Original Paper

Retaining mTeSR1 Medium during Hepatic Differentiation Facilitates Hepatocyte-Like Cell Survival by Decreasing Apoptosis

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Abstract

Background/Aims: Hepatocyte-like cells derived from human pluripotent stem cells could be an important cell source for hepatocyte transplantation. The present study investigated the effect of retaining mTeSR1 medium during hepatic differentiation on hepatocyte-like cells *in vitro*. ***Methods:*** Human embryonic stem cell line H1 were treated with activin A and bone morphogenetic protein 4 (BMP4) for definitive endoderm (DE) cell induction and subsequently treated with BMP2 and fibroblast growth factor 4 (FGF4) for early hepatic cell induction. Hepatocyte growth factor (HGF) and fibroblast growth factor (KGF) were added for early hepatic cell expansion and then mixed with oncostatin-M for maturation. During DE induction, 0%, 25%, 50% and 75% concentrations of mTeSR1 medium were separately added for early hepatic induction and expansion. For optimization, the expression levels of SRY-related HMG-box 17 (SOX17) and forkhead box A2 (FOXA2) at day 4, alpha fetoprotein (AFP) and hepatocyte nuclear factor 4α (HNF4α) at day 15, and albumin (ALB) at day 25 were quantified in differentiated cells by qRT-PCR. The ALB-positive cell proportion was measured by flow cytometry. Functional tests including ALB secretion and indocyanine green (ICG) angiography uptake and release by ELISA, urea production by urea assay kit, and glycogen storage ability by periodic acid Schiff reaction (PAS) staining were performed in the differentiated cells. The induced pluripotent stem (iPS) cells were used to examine whether the optimized method was suitable for differentiating iPS cells. DE and hepatic markers were detected by immunostaining, and functional testing was performed as described above. Flow cytometry with an Annexin V-FITC apoptosis detection kit and fluorescence microscopy with Hoechst 33258 were used to analyze apoptosis in differentiated cells derived from

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H1 cells. **Results:** All differentiated cells with retention of 0%, 25%, 50% and 75% mTeSR1 expressed SOX17, FOXA2, AFP, HNF4α, and ALB, while higher expression levels were observed in differentiated cells in the 0% and 25% groups. The flow cytometry results showed that the proportion of ALB-positive differentiated cells derived from H1 cells was higher in the 25% mTeSR1 group than in other groups. However, no significant difference in ALB secretion, urea production, ICG uptake and release and glycogen storage ability was detected between the 25% and 0% groups. The iPS cells could differentiate into hepatocyte-like cells with 25% mTeSR1 retention. The apoptosis ratio of differentiated cells was lower in the 25% mTeSR1 group than in the 0% mTeSR1 group. **Conclusion:** Retaining 25% mTeSR1 medium during hepatic differentiation has been proposed to increase the percentage of ALB-positive cells and cell survival by decreasing cell apoptosis.

Introduction

Liver dysfunction is one of the most important global health problems, and liver transplantation is the only successful therapy for end-stage liver failure [1, 2]. However, the availability of human liver donors is limited. Hepatocyte transplantation, as an alternative to organ transplantation, restores damaged liver function and provides a potential way to treat a wide range of liver diseases [3]. Nevertheless, the application of primary human hepatocytes has been hindered by a lack of cellular growth, loss of function and dedifferentiation *in vitro* [4]. Thus, the generation of functional hepatocytes from other unlimited sources would be greatly beneficial.

Human embryonic stem (ES) cells (hESCs) are characterized by pluripotency and the capacity for unlimited self-renewal. In addition, growth factors or microRNAs have been reported to contribute to hepatocyte differentiation [5, 6]. Moreover, several studies found that hepatocyte-like cells could be derived from hESCs and that pluripotent stem cells could serve as a potentially inexhaustible source of cells for replacement therapies [7-9]. Thus, in the present study, we focused on the effect of different mTeSR1 culture conditions on the differentiation of human ES cells and induced pluripotent stem cells (iPS) cells to optimize robust and reliable methods of hepatic differentiation *in vitro*.

