Differentiation 79 (2010) 171–181

Contents lists available at ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

Generation of murine hepatic lineage cells from induced pluripotent stem cells

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ARTICLE INFO

Article history:
Received 29 September 2009
Received in revised form
10 December 2009
Accepted 7 January 2010

Keywords:
iPS cells
Reprogram
Murine hepatocytes
Hepatic differentiation
Liver
Regeneration

ABSTRACT

Induced pluripotent stem (iPS) cells can be generated from somatic cells of individuals by retrodifferentiation using defined transcription factors. Similar to embryonic stem (ES) cells, iPS cells can be differentiated into a variety of specific cell types. However, to date, no detailed hepatic differentiation of mouse iPS cells has been reported. In this study, we successfully developed a stepwise protocol to induce hepatic differentiation of iPS cells reprogrammed from mouse tail tip fibroblasts. At day 25 of differentiation, the iPS cell-derived hepatocytes morphologically resemble mouse primary hepatocytes with a distinct polygonal shape. Immunostaining and reverse transcription-polymerase chain reaction analysis revealed expression of specific hepatic markers including alpha-fetoprotein, albumin and alpha-1-anti-trypsin. In addition, these iPS cell-derived hepatocytes successfully demonstrated mature liver cell functions *in vitro*. Furthermore, *in vivo* assays revealed that the mouse iPS cell-derived hepatocytes successfully engrafted into the recipient livers with typical hepatic morphology. Thus, iPS cell-derived hepatocytes may hold great promise as a unique system for basic liver research and liver regeneration in the near future.

1. Introduction

Liver disease is one of the leading causes of death in the United States. Unfortunately, the supply of healthy liver donors is below that needed. This hampers the use of liver transplantation as an effective treatment. The prospect of cell-based therapies for generating liver tissue is promising and may well have utility in treating chronic liver disease and cirrhosis (Souza et al., 2009).

Previous studies have shown that ES cells can be differentiated into mature hepatocytes, but ethical issues and immunogenic complications restrict their use. iPS cells, which are generated from somatic cells by retrodifferentiation using defined transcription factors, are similar to ES cells and may overcome those barriers(Takahashi et al., 2007; Takahashi and Yamanaka, 2006). To date, iPS cells have been used to successfully generate a variety of differentiated cells, including motor neurons, cardiomyocytes, endothelial cells, bone marrow cells, osteogenic cells, adipocytes and pancreatic islets (Choi et al., 2009; Dimos et al., 2008; Mauritz et al., 2008; Tashiro et al., 2009; Tateishi et al., 2008). Recently, two groups reported the generation of functional hepatocytes from human iPS cells *in vitro* (Song et al., 2009; Sullivan et al., 2009); however, the *in vivo* integration into the liver and functional analysis of these human iPS-differentiated hepatocytes were not demonstrated in these two studies.

In this study, we successfully developed a stepwise differentiation method to generate mature hepatocytes from iPS cells reprogrammed from mouse tail tip fibroblasts (TTF). To our knowledge, this is the first report of hepatic differentiation from mouse iPS cells. By monitoring the continuous morphological changes in the differentiated cells and the expression of specific hepatic markers at various differentiation stages, we found that the mouse iPS cells were successfully induced to differentiate into mature hepatocyte-like cells. After 25 days of differentiation, the cells possessed polygonal morphology with one or two prominent nuclei and expressed mature hepatic cell specific markers. Functional tests demonstrated that the iPS cell-derived hepatocytes could store glycogen, uptake and release indocyanine green (ICG), and take up acetylated low density lipoprotein (LDL). One week after intra-portal vein injection, immunohistochemistry analysis showed that the iPS cell-derived hepatocytes were successfully engrafted into the host liver. Our study may shed new light on the burgeoning field of cell replacement to diseased liver tissues.

2. Materials and methods

2.1. Generation of iPS cells

Tail skin tissues of adult C57BL/6 mice expressing green fluorescent protein (GFP) (Jackson lab, Bar Harbor, ME, USA) were minced into small pieces and incubated in tissue culture dishes

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doi:10.1016/j.diff.2010.01.002

with DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) for 7 days. Fibroblasts that migrated out of the skin tissues were digested with 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA), reseeded to new culture dishes, and maintained in DMEM containing 10% FBS. GFP-TTF cells at passage 3–5 were used for iPS cell generation.

Four mouse retroviral constructs pMXs-Oct3/4, pMXs-Sox2, pMXs-Klf4 and pMXs-c-Myc (Addgene, Cambridge, MA, USA) were used for iPS cell production as described (Takahashi and Yamanaka, 2006). Plat-E cells (ATCC, Manassas, VA, USA), maintained in 90% DMEM supplemented with 10% FBS, were plated at 10×10^6 cells per 100 mm dish and were then transfected by retroviral constructs separately with Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) 24 h post plating. The four retrovirus containing supernatants were collected at both 24 and 48 h after transfection, mixed equally and supplemented with 4 μg/ml polybrene. GFP-TTF cells were seeded at 0.5×10^6 cells per 100 mm dish on the day before infection and were infected twice with the mixture of the four retroviruses. Two days after infection, the GFP-TTF cells were harvested and replated at 0.5×10^6 cells per 100 mm dish on Mitomycin C treated CF-1 feeder layers (ATCC, Manassas, VA, USA). The next day the medium was replaced in Knockout DMEM supplemented with 20% FBS, 2 mM L-glutamine, 1% MEM nonessential amino acids solution, 0.1 mM β-mercaptoethanol (all from Invitrogen, Carlsbad, CA, USA) and 1000 u/ml LIF (Chemicon, Billerica MA, USA). The medium was changed every day. On day 10 after infection, 24 colonies were picked and expanded individually for identification of pluripotent stem cell characteristics.

