ORIGINAL

Farnesoid X receptor agonist accelerates ammonium metabolism of mesenchymal stem cell-derived hepatocyte-like cells

Yu Saito, Shuhai Chen, Tetsuya Ikemoto, Hiroki Teraoku, Shinichiro Yamada, Yuji Morine, and Mitsuo Shimada

Department of Surgery, Tokushima University, Tokushima, Japan

Abstract : Background : Accelerating ammonium metabolism of hepatocyte like cells (HLCs) is critical for various functions of hepatocytes. The aim of the present study was to investigate whether Farnesoid X receptor (FXR) agonist, obeticholic acid (OCA), accelerated ammonium metabolism of HLCs, which was derived from adipose derived mesenchymal stem cells (ADSCs). Methods : Human ADSCs were seed in flat bottom plate, then our differentiation protocol was used for 21 days. OCA treatment had been performed in Step3 for 10days. Then, 1) hepatic maturation, 2) urea cycle genes, 3) urea production, and 4) ammonium metabolism was compared depend on the presence or absence of OCA. Results : HLCs had been successfully produced for 21 days. HLCs with OCA showed significantly higher mRNA expressions of AAT than those without OCA. HLCs with OCA showed significantly higher mRNA expressions of urea cycle genes such as SLC25A13, CPS1, and OTC. Urea production was also tended to be upregulated by OCA addition. HLCs with OCA showed significantly higher clearance of NH_4Cl at 6hr and 24 hr after addition of NH4Cl. Conclusion : FXR agonist, OCA, accelerates ammonium metabolism of ADSCs derived HLCs. HLCs could be one of treatment options of hepatic encephalopathy of patients with liver failure or urea cycle disorder in the future. J. Med. Invest. 72 : 54-59, February, 2025

Keywords **:** *mesenchymal stem cells, hepatocyte like cells, Farnesoid X receptor agonist, ammonium metabolism*

INTRODUCTION

Liver transplantation is a life-saving intervention for patients with end-stage liver disease ; however, long-term donor shortages mean that new donor sources are required. Functional hepatocyte-like cells (HLCs) present a viable alternative to whole livers ; HLCs can be differentiated from various stem cells, making them a flexible and reliable source compared with primary hepatocytes (1). Mesenchymal stem cells currently represent the best cell source for hepatocyte differentiation because they do not carry risks associated with genetic damage, rejection, or ethical problems (2, 3). In particular, adipose-derived mesenchymal stem cells (ADSCs) can be obtained with minimal invasiveness and successfully differentiated ; we previously established a new, three-dimensional culture protocol for ADSC-derived HLCs (4). However, currently available techniques do not differentiate HLCs to the functional level of primary hepatocytes.

Hepatocytes have many functions : these include alcohol metabolism, detoxification, ammonia metabolism, glucose metabolism, lipid metabolism, and bile acid production (5). Hepatic encephalopathy, a neurological disorder caused by accumulation of ammonia in severe liver disease, contributes greatly to impaired quality of life in patients with end-stage liver disease. Therefore, ammonia metabolism and clearance is an especially important hepatocyte function (4). In addition to those suffering with liver disease, overcoming dysfunctional ammonia metabolism would have a significant impact in patients with metabolic disorders, such as urea cycle disorder (UCD) (1). Therefore, improving ammonia metabolism in HLCs could significantly impact numerous patient groups.

Farnesoid X receptor (FXR) is a nuclear receptor that induces transcription of fibroblast growth factor 19, which regulates bile acid synthesis and transport, glucose homeostasis, and cholesterol metabolism (6). The FXR/fibroblast growth factor 19 system plays critical roles in the pathogenesis of cholestatic disease (7), nonalcoholic fatty liver (8), poor liver regeneration (9), liver fibrotic changes (10), and hepatocellular carcinoma (11). Obeticholic acid (OCA) is a potent, first-in-class FXR agonist. In recent phase 2 clinical trials for nonalcoholic steatohepatitis (FLINT) (12) and primary biliary cholangitis (POISE) (13), OCA treatment was associated with rapid improvements of serum biochemistry markers. In addition, FXR regulates detoxification of ammonium through ureagenesis in hepatocytes (14).

