Converting Skin Fibroblasts into Hepatic-like Cells by Transient Programming

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ABSTRACT

Transplantation of hepatocytes is a promising therapy for end-stage liver disease, but the availability of functional cells currently precludes its clinical application. We now report a simple transient reprogramming approach to convert fibroblasts into hepatic-like cells. Human skin fibroblasts were treated with fish egg extracts to become the transiently remodeled cells (TRCs). After infected with retroviral EGFP, they were directly injected into the fetal monkey liver, where they underwent in situ differentiation in the hepatic niche. The hepatic-like cells were functional as shown by the synthesis of hepatic markers in vivo, including albumin, cytokeratin-18, and hepatic serum antigen. Similarly, when implanted in the mouse liver, the TRCs were differentiated into hepatic-like cells that synthesize albumin and CK18 and became completely integrated into the liver parenchyma. The potency of TRCs was mechanistically related to the activation of several signal pathways, which reactivate endogenous genes related to cell potency. This study demonstrates the feasibility of a simple and inexpensive epigenetic remodeling approach to convert human fibroblasts into therapeutic hepatic-like cells for the treatment of end-stage liver disease. J. Cell. Biochem. 117: 589–598, 2016.   © 2015 Wiley Periodicals, Inc.

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Although liver transplantation is a standard therapy for patients with end-stage liver disease [Ogawa and Miyagawa, 2009; Kisseleva et al., 2010], many patients cannot benefit from this therapy because of the short supply of donor livers. In those patients who do undergo liver transplantation, the need for chronic immunosuppressive therapy has a detrimental impact on their quality of life. Therefore, new approaches to hepatic replacement therapy are urgently needed.

Transplantation of isolated hepatocytes is a minimally invasive approach as compared with organ transplantation in augmenting the function of the damaged liver [Grompe, 1999; Dhawan et al., 2010]. However, primary hepatocytes do not replicate sufficiently in vitro to generate sufficient cells needed for transplantation [Fitzpatrick et al., 2009]. As a potential alternative, therapeutic hepatocytes might be generated by differentiating specific types of stem cells. Mouse ESCs can be differentiated into mature hepatocytes in vitro [Hamazaki et al., 2001; Jones et al., 2002], but the use of currently available human ES cell lines in patients risks rejection by the patients' own immune system.

Creating "autologous" patient-specific pluripotent stem cells specific for individual patients by therapeutic cloning offers an alternative methodology. Induced pluripotent stem cells (iPSCs) derived from viral transduction of defined factors [Takahashi and Yamanaka, 2006] offer a promising alternative. Recently, Si-Tayeb et al differentiated induced stem cells into hepatocyte-like cells that

display key liver functions in vivo [Si-Tayeb et al., 2010]. However, some hurdles need to be resolved before the use of iPSCs can be translated to the clinic. Moreover, the iPSCs may not become completely differentiated, and the integration of the viral vector used to create the iPSCs remains a potential problem for human use [Okita et al., 2007; Takahashi et al., 2007]. Finally, full reprogramming of human somatic cells is a very inefficient and considerably time-consuming process [Takahashi et al., 2007; Park et al., 2008]. Long periods of cell culture and iPSC selection may lead to subtle genetic and epigenetic abnormalities in iPSCs, like those recently reported in de novo generation of copy number variations [Hussein et al., 2011], somatic coding mutations [Gore et al., 2011], un-erased epigenetic memory [Deng et al., 2009; Kim et al., 2010; Polo et al., 2010], aberrant DNA methylomes [Lister et al., 2011], and altered genomic imprinting [Pick et al., 2009; Stadtfeld et al., 2010].

Alternatively, somatic cells can be reprogrammed directly into hepatic-like cells by viral transduction of transcription factors, without a requirement for embryonic stem (ES) cells or induced pluripotent stem cells [Huang et al., 2011; Sekiya and Suzuki, 2011]. This innovative strategy will allow the production of hepatocytes of diverse genetic backgrounds for medical research and transplantation. However, the reprogramming factors are delivered by lentiviral or retroviral infection. There is a concern that integration of the viral vectors into the target cell genome could potentially disrupt key genes and promote unpredictable cell growth or differentiation [Swenson, 2012].

To overcome these obstacles, we propose to test a simple transient reprogramming approach to convert human skin fibroblasts into functional hepatic-like cells. The therapeutic potential of these induced hepatic-like cells was tested in vivo in both mouse and fetal monkey livers.

MATERIALS AND METHODS

CULTURE OF HUMAN SKIN FIBROBLASTS

Primary human fibroblasts were cultured from foreskin tissues of a healthy male after circumcision. The tissue sections ($1 \times 1\,cm^2$) were digested in DMEM (Invitrogen, CA) containing 200 U/ml collagenase and 300 U/ml hyaluronidase (Sigma, MO), and were further digested with 0.25% trypsin (Invitrogene, CA). After centrifugation, cells (2×10^5/ml) were cultured in DMEM/F12 and used for cell reprogramming. All experimental protocols and procedures were approved by the Institutional Care and Use Committee at Kunming PLA General Hospital (Protocol number: SYXK2007-041). The donor was asked to sign the consent form, but no clinical information, including age, was recorded.

Two human fetal fibroblast cell lines (HBF2 and WSF1), derived from the skin of two human fetuses [Hu et al., 1996, 1997], were also cultured and expanded in the lab for cell reprogramming.

