

Proteomic Comparative Analysis of Pregnancy Serum Facilitating Hepatitis E Virus Replication in Hepatoma Cells

Li Yi ^{1,2}, Yanhong Bi ¹, Wenhai Yu ³, Chenchen Yang ¹, Jue Wang ¹, Feiyan Long ¹, Yunlong Li ¹, Fen Huang ^{1*}, Xiuguo Hua ^{2*}

1. Medical Faculty, Kunming University of Science and Technology, Kunming, China;

2. Agriculture and Biotechnology, Shanghai Jiaotong University, Shanghai, China;

3. Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Kunming, China;

*Correspondence to Fen Huang, E-mail: huangfen6789@163.com, Tel: +86 871 5920776;

Fax: +86 871 5920776; Xiuguo Hua, E-mail: hxg@sjtu.edu.cn, Tel: +86 21 34206928; Fax: +86 21 34206928

Abstract: Hepatitis E virus (HEV) is a common cause of acute hepatitis worldwide, accounting for approximately 25% of deaths among pregnant women. We previously reported that pregnancy serum facilitates HEV replication *in vitro*. However, the differences in host cells with HEV infection induced by pregnancy serum and fetal bovine serum (FBS) are unclear. In this study, differentially expressed proteins were identified in HEV-infected hepatoma cells (HepG2) supplemented with different sera by using isobaric tags for relative and absolute quantitation. Proteomic analysis indicated that HEV infection significantly induced 1014 differentially expressed proteins in HEV-infected HepG2 cells when supplemented with FBS compared with pregnancy serum. Further validation by Western blot confirmed that filamin A, heat-shock proteins 70 and 90, Cytochrome c, and Thioredoxin were associated with HEV infection. This comparative analysis provides an important basis to further investigate HEV pathogenesis in pregnant women and HEV replication.

Keywords: hepatitis E virus; proteomic comparative analysis; pregnancy serum

Introduction

Hepatitis E virus (HEV) is the causative agent of acute fulminant hepatitis. HEV is a zoonotic virus that uses domestic animals as reservoirs. HEV is predominantly transmitted through the fecal–oral route (Arankalle et al., 2001; Okamoto, 2007). This disease infects

about 20 million people and causes 70,000 deaths every year (Rein et al., 2012). The infection is generally acute and largely self-limited, but HEV-infected immunocompromised individuals have a high risk of developing chronic hepatitis (Kenfak-Foguena et al., 2011). The mortality rates of HEV infection are generally low but rather high in pregnant women (up to 30%), especially during their third trimester of pregnancy (Kumar et al., 2004; Labrique et al., 2012; Wedemeyer et al., 2012). During pregnancy, HEV infection can cause serious threats to the mother and fetus. HEV infection transmitted from mother-to-fetus usually causes high neonatal mortality, leading to fetal loss, premature birth, birth of a dead fetus, or icteric acute hepatitis in the newborns (Kar et al., 2002; Kumar et al., 2004). HEV is the main etiologic agent that causes death in pregnant women with acute liver failure in rural Bangladesh (Labrique et al., 2012). Jilani (Jilani et al., 2007) reported an extremely high mortality rate of 65.8% in HEV-infected pregnant women with fulminant hepatic failure. About 58% of the deaths of pregnant women with acute liver disease in hospitals are associated with HEV in Bangladesh (Gurley et al., 2012b). However, the pathogenesis of HEV in pregnant women remains unclear.

