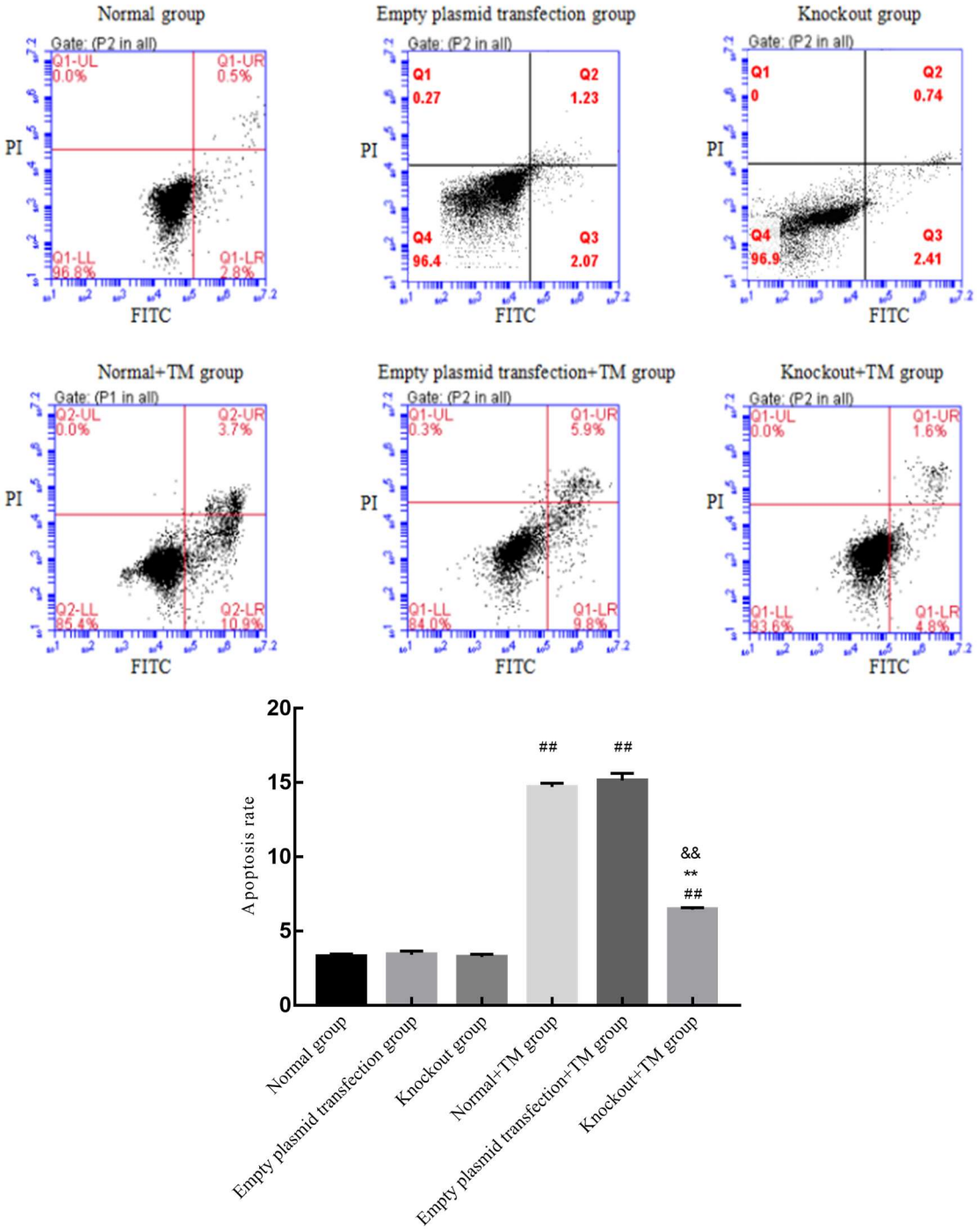


Figure 8. apoptosis rate of different groups (x±s, n = 3, #p < 0.05, ##p < 0.01 compared with the control group, * P < 0.05, ** P < 0.01 compared with the ERS group, &P < 0.05, &&P < 0.01 compared with the sgRNA-NC + ERS group)