Materials and Methods

Cell culture and differentiation

The human ESC line H1 was obtained from WiCell Research Institute (Madison, WI). The passage number of the H1 cells and iPS cells used ranged from 44 to 60 [10, 11]. The H1 hESCs were plated on Matrigel (BD Biosciences, San Jose, CA)-coated plates and maintained in mTeSR1 medium (StemCell Tech., Vancouver, BC, Canada). For endoderm induction, the H1 cells were incubated for 2 days with RPMI 1640 (Invitrogen) plus 0%, 25%, 50% or 75% mTeSR1 medium (StemCell), supplemented with 100 ng/mL activin A and 20 ng/mL BMP4 (both from Peprotech, Rocky Hill, NJ) and then with 100 ng/mL activin A for an additional 2 days. Following treatment with activin A, the differentiated H1 cells were cultured in RPMI 1640 with 30 ng/mL FGF4 and 20 ng/mL BMP2 (both from Peprotech) for 5 days. Next, the differentiated cells were incubated in RPMI 1640 plus 0%, 25%, 50% or 75% mTeSR1 medium (StemCell) with 20 ng/mL HGF and 20 ng/mL KGF (both from Peprotech) for 12 days. Subsequently, the differentiated cells were treated with high-glucose DMEM (Thermo Fisher Scientific, Hampton, NH) with ascorbic acid (1:1000), bovine serum albumin–fatty acid free (1:500), transferrin (1:1000), insulin (1:1000), gentamicin/amphotericin-B (1:1000) (all from Lonza Sales Ltd, Basel, Switzerland), and 10 ng/mL oncostatin-M (OSM; R&D, Minneapolis, MN, USA) for another 10 days. The iPS cell line culture was the same as the method mentioned above.

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RNA isolation and qRT-PCR

Total RNA was isolated from the cultured cells using TRIzol Reagent (Thermo Fisher Scientific) and was reverse transcribed by a PrimeScript RT reagent kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed with GoTaq Green MasterMix (Promega, Madison, WI, USA). Primers were designed as previously described [12] and are listed in Table 1.

Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde for 15 minutes and then blocked and permeabilized with 1% BSA and 0.2% Triton X-100 in phosphate-buffered saline (PBS) at room temperature for 1 h. Next, the cells were incubated with primary antibody overnight at 4°C. The primary antibodies used were goat anti-SOX17 (R&D, AF1924), rabbit anti-FOXA2 (R&D, AF2400), mouse anti-AFP (Santa Cruz Biotechnology, CA, sc-130302), mouse anti-HNF4α (Genetex, San Antonio, TX, GTX62347), mouse anti-ALB (R&D, MAB1455), and mouse anti-CK19 (Genetex, GTX112666), and all were diluted at 1:200. After five washes with PBS, the cells were incubated with FITC-conjugated and TRITC-conjugated secondary antibodies diluted at 1:500 for 1 h at room temperature. Finally, the cells were incubated with 1 μg/mL 4, 6-diamidino-2-phenylindole (Sigma, St. Louis, MO, USA) to stain the cell nuclei. Additionally, the appropriate isotype antibody and normal serum from the species corresponding to the primary antibody were used as negative controls. Cells were analyzed by a confocal fluorescence microscope (Zeiss 710 NLO, Carl Zeiss Microscopy, LLC, Thornwood, NY, USA).

ELISA for ALB

The content of human albumin in the supernatant was determined by a Human Albumin ELISA Kit (Koma Biotech, Seoul, Korea) according to the manufacturer's instructions. Cells were trypsinized and counted with a hemocytometer.

Urea assay

Urea production was detected by a QuantiChrom Urea assay kit (Bioassay Systems, Brussels, Belgium). Cells were trypsinized and counted with a hemocytometer. The supernatant samples were stored at -20°C, and the assay was performed according to the manufacturer's instructions.

PAS staining

Cells in culture dishes were fixed in 70% ethyl alcohol, and staining was performed according to the manufacturer's instructions (Cat. 24200, Polysciences).

Cellular uptake and release of ICG

ICG (TCI, I0535) was dissolved in DMSO to make a stock solution at 50 mg/mL and then freshly diluted in culture medium to 1 mg/mL. After incubation of cells with ICG for 30 min at 37°C, the medium with ICG was discarded, cells were washed with PBS, and cellular uptake of ICG was examined by microscopy. The cells were then returned to the culture medium and incubated for 6 h to release the cellular ICG stain.

Analyses of apoptotic cells by flow cytometry

Annexin V-FITC apoptosis detection was reported previously [13]. An Annexin V-FITC Kit (Beyotime, China) was used to analyze the apoptotic cells. Briefly, cells were collected and incubated with Annexin V-FITC and PI in the provided binding buffer for 30 min in the dark at 4°C. They were then subjected to flow cytometry analysis at an emission wavelength of 488 nm.

