Hepatocyte-Ductal Transdifferentiation Is Mediated by Reciprocal Repression of SOX9 and C/EBP α

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Abstract

Primary hepatocytes rapidly dedifferentiate when cultured *in vitro*. We have studied the mechanism of hepatocyte dedifferentiation by using two culture media: one that maintains hepatocytes in a differentiated state and another that allows dedifferentiation. We show that dedifferentiation involves partial transformation of hepatocytes into cells that resemble biliary epithelial cells. Lineage labeling and time-lapse filming confirm that the dedifferentiated cells are derived from hepatocytes and not from contaminating ductal or fibroblastic cells in the original culture. Furthermore, we establish that the conversion of hepatocytes to biliary-like cells is regulated by mutual antagonism of CCAAT/enhancer binding protein alpha (C/EBP α) and SOX9, which have opposing effects on the expression of hepatocyte and ductal genes. Thus, hepatocyte dedifferentiation induces the biliary gene expression program by alleviating C/EBP α -mediated repression of *Sox9*. We propose that reciprocal antagonism of C/EBP α and SOX9 also operates in the formation of hepatocytes and biliary ducts from hepatoblasts during normal embryonic development. These data demonstrate that reprogramming of differentiated cells can be used to model the acquisition and maintenance of cell fate *in vivo*.

Introduction

THE TERM “DEDIFFERENTIATION” DESCRIBES loss of the cell type-specific characters that define a terminally differentiated cell. Following isolation, primary hepatocytes rapidly dedifferentiate, a process that has hindered *in vitro* modeling of liver disease, regeneration, and liver function, particularly xenobiotic metabolism (Fraczek et al., 2013). We show here that this dedifferentiation results in the partial transdifferentiation of hepatocytes toward a bile duct phenotype. Analysis of this phenomenon can therefore be used as a tool to investigate the mechanism of transdifferentiation (Slack, 2007) and normal liver development.

The adult liver contains many cell types—hepatocytes, biliary epithelial cells, endothelial cells, stellate cells, and Kupffer cells (Si-Tayeb et al., 2010). The epithelial component of the organ is composed of hepatocytes and bile duct epithelial cells (BECs). In normal embryonic development,

hepatocytes and biliary epithelial cells are derived from a bipotential precursor, the hepatoblast (Strick-Marchand and Weiss, 2002; Suzuki et al., 2008; Tanimizu and Miyajima, 2004). Hepatocyte identity is defined by several core transcription factors that form increasingly complex cross-regulatory interactions as development proceeds. Expression of several of the core factors is regulated by CCAAT/enhancer binding protein alpha (C/EBP α) (Kymizi et al., 2006), a key hepatic transcription factor that also controls expression of genes involved in ammonia detoxification and glucose and lipid homeostasis (Inoue et al., 2004). The earliest and most specific marker of biliary cells in the developing liver is SOX9. *Sox9* encodes a transcription factor with a wide variety of developmental functions: It is directly downstream of sex-determining region Y (SRY) in the male sex determination pathway (Kashimada and Koopman, 2010), it is necessary for skeletal development (Bi et al., 1999), and it is expressed in a number of types of tissue-specific stem cells

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(Huch and Clevers, 2011). In the context of the liver, SOX9 regulates bile duct morphogenesis by controlling the maturation of early ductal structures into symmetrical bile ducts (Antoniou et al., 2009).

There have been various reports of partial or complete transdifferentiation of mature hepatocytes to ductal cells. Nishikawa et al. reported the induction of ductal genes in aggregates of primary rat hepatocytes embedded in a collagen gel matrix (Nishikawa et al., 2005). Similarly, mouse hepatocytes allowed to dedifferentiate in culture activate CK19, A6 antigen, and $\alpha 6$ integrin, all of which are normally expressed in mouse bile duct cells (Fougere-Deschatrette et al., 2006). Reprogramming of hepatocytes to ductal cells has also been described *in vivo* using rats with hybrid livers that contained donor-derived clones of hepatocytes expressing dipeptidyl peptidase IV (DPPIV) (Michalopoulos et al., 2005). All of these suggest that hepatocytes can probably differentiate into bile duct cells, but because lineage-tracing analysis was not performed in these studies, it remains possible that biliary cells were derived from contaminating duct or progenitor cells in the original cell population.

Although the likely transdifferentiation of hepatocytes to ductal cells has been previously described, the underlying cellular and molecular mechanisms remain unknown. To address this problem, we have developed an *in vitro* model based on culture in two different media—keratinocyte serum-free medium maintains hepatocyte differentiation for up to 3 weeks, and Dulbecco's Minimal Essential Medium allows rapid dedifferentiation (Li et al., 2007). In this work, we used lineage labeling and time-lapse filming to confirm that hepatocytes dedifferentiate in culture and are not simply overgrown by a minority cell type. We also show that dedifferentiated primary hepatocytes acquire a number of ductal characteristics. Using adenoviral vectors, we find that overexpression of *Sox9* in differentiated hepatocytes can mimic dedifferentiation and induce biliary genes. Conversely, overexpression of *C/ebp α* in dedifferentiated hepatocytes can reverse dedifferentiation and suppress biliary genes. We conclude from this that *C/ebp α* and *Sox9* form a mutually antagonistic system controlling the choice of hepatocyte versus biliary fate, and discuss the relevance of this finding to the generation of hepatocytes and biliary epithelial cells in normal liver development.

