Original Article

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Development of an *in vitro* insulin resistance dissociated model of hepatic steatosis by co-culture system

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SUMMARY The evidence shows that there is an associated relationship between hepatosteatosis and insulin resistance. While some existing genetic induction animal and patient models challenge this relationship, indicating that hepatosteatosis is dissociated from insulin resistance. However, the molecular mechanisms of this dissociation remain poorly understood due to a lack of available, reliable, and simplistic setup models. Currently, we used primary rat hepatocytes (rHPCs), co-cultured with rat hepatic stellate cells (HSC-T6) or human foreskin fibroblast cells (HFF-1) in stimulation with high insulin and glucose, to develop a model of steatosis charactered as dissociated lipid accumulation from insulin resistance. Oil-Red staining significantly showed intracellular lipid accumulated in the developed model. Gene expression of sterol regulatory element-binding protein 1c (*SREBP1c*) and elongase of very-long-chain fatty acids 6 (*ELOVL6*), key genes responsible for lipogenesis, were detected and obviously increased in this model. Inversely, the insulin resistance related genes expression included phosphoenolpyruvate carboxykinase 1 (*PCK1*), pyruvate dehydrogenase lipoamide kinase isozyme 4 (*PDK4*), and glucose-6-phosphatase (*G6pase*) were decreased, suggesting a dissociation relationship between steatosis and insulin resistance in the developed model. As well, the drug metabolism of this developed model was investigated and showed up-regulation of cytochrome P450 3A (*CYP3A*) and down-regulation of cytochrome P450 2E1 (*CYP2E1*) and cytochrome P450 1A2 (*CYP1A2*). Taken together, those results demonstrate that the in vitro model of dissociated steatosis from insulin resistance was successfully created by our co-cultured cells in high insulin and glucose medium, which will be a potential model for investigating the mechanism of insulin resistance dissociated steatosis, and discovering a novel drug for its treatment.

Keywords hepatic steatosis, *in vitro* model, co-culture, dissociation, insulin resistance

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease, with a worldwide prevalence of 25% (*1,2*), which is the spectrum from hepatic steatosis to nonalcoholic steatohepatitis (NASH), which eventually leads to liver cirrhosis and hepatocellular carcinoma (HCC) (*3*). Hepatic steatosis, the initial and benign reversible event of NAFLD, is characterized by the accumulation of fat in at least 5% of hepatocytes and becomes the main feature of all stages of NAFLD from Nonalcoholic fatty liver (NAFL) progressing to NASH (*4*). Importantly, previously accumulated evidence showed that 10-25% of hepatic steatosis patients progress to NASH (*3*). While at the initial stage of NAFLD, modification of lifestyles such as exercise and diet can effectively reverse hepatic steatosis, subsequently protecting hepatic steatosis progress to NASH (*5*). In conclusion, hepatic steatosis is a major concern stage of NAFLD, and dealing with it as early as possible can effectively protect its progress to NASH.

Insulin resistance is a basically pathogenic mechanism of NAFLD, obesity, and type 2 diabetes. Many epidemiology studies have shown that hepatosteatosis is strongly associated with obesity and type 2 diabetes mellitus, indicating that there is a mutual cause-and-effect relationship between hepatosteatosis and insulin resistance (*3,6,7*). For instance, existing

insulin resistance may drive hepatic lipogenesis and subsequently cause steatosis (*8*). Also, the accumulation of lipid species in intracellular hepatocytes can disrupt insulin signaling, leading to cause insulin resistance (*9,10*). In addition, all this cause-and-effect relationship between hepatosteatosis and insulin resistance was evidently confirmed by various genetic or environmental induction animal or *in vitro* models of hepatic steatosis (*3,11-14*). However, there were several genetic or environmental induction animal models challenging this association of hepatosteatosis with insulin resistance. For instance, liver-specific knock out of insulin receptor (*15*), phosphoinositide 3-kinases (*PI3Ks*) (*16,17*), and protein kinase B (*AKT*) (*18*) or liver-specific deletion of phosphatase and tensin homolog (*PTEN*) (*19*) mice clearly display dissociation of hepatosteatosis from insulin resistance. Although the dissociation relationship is apparently displayed by those genetic animal models, the exact mechanism of this dissociation is still largely unknown. Therefore, for clearly elucidating the molecular mechanism of this dissociation relationship between hepatosteatosis and insulin resistance, a new experimental approach, animal model, or even an *in vitro* cell model will be urgently needed to explore or develop.

