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Stable Overexpression of Arginase I and Ornithine Transcarbamylase in HepG2 Cells Improves Its Ammonia Detoxification

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ABSTRACT

HepG2 is an immortalized human hepatoma cell line that has been used for research into bioartificial liver systems. However, a low level of ammonia detoxification is its biggest drawback. In this work, a recombinant HepG2 cell line with stable overexpression of human arginase I (hArgI) and human ornithine transcarbamylase (hOTC), HepG2/(hArgI + hOTC)4, was developed using a eukaryotic dual gene expression vector pBudCE4.1. (1) The hArgI and hOTC enzymatic activity in HepG2/(hArgI + hOTC)4 cells were higher than in the control cells. (2) The ammonia tolerance capacity of HepG2/(hArgI + hOTC)4 cells was three times that of HepG2 cells and 37.5% of that of primary human hepatocytes in cultivation. In the experiment of ammonia detoxification, HepG2/(hArgI + hOTC)4 cells produced 3.1 times more urea (at 180 mM NH_4Cl) and 3.1 times more glutamine (at 120 mM NH_4Cl and 15 mM glutamate) than HepG2 cells, reaching 63.1% and 36.0% that of primary human hepatocytes, respectively. (3) The hArgI and hOTC overexpression did not influence the growth of HepG2 cells and also promoted the expression of other ammonia detoxification associated proteins including glutamine synthetase (GS), arginase II (ArgII), arginosuccinate synthase (ASS) and arginosuccinate lyase (ASL) in HepG2 cells. This work illustrates that the modification reported here made significant progress in the improvement of HepG2 cell function and the HepG2/(hArgI + hOTC)4 cells will provide a better selection for the application of bioartificial liver system. J. Cell. Biochem. 113: 518–527, 2012.

KEY WORDS: BIOARTIFICIAL LIVER; UREA CYCLE; GLUTAMINE

In recent years, the number of patients with various types of liver failure or waiting for a liver transplant has increased continuously. However, the development and application of bioartificial liver, as a bridge to liver transplantation, have lagged behind. One of the obstacles is the lack of appropriate human liver cells. So far, only two kinds of liver cells, primary porcine liver cells [Sauer et al., 2003; van de Kerkhove et al., 2003] and human hepatoma cell line HepG2 C3A [Demetriou et al., 1995; Watanabe et al., 1997; Filippi et al., 2004], have been used clinically and shown to have preliminary effectiveness. The primary porcine liver cell has the advantage of higher biosynthesis and metabolism, but the presence of several defects cannot be ignored: Antigenicity of secreted proteins, possibility of porcine retrovirus infection [Nyberg et al., 2000; Fruhauf et al., 2009], short maintenance time of primary hepatocyte function, complicated preparation process, and higher cost. HepG2 cells are derived from immortalized human liver cancer

Abbreviations used: hArgI, human arginase I; hOTC, human ornithine transcarbamylase; GS, glutamine synthetase; ArgII, arginase II; ASS, arginosuccinate synthase; ASL, arginosuccinate lyase; NH_4Cl, ammonium chloride; CCK-8, Cell Counting Kit 8; ECL, enhanced chemiluminescence; Gln, glutamine; Glu, glutamate; ALB, albumin; TBIL, total bilirubin; ALP, alkaline phosphatase; γ-GT, γ-glutamyltransferase; LDH, lactate dehydrogenase; LD50, median lethal dose; CPS1, carbamoyl phosphate synthase 1.

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cell lines, which have the advantage of very low antigenicity, easy-to-large-scale cultivation, and quality control. However, the disadvantages of HepG2 cells are clear: Carcinogenic potential and significantly lower ammonia detoxification compared to primary liver cells [Wang et al., 1998; Mavri-Damelin et al., 2008]. As for the latter, we believe that HepG2 cells are safer than liver cells of animal origin, particularly after testing for oncogenes.

In terms of clinical application, HepG2 cells are preferable, because the current molecular biology methods can change their biological function. Japanese researchers have used transgenic technology to transfer the mouse glutamine synthetase (mGS) gene into HepG2 cells (almost no ammonia clearance), which resulted in increased ammonia detoxification that reached 20% of that in primary liver cells [Enosawa et al., 2000]. They went on to use these cultured cells in an animal model of liver failure and obtained good therapeutic results [Miyashita et al., 2000]. In 2007, a British group found that the deficiency of two key enzymes, human arginase I (hArgI) and human ornithine transcarbamylase (hOTC) [Mavri-Damelin et al., 2007], in the ornithine cycle resulted in low ammonia detoxification in HepG2 cells.

In the present study, we modified the HepG2 cells by stable overexpression of hArgI and hOTC, to screen out the recombinant HepG2 cells with strong ammonia detoxification as well as their basic functions.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

The HepG2 cells were purchased from American Type Culture Collection (ATCC) and maintained in high glucose DMEM medium supplemented with 10% fetal bovine serum (growth medium) in a 5% CO_2 incubator at 37°C. The reagents were purchased: vector pBudCE4.1, Lipofectamine2000, and Zeocin from Invitrogen, Inc.; restriction enzyme *Sal*I, *Xba*I, *Kpn*I, *Xho*I, and T4 ligase from Biolabs, Inc.; carbamyl phosphate, dithiothreitol, and 2,3-butanedione monoxime from Sigma, Inc.; triethanolamine, ornithine, L-arginine, EGTA, ammonium chloride (NH_4Cl), L-glutamate from YongDa Chemistry, Inc. (China); anti-hArgI (H-52), anti-hOTC (W-17), anit-hCPS1 (H-140), anti-hASS (H-231) and anti-hArgII (H-64) rabbit pAb, anti-hASL (B-1), and anti-β-actin mouse mAb from Santa Cruz Biotechnology, Inc.; anti-hGS mouse mAb from Abcam, Inc.; Cell Counting Kit 8 (CCK-8) from Dōjindo Laboratories (Japan); Glutamine Assay Kit (EGLN-100) from BioAssay Systems, Inc.; and Protease Inhibitor Cocktail Set III from Calbiochem, Inc.

