REPORTS Cell Biology International

Cell Biol. Int. Rep. 17(1):art:e00001.doi:10.1042/CBR20100001

Research Article

Reversible conversion of epithelial and mesenchymal phenotypes in SV40 large T antigen-immortalized rat liver cell lines

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Cite this article as: Takenouchi T, Yoshioka M, Yamanaka N and Kitani H (2010) Reversible conversion of epithelial and mesenchymal phenotypes in SV40 large T antigen-immortalized rat liver cell lines. Cell Biol. Int. Rep. 17(1):art:e00001.doi:10.1042/CBR20100001

Abstract

EMT (epithelial–mesenchymal transition) is a key process in the development of liver fibrosis. This process is also essential for liver morphogenesis in embryonic development. To study the cellular and molecular basis of EMT, we established two phenotypically different SV40 large T antigen-immortalized cell lines from rat hepatocytes. The first cell line, which had an epithelial morphology and was established in DMEM (Dulbecco's modified Eagle's medium)/Ham's F-12 (DF)-based medium (RL/DF cells), expressed CK18 (cytokeratin 18), a marker of parenchymal hepatocytes. The other, a mesenchymal-like cell line established in DMEM-based medium (RL/DMEM cells), expressed αSMA (α-smooth muscle actin), a marker of hepatic myofibroblasts. Epithelial RL/DF cells underwent phenotypic changes, such as increased expression of αSMA, when the culture medium was switched to DMEM-based medium. In contrast, mesenchymal RL/DMEM cells were induced to express the epithelial marker CK18 with a concomitant decrease in αSMA expression when the culture medium was replaced with DF-based medium. These cell lines may provide novel *in vitro* models for the study of the conversion between epithelial and mesenchymal phenotypes during EMT in liver fibrosis and morphogenesis.

Keywords: epithelial–mesenchymal transition; immortalization; liver cell line

1. Introduction

The liver is composed of parenchymal hepatocytes and non-parenchymal cells including BECs (biliary epithelial cells), HSCs (hepatic stellate cells), sinusoidal endothelial cells and Kupffer cells. In some pathological conditions, such as viral hepatitis and hepatic carcinoma, epithelial cells transform into cells with mesenchymal characteristics that can form fibrosis in the liver, or migrate to other organs. This phenotypic and functional conversion is referred to as the EMT (epithelial–mesenchymal transition). This process is also essential for organ morphogenesis in embryonic development (Lee et al., 2006; Gorrell, 2007; Wallace et al., 2008). During EMT, morphological and behavioural transformations of epithelial cells are associated with the induction of mesenchymal cytoskeletal proteins, such as αSMA (α-smooth muscle actin) and vimentin, and a concomitant decrease in the level of epithelial cytokeratins. EMT has been demonstrated to be a major mechanism for the deposition of ECM (extracellular matrix) in renal and pulmonary fibrosis models and is probably involved in the progressive changes towards liver fibrosis.

Activated hepatic myofibroblasts play central roles in the ECM accumulation observed in liver fibrosis. It is well recognized that such myofibroblasts are produced by the transdifferentiation of activated HSCs in the liver (Potter et al., 1999). However, owing to their heterogeneity, the origin and lineage of HSCs are largely obscure. Emerging evidence suggests that hepatocytes and BECs contribute to the production of myofibroblasts through the process of EMT (Gorrell, 2007). Although TGF (transforming growth factor)-β1 and the ECM have been shown as modulators of the EMT process in hepatocytes (Kaimori et al., 2007; Godoy et al., 2009), the molecular mechanisms of EMT in liver fibrosis still remain unclear. So, biologically relevant cellular models for EMT should still be pursued.