PREPARATION OF FISH OOCYTE AND MOUSE LIVER EXTRACTS

Fish oocyte extracts were prepared as previously described [Zhu et al., 2009]. Briefly, fish oocytes with profuse yolk (silver carp) collected from a fish supermarket were frozen in liquid nitrogen, ground by mortar, and extracted with equal volumes of 0.9% sodium

chloride. After passing through two layers of 35 m nylon mesh, the samples were centrifuged and the supernatants were filtered through 0.2 um filters. Protein levels were determined as described [Yao et al., 2003a] and the extracts were diluted using 0.9% sodium chloride to 10 mg protein/ml as stock extracts and preserved at −80C for studies.

IN VITRO CELL TRANSIENT REMODELING OF FIBROBLASTS

The "transiently remodeled cells" (TRCs) were generated using a method with minor modification as previously described [Zhu et al., 2009]. Briefly, fibroblasts were cultured in $60 \times 15\,mm$ uncoated petri dishes (#8609-0160, Fisher, CA) in the reprogramming medium [DMEM/F12 supplemented with 20% Knockout Serum Replacer (KSR), 2 mM non-essential amino acids (NAA), 0.1 μM β-mercaptoethanol (all from Invitrogen, CA), and 4 ng/mL bFGF (Sigma), and 10 ng/ml Leukemia inhibitory factor LIF (Sigma)]. For the reprogramming group, 10 μg protein/ml fish oocyte extracts were added to the medium. For the control group, we simply replaced the oocyte extracts with equal volume of PBS in the same reprogramming medium. After 24–48 hrs of culturing, the sphere-like TRCs were collected and used for the analysis of gene expression and in vivo transplantation. With this simple oocyte extract remodeling, the treated cells acquired the potential to differentiate into other cell types under appropriate differentiation conditions [Zhu et al., 2009].

CELL LABELING BEFORE TRANSPLANTATION

In order to track the transplanted cells in animals, TRCs were first labeled with a lentivirus carrying the EGFP tracking marker. Specifically, EGFP was amplified from the pEGFP vector (Clontech, CA) and cloned into the pCDH-CMV-EF1-Puro vector (System Biosciences, CA). Lentivirus was packaged and concentrated using PEG-IT Virus Precipitation Solution (System Biosciences, CA) as previously described [Zhang et al., 2011]. Exponentially-growing TRCs were infected with lentivirus (1:100) in the presence of 8 μg/ml polybrene for 72 hrs and collected for cell transplantation.

TRANSPLANTATION OF TRCs INTO THE LIVER OF MICE

Male Balb/C mice (6 weeks old), purchased from Kunming Animal Institute (Yuennan, China), were used for examining the in vivo differentiation of TRCs into hepatic-like cells. Mice were anesthetized intraperitoneally with 3% pentobarbital sodium (1 μl/g body weight of mouse). After sterilization with 75% ethanol, a transection incision below the sternum was made and 5×10^7 TRCs were slowly injected into the right lobe of the liver with a 28-gauge needle as previously described [Yao et al., 2003a,b]. Animal studies were approved by the Animal Use Committee of the hospital.

In the absence of immunosuppressive agents, subcutaneous transplanted human embryonic stem cells survived up to seven days in Balb/C mice [Pearl et al., 2011]. We thus collected liver tissues on day 7 without the use of immunosuppressive agents. The collected tissues were immunohistochemically stained for human hepatic markers using anti-human albumin (Invitrogen, CA) and anti-human cytokeratin 18 (Abcam, MA). Total RNA was extracted using RNA extraction kit (Qiagen, GE) and quantitative PCR was used to detect hepatic CK18 and albumin.

ELISA DETECTION OF HUMAN-SPECIFIC ALBUMIN IN THE URINE

To ascertain functionality of the implanted cells, we measured the excretion of human albumin in the urine of the animals as previously described [Yaccoby et al., 2002]. One week following cell transplantation, urinary samples were collected and albumin was measured using a human albumin-specific enzyme-linked immunosorbent assay (ELISA) (Alpha Diagnostic International, TX), following the instruction provided by the manufacturer.

INJECTION OF TRCs INTO THE FETAL MONKEY LIVER

Five healthy pregnant monkeys (Rhesus Macacus) were purchased from Kunming Animal Institute (Yunnan, China) and were housed in the Clinical Animal Center of Kunming Army General Hospital. All animal studies were approved by the Animal Use Committee of the hospital.

To track the transplanted cells in animals, TRCs were first infected with EGFP-lentivirus. For cell transplantation, monkeys during the fourth month of pregnancy were anaesthetized. Under the guidance of B ultrasound, the 2×10^8 GFP-labeled TRCs were directly injected into the fetal liver using 10 cm biopsy needle gauges. Two weeks after delivery, the baby monkeys were sacrificed. Liver samples were collected, fixed in 4% formaldehyde, and used for immunostaining using 5-Bromo-2-deoxy-uridine antibody (Sigma, MO).

EXPRESSION OF STEM CELL RELATED GENES

Total RNA was extracted by TRI-REAGENT (Sigma, MO), and cDNA was synthesized with RNA reverse transcriptase. Gene expression was assessed by RT-PCR [Hu et al., 1995, 1996] or by real time PCR (qPCR) [Chen et al., 2006]. Primers used for PCR amplification included (1) OCT4: #J498 (forward), 5′-ACTGAGGTGCCTGCCCTTCTAGGA-3′ and #J499 (reverse), 5′-CAATTCCTTCCTTAGTGAATGAAGAAC-3′ (NM_002701 DNA sequences 1133-1254); (2) NANOG: #J492 (forward), 5′-TACCTCAGCCTCCAGCAGATGCAAG-3′ and #J493 (reverse), 5′-AGGCCTTCTGCGTCACACCATTGCT-3′ (NM_024865 DNA sequences 571-724); 3() CK18 forward, 5′-GCCACCGGGA-TAGCCGGGGGTCT-3′ and CK18 reverse, 5′-CGGGCATTGTCCACAG-TATTTGCG-3′; (4) CK19 forward, 5′-GATGCTGAAGCCTGGTTCACCA-3′ and CK19 reverse, 5′-GCGCCAGCTGGGCTCCAAAGCGC-3′; (5) Albumin (ALB) forward, 5′-GAAGAAAATTTCAAAGCCTTGGTGT-3′ and CK19 reverse, 5′-GTTTCACGAAGAGTTGCAACTGTG-3′; (6) β-ACTIN: #774 (forward), 5′-CAGGATTTAAAACTGGAACGGTGAAGG-3′ and #775 (reverse), 5′-AATGTGCAATCAAAGTCCTCGGCCACA-3′ (NM_001101 DNA sequences 1346-1442); and 7) 18S forward, 5′-GCGGCTTTGGTGACTCTA-3′ and 18S, 5′-CTGCCTCCTTGGATGTG-3′.

