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nH115a, a novel inhibitor of the La protein: Effect on expression of multiple RNAs in hepatitis B virus-infected hepatoma cells and embryotoxicity profile

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Summary. – The La protein binds to RNA and protects replication of the hepatitis B virus (HBV). We recently developed the compound nH115a, an inhibitor of the La protein that has high stability and anti-HBV activity. However, the mechanism, by which this compound inhibits HBV infection and its safety to embryos, remains unclear. Our goal was to examine the molecular mechanism, by which nH115a inhibits HBV infection, and to characterize its embryotoxicity. Microarray experiments using HepG2. 2. 15 cells (established by transfecting an HBV plasmid into HepG2 hepatoma cells) and bioinformatics analyses were used to measure the effect of nH115a on the expression of lncRNAs, mRNAs, and circRNAs. The embryonic stem cell test was used to assess the embryotoxicity of nH115a. nH115a significantly altered the expression of 2402 lncRNAs, 338 mRNAs, and 559 circRNAs. Gene Ontology (GO) analysis indicated the differentially expressed transcripts functioned in interleukin-2 production, I-SMAD binding, RNA-induced silencing complex (RISC), NLRP3 inflammasome complex assembly, cytoplasmic sequestering of nuclear factor kappa-B (NF-κB), and death receptor binding. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated the most enriched pathways included transforming growth factor-β (TGF-β) signaling, pathways in cancer, ubiquitin mediated proteolysis, p53 signaling, antigen processing and presentation, Fc gamma R-mediated phagocytosis, and B cell receptor signaling. The results of the embryonic stem cell test indicated that nH115a exhibited weak embryotoxicity. In conclusion, immune responses, TGF-β/SMAD signaling, and cancer-related pathways may function in the nH115a-mediated inhibition of HBV replication.

Keywords: hepatitis B virus; La protein; inhibitor; nH115a

Introduction

Chronic hepatitis B (CHB), which is the result of persistent infection by the hepatitis B virus (HBV), is a serious worldwide public health problem, mainly because it can lead to cirrhosis and hepatocellular carcinoma (Sarin *et al*., 2016). It was estimated that more than 240 million people (Schweitzer *et al*., 2015) worldwide are chronically infected with HBV; 65,000 people (Montuclard *et al*., 2015) die each year directly from hepatitis, and many more die from complications of CHB (WHO Guidelines Approved by the Guidelines Review Committee, 2015). HBV is highly infectious and spreads through blood or body fluids. Mother-to-child transmission during childbirth is the most common transmission route in China (Zeng *et al*., 2019). Newborns who test positive to the hepatitis B e antigen (HBeAg) and the hepatitis B surface antigen (HBsAg) have an increased risk of HBV infection (Tiele *et al*., 2018).

The most effective preventive strategy for newborns is active and passive dual immunization, in which infants

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Abbreviations: AMPK = adenosine 5'-monophosphate (AMP)-activated protein kinase; CHB = chronic hepatitis B; DMSO = Dimethyl Sulfoxide; ECVAM = The European Centre for the Validation of Alternative Methods; EST = embryonic stem cell test; GO = Gene Ontology; HBeAg = hepatitis B e antigen; HBsAg = hepatitis B surface antigen; HBV = hepatitis B virus; KEGG = Kyoto Encyclopedia of Genes and Genomes; lncRNAs = long noncoding RNAs; mLIF = murine leukemia inhibitory factor; NF-κB = nuclear factor kappa-B; qRT-PCR = quantitative real-time polymerase chain reaction

receive the HBV vaccine and hepatitis B immunoglobulin (HBIG) treatment within 24 h after birth (Fan *et al*., 2016). However, about 5 to 10% of infants experience immune failure, and this almost always occurs when the mother is HBeAg-positive and has an HBV DNA titer greater than 200,000 IU/ml (Pan *et al*., 2016; Wen *et al*., 2016). The American Association for the Study of Liver Diseases recommended two major categories of antiviral drugs for treatment of CHB: nucleos(t)ide analogues and interferon-α (IFN-α) (Terrault *et al*., 2016). The three analogues recommended during pregnancy are tenofovir (TDF), telbivudine (LDT), and lamivudine (LMV) (Zhou *et al*., 2017). The 2015, U.S. Food and Drug Administration (FDA) classification of drug safety during pregnancy considered LDT and TDF as category B drugs, and LMV as a category C drug. Most guidelines recommend TDF as the first choice for the treatment of pregnant women who have hepatitis B due to its favorable safety and drug resistance profiles (European Association for the Study of the Liver, 2017; Idilman, 2017).

La protein, also called Sjögren syndrome antigen B, is a multifunctional nucleoprotein (Teplova *et al*., 2006) that binds to RNA (Horke *et al*., 2002), protecting RNA from ribozyme degradation (Intine *et al*., 2003). Previous research reported that La protein binds to the stem-loop of HBV RNA and blocks RNA cleavage sites, thus stabilizing the RNA by preventing ribozyme binding and catalysis (Wolin and Cedervall, 2002). Other research also reported significant positive correlations in the levels of HBV proteins and RNAs with La expression (Ni *et al*., 2004). Therefore, agents that block the RNA binding site of the La protein have potential as new anti-HBV drugs.

We previously described the use of virtual screening to identify a novel small molecule inhibitor of the La protein H11. Our results indicated that H11 suppressed the expression of La protein and had strong anti-HBV activity (Tang *et al*., 2012). We subsequently modified the structure of H11 to develop a more stable derivative – *N*-methyl pyrazolo[1,5-a] pyridine-2-carboxamide (nH115a, the chemical formula is shown in Fig. 1) (Tong *et al*., 2019). Our *in vitro* studies indicated that nH115a had antiviral activity similar to that of entecavir, in that it reduced the level of HBV antigens by about 50%. Our mouse experiments indicated that nH115a reduced the levels of HBV DNA by 98.9%, HBsAg by 57.4%, and HBeAg by 46.4%; the inhibitions in the control group (PBS injection group) were much lower (90.8%, 3.8%, and 9.8%) (Tong *et al*., 2019). These results indicated that nH115a has potential as an anti-HBV compound.

