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MicroRNA-145 suppresses hepatocellular carcinoma by targeting IRS1 and its downstream Akt signaling

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ABSTRACT

Accumulating evidences have proved that dysregulation of microRNAs (miRNAs) is involved in cancer initiation and progression. In this study, we showed that miRNA-145 level was significantly decreased in hepatocellular cancer (HCC) tissues and cell lines, and its low expression was inversely associated with the abundance of insulin receptor substrate 1 (IRS1), a key mediator in oncogenic insulin-like growth factor (IGF) signaling. We verified IRS1 as a direct target of miR-145 using Western blotting and luciferase reporter assay. Further, the restoration of miR-145 in HCC cell lines suppressed cancer cell growth, owing to down-regulated IRS1 expression and its downstream Akt/FOXO1 signaling. Our results demonstrated that miR-145 could inhibit HCC through targeting IRS1 and its downstream signaling, implicating the loss of miR-145 regulation may be a potential molecular mechanism causing aberrant oncogenic signaling in HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent malignancy worldwide and the third most common cause for cancer related death [1]. Although various genetic and epigenetic changes leading to HCC have been revealed, the molecular mechanisms underlying liver cancer are not fully elucidated [2]. Recent research advances highlighted the role of dysregulated microRNAs (miRNAs) in HCC [3]. MicroRNAs are a class of highly conserved short non-coding RNAs which silence target genes by inhibiting protein translation or inducing mRNA degradation. Through the influence on target genes, altered miRNA expression is capable to regulate tumor cell growth, apoptosis, angiogenesis and invasion in cancers [4]. In HCC, it becomes evident now that microRNAs can function as tumor suppressors or oncogenes.

MicroRNA-145 (miR-145) is a 22-nt miRNA whose genomic site is located in a fragile region of Chromosome 5q. MiR-145 has been noted for its under-expression in a variety of cancers including lung adenocarcinoma, bladder cancer, colon cancer, ovarian cancer and hepatocellular carcinoma [5–9]. It was reported that miR-145 possessed inhibitory effect on cancer cell proliferation and potentially functioned as a tumor suppressor [10]. Moreover, decreased level of miR-145 expression in hepatocellular carcinoma was correlated with poor histological grade and prognosis [11]. Previous study reported that miR-145 in HCC cells was associated with multiple mediators of insulin-like growth factor (IGF) signaling, which is a frequently over-activated oncogenic pathway in HCCs [12,13]. However, the detailed regulation mechanism of miR-145 in HCC was still under investigation.

In this study, we focused on miR-145 in HCC and its relationship with insulin receptor substrate-1 (IRS1), an intracellular adapter protein which transmits many key extracellular signals like insulin and IGF to downstream intracellular pathways. We demonstrated IRS1 as a direct target of miR-145 using Western blotting and luciferase reporter assay. Further, we observed the effect of miR-145 restoration on cell growth of HCC cell lines and investigated the underlying link to IGF/Akt signaling. Our results would complement current understanding for the role of miR-145 in HCC.

2. Materials and methods

2.1. Tissue specimens and cell culture

Paired liver cancer tissues as well as adjacent liver tissues were obtained from 48 patients who underwent liver resection at the

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First Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, PR China). The pathological diagnoses of all cancer samples were hepatocellular carcinoma. All tissue samples were collected and used according to the ethical guidelines and all procedures were approved by the institutional supervisory committee. Human HCC cell lines including PLC, Huh7 were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. Cells transfected with miR-145 were starved for 24 h prior to the addition of recombinant human IGF-1 (rhIGF-1, Everest Biotech) [14,15], and incubation continued as indicated for individual experiments.