IMMUNOSTAINING

Immunostaining was performed using the method as previously described [Zhu et al., 2009]. Tissue sections were stained with antibodies against hepatocyte specific antigen (HSA) (Santa Cruse Biotechnologies, CA), albumin (Invitrogen, CA), beta-2-micro-globulin (B2M), and cytokeratin 18 (CK18) (Abcam, Cambridge, MA). 3,3′-diaminobenzidine (DAB) chromogenic liquid was purchased from MaiXin Bio-company (Fuzhou, China).

FLOW CYTOMETRIC ANALYSIS

Suspended cells (1×10^6) were fixed with 100 μl of 4% paraformaldehyde for 10–15 min and were incubated with 100 μl 0.5% saponin solution at room temperature for 15 min. After being washed twice with PBS/BSA buffer containing 0.5% saponin, cells were incubated with anti-Oct-3/4-PE and SSEA-4-PE antibodies (BD Biosciences, CA) at room temperature for 30 min. Isotype controls were stained with an isotype-matched control of irrelevant specificity. Quadrant markers based on isotype control and unstained cells were set. Data were acquired with a FACSCalibur flow cytometer and analyzed with CELLQuest software (BD Biosciences, CA).

For GFP-FACS analysis, the liver section containing implanted GFP-TRCs was collected and hepatocytes were isolated by collagenase digestion as previously described [Fiegel et al., 2003; Lange et al., 2006]. After repeated washing with PBS, cells were centrifuged at 500g for 3 min. An aliquot of the cell suspension (0.5 ml) was used for flow cytometry (BD Biosciences, CA) to determine the percentage of GFP-positive cells. Hepatocytes from the fibroblast-implanted liver were collected as a control in FACS analysis.

ACTIVITY OF SIGNAL PATHWAYS

The activity of signal pathways was measured by Western blotting as previously described [Chen et al., 2012]. Briefly, cells were harvested and proteins were separated by 7.5% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to poly-vinylidene fluoride membranes for immunoblotting with the following antibodies: phospho-AS160 (Thr^642, #4288), Phospho-p38 MAPK (Thr180/Tyr182, #28B10), phospho-β-catenin (Thr41/Ser45, #9565) (Cell signaling Technology, MA), and β-ACTIN (#ab3280, Abcam, MA).

STATISTICAL ANALYSIS

Data were analyzed using SPSS software (version 20.0; SPSS, IL) and one-way ANOVA (Bonferroni test) was used to compare statistical differences for variables among treatment groups. Data were expressed as mean ± SD. The comparative CT method was applied in the quantitative real-time RT-PCR assay according to the delta-delta CT method [Zhang et al., 2013; Li et al., 2014]. Results were considered statistically significant at $P < 0.05$.

RESULTS

REPROGRAMMING IN THE MONKEY LIVER

The cell microenvironment (or niche) is able to modify the epigenotype of a number of functional genes, leading to an altered cellular phenotype. As a typical example, intact somatic cells can be reprogrammed when transferred into enucleated oocytes [Chang et al., 2003; Lin et al., 2008]. For example, pretreatment of skin keratinocytes with ES conditioned medium epigenetically changes gene expression, including pluripotent markers *Oct4*, *Sox2*, and *Nanog* [Grinnell and Bickenbach, 2007]. Recently, we reported the induced reprogramming of skin fibroblasts by transient treatment using fish oocyte factors [Zhu et al., 2009]. The treated cells became reprogrammed and mimicked the behavior of mesenchymal stem cells.