We previously reported that pregnancy serum facilitates HEV replication (Bi et al., 2015), but the reasons why pregnancy serum is a better promoter of HEV replication in cells than fetal bovine serum (FBS) are unknown. Viral infections usually alter host cell function to develop a suitable environment for viral replication. Proteomic methods can be used to comprehensively characterize virus–host interactions and thus understand HEV infection and pathogenesis. Several quantitative proteomic analyses have been used to provide specific insights into the gene expression profiles of hepatitis B virus (HBV) (Zhang et al., 2009), hepatitis C virus (HCV) (Yang et al., 2011), and influenza virus (Dapat et al., 2014a). Viral infection of host cells not only can cause changes in transcriptional patterns, cell cycle, cytoskeleton, apoptotic pathways, immune system, and stress response but also induce inflammation (Gurley et al., 2012a). Proteomic analysis is rarely applied in HEV-infected host cells. Shen et al. (Shen et al., 2014) found 31 differentially expressed proteins (10 significantly upregulated and 21 significantly downregulated) in HEV-infected A549 cells compared with uninfected cells. However, the difference between HEV-infected cells supplemented with the sera of pregnant women and non-pregnant women remains unknown. HEV infection in HepG2 cells supplemented with pregnancy serum was more efficient than that supplemented with FBS. Therefore, quantitative proteomic analysis of HEV-infected HepG2 cells supplemented with the sera of pregnant or non-pregnant women may explain why pregnancy serum facilitates HEV replication.

In the present research, differentially expressed proteins in HEV-infected HepG2 cells supplemented with pregnancy serum (from the third trimester) or FBS were analyzed using isobaric tags for relative and absolute quantitation (iTRAQ) to investigate HEV replication-related host proteins. Results indicated that HEV infection resulted in 1014 significantly modulated proteins in HepG2 cells supplemented with different sera. Functional analysis showed that these proteins were involved in cytoskeletal components, immune response, complement activation, metabolism, and cell signaling.

Results

HEV replicated in HepG2 cells supplemented with pregnancy serum

To compare HEV-infected HepG2 cells supplemented with pregnancy serum or FBS, HEV replication was first determined. HEV progeny virion was detected using RT-nPCR in HepG2 cells supplemented with FBS, healthy non-pregnancy serum, or pregnancy serum at 6 dpi (Fig. 1A). HEV ORF2 protein was detected through Western blot with ORF2 specific antibody. Similarly, HepG2 cells supplemented with pregnancy serum significantly facilitated HEV replication, which was consistent with that in HEV-infected A549 cells. However, the HEV ORF2 protein in cells supplemented with FBS was barely detected (Fig. 1B).

Proteomic analysis of host protein changes induced by HEV infection supplemented with different sera through iTRAQ

The host–virus interactions in HEV-infected cells were comprehensively investigated, and cells supplemented with pregnancy serum and FBS were compared. HEV-infected cells were collected at 6 dpi, and proteins were extracted for liquid chromatography mass spectrometry. The quantitative protein ratios were weighed and normalized by the median ratio in Mascot. We only used ratios with $P < 0.05$, and only fold changes of 1.2 were considered significant. Statistical analysis showed that 1511 unique proteins were identified in HEV-infected HepG2 cells with 95% confidence and 1014 proteins were significantly differentially expressed (Fig. 2).

HEV infection upregulated and downregulated 262 and 66 proteins in cells supplemented with FBS, respectively, and upregulated and downregulated 63 and 201 proteins in cells supplemented with pregnancy serum, respectively (Fig. 2). Thus, a large number of the proteins in the host cells changed in expression. Furthermore, the HEV-infected cells supplemented with FBS showed 91 upregulated and 331 downregulated

proteins compared with the HEV-infected cells supplemented with pregnancy serum (Fig. 2). The cells supplemented with pregnancy serum downregulated more host cell proteins than the cells supplemented with FBS.