In general, for understanding the mechanism of NAFLD, animal models were first considered to be used and became the most common application experiment method in laboratory research. However, it is currently acknowledged that NAFLD is a complex multiple hit disease, and the developed *in vivo* animal models could not fully recapitulate the complexity of disease characteristics, thus hindering elucidation of the disease mechanism. In addition, the complex environment *in vivo* obstructs exploration of the exact disease mechanism. Therefore, *in vitro* models of fatty liver disease have been considered and continuously developed during the last few decades. For instance, immortalized cell lines or primary hepatocytes were cultured to develop models and well-established to be used for NAFLD research (*13,20*). Unfortunately, the above models developed from single cell cultures still failed to model the complex NAFLD pathogenesis because *in vivo* NAFLD pathogenesis is caused by numerous cellular communications between hepatocytes and nonparenchymal cells including HSCs, endothelial cells, or Kupffer cells. Hence, co-culture of two or more different cells is an interesting method to model continuous NAFLD pathogenesis, and it succeeded by culture of hepatocytes and HSC, which further found that co-culture with HSC can promote differentiation of the hepatocyte to maintain liver-specific functions and structure (*21*). Moreover, contrary to animal models developed in a hyperglycemic-hyperinsulinemic environment, there are rare and unsuccessful high concentration insulin-glucose induced *in vitro* cell culture models of hepatic steatosis. Consequently, for mimicking the pathogenesis of animal or human NAFLD, developing an *in vitro* hepatosteatosis model by co-culturing cells with a high concentration of insulin-glucose should be further explored, thus helping to investigate the exact molecular mechanisms involved in pathogenesis of NAFLD.

Therefore, in this study, we used rHPCs, co-cultured with HSC-T6 or HFF-1 cells under a high concentration of insulin-glucose conditions to develop an *in vitro* model of hepatic steatosis, which dissociated from insulin resistance for potentially investigating the dissociation mechanism. The developed model displayed obvious intracytoplasmatic lipid droplet accumulation and gradual growth into macrovesicular lipid vacuoles. Notably, the analysis of genes of *de novo* lipogenesis (DNL) significantly showed that the infiltrated fatty was caused by up-regulating *SREBP1c* and *ELOVL6*. Furthermore, the *CYPs*, *PCK1*, *PDK4*, and *G6pase* gene analysis suggested that rHPCs' drug-metabolism function was altered in the co-culture system, and the developed hepatosteatosis was dissociated from insulin resistance. Together, the results indicate that the co-culture system used is an effective approach to develop an *in vitro* insulin resistance dissociated hepatic steatosis model, which will potentially be used to investigate the dissociation mechanism and develop a novel drug for its treatment.

2. Materials and Methods

2.1. Isolation of primary rat hepatocytes

A two-step collagenase perfusion method was used to isolate primary hepatocytes from Wister rats. Briefly, after anesthetizing and sterilizing, the abdominal cavity of rats was opened to expose the portal vein and the inferior vena cava. Sequentially, the catheter was placed into the portal vein, and continuously pre-warmed Hank's Balanced Salt Solution (Gibco, New York, USA) containing EGTA (Coolaber, Beijing, China) and Digestion Medium including Type IV collagenase (Coolaber, Beijing, China) were perfused at 25-30 mL/min. After perfusion, the liver was carefully and integrally gathered and transferred to a culture dish containing Digestion Medium. Then, the liver capsule was torn by ophthalmic forceps and constantly shaked to dissociate liver cells into the medium. After dissociation, the hepatocyte suspension was filtered through a 70 μm filter and separated by centrifugation at a speed of 50 g for 2 min. Finally, Percoll gradient centrifugation was performed to purify the isolated hepatocytes.

2.2. Cells cultures

Isolated hepatocytes were stained with trypan blue and subsequently counted to calculate the number of alive

hepatocytes. After counting, 8×10^5 hepatocytes were seeded on a culture dish and cultured in an adherent medium, which contained DMEM (Gibco, New York, USA), 10% fetal bovine serum (FBS, Gibco, New York, USA), 1% penicillin-streptomycin (Gibco, New York, USA), 1% ITS (Insulin, Transferrin, and Selenium) (Cyagen, Guangzhou, China), 15mM HEPES (1M, Gibco, New York, USA) and 1 μM dexamethasone (Solarbio, Beijing, China). After 4h adhesion, the rat hepatic primary cells (rHPCs) were washed with PBS twice to remove non adhering rHPCs. Then the adhering rHPCs were subsequently cultured for another 20h with an adherent medium. For co-culture with rHPCs, HSC-T6 (rat hepatic stellate cells, purchased from Conservation Genetics CAS Kunming Cell Bank, Kunming, China) and HFF-1(human foreskin fibroblasts, purchased from Stem Cell Bank, Chinese Academy of Sciences, Shanghai, China) were previously cultured in DMEM (Gibco, New York, USA) containing 10% or 15% fetal bovine serum and 1% penicillin-streptomycin. Then HSC-T6 or HFF-1 cells were added into the cultured rHPCs while the medium was switched to steatosis-inducing medium containing DMEM, 10% fetal bovine serum, 1% penicillin-streptomycin, 1% ITS, 15 mM HEPEs, 1 μM dexamethasone and 0.002 μM glucagon (Yuanye Biotechnology, Shanghai, China) for 10d to induce fat accumulation in hepatocytes.

2.3. Hepatocyte Function assessment

To evaluate the function of hepatocytes in the co-culture system, the enzyme-linked immunosorbent assay kit (Ruixin Biotech, Fujian, China) and a biochemical assay kit (Jiancheng bioengineering, Nanjing, China) were used to detect and measure albumin and urea nitrogen. First, the cultured supernatant was collected and centrifuged at 4,000 rpm for 20 min. Then the centrifuged supernatant was used to detect albumin and urea nitrogen following the assay kits manufacturer's recommendations. Finally, the concentration of albumin and urea nitrogen were calculated according to a standard curve and established regression equation.

