

ORIGINAL

Role of Nrf2 signaling in development of hepatocyte-like cells

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Abstract : Generation of hepatocytes from human adipose-derived mesenchymal stem cells (hADSCs) could be a promising alternative source of human hepatocytes. However, mechanisms to differentiate hepatocytes from hADSCs are not fully elucidated. We have previously demonstrated that our three-step differentiation protocol with glycogen synthase kinase (GSK) 3 inhibitor was effective to improve hepatocyte functions. In this study, we investigated the activation of the nuclear factor erythroid-2 related factor 2 (Nrf2) on hADSCs undergoing differentiation to HLC (hepatocyte-like cells). Our three-step differentiation protocol was applied for 21 days (Step 1 : day 1-6, Step2 : day 6-11, Step3 : day 11-21). Our results show that significant nuclear translocation of Nrf2 occurred from day 11 until the end of HLC differentiation. Nuclear translocation of Nrf2 and CYP3A4 activity in the GSK3 inhibitor-treated group was obviously higher than that in Activin A-treated groups at day 11. The maturation of HLCs was delayed in Nrf2-siRNA group compared to control group. Furthermore, CYP3A4 activity in Nrf2-siRNA group was decreased at the almost same level in Activin A-treated group. Nrf2 translocation might enhance the function of HLC and be a target for developing highly functional HLC. *J. Med. Invest.* 70: 343-349, August, 2023

Keywords : HLC, ADSC, differentiation

INTRODUCTION

Globally, more than 1 million people die of liver disease every year (1, 2). For acute or chronic liver failure associated with end-stage liver dysfunction, liver transplantation is regarded as the only available therapy. However, considering the lack of healthy donors, hepatocyte transplantation shows potential as an alternative treatment – except for liver transplantation (3-5). The demand for liver transplantation has significantly outstripped the supply of donor organs. Consequently, multiple attempts to expand the availability of donor organs have been employed, including opt-out organ donation programs, use of suboptimal donor organs [e.g. from deceased cardiac donors or steatotic (fatty) livers], split-donor transplantation, and living-donor liver transplantation (6, 7). Fortunately, unlike most other organs, the introduction of exogenous hepatocytes into the liver parenchyma is a relatively simple undertaking, suggesting that the liver is highly amenable to tissue therapy using induced pluripotent stem cell–derived hepatocytes (8, 9). Several studies showed that human mesenchymal stem cells (MSCs) derived from adipose tissue, bone marrow, or umbilical cord blood are capable of differentiating into hepatocyte-like cells (HLCs) in specialized *in vitro* culture conditions (10-14). Recent studies also demonstrated that hepatocytes derived from human adipose stem cells (hADSCs) are a potentially scalable and applicable alternative to human hepatocytes (13, 15-18). However, a precise mechanism to generate mature HLCs from hADSCs remains unclear.

The nuclear factor erythroid-2 related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) pathway protects cells against oxidative stress. In response to oxidative stress, Nrf2 dissociates from Keap1 and enters the nucleus, whereby it

regulates transcription of hemeoxygenase-1 and other target genes encoding proteins for detoxification, energy metabolism, or other cytoprotective functions (19-23). For example, activated Nrf2 signaling reportedly plays a protective role in liver injury associated with nonalcoholic steatohepatitis, and overexpression of hepatic Nrf2 can functionally restore liver injury in mouse models (24). Furthermore, the Nrf2/farnesoid X receptor/liver X receptor pathway was found to regulate lipogenesis and CYP3A4 expression in murine hepatocytes (25). Here, we investigated the involvement of Nrf2 in differentiation of HLCs from hADSCs.

MATERIALS AND METHODS

Culture and differentiation of hADSCs

For this study, we applied our established three-step differentiation protocol, as previously reported (26). Briefly, hADSCs (StemPro Human Adipose-Derived Stem Cells, R7788-115) purchased from Invitrogen (Waltham, MA, USA) and cultured in MesenPRO RS medium (Thermo Fisher Scientific, USA). hADSCs were passaged three times before use in our three-step differentiation protocol.