Functional analysis of modulated proteins induced by HEV infection

Functional annotations of modulated proteins induced by HEV infection were mainly involved in pathogen infection (51.11%), metabolic pathways (15.84%), and primary immunodeficiency (8.52%). The other proteins were implicated in phagosome, calcium signaling pathway, transcriptional regulation, natural killer cell-mediated cytotoxicity, B-cell receptor signaling pathway, complement and coagulation cascades, and cytoskeleton regulation. The result was similar to that reported in acute HEV infection in a swine model with regulated proteins mainly involved in metabolism, toxicity/inflammatory response, and cytoskeleton trafficking (Rogee et al., 2015). Clusters of orthologous group functional classification is mainly involved in post-translational modification, protein turnover, chaperones, translation, ribosomal structure and biogenesis, energy production and conversion, and cytoskeleton. Gene ontology enrichment analysis showed that most of the identified proteins are phosphoproteins involved in RNA processing, immune system, and infection response. The common modulated proteins (37 proteins) in the HEV-infected HepG2 cells supplemented with different sera or without HEV infection are listed in Table 1.

Validation of proteomic data by Western blot

The HEV-modulated proteins were verified through proteomic analysis. The changes in five proteins involved in cytoskeleton (filamin A), immune responses (HSP70 and HSP90), and apoptosis (CytC and TXN) were analyzed via Western blot at 48 h post-inoculation. GAPDH served as the control.

The actin-binding protein filamin A plays a central role in signal transduction by linking the actin cytoskeleton to various transmembrane proteins to facilitate intracellular communication (Feng and Walsh, 2004). In this study, we proved that the expression of filamin A decreased in HepG2 cells which supplemented with human serum (from non-pregnant and pregnant women), moreover, the expression of filamin A was significant repressed in cells supplemented with pregnancy serum (Figs. 3A and B). In HEV-infected HepG2 cells, the abundance of filamin A decreased in the cells supplemented with FBS at 12, 24h post-inoculation with HEV and in the cells supplemented with non-pregnancy serum at 48h post-inoculation with HEV, whereas that of filamin A significant increased in the cells

supplemented with non-pregnancy serum at 4, 12, 24h post-inoculation with HEV and the HEV-infected cells supplemented with pregnancy serum (Figs. 3A and C). Filamin A also increases in HCV-expressing human hepatoma cells (Ghosh et al., 2011). Moreover, filamin A as an adaptor protein links human immunodeficiency virus-1 (HIV-1) receptors to the actin cytoskeleton remodeling machinery to facilitate virus infection (Jimenez-Baranda et al., 2007).

HSPs are immunogenic molecules that can increase cellular immunity (Kaufmann, 1990). HSP expression is frequently upregulated in hepatocellular carcinomas (HCCs), and the increased HSP levels strengthen cell resistance to apoptosis (Lim et al., 2005). HSP70 significantly increased in HepG2 cells supplemented with human serum. HSP90 also increased in cells supplemented with human serum, however pHSP90 did not increase (Figs. 3A and 3B). Moreover, Both HSP70 and HSP90 almost increased in HEV-infected HepG2 cells supplemented with FBS or non-pregnancy serum while pHSP90 showed inapparent changes (Figs. 3A and 3C). This result is consistent with the observation in HEV-infected A549 cells (Shen et al., 2014). HSP70 and HSP90 also increase in HBV-related HCCs (Lim et al., 2005). However, those increases of HSP70 and HSP90 were not clear in HEV-infected HepG2 cells supplemented with pregnancy serum while pHSP90 had a significant increase (Figs. 3A and 3C).

CytC regulates the intrinsic apoptotic pathway and is altered during viral infections. CytC oxidase (COX) VIC is induced in the early stage of influenza virus infection (Othumpangat et al., 2014). Moreover, the X protein of HBV impairs the mitochondrial respiration chain and energy metabolism through interactions with COX subunit III (Li D, 2015). HIV infection results in the dysregulation of CytC (Eugenin and Berman, 2013). CytC decreased in HepG2 cells supplemented with human serum (Figs. 3A and 3B). However, CytC had a significant increase in the HEV-infected HepG2 cells supplemented with pregnancy serum and in the cells supplemented with FBS and non-pregnancy serum at 4, 24h post-inoculation with HEV (Figs. 3A and 3C). CytC interaction with the HEV capsid protein has been confirmed using the yeast two-hybridization system (Shen et al., 2011). Furthermore, the HEV ORF3 protein is reportedly associated with CytC to preserve mitochondrial potential and prevent CytC release (Moin et al., 2007).