2.2. Teratoma formation

One million mouse iPS cells at passage 20 were treated with 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA), resuspended in 100 μl PBS, mixed with an equal volume of matrigel (Invitrogen, Carlsbad, CA, USA), and were injected subcutaneously into three SCID mice (Jackson Laboratory, Bar Harbor, ME, USA). After 6 weeks, teratomas were dissected, fixed with formalin (Fisher Scientific, Pittsburgh, PA, USA), embedded in Paraffin, and examined histologically after hematoxylin and eosin staining.

2.3. Hepatic differentiation of iPS cells

Mouse iPS colonies (passage 20) were made into a single cell suspension by treatment with 0.25% trypsin-EDTA and embryoid bodies (EBs) were formed by the hanging drop method in DMEM, supplemented with 20% FBS, 0.1 mM β-mercaptoethanol, 2 mM glutamine and 1% MEM nonessential amino acid solution for 2 days. EBs were transferred to petri dishes for another 4 days in suspension and then plated to matrigel coated tissue culture dishes in DMEM plus 10% FBS, 50 ng/ml Activin-A and 50 ng/ml Wnt 3 (Invitrogen, Carlsbad, CA, USA). On the next day, after most of the EBs were attached, the cultures were switched to DMEM supplemented with 1% FBS, 50 ng/ml Activin-A and 50 ng/ml Wnt 3. Medium was changed every day for the following 3 days. To further initiate hepatic differentiation, DMEM supplemented with 10% FBS, 10 ng/ml basic FGF, 1% dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) was utilized in the cultures for 4 days and then 10 ng/ml HGF, 1% DMSO in hepatocyte culture medium (HCM, Invitrogen, Carlsbad, CA, USA) was used for another 5 days. For subsequent hepatic maturation, the cultures were fed with HCM supplemented 10 ng/ml HGF, 10 ng/ml oncostatin M (OSM) and 0.1 μM dexamethasone (Sigma, St. Louis, MO, USA) for another 7 days.

2.4. Immunochemical reagents and procedures

Cells were grown in 24-well plates and fixed in 4% paraformaldehyde in PBS for 10 min, blocked with 5% bovine serum albumin (Sigma, St. Louis, MO, USA) in 0.1% TritonX-100 for 1 h at room temperature, and incubated with primary antibody at 4 °C overnight. 5% donkey serum was used instead of bovine serum albumin when goat primary antibodies were used. The primary antibodies against OCT3/4 (goat, 1:50), Nanog (goat, 1:50), SSEA-1 (mouse, 1:50), AFP (goat, 1:50), ALB (goat, 1:50), AAT (goat, 1:50), CYP1A1 (mouse, 1:50), HNF-4α (goat, 1:50) and Sox17 (goat, 1:50) were all from Santa Cruz Biotechnology, Santa Cruz, CA, USA; E cadherin (mouse, 1:500) and cytokeratin 19 (Rabbit, 1:500) were from Abcam, Cambridge, MA, USA. The specificity of all antibodies used was further verified by the controls included in each experiment.

The cells were stained with PE-conjugated anti-mouse secondary antibody (1:1000, Sigma, St. Louis, MO, USA), anti-goat secondary antibody and anti-rabbit secondary antibody (1:1000, Southern Biotech, Birmingham, AL, USA) at room temperature for 1 h. Nuclei were counterstained with 1 mg/mL 4′, 6-diamidino-2-phenylindole (DAPI, Sigma) for 10 min. The samples were analyzed by a Motic AE31 microscopy equipped with Spot digital camera and software.

2.5. Reverse transcription PCR

Total RNA was extracted using Trizol® (Invitrogen, Carlsbad, CA, USA) and then treated with DNase I at 37 °C for 20 min. cDNA was prepared by reverse transcription of approximately 1 μg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. PCR was performed using the PCR Master Mix Kit (Promega,

Table 1
Polymerase chain reaction primers.

Gene	GenBank ID no.	Primer	Size (base pairs)
AFP	11576	5′-AGGAGGAGTGCTTCCAGACA-3' 5′-TGCGTGAATTATGCAGAAGC-3'	359
AAT	20699	5′-CAGAGGAGGCCAAGAAAGTG-3' 5′-ATGGACAGTCTGGGGAAGTG-3'	430
Albumin	11657	5′-TGAACTGGCTGACTGCTGTG-3' 5′-CATCCTTGGCCTCAGCATAG-3'	719
TTR	22139	5′-AGTCCTGGATGCTGTCCGAG-3' 5′-TTCCTGAGCTGCTAACACGG-3'	440
TDO	56720	5′-AGAGCCAGCAAAGGAGGAC-3' 5′-CTGTCTGCTCCTGCTCTGAT-3'	500
β-actin	11461	5′-ATGGTGGGAATGGGTCAGAAG-3' 5′-CACGCAGCTCATTGTAGAAGG-3'	150