ISSN: 2414-1895 DOI: 10.6919/ICJE.202206_8(6).0007

4. Discuss

The detailed protein structure and function of klhdc7b in normal and cancer cells have not yet been determined. In previous studies, klhdc7b was identified as a potential epigenetic marker, showing promoter hypermethylation in both breast cancer cell lines and cancer tissues, as klhdc7b is hypermethylated on promoters Klhdc7b protein may have anti-tumor or pro-tumor effects, and even have dual effects [20]; Changes in klhdc7b expression have significant effects on acute HCV infection: klhdc7b is highly upregulated during acute HCV infection, and silencing the klhdc7b gene inhibits HCV replication and infectious virus production. Papic N, Maxwell CI, Delker DA et al. Given the role of klhdc7b in actin binding, it is speculated that it plays a role in the cytoskeletal and membrane rearrangement required for efficient HCV RNA replication and secretion of infected virus [21]. and Yahiro K, Ogura K, Tsutsuki H et al. in 2021 showed that CHOP expression was attenuated in subtilase cytotoxin (SubAB)-induced endoplasmic reticulum stress in Hela cells with knockdown of klhdc7b, indicating that subab-induced klhdc7b expression It is regulated by the PERK/ATF4/CHOP/CEBPB signaling pathway, and klhdc7b also controls the expression of CHOP.They believe that klhdc7b is a novel mediator of cell death. Although the function of this gene is unclear, increased expression of klhdc7b has been observed in various tumor cells. However, its expression level is not always higher than in non-tumor tissues [22]. In human breast cancer MCF cells, the proliferation of cells overexpressing klhdc7b is decreased, while that of klhdc7b knockdown cells [3] is increased. In contrast, in their study, subab-induced apoptosis was shown to be significantly reduced in klhdc7b knockdown HeLa cells, suggesting that klhdc7b is associated with cell proliferation. So the role of klhdc7b may be different, depending on different tissues or stimulators [22]. The endoplasmic reticulum is very rich in hepatocytes and is one of the most sensitive tissues for ERS. ERS can cause hepatic insulin resistance, lipid accumulation, inflammation and hepatocyte apoptosis [23]. Therefore, ERS is an intracellular signal of liver homeostasis. , to study endoplasmic reticulum stress plays a crucial role in liver disease. Too low ERS is not conducive to the clearance of misfolded proteins, while too high ERS may activate inflammatory signaling pathways and hepatocyte apoptosis [24]. In the previous study of our group, by constructing shRNA targeting human CHOP gene, the results obtained by RNA-seq high-throughput sequencing found that when L-02 cells undergo endoplasmic reticulum stress, klhdc7b will be associated with the CHOP gene. Silence and significant down-regulation, but the specific regulatory mechanism and function of the klhdc7b gene remain unclear.

In this study, MTT method and flow cytometry were used to detect the cell proliferation rate and apoptosis rate of 6 groups, and the biological function of klhdc7b in non-tumor cells was preliminarily studied. According to the results, klhdc7b can inhibit cell proliferation and promote cell apoptosis when L-02 undergoes endoplasmic reticulum stress; however, under normal cell conditions, it has no significant effect on cell proliferation and apoptosis. . In view of this phenomenon, we speculate that only when L-02 undergoes endoplasmic reticulum stress, some stimulatory factors that promote the function of this gene are active or begin to play its role. At the same time, for the phenomenon of differential expression of klhdc7b before and after interference with CHOP, we can also reasonably predict that it may be regulated by CHOP and that there may be a close relationship between it and endoplasmic reticulum stress. These predictions will be established in our construction. On the basis of stable cell lines with good klhdc7b knockout, more in-depth studies are carried out.

Existing literature reports show that klhdc7b is closely related to tumor cells, and most of them are highly expressed in tumor cells. This phenomenon suggests that klhdc7b may be used as a cancer indicator. However, little research has been done on the regulatory role of klhdc7b in the process of endoplasmic reticulum stress in non-tumor cells or its relationship with CHOP and related factors of the pathway. It is known that klhdc7b may play an important role in the occurrence and development of endoplasmic reticulum stress.Therefore, it has far-reaching significance to study the function and regulatory mechanism of this gene. In this experiment, a stable Klhdc7b knockout L-02 cell line was successfully constructed, which provided an experimental basis for the later study of the regulatory

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mechanism of klhdc7b on ER stress-induced liver injury, and also provided new cell models for the treatment and prevention of clinical liver diseases.

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