Long noncoding RNAs (lncRNAs) are a large group of noncoding transcripts that have more than 200 nucleotides (Heward and Lindsay, 2014; Zhang and Cao, 2016). The lncRNAs function by interacting with RNA, DNA, and proteins, and thereby alter major biological processes, such as interferon responses (Valadkhan and Gunawardane, 2016), cancer progression (Chen, 2016), and viral infections (Yang *et al*., 2019; Moyo *et al*., 2016).

Our goal was to examine the molecular mechanism, by which nH115a inhibits HBV infection and to characterize its embryotoxicity. We used microarray analysis to identify lncRNAs, mRNAs, and circRNAs, whose levels were altered during the nH115a-mediated suppression of HBV, and performed bioinformatics analyses (Gene Ontology [GO] and Kyoto Encyclopedia of Genes and Genomes [KEGG]) to investigate the functions and interactions of the different transcripts.

We also used the embryonic stem cell test (EST) to evaluate the embryotoxicity of nH115a. The European Centre for the Validation of Alternative Methods (ECVAM) (Boess *et al*., 2003) approved EST models as an alternative for determination of the *in vitro* embryotoxicity of drugs and other chemicals (Liu *et al*., 2017). We thus evaluated the embryotoxicity of nH115a by measuring its toxicity to 3T3 cells (IC_{50}3T3) and D3 cells (IC_{50}D3) and its inhibition of D3 cell differentiation (ID_{50}D3).

Materials and Methods

Cell culture and nH115a treatment. Mouse embryonic stem cells (ES-D3, strain 129/Sv+c/+p), mouse embryonic fibroblast cells (MEF-3T3, strain aa: BALB/c), and Kunming White MEF cells (passage 3, P3) were purchased from Shanghai Institute of Life Sciences, Chinese Academy of Sciences. HepG2. 2. 15 cells were donated by the Shanghai Public Health Clinical Center, Affiliated to Fudan University. D3 cells were cultured on MEF feeder layers in high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Gibco), 1% glutamine (Gibco), 1% streptomycin, 1% penicillin (Gibco), 1% nonessential amino acids (Gibco), 0.1 mM β-mercaptoethanol (Sigma), and 1000 U/ml murine leukemia inhibitory factor (mLIF). 3T3 cells were maintained in DMEM with 10% FBS and 100 μg/ml streptomycin and 100 units/ml penicillin. HepG2. 2. 15 cells were established by transfecting an HBV plasmid into HepG2 hepatoma cells to establish stable expression of the HBV virus. HepG2. 2. 15 cells were maintained in DMEM with 380 μg/ml G418 (Gibco). All cells were incubated at 37°C in 5% CO_2 atmosphere. nH115a was dissolved in Dimethyl Sulfoxide (DMSO; Sigma-Aldrich) at 1 M and stored at -20°C. The stock solution was the diluted in complete DMEM to a concentration of 50 μM and filtered through a 0.22-μm membrane for following microarray test.

Cytotoxicity assay. The Cell Counting Kit-8 (CCK-8, Bimake) was used to assess the cytotoxic effects of nH115a on 3T3 cells and ES-D3 cells. Cells were seeded in 96-well plates at a density of 500 cells/well. After 2 h, 200 μl of complete medium containing different concentrations of nH115a (1000, 2000, 4000 μM)

or DMSO (control) were added. On day 3 and day 5, the medium was changed, and the cell viability was assessed on day 10 by measuring A_{450nm} using a Gen5 microplate reader. Concentration-response curves were plotted to determine IC_{50} values.

Differentiation assay. ES-D3 cells naturally differentiate into cardiomyocytes. An assay originally described by Riebeling *et al.* (2015) was used to determine the effect of nH115a on the differentiation of these cells into contracting cardiomyocytes. Different doses of nH115a were dissolved in an embryonic stem cell differentiation medium (H-DMEM medium, without mLIF) for preparation of mono-cellular suspensions. Then, 20 µl of suspension (approximately 750 ES-D3 cells) were placed on the lid of a 100 mm × 20 mm culture dish filled with 5–10 ml PBS as hanging drops for 3 days. When embryoid bodies (EBs) were observed, they were transferred into sterile Petri dishes for 2 days. On day 5, the EBs were plated separately into the wells of a 24-well tissue culture plate (BD Falcon, Erembodegem, Belgium) with different concentrations of nH115a to allow adherence and outgrowth of the EBs and development of spontaneously beating cardiac muscle cells. On day-5, the EBs were separately added to gelatin-coated 24-well plates to allow development of spontaneously beating cardiac muscle cells. On day 10, quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the level of β-MHC (a marker of differentiation). A concentration-response curve was plotted to determine the ID_{50}D3 value.

Classification of embryotoxicity. ECVAM proposed a method that combines three endpoints (IC_{50}D3, IC_{50}3T3, and ID_{50}D3) to classify the embryotoxicity of a test compound (Table 1). Based on the results, a test compound is considered to have strong embryotoxicity (III > I and III > II), weak embryotoxicity (II > I and II > III), or no embryotoxicity (I > II and I > III).

RNA extraction and qRT-PCR. Total RNA from cells was extracted using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Then, it was purified using the NucleoSpin RNA Clean-up XS kit (Cat. #740903, MN, Germany) and the RNase-Free DNase Set (Cat. #79254, QIAGEN, GmbH, Germany). The purity and concentration were determined using a NanoDrop ND-2000 spectrophotometer and the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). RNAs was reverse transcribed into cDNA using PrimeScript RT Master Mix (RR036Q; Takara, Japan). The expression of β-MHC was measured by qRT-PCR using the TB Green Premix Ex Taq (Tli RNaseH Plus) kit (RR420A, TaKaRa, Japan) on the ABI 7900 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendation. Specific primers for GAPDH and β-MHC are listed in Table 2. Relative fold change was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to GAPDH.

