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Human serum activates CIDEB-mediated lipid droplet enlargement in hepatoma cells

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ABSTRACT

Human hepatocytes constitutively express the lipid droplet (LD) associated protein cell death-inducing DFFA-like effector B (CIDEB). CIDEB mediates LD fusion, as well as very-low-density lipoprotein (VLDL) maturation. However, there are limited cell culture models readily available to study CIDEB's role in these biological processes, as hepatoma cell lines express negligible levels of CIDEB. Recent work has highlighted the ability of human serum to differentiate hepatoma cells. Herein, we demonstrate that culturing Huh7.5 cells in media supplemented with human serum activates CIDEB expression. This activation occurs through the induced expression of PGC-1α, a positive transcriptional regulator of CIDEB. Coherent anti-Stokes Raman scattering (CARS) microscopy revealed a correlation between CIDEB levels and LD size in human serum treated Huh7.5 cells. Human serum treatment also resulted in a rapid decrease in the levels of adipose differentiation-related protein (ADRP). Furthermore, individual overexpression of CIDEB was sufficient to down-regulate ADRP protein levels. siRNA knockdown of CIDEB revealed that the human serum mediated increase in LD size was CIDEB-dependent. Overall, our work highlights CIDEB's role in LD fusion, and presents a new model system to study the PGC-1α/CIDEB pathway's role in LD dynamics and the VLDL pathway.

1. Introduction

Cytosolic lipid droplets (LDs) were once thought to be static storage centres for excess neutral lipids. However, it is becoming quite apparent that cytosolic LDs are highly dynamic and play crucial roles in cellular processes, such as lipoprotein assembly and secretion in the liver [1]. Their relevance in liver pathologies is also becoming more evident. For example, the hepatitis C virus (HCV) RNA encodes a core protein that localizes to the LD surface [2–4]. This interaction plays a crucial role in proper viral particle assembly [5]. Increased understanding of the host factors which regulate the dynamics of LDs will prove instrumental in creating new therapeutic angles to combat metabolic disorders [6].

The LD surface serves as a platform for several LD binding proteins, including the well-studied PAT (perilipin, ADRP, TIP47, and related proteins) family and CIDE (cell death-inducing DFF45 like effector) family [7]. These host factors determine the diverse functionality of the LDs. While the PAT family represent well-characterized LD-associated proteins [8], the CIDE proteins have only recently emerged as important regulators of lipid homeostasis [9]. The CIDE family consists of CIDEA, CIDEB, and CIDEC (or FSP27). All members of the CIDE family have functional roles in LD clustering and fusion [9–13]. Interestingly, CIDEB is the only member of the family that is constitutively expressed in the liver and implicated in VLDL maturation [11,14].

Aside from primary cell culture and mice models, there are limited models to study CIDEB in the liver. The commonly used hepatoma cell models (e.g. HepG2 and Huh7 derivatives) expressed low to undetectable levels of CIDEB in comparison to primary hepatocytes [14]. To-date, previous reports have demonstrated that CIDEB mRNA expression is controlled by HNF-4α [15,16] and PGC-1α

Abbreviations: CIDE, cell death-inducing DFFA-like effector; ADRP, adipose differentiation-related protein; CARS, coherent anti-Stokes Raman scattering; PPAR, peroxisome proliferator activated receptor; PGC-1α, PPAR-γ co-activator 1α; HNF-4α, hepatocyte nuclear factor 4α; VLDL, very low density lipoprotein; LD, lipid droplet; TG, triglyceride.

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[17]. HNF-4α is a transcription factor associated with the promotion of cellular differentiation [18]. Furthermore, CIDEB expression in the kidney was found to be down-regulated during renal carcinoma [15]. Overall, these studies suggest CIDEB expression may be down-regulated in hepatoma cells due to their de-differentiated state.

In a recent study, human serum (HS) treatment was shown to induce differentiation in Huh7.5 cells. While Huh7.5 cells maintained in the presence of traditional fetal bovine serum (FBS) are unable to secrete nascent VLDL particles, these HS-treated Huh7.5 cells were able to secrete TG-enriched lipoproteins [19]. Since CIDEB plays an important role in VLDL lipidation [11], we hypothesized that HS-induced differentiation of Huh7.5 cells may rescue VLDL secretion through activation of CIDEB expression. In this report, we demonstrate that HS-induced differentiation in Huh7.5 cells promotes CIDEB expression through up-regulation of PGC-1α. Furthermore, we show the increased expression of CIDEB correlates with increased LD size and a drastic drop in ADRP levels. Our work establishes a new model to study CIDEB's role in hepatic LD morphology and dynamics.