Materials and Methods

Isolation of rat primary hepatocytes

Rats were kept at 25°C, on a 12:12-hr light–dark cycle and given *ad libitum* access to food and water. Animal procedures were conducted according to UK Home Office regulations. Primary rat hepatocytes were isolated from 270- to 300-gram male albino Wistar rats using a two-step collagenase perfusion protocol (Tosh et al., 1988). An 18-gauge cannula (BD Biosciences, Oxford, UK) was inserted into the portal vein, and the liver was perfused with 250 mL of prewarmed perfusion buffer 1 (10 mM HEPES, 5 mM D-glucose, 300 μ M EDTA, and 0.001% Phenol Red in calcium-free Dulbecco's phosphate-buffered saline (D-PBS; pH 7.4, Lonza 17-512) at a flow rate of 25 mL/min. The buffer was then replaced with prewarmed perfusion buffer 2 (30 mM HEPES, 5 mM D-glucose, 1 mM CaCl₂, 0.001% Phenol Red) supplemented with 0.25 mg/mL Worthington type II collagenase (Lorne

Laboratories). Perfusion was continued for approximately 8 min, depending on the activity of the collagenase. Following perfusion, the liver was removed from the animal and dissociated in perfusion buffer 2 (without collagenase) using curved forceps. The resulting cell suspension was filtered through a 30- μ m mesh (Sefar, Bury, UK), and nonviable cells were removed by three rounds of serial differential centrifugation at 50 $\times$ g for 2 min. Finally, the hepatocytes were seeded in attachment medium and allowed to attach at 37°C in 5% (vol/vol) CO₂. After 5–6 hr, the medium was changed to differentiation or dedifferentiation media.

Cell culture

Freshly isolated hepatocytes were allowed to attach in attachment medium [William's Medium E (Sigma), 10% (vol/vol) FBS (Gibco), 2 mM L-glutamine (Lonza), 50 U/mL penicillin (Lonza), 50 U/mL streptomycin (Lonza)]. Differentiated hepatocytes were cultured in KS medium [keratinocyte serum-free medium (Gibco), 2 mM L-glutamine, 50 U/mL penicillin, 50 U/mL streptomycin, 1 μ g/mL fungizone (Gibco), 50 μ g/mL gentamicin (Gibco), 50 μ g/mL bovine pituitary gland extract, and 5 ng/mL human recombinant epidermal growth factor (EGF) and supplemented with either 10 nM or 1 μ M dexamethasone (Dex; Sigma)] (Li et al., 2007). Dedifferentiating hepatocytes were cultured in DS medium [Dulbecco's Modified Eagle Medium (DMEM) (Sigma), 10% (vol/vol) fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/mL penicillin, 50 U/mL streptomycin, 1 μ g/mL fungizone, 50 μ g/mL gentamicin, 50 μ g/mL bovine pituitary gland extract, and 5 ng/mL human recombinant EGF].

Lineage tracing

Hepatocytes of C57BL/6 *Alb-Cre x R26R LacZ* mice were used to trace the origin of dedifferentiating cells in culture. These mice (heterozygous for both transgenes) were created by breeding the parent strains, which were originally provided by Dr. Caroline Chadwick (Manchester University) (*AlbCre*) and Dr. Steve Sheardown (GlaxoSmithKline) (*R26R LacZ*). These experiments were performed under UK Home Office regulations.

For X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) staining, the cultures were fixed at day 8 after isolation in 0.5% glutaraldehyde in PBS for 5 min at room temperature and stained at 37°C in X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 in PBS (pH 7.3) for 6 hr. Cultured hepatocytes of *R26R LacZ* mice, expressing bacterial β -galactosidase ubiquitously, and CD1 wild-type mice, also fixed at day 8, were used as positive and negative controls, respectively.

For time-lapse filming, freshly isolated hepatocytes from wild-type C57BL/6 mice were used. These experiments were conducted at the University of Minnesota under IACUC protocol 1002A78295. Hepatocytes were isolated as above and allowed to adhere in plastic six-well dishes for 5 hr in attachment medium. The medium was then changed to DS medium, and time-lapse microscopy was initiated. The cells were maintained at 5% CO₂ at 37°C in an environment chamber on a Leica DMI6000B microscope. The medium was changed after 48 hr. Images were taken every 8 min for the duration of the experiment using a Retiga 2000R camera

(QImaging). IP Lab 4.0 software was used for microscope and camera control as well as frame assembly into video.

Adenoviruses

Ad-CMV-C/ebpx was obtained from Vector Biolabs (Philadelphia, PA, USA). *Ad-RSV-GFP* was a gift from E. Regardsoe, University of Oxford, UK. *Ad-CMV-Sox9-IRES-eGFP* was constructed in the lab using human *SOX9* cDNA. Hepatocytes were infected after 12 or 24 hr of culture. Infection of differentiated hepatocytes with *Ad-CMV-Sox9-IRES-eGFP* was carried out at a multiplicity of infection (MOI, virus particles per cell) of 2 in KS medium containing 10% FBS. The cells were returned to unsupplemented KS after 1 hr of infection. Dedifferentiated hepatocytes were infected overnight with *Ad-CMV-C/ebpx* at a MOI of 200 in the presence of 5 μ g/mL dextran.

