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# The effect of knockdown of insulin receptor substrates 1 and 2 on glucose and lipid metabolism in human hepatoblastoma cells

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**Abstract:** Hepatic insulin signaling mediated by insulin receptor substrates 1 and 2 (IRS-1 and IRS-2, respectively) plays a central role in the development of type 2 diabetes mellitus. Although the functions of individual components in the signaling network have been extensively studied, our knowledge is still limited with regard to how the signals are integrated and coordinated in the complex network to render their functional roles. To investigate the specific functions of IRS-1 and IRS-2 in regulating liver function in humans, we developed a vector-mediated RNA interference (RNAi) technique in which short hairpin RNAs were used to knock down IRS-1, IRS-2, or both, by 50-60%, in cultured human hepatoblastoma (HepG2) cells. The knockdown of IRS-1 and IRS-2 resulted in upregulation of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK). Decreased IRS-1 was also associated with a decrease in glucokinase (GCK) expression, whereas the knockdown of IRS-2 resulted in the upregulation of lipogenic enzymes, sterol regulatory element-binding protein 1c (SREBP-1c) and cholesterol 7 alpha-hydroxylase (Cyp7a1). Dual-knockdown of IRS-1 and IRS-2 in HepG2 cells was associated with defective GTPase α serine/threonine-protein kinase (Akt) activation. Taken together, our results demonstrate that hepatic IRS-1 and IRS-2 play divergent roles in gene-signaling pathways and have complementary roles in the control of hepatic metabolism.

**Key words:** IRS-1; IRS-2; RNA interference (RNAi); hepatoblastoma cells; glucolipid metabolism

## INTRODUCTION

Insulin resistance, defined as reduced responsiveness of tissues to normal insulin concentrations, is a principal feature of type 2 diabetes, which leads to compensatory hyperinsulinemia [1-3]. The liver plays an essential role in the metabolism of glucose, lipid and energy. In liver cells, impaired insulin sensitivity (insulin resistance) or a dysregulated insulin response, contribute greatly to the development of type 2 diabetes [4,5]. Decreased hepatic insulin sensitivity leads to postprandial hyperglycemia and increased hepatic glucose production, which exacerbates an already deleterious situation of hyperglycemia and chronic hyperinsulinemia in diabetics [4]. In addition to affecting glucose levels, hepatic insulin resistance may also lead to dysregulated lipid synthesis, which can lead to hepatic steatosis and further systemic insulin resistance [6].

Insulin signaling is initiated when insulin binds to the insulin receptor (IR), a receptor tyrosine kinase [7], and is transmitted intracellularly by insulin-receptor substrates [8]. The IRS proteins do not possess intrinsic enzymatic activity and they contribute to signaling through their function as adaptors that organize signaling complexes [9]. There are two major forms of IRS: insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2), and both are highly expressed in the livers of normal mice but are downregulated to various degrees in the livers of diabetic animals and humans [10-14]. Indeed, the decreased expression of IRS proteins or reduced tyrosine

phosphorylation of IRS in liver cells are the key molecular events in the development of insulin resistance and type 2 diabetes [15,16].

The IRS proteins have a high level of homology in their N-termini, which contain two highly conserved domains that contribute to their recruitment to activated upstream receptors. Essential downstream kinase cascades activated by IRS involve the phosphatidylinositol 3-kinase (PI3K)-Akt pathway [17] and the phosphorylation of transcription factor fork head box O1 (FOXO1) [18]. Although IRS-1 and IRS-2 are highly homologous and mediate insulin signaling through the PI3K-Akt signaling pathway, it was suggested that IRS-1 or IRS-2 play a unique role in hepatic insulin action [19,20]. Short hairpin RNA (shRNA) was used to specifically knockdown IRS-1 and IRS-2 in mouse liver and it was shown that hepatic IRS-1 and IRS-2 have complementary roles in the control of PI3K and FoxO1 activity. Single knockdown of IRS-1 or IRS-2 showed that IRS-1 was more closely linked to glucose homeostasis and IRS-2 was more closely linked to lipid metabolism [21]. Only dual-knockdown of IRS-1 and IRS-2 resulted in systemic insulin resistance, glucose intolerance and hepatic steatosis [21]. Individual global knockout of IRS-1 and IRS-2 did not result in an obvious hepatic phenotype, and IRS-1/IRS-2 knockout mice die *in utero* [8,22,23]. IRS-1 knockout mice have no defects in hepatic phosphotyrosine (PY)-associated PI3K activity as the constitutive increase in IRS-2 protein levels is capable of fully compensating for defects in IRS-1 expression [24]. However, IRS-2 knockout mice exhibit a 50% decrease in PY-associated PI3K activity in the liver, with no increase in IRS-1 levels [25]. Mice with liver-specific IRS-1 knockout developed insulin resistance only after refeeding but not during fasting, while IRS-2 knockout mice exhibited insulin resistance in the fasted state [26]. These experiments suggest that insulin signaling adapts to different physiological functions through IRS-1 and IRS-2 signaling with different responses.