Hoechst staining

Hoechst staining was performed using an Apoptosis-Hoechst Staining kit (Beyotime) according to the manufacturer's instructions. In brief, the cells were seeded in 24-well plates and fixed with 4% paraformaldehyde, stained with Hoechst 33528 and then visualized under a fluorescence microscope (Zeiss 710 NLO).

Table 1. Primers for qRT-PCR

Gene	Primers
SOX17	F: GAACGCTTTCATGGTGTGGGCTAAG
	R: CCACGACTTGCCCAGCATCTTGC
FOXA2	F: GGTGATGCACTACCCCGGCTACG
	R: CAGGGGCGAGGCGTCCAGG
AFP	F: GCGAAGCTGCGGCCTCTTCCAG
	R: GCTGTGGCTGCCATTTTTCTGGTGAT
HNF4α	F: AGAAGGCACCAACCTCAACGC
	R: GGGCAGGCTGCTGTCCTCATA
ALB	F: GGTGTTGATTGCCTTTGCTCAGTATC
	R: CCCTTCATCCCGAAGTTCATCGA
GAPDH	F: ATCCTGGGCTACACTGAGCACC
	R: AAGTGGTCGTTGAGGGCAATG

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Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). A Microsoft Excel 2016 database and GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, California) were used to record and analyze all the data. One-way analysis of variance (ANOVA) with a post hoc test or Student's *t*-test were used for data analysis. Differences with a p-value of <0.05 were considered statistically significant.
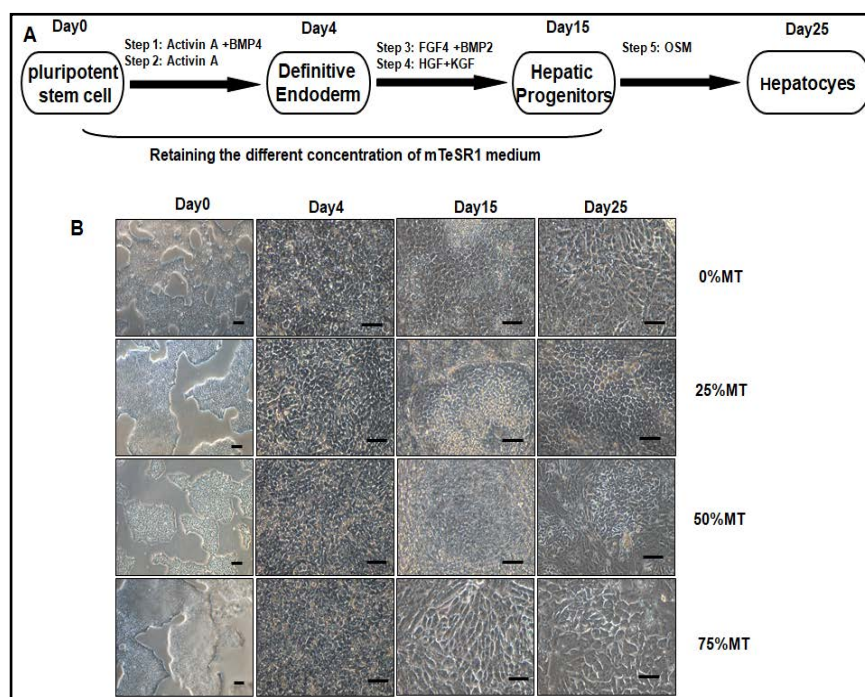
Results

Retaining a 25% concentration of mTeSR1 medium could increase the proportion of ALB-positive differentiated cells derived from H1 cells

Here, we developed a hepatic differentiation method for human pluripotent stem cells, as shown in Fig. 1A. The differentiation protocol was composed of four stages. In the first stage, activin A and BMP4 synergically induced endoderm differentiation. Then, FGF4 and BMP2 were used to induce hepatic specification from endoderm cells, and KGF and HGF were used to expand the early hepatic cells. During these three stages, different concentrations of mTeSR1 medium were retained. In the final stage, the mTeSR1 medium was removed, and OSM was used to facilitate early hepatic cell maturation. Fig. 1B shows the cell morphology changes with time during the differentiation of H1 cells treated with 0%, 25%, 50% or 75% mTeSR1. During differentiation, more dead cells were observed in the group without mTeSR1 than in the other groups (data not shown). At day 4, the cells in the four groups displayed endoderm cell morphology. At days 15 and 21, the group treated with 25% mTeSR1 displayed better cell morphology of early hepatic cells and mature hepatocytes, respectively, than the other groups (Fig. 1B).