2. Materials and methods

2.1. Cell culture and reagents

Adherent cells were cultured as previously described [19]. Huh7 and Huh7.5 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Burlington, ON) supplemented with either HS (Invitrogen, 34005-100, pooled human AB serum, lot number 1215151) or 10% fetal bovine serum (FBS) and 100 nM non-essential amino acids (NEAA), 50 U/mL penicillin, and 50 mg/mL streptomycin. The plasmid expressing CFP was previously described [20]. The human CIDEB gene (GenBank Accession Number AAH35970.1) was cloned from cDNA obtained from the HepG2 hepatoma cell line using primers that are listed in Table S1 for cloning into pCMVβ and pIRES2-EGFP (bicistronic construct expressing EGFP).

2.2. Transfection

siRNA transfections were performed using Lipofectamine RNAiMax (Invitrogen), following manufacturer's protocols. CIDEB (SMARTpool, Thermo Fisher Scientific, Waltham, MA, USA) and negative control (Ambion, Austin, TX) siRNAs were transfected in HS cultured Huh7.5 cells at a concentration of 50 nM for 96 h, followed by a second transfection at 50 nM for 72 h. Plasmid transfections were performed using Lipofectamine 2000 (Invitrogen), following manufacturer's protocols.

2.3. Immunoblot analyses

After treatment with appropriate media, cells were washed twice with PBS and lysed with an SDS lysis buffer consisting of 50 mM Tris–HCl (pH 6.8), 2% SDS, and 10% glycerol. A protease inhibitor cocktail mix (Roche Diagnostics, Penzberg, Germany) was added to each extract. The protein concentration of each sample was quantified using the Bio-Rad DC Protein Assay according to the manufacturer's protocol. Prior to loading, 10% v/v of DTT and bromophenol blue (1:1) were added to each sample, and 30–60 μg/well was loaded onto a SDS–PAGE gel (10% resolving, 4% stacking gel). The resolved proteins were transferred to a Hybond-P (Amersham Biosciences, Piscataway, NJ) polyvinylidene difluoride membrane. The membrane was probed using a mouse anti-CIDEB (sc-101244; 1:200 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti-PGC-1α, (sc-13067; 1:200 dilution; Santa Cruz Biotech. Inc.), goat anti-HNF-4α (sc-6556; 1:1000 dilution; Santa Cruz Biotech. Inc.), mouse anti-ADRP (1:100; Progen Biotechnik, Heidelberg, Germany) or mouse anti-PTP1D (1:10,000 dilution; Sigma, Saint Louis, MO) primary antibodies followed by a secondary (HRP)-conjugated goat anti-mouse, donkey anti-goat, or donkey anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA). Protein bands were visualized by Western Lightning Western Blot Chemiluminescence reagents (GE Healthcare, Baie d'Urfé, QC) according to the manufacturer's protocol.

2.4. RNA isolation and qRT-PCR

RNA isolation from hepatocytes was performed using TRIzol (Invitrogen) as per the manufacturer's protocol. RNA integrity was confirmed by electrophoresis on a 0.8% agarose gel in 1× TBE (Ambion, Austin, TX). For mRNA levels, 500 ng of total RNA was used for cDNA synthesis using the Superscript II kit (Invitrogen, Burlington, ON) according to the manufacturer's protocol. Quantitative PCR (qPCR) was subsequently performed on an iCycler (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad), as per manufacturer's protocol. Primer sequences are listed in Table S1. A 20 μL reaction was assembled according to the manufacturer's protocol. For data analysis, the $2^{-\Delta\Delta Ct}$ method was used, and mean fold changes in expression are shown relative to cells maintained in FBS supplemented media [20].

2.5. CARS microscopy/two-photon fluorescence (TPF) and image analysis

Imaged cells were washed twice with PBS, followed by a 15 min incubation at room temperature with fixing solution (4% formaldehyde, 4% sucrose, 1 mL). The fixed cells were washed twice with PBS for 3 min and then stored at 4 °C in PBS prior to imaging. The CARS microscopy system was setup as previously described [20]. The imaging of TG content was performed as previously described [20]. Lipid droplet counting and sizing was performed using the ImageJ plugin for Particle Counting and Analysis (National Institutes of Health).