In order to further determine the roles of IRS-1 and IRS-2 in human hepatic insulin action, we used a vector-mediated RNAi technique that utilizes shRNAs to substantially and stably knockdown IRS-1 and IRS-2 expression specifically in human HepG2 cells. By knocking down IRS-1 and IRS-2 separately and together in human HepG2 cells, we showed that IRS-1 and IRS-2 work together via mutual compensation to activate the PI3K-Akt signaling pathway, and that they have unique roles in gene regulation.

## MATERIALS AND METHODS

### *shRNA preparation and plasmid construction*

Silencer-validated small interfering RNA (siRNA) targeting human IRS-1 and IRS-2 mRNAs were synthesized by Invitrogen (Shanghai, China) [27]. The sequence of shIRS1 is: 5′-CCC AAG AGC ATG CAC AAA CTT GTG TGC TGT CCG TTT GTG CAT GCT CTT GGG-3′; the sequence of shIRS2 is: 5′-GCG AGT ACA TCA ACA TCG ATT GTG TGC TGT CCT CGA TGT TGA TGT ACT CGC-3′; the sequence of scrambled shRNA is: 5′-GGC GCA GTA GTA AGC TCT TGT GTG TGC TGT CCA AGA GCT TAC TAC TGC GCC-3′. The scrambled nontargeting siRNA was used as a negative control. The shRNA sense oligonucleotides contained a 4-nucleotide overhang to create a restriction site plus G, followed by a 21-nucleotide sense siRNA sequence, a 11-nucleotide loop (GTGTGCTGTCC), a 21-nucleotide reverse complementary antisense siRNA sequence, and a polymerase III terminator (TTTTTA). The complementary antisense oligonucleotides contained a four-nucleotide overhang at the 5' terminus to create another restriction site. DNA sequences containing a small hairpin structure were synthesized, annealed, and inserted into the digested pGeneSil-1 vector with *Bam*HI and *Hind*III. Both shIRS1 and shIRS2 were then inserted into digested pGeneSil-2 vector with *Bam*HI, *Hind*III site and *Nhe*I, *Xho*I site. Successful construction of IRS1U6, IRS2U6 and IRS1/2U6 was confirmed by DNA sequencing. All restriction endonucleases were purchased from TaKaRa (Dalian, China).

### *Cell culture and reagents*

Human hepatoblastoma cells (HepG2) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Shanghai, China) with 10% fetal bovine serum (FBS; Gibco, Shanghai, China) and penicillin-streptomycin (penicillin: 10000 U/mL, streptomycin: 10000 ng/mL; Invitrogen, Shanghai, China). Freshly trypsinized HepG2 cells were suspended at 5-$10^5$ cells/mL in standard HepG2 culture medium and seeded

at a density of $10^6$ cells per well in standard six-well tissue-culture plates. After seeding, the cells were incubated at 37°C in a 90% air/10% $CO_2$ atmosphere, and 2 mL of fresh medium was supplied every other day to the cultures after removal of the supernatant. The HepG2 cells were cultured in standard medium for 5-6 days to achieve 90% confluence before treatment. Human insulin was purchased from Sigma-Aldrich (Shenyang, China) and was stocked in HEPES buffer. We treated the cells with insulin at a concentration of 1 nM to mimic physiological concentrations. Cells were deprived of serum for 16 h before insulin treatment.