In order to create a simple approach to directly convert human fibroblasts into functional hepatic-like cells, we transiently treated human fibroblasts with fish oocyte extracts (Fig. 1A). The factors in the extracts would induce the expression of stemness genes [Zhu et al., 2009] and reprogram cells into "transiently remodeled"
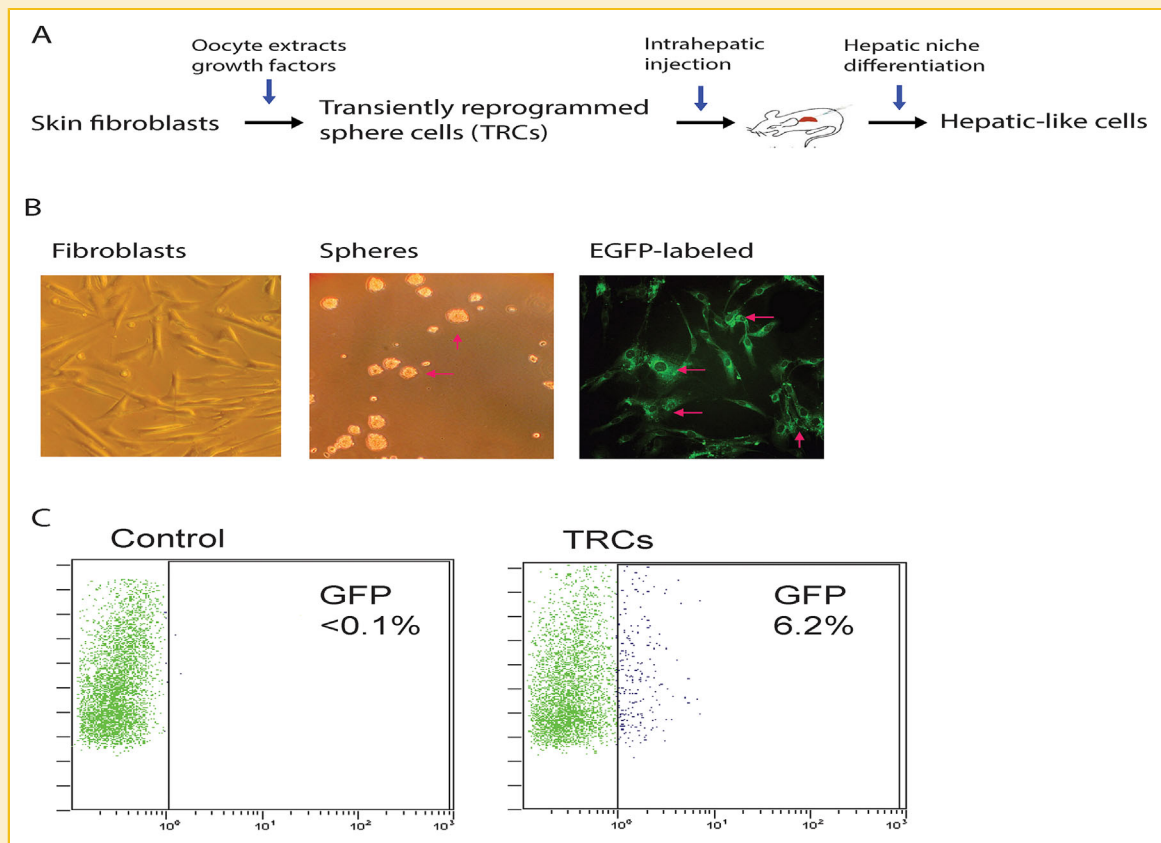
Fig. 1. Transplantation of TRCs in the fetal monkey liver. (A) Schematic diagram of transient reprogramming of fibroblasts into hepatic cells. After treatment with oocyte extracts, the sphere-like TRCs are transplanted into the liver, where they are differentiated into hepatic-like cells. TRCs: transiently remodeled cells. (B) Generation of hepatic-like cells by transient reprogramming. In the presence of oocyte extracts and growth factors (bFGF, LIF), sphere-like TRC cells formed in the attachment-free petri dish. The TRCs were infected with EGFP retrovirus prior to transplantation into the fetal monkey liver. Fetal livers were collected, fixed in 4% formaldehyde, and used for immunohistochemical staining or directly examined for EGFP fluorescence. Note the polygonal shape of hepatocytes (red arrows). Scale bars: 100 μm. Fetal hepatic cells were collected, FACS analysis of GFP in Livers. (C) Distribution of implanted TRCs in the monkey liver. Hepatocytes were isolated by collagenase digestion from the GFP-TRC-containing liver section and subjected to flow cytometry. Hepatocytes from the non-implanted liver section were used as the FACS control.

cells (TRCs). To track the transplanted cells, we infected TRCs with a retrovirus carrying the EGFP tracking marker. The GFP-labeled, sphere-like TRCs were then directly injected into the fetal monkey liver under the guidance of B ultrasound. We found that the injection of TRCs did not affect fetal development as all fetal monkeys were delivered at full term. After delivery, liver biopsies were performed on the newborn monkeys for analysis of human hepatic markers.

Using fluorescent EGFP as a marker, we found that the TRCs differentiated into the typical polygonal shape of hepatocyte-like cells, which were completely integrated into the fetal monkey liver parenchyma (Fig. 1B). The TRC-implanted liver section was collected and the FACS analysis showed that about 6.2% cells were EGFP-positive (Fig. 1C).

GENERATION OF HEPATIC-LIKE CELLS BY TRANSIENT REPROGRAMMING

To examine the function of these newly formed hepatocyte-like cells in the liver of the recipient fetal monkey, we used human-specific antibodies to immunohistochemically stain liver tissues for the expression of hepatic markers. As expected, the control monkey liver

tissues were negative for staining with the human-specific albumin antibody. However, transplanted cells located near the portal vein stained positive for human-specific albumin (Fig. 2A).

Expression of cytokeratin-18 (CK18) is a characteristic phenotype of adult parenchymal liver cells. Using human-specific anti CK-18 antibody, we could not detect the immunostaining signals in the control monkey liver. In contrast, extensive CK-18 expression was detected in the transplanted cells in close vicinity to vascular structures (Fig. 2B). Similarly, the transplanted cells also stained positively for hepatocyte-specific antigen (HAS, Fig. 2C) and beta-2-microglobulin (B2M, Fig. 2D). Thus, the TRCs have the potential to form hepatocyte-like cells when injected directly into the liver of fetal monkeys.

FORMATION OF HEPATIC-LIKE CELLS IN THE MOUSE LIVER

We then tested the ability of the TRCs to differentiate into hepatic cells in a mouse model. The TRCs were directly transplanted into the right lobe of the mouse liver. At the end of the study, mouse liver tissues were collected for the detection of hepatic markers by immunostaining.