Fig. 1. Generation of hepatocyte-like cells derived from H1 cells. Note: (A) The schema of the stepwise differentiation protocol. Cells were treated with 100 ng/mL activin A and 20 ng/mL BMP4 for 2 days and then with 100 ng/mL activin A for 2 days to induce the formation of definitive endoderm. Then, cells were treated with 20 ng/mL BMP2 and 30 ng/mL FGF4 for 5 days to induce early hepatic cells and with 20 ng/mL HGF and 20 ng/mL KGF for 7 days to expand them. During the differentiation protocol above, RPMI 1640 medium plus a 0%, 25%, 50% or 75% concentration of mTeSR1 medium was used. In the mature stage, high-glucose DMEM with 10 ng/mL oncostatin-M was used for 10 days. (B) The cells on days 0, 4, 15 and 25 in bright-field microscopy. Scale bar, 100 μm.
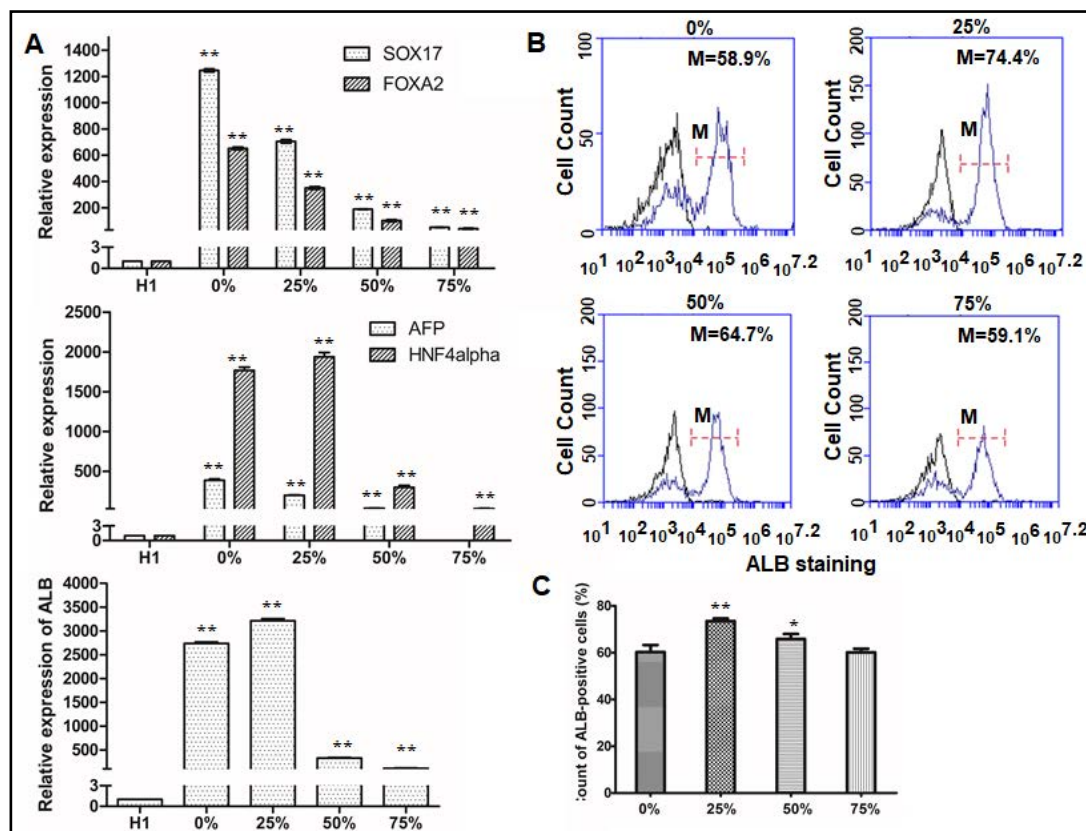
Fig. 2. Characterization of differentiated cells derived from H1 cells with the indicated markers. Note: (A) The expression of SOX17 and FOXA2 at day 4, AFP and HNF4α at day 15 and ALB at day 25 were detected in differentiated cells by qRT-PCR. The relative expression levels were normalized to an internal control. The data are expressed as the mean ± SD. **p=0.000 vs. H1 cell line. (B) Representative flow cytometry profile and (C) the average number of albumin-positive H1-derived hepatocytes in two independent differentiation experiments. *p<0.05, **p=0.000 vs. control group (0%).