In the present paper, we describe simple and efficient models of the transition between epithelial and mesenchymal phenotypes using two phenotypically different SV40LT (SV40 large T antigen)-immortalized rat liver cells. The conversion of epithelial and mesenchymal phenotypes can be simply induced in these cell lines by switching the type of culture medium, and the converted phenotypes can be reversed by returning them to the original culture medium. This is a unique *in vitro* system in which EMT can be reversibly observed by only switching the type of growth medium using SV40LT-immortalized cell lines derived from primary rat hepatocytes.

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Abbreviations used: BEC, biliary epithelial cell; CK18, cytokeratin 18; CK19, cytokeratin 19; DMEM, Dulbecco's modified Eagle's medium; DF, DMEM/Ham's F-12; ECM, extracellular matrix; EGF, epidermal growth factor; EMT, epithelial–mesenchymal transition; FBS, fetal bovine serum; HBSS, Hanks balanced salt solution; HSC, hepatic stellate cell; αSMA, α-smooth muscle actin; SV40LT, SV40 large T antigen; TGF, transforming growth factor; VE-cadherin, vascular endothelial cadherin; ZO-1, zonula occludens 1.

2. Materials and methods

2.1. Isolation of hepatocytes from the rat liver

Adult male Sprague–Dawley rats (10–15 weeks old) were obtained from CLEA Japan, and kept in the animal facility in the National Institute of Animal Health according to institutional guidelines for experimental animals. Primary hepatocytes and non-parenchymal cells were isolated from the rat liver by the two-step liver-perfusion method (Seglen, 1976). In brief, adult rat livers were perfused *in situ* through the portal vein first with Ca^{2+}/Mg^{2+}-free HBSS (Hanks balanced salt solution) containing 0.5 mM EGTA and 10 mM Hepes (pH 7.2) (Hepes-buffered HBSS) and secondly with Hepes-buffered HBSS containing type I collagenase (0.5 μg/ml) and $CaCl_2$ (0.7 μg/ml) at 37°C. Then, the livers were removed, dissected out and minced. The liver suspension was sedimented to remove the larger debris, and filtered through gauze. The collected solution was centrifuged at 50 ***g*** for 1 min, and the pellet was resuspended in ice-cold MEM (minimum essential medium). After repeating the centrifugation and re-suspension steps twice, the parenchymal hepatocyte fraction was obtained. Non-parenchymal cells were obtained from the supernatant after centrifugation of the parenchymal hepatocytes. The cells were separated by centrifugation on a 16% Nycodenz (Nycomed AS) gradient at 600 ***g*** for 20 min at 4°C, and cultured as described below.

The purified hepatocytes were suspended in two different culture media. The DMEM (Dulbecco's modified Eagle's medium)-based medium consisted of DMEM supplemented with 10% FBS (fetal bovine serum) (HyClone), and the DF-based medium consisted of a 1:1 mixture of DMEM and Ham's F-12 supplemented with 10% FBS, recombinant human EGF (epidermal growth factor; 50 ng/ml) (Sigma), recombinant human bFGF (basic fibroblast growth factor; 50 ng/ml) (Sigma), sodium selenite (10^{-9} M), insulin (10 μg/ml), transferrin (100 μg/ml), progesterone (20 nM) and putrescine (100 μM). The non-parenchymal cells were suspended in DMEM-based medium.

2.2. Immortalization of rat hepatocytes and HSCs

Dissociated hepatocytes (1×10^6) were seeded into 100 mm tissue culture dishes (Becton Dickinson Labware) containing DMEM- or DF-based medium. The next day, FuGENE® HD transfection reagent (Roche Diagnostics) was used to transfect the primary cultured cells with pSV3neo (A.T.C.C. no. 37150) which encodes the SV40LT gene and the *neo* gene for resistance to the drug G418 (Invitrogen), to immortalize the cells. After selection with medium containing G418 (400 μg/ml), the resistant colonies were formed and subsequently cloned after the collection of colonies with glass cloning rings. Two phenotypically different cell lines established from each culture condition, mesenchymal RL/DMEM and epithelial RL/DF cells, were characterized in the present study.