For the transfection experiment, cells were plated in a 3.5-cm plate to achieve 80-90% confluence within 24 h in HepG2 culture medium without penicillin/streptomycin. The cells were transfected with lipofectamine 2000 (Invitrogen, Shanghai, China) according the manufacturer's instructions at a ratio of 3:1 transfection reagent (mL): DNA (mg). Cells were passaged 24 h after transfection. Selection medium DMEM (high glucose supplemented with 10% FBS and 400 ng/mL G418) replaced the standard HepG2 culture medium after cell adhesion. Cells for stable selection were obtained by G418 selection for 10 days, when the non-transfected cells died completely, and cells for transient transfection were harvested 24 h after transfection.

### *Real-time quantitative PCR analysis*

Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen, Shanghai, China) according to the manufacturer's protocol. RNA quality and quantity were evaluated by UV spectrophotometry at 260 and 280 nm. The RNA was reverse transcribed to cDNA using the Revert Aid H Minus First Strand cDNA Synthesis Kit (Fermentas, Beijing, China) according to the manufacturer's instructions. The cDNA was either analyzed immediately or stored at -20°C until use. Quantitative PCR amplification was performed using the ABI 7500HT fast real-time PCR System (ABI Company, Beijing, China), and the SYBR Green I fluorescent dye method was used to quantify the cDNA (TaKaRa, Dalian, China). The primers used for quantitative RT-PCR analyses of human IRS-1 (5′-TCCACCTCGGATTGTCTCTT-3′ and 5′-AGGGACTGGAGCCATACTCA-3′), human IRS-2 (5′-CCACTCGGACAGCTTCTTCT-3′ and 5′-AGGATGGTCTCGTGGATGTT-3′), and human GAPDH (5′-AACTTTGGTATCGTGGAAGGA-3′ and 5′-CAGTAGAGGCAGGGATGATGT-3′) were synthesized by Invitrogen. RT-PCR was performed as described previously and normalized to GAPDH expression levels [28].

### *Western blot analysis and immunoprecipitation*

HepG2 cells were lysed as described previously [28]. Total protein levels were quantified using the bicinchoninic acid (BCA) assay kit from Pierce Biotechnology (Rockford, USA). Twenty to 40 μg of total protein were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels (Bio-Rad, Hercules, USA), transferred to PVDF membranes, and probed with primary and secondary antibodies. Biotinylated protein ladders (Invitrogen, Shanghai, China) were loaded into one well of each SDS-PAGE gel. Antibody detection was performed using the enhanced chemiluminescence kit from Pierce Biotechnology (Rockford, USA). The Western blots were quantified using Quantity One software (Bio-Rad, Shanghai, China).

Anti-IRS1, anti-IRS2 and anti-β-actin antibodies were from Sigma-Aldrich (Shenyang, China). Anti-PEPCK, anti-F-1,6-BP and anti-GCK were purchased from Abcam Technology Inc (Cambridge, UK). Rabbit polyclonal anti-Akt, anti-phospho Akt (S473), anti-SREBP-1, anti-LXRA, anti-Abcg8 and anti-Cyp7a1 were purchased from Cell Signaling Technology (Boston, USA). Secondary anti-rabbit and anti-mouse antibodies were purchased from Pierce Biotechnology (Rockford, USA). For Western blotting, anti-IRS1, anti-IRS2, anti-β-actin, anti-PEPCK, anti-F-1,6-BP, and anti-GCK, anti-Akt, anti-LXRA, anti-Abcg8 and anti-Cyp7a1 antibodies were diluted 1:1000. Anti-phospho Akt (S473) and anti-SREBP-1 antibodies were diluted 1:500. Secondary anti-rabbit and anti-mouse antibodies were diluted 1:2000.

### *Statistical analysis*

All experiments were performed at least three times and representative results are shown. All data, unless specified, are presented as the mean±SD for the indicated number of experiments. One-way ANOVA

with Student's t test was used to evaluate statistical significances between the different treatment groups. P<0.05 indicates significant differences.

## RESULTS

### RNAi caused specific knockdown of IRS-1 and IRS-2 in HepG2 cells

RNA interference can regulate gene expression by inhibition of translation or by degradation of mRNA. To confirm that mRNA levels were decreased, the levels of expression of IRS-1 and IRS-2 mRNAs were measured by quantitative RT-PCR after G418 selection. The results showed that IRS-1 mRNA was decreased by 58% when an IRS1U6 or IRS1/2U6 vector was used. Similarly, the IRS2U6 vector caused a 54% drop in IRS-2 mRNA levels when used alone, and a 67% decrease when the IRS1/2U6 vector was used (Fig. 1A and B).