2.4. Oil Red O staning

For detecting accumulated lipid in intrahepatocytes, the Oil Red O stain kit (Solarbio, Beijing, China) was chosen. Following the kit manufacturer's instructions, the co-cultured cell models were washed twice with PBS, fixed in ORO Fixative solution for 30 min, washed twice again with PBS and 60% isopropanol, stained with ORO stain for 20 min and counterstained with Mayer hematoxylin for 2 min, and then washed with ORO buffer for 1 min. Finally, the images were captured using a light microscope (Leica Microsystems, Wetzlar, Germany). Representative photomicrographs are shown.

Table 1. Primer sequences used for PCR

Genes	Sequences
CYP2E1	F: 5'-TGTTTCTGCTCCTGTCTGCTATTCTG-3'
	R: 5'- TGGGATACTGCCAAAGCCAACTG-3'
CYP1A2	F: 5'- ACCATCTAATCAGCAAGTTCCAGAAGC-3'
	R: 5'- CCGATGACATTAGCCACCGATTCC-3'
CYP3A	F: 5'- CCGATGACATTAGCCACCGATTCC-3'
	R: 5'- TCCTCGTGCTCCTGTATCTGTATGG-3'
G6pase	F: 5'- AGGTGGTGGCTGGAGTCTTGTC-3'
	R: 5'- CTCTGGAGGCTGGCATTGTAGATG-3'
PDK4	F: 5'- GTTCTGAGGCTGATGACTGGTGTATC-3'
	R: 5'- GCACTGCCGTAGACCCACTTTG-3'
PCK1	F: 5'- GTGGAAAGTTGAATGTGTGGGTGATG -3'
	R: 5'- GTCTTAATGGCGTTCGGATTTGTCTTC -3'
Fads2	F: 5'- GAAGAAGACTGCTGAGGACATGAACC -3'
	R: 5'- CCATTGCCGAAGTACGAGAGGATG -3'
FASN	F: 5'- GTGTGGTAGGCTTGGTGAACTGTC-3'
	R: 5'- GTGAGATGTGCTGCTGAGGTTGG-3'
Dgat2	F: 5'- ACTCCTCTTCTCCAATCTGAGCCTAC-3'
	R: 5'- TGTGTTCACGATGCCAATCTCCAG-3'
ACC2	F: 5'- GAGTCCATCTTCCTGTCAGCCATTG -3'
	R: 5'- CGCCATACAGACGACCTTGTTAGC -3'
SCD1	F: 5'- TGTCAAAGAGAAGGGCGGAAAGC -3'
	R: 5'- CAGGATGAAGCACATGAGCAGGAG -3'
SREBP1	F: 5'- GCCTCATCTGATTGCCATCCTTCC-3'
	R: 5'- TCAACATACCGCACAAGGCAGAAG-3'
ELOVL6	F: 5'- CTTCCTCTTCCTCAACTTCTACACTCG-3'
	R: 5'- TTCTCTGACTTGTTCACACCGTTCG-3'
ChREBP	F: 5'- GCTGAACAACGCCATCTGGAGAG-3'
	R: 5'- GCAGAGGAGTTACGAAGCCACATAC-3'
GAPDH	F: 5'- TCTCTGCTCCTCCCTGTTCT -3'
	R: 5'- CCGATACGGCCAAATCCGTT -3'

F, Forward, R, Reverse.

2.5. RNA isolation and qRT-PCR

Total RNAs of cell models were extracted using TRIZOL reagent (Invitrogen, California, USA) according to the manufacturer's instructions. Then 1μg of extracted RNA was reverse transcribed into cDNA following the manufacturer's instructions of RevertAid First Stand cDNA Synthesis Kit (Thermo Scientific, Massachusetts, USA). Then, the cDNAs were quantified by performing quantitative real-time PCR using SYBR Green Master Mix (Genstar, Beijing, China) on ABI StepOnePlus real-time PCR system (Applied Biosystems, Waltham Mass, USA). All sense and antisense primers used for the PCR assays are provided in Table 1. All q-PCR data was collected and calculated as described previously (*22*).

2.6. Statistical analysis

SPSS ver. 20.0 (IBM, Armonk, New York, USA) software was used to perform One-way ANOVA Test for multiple comparisons. Statistical analysis data is displayed as bar graph using GraphPad prism5 (GraphPad Software, California, USA). Also, the data is shown as mean ± standard deviation. Meanwhile, statistically significant values of all the data were set as $P < 0.05$.