Using human-specific antibodies, we detected hepatic-like cells in the mouse liver, which became completely integrated into the mouse
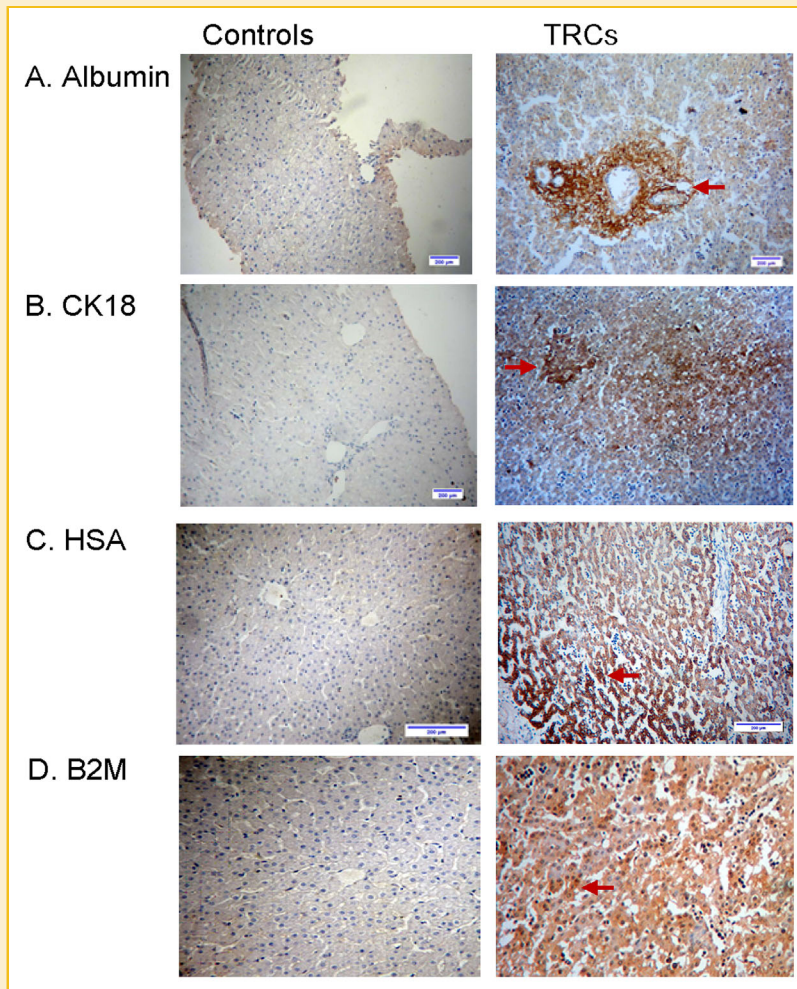
Fig. 2. Formation of functional hepatic–like cells in the fetal monkey liver. Fetal livers were collected and used for immunostaining with antibodies against human albumin (B), cytokeratin 18 (Ck18) (C), hepatocyte–specific antigen (HSA)(D), and beta–2–microglobulin (B2M)(D). Scale bars: 100 μm.

liver parenchyma, particularly around the portal vein zones. The hepatic-like cells are functional, staining positive for human albumin and CK18 (Fig. 3A). No human albumin or CK18 was detectable in the liver of control mice. Similarly, PCR also detected hepatic markers, including albumin, CK18, and CK19 (Fig. 3B).

To further examine the function of the implanted cells, we used a human-specific ELISA kit to quantitate the albumin secreted in the urine. As seen in Figure 3C, only the background signal was detected in the fibroblast-implanted mice. However, a significantly higher than background level of albumin was present in the TRC-implanted mice. Taken together, these data suggest that after exposure to the hepatic environment, TRCs acquire the potential to differentiate into hepatic-like cells in transplanted mice.

REACTIVATION OF STEMNESS GENES BY TRANSIENT REMODELING

We then explored the epigenetic mechanisms by which the transient reprogramming process affects differentiating potential in treated fibroblasts by examining the expression of endogenous genes that are related to cell potency. As previously reported in mouse

fibroblasts [Zhu et al., 2009], we found that human fibroblasts also responded to this short reprogramming stimulus by reactivating stemness genes. Following a short exposure to low concentrations of fish oocyte extracts, cells proliferated more rapidly than did control cells. There was a dramatic increase in *OCT4* gene expression as measured by RT-PCR in the two human fibroblast cell lines (HBF2 and WSF1) (Fig. 4A, lanes 2–3, 7–8) and by real-time PCR in human primary preputial fibroblasts (Fig. 4B). Using real-time PCR quantitation, we found that *NANOG* was also upregulated in treated primary fibroblasts (Fig. 4C). Using FACS analysis, we also showed that the expression of pluripotent cell surface markers SSEA-4 and OCT4 increased in treated cells (Fig. 4D). Our previous studies showed that expression of these stemness genes was able to last for more than 96 hrs after induction [Zhu et al., 2009].