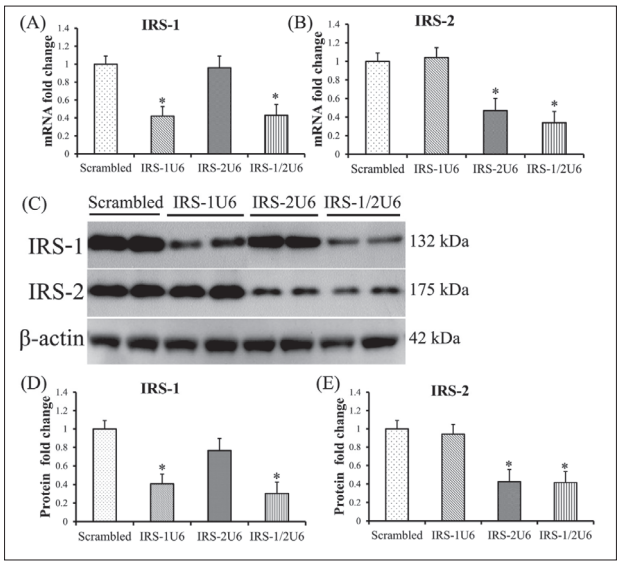


**Fig. 1.** RNAi vector caused specific knockdown of IRS-1 and IRS-2 in HepG2 cells. **A** – Quantitative RT-PCR analysis of IRS-1 mRNA levels in HepG2 cells after treatment with shRNA vector. **B** – Quantitative RT-PCR analysis of IRS-2 mRNA levels in HepG2 cells after treatment with shRNA vector. **C** – Western blots of IRS-1, IRS-2, and actin in HepG2 cells treated with shRNA vector. **D** – Corresponding densitometric analyses of the protein bands of interest/actin shown as relative values (Fig. 1C, presented as means±SD). **E** – Corresponding densitometric analyses of the protein bands of interest/actin shown as relative values (Fig. 1C, presented as means±SD). Statistical analysis was performed on IRS-1U6, IRS-2U6 and IRS-1/2U2 vs scrambled, respectively. *P<0.05 indicates significant differences.

Following transfection with a vector expressing shRNAs against IRS-1 (IRS1U6), we observed an average 60% decrease in IRS-1 protein levels in HepG2 cells as compared with HepG2 cells transfected with a control vector that expressed scrambled shRNAs (Fig. 1C). No associated decreases in actin and IRS-2 protein expression were observed. Similarly, a recombinant RNAi vector against IRS-2 (IRS2U6) specifically reduced IRS-2 protein expression by approximately 60% without altering IRS-1 levels or actin controls (Fig. 1C, D and E).

The use of vector shRNAs to knockdown multiple genes simultaneously is feasible. However, we were concerned that dual knockdown could result in greater nonspecific effects than the single vector treatment. We found that treatment with IRS1/2U6 vector caused substantial knockdown of both IRS-1 and IRS-2, with decreases of 70% and 60%, respectively, without inducing decreases in the levels of actin protein (Fig, 1C, D and E).

### Downregulated IRS protein expression in HepG2 cells results in changes in the expression of glycometabolism genes

Using Western blot analysis, we measured the protein levels of two key gluconeogenic enzymes, PEPCK and fructose-1,6-bisphosphatase (F-1,6-BP) (Fig. 2A, B, and C). PEPCK was upregulated in double-knockdown HepG2 cells 1.7-fold. F-1,6-BP was upregulated in double-knockdown HepG2 cells 1.2-fold, which was not statistically significant.

Interestingly, treatment with IRS1U6 or IRS2U6 alone did not increase the protein levels of PEPCK and F-1,6-BP. Glucokinase (GCK) plays a key role in regulating glucose homeostasis, and GCK gene mutations in humans cause maturity onset diabetes of the young, type 2 (MODY-2). The MODY phenotype can arise partly from dysregulated hepatic glucose disposal, as mice with liver-specific ablation of glucokinase exhibit severe hyperglycemia [29]. Thus, we measured GCK expression in HepG2 cells treated with RNAi vector and found that GCK expression was closely related to IRS-1 expression (Fig. 2A and D). Treatment with IRS1U6 caused a 50% drop in GCK expression, while dual treatment resulted in a 46% decrease in GCK levels. Treatment with IRS2U6 resulted in a modest