2.2. Transfection

MiR-145 mimic, IRS1 siRNA and negative control RNA were purchased from Gene Pharma (Shanghai, China). Transfection was carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

2.3. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Isolation of total RNA from cells and clinical samples was performed using TRIZOL reagent (Invitrogen) following the instructions of the manufacturer. cDNAs were synthesized using Reverse Transcription System (Promega). MicroRNA was reversely transcribed using One Step Prime script miRNA cDNA Synthesis Kit and then quantified by real-time RT-PCR using SYBR Premix Ex Taq (TaKaRa). All PCR reactions were detected by ABI 7500 Fast system (Applied Biosystems, CA, USA). Endogenous U6 or GAPDH RNA levels were determined simultaneously for normalization using the delta delta Ct method. The primers used were followings: U6 (HmiRQP9001, GeneCopoeia, Inc), miR-145 (HmiRQP0192, GeneCopoeia, Inc), GAPDH (forward 5′-AAGGTGAAGGTCGGAGTC A-3′ and reverse 5′-GGAAGATGGTGATGGGATTT-3′), IRS1 (forward 5′-CAACTGGACATCACAGCAGAA-3′ and reverse 5′-ACTGAAATGGAT GCATCGTACC-3′).

2.4. Cell growth and colony formation assays

Cells were plated into 96-well plates at a density of 3000 cells per well and cultured at the indicated time points (24, 48 and 72 h) when cell growth was estimated by Cell Counting Kit-8 (CCK-8) (DoJinDo). For colony formation assay, cells were seeded in 6-well plate at a low density (1000 cells/per well) and cultured for 7 days. Then cells were fixed with 4% paraformaldehyde for 20 min and counted after staining with 1% crystal violet. The experiments were carried out in triplicate wells for at least three times.

2.5. Cell cycle analysis by flow cytometry

Cells were detached, washed with cold PBS and fixed with 70% ethanol at 4 °C overnight. Staining for DNA content was performed using a DNA Prep stain kit (Beckman Coulter, Fullerton, CA, USA). Cell cycle was measured by BD LSR II Flow Cytometry System with FACS Diva software (BD Bioscience, Franklin Lakes, USA).The data were analyzed using computational software ModFit LT.

2.6. Western blotting

Protein was extracted from cells using RIPA buffer. After centrifugation for 15 min at 4 °C at 14000*g*, upper supernatant was collected and protein concentration was measured with bicinchoninic acid (BCA) method. An amount of 30 μg protein was electrophoresed in SDS–polyacrylamide gels (Invitrogen) and transferred to polyvinylidenedifluoide membranes (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk, membranes were incubated with specific primary antibodies overnight. Then the membrane was washed for three times with TBST buffer, incubated with the corresponding horseradish peroxidase (HRP) -conjugated secondary antibody for 1 h and then developed onto X-ray films (Denville Scientific) using chemiluminescent reagents. Primary antibodies were as follows: IRS1 (#1692-1, Epitomics), FOXO1 (#1874-1, Epitomics), cyclinD1 (#2261-1, Epitomics), CDK6 (#3524-1, Epitomics), Akt (#1085-1, Epitomics) and pAkt (#2118-1, Epitomics), pFOXO1 (#9461, cell signaling), β-actin (#4967, cell signaling).

2.7. Luciferase assays

The 3′-UTR of human IRS1 was amplified by PCR using the following primers: forward primer: 5′-TGTGCTCGAGTAGGGCCATTCT TTTGCCCA-3′; reverse primer: 5′-GTTTTGCGGCCGCAGAGCAGAAAT GAACAGACTGGA-3′. Then the fragment was cloned into psicheck-2 vector and was confirmed by sequencing. MUT 3′-UTR of IRS1 was constructed from wild-type 3′-UTR of IRS1 using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol, and was verified by sequencing. For luciferase assay, 293T cells were plated on 24-well plates and transfected with 100 ng of luciferase reporter vectors (WT, MUT) and 50 pmol of miR-145 or negative control precursor. Forty-eight hours after transfection, firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay (Promega).

2.8. Statistical analysis

Data were expressed as mean ± SD, Student's *t*-test or ANOVA was used when appropriate. A *P*-value <0.05 was considered to be statistically significant.