Microarray analysis. The microarray experiments were performed as previously described (Pan *et al.*, 2018). In brief, total RNA was amplified and labeled using the Low Input Quick Amp WT Labeling Kit and the RNA Spike-In Kit, One Color (Agilent technologies, Santa Clara, CA, US). Then, the labeled cRNAs were purified using the RNeasy mini kit (QIAGEN, GmBH, Germany) and hybridized onto Agilent Sure Print G3 Mouse GE V2 8×60 K Microarray and Sino human lncRNA array V3.0 for 17 h. After washing, each slide was scanned using the Agilent Microarray Scanner (Cat. #G2565CA, Agilent Technologies, Santa Clara, CA, USA). Raw data were extracted and normalized to correct for measurement errors. All lncRNAs, mRNAs, and circRNAs with altered expression were identified by use of scatter plots, volcano plots, and heatmaps. A 2.0-fold or more difference in expression with a P-value of 0.05 was considered significant.

Prediction of lncRNA targets. Genes that were less than 10 kb from the lncRNAs were selected as cis-target genes. The Basic Local Alignment Search Tool database was used to identify genes with complementary or similar sequences to the lncRNAs. Based on RNA duplex energy, sequences above a threshold value ($e \leq -30$) were considered as trans-target genes.

GO and KEGG analysis: GO was used for classification of the function of genes and gene products into three domains: molecular function, biological process, and cellular component. KEGG was used for the systematic analysis of gene function and genomic information. Differentially expressed mRNAs were analyzed using Fisher's exact test with the cluster Profiler

Table 1. ECVAM linear discriminant functions used to determine embryotoxicity

Classification	Linear discriminant formula
I	$5.916Ig(IC_{50}3T3) + 3.500Ig(IC_{50}D3) - 5.307[(IC_{50}3T3 - ID_{50}D3)/IC_{50}3T3] - 15.72$
II	$3.651Ig(IC_{50}3T3) + 2.394Ig(IC_{50}D3) - 2.033[(IC_{50}3T3 - ID_{50}D3)/IC_{50}3T3] - 6.85$
III	$-0.125Ig(IC_{50}3T3) + 1.917lg(IC_{50}D3) + 1.500[(IC_{50}3T3 - ID_{50}D3)/IC_{50}3T3] - 2.67$

Table 2. Specific primers for GAPDH and β-MHC

Primer name	Forward primer	Reverse primer
GAPDH	CCTTCCGTGTTCCTACCC	CAACCTGGTCCTCAGTGTAG
β-MHC	GCCCCTCCTCACATCTTCTCC	CAGGGTTGGCTTGGATGATTT

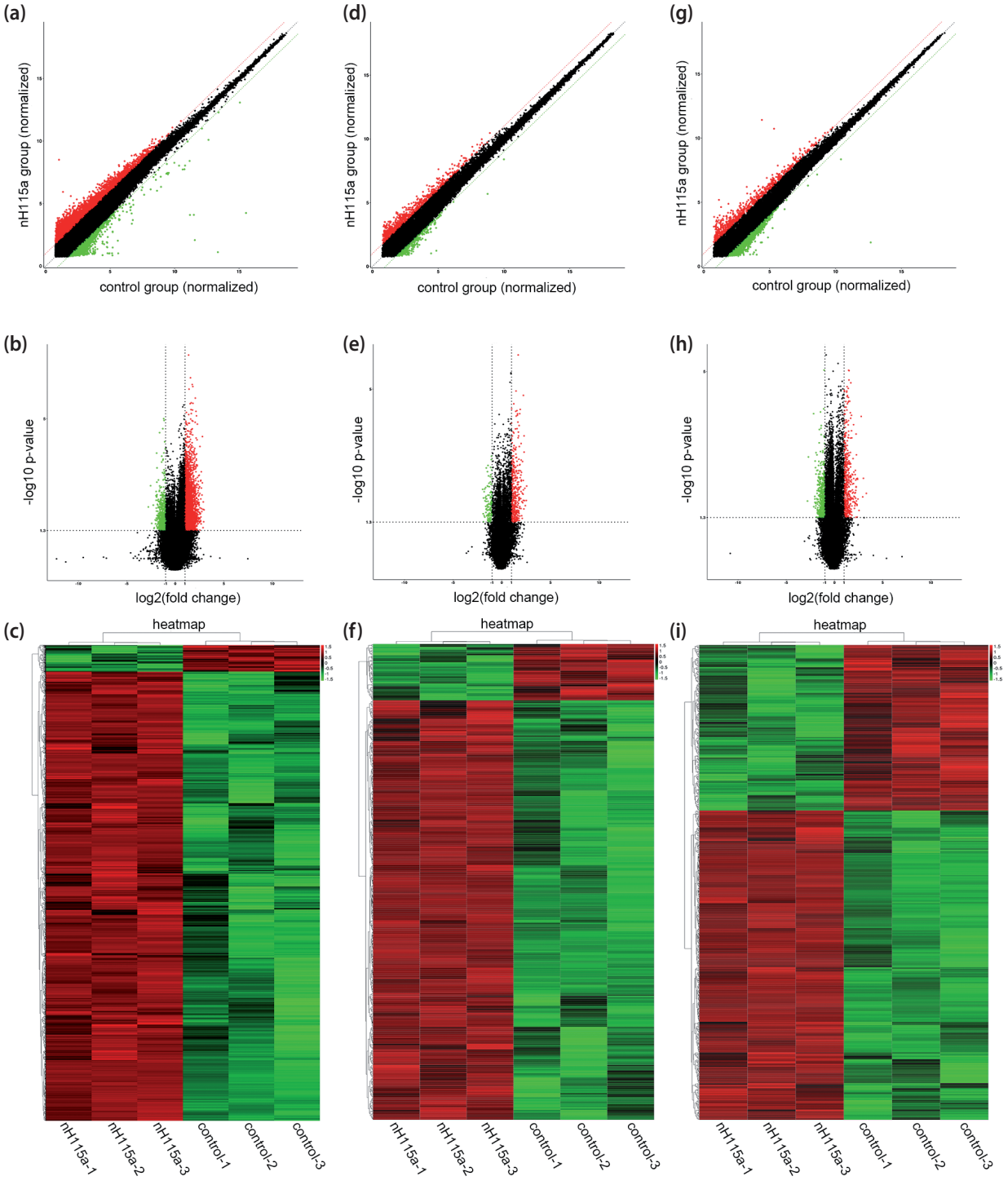

Fig. 1

Microarray profiling of nH115a-treated HepG2. 2. 15 cells

Scatter plots, volcano plots, and hierarchical clustering of differentially expressed lncRNAs **(a, b, c)**, mRNAs **(d, e, f),** and circRNAs **(g, h, i).** Scatter plots: X-axis indicates normalized signal values from the control group, Y-axis indicates normalized signal values from the nH115a group, red dots indicate genes upregulated by 2-fold or more, and green dots indicate genes downregulated by 2-fold or more. Volcano plots: X-axis indicates fold-change, Y-axis indicates P value, vertical lines indicate a 2.0-fold increase or decrease, horizontal line indicates a P-value of 0.05, red dots indicate genes upregulated by 2-fold or more, and green dots indicate genes downregulated by 2-fold or more. Heatmaps: Red indicates high relative expression, green indicates low relative expression, and lines on the top and left indicate functional relationships.