2.6. Statistics

Student's *t*-test was used to analyze the data, and *p*-values less than 0.05 were deemed significant.

3. Results

3.1. Human serum activates the expression of CIDEB and PGC-1α

Previous work demonstrated that human serum (HS) treatment over a period of 30 days gradually rescued ApoB lipidation and VLDL secretion in hepatoma cells [19]. We sought to determine how HS treatment and hepatoma cell differentiation affects CIDEB expression. We treated Huh7.5 cells with HS supplemented media during a similar time course and isolated protein or RNA samples. qRT-PCR analysis revealed that, after 14 and 21 days of HS treatment, mRNA levels of CIDEB increased over five-fold compared to FBS treated Huh7.5 cells. This increase was less prominent, but still significantly higher than FBS treated cells, at later time points (after 30 days). Western blot analysis revealed a maximal induction of CIDEB expression after 21 days of HS treatment.

We hypothesized that HNF-4α, a transcriptional regulator of CIDEB, may be responsible for its up-regulation as the transcription factor is a known promoter of hepatic differentiation [18]. Surprisingly, there were only modest changes in HNF-4α levels (Fig. 1A,

B). However, PGC-1α, another known positive regulator of CIDEB expression, showed dramatically increased levels of expression upon HS treatment. PGC-1α mRNA levels were consistently increased over five-fold during the 30 day treatment, and displayed an over 13-fold increase on day 14. Maximum PGC-1α protein levels were observed after 21 days of HS treatment (Fig. 1A, B). This correlation between CIDEB and PGC-1α strongly suggests HS-differentiated Huh7.5 cells activate CIDEB expression in a PGC-1α dependent manner.

A recent study proposed opposing roles of CIDEB and ADRP, another LD-associated protein, in regulating lipid homeostasis [21]. While CIDEB had a positive influence on VLDL maturation, ADRP was reported to have a negative effect on VLDL secretion [21]. In the latter study, *Cideb* −/− mice demonstrated an increase in ADRP levels [21]. We wished to examine whether a similar inverse relationship existed in the HS model. Interestingly while ADRP mRNA expression was increased, there was a drastic drop in ADRP protein levels (Fig. 1A, B). HS treatment has been shown to increase PPAR-α and PPAR-γ levels [19]; therefore, the observed increase in ADRP mRNA levels is consistent with the presence of an active PPAR response element in the promoter of ADRP [22].

We independently overexpressed CIDEB in Huh7 cells (Fig. S1A). Western blot analysis revealed that CIDEB overexpression resulted in a significant down-regulation of ADRP protein levels. Overall, our data suggests that HS treatment induces CIDEB expression through a PGC-1α dependent mechanism; furthermore, CIDEB overexpression is sufficient to down-regulate ADRP protein levels.

3.2. Human serum differentiated hepatoma cells display larger LDs

CIDEB and other members of the CIDE family (CIDEA and CIDEC/FSP27) have previously been shown to promote LD clustering and fusion [10–12]. Since HS supplementation activated CIDEB expression, we sought to analyze its effect on the size of LDs using coherent anti-Stokes Raman scattering (CARS) microscopy. CARS microscopy enables label-free imaging of cytoplasmic LDs in cells [23]. Our results demonstrated that HS treatment induces larger LDs in Huh7.5 cells (Figs. 2 and S2). Specifically, a gradual increase in average LD diameter was observed as the duration of HS treatment was carried out over the period from 14 to 30 days, in comparison to cells maintained in FBS supplemented media (Fig. 2B). Analysis of the LD size distribution revealed that HS treatment promotes the formation of LDs with a diameter greater than 1.0 μm, while the proportion of LDs with a diameter less than 0.5 μm was greatly reduced (Fig. 2C).