3. Results

3.1. MiR-145 expression is down-regulated in HCC tissues and inversely related with IRS1 levels

In 34 of 48 HCC cases, quantitative reverse transcription polymerase chain reaction showed miR-145 levels were down-regulated when compared with corresponding adjacent nonmalignant liver tissues. Significant down-regulation (>0.5-fold reduction) of miR-145 was observed in 22 of 48 HCC tissues (45.8%) (Fig. 1A). On the contrary, IRS1 expression level was increased in 66.7% (32/48) of HCC tissues, of which 26 tissue samples showed more than two folds elevation (Fig. 1B). These findings were examined as statistically significant by paired *t*-test (Fig. 1C and D). Further, we compared the expression level of IRS1 in HCC tissue groups classified by miR-145 expression level. We found that the expression levels of IRS1 were higher in low level miR-145 expression HCC group than those in high level miR145 group (Fig. 1E). These results indicated that decreased miR-145 expression is a frequent event in human HCC, and it was closely associated with IRS1 over-expression.

3.2. MiR-145 directly targets IRS1 in HCC cells

We further examined the expression level of endogenous miR-145 in Huh7 and PLC HCC cell line cells by qRT-PCR and it showed that miR-145 in HCC cell line was greatly down-regulated compared to non-tumoral liver tissues (Fig. 2A). Transfection of synthetic miR-145 mimic RNA was able to restore its expression near to the physiological status in Huh7 and PLC HCC cell line cells (Fig. 2A). To demonstrate the relationship between miR145 and

