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. In this study, we investigated the role of nuclear factor erythroid-2 related factor 2 (Nrf2) in differentiation of hepatocyte-like cells (HLCs). We used our established three-step differentiation protocol to develop HLCs from hADSCs. Significant nuclear translocation of Nrf2 occurred from day 11 (Step 2) until the end of HLC differentiation. There were no significant differences in Nrf2 translocation rates among the four experimental groups (activin-A, GSK3 inhibitor, Nrf2 siRNA, and control) at day 6 (end of Step 1). Nuclear translocation of Nrf2 in the GSK3 inhibitor-treated group was obviously higher than the other groups at day 11 (Step 2). Moreover, nuclear translocation of Nrf2 in the GSK3 inhibitor-treated group was notably higher than the other groups during Step 3. CYP3A4 activity (Luciferin-IPA assay) of the GSK3 inhibitor-treated group was significantly higher than the other three groups. Nrf2 was activated during differentiation of HLCs, and inhibition of Nrf2 delayed maturation and impaired the function of HLCs. Thus, Nrf2 might be a notable target for developing highly functional human HLCs.

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 CYP3A expression in murine hepatocytes ²⁵. Here, we investigated the involvement of Nrf2 in differentiation of HLCs from hADSCs.

Methods

Isolation and culture 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. The following day, 1% insulin-transferrin-selenium (ITS, Sigma-Aldrich) was added to the medium. 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) 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 Triton100 (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 (Lot No. 1726587; Life Technologies, Waltham, MA, USA), and sealed with ProLong Gold Antifade Reagent with DAPI (Invitrogen). Finally, slides were observed and imaged with a fluorescence microscope (Keyence Corporation, Itasca, IL, USA). The integrated density of immunofluorescence was quantified with ImageJ

(<http://imagej.nih.gov>) and analyzed with GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA).

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

Total RNA was isolated with an 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.

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.

Ammonia Assay

Cells were seeded into 96-well microtiter plates and incubated with 1.5 mM ammonium for 0, 12, or 24 h. Next, 100 μ L of diluted ammonium chloride standards or supernatant was added to plates. After incubating with work liquid from Ammonia Assay Kit (Cell Biolabs, San Diego, CA, USA) for 30 min at room temperature. The concentration of ammonium was detected by reading the absorbance values of each well at 630–670 nm 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 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 *SOX2* ($p < 0.001$) and *OCT4* ($p < 0.01$), as well as significant increases of endoderm-specific genes such as *SOX17* ($p < 0.001$) and *CXCR4* ($p < 0.001$) at day 6 (end of Step 1) (Fig. 2A). Next, the elevation of hepatocyte-related gene *AFP*, a hepatoblast marker, was confirmed during Step 2 (Fig. 2B). Finally, expression of the hepatocyte gene *ALB*, a marker of hepatocyte maturation, was confirmed during Step 3 (Fig. 2C).

Hepatic function was increased in HLCs

Expression of genes indicative of the ammonium clearance function of hepatocytes, namely *CPS1* ($p < 0.001$), *SLC25A1*, and *AAT1* ($p < 0.05$), were notably increased in HLCs compared with hADSCs, although there was still a great distance to reach levels observed in primary hepatocytes (Fig. 3A–C). 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 assay results presented the same trend for HLCs compared with hADSCs and primary hepatocytes ($p < 0.01$) (Fig. 3E).

Nuclear translocation of Nrf2 increased during differentiation of HLCs

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 day 11 (Step 2) during hepatoblast differentiation (Fig. 4).

Knockdown of Nrf2 delayed differentiation into HLCs

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. 5). We also investigated the effect of inhibiting GSK3, which is involved in regulating downstream β -catenin and Nrf2 signaling, by adding CHIR99021 during Step 1²⁷. Activin A, a multifunctional cytokine that plays important roles in the microenvironment of hepatocyte phenotypes and regional endoderm specification¹, was also added during Step 1 as a second control group. Nuclear translocation of Nrf2 was investigated among the four groups: untreated hADSCs, GSK3 inhibitor-treated hADSCs, activin A-treated hADSCs, and Nrf2 siRNA-treated hADSCs. No differences in nuclear translocation of Nrf2 were observed between the four groups at day 6 (end of Step 1; Fig. 6A). Nuclear translocation of Nrf2 in the GSK3 inhibitor-treated group was obviously higher than untreated and siRNA-treated groups. Although nuclear translocation of Nrf2 was higher in activin A-treated cells, it was much higher in the GSK3 inhibitor-treated group (Fig. 6B). After Step 3, nuclear translocation of Nrf2 in the GSK3 inhibitor-treated group was notably higher than the other three groups (Fig. 6C).