In Step 1, definitive endoderm differentiation was induced by incubating cells with Dulbecco's Modified Eagle's Medium with F-12 Supplement containing 0.5 mg/mL bovine serum albumin fraction V (BSA ; Sigma-Aldrich, Tokyo, Japan) and 2 μ M CHIR99021 [glycogen synthase kinase 3 (GSK3) inhibitor ; Selleck Chemicals, Houston, TX, USA) for 24 h from day 3. The following day, 1% insulin-transferrin-selenium (ITS, Sigma-Aldrich) was added to the medium and cells were cultured for 2 days. In Step 2, subsequent hepatoblast differentiation was promoted by changing the medium to Minimum Essential Medium with non-essential amino acids (Thermo Scientific) supplemented with 0.5 mg/mL BSA, 1% ITS, 20 ng/mL bone morphogenic protein 2 (PeproTech, Rocky Hill, NJ, USA), and 30 ng/mL fibroblast growth factor 4 (FGF4, PeproTech) for 5 days. In Step 3, hepatocyte differentiation is stimulated by treating cells with 20 ng/mL hepatocyte growth factor (HGF, PeproTech)

Received for publication August 1, 2022 ; accepted March 26, 2023.

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for 5 days, followed by 20 ng/mL HGF, 10 ng/mL oncostatin M (PeproTech), and 1×10^{-6} M dexamethasone (Sigma-Aldrich) for another 5 days.

In the Nrf2-knockdown group, hADSCs were transfected with an Nrf2 siRNA (10 nmol/L, S9492; Applied Biosystems, Waltham, MA, USA) using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions.

Immunofluorescence

We cultured the cells in chamber slides (Matsunami, Lot No. 191029) for immunofluorescence. Next, we removed the covers of chamber slides, washed the cells in the bottom with phosphate-buffered saline (PBS, 4°C), and fixed slides with 4% paraformaldehyde. After washing with PBS, slides were treated with Triton X-100 (Lot No. 308T1683; Kanto Chemical Co., Tokyo, Japan), washed again, and incubated with an anti-Nrf2 primary antibody (ab62352; Abcam, Cambridge, UK) overnight at 4°C. The next day, slides were washed and incubated with a fluorophore-conjugated secondary antibody (A21428; Life Technologies, Waltham, MA, USA), and sealed with ProLong Gold Antifade Reagent with DAPI (P36931; Thermo Fisher Scientific, MA, USA). Finally, slides were observed and imaged with a fluorescence microscope (Keyence Corporation, Itasca, IL, USA). Nrf2 translocation was defined when the color showed yellow (blue; nucleus, red; Nrf2, yellow: merged red and green) (27-29). The translocation ratio of Nrf2 was obtained from the number of Nrf2 nuclear stained cells (yellow) divided by the number of DAPI stained cells (DAPI) in five independent $\times 40$ magnification.

RNA isolation and real-time PCR (RT-PCR)

Total RNA was isolated with a RNeasy Mini Kit (Qiagen, Hilden, Germany) and subsequently synthesized into complementary DNA with a reverse transcription kit (Applied Biosystems). RT-PCR was performed in triplicate using a StepOnePlus Real-Time PCR System (Applied Biosystems). Human *GAPDH* (4352339E, Applied Biosystems) was used as an internal control.

CYP3A4 activity assay

CYP3A4 enzyme activity was assessed by a P450-Glo assay (Promega, Madison, Wisconsin) in accordance with the manufacturer's instructions. Luminescence was measured with a luminometer (2104-0010; Envision, Waltham, Massachusetts).

Urea Assay

Culture supernatants of HLCs from each step were isolated and measured using a urea nitrogen kit (BioAssay Systems, San Francisco, CA, USA) to quantify urea production. Optical density values of sample plate wells were detected with a plate reader.