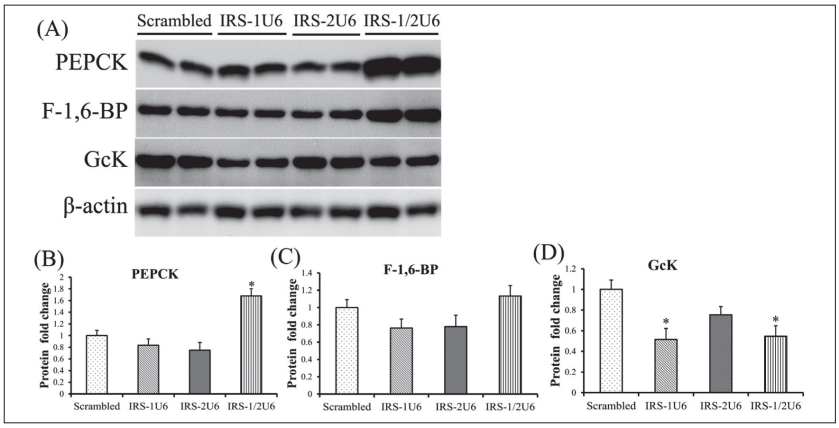


**Fig. 2.** Downregulated IRS protein expression in HepG2 cells resulted in changes in the expression of glycol-metabolism genes. **A** – Western blots of PEPCK, F-1,6-BP, and GCK in HepG2 cells treated with shRNA vector. **B** – Corresponding densitometric analyses of the protein bands of PEPCK/actin. **C** – Corresponding densitometric analyses of the protein bands of F-1,6-BP/actin. **D** – Corresponding densitometric analyses of the protein bands of GCK/actin. Statistical analysis was performed on IRS-1U6, IRS-2U6 and IRS-1/2U2 vs scrambled, respectively. *P<0.05 indicates significant differences.

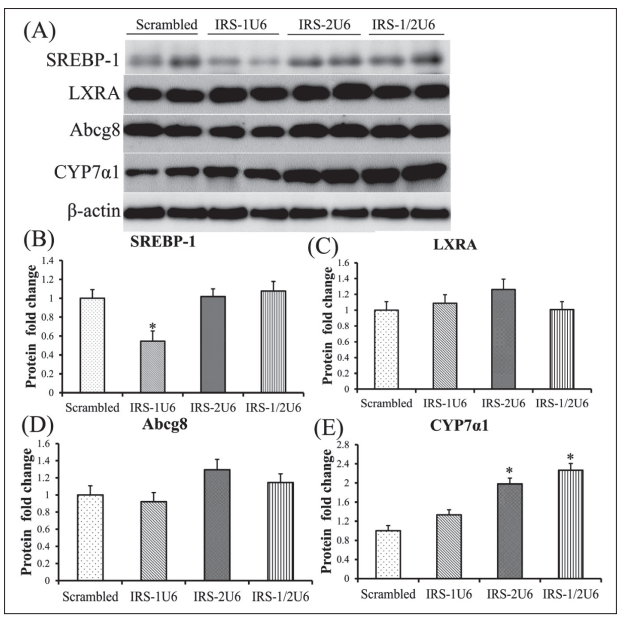


**Fig. 3.** Downregulated IRS protein expression in HepG2 cells resulted in changes in the expression of lipogenic genes. **A** – Western blots of SREBP-1, LXRA, Abcg8 and Cyp7a1 in HepG2 cells treated with shRNA vector. **B** – Corresponding densitometric analyses of the protein bands of SREBP-1/actin. **C** – Corresponding densitometric analyses of the protein bands of LXRA/actin. **D** – Corresponding densitometric analyses of the protein bands of Abcg8/actin. **E** – Corresponding densitometric analyses of the protein bands of Cyp7a1/Actin. Statistical analysis was performed on IRS-1U6, IRS-2U6 and IRS-1/2U2 vs scrambled, respectively. *P<0.05 indicates significant differences.

23% downregulation of GCK, which was not statistically significant (Fig. 2A and D).