To further confirm hepatic differentiation, real-time PCR was used to detect markers of endoderm cells, hepatic precursor cells, and mature hepatocytes (Fig. 2A). The differentiated cells in all the groups showed high expression levels of endoderm markers SOX17 (0%: 1246.036 ± 11.300, 25%: 705.692 ± 13.106, 50%: 189.032 ± 2.141, 75%: 50.907 ± 2.102, H1 cells normalized as 1) and FOXA2 (0%: 651.779 ± 10.388, 25%: 350.850 ± 10.049, 50%: 101.651 ± 8.112, 75%: 40.636 ± 4.150, H1 cells normalized as 1) at day 4, hepatic precursor markers AFP (0%: 387.644 ± 17.295, 25%: 198.390 ± 5.310, 50%: 35.623 ± 3.272, 75%: 0 ± 0, H1 cells normalized as 1) and HNF4α (0%: 1766.782 ± 41.985, 25%: 1937.499 ± 54.023, 50%: 298.390 ± 24.173, 75%: 31.779 ± 5.0380, H1 cells normalized as 1) at day 15, and mature hepatocyte marker ALB (0%: 2737.499 ± 31.250, 25%: 3212.072 ± 45.122, 50%: 328.221 ± 13.114, 75%: 114.615 ± 5.297, H1 cells normalized as 1) at day 21. These results indicated that hepatocyte-like cells could be obtained from all the groups treated with or without mTeSR1 medium, although the efficiency might be different. To detect the differentiation efficiencies of the four groups, the proportions of ALB-positive cells were analyzed using flow cytometry (Fig. 2B, 2C). Fig. 2B shows that the group with 25% mTeSR1 medium displayed a higher percentage of ALB-positive cells than the other groups (0%: 58.9%; 25%: 74.4%; 50%: 64.7%; 75%: 59.1%). Further analysis of replicates confirmed the results above (Fig. 2C) (0%: 60.24% ± 3.01%; 25%: 73.47% ± 1.15%; 50%: 65.82% ± 2.18%; 75%: 60.12% ± 1.56%). These results suggested that retaining 25% mTeSR1 medium facilitated the increased proportion of ALB-positive hepatocyte-like cells derived from H1 cells.

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Functional analysis of hepatocyte-like cells derived from H1 cells via the differentiation method including 25% mTeSR1 medium

We mainly compared the functions of the 0% and 25% groups and found that the 25% group displayed the highest percentage of ALB-positive cells, while the 0% group displayed the highest rate as a control. Next, the ALB secretion activity was detected. The ALB secretion of the 25% group approached 2.334 ± 0.127 mg/L, while that of the 0% group was 2.084 ± 0.097 mg/L (p=0.073, Fig. 3A). Then, the urea production level was also detected in the media with an ELISA, and urea production levels were 92.415±5.737 mg/L in

Fig. 3. Functional testing of the mature differentiated cells derived from H1 cells. Note: (A) ALB secretion of differentiated cells was detected by ELISA at day 25; (B) urea production by differentiated cells was detected by a urea assay kit on day 25; (C) ICG uptake and release; and (D) PAS staining of the differentiated cells was also detected at day 25.

the 25% group and 89.382 ± 3.925 mg/L in the 0% group (p=0.492, Fig. 3B). Moreover, glycogen storage was detected by PAS staining in the differentiated cells. Glycogen content was increased significantly in the differentiated cells compared with the undifferentiated cells, but no significant difference was observed between the 25% and 0% groups (Fig. 3C). The ICG uptake and release of differentiated cells were also detected (Fig. 3D). The differentiated cells of both groups exhibited green staining, almost all of the cells released ICG after 12 h in complete medium with the withdrawal of ICG, and no significant difference was observed between the 25% and 0% groups. These results showed that similar functional activity of the hepatocyte-like cells was observed in the 25% and 0% groups.