Statistical analysis

Data analysis was performed with GraphPad Prism 5 software, which was used to conduct one-way ANOVA and Mann-Whitney U tests. All data are presented as mean \pm standard deviation. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Differentiation of HLCs from hADSCs

First, we differentiated hADSCs to HLCs according to our established three-step protocol (26) (Fig. 1A). The morphology of HLCs changed during each step; eventually, HLCs adopted a round shape compared with the long, spindle-like shape of hADSCs (Fig. 1B). We next evaluated expression of relevant genes to confirm the HLC differentiation. Our results confirmed significant decreases of stemness markers such as SOX (SRY-Box Transcription Factor) 2 ($p < 0.001$, Fig. 2A) and OCT4 (octamer-binding transcription factor 4) ($p < 0.01$, Fig. 2B), as well as significant increases of the expression of endoderm-specific genes such as SOX17 ($p < 0.001$, Fig. 2C) and CXCR4 (C-X-C Chemokine Receptor Type 4) ($p < 0.001$, Fig. 2D) at day 6 (end of Step 1). Next, the elevation of hepatocyte-related gene AFP, a hepatoblast marker, was confirmed during Step 2 (Fig. 2C). Finally, expression of the hepatocyte gene ALB, a marker of hepatocyte maturation, was confirmed during Step 3 (Fig. 2D).

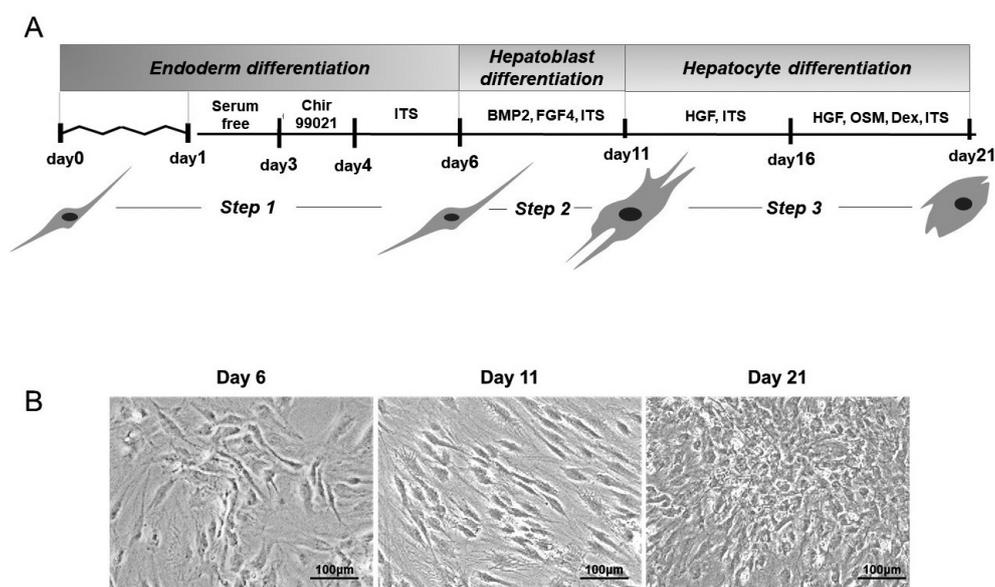


Figure 1. Differentiation of HLCs from hADSCs. (A) Three-step protocol for differentiation of HLCs. (B) Morphology of HLCs at day 6, day 11, and day 21.

Gene expression and hepatocyte functions

Expression of genes indicative of the ammonium clearance function of hepatocytes, namely CPS1 (Carbamoyl phosphate synthase 1) ($p < 0.001$, Fig. 3A), SLC25A1 (Fig. 3B), and AAT (alpha-1 antitrypsin) ($p < 0.05$, Fig. 3C), were notably increased in HLCs compared with hADSCs, although there was still a

great distance to reach levels observed in primary hepatocytes. Similar results were observed for CYP3A4 activity, as indicated by significantly increased expression of Luciferin-IPA in HLCs at day 21 ($p < 0.001$, Fig. 3D). Urea synthesis was also higher in HLCs at Day 21 compared with hADSCs ($p < 0.001$, Fig. 3E) but lower than primary hepatocytes.