ESTABLISHMENT OF RECOMBINANT HepG2 CELL LINE WITH STABLE hARGI AND/OR hOTC OVEREXPRESSION

The RNA of normal human liver tissues from the non-tumor part of hepatic resection in a patient with hemangioma was extracted by Trizol (Invitrogen) and further was reverse-transcribed into hArgI and hOTC cDNA by different primers: hArgI-F (5′-CGGGGTACCA-TGAGCGCCAAGTCCAGAAC-3′) and hArgI-R (5′-CCGCTCGAGTCT-TACTTAGGTGGGTTAAGGTAGTC-3′), which contain *Kpn*I and *Xho*I sites (underlined); hOTC-F (5′-ACGCGTCGACAATGCTGTT-TAATCTGAGGATCCTG-3′), and hOTC-R (5′-GCTCTAGATCAAAA-TTTAGGCTTCTGGAGC-3′), which contain *Sal*I and *Xba*I sites (underlined). And then the hArgI and hOTC cDNA were cloned into the pBudCE4.1 vector MCS region under EF-1 and CMV promoter, respectively. These recombinatant plasmids were used to transform the competence *E. coli* DH5α that were cultured in the low-salt LB medium (containing 50 μg/ml Zeocin) and then the anti-Zeocin clones were obtained. After sequencing, these recombinatant plasmids including pBudCE4.1, pBudCE4.1/hArgI, pBudCE4.1/hOTC, and pBudCE4.1/hArgI + hOTC were used to transfect HepG2 cells by Lipofectamine 2000, respectively. After 2 weeks selection, anti-Zeocin colonies were expanded and maintained in growth medium. The protein expression of hArgI and hOTC in anti-Zeocin colonies were detected by Western blotting. Forty micrograms protein of cell extracts were run on 10% SDS–PAGE gels and electro-blotted to PVDF or NC membranes, which were then visualized by anti-hArgI, hOTC, hGS, hArgII, hCPS1, hASS, and hASL antibody, followed by HRP-labeled anti-mouse or anti-rabbit IgG and detected by enhanced chemiluminescence (ECL). The relative amount of each protein band was quantified as a ratio to the β-actin band indicated underneath each gel using the densitometric scanning software Quantity One (Bio-Rad). The data are expressed as the mean ± SD of the three independent experiments.

DETERMINATION OF hARGI AND hOTC ACTIVITY

(1) The activity of arginase was determined by measuring urea contents in the culture medium as previously described [Corraliza et al., 1994; Zharikov et al., 2008]. We modified the method: Cells were grown to confluence in 100-mm dishes, rinsed twice with ice-cold PBS, and then scraped into 100 μl buffer containing 50 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, and 1:200 protease inhibitor, and the total protein was extracted by three freeze–thaw cycles. Protein (0.2–6.4 mg) was taken to EP tubes, then the 50 μl L-arginine (0.5 M, pH 9.7) was added and cultured at 37°C for 1 h. The hydrolysis reaction of L-arginine by arginase was stopped by adding 25 μl of 1:3 sulfuric acid/phosphoric acid (by volume), and urea production was determined by adding 25 μl α-isonitrosopropiophenone (9% in absolute ethanol), incubating at 100°C in the dark for 45 min, and measured absorbance at 490 nm.

(2) The OTC enzyme activity was measured as described [Wu, 1995] with modifications. Cells were harvested by scraping into 200 μl mitochondria lysis buffer (0.5% Triton, 10 mM HEPES, pH 7.2, 2 mM dithiothreitol), and total protein was extracted by three freeze–thaw cycles. Briefly, 0.1–3.2 mg protein were added to EP tubes, then added 50 μl reaction mixture (5 mM ornithine, 15 mM carbamyl phosphate, and 270 mM triethanolamine, pH 7.7, which was incubated at 37°C for 30 min. Reactions were stopped by adding 25 μl of 1:3 sulfuric acid/phosphoric acid (by volume). Citrulline production was then determined by adding 25 μl of 3% 2,3-butanedione monoxime, incubating at 100°C in the dark for 15 min, and measuring absorbance at 490 nm.

MEASUREMENT OF CELL TOLERANCE TO AMMONIA POISONING

1×10^5 of cells were resuspended in 100 μl growth medium and added to 96-well culture plates. Cells were incubated at 37°C for 12 h, then the supernatant was replaced by 100 μl growth medium or STD buffer containing (in mM) 147 Na^+, 5.0 K^+, 131 Cl^-, 1.3 Mg^{2+}, 1.3 SO_4^{2-}, 2 Ca^{2+}, 25 HCO_3^-, 15 HEPES, 20 D-glucose at pH 7.4 with

0–540 mM NH_4Cl. The cell viability was measured by CCK-8 solution 12 h later. Briefly, the supernatant was removed and 100 μl of 1:10 CCK-8 solution (by volume) was added to the wells, and 2 h later the absorbance was measured at 490 nm.

MEASUREMENT OF UREA PRODUCTION IN CELLS

1×10^5 of cells were resuspended in 100 μl growth medium and added to a 96-well culture plate. Cells were incubated at 37°C for 12 h, then the supernatant was replaced by 100 μl growth medium or STD buffer with 0–540 mM NH_4Cl. After another 12 h, 50 μl supernatant was used for the detection assay. Twenty-five microliters of 1:3 sulfuric acid/phosphoric acid (by volume) was added and urea production was determined by adding 25 μl α-isonitrosopropriophenone (9% in absolute ethanol), incubating at 100°C in the dark for 15 min, and measuring absorbance at 490 nm [Zharikov et al., 2008]. Meanwhile, the standard curve and regression equation for detection were produced using a series of urea standards with different concentration, which were used to calculate the urea concentration in the samples.

MEASUREMENT OF GLUTAMINE PRODUCTION IN CELLS

1×10^5 of cells were resuspended in 100 μl growth medium and added to a 96-well culture plate. Cells were incubated at 37°C for 12 h, then the supernatant was replaced by 100 μl STD buffer with 120 mM NH_4Cl and in the presence of 0–25 mM glutamate (Glu). The supernatant was collected for Glutamine (Gln) detection assay 12 h later using Glutamine Assay Kit (EGLN-100) according to the manufacture's protocol. The standard curve and regression equation for detection were produced using a series of Gln standards the kit provided, which were used to calculate the Gln concentration in the samples.

MEASUREMENT OF CELL PROLIFERATION AND BASIC FUNCTIONS

2×10^3 and 8×10^5 cells in 100 μl growth medium were added to a 96-well plate (five replicates for each observation point) and a 12-well plate (three replicates for each observation point) and cultured at 37°C, respectively. After culturing for 24, 48, and 72 h, detection was carried out as follows. The 96-well plate was used to measure cell proliferation. The supernatant in the 96-well plate was discarded and replaced with 100 μl CCK-8 solution. OD450 was measured for every well in a microplate reader. The 12-well plate was used to detect the basic functions of liver cells. The supernatant in the 12-well plate was collected and sent to the laboratory department in our hospital for detection of biochemical index, including albumin (ALB), total bilirubin (TBIL), alkaline phosphatase (ALP), γ-glutamyltransferase (γ-GT), lactate dehydrogenase (LDH), and glucose, by using a specific protein detection instrument and an automatic biochemical analyzer.

