European Journal of Cell Biology 94 (2015) 603–610

Contents lists available at ScienceDirect

European Journal of Cell Biology

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

Research paper

Transcription factor-mediated reprograming of fibroblasts to hepatocyte-like cells

Behshad Pournasr[a,b], Mohammad Hassan Asghari-Vostikolaee[c], Hossein Baharvand[a,b,*]

[a] Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
[b] Department of Developmental Biology, University of Science and Culture, ACECR, Tehran, Iran
[c] Animal Core Facility, Reproductive Biomedicine Research Center, Royan Institute for Biotechnology, ACECR, Tehran, Iran

ARTICLE INFO

Article history:
Received 13 April 2015
Received in revised form 4 September 2015
Accepted 21 October 2015

Keywords:
Hepatocyte-like cells
Direct conversion
Hepatic-specific transcription factors
Yamanaka factors

ABSTRACT

Direct conversion by overexpression of defined transcription factors (TFs) is a promising new method that can generate desired cell types from abundant, accessible cells. While previous studies have reported hepatic generation from fibroblasts, tremendous interest exists in the understanding of hepatic reprograming and its applicability in regenerative medicine. Here, we show that overexpression of Yamanaka factors can induce reprograming of mouse fibroblasts into cells that closely resemble hepatocytes in vitro in the presence of an optimized hepatic growth medium. By screening the effects of 20 candidate transcription factors, we identified a combination of three TFs (*Hnf4a*, *Cebpa*, and *Nr1i2*) that can convert fibroblasts into a hepatic fate. These factors in conjunction with Yamanaka factors increase the efficiency of hepatic reprograming. The induced hepatocyte-like (iHep) cells have multiple hepatocyte-specific characteristics; express hepatocyte-specific markers, glycogen storage, albumin secretion, urea production, as well as low-density lipoprotein and indocyanin green uptake. Production of iHep cells by these novel approaches may bring new insights into the molecular nature of hepatocyte differentiation and future cell-based therapeutics for liver diseases.

1. Introduction

The establishment of desirable functional cell types is a long-standing major goal for basic and pharmaceutical research in cell therapy and regenerative medicine. Although conventional methods of cellular derivation, such as embryonic or induced pluripotent stem (iPS) cell directed differentiation have been extensively reported for this purpose and progressed rapidly in recent years (Tabar and Studer, 2014), there has also been great interest in the development of alternative strategies for obtaining functional cells. Transdifferentiation or lineage reprograming is an attractive strategy to generate valuable but not easily accessible cells such as hepatocytes. This strategy can be defined as the direct conversion of one somatic mature cell type into another functional mature or progenitor cell type in the absence of reprograming into or through a pluripotent intermediate (Pournasr et al., 2011). Direct conversion presents several advantages: lack of tumorigenic risk, fast conversion rate, and repair of injured tissues by in vivo reprograming (for review see Ben-David and Benvenisty, 2011; Mirakhori et al., 2014; Vierbuchen and Wernig, 2011). There are two strategies for cell fate conversion, either by a lineage-instructive transcription factor (TF) approach or an initial epigenetic activation phase approach by Yamanaka factors followed by appropriate developmental cues that do not go through the pluripotent intermediate (for review see Xu et al., 2015). Until now, hepatic cells have been generated by hepatic-instructive TFs in mice (Huang et al., 2011; Sekiya and Suzuki, 2011) and human (Du et al., 2014; Huang et al., 2014; Kogiso et al., 2013; Zhu et al., 2014) fibroblasts. However, numerous questions about direct conversion into a hepatic fate remain unclear. In this study, we address three questions. Can transient expression of the four Yamanaka factors under hepatoinductive conditions lead to a hepatic fate? Which new cocktail of hepatic TFs direct lineage reprograming? Can overexpression of our hepatic-specific TFs combined with Yamanaka factors improve yield and efficiency?

2. Materials and methods

2.1. Cell cultures

Limbs were isolated from 13.5-day old post coitum mouse embryos. Limb fibroblasts and 293 T cells (Life Technologies, R700-07) were cultured in Fib medium that consisted of Dulbecco's

* Corresponding author at: Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, P.O. Box 19395-4644 Tehran, Iran. Tel.: +98 21 22306485; fax: +98 21 23562507.
E-mail address: Baharvand@RoyanInstitute.org (H. Baharvand).

http://dx.doi.org/10.1016/j.ejcb.2015.10.003
0171-9335/

Modified Eagle's medium/high glucose (DMEM/HG, Life Technologies, 12800116), that contained 10% fetal bovine serum (FBS, Life Technologies, 16140-071) and 2 mM L-glutamine (Life Technologies, 25030). For 293 T cells, we added 500 μg/ml Geneticin (Life Technologies, 10131027) to the medium.

2.2. Virus production

The CDS sequence of interested genes were synthesized and transferred to a vira-power plenti3.6/TO/V5 DEST backbone. Maxiprep was performed on all genes, and then maintained at −20 °C. The inducible polycistronic TetO-FUW-OSKM (a gift from Rudolf Jaenisch, Addgene plasmid # 20321) Lentivirus production was performed according to the manufacturer's protocol. One day before transfection for all needed vectors, we placed 5×10^6 cells separately into 10 cm culture dishes. The next day transduction was performed according to the protocol described in the manual. Briefly, the cell media was changed with 5 ml complete media and 2% FBS without antibiotics. In two separate 5 ml tubes we added 1.5 ml of basal medium followed by the addition of 36 μl lipofectamine. To the first tube we added 12 μg of packaging vector and the desired vector to the other tube. After a 5-min incubation period, the tubes were combined and allowed to incubate for another 20 min. We added the complete 3 ml mix to the culture plates in a drop-wise manner. From this step, the cells must be maintained in a Biosafety level II culture room and manipulated as biohazard materials. The next day the medium was replaced by complete medium that contained 10% FBS. This medium was collected after 24 and 48 h, then centrifuged at 20,000 rpm (Centrifuge, Sigma-Aldrich 3K30) for 2 h after which the pellets were dissolved in 100–300 μl basal media, aliquoted and maintained at −70 °C. Titration was performed on the HT1080 cell line according to the company's manual (Life Technologies, MAN0000273).