TXN is a key component in the link between redox regulation and disease pathogenesis (Yoshihara E, 2014). TXN is mainly involved in the regulation and activation of NF- κ B and other transcription factors; TXN also displays antiviral, anti-inflammatory, anti-apoptotic, cell proliferative activities (Yoshida et al., 2003). Nakamura (Nakamura et al., 2002) found

that TXN plays a protective role on influenza virus. The protective mechanism of TXN can be attributed to its potent anti-oxidative and anti-inflammatory activities. TXN domain-containing protein 5 is reportedly be associated with HCV replication (Real et al., 2013). Meanwhile, recombinant human TXN-1 significantly improves the survival rate and attenuates lung histological changes in the murine model of influenza pneumonia (Yashiro et al., 2013). In the present study, TXN decreased in HepG2 cells supplemented with human serum (Figs. 3A and 3B). However, TXN increased in HEV-infected HepG2 cells supplemented with human serum (Figs. 3A and 3C). The increased TXN protein in the HEV-infected cells revealed that TXN may be involved in the antiviral host process.

Discussion

Efficient HEV culture has been developed in PLC/PRF/5 (human hepatoma cells) and A549 cells (human lung cancer cells) (Takahashi et al., 2007), which facilitates the study on the molecular mechanism and pathogenesis of HEV. However, HEV infection is feeble in another hepatocellular carcinoma cell line (HepG2) when supplemented with FBS. HEV RNA was efficiently transcribed in the HEV-infected HepG2 cells by RT-nPCR, but the HEV ORF2 protein was rarely detected through Western blot. Some post-transcription inhibition possibly occurred, or the synthesis of the capsid protein of HEV was blocked during the later stage of biosynthesis in the viral life cycle. Surprisingly, HEV replication was highly efficient in the HepG2 cells supplemented with pregnancy serum. A significant and quantitative change occurred when the cells were supplemented with different sera, especially the serum from women in their third trimester. These protein changes must be identified using quantitative, sensitive, and high-throughput methods, such as iTRAQ, which has been applied in many host–virus interaction studies, including HIV-or HBV-infected patients (Bora et al., 2014; Qi et al., 2013) or influenza virus-infected A549 cells (Dapat et al., 2014b).

The pathogenesis of HEV infection in pregnant women remains unknown. We previously reported that pregnancy serum significantly facilitates HEV replication in vitro, but the viral–host interaction remains unclear. In general, viruses alter the signaling pathways of host cells to create a suitable environment for viral infection and replication. Therefore, a comprehensive proteomic analysis should be performed to understand the differentially expressed proteins in HEV-infected cells supplemented with different sera. In the present study, 1511 unique proteins were identified in the HEV-infected HepG2 cells; 1014 of these proteins significantly changed. Proteomic analysis indicated that HEV infection was involved in changes in gene molecular functions, cellular component, and biological process. Cells

supplemented with FBS induce more upregulated proteins than those supplemented with pregnancy serum (262 versus 63). By contrast, cells supplemented with pregnancy serum inhibit a large number of proteins of host cells (63 versus 201), which are mainly associated with immune and inflammation system, energy metabolism, and cytoskeleton.

In this study, five proteins associated with virus replication were further validated through Western blot. iTRAQ and Western blot analyses revealed that the cells supplemented with pregnancy serum shared nearly complete different results compared with those supplemented with FBS. Results suggested that HEV in the pregnancy serum-supplied cells may regulate different proteins (more down-regulated proteins) of host cells to facilitate viral replication.