## Downregulated IRS protein expression in HepG2 cells results in changes in the expression of lipid metabolism genes

The inappropriate activation of gluconeogenesis in insulin-resistant states is often accompanied by defects in hepatic lipid regulation. We measured the expression of key lipogenic genes such as Srebp1c in response to IRS-1 and IRS-2 knockdown by vector shRNAs. IRS-2 knockdown alone increased hepatic Srebp1 expression 1.7-fold, whereas decreased hepatic IRS-2 expression and dual knockdown also increased hepatic Srebp1 expression (Fig. 3A and B).

Insulin can also regulate lipogenic gene transcription through pathways independent of the IRS proteins, most notably through liver X receptor A (LXRA) activation [30]. Using Western blot analysis, we found that LXRA protein levels were unchanged after treatment with IRS1U6 or IRS1/2U6 (Fig. 3A and C, respectively), while treatment with IRS2U6 alone resulted in a modest 24% upregulation of LXRA, which was not statistically significant (Fig. 3A and C). However, these results do not preclude the possibility that LXRA activity may be increased. As a proxy for LXR activation, we measured the expression of downstream LXRA genes, such as Abcg8 (Fig. 3A and D), and cytochrome P450, isoform 7A1 (Cyp7a1; Fig. 3A and E). Abcg8 protein levels were unchanged after treatment with IRS1U6 or IRS1/2U6 (Fig. 3A and D, respectively), while treatment with IRS2U6 alone resulted in a modest 26% upregulation of Abcg8, which was not statistically significant (Fig. 3A and D). IRS-1 knockdown alone did not have any effect on Cyp7a1 expression and decreased hepatic IRS-2 expression alone was sufficient to induce a 1.9-fold upregulation of Cyp7a1. Moreover, dual knockdown of IRS-1 and IRS-2 resulted in a 2.2-fold increase in Cyp7a1 genes when compared with the control shRNA treatment (Fig. 3A and E).

### Significant defects in Akt activation in HepG2 cells treated with IRS1U6 and IRS2U6

To better understand the molecular mechanisms of these physiological defects, we measured Akt phosphorylation in HepG2 cells. Akt phosphorylation was decreased only in HepG2 cells with dual defects in IRS-1 and IRS-2 expression (Fig. 4A). The administration of either IRS1U6 or IRS2U6 alone did not result in any detectable changes in Akt activation when compared with the control shRNA treatment (Fig. 4B). However, concomitant treatment with IRS1U6 and IRS2U6 caused a 50% decrease in serine 473 phosphorylation of Akt compared with the control shRNA treatment (Fig. 4B).

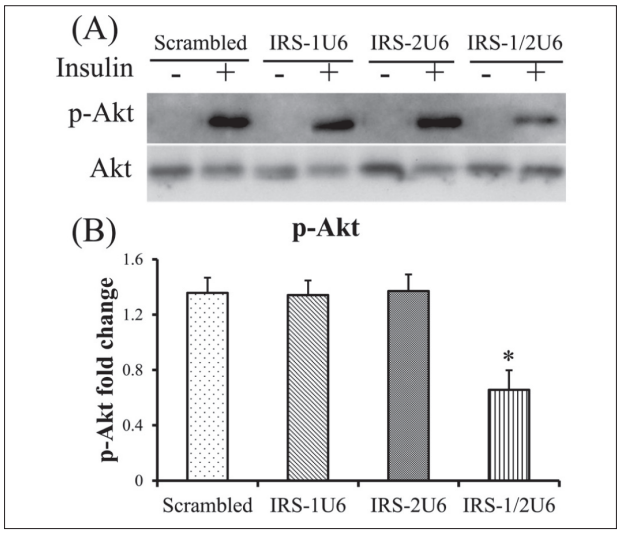


**Fig. 4.** Significant defects in Akt activation in HepG2 cells treated with IRS1U6 and IRS2U6. **A** – Western blots of p-Akt and total Akt in HepG2 cells treated with shRNA vector. **B** – Corresponding densitometric analyses of the protein bands of p-Akt/total Akt. Statistical analysis was performed on IRS-1U6, IRS-2U6 and IRS-1/2U2 vs scrambled, respectively. *P<0.05 indicates significant differences.

## DISCUSSION

In the present study, we utilized an *in vitro* RNAi technique that included a vector to deliver shRNAs against IRS-1, IRS-2, or both in human hepatoblastoma cells. This vector-mediated RNAi technique allowed us to stably knockdown the expression of IRS-1 and IRS-2 in human hepatoblastoma cells, and to define their individual roles in hepatic insulin signaling and hepatic metabolism.