Non-parenchymal cells containing HSCs were seeded into six-well plates (Becton Dickinson Labware) in DMEM-based medium. The next day, pSV3neo was transfected into the cells using FuGENE® HD reagent to immortalize them. After selection with G418, resistant colonies consisting of typical fibroblastic cells were collected, expanded and characterized (RNPC cells).

For the passage, the cells were harvested from the dishes by treating them with 0.01% trypsin, and 1×10^5 cells were seeded into new 35 mm tissue culture dishes at intervals of 3–4 days. Cell numbers were determined using a haemocytometer, and cumulative population doublings were calculated as described previously (Takenouchi et al., 2007, 2009).

2.3. Immunocytochemistry

The immortalized cells were seeded into eight-well chamber slides (5×10^4 cells/well) (Becton Dickinson Labware) containing DMEM- or DF-based medium. After 1–2 days of culture, the cells were washed with PBS and fixed with 3.7% formalin in PBS for 15 min. After permeabilization with 1% Triton X-100 in PBS for 10 min followed by blocking with 5% normal goat serum and 1% BSA (fraction V) in PBS for 30 min, the cells were incubated with primary antibodies at a 1:200 dilution for 1 h at room temperature (25°C) in a humidified box. The primary antibodies used were as follows: mouse monoclonal anti-CK18 (cytokeratin 18; Chemicon International), mouse monoclonal anti-CK19 (cytokeratin 19; Progen), mouse monoclonal anti-OX41 antigen (Chemicon International), mouse monoclonal anti-αSMA (Progen), mouse monoclonal anti-ZO-1 (zonula occludens 1; Sanko-Junyaku), mouse monoclonal anti-VE-cadherin (vascular endothelial cadherin; Alexis Biochemicals) and mouse monoclonal anti-desmin (Chemicon International). After rinsing the slides with PBS containing 0.05% Tween 20, the EnVision system (Dako) was used to visualize the antibody–antigen reaction according to the manufacturer's protocol. The immunostained slides were observed under a microscope (Leica).

3. Results and discussion

3.1. Establishment of immortalized cell lines from rat hepatocytes

After dissociation by collagenase perfusion, followed by sedimentation by low-speed centrifugation, typical binuclear hepatocytes were obtained at purities of more than 95% and cultured in either DMEM- or DF-based medium. After transfection of SV40LT followed by selection with G418, we obtained several G418-resistant colonies in both culture conditions. The number of G418-resistant colonies obtained from DF-based medium was higher than that obtained from DMEM-based medium, possibly due to the presence of several growth factors and supplements in the former medium. All of the G418-resistant colonies were composed of cells with very similar epithelial-like morphologies. Therefore we chose two representative cell lines, RL/DMEM and RL/DF cells (one from each culture condition) for the present study. As for proliferative ability, RL/DMEM cells proliferated at a doubling time of approx. 24 h and were stably passaged for more than 100 population doublings after the isolation of a single colony (Figure 1A), and RL/DF cells were similarly passaged for at least