Knockdown of Nrf2 impaired hepatic function of HLCs

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. 6D). However, this result was cancelled by the addition of siRNA Nrf2.

Discussion

In the present study, we established human HLCs derived from hADSCs according to a three-step protocol¹⁵. Nrf2 was activated during the procedure, and knockdown of Nrf2 delayed the maturation and reduced specific functions of HLCs. Thus, Nrf2 might be a notable target for developing highly functional human HLCs.

Generation of hepatocytes 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 an recombinant peptide μ -piece²⁶. However, mechanisms underlying the differentiation of hepatocytes from hADSCs are not well elucidated. Differentiation of definitive endoderm is an essential and critical early step to generate hepatocytes from hADSCs. Thus, efficient and reproducible production of definitive endoderm in differentiation cultures plays a key role in developing high quality and homogeneous hepatocytes.

We previously reported that Nrf2, a transcription factor that regulates cellular defenses against oxidative stress²⁸, protects against liver ischemia/reperfusion injury²⁹ and calcineurin inhibitor-induced nephrotoxicity³⁰ by inhibiting oxidative stress, inflammation, and apoptosis. In addition, Nrf2 maintains optimal levels of intracellular reactive oxygens species to regulate adipocyte differentiation³¹. Moreover, Nrf2 overexpression in MSCs induce stem-cell marker expression and enhances osteoblastic differentiation by preventing apoptosis under oxidative stress³². Considering this perspective, we anticipated a role for Nrf2 in regulating hADSC differentiation.

Wnt signaling is key for the differentiation of MSCs³³. Wnt/ β -catenin signaling regulates foregut endoderm fate, proliferation, and morphogenesis³⁴. 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 hepatocytes³⁵. GSK3 inhibitors upregulate transcription factors involved in specification of definitive endoderm, namely GATA4, FOXA2, SOX17, and CXCR4¹⁵. 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 Nrf2²⁷. 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 C²⁷. 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 cells¹⁵, the role of Nrf2 seems to be not vital for endoderm differentiation. In line with this notion, application of the GSK3 inhibitor CHIR99021 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.

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 microenvironment³⁶. 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 lineage³⁷. 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 FGFR4-GSK3 β -Nrf2 pathway. Second, we did not investigate the effect of a specific Nrf2 inducer, such as dimethyl fumarate. 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 delayed the maturation and impaired specific functions of HLCs. Our findings suggest that Nrf2 is a notable target for developing highly functional human HLCs.

Declarations

Data availability

The current datasets are deposited in publicly available repositories.

Acknowledgements

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Author Contributions

C.T., Y.M., T.I., and M.S. contributed to the conception and designation of these experiments. C.T., L.G., S.Y., K.M. and S.O. performed the experiments. C.T., L.G. and Y.M. wrote the manuscript. All authors discussed the results and approved the manuscript.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

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. hADSCs gradually changed their morphology and HLCs at day 21 appeared to have a round shape.

Figure 2

Figure 2

Hepatic-specific gene expression during three-step differentiation of hADSCs into HLCs. (A) Step 1: mRNA expression of SOX2, OCT4, SOX17, and CXCR4 in HLCs at day 6 compared with hADSCs. Because DE were generated after Step 1, SOX2 and OCT4 were lower, and SOX17 and CXCR4 were higher in HLCs compared with hADSCs. (B) Step 2: mRNA expression of AFP in HLCs at day 11 compared with hADSCs. Expression of AFP, a hepatoblast marker, was higher in HLCs after Step 2. (C) Step 3: ALB mRNA expression in HLCs was higher at day 21 compared with hADSCs. Expression of ALB, a hepatocyte maturation marker, was highest in HLCs at day 21 (after Step 3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3

Figure 3

Expression of hepatic-specific genes in HLCs. mRNA expression of (A) CPS1, (B) SLC25A1, and (C) AAT in HLCs at day 21 compared with hADSCs and primary hepatocytes during Step 3. (D) CYP3A4 activity was higher in HLCs at day 21 compared with hADSCs, but lower than primary hepatocytes. (E) Urea Assay of HLCs at day 21 compared with hADSCs and primary hepatocytes. Urea synthesis was higher in HLCs at day 21 compared with hADSCs, but lower than primary hepatocytes. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4

Figure 4

Nuclear translocation of Nrf2 in each step. Immunofluorescence staining of Nrf2 expression in HLCs at day 6 compared with day 11 and day 21. Significant translocation occurred from day 11 until the end of HLC differentiation.

Figure 5

Figure 5

Morphology of HLCs in siRNA-Nrf2. Morphology of HLCs at day 21 compared with hADSCs, HLCs, and Nrf2 siRNA-treated HLCs. Nrf2 siRNA-treated HLCs did not appear to have a round shape.

Figure 6

Figure 6

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). (D) 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.