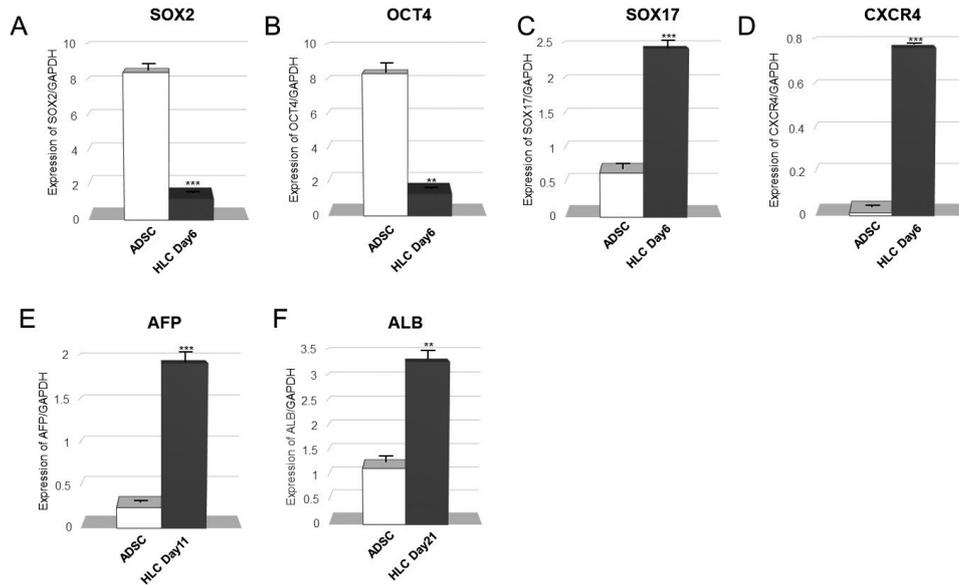


Figure 2. Hepatic-specific gene expression during three-step differentiation of hADSCs into HLCs. Step 1: mRNA expression of SOX2 (A), OCT4 (B), SOX17 (C), and CXCR4 (D) in HLCs at day 6. Step 2: mRNA expression of AFP, a hepatoblast marker, in HLCs at day 11 (E). Step 3: mRNA expression of ALB, a hepatocyte maturation marker, in HLCs day 21 (F). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