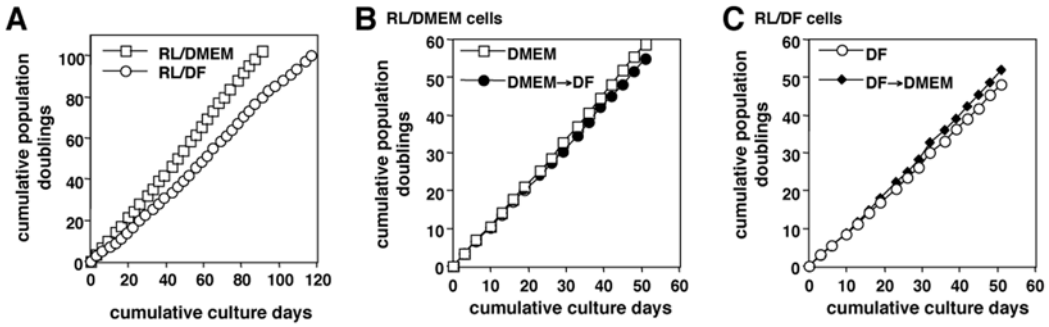

Figure 1 **Plots of cumulative population doublings of RL/DMEM and RL/DF cells against cumulative culture days**
The RL/DMEM and RL/DF cells were plated at 1×10^5 cells in 35 mm cell culture dishes in DMEM- and DF-based medium respectively. The cells were passaged at intervals of every 3 or 4 days just before reaching confluency. Then, the cells were harvested after trypsinization, and cell numbers were determined by haemocytometer counts. The proliferation rate of RL/DMEM cells was faster than that of the RL/DF cells (**A**). When the RL/DMEM cells were cultured in DF-based medium, the doubling time of these cells became gradually longer than that in DMEM-based medium (**B**). When the RL/DF cells were cultured in DMEM-based medium, the doubling time of these cells became gradually shorter than that in DF-based medium (**C**).

100 population doublings, but they proliferated at a longer doubling time of approx. 30 h (Figure 1A).

Next, we investigated the effect of switching the medium type on the proliferation of both cell lines. When the RL/DMEM cells established in DMEM-based medium were transferred to DF-based medium, their growth ability was slightly decreased compared with that in culture under the original medium (Figure 1B). In contrast, the growth ability of RL/DF cells was slightly enhanced when the cells were cultured in DMEM-based medium (Figure 1C). Collectively, these results suggest that both immortalized cell lines exhibited higher proliferative capacity in DMEM-based medium, whereas their growth rates were slightly retarded in DF-based medium. It is possible that EGF, a supplement in the DF-based medium, affects the proliferation of these cells by promoting the expression of hepatic phenotypes in these cells because this growth factor is used in primary cultures of hepatocytes to maintain their differentiated state (de Juan et al., 1992).

3.2. Immunocytochemical characterization of RL/DMEM and RL/DF cells

Two representative rat hepatocyte-derived cell lines were characterized by immunocytochemistry. We used several antibodies that detect cell-type-specific marker proteins of liver cells: CK18 for hepatocytes, CK19 for BECs, OX41 for Kupffer cells, desmin for quiescent HSCs, αSMA for hepatic myofibroblasts and activated HSCs, ZO-1 for epithelial lineage cells and VE-cadherin for sinusoidal endothelial cells (Hozumi et al., 1994; Braet et al., 2004; Wallace et al., 2008).

The RL/DMEM cells exhibited an epithelial-like morphology (Figure 2A). However, they were negative for CK18 and CK19, even though this cell line was established from a primary culture of purified parenchymal hepatocytes (Figure 2A). The cell–cell adhesion boundaries of RL/DMEM cells were immunostained with the anti-ZO-1 antibody, suggesting that they still retain some characteristics of epithelial cells (Figure 2A). The RL/DMEM cells were completely negative for OX41, VE-cadherin and desmin, suggesting that they do not have any characteristics of Kupffer cells, sinusoidal endothelial cells or quiescent HSCs. Strikingly, the RL/DMEM cells were strongly positive for αSMA, a marker of hepatic myofibroblasts and activated HSCs (Figure 2A). From these findings, it can be considered that RL/DMEM cells have properties not only of epithelial cells, but also cells of the mesenchymal lineage.

The RL/DF cells also exhibited an epithelial-like morphology (Figure 2B). As expected, the RL/DF cells were strongly positive for CK18, suggesting that they originated from parenchymal hepatocytes. Only a small population of RL/DF cells were positive for CK19, but these cells were completely negative for desmin, αSMA, OX41 and VE-cadherin (Figure 2B). In addition, the adhesion zones between the cells were immunostained with the anti-ZO-1 antibody (Figure 2B). These results suggest that RL/DF cells retain the phenotypic characteristics of epithelial cells, such as parenchymal hepatocytes, but not BECs (Wells et al., 1997). It is also considered that they do not originate from HSCs, Kupffer cells or sinusoidal endothelial cells.