3. Results

3.1. rHPCs and HSC-T6 or HFF-1 cells (5:1 ratio) co-culture promotes lipid droplets accumulation

For optimizing co-culture system to develop hepatocyte steatosis, different ratios of rHPCs and HSC-T6 or HFF-1 cells were co-cultured. As shown in Figure 1, the intracellular rHPCs appeared with fat deposition on day 3 and significantly increased with time. However, the proportion of accumulation lipid droplets in a ratio of 3:1 and 4:1 was obviously lower than 5:1 and 6:1 groups, which means that greater hepatocyte numbers in the co-culture system will promote lipid droplets accumulation in hepatocytes. Meanwhile, the optical microscope results showed that the micro lipid droplets gradually grew into macro lipid droplets with increased co-culture time, especially in the ratio 5:1 group. (Figure 1) In line with optical microscope results, Oil Red O staining also showed that the fat deposition gradually increased in intracellular rHPCs (Figure 2A) and continuedly grew into a macro lipid droplet, particularly in the ratio 5:1 group. (Figure 2B) Together, the results indicate that the rHPCs and HSC-T6 or HFF-1 cells (5:1 ratio) co-culture can significantly promote rHPCs intracellular lipid droplets accumulation.

3.2. rHPCs and HSC-T6 or HFF-1 cells co-culture protects hepatocellular functions and alters cytochrome P450s genes expression

Maintaining hepatocellular functions *in vitro* plays a pivotal role in developing liver disease models. Therefore, in order to confirm that the functions of the isolated rHPCs were kept in this developed steatosis model, the secreted albumin, urea nitrogen and gene expression of *CYP3A* were measured. The results showed that the albumin was stably secreted until day 7 in the rHPCs and HSC-T6 cells co-culture model, and then it was significantly decreased on day 10 compared to day 0. (Figure 3A) Similarly, in the rHPCs and HFF-1 cells co-culture model, the secreted albumin did not show a significant decline on days 3, 5, 7 or even 10 when compared to day 0. (Figure 3B) Contrary to albumin, the secretion of urea nitrogen was significantly increased on days 3, 5, 7 and 10 when compared to normal hepatocytes (day 0) (Figure 4) In line with urea nitrogen secretion, the gene expression of *CYP3A*,

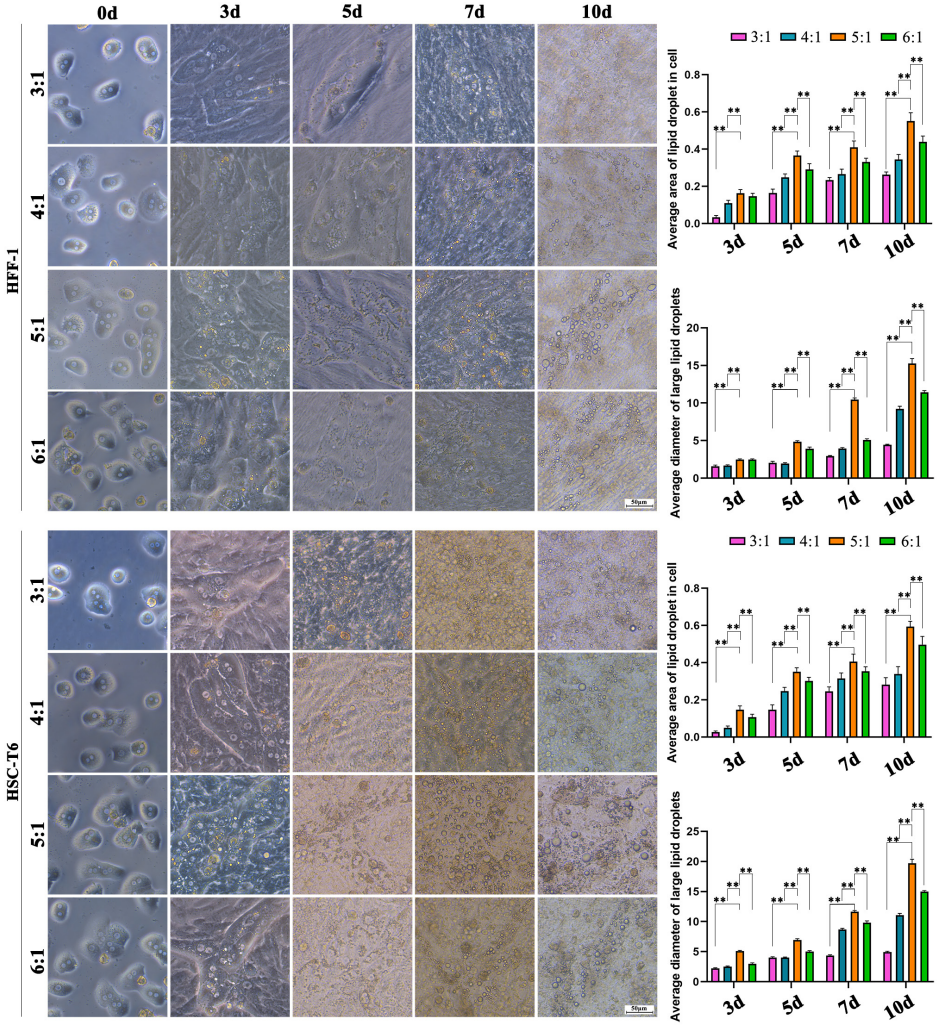

Figure 1. Optical microscope observation of fat deposition on different hepatocytes: HSC-T6 / HFF groups. All values are shown as mean ± standard deviation. ($^{*}P < 0.05$, $^{**}P < 0.01$). Bar = 50μm.