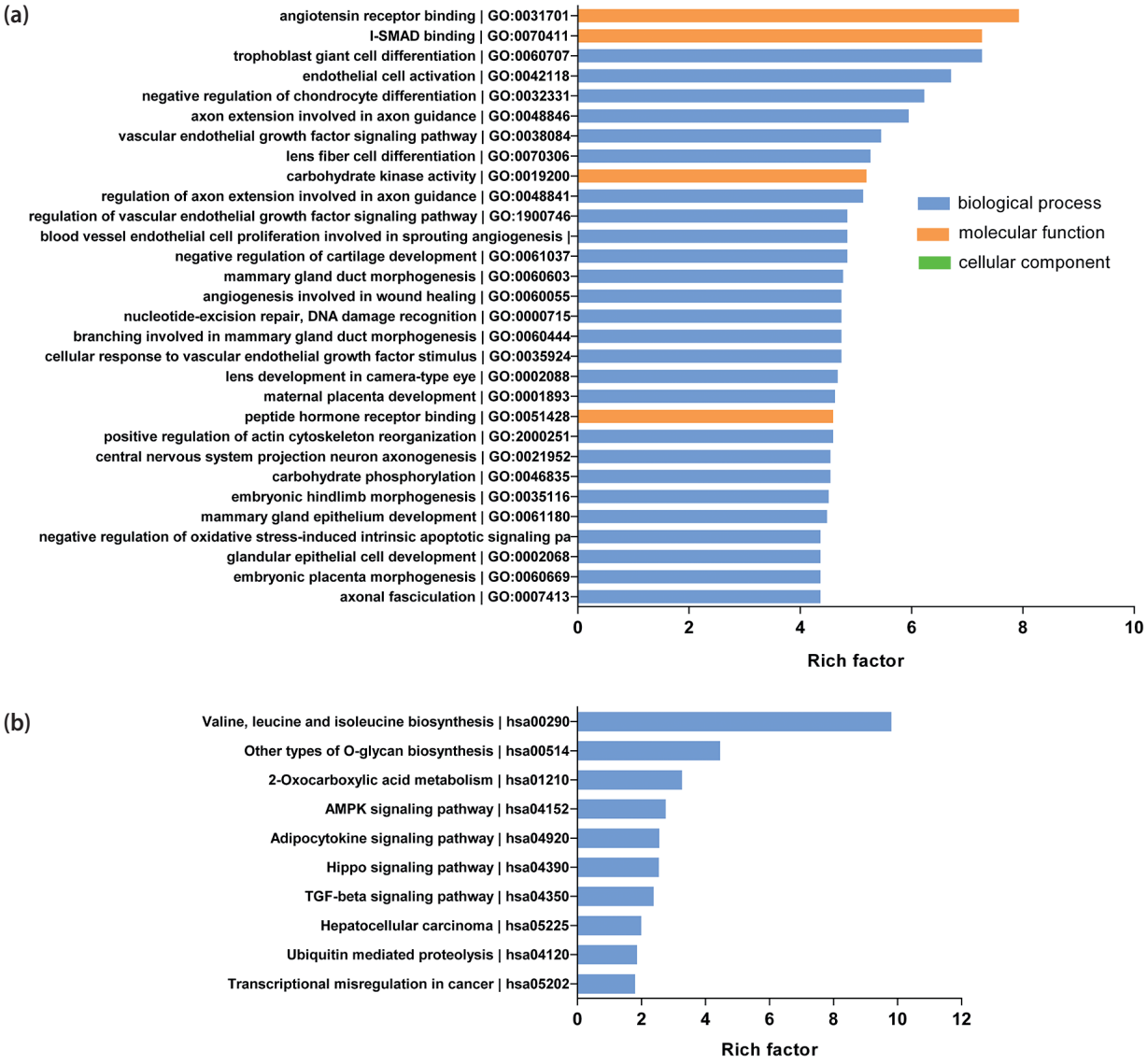

Fig. 2

GO (a) and KEGG (b) analysis of cis-target mRNAs by differentially expressed lncRNAs
Each vertical axis shows the GO or KEGG category and each horizontal axis shows the enrichment score. There were significant differences in all indicated categories (all P <0.05).

packages from R/Bioconductor. The 30 GO terms with the largest enrichment factors were shown in plots.

Data analysis. Three independent replicates were performed for each experiment. Statistical analysis was performed using SPSS software version 22.0 (SPSS, Inc., Chicago, IL, USA). All results were expressed as the means ± standard deviations (SDs). Student's *t*-test was used for comparisons of two groups. A P-value below 0.05 was considered significant.