Previous reports have suggested that IRS-1 and IRS-2 have unique individual roles in regulating key genes in glucose and lipid homeostasis, although they work in a complementary fashion to maintain PI3K signaling [11,13]. In mouse liver, single knockdown of IRS-1 caused a significant increases in mRNAs for the essential gluconeogenic enzymes, PEPCK and F-1,6-BP [21]. However, in this study, the increased expression of the PEPCK gene was a consequence of the marked downregulation of both IRS-1 and IRS-2. In addition, the expression of F-1,6-BP was not changed significantly following knockdown of IRS-1 or IRS-2. This difference may be the result of a dosage effect as in the current study IRS-1 and IRS-2 were knocked down by about 60-70%, while in a previous study they were knocked down by about 70-80%. It was shown that IRS-1 can regulate GCK mRNA levels in cultured hepatocytes by activating PI3K [12]. Our data also indicate that IRS-1 is more prominent than IRS-2 in the regulation of GCK expression, as IRS1U6, either alone or in combination with IRS2U6, caused about a 50% decrease in GCK expression.

It is known that decreased expression of IRS-2 in the liver alone is sufficient to elevate the levels of Srebf1 and can occur independently of the known positive regulatory effects of insulin on Srebf1 expression [31]. In IRS-2 knockout mice, the expression of SREBP-1 was upregulated, and in primary hepatocytes from SREBP-1 knockout mice, the expression of IRS-2 was upregulated [6,32,33]. Our results showed that decreased IRS-2 increased Srebf1 expression in HepG2 cells. The LXR signaling pathway is a distinct pathway that contributes to dysregulated lipogenesis and hepatic steatosis. Our data suggest that the expression of LXRA was not statistically significantly changed, while the expression of Cyp7a1, which is a downstream LXRA gene, was increased. These results preclude the possibility that LXRA activity may be increased. As insulin-stimulated LXR activity increased SREBP-1c transcription via a signaling-independent mechanism [30], increased Srebf1 expression may be the result of increased LXR activity.

PI3K/Akt signaling is incorporated in the main signaling pathway for insulin-dependent regulation of glucose metabolism [34]. After insulin activates its receptors, PI3K must recruit an insulin substrate protein to promote Akt Ser473 phosphorylation [35]. IRS-1

and IRS-2 play complementary roles in the liver at the level of intermediate signaling of PI3K activation. Akt is the main downstream gene of PI3K, and Akt phosphorylation and insulin-stimulated Akt activity can correspond to PI3K activity [36]. Compensatory mechanisms occur in the livers of IRS-1 knockout mice, where IRS-2 expression significantly increases to maintain total PI3K activity and Akt activation [24]. IRS-2 knockout mice do not exhibit this increase in IRS-1-associated PI3K activity in the liver, whereas IRS-2 knockdown results in about a 50% decrease in hepatic Akt activation [37]. The reduction in 60-70% in IRS-1 or IRS-2 individually does not perturb total Akt activity as a reciprocal increase in PI3K activity through the unaltered IRS antipode helps to maintain full PI3K signaling. Reductions in IRS-1 and IRS-2 simultaneously disturb the balance of IRS, whereas Akt phosphorylation and insulin-stimulated Akt activity were decreased in human hepatoblastoma cells, with dual defects in IRS-1 and IRS-2 expression.

## CONCLUSION

The present study shows that dual downregulation of IRS-1 and IRS-2 increased the expression of PEPCK gene, and that a reduction in IRS-1 caused an approximately 50% decrease in GCK expression. The decrease in IRS-2 increased the expression of Srebf1 and Cyp7a1 in human hepatoblastoma cells. Insulin-stimulated Akt activity was decreased in human hepatoblastoma cells with dual defects in IRS-1 and IRS-2 expression. Thus, the differential modulation of hepatic IRS expression and signaling may represent an important component of the molecular pathophysiology that underlies both type 2 diabetes mellitus and the metabolic syndrome.

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**Author contributions:** Tianfei Yu and Ming Li collected the data and contributed equally to the paper. Huiying Dong, Pengyu Xie and Wanzhu Sun collected the data and performed the statistical analysis of the data. Tianfei Yu, Bo Meng and Yanshuang Mu prepared the manuscript. Yanshuang Mu conceived the study. All authors contributed to and approved the final manuscript.

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