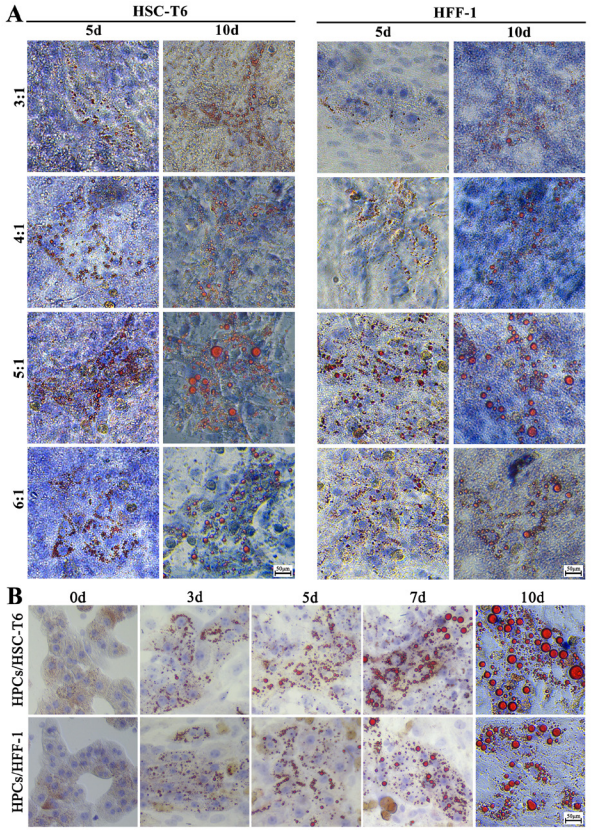

Figure 2. Oil Red O staining for developed steatosis model. A: Effect of hepatocytes ratio on fat deposition. **B**: Oil Red O staining detects the fat deposition and the change in lipid droplet size in ratio of 5:1 group (hepatocytes: HSC-T6 / HFF). Bar = 50μm.

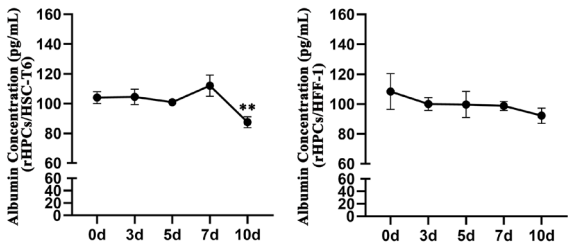

Figure 3. The secretion of albumin from hepatocytes in the developed steatosis model. All values are shown as mean ± standard deviation. ($^{*}P < 0.05$, $^{**}P < 0.01$).

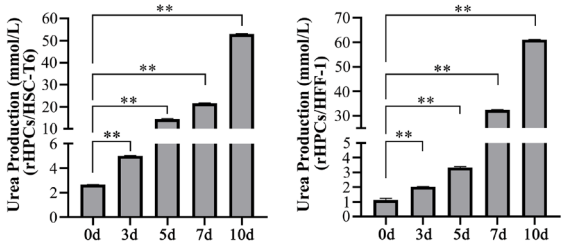

Figure 4. The secretion of urea nitrogen from hepatocytes in developed steatosis model. All values are shown as mean ± standard deviation. ($^{*}P < 0.05$, $^{**}P < 0.01$).

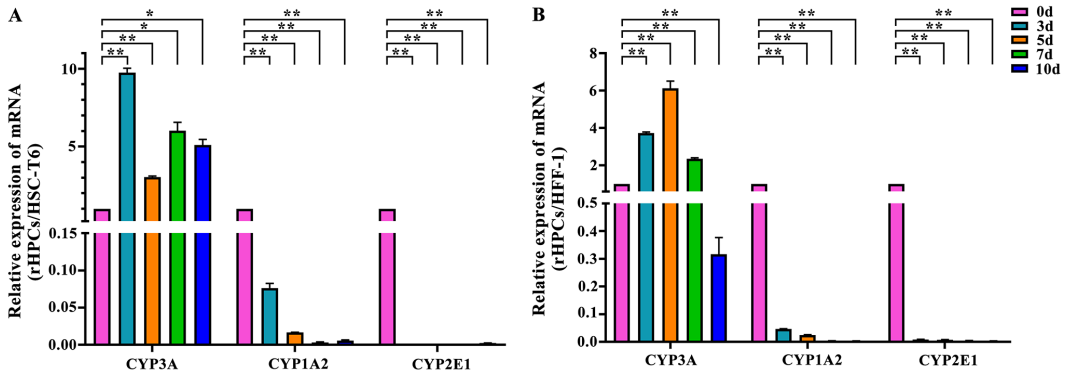

Figure 5. The genes expression of *CYP3A*, *CYP1A2* and *CYP1E* in developed steatosis model. All values are shown as mean ± standard deviation. ($^{*}P < 0.05$, $^{**}P < 0.01$).

quantitatively the most abundant component of human P-450, was also increased. The result showed that it was significantly up-regulated with intracellular lipid accumulation, and reached the highest level on day 3, after which its expression was decreased but higher than normal hepatocytes (day 0). (Figure 5A) However, in the HFF-1 co-culture system, although the *CYP3A* mRNA expression level was also increased, its highest level s appeared on day 5, then began to decrease and significantly was down-regulated compared to normal hepatocytes on day 10. (Figure 5B) In conclusion, the above results suggest that HSC-T6 or HFF-1 cells co-cultured with rHPCs can maintain or even enhance hepatocellular functions *in vitro*.