Antibodies

The primary antibodies were obtained and diluted as follows: Mouse anti-vimentin, rabbit anti-albumin, rabbit anti-transferrin (1:300, DAKO, Ely, UK), sheep anti-UDP-glucuronosyl-transferase (UGT) (1:500, Cypex Ltd., Dundee, UK), rabbit anti-hepatocyte nuclear factor 4 alpha (HNF4 α) (1:100, Santa Cruz Biotechnology, Santacruz, CA), rabbit anti-carbamoylphosphate synthetase-1 (CPS-1) (1:500, a generous gift from Dr. Wouter Lamers, University of Amsterdam, The Netherlands), mouse anti-glutamine synthetase (GS) (1:300, BD-Transduction Labs, Cowley, UK), rat anti-platelet/endothelial cell adhesion molecule-1 (PECAM) (1:200, BD-PharMingen, San Diego, CA), mouse anti-smooth muscle actin (SMA) (1:100), mouse anti-OV6 (1:50, a generous gift from Professor Stewart Sell, Ordway Research Institute and Wadsworth Center, Albany, NY, USA), mouse anti-CK20 (Abcam, Cambridge, UK), rabbit anti-Sox9 (1:100, Millipore), and sheep anti-UGT (1:200, Cypex, Scotland, CYP601). The following secondary antibodies were used at a concentration of 1:200: Anti-rabbit fluorescein isothiocyanate (FITC), anti-rabbit Texas Red, anti-sheep Texas Red, anti-mouse FITC, and anti-rat FITC (all Vector, Peterborough, UK). Alexa Fluor[®] 488-conjugated anti-rabbit immunoglobulin G (IgG; Invitrogen) was used at a dilution of 1:300. FITC-conjugated *Dolichos biflorus* agglutinin (DBA; Vector) was used at a dilution of 1:500 and 4,6-diamidino-2-phenylindole (DAPI; Sigma) was used at 1:1000 to detect the nuclei.

For western blotting, rabbit anti-SOX9, rabbit anti-C/EBP α (Santa Cruz, sc-61), and goat anti-LaminB1 (Santa Cruz, sc-30264) were used at 1:1000. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, anti-goat IgG (Santa Cruz) and anti-mouse IgG (Vector Laboratories) were used at 1:1000.

Immunocytochemistry

Hepatocytes were cultured on coverslips and fixed in 4% (wt/vol) paraformaldehyde (pH 7.4) for 20 min at room temperature. To permeabilize the cells, they were incubated in 0.1% (vol/vol) Triton X-100 in PBS for 20 min at room temperature. Each sample was blocked in 2% (wt/vol) blocking buffer (Roche) for 1–2 hr and incubated with primary antibody overnight at 4°C. After 3 $\times$ 15-min washes in PBS, the cells were exposed to a fluorochrome-conjugated

secondary antibody for 2 hr in the dark at room temperature. Excess antibody was removed by 3 $\times$ 15-min washes in PBS, and the nuclei were stained by incubation in 500 ng/mL DAPI (Sigma) for 10 min at room temperature. The coverslips were rinsed briefly three times in PBS and mounted in Gel-Mount TM (superscript) mounting medium (Biomedica, Foster City, CA, USA).

Adult rat liver was fixed in 4% (wt/vol) paraformaldehyde overnight at 4°C and processed sequentially through the following: PBS, 50% ethanol, 70% ethanol, 90% ethanol, 2 $\times$ 100% ethanol, 2 $\times$ Histoclear (Fisher Scientific), and 3 $\times$ paraffin wax for 1 hr each. The tissue was embedded in paraffin wax and sectioned into 4- μ m ribbons. For immunostaining, sections were dewaxed and rehydrated by sequential incubation in 2 $\times$ Histoclear for 7 min each and then 2 $\times$ 100%, 95%, 90%, 70%, and 50% ethanol for 1 min each. The tissue was permeabilized in 0.5% (vol/vol) Triton X-100 in PBS for 30 min at room temperature. For antigen retrieval, sections were incubated in 1 $\times$ EDTA heat-induced epitope retrieval buffer (Fisher Scientific) in distilled water for 30 min at 90°C and then cooled to room temperature. Endogenous peroxidases were quenched using Dako EnVision peroxidase block (Dako) according to the manufacturer's instructions. The sections were blocked in 2% blocking buffer (Roche) for 1 hr at room temperature and then incubated with 1:50 anti-SOX9 antibody overnight at 4°C. Following 3 $\times$ 10-min washes in PBS, the samples were 3,3'-diaminobenzidine (DAB)-stained using a Dako Envision kit according to the manufacturer's instructions. The sections were counterstained with 0.2% Light Green in 95% ethanol for 45 sec and then dehydrated by sequential incubation in two baths of 100% ethanol for 1 min each, and 2 $\times$ Histoclear for 2 min each. Slides were mounted in DPX Mounting Medium.