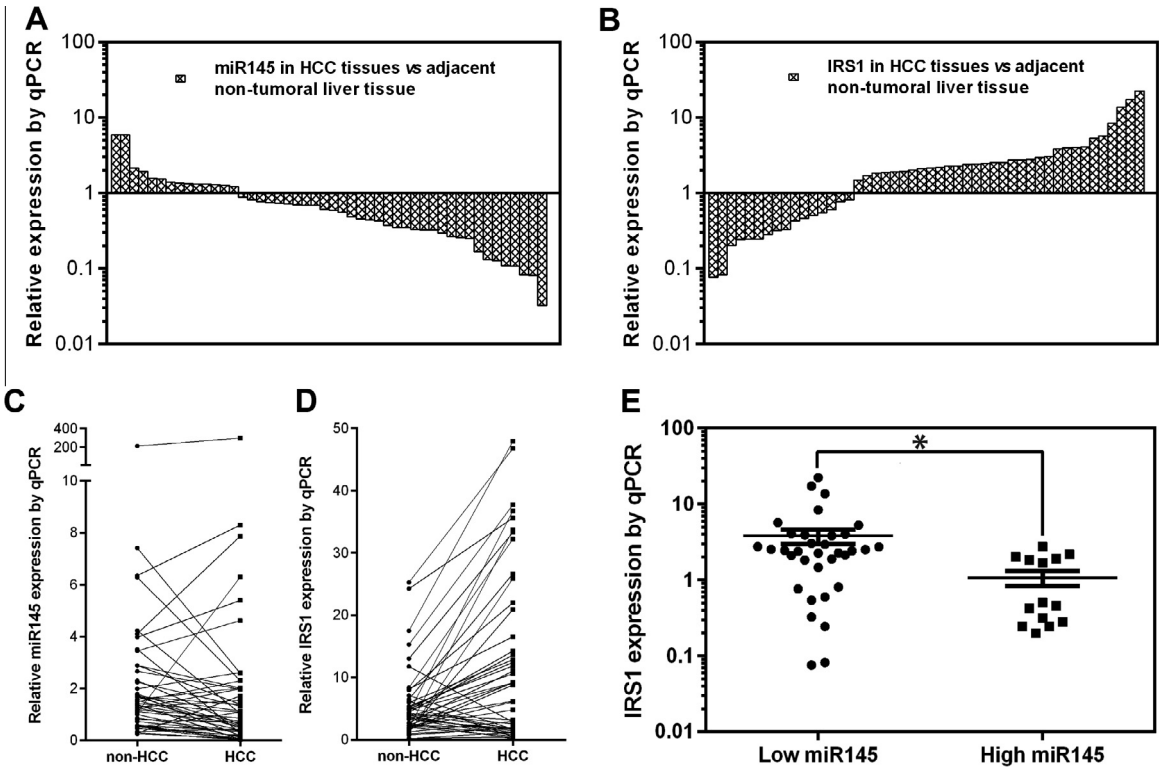

Fig. 1. MiR-145 and IRS1 expression levels in Hepatocellular carcinoma (HCC) tissues by quantitative reverse transcription-polymerase chain reaction (RT-PCR). (A) MiR-145 expression level was decreased in 34 of 48 HCC tissues. (B) IRS1 expression level was increased in 32 of 48 HCC tissues. (C) Paired comparison showed MiR-145 was downregulated in HCC tissues contrast to adjacent non-tumor tissues, $^{*}P < 0.001$. (D) Paired comparison showed IRS1 was overexpressed in HCC tumors compared to adjacent non-tumoral tissues. $^{*}P < 0.001$. (E) Expression levels of IRS1 were higher in low miR-145 HCC group (34 cases) than those in high miR145 group (14 cases). $^{*}P < 0.05$.