Given this background, we investigated whether including the FXR agonist OCA during differentiation enhances ammonium metabolism in HLCs.

Abbreviations :
Farnesoid X receptor ; FXR, obeticholic acid ; OCA, hepatocyte like cells ; HLCs, adipose derived mesenchymal stem cells ; ADSCs, embryonic stem cells ; ESCs, mesenchymal stem cells ; MSCs, hepatic encephalopathy ; HE, urea cycle disorder ; UCD, fibroblast growth factor ; FGF, nonalcoholic steatohepatitis ; NASH, primary biliary cholangitis ; PBC, hepatocyte growth factor ; HGF, small heterodimer partner ; SHP, cholesterol 7 alpha-hydroxylase ; CYP7A1, apolipoprotein E ; ApoE, alpha-1 antitrypsin ; AAT, solute carrier family 25 member 13 ; SLC25A13, carbamoyl phosphate synthetase ; CPS 1, ornithine transcarbamylase ; OTC, branched chain amino acids ; BCAA

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Address correspondence and reprint requests to Yu Saito, MD, PhD, FACS, Department of Surgery, Tokushima University, 3-18-15 Kuramoto-cho, Tokushima, 770-8503, Japan. E-mail : saito.yu.1001@tokushima-u.ac.jp

MATERIAL AND METHODS

Ethics approval

The study was reviewed and approved by Tokushima University Hospital ethics committee and corresponding regulatory agencies (Tokushima Clinical Trial Management System Number ; 3090). All animal experiments conformed to the internationally accepted principles for the care and use of laboratory animals and were approved by the Ethical Committee of Tokushima University.

Isolation and culture of ADSCs

Human ADSCs were cultured in STEMPRO (Life Technologies, Tokyo, Japan) (15).

Generation of HLCs

HLCs were generated as previously reported (4) (Figure 1). Briefly, human ADSCs were seeded into 96-well plates (2.0×10^4 per well) and incubated with serum-free medium for 48 h. A three-step differentiation protocol was used as follows : 1) definitive endoderm differentiation ; 2) hepatoblast differentiation ; and 3) hepatocyte differentiation. To investigate the effect of an FXR agonist on differentiation, cells were treated with 10 μM OCA (FUJIFILM, Tokyo, Japan) for 10 days during the third step of differentiation ; differentiation medium was changed every 2 days.

Real-time RT-PCR of HLCs

RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA, USA). RNA was reverse-transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Tokyo, Japan). RT-qPCR was performed using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). *FXR1* (Hs010196861_g1), small heterodimer partner (*SHP*) (Hs00222677_m1), cholesterol 7 alpha-hydroxylase (*CYP7A1*) (Hs00167982_m1) , apolipoprotein E (*APOE*) (Hs00171168_m1), alpha-1 antitrypsin (*AAT*) (Hs00165475_m1), solute carrier family 25 member 13 (*SLC25A13*) (Hs01573625_m1), carbamoyl phosphate synthetase 1 (*CPS1*) (Hs00157048_m1), and ornithine transcarbamylase (*OTC*) (Hs00166892_m1) TaqMan primers were used. *GAPDH* (Hs02786624_g1) was used as an internal control for normalization. Expression levels of all genes were calculated relative to *GAPDH*.

Urea production assay

For urea quantitation, medium was collected 48 h after the medium change and analyzed using a Urea Assay Kit (BioAssay Systemsm, Hayward, CA, USA) in accordance with the manufacturer's instructions. Optical absorbance was measured using a SpectraMax i3 (Molecular Devices, San Jose, CA, USA) with SoftMax Pro 7 software (Molecular Devices, San Jose, CA, USA). Data were normalized to cell number.