CULTIVATION OF PRIMARY HUMAN HEPATOCYTES

The primary human hepatocytes were isolated from the fresh resected human liver of a patient with hepatic hemangioma. The study was approved by the Medical Human Investigation Committee of Fujian Medical University. Informed consent was obtained from the patient. The resected liver tissue (20 g) was washed with ice-cold PBS, cut into pieces with sterile scissors, placed within a sterile stainless steel gauze, and ground with the blunt end of a syringe. The cells were filtered out of the gauze, collected by the sterile centrifuge tube, and centrifuged for 5 min at 1,000*g*. The supernatant was removed and the cells were resuspended in 5 ml sterile erythrocyte lysis buffer for 10 min at room temperature, and centrifuged for 5 min at 1,000*g*. After repeating the above steps twice, the cells were resuspended in 5 ml growth medium. The primary human hepatocytes (10^5/well) in 100 μl medium were added to a 96-well culture plate, and incubated at 37°C with 5% CO_2.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS 11.5 for Windows. One-way analysis of variance (ANOVA) followed by Fisher's test or Tamhane's T2 test (if equal variance is not assumed) was used for comparison of data from different groups. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

SELECTION OF RECOMBINANT HepG2 CELLS WITH STABLE OVEREXPRESSION OF hARGI AND/OR hOTC

Full-length cDNA of hArgI and hOTC were amplified by RT-PCR from human liver cells (Fig. 1A), and then the hArgI or/and hOTC DNA fragments were cloned into the MCS in the pBudCE4.1 vector to produce the recombinant plasmids pBudCE4.1/hArgI, pBudCE4.1/hOTC, and pBudCE4.1/hArgI + hOTC (Fig. 1B). Subsequently, these recombinant plasmids were transduced into the HepG2 cells by liposomes. After cultured in the growth medium that contained Zeocin, nine resistant cell clones were obtained: HepG2/con, HepG2/hArgI1, HepG2/hArgI2, HepG2/hOTC1, HepG2/hOTC2, HepG2/(hArgI + hOTC)1, HepG2/(hArgI + hOTC)2, HepG2/(hArgI + hOTC)3, hOTC)3, and HepG2/(hArgI + hOTC)4. Furthermore, Western blotting showed that hArgI expression in HepG2/hArgI1 cells was greater than in HepG2/hArgI2 cells, hOTC expression in HepG2/hOTC2 cells was greater than in HepG2/hOTC1 cells, and coexpression of hArgI and hOTC in HepG2/(hArgI + hOTC)4 cells was greatest (Fig. 1C). Finally, HepG2, HepG2/con, HepG2/hArgI1, HepG2/hOTC2, and HepG2/(hArgI + hOTC)4 cells were identified again by Western blotting (Fig. 1D), and were used in all subsequent studies.

hARGI AND hOTC ENZYME ACTIVITIES

To investigate whether hArgI and/or hOTC overexpression in HepG2 cells is functional or not, the proteins of five cell lines were extracted, and hArgI and hOTC enzyme activities were detected by biochemical methods, as shown in Figure 2A,B.

COMPARISON OF TOLERANCE TO AMMONIA

To compare the tolerance to an ammonia environment, five cell lines were cultured for 12 h in growth medium (with Gln) and standard buffer solution (without Gln) with different concentrations of ammonia. Cell viability was detected by CCK-8 assay, as shown in Figure 3A,B.

COMPARISON OF AMMONIA DETOXIFICATION BY CELLS

It is well known that there are two pathways of ammonia metabolism (urea cycle and Gln synthesis) in cells, so the production