Although the RL/DF cells retained some characteristics of parenchymal hepatocytes, we did not detect any significant levels of albumin expression at either the mRNA or protein levels by RT (reverse transcription)–PCR and immunoblotting (data not shown). This suggests that at least some major hepatocyte-specific functions of RL/DF cells may have been suppressed or lost during the course of immortalization, as described previously in other hepatic cell lines (Donato et al., 2008). Further investigation will be required to determine the experimental conditions in which RL/DF cells can be induced to express the functions of mature hepatocytes, such as albumin production, either by supplementation of specific factors, being cultured in specific ECM conditions or co-cultivation with other cell types in the liver.

3.3. Reversible conversions between epithelial and mesenchymal phenotypes in RL/DMEM and RL/DF cells

RL/DMEM cells retained a cobblestone-like morphology and formed an epithelial-like monolayer cell sheet, and yet strongly

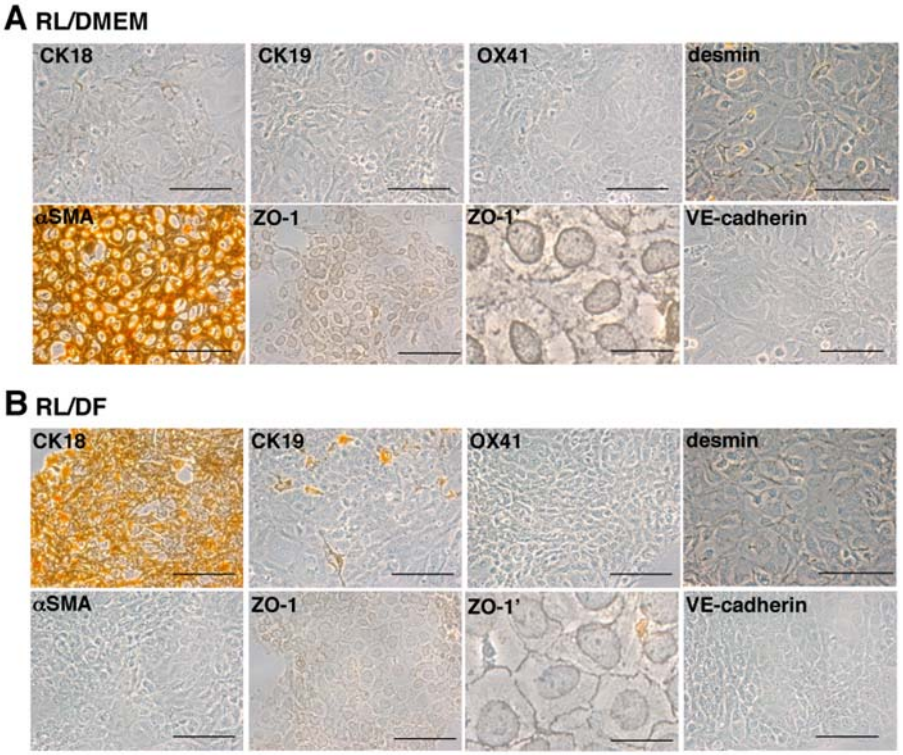

Figure 2 **Immunocytochemical characterization of RL/DMEM and RL/DF cells**
The RL/DMEM (**A**) and RL/DF cells (**B**) were plated in eight-well chamber slides in DMEM- and DF-based medium respectively. The next day, the cells were fixed and stained with specific antibodies against CK18, CK19, OX41, desmin, αSMA, ZO-1 or VE-cadherin. ZO-1′ is a magnified picture of ZO-1. Cell nuclei were non-specifically stained with the anti-ZO-1 antibody. Scale bar=100 μm, except for ZO-1′ (scale bar=25 μm).