Ammonium metabolism assay

After the addition of ammonium chloride (NH_4Cl), ammonium metabolism was evaluated via changes in ammonium concentration in cell culture supernatants over a 24-h period. At 0 h, 300 μM of NH_4Cl (FUJIFILM Wako Pure Chemical Corporation) was added to culture dishes containing 100 differentiated embryoid bodies in suspension. Next, supernatants were collected and used in an Ammonia Assay Kit (Cell Biolabs, San Diego, CA, USA) to measure ammonium concentrations at 2-, 6-, and 24-h after NH_4Cl addition.

Statistical analysis

All results are presented as mean ± standard deviation. Multiple group comparisons were performed using one-way analyses of variance followed by the Bonferroni procedure. Comparisons between two groups were performed with the Mann-Whitney U-test. All statistical analyses were performed using JMP 8.0.1 statistical software (SAS Campus Drive, Cary, NC, USA). $P<0.05$ was considered statistically significant.

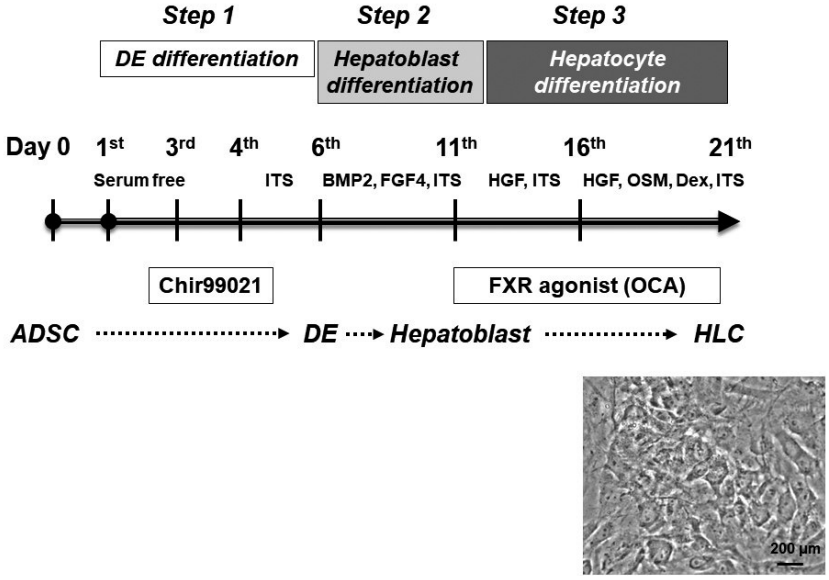

Figure 1. Differentiation protocol of HLCs
HLC differentiation protocols can be divided into three (3) differentiation steps ; definitive endoderm differentiation (Chir99021) ; hepatoblast differentiation ; and hepatocyte differentiation using the growth factors and cytokines known to be necessary for liver development. FXR agonist, OCA, was added into Step3.

RESULTS

Maturation of HLCs

ADSCs successfully differentiated into HLCs during the 21-day protocol. At the 21-day endpoint, HLCs treated with OCA had significantly higher mRNA expression of *AAT* than untreated cells (1.52±0.004 vs 1.78±0.007, Control vs OCA group, $p<0.01$) (Figure 2).

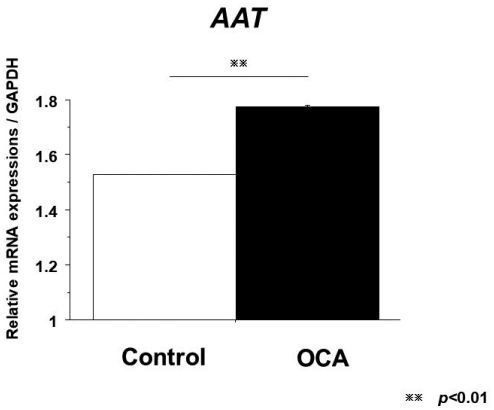

Figure 2. mRNA expressions of AAT in HLCs
HLCs with OCA showed significantly higher mRNA expressions of AAT than those without OCA (1.52±0.004 vs. 1.78±0.007, Control vs. OCA group, $p<0.01$).