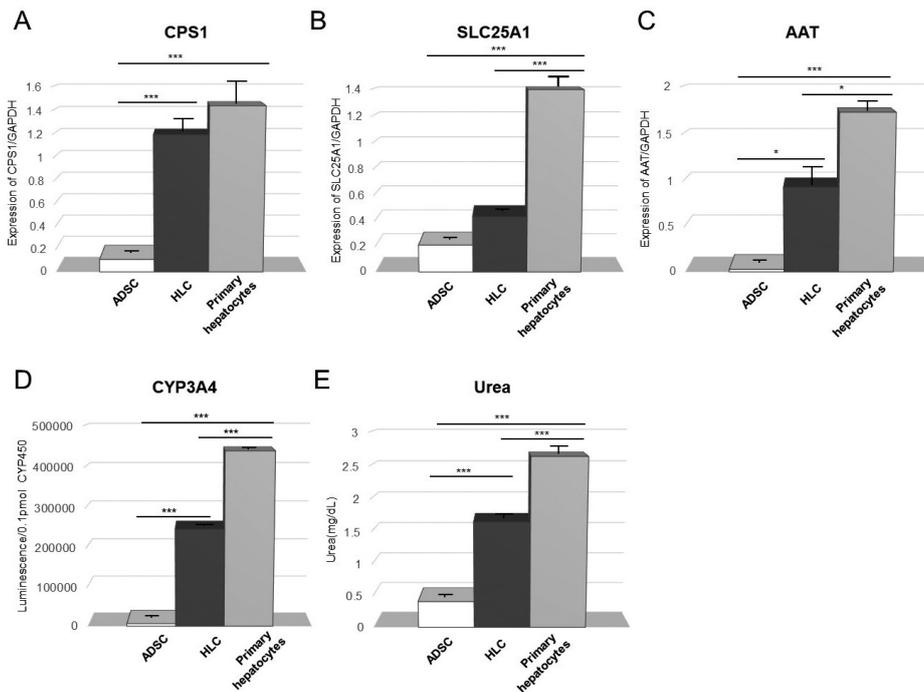


Figure 3. Expression of hepatic-specific genes in HLCs. mRNA expression of (A) CPS1, (B) SLC25A1, and (C) AAT in HLCs at day 21. (D) CYP3A4 activity at day 21. (E) Urea Assay of HLCs at day 21. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Nuclear translocation of Nrf2 increased during differentiation of HLCs

We assessed the distribution of Nrf2 (cytoplasmic or nuclear) during the 21 days of differentiation by immunofluorescence (Fig. 4A). Nuclear translocation of Nrf2 was not observed at day 6 (end of Step 1) during definitive endoderm differentiation. However, nuclear translocation of Nrf2 was notably increased at during hepatoblast differentiation compared to Step 1 (Fig. 4B, $P < 0.001$). The rate of nuclear translocation of Nrf2 was still highly observed at day21 (Step 3) but there was no difference between Step 2 and Step 3.

Nuclear translocation of Nrf2 in the presence of Activin A or GSK3 inhibitor

Our protocol used CHIR99021 (GSK3 inhibitor) to induce definitive endoderm differentiation during Step 1. We also investigated the effect of Activin A, a multifunctional cytokine, that plays important roles in the microenvironment of hepatocyte phenotypes and regional endoderm specification (1). Activin A was generally used for definitive endoderm differentiation during Step 1. Nuclear translocation of Nrf2 was investigated among the three groups: untreated hADSCs, GSK3 inhibitor-treated hADSCs, Activin A-treated hADSCs. No differences in nuclear translocation of Nrf2 were observed between the three groups at day 6 (end of Step 1; Fig. 5A). Although nuclear translocation of Nrf2 was observed in Activin A-treated cells, it was significantly higher in the GSK3 inhibitor-treated group compared to untreated hADSCs ($p < 0.001$) and Activin A-treated hADSCs ($p < 0.001$) at day 11 (end of Step 2; Fig. 5B and 5D). Nuclear translocation of Nrf2 in the GSK3 inhibitor-treated group was still significantly higher than the other three groups (Fig. 5C and 5E, $p < 0.001$).

Effect of knockdown of Nrf2 in differentiation of HLC

We next knocked down Nrf2 expression during Step 1 with siRNA. The results of morphological analyses showed that cells in the siRNA-treated group at day 21 were not completely differentiated into HLCs (Fig. 6A). Nrf2-siRNA treated hADSCs at day 21 did not have a spindle shape and had spindle form. Functional comparison of HLCs with a CYP3A4 activity assay (Luciferin-IPA) revealed that the GSK3 inhibitor-treated group had significantly higher activity than the other three groups (p

< 0.05) (Fig. 6B). However, this result was cancelled by the addition of siRNA Nrf2. CYP3A4 activity in Nrf2-siRNA treated hADSCs was almost same in that in Activin A-treated hADSCs.

DISCUSSION

In the present study, we established human HLCs derived from hADSCs according to a three-step protocol (15). Nrf2 was activated during the procedure, and the maturation of HLCs was delayed in Nrf2-siRNA group. Thus, Nrf2 might be a target for developing highly functional human HLCs.

Generation of HLCs derived from hADSCs could be a promising alternative source of human hepatocytes. Of the donor sources of hepatocytes proposed, including induced pluripotent stem cells, embryonic stem cells, and MSCs, MSCs may be best cell source because they can overcome problems associated with genetic damage, rejection, and ethics. In particular, ADSCs – a type of MSC derived from fat tissues – can be collected from patients with minimal invasiveness. We previously reported the effectiveness of a three-dimensional culture system for HLCs generated from hADSCs with a recombinant peptide μ -piece (26). However, mechanisms underlying the differentiation of hepatocytes from hADSCs are not well elucidated.