We transfected Huh7 cells with a bicistronic CIDEB construct expressing EGFP and imaged CIDEB-overexpressing cells using simultaneous two-photon microscopy and coherent anti-Stokes Raman scattering (CARS) microscopy. Two-photon fluorescence enables GFP-mediated visualization of cells expressing the CIDEB constructs. The cells were fixed 48 h post-transfection and were subsequently imaged (Fig. S1B). This experiment was performed in the presence of oleic acid, as a previous report suggested incubation with oleic acid decreases the cytotoxicity during overexpression of the CIDE family proteins [24], by promoting their localization to LDs. As expected, compared to mock transfections, wildtype CIDEB (WT) transfection resulted in significantly larger LDs. Since CIDEB can independently induce LD enlargement, our data suggests that HS-activated CIDEB expression promotes an increase in LD size.

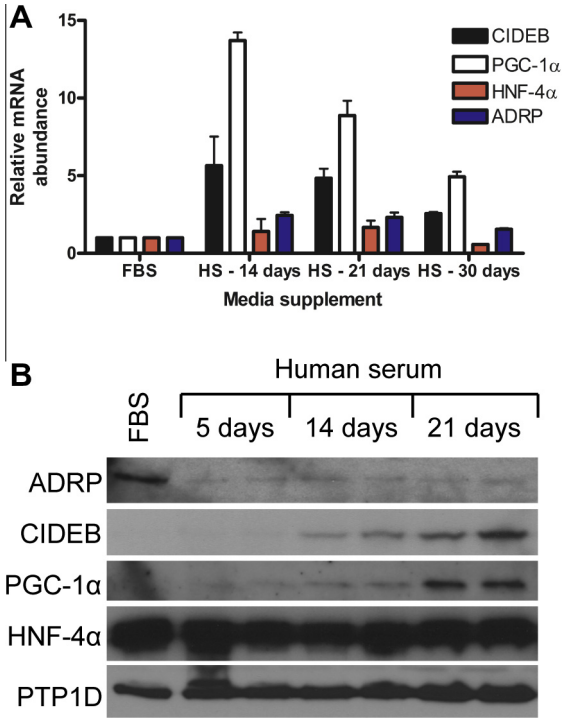

Fig. 1. Human serum activates CIDEB expression. Huh7.5 cells were cultured in media supplemented with either FBS or human serum (HS). Total RNA and protein samples were isolated at the indicated time points. (A) qRT-PCR was performed for PGC-1α, CIDEB, ADRP, and HNF-4α levels. Relative RNA levels are shown after normalization by 18S rRNA levels. Error bars represent the standard deviation. (B) Western blotting was performed for CIDEB, PGC-1α, ADRP, and HNF-4α levels. PTP1D is shown as a loading control. A representative blot is shown. Results are representative of three independent trials.

3.3. CIDEB siRNA knockdown impairs human serum activated LD fusion

In order to determine whether CIDEB played a key role in HS differentiation's induction of larger cytosolic LDs, we transfected Huh7.5 cells with control or CIDEB siRNAs during 7 days of HS-supplemented media treatments (Figs. 3 and S2). The HS treatment was limited to seven days as differentiation of the hepatoma cells decreased their transfection efficiency. Western blot analyses confirmed knockdown of CIDEB in the siRNA transfected cells (Fig. 3A). CARS analysis revealed that CIDEB knockdown impaired the HS's ability to increase LD size (Fig. 3B). The average LD diameter decreased from (823.3 ± 0.8) nm in control siRNA transfected cells to (694.1 ± 0.9) nm in the CIDEB siRNA transfected cells ($p < 0.0001$) (Fig. 3C). Furthermore, a comparison of the LD size distribution in the two sets of cells (Fig. 3D) revealed that CIDEB knockdown resulted in a decrease of LDs with diameter greater than 1 μm, and an increase in LDs with diameter less than 0.5 μm. Collectively, our data demonstrates that HS differentiation induces LD fusion in a CIDEB-dependent manner.

4. Discussion

Recent findings have established LDs as dynamic organelles which serve as protein scaffolds that determine their diverse functions [1]. Furthermore, morphological differences in the cytosolic LDs can largely be attributed to their associated proteins [7]. We observed that HS differentiated hepatoma cells possess significantly larger LDs (Fig. 2). Our data suggests this is largely due to an increase in CIDEB expression (Fig. 1) as previous work has shown that CIDEB overexpression can independently induce an increase in LD diameter [10], likely through increased LD fusion and clustering in a similar fashion to CIDEA and CIDEC/FSP27 [10,12,13]. This is supported by the fact that siRNA-mediated CIDEB knockdown impairs HS activated LD enlargement (Fig. 3).