Furthermore, in humans, CYPs, which function as powerful detox enzymes play a vital role in drug metabolism and bioactivation. Therefore, in addition to CYP3A, other CYPs genes expression such as CYP1A2, which constitutes approximately 15% of total hepatic CYP enzymes, and *CYP2E1*, a major hepatic CYP enzyme involved in the metabolism and bioactivation of several drugs and toxicants, were also analyzed in this

study developed models. Hence the results showed that they were continually and obviously decreased in all HSC-T6 or HFF-1 developed models. (Figure 5A and B) Thus the results suggest that although the hepatocellular functions were protected in developed models, the related drug metabolism CYPs genes expression was altered.

3.3. The lipogenesis gene of *SREBP1c* and *ELOVL6* contributes to hepatosteatosis

Previous studies have reported that liver fat accumulation was linked to DNL (*23*). Therefore, in our developed model, we observed whether the DNL affects contributes to develop hepatosteatosis. To better understand that, the lipogenesis genes expression in the steatotic rHPCs were analyzed, and the results showed that only *SREBP1c*, generally recognized as master activator of lipid synthesis, and *ELOVL6*, an enzyme catalyzing the elongation of palmitate to stearate, were up-regulated. (Figure 6A, B) The up-regulation of *SREBP1c* was basically consistent between the HSC-T6 co-culture system and the HFF-1 system, while the *ELOVL6* up-regulation state was distinctly different between the two cells co-culture system. Because in the HSC-T6 system, *ELOVL6* was sustainably up-regulated from the initial to the day of 10, but in the HFF-1 system, *ELOVL6* genes expression was decreased on days of 3 and 5, and then it continued to increase. On the contrary, other DNL genes including carbohydrate responsive element binding protein (*ChREBP*), diacylglycerol acetyltransferase 2 (*Dgat2*), stearyl CoA desaturase 1 (*SCD1*), sstearyl CoA carboxylase 2 (*ACC2*), fatty acid synthase (*FASN*), and fatty acid desaturase 2 (*Fads2*) were down-regulation in all rHPCs and HSC-T6 or HFF-1 co-culture system. (Figure 6C) Consequently, expression of the above genes demonstrates that *SREBP1c* and *ELOVL6* were correlated with rHPCs steatosis development in the HSC-T6 or HFF-1 co-culture system.

3.4. The hepatosteatosis is dissociated from insulin resistance

Steatosis is strongly associated with insulin resistance and there is a mutual cause-and-effect relationship between hepatosteatosis and insulin resistance (*24*). Therefore, to better understand the relationship between hepatosteatosis and insulin resistance in our co-culture model, we examined the level of genes of *PCK1*, encoding the main checkpoint enzyme for the control of gluconeogenesis, *PDK4*, and G6pase, encoding an important enzyme for glycogenolysis and glucose production. The analysis results showed that gene expression of *PCK1*, *PDK4*, and *G6pase* were significantly decreased compared to normal hepatocytes in all rHPCs and HSC-T6 or HFF-1 co-culture systems, (Figure 7) suggesting that in those developed models, the