We previously reported that Nrf2, a transcription factor that regulates cellular defenses against oxidative stress (30), protects against liver ischemia/reperfusion injury (31) and calcineurin inhibitor-induced nephrotoxicity (32) by inhibiting oxidative stress, inflammation, and apoptosis. In addition, Nrf2 maintains optimal levels of intracellular reactive oxygen species to regulate adipocyte differentiation (33). Moreover, Nrf2 overexpression in MSCs induce stem-cell marker expression and enhances osteoblastic differentiation by preventing apoptosis under oxidative stress (34). Considering this perspective, we anticipated a role for Nrf2 in regulating hADSC differentiation.

Wnt signaling is key for the differentiation of MSCs (35). Wnt/ β -catenin signaling regulates foregut endoderm fate, proliferation, and morphogenesis (36). Previous studies revealed that Wnt signaling is required to specify definitive endoderm from hADSCs, and manipulations of Wnt signaling through use of GSK3 inhibitors have been applied to direct differentiation of definitive endoderm and hepatocytes (37). GSK3 inhibitors upregulate transcription factors involved in specification of

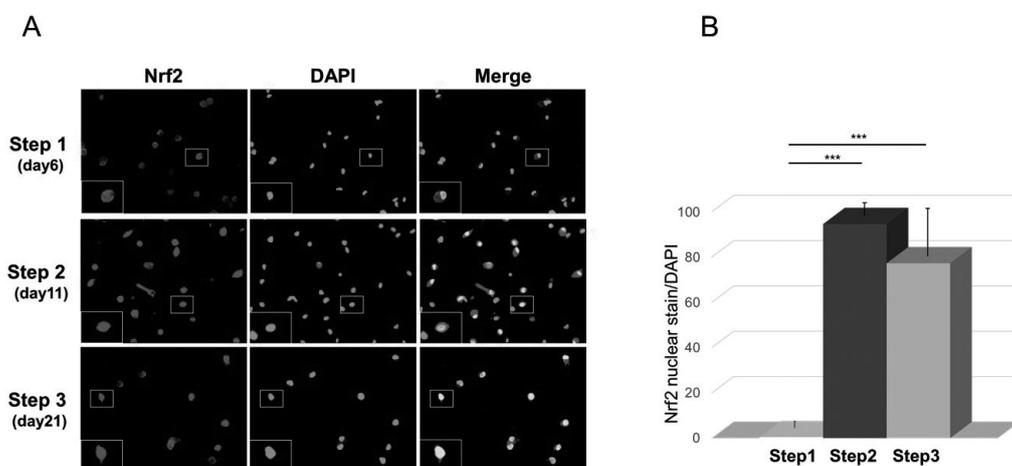


Figure 4. Nuclear translocation of Nrf2 in each step. (A) Immunofluorescence staining of Nrf2 expression in HLCs at each step. (B) Quantification and graphical presentation of the Nrf2 translocation rates. *** $P < 0.001$.