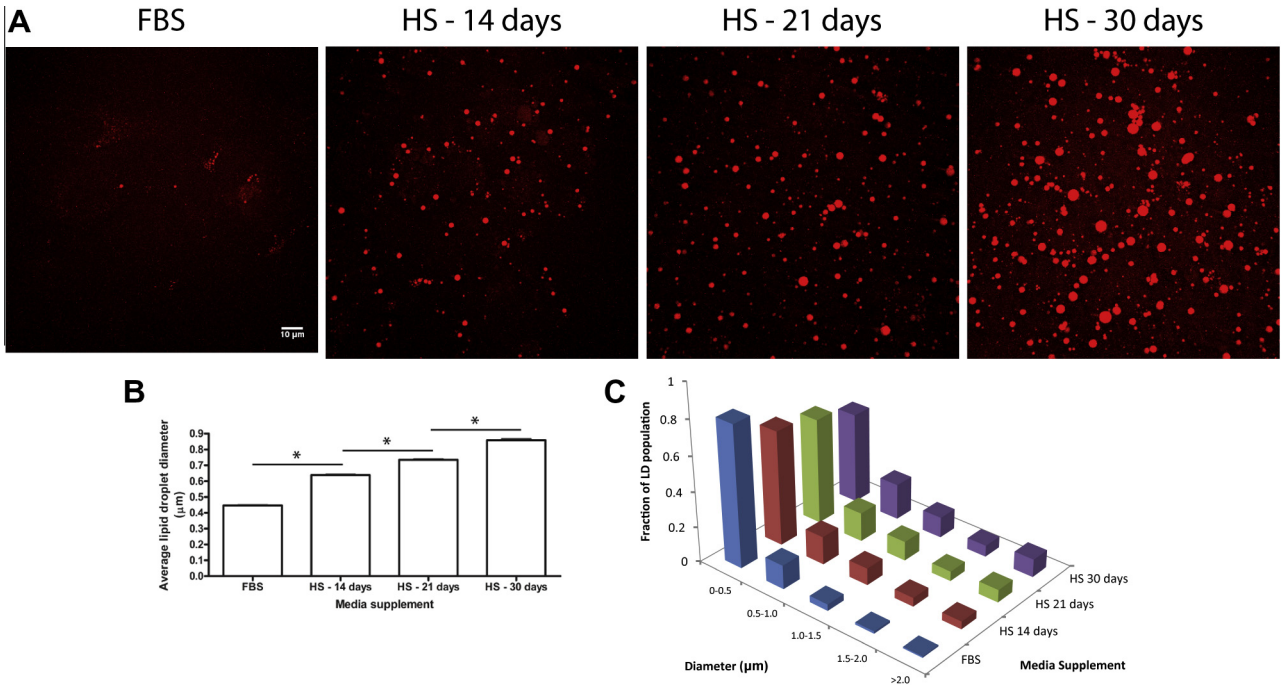

Fig. 2. Human serum promotes enlargement of LDs. Huh7.5 cells were cultured in media supplemented with either FBS or human serum (HS). At the indicated time points, cells were fixed and imaged with CARS microscopy. (A) Representative CARS images are shown for FBS and HS treated cells at various time points. Scale bar represents 10 μm. (B) Average diameter and (C) size distribution of LDs are shown. Error bars represent standard error of the mean ($n \geqslant 900$ LDs) ($^*p < 0.0001$).