Urea cycle gene expression and urea production

HLCs treated with OCA had significantly higher mRNA expression of urea cycle genes such as *SLC25A13* (1.47±0.002 vs 1.78±0.005, Control vs OCA group, $p<0.01$), *CPS1* (1.48±0.006 vs 1.50±0.005, Control vs OCA group, $p<0.01$), and *OTC* (1.63±0.04 vs 1.92±0.02, Control vs OCA group, $p<0.01$) (Figure 3). In line with gene expression changes, urea production tended to be upregulated by the addition of OCA during differentiation (312±22 vs 381±47, Control vs OCA group, $p=0.08$) (Figure 4).

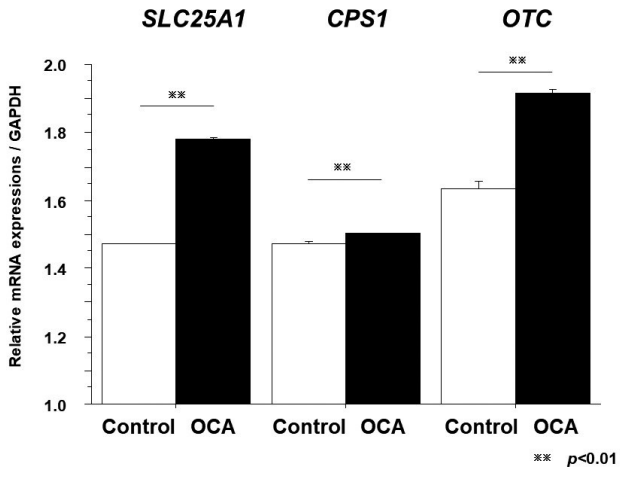

Figure 3. Urea cycle genes in HLCs
HLCs with OCA showed significantly higher mRNA expressions of urea cycle genes such as SLC25A13 (1.47±0.002 vs. 1.78±0.005, Control vs. OCA group, $p<0.01$), CPS1 (1.48±0.006 vs. 1.50±0.005, Control vs. OCA group, $p<0.01$), and OTC (1.63±0.04 vs. 1.92±0.02, Control vs. OCA group, $p<0.01$).

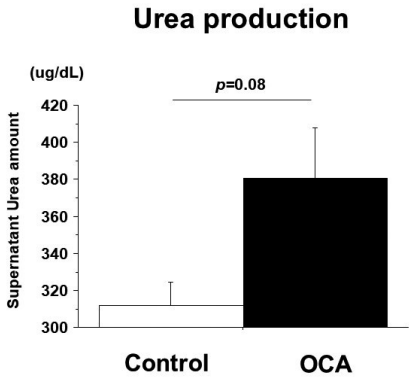

Figure 4. Urea production in HLCs
Urea production was tended to be upregulated by OCA addition (312±22 vs. 381±47, Control vs. OCA group, $p=0.08$).

Ammonium metabolism

All HLCs metabolized NH_4Cl ; however, HLCs treated with OCA had significantly less ammonium remaining in culture media, suggesting higher metabolism and clearance levels of NH_4Cl, 6 h (155±2 vs 118±4, Control vs OCA group, $p<0.01$) and 24 h (128±2 vs 87±2, Control vs OCA group, $p<0.01$) after addition of NH_4Cl (Figure 5).

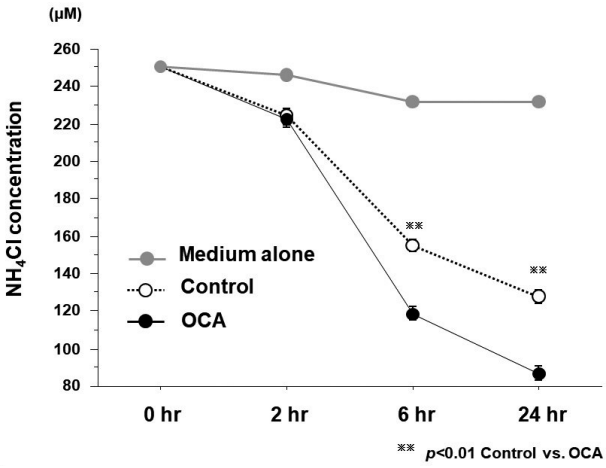

Figure 5. Ammonium metabolism in HLCs
HLCs with OCA showed significantly higher clearance of NH_4Cl at 6hr (155±2 vs. 118±4, Control vs. OCA group, $p<0.01$) and 24 hr (128±2 vs. 87±2, Control vs. OCA group, $p<0.01$) after addition of NH_4Cl.