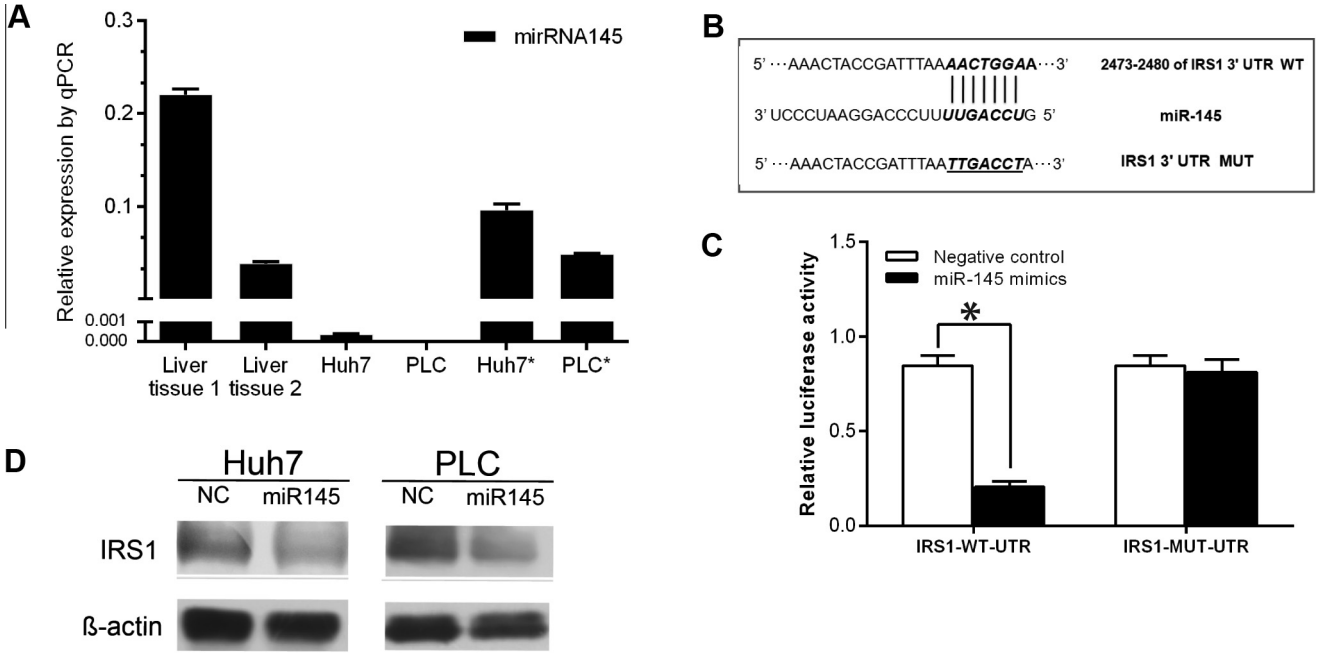

Fig. 2. IRS1 is a direct target of miR-145. (A) Examination of endogenous miR-145 levels in Huh7 and PLC cells showed significant miR-145 down-regulation when compared with normal livers by qRT-PCR. *Huh7 and PLC cells with miR-145 mimic transfection. (B) The putative miR-145 binding sequence in the 3′UTR of miR-145 mRNA. Mutation was generated on the IRS1 3′UTR sequence in the complementary site for the seed region of miR-145. (C) Luciferase reporter assay in 293T cells transfected with reporter vectors containing wild type or mutant 3′UTR of IRS1. Relative firefly luciferase expression was normalized to renilla activity after transfection. $^{*}P < 0.001$. (D) IRS1 protein levels are down-regulated in Huh7 and PLC cells transfected with miR-145 mimics.

IRS1, putative miR-145 binding site was found in the 3′-UTR of IRS1 mRNA by computational algorithms (Fig. 2B) and was cloned into the dual-luciferase reporter vector. Then we co-transfected either miR-145 or negative control RNA with the dual-luciferase expression vector containing wild-type or mutant 3′UTR fragment of IRS1, respectively. We found that exogenous miR-145