Results

Effect of nH115a on differential expression of RNAs in HepG2. 2. 15 cells

We initially used microarray analysis to examine the effect of nH115a on the expression of lncRNAs, mRNAs, and circRNAs in HepG2. 2. 15 cells, and presented these results as scatter plots (Fig. 1a, d and g) and volcano plots (Fig. 1b, e and h). Compared to the control (DMSO) group, the nH115a group had 2268 upregulated and 134 downregulated lncR-

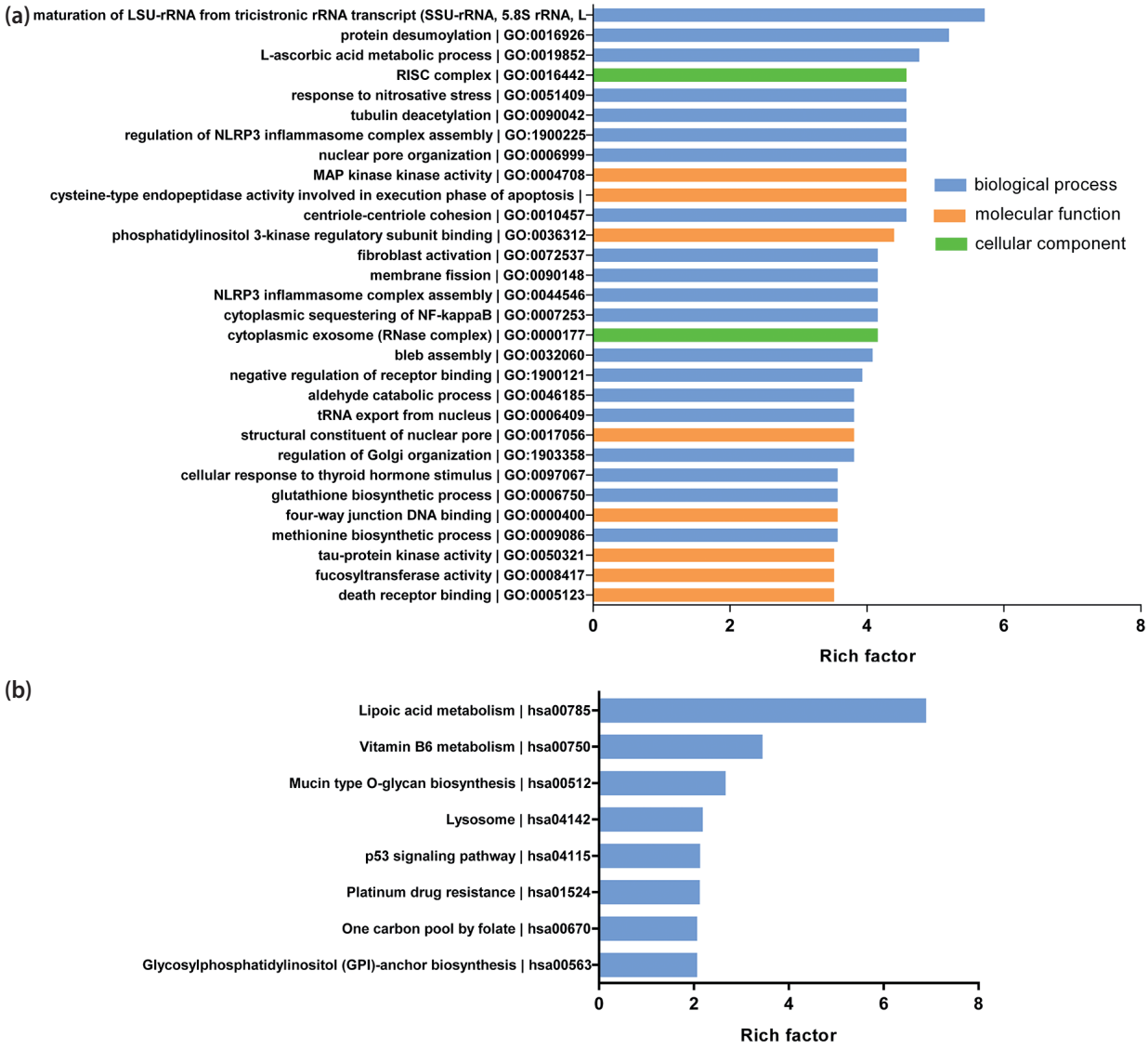

Fig. 3

GO (a) and KEGG (b) analysis of trans-target mRNAs by differentially expressed lncRNAs
Each vertical axis shows the GO or KEGG category and each horizontal axis shows the enrichment score. There were significant differences in all indicated categories (all $P < 0.05$).

NAs, 298 upregulated and 40 down-regulated mRNAs, and 364 upregulated and 195 downregulated circRNAs. Among the differentially expressed lncRNAs, NONHSAT114917.2 was the most upregulated and NONHSAT008884.2 was the most downregulated. Among the mRNAs, SPDYE4 (NM_001128076) was the most upregulated and CDHR1 (NM_033100) was the most downregulated. Among the circRNAs, hsa_circ_0091618 was the most up-regulated and hsa_circ_0047158 was the most down-regulated. Overall, these results indicated that nH115a treatment affected the levels of many different RNAs and led to more up-regulated than down-regulated transcripts. It is particularly notable that there were 2268 upregulated lncRNAs following nH115a treatment. We used hierarchical clustering to determine the relationships of the differentially expressed genes (Fig. 1c,f and i). These results suggested that transcripts with similar functions clustered together.

GO and KEGG analyses of differentially expressed lncRNAs

We next examined the possible functions of the differentially expressed lncRNAs using GO and KEGG analy-