TRANSIENT REMODELING INDUCES MULTIPOTENCY THROUGH THE ACTIVATION OF SIGNAL PATHWAYS

We then examined the molecular mechanisms underlying the induced multipotency by examining signaling pathways that may
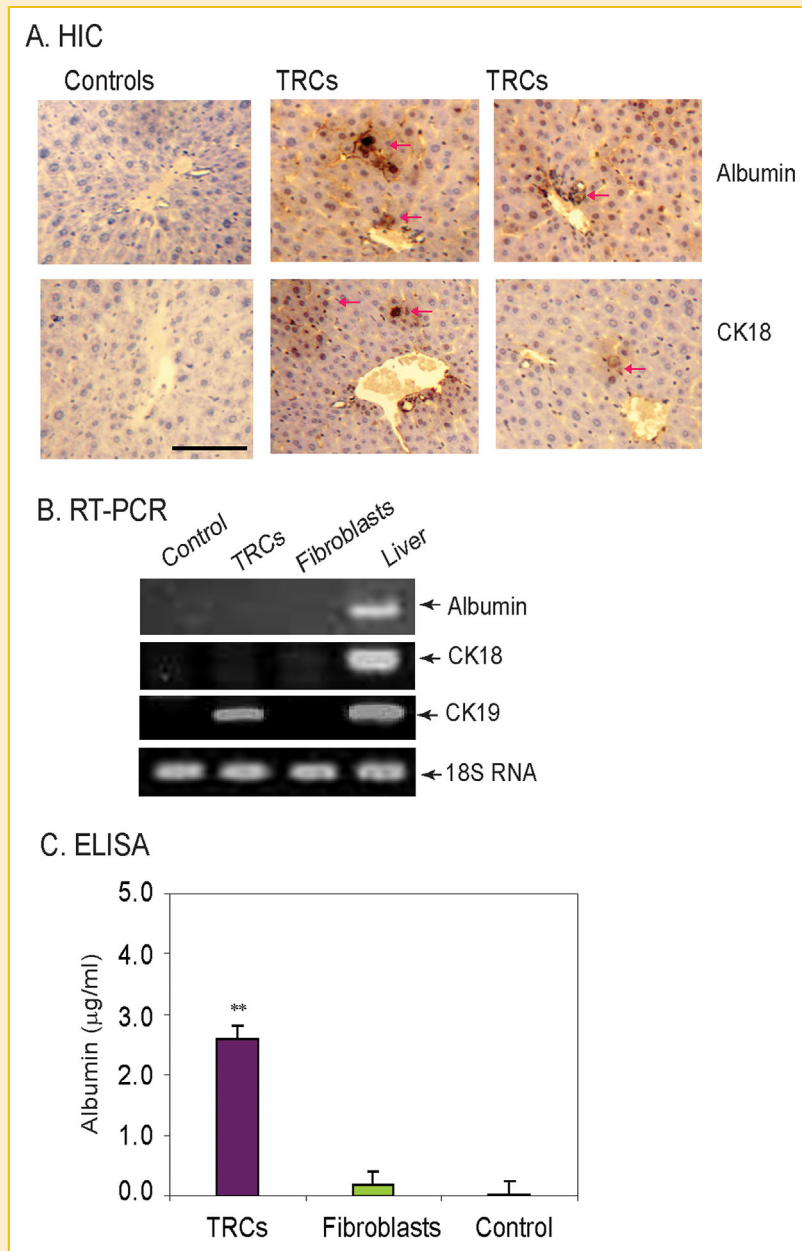
Fig. 3. Differentiation of TRCs into hepatocyte–like cells in the mouse liver. (A) Histoimmunochemical (HIC) staining of albumin and CK18 in the TRC–transplanted mouse liver. Scale bars: 100 μm. (B) PCR quantitation of hepatic markers (albumin, CK18, CK19). 18S RNA was used the PCR control. (C)ELISA of the secreted albumin in the mouse urine. One week after TRC transplantation, urinary samples were collected and albumin was measured using a human albumin–specific enzyme–linked immunosorbent assay (ELISA). ** $P < 0.01$ as compared with control groups.

participate in the regulation of stem cell renewal and cell proliferation [Binetruy et al., 2007; Chen et al., 2012; Kuhl and Kuhl, 2013]. Following transient cell remodeling, the MAPK pathway was significantly activated. Active phosphorylated p38 was significantly upregulated in treated cells as compared with control fibroblasts (Fig. 5A, top panel, lanes 2, 4, 6), suggesting a role for the upregulated p38 MAPK pathway in this transient reprogramming model.

We also observed a moderate upregulation of the AKT and Wnt pathways following cell reprogramming (Figs. 5B and C), suggesting

that these signaling pathways may also coordinate with the p38 MAPK pathway to enhance cell potency.

