Research Article

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Directed differentiation of murine-induced pluripotent stem cells to functional hepatocyte-like cells

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Background & Aims: Induced pluripotent stem (iPS) cells exert phenotypic and functional characteristics of embryonic stem cells even though the gene expression pattern is not completely identical. Therefore, it is important to develop procedures which are specifically oriented to induce iPS cell differentiation.

Methods: In this study, we describe the differentiation of mouse iPS cells to hepatocyte-like cells, following a directed differentiation procedure that mimics embryonic and fetal liver development. The sequential differentiation was monitored by real-time PCR, immunostaining, and functional assays.

Results: By sequential stimulation with cytokines known to play a role in liver development, iPS cells were specified to primitive streak/mesendoderm/definitive endoderm. They were then differentiated into two types of cells: those with hepatoblast features and those with hepatocyte characteristics. Differentiated hepatocyte-like cells showed functional properties of hepatocytes, such as albumin secretion, glycogen storage, urea production, and inducible cytochrome activity. Aside from hepatocyte-like cells, mesodermal cells displaying some characteristics of liver sinusoidal endothelium and stellate cells were also detected.

Conclusions: These data demonstrate that a protocol, modeled on embryonic liver development, can induce hepatic differentiation of mouse iPS cells, generating a population of cells with mature hepatic phenotype.

Introduction

Hepatocytes are essential for the pharmaceutical industry in order to test drug metabolism and to develop new therapeutic strategies for liver diseases. Two main reasons lay behind the slow development of cell therapies for liver diseases. First, there is a shortage of liver organs from which hepatocytes can be isolated. Secondly, it is difficult to culture hepatocytes while maintaining their differentiated state [1–3]. Aside from these, the lack of prognostic animal models and standardized preclinical practices has also restrained the field. Stem cells have been proposed as an ideal cell source to generate unlimited numbers of hepatocytes [4]. For this reason, it is crucial to develop robust differentiation methods for human and mouse stem cells into hepatocytes and investigate the potential of these cells *in vitro* and in preclinical animal models.

The generation of induced pluripotent stem (iPS) cells from terminally differentiated adult cell types has been recently described, first from mouse cells and then from human and rat cells [5–8]. IPS cells were initially generated by using viral vectors of specific transcription factors that turn on the transcriptional regulatory circuit of pluripotent cells. More recently, iPS cells have also been generated by non-integrating methods, both alone and in combination with small molecules that affect methylation or acetylation, mimic the Wnt-signaling pathway, or modulate the transforming growth factor (TGF)β pathway [8–10]. iPS cells have many characteristic features of embryonic stem (ES) cells: they have the ability to create chimeras with germ line transmission and tetraploid complementation, and they can also form teratomas containing various cell types from three embryonic germ layers [5,6,11]. On the other hand, recent reports demonstrate that iPS and ES cells are not identical [12]. The possibility of generating pluripotent cells from any kind of adult cell has raised the potential of creating patient-specific cells immunologically compatible with the host for cell therapy approaches. It also opens the possibility to easily create disease models in order to investigate the role of particular genes in liver development [13]. Differentiation of iPS cells into hepatocyte-like cells has been recently described [14,15]. However, the potential of mouse iPS cells to differentiate into hepatocyte-like cells by mimicking embryonic and fetal liver development has not been fully investigated.

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Abbreviations: iPS, induced pluripotent stem; TGF, transforming growth factor; ES, embryonic stem; PS, primitive streak; ME, mesendoderm; DE, definitive endoderm; Afp, alpha-fetoprotein; FGF, fibroblast growth factor; Hnf, hepatocyte nuclear factor; Krt, keratin; FBS, fetal bovine serum; FCS, fetal calf serum; DMEM, Dulbecco's Modified Eagle Medium; MEF, mouse embryonic fibroblasts; BMP, bone morphogenic protein; HGF, hepatocyte growth factor; Ttr, transthyretin; Aat, alpha-1 antitrypsin; Tat, amino-transferase; G6p, glucose-6-phosphate; PH, partial hepatectomy.

Liver embryonic development has been extensively studied. Cells from the inner cell mass will form the epiblast that under the effect of Nodal and Cripto signaling, will be specified to form the primitive streak (PS), mesendoderm (ME), and definitive endoderm (DE), while outer cells will form the primitive endoderm or hypoblast [16,17]. Primitive and definitive endoderms express a number of genes in common such as *Foxa2*, *Gata4*, *Gata6*, alpha-fetoprotein (*Afp*), or *Sox17*. However, definitive and visceral endoderm can be discriminated by the expression of *goosecoid* (expressed in definitive endoderm, as well as anterior visceral endoderm but not in the rest of primitive endoderm), *Cxcr4*, *Eomes*, and *Mixl1* expressed only in (mese)endoderm but not in primitive endoderm and *Sox7*, which is only expressed in primitive endoderm [18–20]. Mesodermal cytokines produced by the adjacent cardiac mesoderm and septum transversum will induce foregut specification into hepatic endoderm, and following fibroblast growth factor (FGF) signaling will grow into the liver bud [16,21]. Hepatoblasts expressing *Afp*, hepatocyte nuclear factor *(Hnf)4*, and keratin *(Krt)19* will be specified into cholangiocytes and hepatocytes [22,23].

Here, we describe the differentiation of mouse iPS cells into functional hepatocyte-like cells following a hepatocyte differentiation protocol that mimics embryonic liver development by inducing the differentiation of pluripotent cells to form definitive endoderm, hepatoblasts, and hepatocyte-like cells. Although iPS cell progeny is a mixture of cells with immature and more mature hepatic characteristics, a fraction of the progeny has acquired phenotypic and functional characteristics of adult hepatocytes.