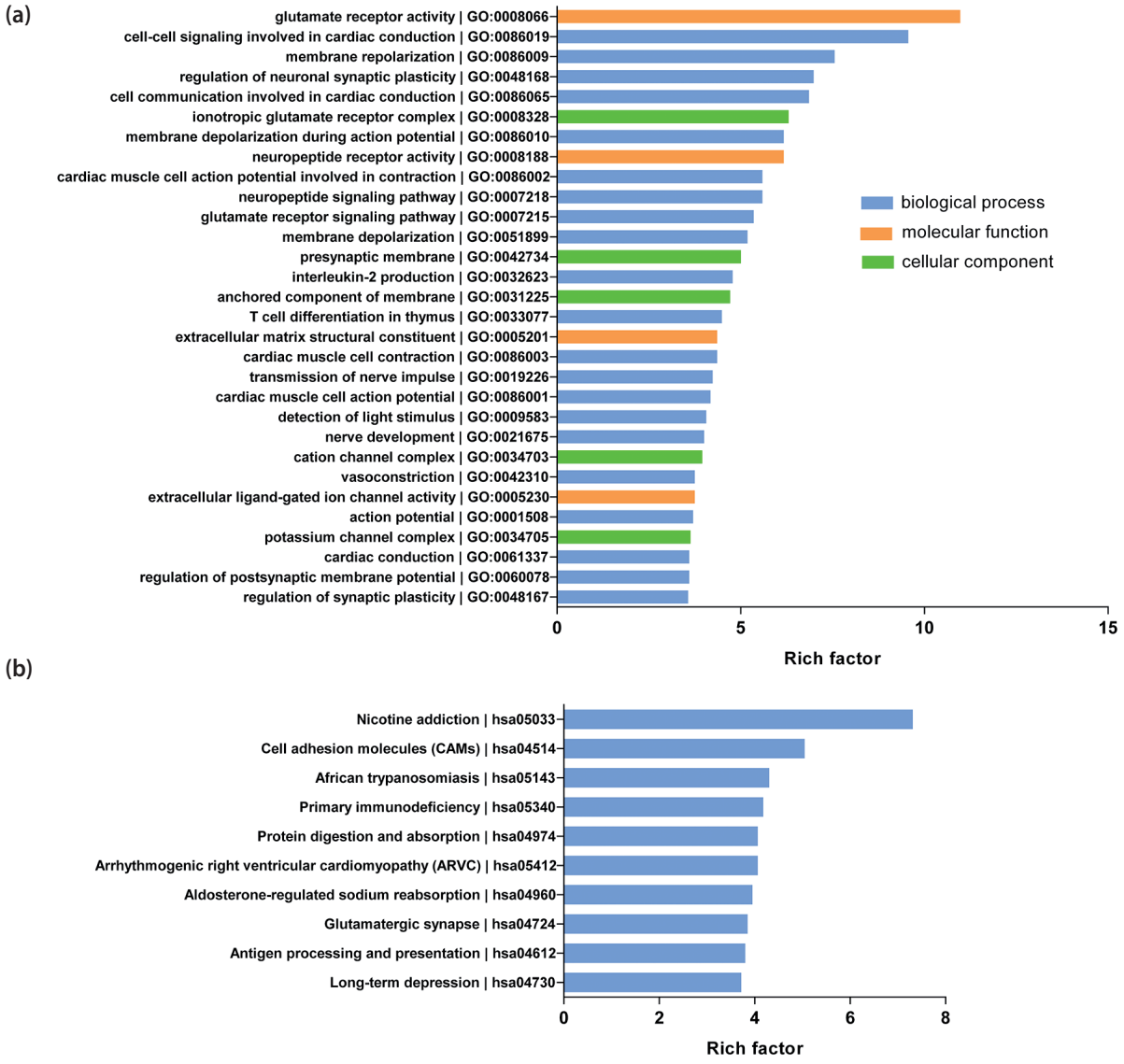

Fig. 4

GO (a) and KEGG (b) analysis of differentially expressed mRNAs

Each vertical axis shows the GO or KEGG category and each horizontal axis shows the enrichment score. There were significant differences in all indicated categories (all $P < 0.05$).

sis of their cis-target mRNAs and trans-target mRNAs. There were 1408 cis-target mRNAs. GO analysis (Fig. 2a) of these mRNAs indicated the differentially expressed lncRNAs were related to many biological processes, such as nucleotide-excision repair, DNA damage recognition, positive regulation of actin cytoskeleton reorganization, and negative regulation of the oxidative stress-induced intrinsic apoptotic signaling pathway. The major molecular functions of the differentially expressed lncRNAs were angiotensin receptor binding and I-SMAD binding. We also performed KEGG analysis to examine the possible anti-HBV mechanism of nH115a (Fig. 2b). The ten most enriched pathways included transforming growth factor-β (TGF-β) signaling, adipocytokine signaling, Hippo signa-ling, and Adenosine 5'-monophosphate-activated protein kinase (AMPK) signaling. The significantly enriched pathways were related to cancer, transcriptional mis-regulation in cancer, and ubiquitin-mediated proteolysis.

There were 5293 trans-target mRNAs. GO analysis (Fig. 3a) of these mRNAs indicated that the differentially expressed lncRNAs were involved in regulation of NLRP3 inflammasome complex assembly, cytoplasmic sequestering of NF-κB, NLRP3 inflammasome complex assembly, RISC complex, and death receptor binding. KEGG analysis