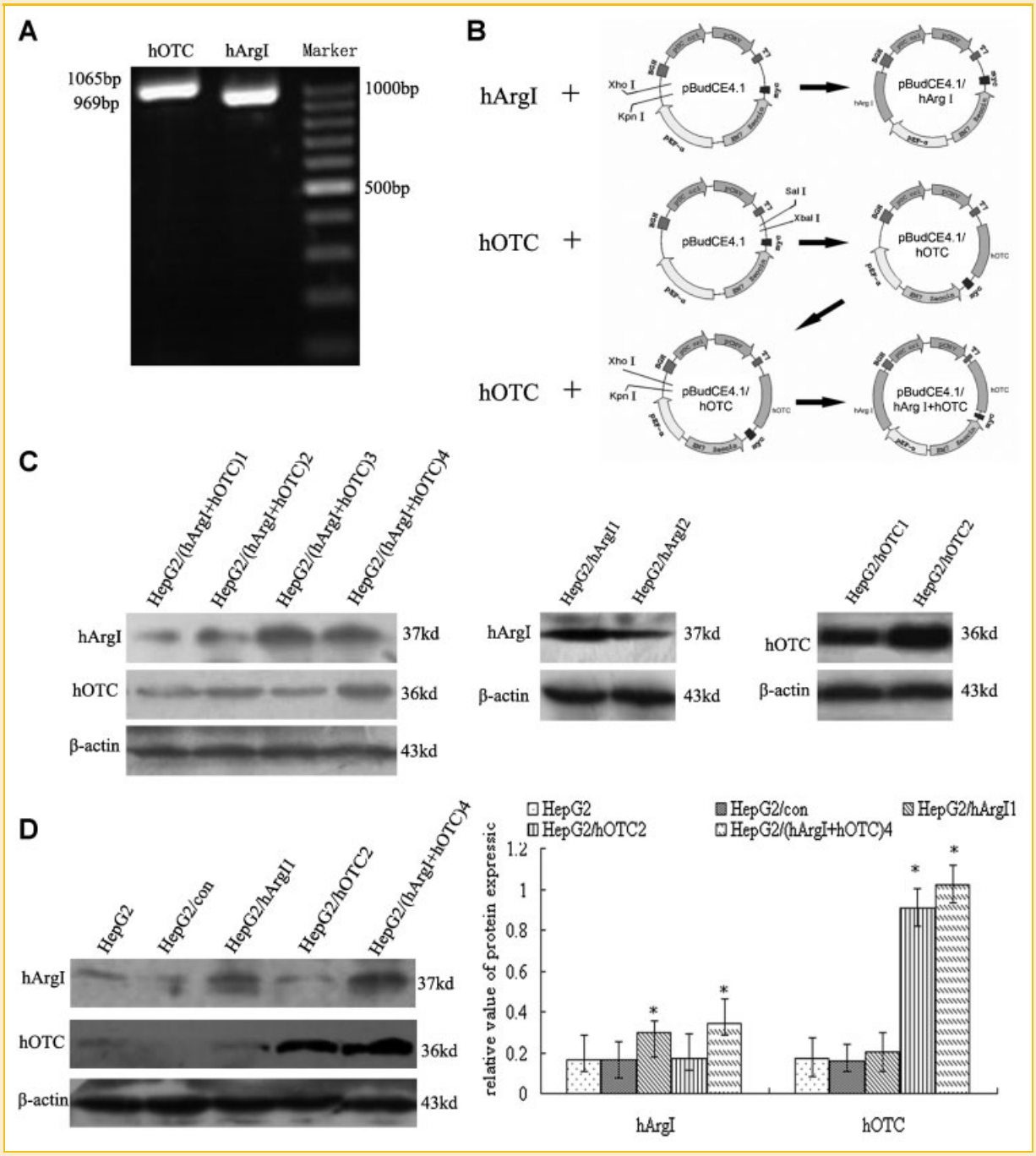

Fig. 1. Selection of recombinant HepG2 cells with stable overexpression of hArgI and/or hOTC. A: Full-length cDNA of hArgI and hOTC were amplified by RT-PCR from human liver cells. B: The construction process of the recombinant plasmids pBudCE4.1/hArgI, pBudCE4.1/hOTC, and pBudCE4.1/hArgI + hOTC. C: hArgI, hOTC, and β-actin protein in HepG2/hArgI1, HepG2/hArgI2, HepG2/hOTC1, HepG2/hOTC2, HepG2/(hArgI + hOTC)1, HepG2/(hArgI + hOTC)2, HepG2/(hArgI + hOTC)3, and HepG2/(hArgI + hOTC)4 cells were detected by Western blotting. D: Relative expression levels of hArgI and/or hOTC protein in the representative cell clones that were selected for subsequent experiments.

of urea and Gln were used to evaluate the capacity for ammonia detoxification in cells. Urea and Gln synthesis in these cells is shown in Figure 4A,B. The median lethal dose (LD50) of NH_4Cl in each cell line was different (as shown in Fig. 3, the LD50 for HepG2 cells was 60 mM, and HepG2/(hArgI + hOTC)4 cells was 180 mM), therefore, we chose 120 mM NH_4Cl as the unified concentration for Gln production to guarantee that HepG2 cells were still alive after the experiment.

COMPARISON OF CELL PROLIFERATION

To investigate the effect of hArgI and/or hOTC overexpression on cell proliferation and other synthetic functions of HepG2 cells, the

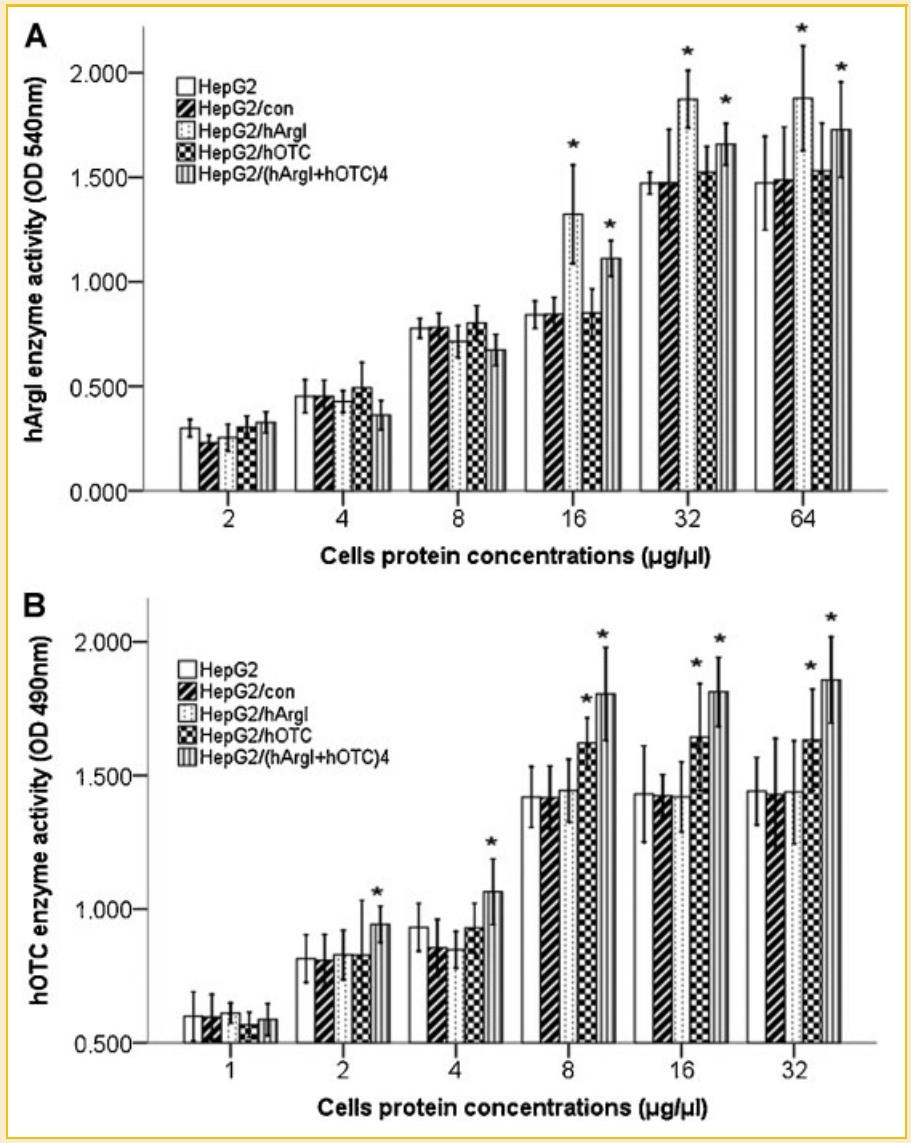

Fig. 2. hArgI and hOTC enzyme activity at different concentrations of proteins extracted from five cell lines. A: The hArgI enzyme activity increased with protein concentration in all cell lines, and that in HepG2/hArgI1 and HepG2/(hArgI + hOTC)4 cells was significantly higher than that in HepG2, HepG2/con, and HepG2/hOTC2 cells when protein concentration was from 16 to 64 μg/μl ($^{*}P < 0.05$, n = 3). B: The hOTC enzyme activity increased with protein concentration in all cell lines, and that in HepG2/hOTC2 (protein concentration 8–32 μg/μl) and HepG2/(hArgI + hOTC)4 cells (protein concentration 2–32 μg/μl) was significantly higher than that in HepG2, HepG2/con, and HepG2/hArgI1 cells ($^{*}P < 0.05$, n = 3). This suggested that the transduction of hArgI and hOTC genes resulted in the increased expression of hArgI and hOTC as well as function (activity) in HepG2 cells.