2.3. Media

Reprograming medium: This medium consisted of standard mouse embryonic stem cell medium without leukemia inhibitory factor (LIF) but included Jack-stat inhibitor (0.5 μM).

Hepatic induction medium 1 (*HIM*1): This medium contained L15 (Life Technologies, 41300070) and ITS (Life Technologies, 41400045) that started from 1% and decreased to 0% during the culture period. Either activin A (R&D Systems, 338AC, 100 ng/ml), follistatin (R&D Systems, 769FS, 100 ng/ml) or epidermal growth factor (EGF, R&D Systems, 2028-EG) were added according to the experiment conducted.

Hepatoinductive medium 2 (*HIM*2): This medium contained 50% L15 and 50% hepatocyte culture medium (HCM, Lonza, CC-4182) with fibroblast growth factor 4 (FGF4, R&D Systems, 235F4, 20 ng/ml), hepatocyte growth factor (HGF, R&D Systems, 294HG, 30 ng/ml), oncostatin M (OSM, R&D Systems, 295OM, 35 ng/ml), dexamethasone (Dex, Sigma-Aldrich, D2915, 7.4 mg/l) and nicotinamide (NicA, Sigma, N0636, 100 mg/l).

2.4. RNA isolation and real time RT-PCR

Total RNA was extracted using TRI Reagent® (Sigma-Aldrich, T9424) from cells for qRT-PCR analysis as previously described (Tahamtani et al., 2013). cDNA was produced by the RevertAid First Strand cDNA Synthesis Kit (Fermantas, K1632) according to the manufacturer's instructions.

Duplicate qRT-PCR reactions were performed with the SYBR Green Master Mix (Takara Bio, Inc., RR081Q) with a real-time PCR system (Corbett Life Science; Rotor-Gene 6000) and analyzed with Rotor-Gene 6000 analysis software (Corbett Life Science; version 1.7). The samples were collected from either two or three independent biological replicates. The expression level of desired genes was normalized to *Gapdh* or the corresponding mouse fibroblasts as the starting cells or primary hepatocytes. Analysis was performed by the comparative CT method. Primers are listed in Supplementary Table 1.

2.5. Flow cytometry analysis

Single cells were enzymatically prepared and subsequently fixed in 4% paraformaldehyde (Sigma-Aldrich, P6148) for 20 min at room temperature (RT). For permeabilization, cells were treated with 0.1% Triton X-100 for 5 min at RT and then incubated overnight with primary antibodies at the proper dilution in blocking buffer at 4 °C. Blocking buffer contained PBS, 10% of secondary host serum, and 0.5% BSA. The next day diluted secondary antibodies were added to each sample after washing followed by a 1 h incubation period at 4 °C. A BD-FACS calibur flow cytometer (FACS Calibur) was used to determine the percentage of positive cells. Analyses were undertaken on three independent biological experiments for each marker. Acquired data were analyzed with FlowJo version 7.8 software. Antibodies used in the experiment are listed in Supplementary Table 2.

2.6. Immunofluorescence staining

We evaluated the cells for expression of albumin (Alb) and α-fetoprotein (AFP). Cells were washed with PBS and then fixed in 4% paraformaldehyde for 20 min at RT. After washing, the cells were permeabilized with 0.1% Triton X-100 for 10 min, then blocked in 10% secondary antibody host serum and 0.5% BSA for 1–2 h at RT with agitation. After washing with PBS-Tween, the cells were incubated overnight with primary antibodies at 4 °C with shaking. Following the incubation period, cells were washed and incubated with secondary antibodies for 1 h at 4 °C. The nuclei were counterstained with DAPI (Sigma-Alrich; D8417). We observed the plates under a fluorescent microscope (IX71; Olympus). The antibodies are listed in Supplementary Table 2.

2.7. Periodic acid-Schiff staining

The cells were oxidized in 1% periodic acid for 5 min after fixation in 4% paraformaldehyde and then rinsed in distilled H_2O. Next, cells were treated with periodic acid-Schiff (PAS) staining for 15 min; for color development cells were treated in distilled H_2O for 5–10 min. The plates were observed under a light microscope (BX51; Olympus).

2.8. Low-density lipoprotein uptake

Cells were incubated for 4–6 h with acetylated low-density lipoprotein (LDL) labeled with 1,1′-dioetadeeyl-3,3,3′,3′-tetramethylindocarboeyanine perchlorate (DiI-Ac-LDL; Biomedical Technologies Inc., 9202K08). The test was performed according to the manufacturer's instructions and cells were visualized using a fluorescent microscope (IX71; Olympus).

2.9. Indocyanin green uptake

Cells were incubated with indocyanin green (ICG, CardioGreen; Sigma-Aldrich, 12633) in basal medium for 1 h in an incubator under standard conditions. ICG uptake was visualized with light microscopy (BX71; Olympus).