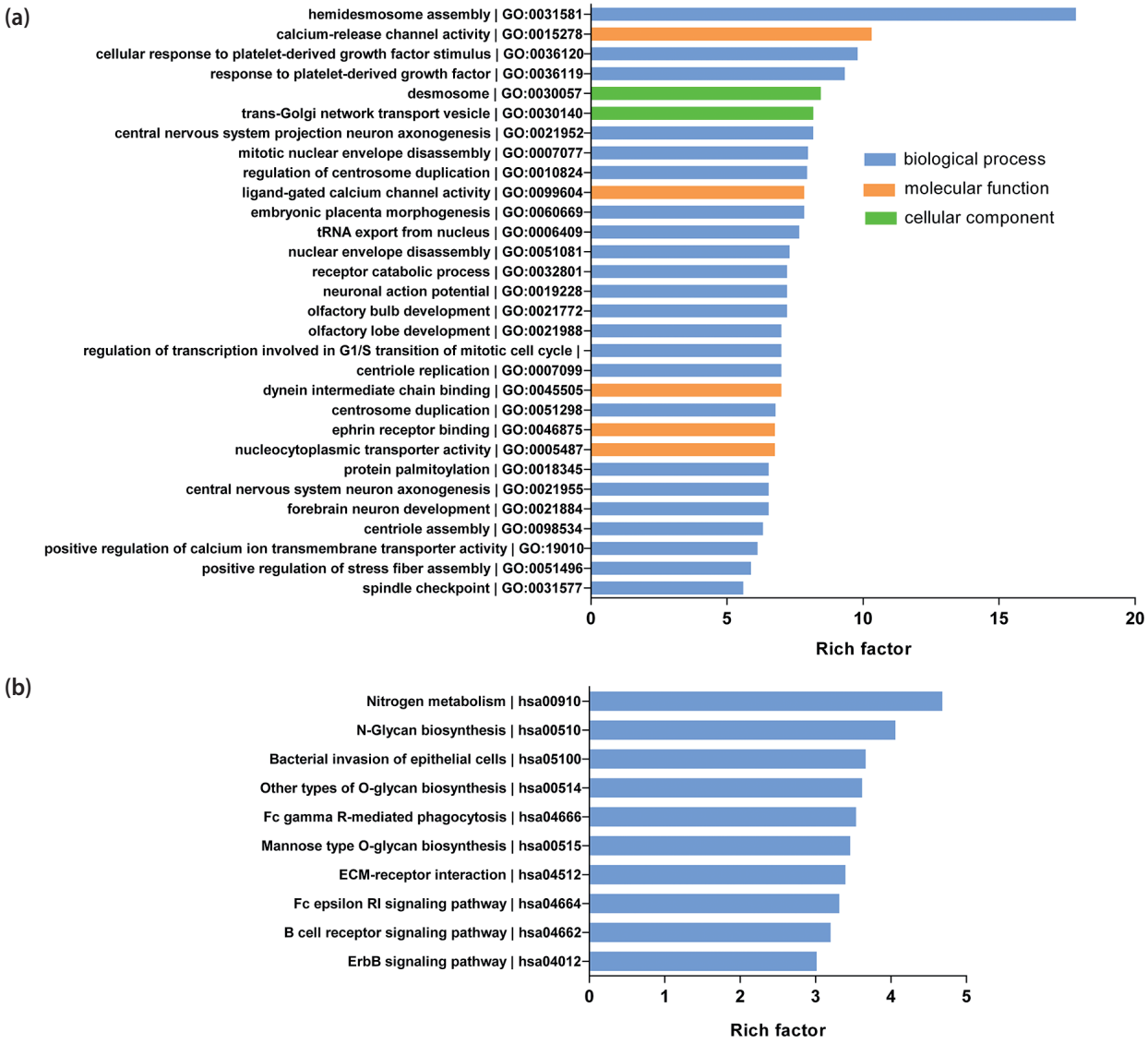

Fig. 5

GO (a) and KEGG (b) analysis of differentially expressed circRNAs
Each vertical axis shows the GO or KEGG category and each horizontal axis shows the enrichment score. There were significant differences in all indicated categories (all P <0.05).

of these trans-target mRNAs indicated that the differently expressed lncRNAs were mostly related to lysosome metabolism and the p53 signaling pathway (Fig. 3b).

GO and KEGG analyses of differentially expressed mRNAs

We performed the same analyses for differentially expressed mRNAs (Fig. 4a,b). These results showed that the mRNAs with significant differential expression were enriched in the glutamate receptor signaling pathway and interleukin 2 production. The cell adhesion molecules (CAMs) and antigen processing and presentation were among the major KEGG pathways. The enrichment of several mRNAs related to antigen processing and presentation (*KLRC1*, *HSPA6*, *CD8B*, and *KIR3DL3*) suggests that further studies should examine the functions of these genes in patients with CHB.

GO and KEGG analyses of differentially expressed circRNAs

GO analysis indicated the differentially expressed circRNAs were associated with numerous biological

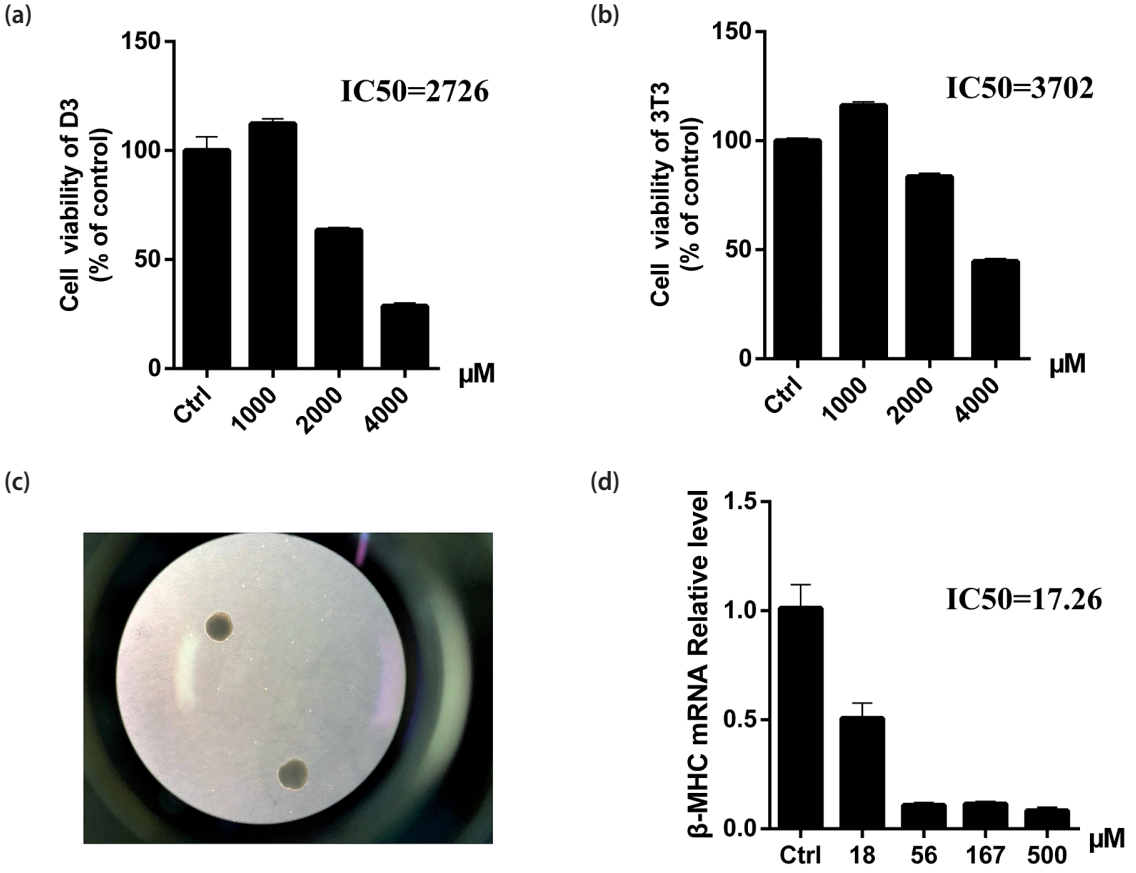

Fig. 6

Embryonic stem cell test

Effect of nH115a concentration on viability of ES-D3 cells (**a**; IC_{50}: 2726 μM) and 3T3 cells (**b**; IC_{50}: 3702 μM); After hanging drop culture for 3 days, suspension culture for 2 days, and adherent culture for 5 days, ES-D3 cells were differentiated into cardiomyocyte cells (**c**); expression of β-MHC (differentiation marker) in D3 cells (**c, d**; ID_{50}: 17.26 μM).

processes (Fig. 5a). These included hemidesmosome assembly, regulation of centrosome duplication, tRNA export from the nucleus, centriole replication, centrosome duplication, and regulation of transcription involved in the G1/S transition of mitosis. KEGG analysis indicated enrichment of Fc gamma R-mediated phagocytosis, Fc epsilon RI signaling, B cell receptor signaling, and ErbB signaling (Fig. 5b).