Western blotting

For extraction of nuclei, two 10-cm dishes of primary rat hepatocytes were scraped on ice in 1 mL of ice-cold cytosolic extraction buffer [20 mM HEPES, 10 mM KCl, 1 mM EDTA, 10% glycerol (pH 7.9) plus protease inhibitor cocktail (4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin, Sigma P8340)]. To ensure efficient disruption of plasma membranes, the lysate was incubated for 30 min at 4°C on a rotator and then pulled 10 times through a 27-gauge needle. Nuclei were collected by centrifugation at 16,300 $\times$ g at 4°C for 5 min and washed three times by repeated resuspension in 500 μ L of cytosolic extraction buffer and centrifugation. The final pellet was resuspended in 200 μ L of ice-cold nuclear extraction buffer [20 mM HEPES, 10 mM KCl, 400 mM NaCl, 1 mM EDTA, 20% glycerol (pH 7.9) plus protease inhibitor cocktail] and incubated for 1 hr at 4°C on a rotator to lyse the nuclei. Samples were then centrifuged at 16,300 $\times$ g for 15 min at 4°C, and the supernatant retained as the nuclear fraction.

The protein concentration of nuclear extracts was determined using the Bio-Rad protein assay (Bio-Rad, UK). Normalized protein samples were mixed with an equal volume of 2 $\times$ sample buffer [125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.2 M dithiothreitol (DTT), 0.02% Bromophenol Blue] and denatured at 100°C for 5 min. A 10 μ g amount of sample was loaded onto a 5% or 10% Tris-HCl Criterion[™] Precast Gel (BioRad) alongside a

Precision Plus Dual-Colour standard (Bio-Rad) and subjected to electrophoresis at 100V in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). The protein was wet transferred onto a Immobilon-PTM polyvinylidene fluoride (PVDF) 0.2- μ m membrane (Bio-Rad) for 2 hr at 200 mA in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Blots were stained with Ponceau S [0.6% (wt/vol) Ponceau S (Sigma), 2.8% acetic acid] to detect the protein and then blocked in 5% (wt/vol) skimmed milk (Marvel) in PBS-T (0.1% Tween-20 in PBS) overnight at 4°C. The membranes were exposed to primary antibody diluted in 3% milk in PBS-T for 2 hr at room temperature. After 3 $\times$ 15-min washes in PBS-T, blots were incubated with a HRP-conjugated secondary antibody diluted in 3% milk in PBS-T for 1 hr at room temperature. The membranes were then washed in PBS-T for 3 $\times$ 15 min and covered in ECL detection reagent (GE Healthcare, UK) for 5 min at room temperature. Bound secondary antibody was detected by ECL chemiluminescence (GE Healthcare), which was detected by autoradiography on MIDSCI Classic Blue Autoradiography film BX (Cole-Parmer, UK).

Reverse transcription polymerase chain reaction

Primers were designed using Primer3 software (Rozen and Skaletsky, 2000) and synthesized by Eurofins MWG Operon (Ebersberg, Germany) and are shown in Table S1 (Supplementary Data are available at www.liebertpub.com/cell/). Total RNA was extracted using TRI ReagentTM (Sigma) according to the manufacturer's instructions. To remove genomic DNA contamination, 5 μ g of RNA was treated with 0.2U/ μ L of Ambion RNase-free DNase I (Applied Biosystems, UK) for 30 min at 37°C. The DNase was inactivated by a further incubation at 65°C for 10 min. DNase I-treated RNA (2 μ g) was reverse transcribed by Superscript II Reverse Transcriptase according to the manufacturer's instructions. Then 100 ng of cDNA was amplified using ReddyMixTM PCR Master Mix (1.5 mM MgCl₂) with KCl buffer (Fisher Scientific) and specific primers under the following conditions: Initial denaturation 94°C for 5 min, PCR (22–30 cycles) denaturation 94°C for 60 sec, annealing at 56°C (or 60°C for *Hnf6*) for 60 sec, elongation at 72°C for 60 sec, and final extension at 72°C for 5 min. PCR products were run on a 2% agarose gel.

Image collection

Images of cells in culture were collected on a DMIRB fluorescent microscope (Leica) with a SPOTTM digital camera. A DMRB microscope (Leica) and Nikon digital camera were used to collect all other fluorescent and bright-field images. Figures were assembled in Photoshop 7.0.1. Only linear adjustments of brightness and contrast were made, and any adjustments were applied equally to comparable panels.

Results

Characterization of freshly isolated liver cells

To establish the purity of the hepatocyte population obtained by collagenase digestion, we fixed freshly isolated rat hepatocytes and determined the expression of hepatocyte, ductal, endothelial, and connective tissue markers by immunohistochemistry. The isolated cells represent a relatively pure population of parenchymal hepatocytes that express transfer-

rin and are derived from both the periportal and perivenous zones of the liver (expressing CPS and GS, respectively) (Fig. 1A, B). No staining for PECAM-1, SMA, or DBA was observed, indicating that there were no contaminating populations of endothelial cells, connective tissue, or intrahepatic ductal cells, respectively. A small subpopulation of vimentin-positive cells was present that comprised $0.039 \pm 0.009\%$ of the total. These cells were most often found within clumps of hepatocytes, and their numbers were reduced by ensuring that a single-cell suspension of hepatocytes was obtained.