expressed αSMA (Figure 3A, upper panels). The expression of αSMA is well-defined as a marker of hepatic myofibroblasts, which are principally produced by the transdifferentiation of activated HSC. However, recent studies have revealed that hepatocytes and BECs are able to transdifferentiate into myofibroblasts through the EMT process (Gorrell, 2007). On the basis of these previous findings, it is considered that RL/DMEM cells may have been transformed from parenchymal hepatocytes or hepatoblasts through EMT to myofibroblast-like cells during the immortalization process in DMEM-based medium. In addition, it was reported that the differentiated status of hepatocytes fluctuates in *in vitro* culture, depending on the signalling pathways triggered by growth factors or the ECM (Godoy et al., 2009). So we examined whether RL/DMEM cells have the capability to undergo reversible transitions between epithelial and mesenchymal phenotypes.

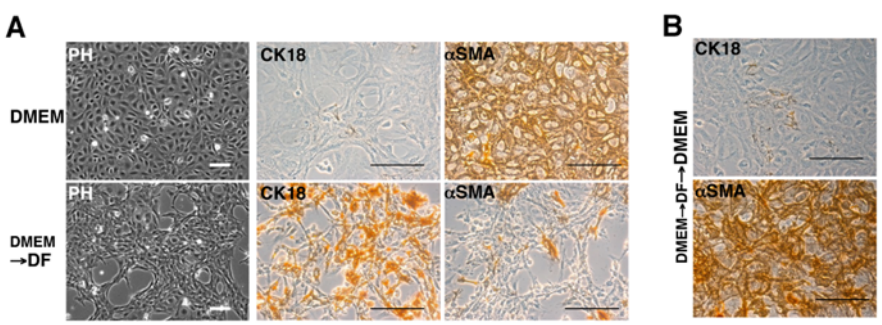

Figure 3 **Expression of CK18 and αSMA in RL/DMEM cells cultured in DMEM- and DF-based medium**
The morphology of the live cells was observed under a phase-contrast microscope [PH in (**A**)]. The RL/DMEM cells were routinely passaged in DMEM-based medium [DMEM in (**A**)]. The cells were cultured for 11 days after the replacement of the medium with a DF-based medium [DMEM→DF in (**A**)]. These cells were then returned to DMEM-based medium to determine their plasticity (**B**). The cells were plated in eight-well chamber slides in the conditions indicated, fixed and stained with specific antibodies against CK18 or αSMA. Scale bar=100 μm.

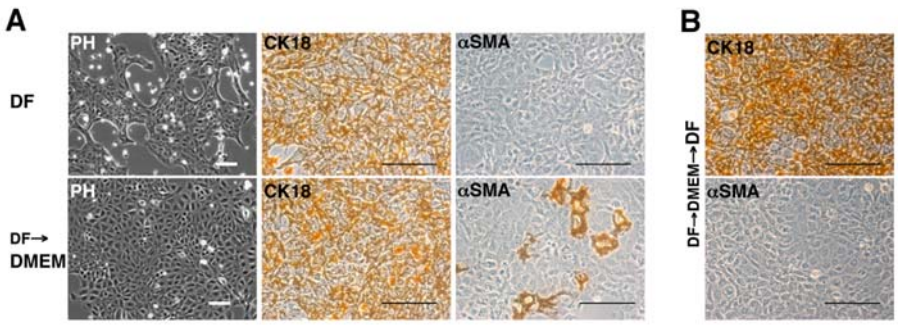

Figure 4 **Expression of CK18 and αSMA in RL/DF cells in DF- and DMEM-based medium**
The morphology of the live cells was observed under a phase-contrast microscope [PH in (**A**)]. The RL/DF cells were routinely passaged in DF-based medium [DF in (**A**)]. The cells were also cultured for 11 days after replacement of the medium with a DMEM-based medium [DF→DMEM in (**A**)]. These cells were then returned to DF-based medium to determine their plasticity (**B**). The cells were plated in eight-well chamber slides under the conditions indicated, then fixed, and stained with specific antibodies against CK18 or αSMA. Scale bar=100 μm.