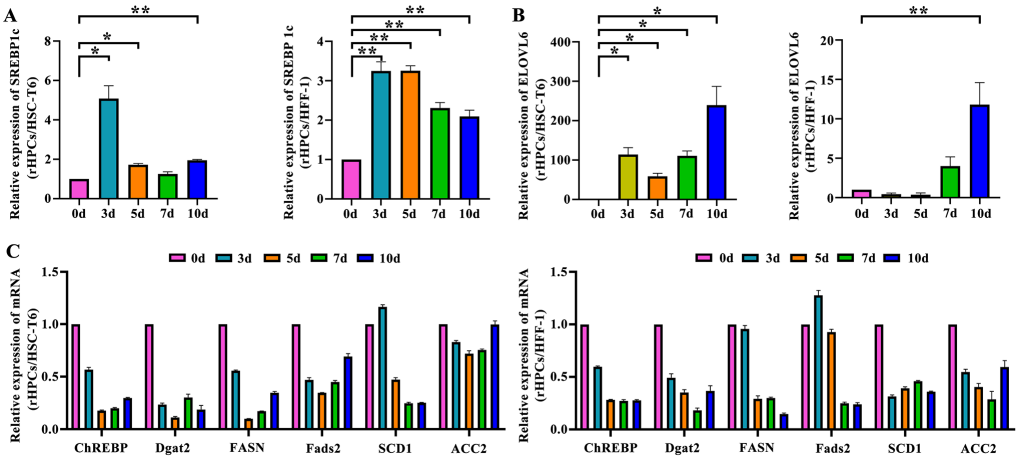

Figure 6. The *de novo* lipogenesis genes expression in developed steatosis model. A, B: the mRNA levels of *SREBP1c* and *ELOVL6* gene, as quantified by qRT-PCR, in hepatocytes. **C**: the other genes expression, including *ChREBP*, *Dgat2*, *FASN*, *Fads2*, *SCD1* and *ACC2*, in hepatocytes. All values are shown as mean ± standard deviation. ($^{*}P < 0.05$, $^{**}P < 0.01$).

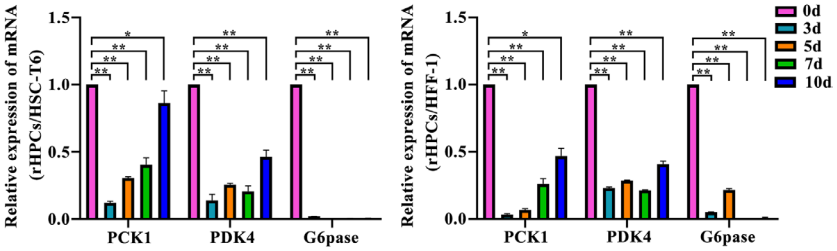

Figure 7. The genes expression of *PCK1*, *PDK4* and *G6pase* in developed steatosis model. All values are shown as mean ± standard deviation. ($^{*}P < 0.05$, $^{**}P < 0.01$).

hepatosteatosis is dissociated from insulin resistance.

4. Discussion

Epidemiology studies have reported that there is a mutual cause-and-effect relationship between hepatosteatosis and insulin resistance (*3,11-14*). However, this relationship in NAFLD was challenged by some genetic animal models and clinical patients (*15-19,25,26*), and the exact mechanistic basis of this dissociation relationship is still incompletely understood due to the lack of an ideal and simplistic setup model to elucidate this dissociation mechanism. Therefore, in this study, we used a co-culture system to develop an *in vitro* hepatic steatosis cell culture model. We found that the rHPCs and HSC-T6 or HFF-1 cells (5:1 ratio) co-culture can protect hepatocytes function and significantly promote rHPCs intracellular lipid droplets accumulation. Also, the developed hepetasteatosis was correlated with gene expression of *SREBP1c* and *ELOVEL6* and, importantly, was dissociated from insulin resistance due to the down-regulation of gene expression of *PCK1*, *PDK4* and *G6pase*. All results suggest that our approach used would be an ideal and simplistic method to develop an insulin resistance dissociated hepatosteatosis model, which will be a suitable *in vitro* model to elucidate the dissociation mechanism.

Generally, the disease of NAFLD happening and progression is a complex and multicellular event, because the liver fat accumulation, and inflammation or fibrosis is a result of the interaction between two or more cells (*13*). Therefore, in this study, we cultured rHPCs and HSC-T6 to develop steatosis and explore this interaction. As well to declare that the induced results such as the gene expression of DNL, *PCK1*, *PDK4*, *G6pase*, and CYPs were not coming from HSC-T6 cells, the heterogenous HFF-1cells were cultured with rHPCs and set as a control for HSC-T6 developed models. Then the results showed that the co-culture can prolong rHPCs culture *in vitro* to 15d compared to rHPCs culture alone (data not shown). In addition, the co-culture can protect hepatocyte functions, which may be due to cell contact and soluble factors production from HSC-T6 or HFF-1. Previous studies have proved that co-culture hepatocytes with HSC can maintain hepatocyte liver-specific functions and structure through cell contact and soluble factors (*21,27*). Similarly, previous study also showed that HFF-1 was a feeder cell that can maintain stem cell features (*i.e.*, pluripotency, immortality, and unlimited undifferentiated proliferation capability) when they were cultured with stem cells (*28*). Moreover, in this study we used high insulin and glucose medium, not free fatty acid (*13,29,30*), to develop steatosis, which was enlightened by developing animal NAFLD models through dieting low fat/high carbohydrate to mimic clinical patients hyperglycemic-hyperinsulinemic pathogenicity environment, and also to avoid free fatty acid to cause a negative effect on hepatocytes by generating cytotoxicity and caspase-dependent apoptosis (*31,32*).