The differentiation method retaining 25% mTeSR1 medium could facilitate the generation of functional hepatocyte-like cells derived from iPS cells

To further confirm the novel hepatic differentiation method, we used an iPS cell line to generate hepatocyte-like cells with 25% mTeSR1 medium retained during differentiation. Fig. 4A shows the cell morphology changes during the differentiation of iPS cells. The differentiated cells displayed endoderm cell morphology at day 4, early hepatic cell morphology at day 15, and mature hepatocyte morphology at day 25. Further immunofluorescence results showed that the differentiated cells expressed the endoderm markers SOX17 and FOXA2 on day 4, the hepatic precursor markers AFP and HNF4α on day 15, and the mature hepatocyte marker ALB on day 25 (Fig. 4B). The ALB secretion of the differentiated cells approached 1.82 ± 0.091 mg/L (Fig. 4C), and the urea production levels were 77.608 ± 3.880 mg/L (Fig. 4D). Moreover, the differentiated cells also displayed glycogen storage ability (Fig. 4E) and ICG
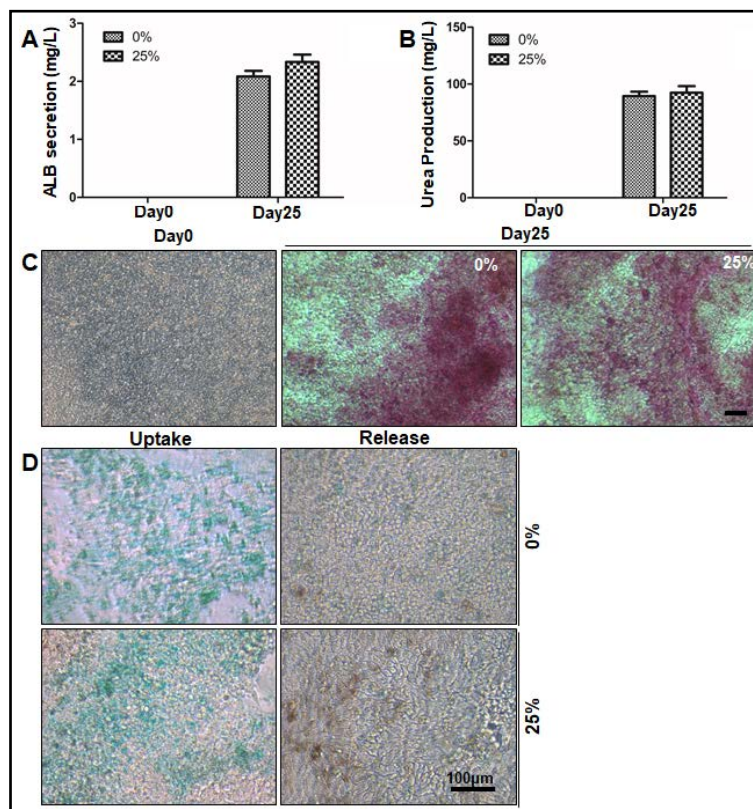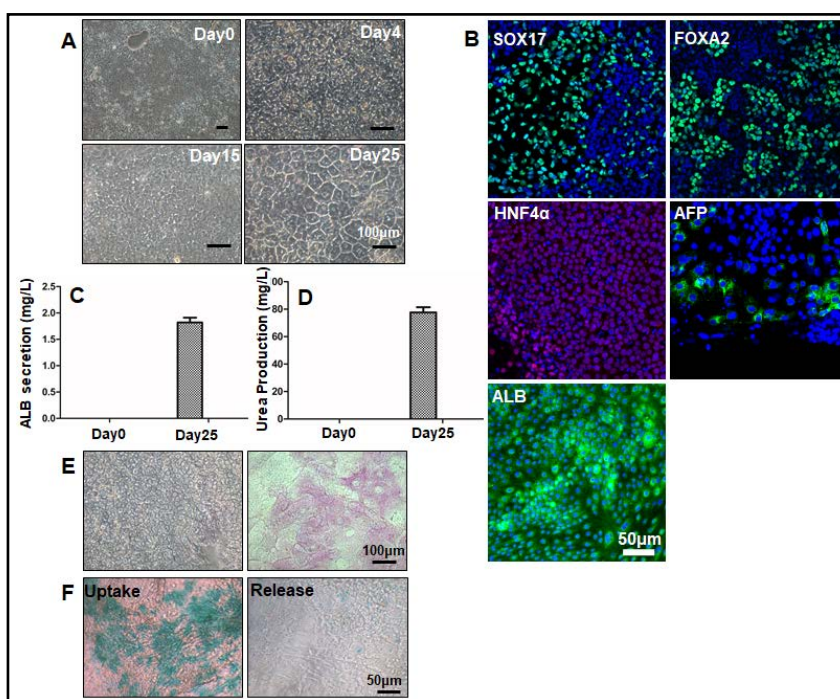
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Fig. 4. Characterization of differentiated cells derived from iPS cells with 25% concentration of mTeSR1 medium during hepatic differentiation. Note: (A) The cells on days 0, 4, 15 and 25 in bright-field microscopy; (B) the expression levels of SOX17 and FOXA2 at day 4, AFP and HNF4α at day 15 and ALB at day 25 were detected in differentiated cells by immunostaining; (C) ALB secretion by differentiated cells was detected by ELISA at day 25; (D) urea production by differentiated cells was detected by a urea assay kit on day 25; (E) PAS staining and (F) ICG uptake and release of the differentiated cells were also detected at day 25.
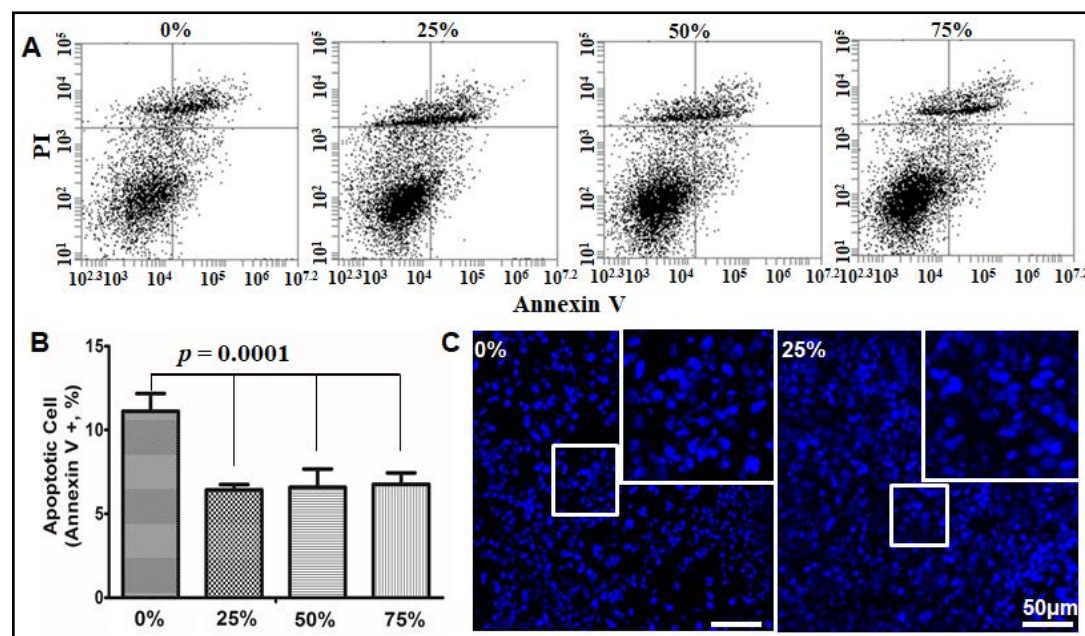