Swine HEV-infected liver was analyzed through 2D differential gel electrophoresis to identify significant alternations in 10 protein spots in an HEV-infected organ; this analysis further confirmed that apolipoprotein E (Apo E) and ferritin heavy chain are regulated by HEV infection (Lee et al., 2011). Moreover, Rogee et al. (Rogee et al., 2015) identified 61 modulated proteins (Apo E and prohibiting) in the HEV-infected liver of swine; these proteins are known to be involved in other viral life cycles. In the present study, Apo E was also identified in HEV-infected HepG2 cells, indicating that Apo E may be important in HEV infection. Furthermore, CytC was also identified to interact with both HEV ORF2 and ORF3 proteins (Moin et al., 2007; Shen et al., 2011) to promote viral replication.

In conclusion, the differentially expressed proteins in HEV-infected HepG2 cells supplemented with FBS or pregnancy serum were screened using the iTRAQ method. A total of 1014 significantly differentially expressed proteins were obtained. This study is the first to use a high-throughput proteomic method to analyze the host cell protein changes in HEV-infected HepG2 cells. Furthermore, the HEV-infected HepG2 cells supplemented with pregnancy serum were compared with the cells supplemented with FBS to explore the pathogenesis of HEV in pregnant women. These data provided many resources to study the interactions between HEV and host cells, although further works are required to discover the molecular mechanism and pathogenesis of HEV.

Methods

Ethical statement

All serum was collected from patients during hepatitis E outbreaks for epidemic investigations in 2012 in Kunming City, China. This study was approved by medical ethics committee of Medical Faculty, Kunming University of Science and Technology to recruit

pregnant women, healthy adult, no-pregnant women and children to attend this study. Because of the large number of patients (more than one thousand) were investigated, written informed consent of patient were not obtained, but only the patient approved to attend this study verbally were recorded and included in the present study. The study protocol was approved by the Institutional Review Board (IRB) of the Inje University Sanggye-Paik Hospital (SPIRB 13-037, as a central IRB for this multicenter study, which waived the need for written or oral informed consent from the participants. Patients with the hepatitis A virus, hepatitis B virus, HCV, and human immunodeficiency virus were excluded.

Virus and cells

HEV positive swine fecal samples containing HEV genotype 4 (GenBank accession no. KJ155502) were obtained from Kunming City, Yunnan Province, China (17). The fecal samples was converted to 10% (w/v) suspensions in DEPC-H₂O and centrifuged at 12,000×g at 4 °C for 10min, filtered through 0.22 μm microfilters before viral inoculation, and treated with penicillin and streptomycin for 1 h. The suspension was then stored in liquid nitrogen until use. The viral titers of 1.0×10⁶ copies/ml determined using real-time quantitative PCR (qPCR).

Human hepatoma cell line (HepG2) and human lung carcinoma cell lines (A549) were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C under 5% CO₂.

Virus inoculation

Cells were planted in six-well microplates 24 h before virus inoculation, at ~50% confluence were supplemented with 10% FBS (FBS group), 10% mixed serum of healthy non-pregnant women (Healthy non-pregnant women group), 10% mixed serum of third-trimester of pregnancy (Third trimester pregnancy group). The protocol for HEV inoculation was according to the previously study (11). HEV infected A549 cells served as HEV replication control. In brief, monolayer cells were washed thrice and inoculated with 0.2 mL of the filtered virus inoculum and 30 mM MgCl₂ (final concentration) for 1 h. The solution was removed after inoculation, and fresh maintain medium containing 2% FBS, 2% healthy non-pregnant women serum or 2% third trimester of pregnancy serum was separately added. The cells were collected either for proteomic analysis or viral replication determination 6 days post-inoculation (dpi).

Detection of HEV RNA through RT–nPCR

HepG2 cells were collected after three freeze–thaw cycles. Total RNA was extracted using Trizol in accordance with the manufacturer’s directions. Reverse transcription nested polymerase chain reaction (RT–nPCR) analysis was performed using Avian Myeloblastosis Virus Reverse Transcriptase XL (Takara) in accordance with the manufacturer’s directions. The HEV RNA (ORF2) in the supernatant of cells supplemented with FBS, non-pregnancy serum, or pregnancy serum was detected using RT–nPCR as previously described (Huang et al., 2002).