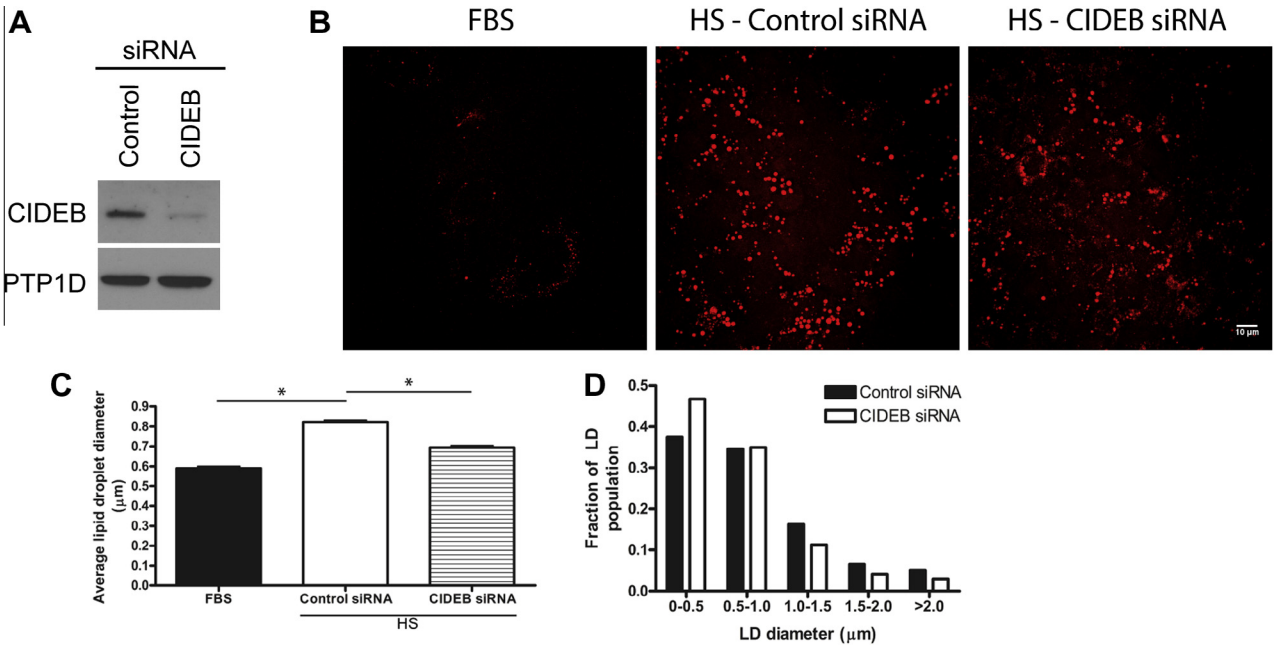

Fig. 3. CIDEB knockdown impairs human serum activated LD enlargement. Huh7.5 cells were cultured in FBS or human serum (HS) supplemented media. HS cultured cells were transfected with 50 nM control or CIDEB siRNAs for 7 days. (A) Western blot validating CIDEB knockdown. Immunoblotting was performed for CIDEB and PTP1D (loading control). (B) Cells were fixed and imaged with CARS microscopy. Representative CARS images are shown. Scale bar represents 10 μm. (C) Average diameter and (D) size distribution of LDs are shown. Error bars represent standard error of the mean ($n \geqslant 2700$ LDs) ($^*p < 0.0001$).

Overall, our data demonstrates that HS induces LD enlargement in a CIDEB dependent manner (Fig. 4).

A recent study demonstrated that HS treatment rescues VLDL lipidation in Huh7.5 cells [19]. The mechanism by which this occurs remains unclear. Our work herein reveals that HS differentiated hepatoma cells exhibit up-regulated CIDEB and PGC-1α levels (Fig. 1). A recent study elucidated that overexpression of PGC-1α could independently stimulate VLDL assembly in a CIDEB-dependent manner [17]. Moreover, the up-regulated CIDEB expression correlates with previous observations of HS-mediated