Dedifferentiation of hepatocytes

Hepatic gene expression was compared in differentiated and dedifferentiated hepatocytes by reverse transcription polymerase chain reaction (RT-PCR) and immunostaining. Following 5–6 hours in attachment medium, the cells were cultured in either KS medium or DS medium until day 7. Hepatocytes in KS medium maintained an epithelial morphology throughout the culture period and continued to transcribe *Cps1*, *Gck*, *Cebpx*, and *Cebpb* at levels equivalent to freshly isolated cells (Fig. 1A, C). Immunostaining showed that they maintained their characteristic hepatic proteins [albumin, transferrin, UDP-glucuronosyltransferase (UGT), and HNF4] (Fig. 1D). In contrast, DS medium promoted dedifferentiation to a mesenchymal morphology, associated with rapid loss of *Cebpx*, *Cps1*, and *Gck* (Fig. 1D). At the protein level, albumin, transferrin, UGT, and HNF4 were all lost, and strong staining of vimentin appeared.

Lineage analysis of dedifferentiated hepatocytes

Whenever a change of phenotype occurs in cultured cells, it is necessary to demonstrate that the effect is not due to selection. This is because a rapidly growing minority cell population could become a significant proportion of the total cells after a 7-day culture period. Mouse hepatocytes undergo dedifferentiation in a similar manner to rat hepatocytes (Klaunig et al., 1981), so we designed a lineage experiment using *Alb-Cre;R26R LacZ* mice. In these mice, Cre recombinase is expressed in hepatocytes, which have activated the *albumin* promoter, but not in other cell types. The non-hepatocytes present in small numbers in the original culture are not labeled. Hepatocytes were isolated from *Alb-Cre R26R LacZ* mice using the method described for rat hepatocytes, and cultured in DS medium. By day 8 of culture, the vast majority of the cells with mesenchymal morphology were X-Gal positive and therefore must have been derived from hepatocytes that once expressed albumin, rather than being derived by the expansion of the small subpopulation of vimentin-positive cells (Fig. 2A–C). A few of the cells with mesenchymal morphology were X-Gal negative, but this is also true in positive control cultures set up from *Rosa 26* mice in which all cell types express β -galactosidase constitutively from the same promoter. This comparison shows that there is no overgrowth by nonhepatocytes.

To complement the lineage-tracing technique, we also followed dedifferentiating hepatocytes from wild-type C57BL/6 mice in culture using time-lapse photography. This allows continual monitoring of dedifferentiation for periods up to 4.85 days. Videos from multiple hepatocyte isolations and individual culture areas were viewed and frames from a representative culture are shown in Figure 2D–F. The

FIG. 1. Hepatocytes in KS and DS culture media. (A) Phase-contrast views of rat liver cells immediately after isolation and after culture in KS or DS medium. Scale bars, 50 μ m. (B) Immunostaining of liver cell markers in freshly isolated cells. Antibodies are green and DAPI blue. Scale bars, 50 μ m. (C) RT-PCR of key mRNAs in hepatocytes cultured in differentiation or dedifferentiation conditions. In KS medium, liver gene expression is maintained, whereas cells in DS medium downregulate transcription of *Cps1*, *Gck*, and *C/ebpa*, but maintain *C/ebpb* expression. Controls include a no reverse transcriptase (RT) reaction and a negative control reaction (negative) that did not contain sample. Cycle numbers: β -actin, 22; *Cps1*, 23; *Gck*, 28; *C/ebpa*, 28; *C/ebpb*, 28. (D) Immunostaining of proteins in hepatocytes cultured in differentiation or dedifferentiation conditions. Cells were cultured for 7 days in KS (1 μ m Dex) or DS, and then fixed and immunostained for albumin (alb, green), transferrin (TF, red), UGT (red), HNF4 (green), and vimentin (vim, green). Nuclei were stained with DAPI. Scale bars, 10 μ m.

complete video is shown in the Supplementary Data. Over the course of this culture, cells exhibiting typical hepatocyte morphology, evident after initial plating (Fig. 2D), are seen to become both progressively more fibroblastic in morphology and more motile as the culture advances. Very few cells of the starting hepatocyte population are lost by cell death in the course of the culture, and no rapidly dividing minority cell population is evident. The video data support the idea that, when cultured in DS medium, hepatocytes undergo a rapid change to a mesenchymal morphology.