Western blot

Protein changes in the HEV-infected HepG2 cells were identified through Western blot. Cells were collected at 6 dpi and lysed in radio immunoprecipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, pH 8.0, 30 mM NaF, 1 mM Na₃VO₄, 40 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, protease inhibitors, 10% glycerol, and 1% Nonidet-P40). Proteins were analyzed through 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% skimmed milk, and the membrane was separately incubated with primary antibodies, including HEV ORF2 (Millipore, 1:1000 dilution), filamin A, Cytochrome c (CytC), heat-shock proteins (HSPs) 90 and 70, plasmid HSP90, and Thioredoxin (TXN; Bioworld, 1:1000 dilution) at 4 °C overnight. Horseradish peroxidase-conjugated IgG was used as a secondary antibody (Promega, 1:10,000 dilution). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein served as the loading control. The bands were exposed to X-ray films by using an Immobilon ECL kit (Millipore).

Statistical analysis

All experiments were performed at least three times. Data are presented as mean ± SD. Statistical analysis was performed on Western blot by using GraphPad Prism software, and P values were calculated using Student’s t-test to determine the significance of differences between two or more groups. P < 0.05 was considered to indicate statistical significance.

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Figure legends

Fig. 1 HEV Replication in HepG2 cells.

(A) HEV RNA was detected through RT-nPCR. (B) HEV replications were determined via Western blot with anti-HEV antibodies (ORF2) in HepG2 cells or A549 cells supplemented with different sera at 6 dpi. FBS represented cells supplemented with fetal bovine serum, FPS represented cells supplemented with serum in their first trimester of pregnancy, PS represented cells supplemented with serum in their third trimester of pregnancy. GAPDH served as the loading control.

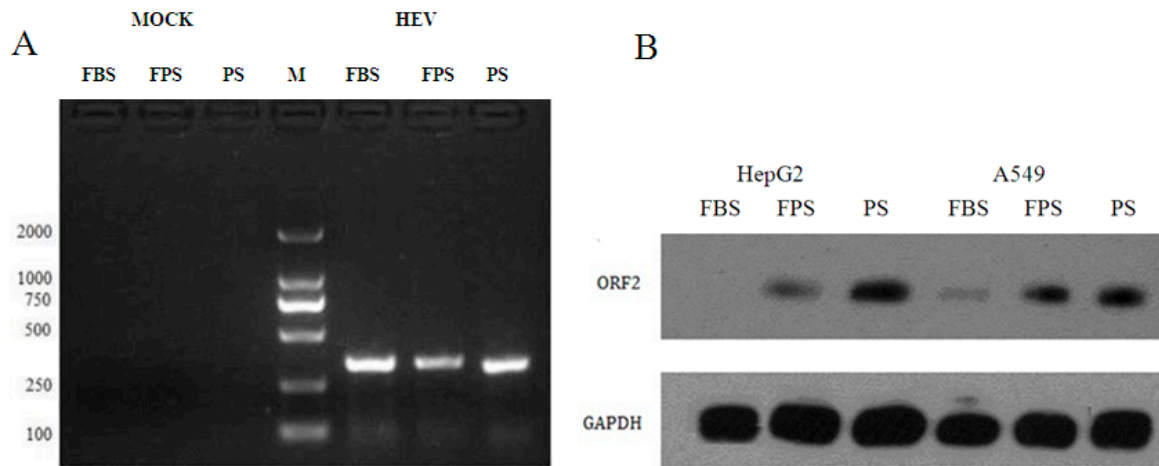


Fig. 2 HEV infection induced differential protein expression in HepG2 cells.