When the RL/DMEM cells were transferred to DF-based medium and maintained for 11 days in culture, the expression of CK18 in these cells dramatically increased, whereas the expression of αSMA was markedly decreased (Figure 3A, lower panels). Their morphology became more slender, and the cobblestone-like monolayer shrank slightly in DF-based medium (Figure 3A). This morphological change suggests an increase in the cell–cell interaction of RL/DMEM cells, resulting in a lower affinity for the dish surface in DF-based medium. The morphological alteration and the cytoskeletal protein shifts were apparent at day 4 after the switch of the original medium to the DF-based medium. When the culture conditions were returned to the DMEM-based medium, the expression of both cytoskeletal protein markers returned to the original pattern in RL/DMEM cells after 4 days of culture, i.e. positive for αSMA and negative for CK18 (Figure 3B). Collectively, these findings suggest that RL/DMEM cells can be induced to undergo reversible conversion between mesenchymal and epithelial phenotypes by switching the type of culture medium.

Similarly, we examined the ability of RL/DF cells to exhibit phenotypic conversion between epithelial and mesenchymal phenotypes. The monolayer formed by RL/DF cells was cobblestone-like in morphology, but shrank slightly as observed in RL/DMEM cells cultured in DF-based medium (Figure 4A, upper panels). When the RL/DF cells were transferred to DMEM-based medium and maintained for 11 days in culture, the αSMA expression increased in some, but not all, cells, whereas the expression level of CK18 did not change (Figure 4A, lower panels). When the medium was returned to a DF-based medium, the αSMA-positive cells disappeared (Figure 4B). These findings suggest that a reversible epithelial–mesenchymal phenotypic conversion occurs in at least some populations of RL/DF cells.

3.4. Incapability of immortalized HSCs to undergo phenotypic conversion

For comparison, we established SV40LT-immortalized cell lines from the non-parenchymal cell fraction of rat livers and examined their ability to undergo phenotypic conversion. After selection with G418 treatment, a single collected colony was expanded as a cell line, RNPC cells. RNPC cells exhibited fibroblastic morphology in DMEM-based medium and were completely negative for CK18 staining (Figure 5A, upper panels). They strongly expressed αSMA and also expressed desmin at a lower intensity (Figure 5A, lower panels). These results suggest that RNPC cells are characterized as activated HSCs, although they retained some characteristics of quiescent HSCs. When the RNPC cells were transferred to DF-based medium, neither morphological change nor the induction of