five cell lines (2×10^3 cells) were cultured for 1, 2, and 3 days in a 96-well plate for detecting cell proliferation (Fig. 5), and 8×10^5 cells were added to a 12-well plate to measure ALB, TBIL, γ-GT, ALP, and glucose in the supernatant at 1, 2, and 3 day. As shown in Table I, on the day 1 after culture, the glucose level in the supernatant of HepG2 and HepG2/con cells was significantly lower than that of HepG2/hArgI1, HepG2/hOTC2, and HepG2/(hArgI + hOTC)4 cells ($^{a}P < 0.05$). On the days 2 and 3 after culture, HepG2/hOTC2 cells had higher glucose levels than HepG2, HepG2/con, HepG2/hArgI1, and HepG2/(hArgI + hOTC)4 cells ($^{b}P < 0.01$), and ALB levels in the supernatant of HepG2/(hArgI + hOTC)4 cells were markedly higher than those in HepG2, HepG2/con, HepG2/hArgI1, and HepG2/hOTC2 cells ($^{c}P < 0.01$). There were no significant differences in TBIL, γ-GT, and ALP values among the supernatants of these five cell lines at all time points ($P > 0.05$).

EFFECTS OF hARGI AND hOTC OVEREXPRESSION ON OTHER KEY ENZYMES OF UREA METABOLISM AND GLUTAMINE SYNTHESIS

Overexpression of hArgI and hOTC in HepG2 cells markedly improved the capacity for ammonia tolerance and detoxification. However, we could not determine whether overexpression of hArgI and hOTC entirely contributed to all of the improvement or affected expression of other enzymes involved in production of urea and Gln. Therefore, we investigated the expression of ammonia-detoxifica-

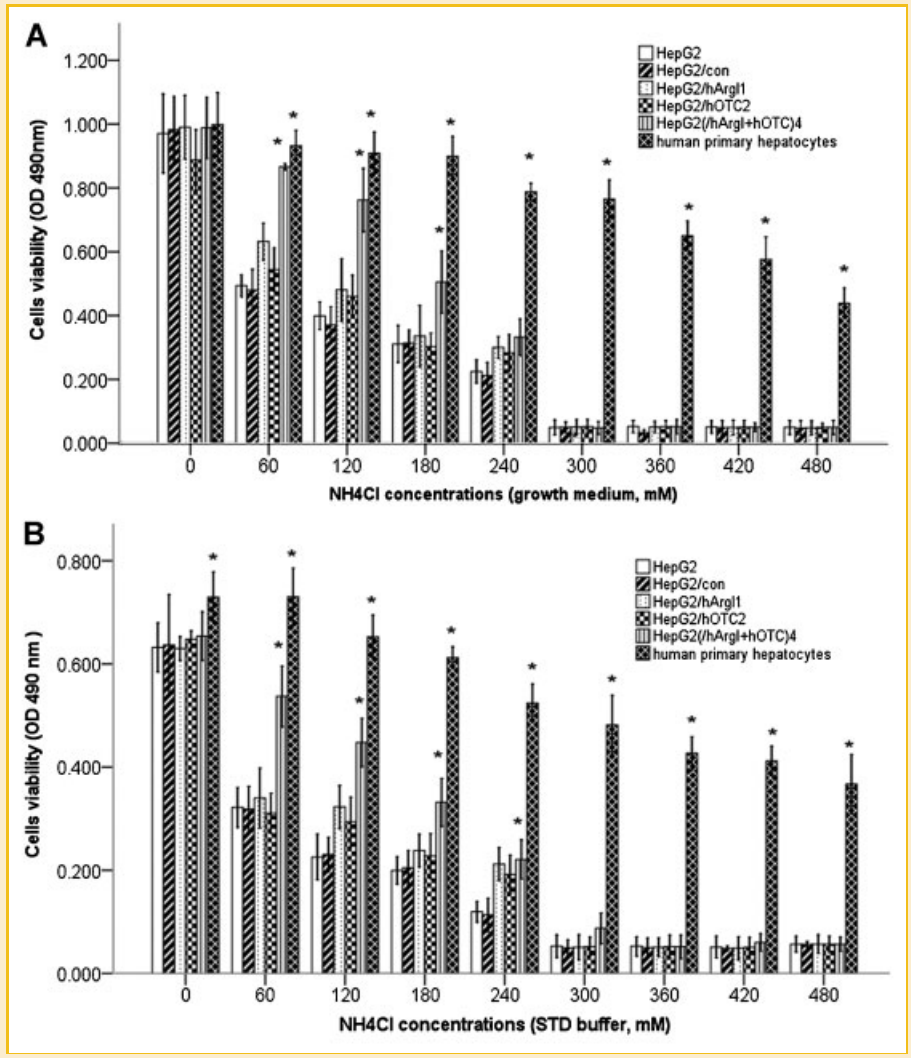

Fig. 3. Cell viability at different concentrations of NH_4Cl. NH_4Cl killed cells in a dose-dependent manner. Cell viability in growth medium was higher than that in STD buffer under the same conditions. $^*P < 0.05$ versus HepG2, HepG2/con, or HepG2/hOTC2 cells ($n = 3$). The LD50 of NH_4Cl for HepG2, HepG2/con, HepG2/hArgI1, HepG2/hOTC2, HepG2/(hArgI + hOTC)4 cells, and human primary hepatocytes was 60, 60, 120, 120, 180, and 480 mM, respectively. The capacity for ammonia tolerance in HepG2/(hArgI + hOTC)4 cells was three times that of HepG2 cells, reaching 37.5% of that of human primary hepatocytes.

tion-associated protein in HepG2 cells, hGS, hArgII that is involved in direct synthesis of urea and the other key enzymes in ornithine cycle including carbamoyl phosphate synthase 1 (hCPS1), argino-succinate synthase (hASS), and arginosuccinate lyase (hASL), as shown in Figure 6.

DISCUSSION

For patients suffering from liver failure, the most important factor is increased ammonia concentration. After passing through the blood–brain barrier to reach brain, ammonium ions induces brain edema and it is the important pathological factor causing hepatic encephalopathy [Albrecht and Dolinska, 2001]. Therefore, to avoid injury of higher blood-ammonia on brain and other organs, ammonia should first be removed. Only decreasing the concentration of ammonia in blood can effectively inhibit Gln production in the brain. Blood dialysis is an alternative method. However, bioartificial liver makes use of the biological detoxification and synthesis functions of liver cells, which better reflects its value in the treatment of liver failure. This is the purpose of establishing the strain HepG2 cell with high expression of hArgI and hOTC.