Mock represented HepG2 cells uninfected with HEV; HEV_{FBS} represented HEV-infected HepG2 cells supplemented with FBS; HEV_{PS} represented HEV-infected HepG2 cells supplemented with pregnant woman serum (from the third trimester).

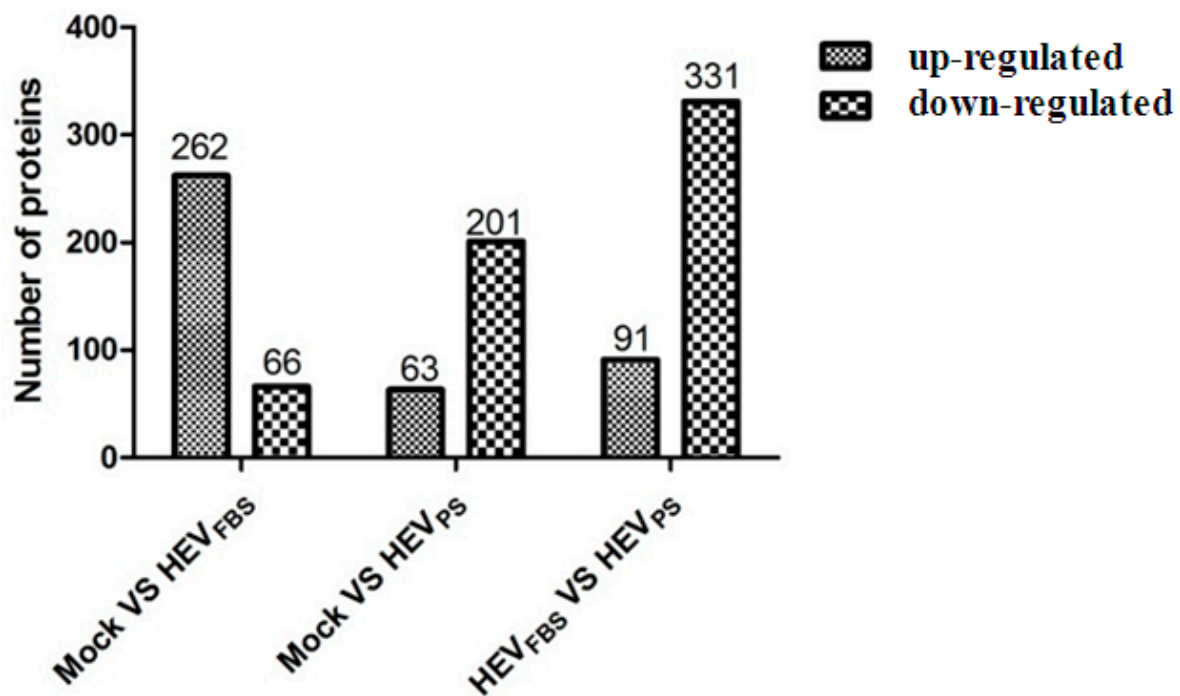
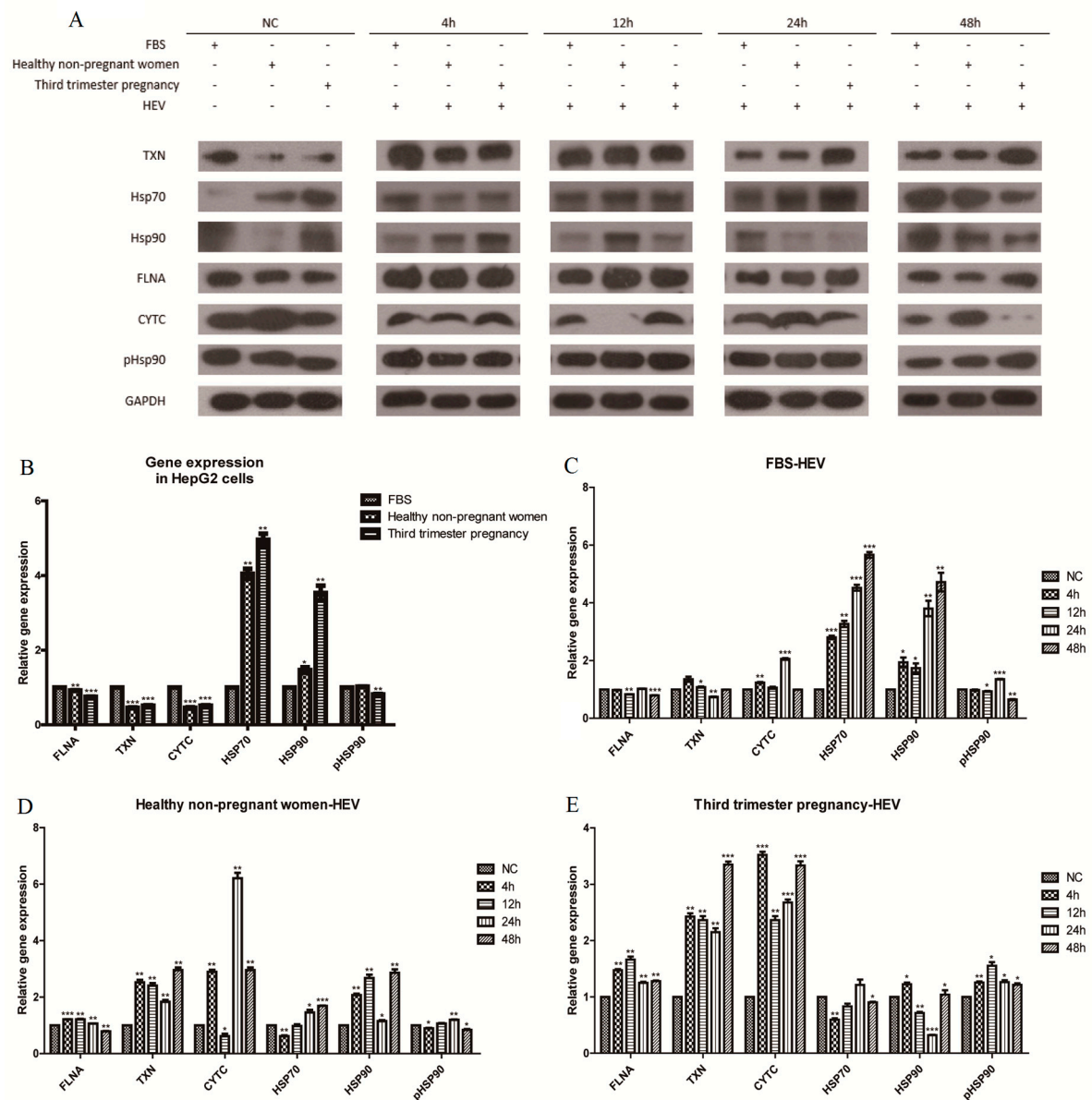


Fig. 3 Validation of the five HEV infection-modulated proteins through Western blot assay.

(A) HepG2 cells supplemented with indicated serum inoculated with HEV were collected at 0, 4, 12, 24, 48 h post-inoculation; Filamin A, HSP70, HSP90, CytC, and TXN were analyzed through Western blot with specific antibody. GAPDH served as the loading control. (B) Serum relative changes in the expression of proteins analyzed using GraphPad Prism

software were indicated and normalized with GAPDH. (C) Time relative changes in the expression of proteins analyzed using GraphPad Prism software were indicated and normalized with GAPDH. FBS represented HEV-infected HepG2 cells supplemented with FBS; healthy non-pregnant women represented HEV-infected HepG2 cells supplemented with the serum of healthy non-pregnant women; third trimester pregnancy represented HEV-infected HepG2 cells supplemented with the serum of pregnant women in their third trimester.



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