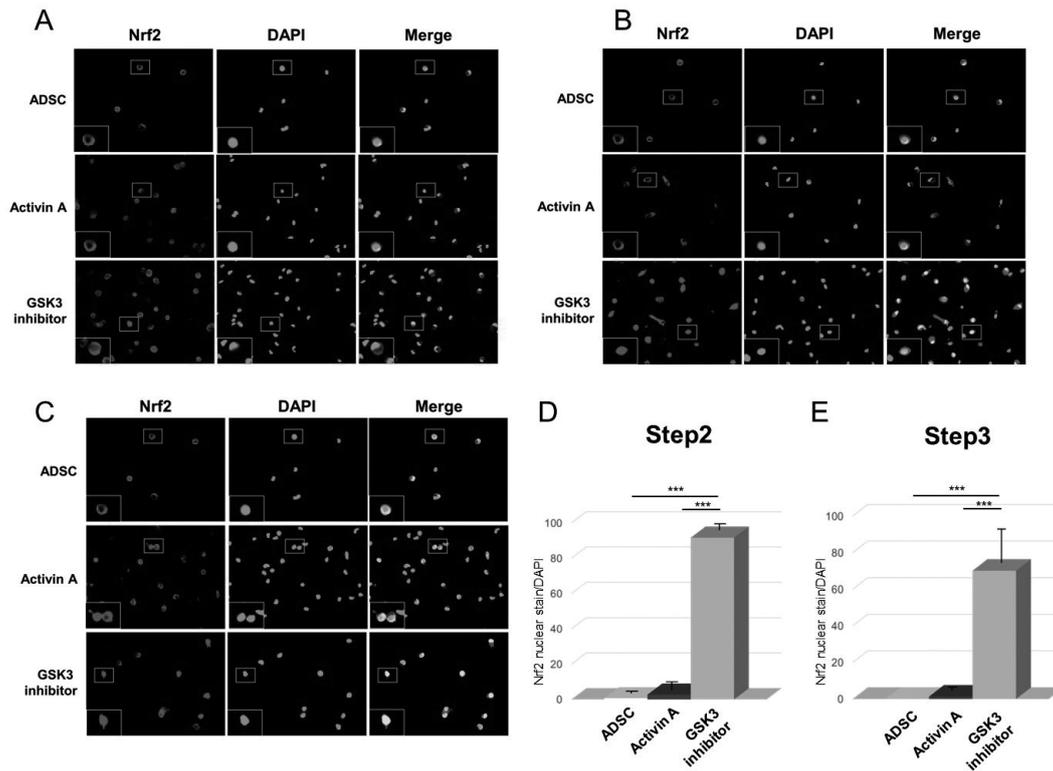


Figure 5. Nuclear translocation of Nrf2 in each group. Immunofluorescence staining of Nrf2 expression following treatment with GSK3 inhibitor, Activin A, or GSK3 inhibitor with Nrf2 siRNA at day 6 (A), day 11 (B), and day 21 (C). Quantification and graphical presentation of the Nrf2 translocation rates at day 11 (D) and day 21 (E). *** P < 0.001.