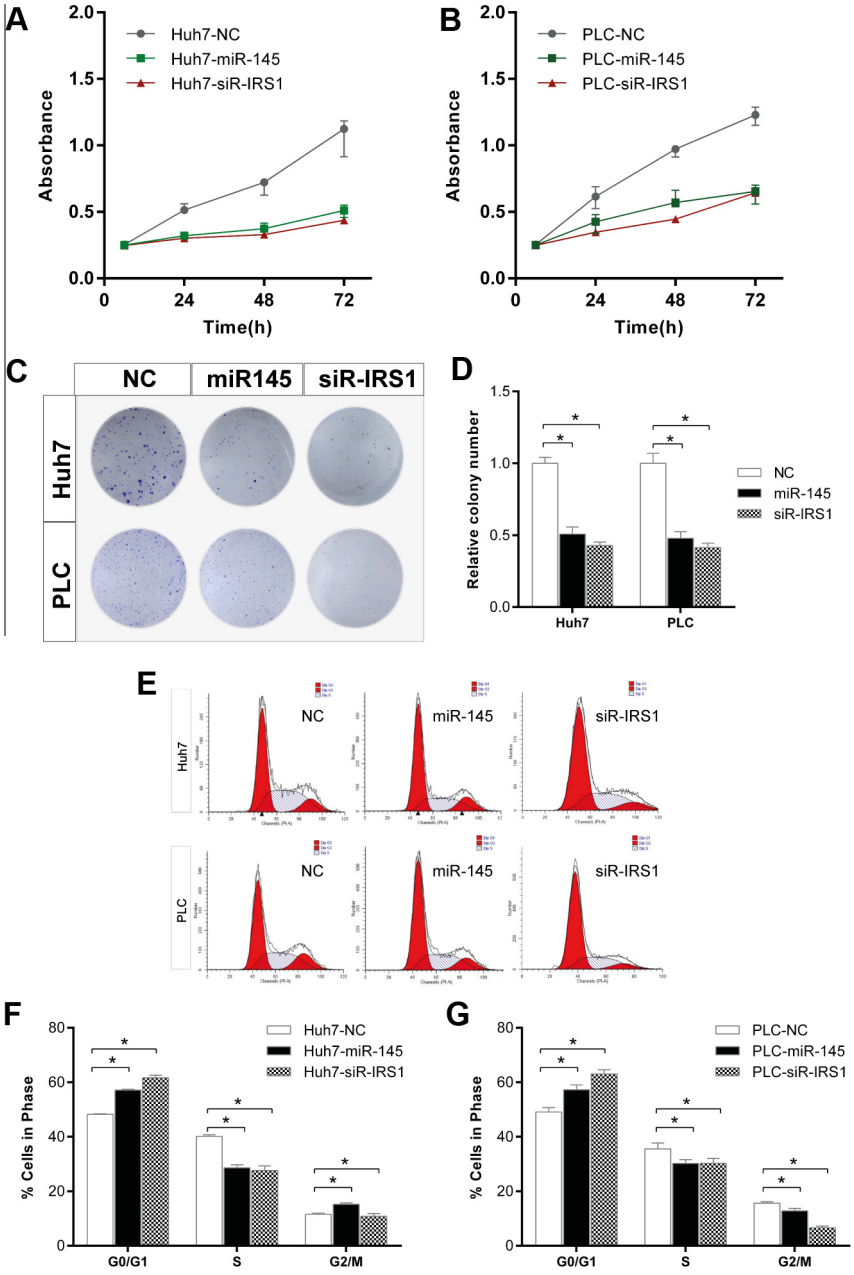

Fig. 3. Restored expression of miR-145 suppressed HCC cell proliferation and growth. (A, B) Cell growth of Huh7 and PLC HCC cells were significantly inhibited under miR-145 mimics and IRS1 siRNA transfection treatment. $^*P < 0.001$. (C) Colony formation assay in Huh7 and PLC HCC cells under miR-145 mimics and IRS1 siRNA transfection treatment. (D) Quantitative analysis of colonies formed by Huh7 and PLC HCC cells, $^*P < 0.01$. (E) Representative flow cytometry analysis of cell cycle of Huh7 and PLC cells. (F, G) The alteration of cell cycle distribution was analyzed. The experiment was repeated in triplicate. MiR-145 restoration and IRS1 silencing induced a significant accumulation of G1-phase cells and blocked G1/S transition. $^*P < 0.01$.

expression obviously suppressed the luciferase activity of wide-type IRS1 site, but the activity of the mutant IRS1 site was not affected (Fig. 2C), which suggested that IRS1 is directly targeted by miR-145. Moreover, we transfected miR-145 into Huh7 and PLC HCC cells, and the Western blotting showed that IRS1 protein expression was greatly decreased under miR-145 treatment (Fig. 2D), which was in consistent with the above mentioned finding in HCC tissues.

3.3. Restored expression of miR-145 suppressed HCC cell proliferation and growth

To determine the functional effect of miR-145 in HCC cells, we restored miR-145 expression by transfecting miR-145 mimics to HCC cell lines. For examining whether miR-145 acts via modulating IRS1, we knockdowned the IRS1 expression of HCC cells by siRNA treatment, and then its effect was compared to restored miR-145 expression. Cell growth assay showed that restored expression of miR-145 could efficiently inhibit proliferation of Huh7 and PLC cells (Fig. 3A and B). The inhibitory effect of miR-145 on HCC cells was further affirmed by colony formation assay. In contrast to cell lines treated by negative control, the colonies formed by miR-145 transfected HCC cells were markedly fewer and smaller (Fig. 3C and D).

To study the mechanisms of miR-145 contributing to growth inhibitory effects, we transfected Huh7 and PLC cells with miR-145 to examine cell cycle alterations subsequently. Flow cytometry analysis revealed that expression of miR-145 increased the number of cells in G0/G1 phase of cell cycle (48.39–57.12%), while the *S* phase decreased from 39.16% to 28.63% in Huh7 cells

(Fig. 3E and F). Similar results were found in PLC cells with an increase in G0/G1 phase (48.55–57.4%) and a decrease in *S* phase (34.77–30.64%) (Fig. 3E and G). These results indicated miR-145 triggered G1/*S* transition arrest of HCC cells.