Fig. 5. Apoptosis was investigated in differentiated cells derived from H1 cells at day 25. Note: Flow cytometry analysis using Annexin V-FITC and propidium iodide (PI) double staining was performed to detect the apoptosis ratio. (A) the typical images; (B) statistic results of the apoptosis ratio (n=3); (C) cells stained with Hoechst 33258 were observed by fluorescence microscopy.

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uptake and release (Fig. 4F). These data suggested that the improved differentiation method, in which 25% of the mTeSR1 medium was retained, was appropriate for differentiating iPS cells into hepatocyte-like cells.

Retaining 25% of the mTeSR1 medium during hepatic differentiation could decrease cell apoptosis

During hepatic differentiation of H1 cells, we observed that the cell morphology of the 25% mTeSR1 group was better and had fewer dead cells than the other groups. Thus, we hypothesized that retaining 25% of the mTeSR1 medium during hepatic differentiation might decrease cell apoptosis. To test this hypothesis, flow cytometry was used to quantify apoptosis in differentiated cells. At day 25, the differentiated cells with 0%, 25%, 50% or 75% mTeSR1 were stained with the early apoptotic marker Annexin V-FITC. The populations of the Annexin V-FITC-positive cells were 11.12% ± 1.05% in the 0% group, 6.43% ± 0.32% in the 25% group, 6.58% ± 1.09% in the 50% group, and 6.77% ± 0.67% in the 75% group (Fig. 5A, 5B). Then, we mainly focused on the apoptotic nuclei morphology of the 0% and 25% groups using Hoechst 33258 staining. As shown in Fig. 5B, most cells in the 25% group had regular nuclei, with only a few showing apoptotic nuclei with condensed chromatin, while increased apoptotic nuclear morphology was shown in the 0% group. These results indicated that retaining 25% of the mTeSR1 medium during hepatic differentiation could decrease cell apoptosis.