Expression of FXR1 and its target genes

HLCs treated with OCA had significantly higher mRNA expression of *FXR1* than undifferentiated ADSCs and cells differentiated without OCA (0.59±0.07 vs 0.98±0.03 vs 1.13±0.06, ADSC vs Control vs OCA group, $p<0.01$). HLCs treated with OCA had significantly higher mRNA expression of *FXR1* target genes than the control group, including *SHP* (0.73±0.57 vs 1.39±0.63, Control vs OCA group, $p=0.09$), *CYP7A1* (0.98±0.15 vs 1.28±0.09, Control vs OCA group, $p<0.01$), and *APOE* (1.04±0.11 vs 1.40±0.09, Control vs OCA group, $p<0.01$) (Figure 6).

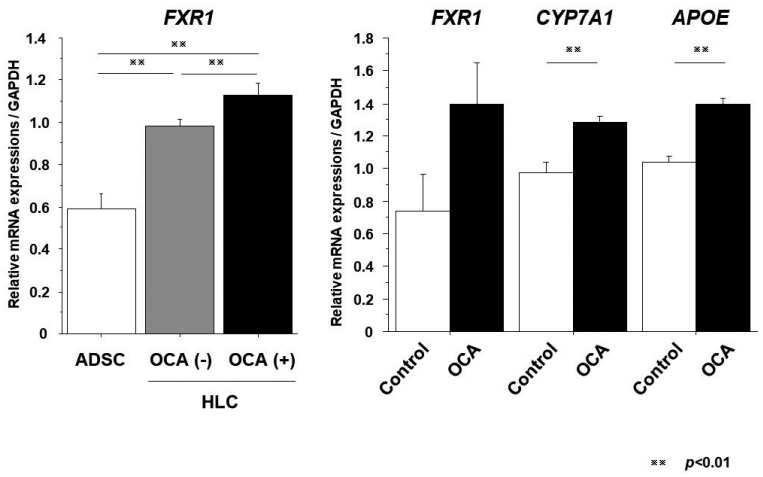

Figure 6. FXR1 and its target genes
HLCs with OCA showed highest mRNA expression of *FXR1* (0.59±0.07 vs. 0.98±0.03 vs. 1.13±0.06, ADSC vs. Control vs. OCA group, $p<0.01$). HLCs with OCA showed higher mRNA expression of its target genes such as *SHP* (0.73±0.57 vs. 1.39±0.63, Control vs. OCA group, $p=0.09$), *CYP7A1* (0.98±0.15 vs. 1.28±0.09, Control vs. OCA group, $p<0.01$), and *APOE* (1.04±0.11 vs. 1.40±0.09, Control vs. OCA group, $p<0.01$).

DISCUSSION

In this study, we demonstrated that the FXR agonist OCA stimulates hepatic maturation of HLCs and enhances their ammonium metabolism. This is the first report describing the use of an FXR agonist during differentiation of HLCs, especially relating to ammonium metabolism. This phenomenon could be critical for accelerating the use of HLC transplantation for patients with liver failure or UCD.

Although HLCs exhibit various hepatic functions, these exist at a lower level than those observed in primary human hepatocytes. Therefore, maturation of HLCs using the addition of compounds, gene manipulation, and three-dimensional/organoid/co-culture systems is an active area of research (1), as previously reported. Among these methods, the addition of compounds during differentiation is the simplest. Previously, use of compounds that stimulate cAMP signaling (16), adrenergic receptor agonists (17), valproic acid (18), insulin-like growth factor 1 (19), and functional proliferation hits (20) have improved maturation of HLCs. However, these compounds also enhance albumin synthesis and cytochrome activities in HLCs ; upregulation of these functions is not necessary in patients with UCD, because only the enzymes required to convert nitrogen from ammonia into urea are lacking (21, 22).