DISCUSSION

In this study, we have demonstrated the feasibility of an economic and efficient approach to generate functional human hepatic-like cells from skin fibroblasts. Instead of using virally delivered defined factors as in induced pluripotent stem cells (iPSCs) [Takahashi and
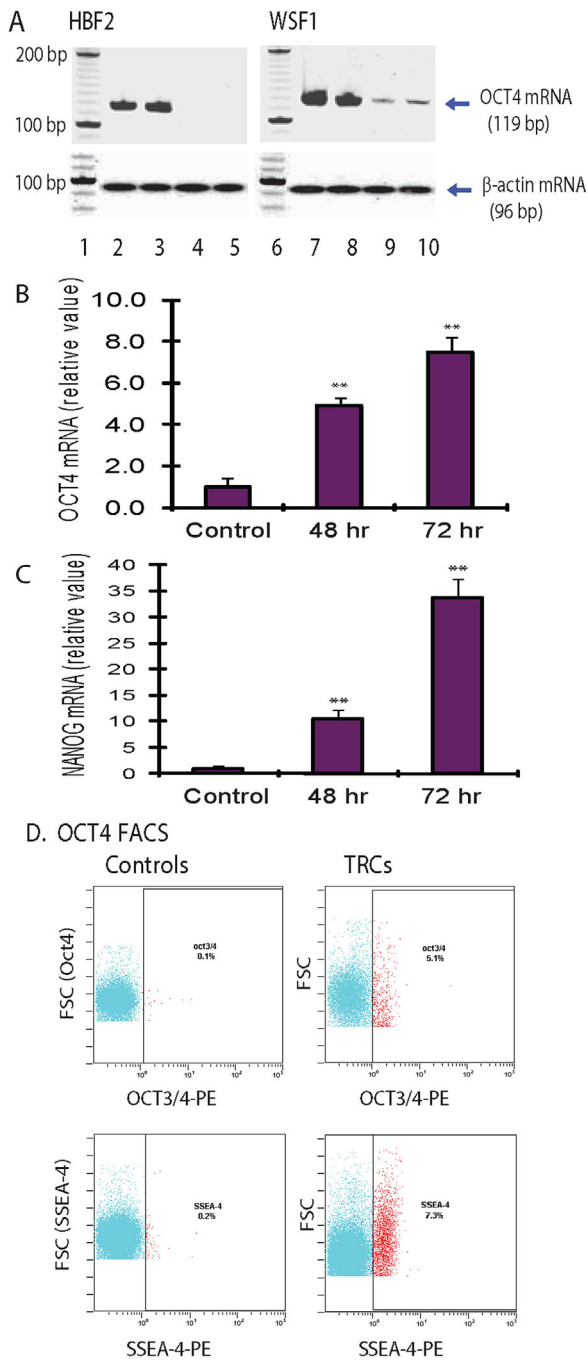
Fig. 4. Activation of stemness genes in TRCs. (A) RT–PCR of *OCT4* mRNA abundance in induced cells derived from fetal human skin HBF2 and WSF1 fibroblasts. Treated cells were collected at 48 hr following extract treatment and were used for PCR quantitation. β-ACTIN was used as the internal control. Lanes 1,6: 100 bp marker; lanes 2–3, 7–8: TRCs; lanes 4–5, 9–10: control cells. Note the very weak (lanes 7–8) or no (lanes 2–3) *OCT4* in control fibroblasts. (B–C) Real time PCR of *OCT4* (B) and *NANOG* (C) mRNA in induced cells derived from primary preputial fibroblasts. *OCT4* and *NANOG* were determined by a "delta Ct and delta–delta Ct" calculation with reference to *GAPDH* control. Gene expression was normalized and presented using the control as 1. **P < 0.01 as compared with the control group. (D) FACS analysis of OCT4 and SSEA-4 in TRCs.

Yamanaka, 2006], we transiently remodeled skin fibroblasts by briefly exposing the cells to a pluripotent environment supplemented with fish oocyte extract. After this transient exposure, human skin fibroblasts become at least partially reprogrammed as demonstrated by the activation of a set of genes characteristic of undifferentiated stem cells and the ability to differentiate in the liver into hepatic-like cells that synthesize human albumin and other hepatic antigens. The alterations in gene expression profiles and cell plasticity implicate global remodeling of chromatin by this short period of cell reprogramming. Notably, *OCT4* and *NANOG*, which are expressed in ES cells to maintain pluripotency [Nichols et al., 1998; Niwa et al., 2000; Cavaleri and Scholer, 2003; Mitsui et al., 2003; Chambers et al., 2007; Zhang et al., 2013], were reactivated in these reprogrammed cells.

Using the same approach, we previously demonstrated that transiently reprogrammed cells resemble mesenchymal stem cells in morphology and are able to be differentiated into multiple functional cell types, including insulin-secreting islets, beating myoblasts, skeletal muscle, adipocytes, and neural cells, suggesting a potential for multiple lineage differentiation and acquisition [Zhu et al., 2009]. Thus, transient cellular remodeling by fish oocyte extract induces cellular reprogramming similar to that seen in other types of multipotent stem cells, such as mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. The approach used in this study may greatly simplify the process of cell reprogramming.
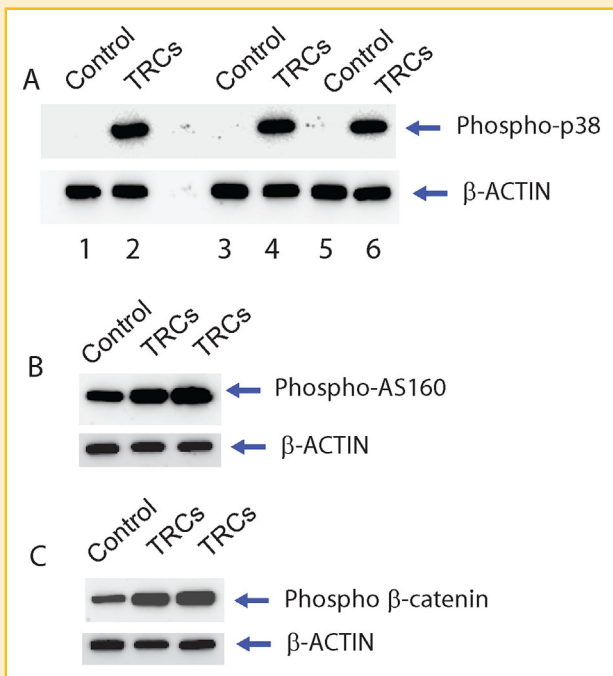

Fig. 5. Activation of signal pathways in TRCs. Following the transient treatment of fish egg extracts, TRCs were collected and lysed. The activity of the signal pathway components was measured by Western blotting. β-ACTIN was used as the internal control.

In iPSCs, the somatic nucleus is fully reprogrammed to become pluripotent by virally delivered defined factors [Takahashi and Yamanaka, 2006]. As a result, the cells behave like embryonic stem cells (ESCs). However, full reprogramming of somatic cells into iPSCs is a time-consuming and very inefficient process, and none of the currently existing iPS approaches is able to generate sufficient cells for clinical application within a short period of time.