In all these assays, we found that IRS1 knockdown had similar anti-growth effects as restoration of miR-145, which indicated miR-145 may function through down-regulating IRS1 (Fig. 3A–G).

3.4. MiR-145 induced IRS1 under-expression potentially reduced downstream AKT signaling

It is known that Akt signaling is one of the major molecular pathways under IRS1 and Akt phosphorylates and affects multiple downstream effectors, including FOXO1 which plays an important role in control of cell proliferation [16]. In this study, we found phosphorylated Akt and FOXO1 were both markedly reduced after transient miR-145 expression in the HCC cell line Huh7 and PLC (Fig. 4A), which suggested that miR-145 suppressed Akt signaling via IRS1 reduction. Moreover, Western blotting showed that the expression of cyclin D1, a key cell cycle initiator under FOXO1 regulation, decreased as a consequence of down-regulated Akt/FOXO1 phosphorylation. Our experiments also showed that the effect of miR-145 on IRS1/Akt/FOXO1 signaling activity was fully in accordance with IRS1 silencing treatment (Fig. 4A). Moreover, we found that IGF-1 stimulation could reverse the effect of miR-145 on IGF signaling pathway in HCC cells (Fig. 4B), which validated the mechanism of miR-145 modulating IRS1/Akt/FOXO1 axis, as summarized in Fig. 4C.

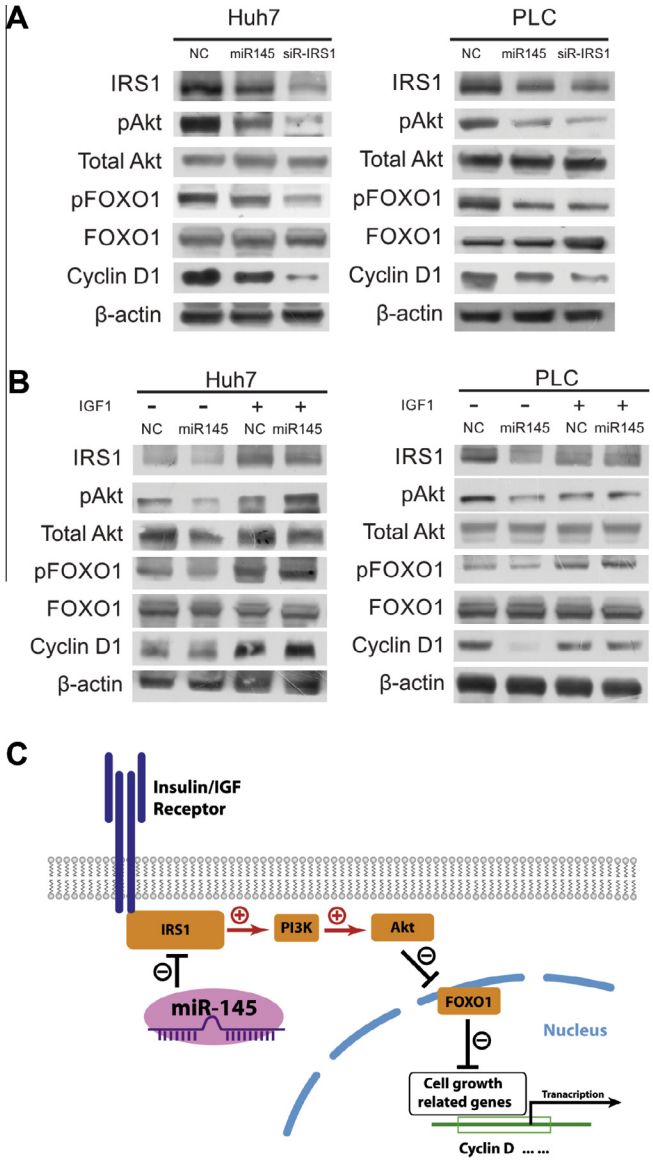

Fig. 4. The effect of miR-145 on Akt/FOXO1 pathway. (A) Over-expression of miR-145 or silencing of IRS1 markedly decreased the phosphorylation level of Akt and FOXO1 and lower cyclin D1 expression. (B) Stimulation of rhIGF-1 (125 ng/ml) for 24 h could reverse the effect of miR-145 on the phosphorylation of Akt and FOXO1 as well as the expression of cyclin D1. (C) The hypothetical diagram of miR-145 in regulating IRS1/Akt/FOXO1 pathway.