It is well known that ammonia metabolism involves two major pathways [Dejong et al., 1996; Scaglia et al., 2004]. One is Gln synthesis. Here, ammonia is catalyzed by glutaminase in renal tubular epithelial cells to generate Gln that is directly excreted into the urine. The other pathway is urea synthesis, which is mainly performed in the liver. The HepG2 cell line is an immortalized human liver cell line that has been used for research into bioartificial liver systems [Enosawa et al., 2006; Nibourg et al., 2010]. Unfortunately, a low level of ammonia detoxification is its biggest drawback [Mavri-Damelin et al., 2007]. There are two possible

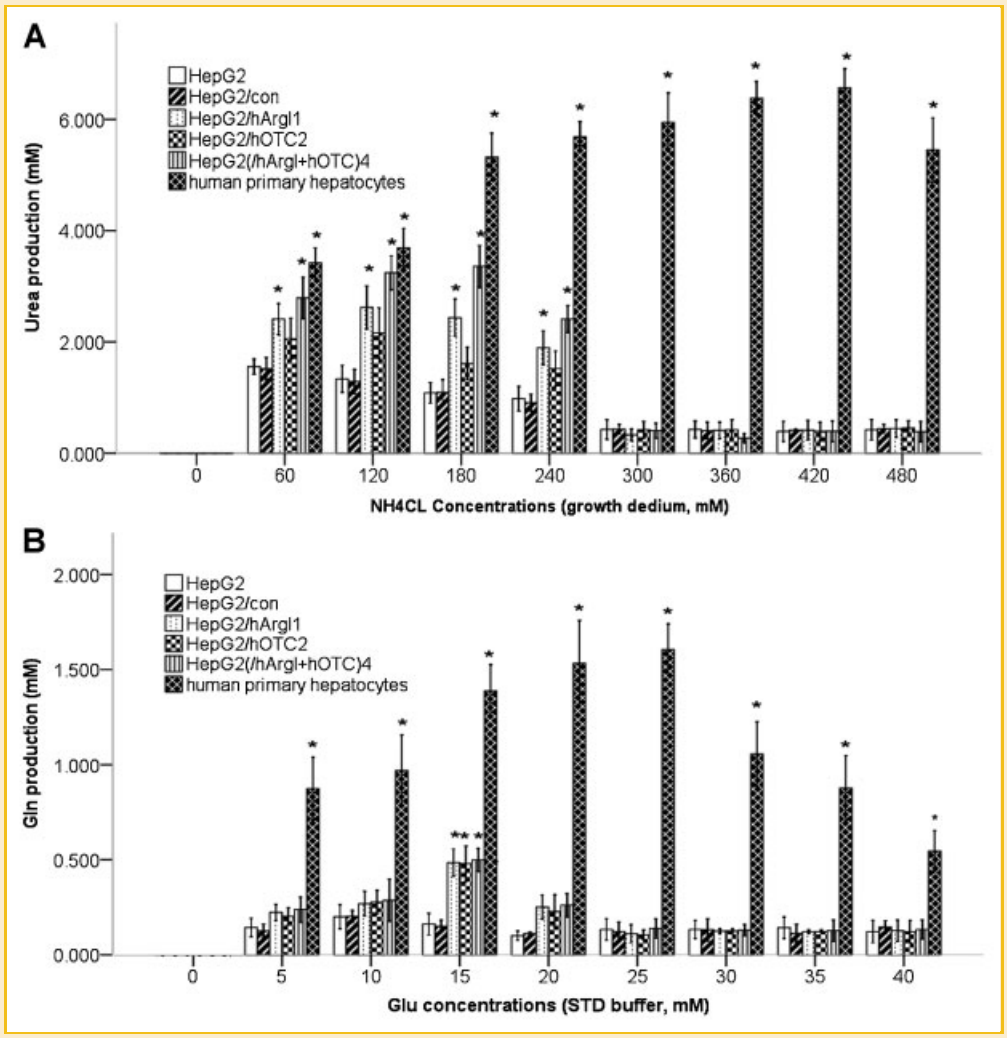

Fig. 4. Urea and Gln production in cells with different concentrations of ammonia. A: With increasing ammonia concentration, urea production in HepG2/hArgI cells, HepG2/(hArgI + hOTC)4 cells, and primary human hepatocytes increased at the beginning and decreased later; *denotes a significant difference compared to HepG2 and HepG2/con cells ($P < 0.05$, $n = 3$). When NH_4Cl concentration was 180 mM, urea production in HepG2/(hArgI + hOTC)4 cells reached a maximum value of 3.358 mM, and the corresponding value for HepG2 cells and human primary hepatocytes was 1.084 and 5.324 mM. Urea production in HepG2/(hArgI + hOTC)4 cells was 3.1 times that of HepG2 cells and 63.1% that of human primary hepatocytes. B: With 120 mM NH_4Cl and increasing Glu concentration, Gln production in these cells presented increased at beginning and decreased later; *denotes a statistically significant difference compared to HepG2 and HepG2/con cells ($P < 0.05$). When Glu concentration was 15 mM, Gln production in HepG2/(hArgI + hOTC)4 cells reached a maximum value of 0.5 mM, and the corresponding value for HepG2 cells and human primary hepatocytes was 0.162 and 1.387 mM. Gln production in HepG2/(hArgI + hOTC)4 cells was 3.1 times that of HepG2 cells and 36.0% that of human primary hepatocytes.

explanations for this: (1) hArgI expression is inhibited by hepatocyte nuclear factor 4, which is a transcription factor that is highly expressed in HepG2 cells [Chowdhury et al., 1996]; and (2) the lack of hOTC expression that is due to methylation of the gene [Delers et al., 1984].

Mavri-Damelin et al. [2007] has observed the impact of the transient transduction of hArgI and hOTC on HepG2 cells, but we think that this research was limited. This is because transient transduction of hArgI and hOTC could not maintain stable and high expression of the two enzymes in HepG2 cells, which is not conducive to studying the real impact of hArgI and hOTC on cells and the potential value of BAL application. To this end, we aimed to construct HepG2 cells with stable overexpression of hArgI and hOTC. The present study differed from that of Mavri-Damelin, in that the two key genes were transduced simultaneously into HepG2 cells by a eukaryotic dual gene expression vector, and very low expression of hArgI and hOTC mRNA and protein was observed before gene transduction.