As an alternate approach, we have shown that somatic cells can also be partially or transiently remodeled as the TRCs. Unlike the iPSC full reprogramming, TRCs do not show altered morphology. In cell culture, TRCs are similar to bone marrow mesenchymal stem cells and other mesenchymal stem cells in both cell morphology and multipotency. Thus, this transient cell remodeling by fish oocyte extract enhances the cell plasticity of skin fibroblasts. The remodeled cells become multipotent by expressing several genes associated with stem cells, such as *OCT4* and *NANOG*. Of critical importance, the induced cells can be differentiated *in vivo* into hepatocyte-like cells that synthesize human liver proteins, indicating that these TRCs may be useful for hepatic regeneration therapy.

Partial cell remodeling has also been observed in other systems. For example, pretreatment of skin keratinocytes with ES conditioned medium epigenetically changes gene expression, including pluripotent markers *Oct4*, *Sox2*, and *Nanog* [Grinnell and Bickenbach, 2007]. Alteration of the cell microenvironment (or cell niche) by incubating with Xenopus oocyte extracts or embryonic stem cell extracts can significantly modify the epigenotypes of a group of functional genes [Martys et al., 1995; Tada et al., 2001; Hansis et al., 2004; Taranger et al., 2005], leading to an altered cell phenotype. Thus, transient or partial cell remodeling may be used to enhance cell plasticity and replace the time-consuming iPS induction approach to create potent cells useful for cell therapy.

A major advantage of the approach used in this study is that the efficiency in producing TRCs is extremely high. In contrast, the existing strategies using defined factors are generally inefficient. In addition, the remodeling process of the TRCs is simple, consisting of a short exposure of cells to fish oocyte extracts in the medium. Potentially, this methodology can be used for large scale production of cells for clinical studies. Furthermore, the methodology appears to be free of the many safety concerns inherent in using virally infected cells.

It also should be emphasized that complete differentiation is required for clinical testing of embryonic stem cells as undifferentiated ES cells may pose a risk of tumorigenesis. Similarly, safety issues regarding iPSCs also need to be resolved before they can be given to human subjects. In contrast, the induced TRCs behave more like bone marrow stromal cells and umbilical cord blood mesenchymal stem cells, which have been extensively tested in both animal models and human clinical trials. They are recognized as safe for treating patients with a variety of diseases, and therefore, we predict that the TRCs may also be relatively safe for human use. Furthermore, fibroblasts can be cultured directly from a patient's skin biopsy and easily expanded to large scale in the lab, mitigating the concern of transplant rejection by the host's immune system.

The mechanisms underlying the acquired differentiation potential of skin fibroblasts following transient reprogramming remain elusive. As a first step to explore the epigenetic mechanisms involved, we examined several signaling pathways that may participate in the regulation of cell stem renewal. We showed that the p38 signaling pathway was activated following cell remodeling. The activation of MAPKs requires phosphorylation of a tyrosine and a threonine residue by dual-specificity MAPKKs and results in their translocation to the nucleus. p38 MAPK is directly activated by MKK3, MKK6, and MKK4 [Brancho et al., 2003], which are activated by phosphorylation of two serine residues. Mitogen activated protein kinases (MAPKs) mediate regulation of cell growth, survival and death in response to different extracellular stimuli, such as growth factors and cytokines, via phosphorylation-driven activation of transcription factors [Treisman, 1996; Robinson and Cobb, 1997]. Thus, it seems that cell remodeling may alter the cellular niche [Zhu et al., 2009] by upregulating the p38 mitogen-activated protein kinase pathway.

Phosphatidylinositol-3 kinases (PI3K) constitute a lipid kinase family characterized by their ability to phosphorylate inositol ring 3′-OH groups in inositol phospholipids, initiating the cascade response of several signaling pathways in response to various stimuli. Following transient reprogramming, the active form of Thr 642-phophorylated AS160 is significantly upregulated. Thus, AS160 may function as an early responding gene of the PI3K-AKT signaling pathway, mediating the transient reprogramming in this model as previously demonstrated in our T3-iPSC model [Chen et al., 2012]. In addition, we also observed the upregulation of the active form of phophorylated β-catenin, an important component of the Wnt signal pathway. Further studies are needed to examine how these signaling pathways are coordinated in initiating cell remodeling.

There is an evidence that hematopoietic stem cells (HSCs) can generate the hepatocyte lineage in rodent models of liver disease and regeneration. It has been long argued if the hepatic phenotype is derived from the transdifferentiation-induced de novo generation of hepatocytes or from cell fusion-induced genetic reprogramming of resident hepatocytes (see reviews [Austin and Lagasse, 2003; Vainshtein et al., 2014]). The specific mechanism underlying the formation of hepatic-like cells in this model is still unclear. Cytogenetic analysis of the sorted GFP-positive cells from the implanted liver section shows a typical diploid karyotype analysis (Fig. S1). It is assumed that the implanted TRCs would become in situ differentiated in the hepatic niche. However, after implantation, some TRCs showed variable nucleus size (Fig. 3A). Thus, we cannot completely exclude the possibility of occurrence of cell fusion in some of the implanted TRCs. Future studies are required to address if cell fusion is a potential mechanism in this TRC model. Regardless of whether cell fusion or differentiation actually takes place, TRCs may offer a potential alternative in hepatic cellular therapy.

In conclusion, this study demonstrates that skin fibroblasts may become capable of differentiating into cells that have the morphology and functional characteristics of hepatocytes following a simple *in vitro* remodeling approach. In fetal monkey and mouse livers, these cells form integrated functional hepatic-like cells that synthesize human-specific albumin and hepatocyte-specific biomarkers. Thus, these TRCs hold considerable promise for use as hepatocyte alternatives in cellular transplant therapies for the treatment of end-stage liver disease. It should be noted that TRCs can weakly form teratomas after transplanted into the dorsal flank of

nude mice [Zhu et al., 2009]. Thus, the safety issue of TRCs in hepatic cellular therapy needs to be addressed in future preclinical studies.

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