Hepatic DNL plays a vital role in contributing to steatosis. As well, in this successful developed model, we proved that its steatosis development was regulated by gene expression of *SREBP1c* and *ELOVL6*, which is in line with previous reports, which showed that *SREBP1c* is a key transcription factor regulating lipid metabolism (*33,34*). However, those studies have shown that its regulation is through increasing its downstream fatty acid synthesis genes including *ACC, FASN*, *SCD1* and *ELOVL6* (*35,36*). While in our model, we only found the *ELOVL6* gene was up-regulated, and the other lipogenesis genes such as *ACC*, *FASN*, *SCD1*, *Fasd* and *Dgat2* were not increased. Consistent with those gene expressions, *ChREBP*, another major factor that regulates fatty acid synthesis, also showed down-regulation, which means that the down-regulation of *ACC*, *FASN*, *SCD1*, *Fasd* and *Dgat2* may be regulated by *ChREBP*. Because previous studies have reported that *ChREBP* is also the upstream regulator of *ACC*, *FASN*, *SCD1* and *Fasd* (*35,36*). Therefore, in our model, hepatosteatosis development may be regulated through *SREBP1c* and *ELOVL6* gene expression, not *ChREBP*, *ACC*, *FASN*, *SCD1* and *Fasd*. While its exact mechanism needs to be further explored.

Insulin resistance has been characterized as the crucial pathophysiological factor and tightly associated with every stage of NAFLD (*3,6-8*). However, this association was challenged by clinical and genetic animal studies (*15-19,25,26*), showing that hepatosteatosis is dissociated from insulin resistance. As well, in this study, we used the co-culture system successfully to develop hepatosteatosis, and further gene detection of *SREBP1c*, *ELOVEL6*, *PCK1*, *PDK4* and *G6pase* evidence confirmed that hepatosteatosis was dissociated from insulin resistance. The dissociation in this model was strongly confirmed by down-regulation of *DGAT2*. Because in previous studies, they found overexpression of *DGAT2* can cause hepatic insulin resistance in mice models of severe hepatic steatosis (*37,38*). Moreover, in insulin resistance obesity, the study also found that mice with deletion of X-box-binding protein-1(*XBP-1*) can lead to develop insulin resistance in HFD-dieted conditions (*39*), and the other study further found that the up-regulation of IκB kinase beta (IKKβ) activity in the liver of obese mice can increase XBP1s activity and reduce ER stress, resulting in a significant improvement in insulin sensitivity (*40*). In line with the above results, we also found that the gene expression of *XBP-1s* was up-regulated and the other ER stress-related genes were down-regulated in our developed model (data not shown). Therefore, we hypothesize that the mechanism of this insulin resistance dissociated from

hepatosteatosis might be caused by up-regulated XBP-1 or even by XBP-1 regulating its downstream pathways. Hence, the exact mechanism of this developed model should be further investigated in subsequent studies.

Cytochrome P450, mainly in families CYP1, CYP2, and CYP3, is the majority of enzymes in the liver to metabolize hepatically cleared drugs (*41*). Noteworthily, 75% of enzymatic reactions that occur in the metabolism of drugs are related to cytochrome P450s. As well, five genes of P450 are involved in the metabolism of more than 90% of the small-molecule drugs in use today (*42*), which suggests that P450s play a crucial role in considering new drug development. Therefore, in this study, we detected the genes expression of P450 including *CYP3A*, *CYP1A2*, and *CYP2E1*. The results showed that the expression of *CYP3A* was up-regulated, which was in line with the Onni Niemela et. al. clinical study results (*43*). However, this result is contrary to other recent clinical studies (*44*), leading to this opposite finding which may potentially be due to the two cells co-culture or insulin used in the culture system. Because previous studies have reported that co-cultured hepatocytes with endothelial cells can provide an optimal trophic support for the hepatocytes, which can improve functionality of hepatocytes such as cytochrome P450 activity and albumin secretion (*45,46*). As well, the other studies also have reported that the increase of *CYP3A* was caused by up-regulating of *NR1I2* (nuclear receptor subfamily 1, group I, member 2, also known as pregnane X receptor or *PXR)* transcript which could be overexpressed by induced insulin (*47,48*). Hence, the *CYP3A* increased in our developed model may be due to HSC-T6 of HFF-1 cells support or overexpression of *PXR* by induced insulin. On the contrary to expression of *CYP3A*, *CYP1A2* and *CYP2E1* were significantly down-regulated, and this result is in line with *in vitro* and clinical studies which showed that mRNA levels of *CYP1A2* and *CYP2E1* were decreased in hepatocytes or humans with progressive stages of NAFLD (*29,30,49*). Therefore, the expression characteristics of P450 in this developed model could guide development of novel therapeutic drugs for treating NAFLD, which is dissociated fatty accumulation from insulin resistance.

5. Conclusion

This study describes a co-culture approach to develop an *in vitro* model of steatosis, and validates that the hepatosteatosis was dissociated from insulin resistance. By co-culture rHPCs with HSC-T6 or HFF-1 in high insulin and glucose medium, we developed a simplistic set up of an *in vitro* hepatosteatosis model, and genes expression of *SREBP1c*, *ELOVL6*, *PCK1*, *PDK4* and *G6pase* validated that the model is successful and deeply shows that the steatosis is dissociated from insulin resistance. Taken together, these studies evidence that a high insulin and glucose stimulated co-culture system is a valuable approach for developing an *in vitro* hepatosteatosis model, which is dissociated from insulin resistance. In addition, this developed model will be a potential tool for investigating the molecular mechanism of the case of NAFLD, which dissociated from insulin resistance, thereby finding an effective and accurate therapeutic target for treatment.

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