Embryotoxicity of nH115a

We used the EST to evaluate the embryotoxicity of nH115a (Fig. 6). We first cultured 3T3 and ES-D3 cells, and then treated these cells with different concentrations of nH115a for 10 days. For both types of cells, viability decreased as nH115a concentration increased (Fig. 6a,b). The IC_{50}3T3 at 24 h was 3702 μM (647.5 mg/l) and the IC_{50}D3 at 24 h was 2726 μM (477 mg/l).

ES-D3 cells spontaneously differentiate into cardiomyocytes in the absence of mLIF. We measured cell differentiation by use of qRT-PCR to determine the expression of β-MHC, a maker of cardiomyocyte development (Fig. 6c,d). The results indicated that cell differentiation decreased as the level of nH115a increased, with an ID_{50}D3 of 17.26 μM (3.02 mg/l). We then used these three IC_{50} values with the EST linear discriminant equations to calculate embryotoxicity (Table 1). The results (II > I and II > III) indicated that nH115a had 'weak' embryotoxicity.

Discussion

Currently available therapies effectively suppress viral replication, but their efficacy is limited because of drug resistance and ineffective removal of the cccDNA reservoir (Pan *et al.*, 2016). Therefore, it is of great significance to

develop new anti-HBV drugs with different mechanisms of action. The La protein facilitates HBV replication because it binds to and improves the stability of HBV RNA, thereby preventing its degradation by host cells. This suggests that the La protein may be a potential target for the treatment of HBV infection. nH115a is a derivative of H11 (an inhibitor of HBV) that has improved *in vivo* stability and antiviral activity. However, the antiviral mechanism of nH115a has not been elucidated. The present study examined the mechanism by which nH115a inhibited HBV replication and its embryotoxic profile.

Compared with DMSO control group, a large number of transcripts were found to be abnormally expressed in nH115a-treated group. It is worth noting that the expression patterns of lncRNA are consistent with mRNA in that they have more up-regulated transcripts than down-regulated transcripts. These results suggest there may be functional connections between the differentially expressed lncRNAs and mRNAs during the pathogenesis of HBV infection.

Previously published studies have reported that AMPK signaling pathway inhibits HBV replication through controlling cellular autophagy (Xie *et al.,* 2016; Wang *et al*., 2020). TGF-β signaling plays an important role in HBV infection and can trigger HBV cccDNA degradation through activation-induced cytidine deaminase-dependent deamination (Liang *et al*., 2015; Qiao *et al*., 2016). Considering the antiviral activity of nH115a, our findings unsurprisingly revealed enrichment in AMPK, TGF-β pathways and I-SMAD GO term. Furthermore, agonism of FFAR2 has been proved to ameliorate HBx-induced oxidative stress (He *et al*., 2020). Here, we found the FFAR2 was significantly upregulated in nH115a-treated HepG2.2.15, revealing its role in HBV infection.

In contrast to the well-known adaptive immune response, the detailed role of innate immune response in HBV infection is controversial. HBV seems to be able to evade interferon-based innate reactions (Yoneda *et al*., 2016; Mutz *et al*., 2018). Recently, many studies have proved that the NF-κB and Hippo signaling pathways harbor a regulatory task in innate immune response to HBV infection (Hesari *et al*., 2018; Luo *et al*., 2021). Chronic HBV infection suppressed NLRP3 expression via suppressing NF-κB pathway and reactive oxygen species (ROS) production (Yu *et al*., 2017). Our results showed that differentially expressed transcripts are enriched in Hippo, NF-κB pathway, NLRP3 inflammasome complex assembly, as well as antigen processing and presentation, indicating that nH115a may exert antiviral effects by regulating innate and adaptive immune responses.

We also found that the target genes of differentially expressed LncRNA are enriched in cancer-related pathways, such as hepatocellular carcinoma, transcriptional mis-regulation in cancer and p53 signaling pathway. It is interesting that La protein is also involved in cell cycle (Huang and Tang, 2020), invasion, apoptosis (Stavraka and Blagden, 2015) and Epithelial-mesenchymal transition (EMT) tumor processes (Petz *et al*., 2012). In our previous study, La protein contributes to cells proliferation and migration and serves as a potential therapeutic target for hepatocellular carcinoma (Pan *et al*., 2020). Therefore, it is of clinical significance to study whether La protein plays a role in hepatocellular carcinoma via regulation of HBV expression. At the same time, these results suggest that whether nH115a has anti-tumor activity remains to be established.

When considering the administration of a drug to a pregnant woman with CHB, the safety of the drug to the fetus is a major concern. However, it is also important for a woman with CHB to reduce the HBV DNA titer to lower the risk of transmission to the newborn. The results of our embryonic stem cell test indicated that nH115a exhibited 'weak' embryotoxic effects. These results suggest that nH115a should be examined in future studies that assess its potential for use as a novel anti-HBV drug for pregnant women.

In conclusion, we found that nH115a, a novel inhibitor of the La protein, exhibited weak embryotoxicity and may mediate the inhibition of HBV replication by regulating immune responses, TGF-β/SMAD signaling, and cancer-related pathways. Our study provides novel mechanism for nH115a-mediated HBV replication inhibition. However, there are several questions that are yet to be solved. These differentially expressed transcripts need more experiments to prove their expression level changes. The anti-HBV mechanism of nH115a obtained by bioinformatics analysis also needs further experimental studies by interference with related pathways.

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