Dedifferentiated hepatocytes express biliary genes

Although the transformation of hepatocytes to a mesenchymal morphology is conventionally described as “dedifferentiation,” we found that genes whose expression is characteristic of biliary epithelial cells were upregulated. *Sox9*, which encodes a transcription factor regulating bile duct development, was rapidly induced within 24 hr of DS culture and its level progressively increased thereafter (Fig. 3A). In addition, dedifferentiated hepatocytes expressed various characteristic ductal products: *Osteopontin*, encoding

FIG. 2. Dedifferentiated cells are derived from hepatocytes. (A–C) *Albi-Cre x R26R LacZ* hepatocytes were fixed and stained with X-Gal. (A) One-day culture. (B) Eight-day culture in DS medium. (C) Hepatocytes from CD1 mouse, 8-day culture in DS medium (negative control). Scale bars, 100 μ m. (D–F) Time-lapse microscopy was used to monitor the acquisition of mesenchymal morphology by freshly isolated mouse hepatocytes in DS culture. D–F are frames taken from a video lasting 4.85 days. (D) Hepatocytes after initial plating with five individual cells marked by arrows that were followed during the time lapse. (E) After 29 hr in culture, the labeled cells have acquired fibroblast-like morphologies. (F) This is maintained after 53 hr of culture; cell 4 is not dead but dividing.

an extracellular matrix protein, *Ck7* and *Ck19*, encoding ductal cytokeratins, and *Cx43*, encoding a gap junction protein. Some of these markers are also found in hepatic myofibroblasts, but for various reasons we consider that the phenotype is duct-like (see Discussion). Low-level expression of *Sox9* and *Ck19* was occasionally found in KS-cultured cells by day 5, indicating incipient dedifferentiation, but was never present on day 1. SOX9 protein was detectable both by western blotting and immunostaining (Fig. 3B, C). CK20, a ductal cytokeratin, and OV6, an antigen present on developing bile ducts, were also observed in dedifferentiated cells by immunostaining (Fig. 3D).

Duct-like cells are derived from differentiated hepatocytes

Immunohistochemistry of adult rat liver confirmed that SOX9 is expressed specifically in intrahepatic bile ducts and not in hepatocytes (Fig. 3E). Combined with the observation that SOX9 regulates development of murine intrahepatic bile ducts (Antoniou et al., 2009), this suggests that SOX9 may mediate the upregulation of ductal genes such as *Ck19* and *Osteopontin* during hepatocyte dedifferentiation (Fig. 3A).

To exclude the possibility that the induction of ductal genes in DS cultures occurred in a nonhepatocyte population, or simply reflected proliferation of contaminating duct cells, the hepatocyte lineage was traced in the short term by immunostaining for the hepatocyte-specific enzyme UGT. In some cells, residual UGT protein still remains immunodetectable for up to 5 days, suggesting that this liver enzyme is one of the last to be lost in the course of dedifferentiation. Examination of DS cells in the early phase of dedifferentiation showed that UGT and SOX9 were extensively coex-

pressed, confirming that *Sox9* was indeed induced in the dedifferentiating hepatocytes (Fig. 3F).

SOX9 activates ductal genes and represses hepatic genes in differentiated hepatocytes

Hepatocyte dedifferentiation and upregulation of ductal markers correlated with the repression of *C/ebp α* and induction of *Sox9*. However dedifferentiation is a complex process involving up- and downregulation of numerous genes. Therefore, to demonstrate a causal relationship between changes in *C/ebp α* and *Sox9* expression and reprogramming, we employed adenoviral vectors to express these two factors in dedifferentiated and differentiated hepatocytes, respectively. Hepatocytes were cultured for 12 hr in KS medium and then were infected on day 1 with an adenoviral vector expressing either *Sox9* (*Ad-Sox9*) or *GFP* (*Ad-GFP*). By day 5, *Ck19*, *Osteopontin*, and *Cx43* were induced in the *Sox9*-expressing cells (Fig. 4A). Furthermore, differentiated hepatocytes infected with *Ad-Sox9* downregulated expression of *C/ebp α* , *C/ebp β* , *Gck*, and, to a lesser extent, *Cps1* (Fig. 4B). Thus, in addition to activating the duct gene expression profile, *Sox9* can contribute to the suppression of the hepatocyte phenotype.

C/EBP α represses ductal gene expression in dedifferentiated hepatocytes

To establish whether repression of *C/ebp α* is required for the induction of biliary genes, hepatocytes were kept in DS medium for 12 hr then infected on day 1 with *Ad-C/ebp α* or *Ad-GFP*. Restoration of *C/ebp α* expression repressed transcription of *Sox9* and *Ck19*, although effects on *Osteopontin* and *Cx43* were limited (Fig. 4C). It also partially rescued transcription of its target *Cps1* (Fig. 4D).

These results show that SOX9 and C/EBP α are mutually antagonistic. Each represses the expression of the other. The antagonism is reflected in the reciprocal regulation of hepatic and biliary gene expression, *Sox9* promoting ductal differentiation whereas *C/ebp α* promotes hepatic differentiation. The dedifferentiation of hepatocytes in culture may therefore facilitate reprogramming to biliary cells because the loss of C/EBP α relieves repression of *Sox9*, which in turn can activate downstream ductal genes.