In the present study, we focused on hepatocyte ammonium metabolism, since regulating this function could have significant clinical impact. In patients with chronic liver disease or acute liver failure, high ammonia levels can lead to hepatic encephalopathy : this is a common and serious complication that contributes greatly to impaired quality of life, morbidity, and mortality (23). Many therapeutics aim to reduce the production and absorption of ammonia. These include lactulose, probiotics, rifaximin, and fecal matter transplantation (24-27). Another approach is to stimulate ammonia clearance. This has been attempted previously using L-ornithine, L-aspartate, and ornithine phenylacetate supplementation in residual hepatocytes and myocytes (28), and branched chain amino acids in muscle (29). Our results suggest that transplantation of HLCs could be a new ammonia clearance strategy.

FXR regulates amino acid catabolism and detoxification of ammonium via ureagenesis and glutamine synthesis in primary hepatocytes (14). We found that adding OCA during HLC differentiation significantly upregulated urea cycle genes. Furthermore, HLCs differentiated in the presence of OCA exhibited significantly higher clearance of NH_4Cl. In mice, FXR binds in proximity of the transcription start sites of *Cps1*, *Glul*, *Ass1*, and *Asl*, indicating that FXR directly regulates transcription of these urea cycle and glutamine synthesis genes (14). Since we observed upregulation of both *FXR1* and its target genes, this appears to be one mechanism by which OCA regulates ammonium metabolism.

In 2020, successful transplantation of ESC-derived HLCs into a patient with an inborn urea cycle disorder was reported by the National Center for Child Health and Development in Tokyo, Japan (unpublished at present). In this case, HLC transplantation was performed as a bridging therapy following liver transplantation, and might be the world's first report of ESC-derived HLCs transplantation. In the present study, we used ADSCs ; compared with ESCs, ADSCs can be collected with minimal invasiveness, thus enabling repeated auto-transplantation. This suggests that ADSC-derived HLCs differentiated with OCA could offer an accessible and effective treatment for urea cycle disorders in the future.

One limitation of this study is that *in vivo* experiments (i.e., HLC transplantation) were not performed, which would require the use of a urea disorder mouse model and development of mouse-specific HLC differentiation protocols. A second limitation is the lack of comparison of OCA-treated HLCs with an ammonia-lowering therapy (e.g., rifaximin, lactulose, and BCAA). Finally, our methods are not yet xeno/ antigen-free, or scalable to mass culture, both of which are necessary for clinical applications. However, development of these experimental systems are currently underway.

CONCLUSIONS

In conclusion, addition of the FXR agonist OCA during differentiation enhanced ammonium metabolism of ADSC-derived HLCs. Although further investigations are necessary, HLCs

could be an effective treatment option for hepatic encephalopathy of patients with liver failure or UCD in the future.

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All author(s) declare that they have no sources of funding for the research.

AUTHOR CONTRIBUTIONS

Conceptualization ; Y.S., T.I. and M.S., Methodology ; S.C. and Y.S., Formal analysis ; S.C., H.T., S.Y. and Y.S., Investigation ; S.C., Writing—original draft preparation ; Y.S., Writing—review and editing ; Y.S., and M.S., Supervision ; Y.S., T.I, Y.M. and M.S., Project administration ; M.S. All authors have read and agreed to the published version of the manuscript.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was approved by the Tokushima University Hospital ethics committee and the corresponding regulatory agencies. All experiments were carried out in accordance with approved guidelines (Tokushima Clinical Trial Management System Number 3090). Informed consent was obtained from all participants.

INFORMED CONSENT STATEMENT

There are no human subjects in this article and informed consent is not applicable.

DATA AVAILABILITY STATEMENT

The data sets used and/or analyzed during this study are available from the corresponding author on reasonable request.

CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FOOTNOTE

Statement of human and animal rights this article does not contain any studies with human or animal subjects.

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