Discussion

The directed differentiation of human ES or iPS cells into hepatocytes could provide a renewable source of exogenous hepatocytes for drug toxicity testing and cell-based therapeutics [14]. However, the ability to use primary hepatocytes either for therapeutic purposes or basic research has been frustrated by their tendency to rapidly dedifferentiate and lose most hepatic functions after growth in a tissue culture environment [15]. The need to expand primary hepatocytes purified from donor liver tissue could be avoided by using ES and iPS cells for their indefinite proliferation without a loss of potency.

Some growth factors were reported to be important in hepatic differentiation. Activin A and BMP4 were used for definitive endoderm (DE) formation, which was the first and one of the most crucial steps in the hepatic differentiation of ES/iPS cells [16, 17]. In our study, we also found high expression levels of SOX17 and FOXA2. For hepatic progenitor differentiation, BMP2, FGF, HGF, and KGF were used with high AFP and HNF4α expression levels, while for hepatic maturation, oncostatin-M was used to induce ALB [18-24]. However, in this study, the number of dead cells was substantial within 3 days after differentiation, and the hepatocyte-like cells could not survive beyond 25 days. To improve the cell survival rate, we tried adding different concentrations of mTeSR1 medium to the stages of DE and hepatic progenitor differentiation. Interestingly, we found that fewer cells died during differentiation and that hepatocyte-like cells could survive well beyond day 35 in the mTeSR1 medium treatment groups. The expression levels of the DE markers and hepatic progenitor markers were slightly different between the 0% and 25% groups. However, the 25% mTeSR1 group showed a higher proportion of ALB-positive cells than the other groups, which was generally consistent with a previous study [25]. Similar hepatocyte functional activities were found in the 0% and 25% mTeSR1 groups. Moreover, iPS cells could also be differentiated into hepatocyte-like cells using this method with 25% mTeSR1 and could survive well beyond 40 days in our experiment. Evidence shows that culture with low serum concentrations could improve ICG uptake function as well as mature hepatic marker expression [26]. In the present study, we did not observe a significant difference in hepatic marker expression and function between the 0% and 25% mTeSR1 groups. However, we found that retaining 25%

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of the mTeSR1 medium during hepatic differentiation could decrease hepatocyte-like cell apoptosis. Taken together, these results suggest that retaining 25% of the mTeSR1 medium during hepatic differentiation could increase the proportion of ALB-positive cells and cell survival by decreasing cell apoptosis.

The components of the mTeSR1 medium is complicated, including the growth factors, vitamins, lipids, amino acids, trace minerals, inorganic salts *et al*. It is difficult for us to illuminate which component play a primary role. One of the components caused our attention. bFGF can synergize with Noggin, an antagonist of BMPs, to maintain human ES cell culture via inhibition of caspase activation [27, 28]. bFGF and Activin A function to promote survival and proliferation of single iPS cells in conditioned half-exchange mTeSR1 medium [29]. Furthermore, acidic FGF (aFGF) and bFGF produced by the adjacent cardiac mesoderm could induce the hepatocyte lineage [22]. During this stage, BMPs produced by the transversum mesenchyme could promote the ability of endoderm to respond to FGFs [22]. bFGF is used for hepatic specification [30]. We also concern about the other components, TGF-β1 and insulin. TGF-β1 could induce apoptosis in liver, however, insulin could inhibit it [31-33], which maybe the apoptosis induced by TGF-β1 could not be observed in our experiment. Thus, we speculated that the complicated mechanisms of the bFGF, BMPs and Activin A might exist to regulate the cells survival in definitive endoderm specification and to facilitate hepatic specification in our experiment, in which both the antagonism and synergism of the growth factors were involved.

There are also some limitations in this study. We did not investigate the specific components of mTeSR1 medium or the molecular factors involved in regulating hepatocyte-like cell survival. Thus, we do not know whether retaining 25% of the mTeSR1 medium is appropriate for all hepatic differentiation methods. Nonetheless, this report offers a clue for mTeSR1 medium culture conditions during hepatic differentiation, expanding our knowledge of hepatocyte induction *in vitro*.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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