In this study, overexpression of hArgI and/or hOTC was found in HepG2/hArgI1, HepG2/hOTC2, and HepG2/(hArgI + hOTC)4 cells, and the corresponding enzyme activity, and ammonia tolerance and detoxification were also improved more significantly in these cells than in HepG2 cells, particularly in the HepG2/(hArgI + hOTC)4 cells. We offer the following speculation on the mechanism. First, transduction of the hArgI and hOTC genes improved significantly the low expression of hArgI and hOTC in HepG2 cells, which led to complete recovery of the urea metabolic pathway and increases urea synthesis. Both ArgI and OTC have smaller Michaelis constant Km

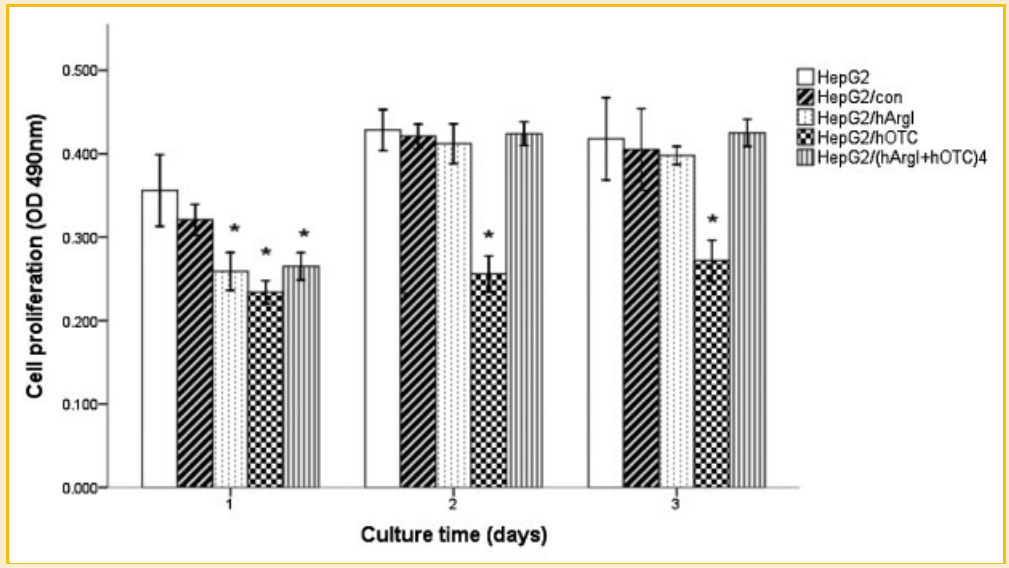

Fig. 5. Cell proliferation curves. On day 1 after culture, the proliferation of HepG2/hArgI1, HepG2/hOTC2, and HepG2/(hArgI + hOTC)4 cells was significantly lower than that of HepG2 and HepG2/con cells ($^{*}P < 0.05$, n = 3). On days 2 and 3 after culture, the proliferation of HepG2/hOTC2 cells was markedly lower than that of HepG2, HepG2/con, HepG2/hArgI1, and HepG2/(hArgI + hOTC)4 cells ($^{*}P < 0.01$, n = 3), and there were no significant differences among these four cells ($P > 0.05$).

[Morizono et al., 1997; Cheng et al., 2007], which has higher affinity with substrates and higher catalytic functions. Thus, in vitro experiments can show better catalytic activity. Meanwhile, it also increased the expression of hASS and hASL; two of the other key enzymes in the urea pathway. This shows that improvement of hArgI and hOTC expression in HepG2 cells is essential for urea metabolism. Second, HepG2/(hArgI + hOTC)4 cells produced more Gln in the presence of high concentrations of ammonia and different concentrations of Glu, and cell viability increased with the level of Gln. This shows that transduction of hArgI and hOTC promotes another path of ammonia metabolism, namely, the reaction of Gln synthesis catalyzed by hGS [Tang et al., 2008]. Our Western blotting results also suggest increased hGS protein expression in HepG2/(hArgI + hOTC)4 cells. This confirms that Gln could be used to protect cells, which is consistent with the study of Nakamura [Nakamura and Hagen, 2002]. Third, it is known that there are two metabolic pathways to produce urea in cells: one is the urea cycle for ammonia metabolism (which involves hArgI; cytosolic localization), and the other is direct synthesis of urea (which involves hArgII; mitochondria localization) [Cederbaum et al., 2004]. Transduction of hArgI and hOTC might also promote another path of urea synthesis, in a reaction that is catalyzed directly by hArgII [Ash, 2004]. hArgII protein expression was also higher in the HepG2/(hArgI + hOTC)4 cells than the HepG2 cells. HepG2 cells can express hArgII [Mavri-Damelin et al., 2008], and this study confirms this finding. Under normal conditions, hArgI should express liver cells, and hArgII expresses mainly the extrahepatic system (e.g., kidney cells). However, the HepG2 cell is a strain of immortalized hepatoblastoma cells. Its mechanism in regulating the expression of hArgII may change. In addition, an important explanation is that we

TABLE I. Biochemical Index in the Supernatant of Cells

Cell line	Culture time (day)	ALB (mg/L)	TBIL (μmol/L)	γ-GT (IU/L)	ALP (IU/L)	Glucose (mmol/L)
HepG2	1	11.5 ± 1.03	8.4 ± 0.97	5.3 ± 1.53	49.0 ± 3.60	14.4 ± 1.03[a]
	2	22.5 ± 1.41	8.2 ± 0.66	7.7 ± 1.53	38.7 ± 2.52	8.7 ± 1.11
	3	30.8 ± 2.80	6.8 ± 1.56	7.0 ± 1.00	46.0 ± 2.65	7.4 ± 0.50
HepG2/con	1	11.7 ± 1.00	8.1 ± 1.39	5.0 ± 1.00	49.3 ± 3.51	14.8 ± 1.01[a]
	2	21.5 ± 1.05	7.6 ± 0.75	7.7 ± 1.53	40.3 ± 2.52	8.6 ± 1.08
	3	21.9 ± 1.25	6.9 ± 1.41	7.7 ± 1.53	46.3 ± 3.51	7.0 ± 0.43
HepG2/hArgI1	1	12.0 ± 1.31	8.6 ± 0.84	5.7 ± 1.53	49.3 ± 5.69	17.5 ± 0.90
	2	22.2 ± 2.32	8.0 ± 1.20	6.7 ± 1.15	41.0 ± 2.00	9.1 ± 1.42
	3	24.3 ± 0.37	7.6 ± 0.65	6.7 ± 1.53	46.0 ± 2.00	8.0 ± 0.44
HepG2/hOTC2	1	11.8 ± 0.87	8.3 ± 1.11	5.7 ± 2.08	49.7 ± 3.06	18.1 ± 1.17
	2	12.6 ± 1.32	7.7 ± 0.56	7.7 ± 1.53	38.0 ± 2.00	16.2 ± 1.77[b]
	3	20.7 ± 0.64	6.8 ± 0.91	7.7 ± 1.53	47.3 ± 1.53	13.9 ± 0.96[b]
HepG2/(hArgI + hOTC)4	1	12.6 ± 1.16	8.5 ± 1.24	6.0 ± 1.00	50.7 ± 3.06	18.2 ± 1.69
	2	26.5 ± 0.99[c]	8.4 ± 0.67	6.3 ± 1.53	42.3 ± 4.04	9.2 ± 1.17
	3	59.4 ± 1.08[c]	7.3 ± 1.13	6.0 ± 1.00	47.3 ± 4.04	7.1 ± 0.67