Discussion

Identity of dedifferentiated cells

In this work, we have considered that the product of dedifferentiation of hepatocytes cultured for several days in DS medium is a bile duct-like cell, or at least a partial transformation toward this phenotype. This is based on the observed upregulation of *Sox9* and on the appearance of the ductal markers cytokeratins 7, 19, and 20, OV6, osteopontin, and *Cx43*. But another possibility for the identity of the dedifferentiated hepatocytes is that they are activated stellate cells. The hepatic stellate cells, also known as Ito cells, are located in the subendothelial space, and following liver damage they become activated and proliferate, eventually causing cirrhosis of the liver (Friedman, 2008). When activated they are known as myofibroblasts. These cells are known to share some of the same gene and protein expression profile as dedifferentiated hepatocytes, including CK19, osteopontin, and *Cx43*

FIG. 3. Ductal differentiation of dedifferentiated hepatocytes. (A) Dedifferentiated hepatocytes express ductal gene products. *Sox9*, *Ck19*, *Ck7*, *Osteopontin*, and *Cx43* were upregulated in dedifferentiated, but not in differentiated, cells. Cycle numbers: β -actin, 22; *Sox9*, 30; *Ck19*, 30; *Ck7*, 30; *Osteopontin*, 25; *Cx43*, 30. (B) Western blots of nuclear extracts. During dedifferentiation, C/EBP β protein is reduced and SOX9 is increased. (C) SOX9 is upregulated in dedifferentiated hepatocytes. SOX9 immunostain is green; DAPI is blue. KS cells are SOX9-negative after 24 hr. By 5 days, some SOX9 is present in the differentiated hepatocytes, but much greater expression is observed in the dedifferentiated cells. Scale bar, 50 μ m. (D) OV6 and CK20 in dedifferentiated cells. Immunostain green, DAPI, blue. Scale bars, 10 μ m. (E) In adult rat liver, SOX9 is expressed in biliary epithelial cells but not in hepatocytes (immunostain with DAB detection, brown). In the small bile duct (left), all nuclei are positive; in the larger one (right), some nuclei have lost SOX9. Scale bar, 25 μ m. (F) Hepatocytes cultured for 5 days in DS medium were immunostained for SOX9 (green) and UGT (red) and counterstained with DAPI (blue). At this time, SOX9 and UGT are often coexpressed, including in binucleate cells (star, in merge panel). Scale bars, 50 μ m.

(De Minicis et al., 2007; Fischer et al., 2005; Lim et al., 2002). Most significantly, they can also upregulate *Sox9* on activation (Piper Hanley et al., 2008). Although a myofibroblast identity is a definite possibility, on balance we consider that a ductal identity is more likely.

There are various reasons for this. First, a recent lineage labeling study has shown that the stellate cells are of mesodermal (septum transversum) origin (Asahina et al., 2011). We know from our lineage labelling studies that the dedifferentiated/bile duct-like cells are derived from hepatocytes.

FIG. 4. Ectopic *Sox9* induces ductal genes, whereas *C/ebp α* suppresses them. (A) Effects of *Ad-Sox9* on differentiated hepatocytes cultured in KS medium. Controls include freshly isolated hepatocytes (isolate), day-5 DS hepatocytes (positive), and reactions without reverse transcriptase (–RT) or without sample (negative). Cycle numbers: β -actin, 22; *Sox9*, 28; *Ck19*, 30; *Osteopontin*, 30; *Cx43*, 30; *Ck7*, 30; *Hnf1 β* , 30. (B) *Ad-Sox9* represses expression of *C/ebp α* in differentiated hepatocytes. Cycle numbers: β -actin, 22; *Sox9*, 28; *C/ebp α* , 28; *C/ebp β* , 28; *Cps1*, 27; *Gck*, 27. (C) *Ad-C/ebp α* represses induction of *Sox9* and *Ck19* in dedifferentiating hepatocytes. Cycle numbers: β -actin, 22; *C/ebp α* , 28; *Sox9*, 27; *Ck19*, 27; *Osteopontin*, 25; *Cx43*, 27. (D) *Ad-C/ebp α* restores expression of *Cps1* in dedifferentiating hepatocytes. Cycle numbers: β -actin, 22; *C/ebp α* , 28; *Cps1*, 25; *Gck*, 30.

A transformation across germ layers is not impossible, but it seems less likely that one between developmentally related cell types such as hepatocytes and bile duct cells. Second, the stellate cells contain numerous lipid droplets, many of which are retained after activation (Friedman, 2008), while our dedifferentiated cells actually lose the few droplets apparent in the original hepatocytes. Finally, the rate of *Sox9* appearance on activation of stellate cells (Piper Hanley et al., 2008) seems somewhat slower than described here.

C/EBP α is required to maintain lineage commitment in adult hepatocytes

The reciprocal repression of synthesis of *C/EBP α* by *Sox9* and of *SOX9* by *C/ebp α* has implications both for the mechanism of hepatocyte dedifferentiation in culture and for the maintenance of lineage commitment in adult liver *in vivo*. The expression of ductal genes during dedifferentiation is likely to reflect the downregulation of *C/ebp α* , which

in turn relieves transcriptional repression of *Sox9*. This suggests that *in vivo*, *C/EBP α* -mediated repression of *Sox9* may be continuously required to maintain hepatocyte differentiation. A requirement for active maintenance of lineage commitment by cell type-specific transcription factors has been described in several other systems. For example, conditional deletion of *Pax5* causes pro-B cells cultured *in vitro* to differentiate into macrophages on exposure to the myeloid cytokine macrophage colony-stimulating factor (M-CSF) (Mikkola et al., 2002). Similarly, deletion of *FoxL2* in adult female mice results in transdifferentiation of granulosa cells (somatic cells that support the oocytes), to male-specific Sertoli cells (Uhlenhaut et al., 2009).