4. Discussion

Emerging role of dysregulated microRNAs in cancer has been proved by many researches. Therefore, better understanding of miRNA in pathogenesis of malignancy may help find more effective cancer therapy. Under physiological condition, miR-145 is reported to regulate biological processes in determining vascular smooth muscle cell phenotype and stem cell differentiation [17,18]. Moreover, there are growing evidences indicating miR-145 acts as a tumor suppressor in various tumors including HCC. The precursor sequence of miR-145 is located on Chromosome 5q, which has been showed to be a frequent loss of heterozygosity (LOH) linked to hepatocarcinogenesis [19]. Previous study in microRNA profiling of HCV associated HCC showed that miR-145 was progressively down-regulated from cirrhosis via dysplastic nodules to HCCs and even further to metastasis [11]. It is reasonable to postulate that miR-145 contributes to the development of HCC with enhanced malignant potential. Our study confirmed that miR-145 under-expression was a frequent event in human HCCs from varied etiologic background. Restored expression of miR-145 was able to suppress cell proliferation and induce cell cycle arrest in hepatocellular carcinoma cells, which further demonstrated the tumor suppressing function of miR-145 in HCC.

It is well known that IRS1 over-expression occurs as an early event in hepatocarcinogenesis and promotes cancer proliferation and invasion [20–22], and our study has proved that IRS1 silencing could inhibit the growth of HCC cell lines. Although IRS1 expression presents at low levels in normal hepatocytes, IRS1 protein over-expression was commonly identified in HCC tumor tissue and cell lines [23]. IRS1 was predicted to be one target of miR-145 and it was affirmed in our current study. We showed by quantitative RT-PCR that miR-145 was decreased in HCC tissue samples compared to adjacent non-tumor tissues while IRS1 was inversely over-expressed. The analysis of IRS1 expression levels between low and high of miR-145 expressing HCC tissue samples was pretty meaningful, and further validation by luciferase reporter assay and Western blotting demonstrated the relevance between miR-145 and IRS1 in advance.

IRS1 transmits signals from insulin or IGF receptor and activates PI3K/Akt and MAPK pathways, both of which were critical in mitogenesis and oncogenesis [23,24]. In our study, we focus on the effect of down-regulated IRS1 on Akt signaling, for constitutive activation of Akt signaling was reported in 40–60% of human HCC [2]. Phosphorylated Akt can drive cellular proliferation and protect cells from apoptosis by phosphorylating its downstream mediators or effectors. FOXO1 and FOXO family are downstream targets of Akt and transcriptionally activated genes that promote cell apoptosis, cell-cycle arrest, and turn down cellular metabolism. Akt inhibits FOXO1 function by phosphorylating FOXO1 and then prompting its nuclear exclusion, which down-regulate FOXO1-mediated transcription activity [25–27]. Our study suggested that miR-145 induced IRS1 reduction could decrease the phosphorylation of Akt and in turn sustained FOXO1 activity. It was consistent with the findings that cyclin D1 was decreased in

miR-145 treated HCC cells and therefore cell cycle was slowed down. Based on the importance of Akt/FOXO1 axis in cells, we may further propose that miR-145 could play a more critical role in regulating cellular metabolism, differentiation, and transformation [28].

In summary, our study suggested that miR-145 regulates IRS1 expression in HCCs and modulates IRS1/Akt activity, which is in control of tumor growth and progression. Our findings would provide more detailed miR-145 related mechanism in HCC and may implicate some clues for future development of diagnostic and therapeutic application.

Acknowledgments

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