Methods

IPS cell generation

IPS cells were generated using the protocol described by Takahashi et al. [24] from the adherent fraction of the bone marrow (iPS lines J3 and J23) from Oct4-GFP mice (a kind gift from Dr. R. Jaenisch)[25] and from tail clip fibroblasts (iPS line A1) of Tg(Pou5f1-EGFP)2Mnn (CBA/CaJxC57BL/6J) mice. All isolations were approved by the ethical committee for the use of animals in research of The Catholic University of Leuven, Belgium. Briefly, bone marrow adherent cells were cultured in differentiation medium supplemented with 10 ng/ml mouse epidermal growth factor (Sigma, Bornem, Belgium), 10 ng/ml human platelet-derived growth factor-ββ (R&D Systems, Abingdon, UK), 1000 U/ml murine Leukemia Inhibitory Factor (mLIF) (Chemicon, Millipore, Brussels, Belgium), and 2% fetal bovine serum (FBS) (Hyclone, Breda, The Netherlands). Tail clip was obtained from Oct4-GFP mice, minced, and cultured on a gelatin-coated plate in fibroblast expansion medium: high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen, Merelbeke, Belgium), 10% FBS (Hyclone), 1% L-glutamine (Gibco), and penicillin/streptomycin (100 and 1000 U, respectively, Gibco). Cells that grew out of the tissue were cultured and expanded until transduced with retrovirus.

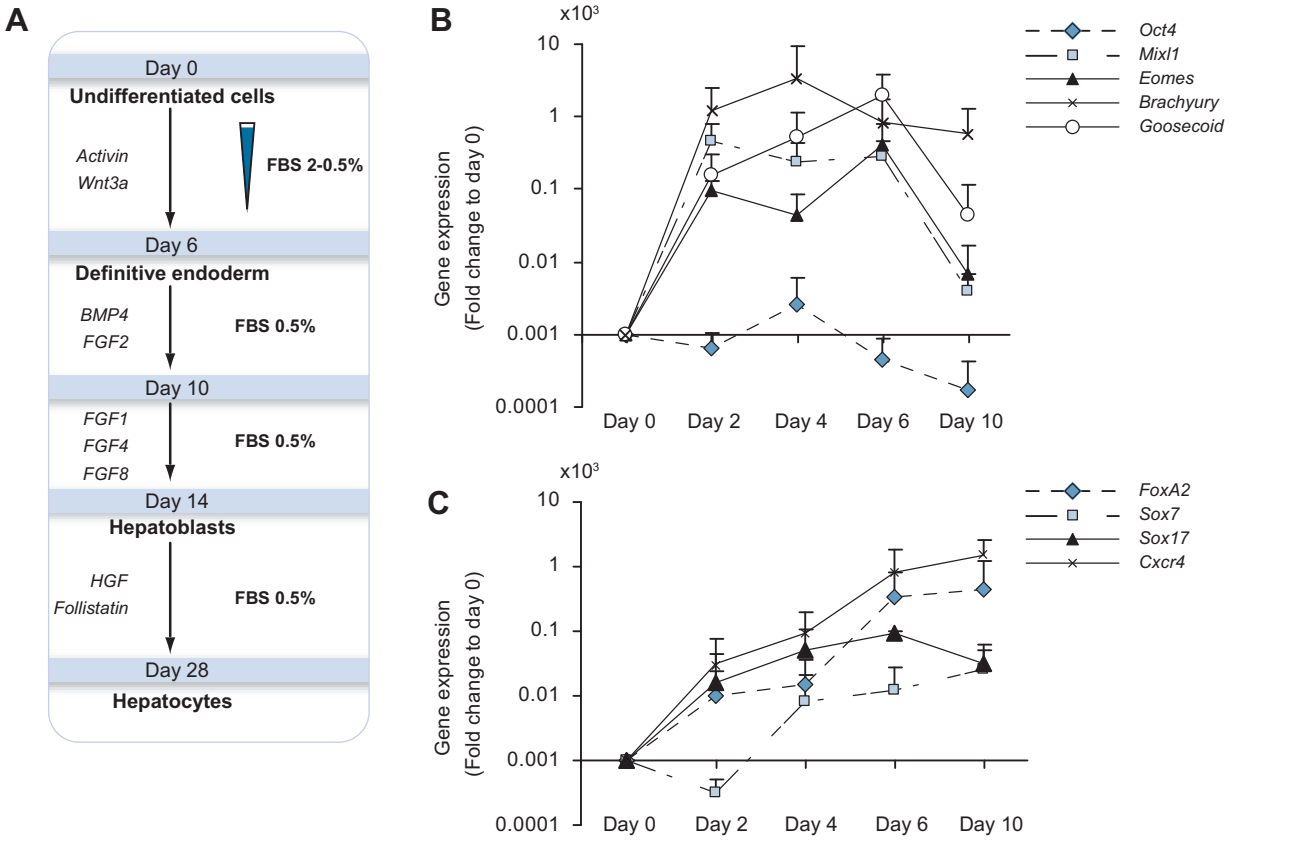

Fig. 1. Gene expression analysis at early stages of differentiation. (A) Schematic diagram of the differentiation protocol. (B) Gene expression analysis by real-time RT-PCR of key genes expressed in primitive streak/mesendoderm at days 0, 2, 4, 6, and 10 of differentiation, and (C) gene expression analysis by real-time RT-PCR of key genes expressed in definitive endoderm and primitive endoderm at days 0, 2, 4, 6, and 10 of differentiation. Results are shown as mean fold change respect to day 0 ± standard deviation of three independent differentiations.