Reprogramming of hepatocytes to biliary-like cells recapitulates development

Reprogramming of differentiated cells is usually achieved experimentally by overexpressing transcription factors that

regulate embryonic development of a particular tissue type in adult cells of a different tissue type. For example, a combination of PDX1, NGN3, and MAFA can convert pancreatic exocrine cells to insulin-secreting β -like cells (Zhou et al., 2008). SOX9 is an important regulator of embryonic bile duct development (Antoniou et al., 2009). Development of murine intrahepatic biliary ducts (IHBDs) begins with the appearance of a single layered ring of SOX9-expressing cells around branches of the portal vein. Thus, SOX9 is the earliest specific marker of IHBD differentiation. As development proceeds, focal areas of the SOX9-positive ring of cells become bilayered, giving rise to asymmetrical ductal structures that are lined on the portal side by biliary cells and on the parenchymal side by hepatoblasts. The hepatoblasts differentiate into biliary epithelial cells to form symmetrical, SOX9-positive bile ducts. However in the absence of *Sox9*, the maturation of asymmetrical ductal structures to symmetrical ducts is delayed. The ability of SOX9 to induce expression of ductal genes in hepatocytes is therefore consistent with its role in normal liver development.

Mutual antagonism of C/EBP α and SOX9 determines hepatoblast fate

Cross-antagonism of lineage-instructive transcription factors often underlies binary differentiation of progenitor cells. In hematopoietic development, for example, an antagonistic interaction between PU.1 and GATA1 controls differentiation of myeloerythroid precursors into myeloid and erythroid lineages (Graf and Enver, 2009). Similarly, mutual repression of FOXC2 and PAX3 regulates differentiation of vascular smooth muscle and skeletal muscle from multipotent cells of the dorsal somite (dermomyotome) (Lagha et al., 2009). Our analysis of the reprogramming of hepatocytes to duct-like cells may therefore be used to make predictions about the acquisition of hepatocyte and biliary fate during development. Because C/EBP α and SOX9 activate hepatic and ductal gene sets, respectively, and are mutually antagonistic, these two factors may determine the direction of hepatoblast differentiation in normal development. Consistent with this idea, C/EBP α , which is downregulated in wild-type biliary cells by e18.5, persists on both the portal and parenchymal sides of primitive ductal structures in *Alfp-Cre;Sox9^{fl/fl}* animals (Antoniou et al., 2009). This continued expression of C/EBP α correlates with delayed maturation of asymmetrical ductal structures to symmetrical ducts. Yamasaki et al. have previously shown that in *C/ebp α* knockout mice pseudoglandular structures coexpressing hepatocyte and biliary markers develop throughout the liver parenchyma (Yamasaki et al., 2006). These authors suggested that the ectopic expression of ductal genes is a result of elevated expression of *Hnf6* and its target *Hnf1 β* . However, our data imply that upregulation of *Hnf6* and *Hnf1 β* may occur as a consequence of the induction of *Sox9*, which is repressed by C/EBP α in wild-type cells.

Dedifferentiation may promote reprogramming of hepatocytes into liver progenitor cells

Furuyama et al. (2011) demonstrated by genetic lineage tracing that physiological cell replacement in adult liver depends on *Sox9*-expressing progenitors located in the biliary tree. Therefore, it is possible that dedifferentiated hepatocytes, which are SOX9-positive, have the potential to

acquire stem cell-like properties. There is a recent report of the derivation of cells with liver progenitor properties from dedifferentiated hepatocytes (Chen et al., 2012). Consistent with this idea, induction of epithelial-to-mesenchymal transition (EMT) in human mammary epithelial cells gives rise to cells that both express stem cell markers and have an increased ability to form mammospheres in suspension culture (Mani et al., 2008). However a marker that could be used to uniquely identify actively self-renewing stem cells in DS cultures is yet to be described (Huch and Clevers, 2011).

In summary, the reprogramming of differentiated cells can represent a useful model of cell fate acquisition and maintenance. Conversion of hepatocytes into biliary-like cells is likely to be controlled by reciprocal repression of C/EBP α and SOX9, which induce hepatocyte- and duct-specific genes, respectively. During dedifferentiation, downregulation of C/EBP α relieves repression of *Sox9*, and SOX9 in turn can activate transcription of downstream biliary genes. *In vivo*, C/EBP α -mediated repression of *Sox9* probably maintains lineage commitment of adult hepatocytes by blocking activation of the ductal program, and antagonistic expression of C/EBP α and SOX9 may also control hepatoblast differentiation in normal development. Mutual antagonism of lineage-activating transcription factors is found in a range of tissue contexts. In terms of practical applications, it is therefore likely that a phase of dedifferentiation can be used to improve the efficiency of experimental cell reprogramming.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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