[a]denotes a significant difference compared to HepG2/hArgI1, HepG2/hOTC2 and HepG2/(hArgI+hOTC)4 cells ($P < 0.05$, n = 3).
[b]denotes a significant difference compared to HepG2, HepG2/con, HepG2/hArgI1 and HepG2/(hArgI+hOTC)4 cells ($P < 0.05$, n = 3).
[c]denotes a significant difference compared to HepG2, HepG2/con, HepG2/hArgI1 and HepG2/hOTC2 cells ($P < 0.05$, n = 3).

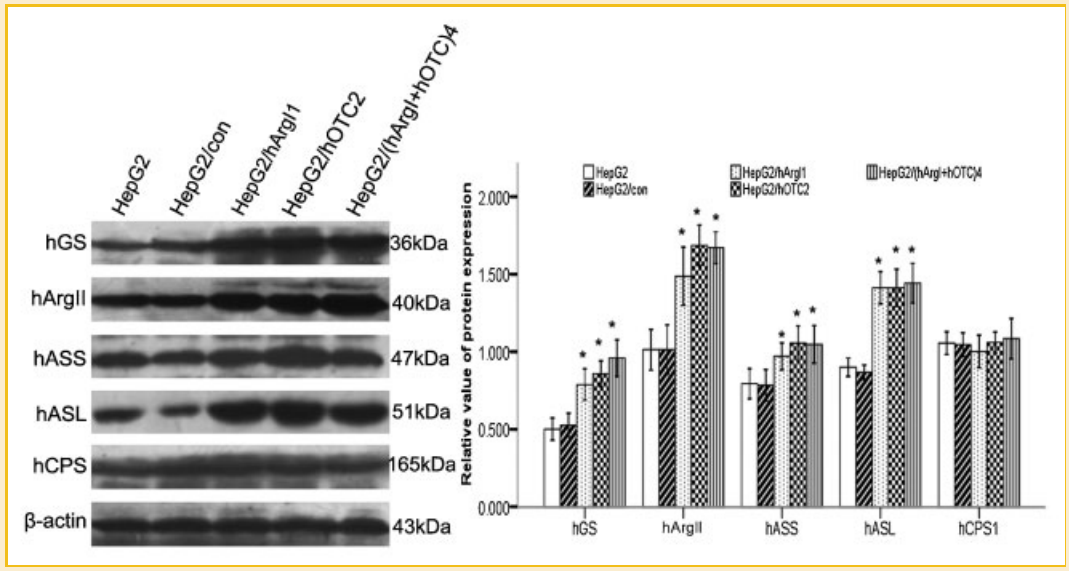

Fig. 6. Effect of hArgI and/or hOTC overexpression on ammonia detoxification associated proteins in HepG2 cells. hArgI and/or hOTC overexpression promoted expression of ammonia-detoxification-associated proteins in HepG2 cells. The comparisons of relative level are as follows: hGS: (1) population variance $F = 14.739$ ($P = 0.000$); (2) group comparison $^{*}P < 0.05$, versus HepG2, HepG2/con; hArgII: (1) population variance $F = 16.237$ ($P = 0.000$); (2) group comparison $^{*}P < 0.05$, versus HepG2, HepG2/con; hASS: (1) population variance $F = 4.890$ ($P = 0.019$); (2) group comparison $^{*}P < 0.05$, versus HepG2, HepG2/con; hASL: (1) population variance $F = 27.628$ ($P = 0.000$); (2) group comparison $^{*}P < 0.05$, versus HepG2, HepG2/con; hCPS1: (1) population variance $F = 0.321$ ($P = 0.858$).

conducted an in vitro experiment of tolerance to ammonia environments in shorter periods (culturing for 12 h). NH_4Cl was dissolved in a culture medium with stable pH and standard buffer; this is more ideal than the plasma environment of patients suffering from liver failure.

In this study, we observed that only the proliferation of HepG2/hOTC2 cells was significantly lower than that of other cells, and proliferation of HepG2/(hArgI + hOTC)4 cells was also slightly lower than that of HepG2 cells (although not significantly). This might be the hOTC overexpression in HepG2/hOTC2 cells resulted in increased synthesis of citrulline, but decreased synthesis of polyamines that affect cell proliferation [Wallace et al., 2003; Pegg, 2006]. In addition, the effect of hArgI overexpression was not as obvious as that of hOTC on inhibition of cell proliferation, despite the absence of hArgI is thought to be beneficial to the shift of nitric oxide substrate (NO) synthesis or/and agmatine synthesis from arginine, and thus is conducive to cell proliferation [Mavri-Damelin et al., 2007].

In assessing the basic functions of liver cells (including synthesis of ALB, TBIL, γ-GT, ALP, and glucose), we found that the level of glucose in the supernatant of HepG2/hOTC2 cells after 3 days culture was significantly higher than in other cells. This phenomenon might be attributed to the slow proliferation and inadequate consumption of nutrients including glucose in the HepG2/hOTC2 cells. Comparison of other indicators between the five cell lines showed no significant difference, indicating that the effects of transduction of hArgI and hOTC on TBIL, ALP, and γ-GT synthesis were not obvious. We found that the HepG2/(hArgI + hOTC)4 cells had a stronger function of ALB synthesis than the other cells had. ALB synthesis is the most important indicator for the biosynthesis of liver cells. Therefore, we will investigate its mechanism further.

Although the capacity for ammonia tolerance and ammonia detoxification in HepG2/(hArgI + hOTC)4 cells did not reach that of human primary hepatocytes, this modification using hArgI and hOTC genes in this study made significant progress in the improvement of HepG2 cell function. The practical applications will be investigated in our follow-up animal experiments. We believe that the HepG2/(hArgI + hOTC)4 cells will not only lay a solid foundation for future research, but also provide a better selection of materials for the development of bioartificial liver systems.

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