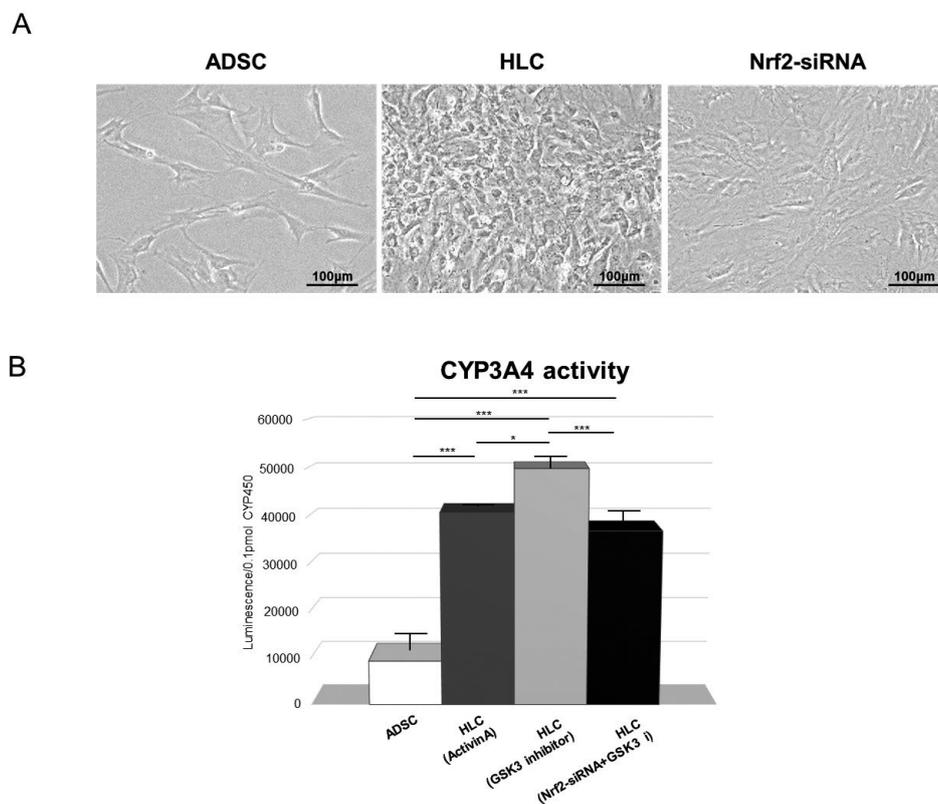


Figure 6. Morphology of HLCs in siRNA-Nrf2. (A) Morphology of HLCs at day 21 (B) CYP3A4 activity was highest in the GSK3 inhibitor-treated group. However, this effect was cancelled by Nrf2 siRNA. * P < 0.05, ** P value < 0.01, *** P < 0.001.

definitive endoderm, namely GATA4 (GATA Binding Protein 4), FOXA2 (Forkhead Box A2), SOX17, and CXCR4 (15). In this study, we used the GSK3 inhibitor (CHIR99021) in Step 1 and successfully acquired homogenous hepatic progenitor cells. Notably, GSK3 β is a negative regulator of Nrf2 (38). Exposure of cells to inducers of antioxidant response elements (AREs), such as oxidative stress, leads to the dissociation of Nrf2 from its cytosolic inhibitor Keap1 in a process mediated by protein kinase C (38). After nuclear translocation, Nrf2 promotes the AREs of many cytoprotective genes in combination with other transcription factors, such as small musculoaponeurotic fibrosarcoma, activation transcription factor 4, and polyamine-modulated factor 1. Nuclear export of Nrf2 is regulated by the tyrosine kinase Fyn. Moreover, GSK3 β acts upstream of Fyn kinase to control nuclear export and subsequent proteasomal degradation of Nrf2. Because a previous report showed an equal ability of CHIR99021 and Activin A to induce hepatic progenitor cells (15), the role of Nrf2 seems to be not vital for endoderm differentiation. In line with this notion, application of the GSK3 inhibitor did not significantly enhance Nrf2 nuclear translocation during Step 1 in the present study compared with Activin A. However, knockdown of Nrf2 during Step 1 resulted in a longer time for HLCs to mature in the present study, implicating the importance of the Nrf2 pathway in promoting HLC differentiation. Moreover, rates of Nrf2 nuclear translocation were higher in the presence of the GSK3 inhibitor compared with Activin A in Step 2. Thus, Nrf2 may play an important role in hepatoblast differentiation not in definitive endoderm differentiation.

Another possible mechanism is FGFR4-GSK3 β signaling, which promotes translocation of Nrf2 to the nucleus. FGFR4 receptors on the surface of hepatocellular carcinoma cells were activated by FGF19 through inactivation of GSK3 β in the tumor microenvironment (39). Although the role of the FGFR4-GSK3 β -Nrf2 pathway under basal conditions is unknown, the homodimer of FGFR4 binds with FGF subfamily members including FGF4 or FGF19. We used FGF4 for hepatoblast differentiation in Step 2. FGF4 is the key cytokine required for differentiation of MSCs into a hepatic lineage (40). Nuclear translocation of Nrf2 mainly occurred during Step 2. Taken together, the FGFR4-GSK3 β -Nrf2 pathway may play a key role in hepatoblast differentiation, although further investigation is needed.

As a limitation of the present study, we did not investigate other molecules in the Wnt signaling pathway or GFR4-GSK3 β -Nrf2 pathway. Second, we did not investigate the effect of a specific Nrf2 inducer, such as dimethyl fumarate. Furthermore, only single siRNA was used in this study. Third, we did not perform transplantation of HLCs in this study to verify their actual function *in vivo*.

In conclusion, we demonstrated that Nrf2 was activated during differentiation of HLCs, especially during the hepatoblast differentiation step, whereas knockdown of Nrf2 might delay the maturation and impaired specific functions of HLCs. Our findings suggest that Nrf2 is a target for developing highly functional human HLCs.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

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