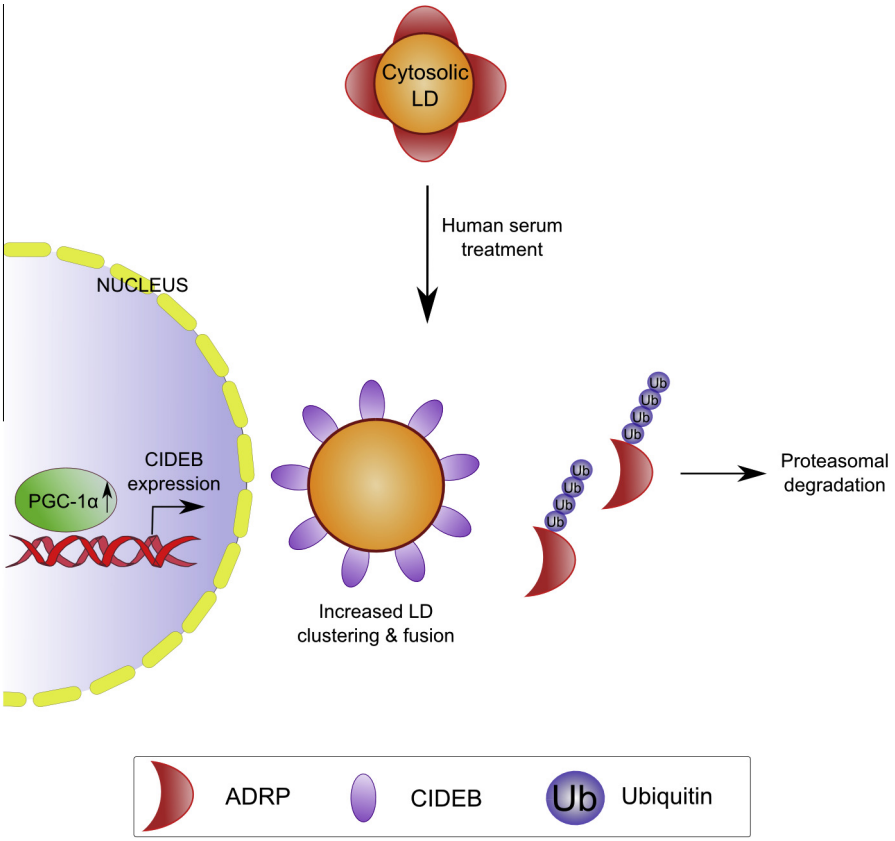

Fig. 4. Proposed model of human serum's influence on LD morphology. Our data suggests a model by which human serum differentiation of hepatoma cells up-regulates PGC-1α levels. In the nucleus, PGC-1α transcriptionally activates CIDEB expression. CIDEB outcompetes ADRP for binding of cytosolic LD surfaces and promotes LD clustering and fusion. In the absence of LD binding, ADRP is ubiquitinated and sent to the proteasome pathway for degradation.

rescue of VLDL secretion in Huh7.5 cells [19]. Taken together, this suggests that HS rescues proper lipidation and secretion of VLDL particles by activating the PGC-1α/CIDEB pathway.

Furthermore, the CIDEB induced down-regulation in ADRP protein levels may also contribute to the rescue of proper VLDL maturation as ADRP inhibits secretion of VLDL particles [25]. This is further supported by a previous study demonstrating that ADRP silencing in *Cideb* −/− mice restores VLDL lipidation [21]. However, the drop in ADRP protein levels suggests that ADRP translation or protein stability is being compromised during HS differentiation. Previous work has shown that, in the absence of LD binding, ADRP is rapidly degraded via the ubiquitin–proteasome system [26]. Our data suggests that HS-induced CIDEB out-competes ADRP for binding at the surface of cytosolic LDs, resulting in ADRP degradation (Fig. 4).

To-date, there are limited cell culture models which enable examination of hepatic lipid metabolism. Hepatoma cell lines, such as HepG2 and Huh7 cells, lack proper assembly and secretion of VLDLs [27], limiting their value in studying hepatic metabolism and liver disease. In fact, the Huh7 cell line and its derivatives (e.g. Huh7.5) represent one of the main cell culture models to study hepatitis C virus (HCV) infection due to their permissivity to the entire viral life cycle. However, the infectious viral particles which are produced via this HCV cell culture model lack the ApoB association and lipidation that is observed in patients [28]. It has been proposed that proper VLDL maturation requires a secondary lipidation step [29], potentially mediated by CIDEB. Improper lipidation of ApoB results in ApoB misfolding and degradation [29]. Therefore, the insufficient ApoB lipidation due to a lack of CIDEB expression in Huh7 cells may explain the lack of ApoB associated with cell culture-derived HCV particles. Additionally, a lack of CIDEB expression may account for the poorly lipidated viral particles secreted from the Huh7 family of cell lines [28]. This is consistent with the observed decrease in the density of HCV particles secreted from HS treated hepatoma cells, which is suggestive of TG enrichment of VLDLs [19]. Future work using the HS induced differentation model should enable examination of CIDEB's role in HCV particle lipidation.

In conclusion, we have characterized a recently developed culturing method for hepatoma cells as a model for studying the role of CIDEB in LD dynamics and the VLDL pathway. Our work highlights the activation of the PGC-1α/CIDEB pathway as a key mechanism by which HS treatment induces LD fusion and proper VLDL assembly. This cell culture system should prove highly useful in future studies of LD dynamics and VLDL secretion in the context of liver physiology and disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.080.

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