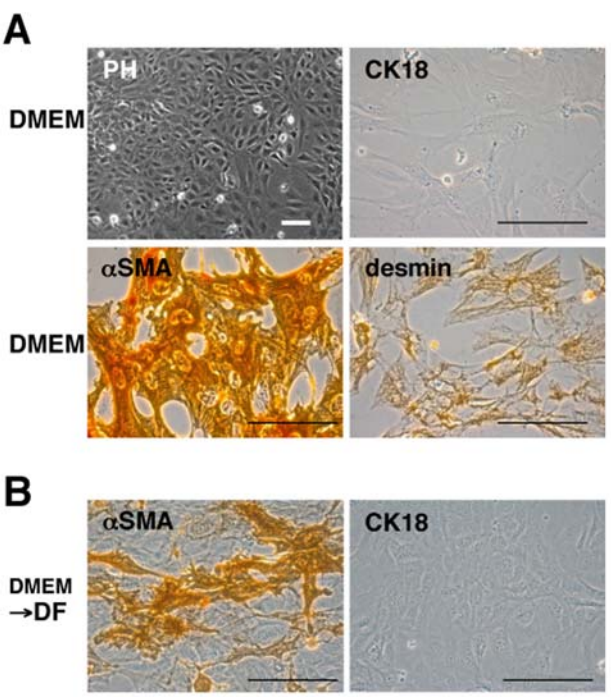

Figure 5 **Immunocytochemical characterization of RNPC cells**
The morphology of the live cells was observed under a phase-contrast microscope [PH in (**A**)]. The RNPC cells were plated in eight-well chamber slides in DMEM-based medium, then fixed and stained with specific antibodies against CK18, αSMA and desmin (**A**). The cells were also cultured for 9 days after the replacement of the medium with a DF-based medium, then fixed and stained with specific antibodies against CK18 and αSMA (**B**). Scale bar=100 μm.

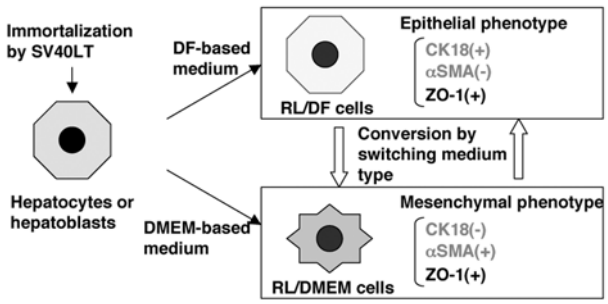

Figure 6 Possible origins of SV40LT-immortalized rat liver cells
Phenotypic conversion of the cells can be induced by switching the type of culture medium in a reversible manner.

CK18 expression was observed after 9 days of culture (Figure 5B), except for a marginal reduction in the number of αSMA-positive cells as observed in RL/DMEM cells (Figures 3 and 5).

Although RL/DMEM and RNPC cells share some mesenchymal properties, such as αSMA expression in DMEM-based medium, these cells exhibited differences in their ability to undergo phenotypic conversion upon the replacement of the culture medium with DF-based medium. This implies that DF-based culture medium may not be sufficient to convert mature mesenchymal cells, such as RNPC cells, into epithelial cells. In addition, RL/DMEM cells might not fully differentiate into mesenchymal lineage cells. As RL/DMEM cells retain at least some epithelial properties, such as ZO-1 expression, the ability to undergo reversible conversion may be associated only with cells in epithelial lineages, but not those in mature mesenchymal lineages.

Primary hepatocytes from rats and mice have often been used for the *in vitro* study of EMT in the liver (Pagan et al., 1997; Kaimori et al., 2007; Zeisberg et al., 2007; Kojima et al., 2008; Nitta et al., 2008). Since primary hepatocytes maintain their morphological and physiological properties for only a limited duration, hepatoma cell lines, such as human HepG2 and PLC/PRF/5 cells, are alternatively utilized (Wu et al., 2006; Matsuo et al., 2009; Slany et al., 2010). Although these cell lines possess a potent proliferative ability, they only partially maintain functional characteristics of hepatocytes. The present study reveals that SV40LT-immortalized cells derived from primary rat hepatocytes can be an intriguing alternative for the *in vitro* model of hepatocyte EMT. To our knowledge, this is the first report demonstrating the reversible conversion between epithelial and mesenchymal phenotypes in SV40LT-immortalized liver cells. As TGF-β1 is generally known as a potent stimulator of hepatocyte EMT, TGF-β1 endogenously produced from the immortalized cells might be involved in their phenotypic conversion in our system, as observed in primary mouse hepatocytes exposed to hypoxic conditions (Copple, 2010).

In conclusion, we have demonstrated in the present study that two different SV40LT-immortalized rat liver cell lines, RL/DMEM and RL/DF, have the capability to undergo reversible conversions between mesenchymal and epithelial phenotypes, which can be induced by switching the type of culture medium used (Figure 6). Therefore the present experimental systems using these immortalized rat liver cell lines might be useful for the study of the cellular and molecular mechanisms of the phenotypic conversion of hepatocytes during differentiation and disease conditions. These models also show promise for the investigation of the mechanisms of the early stages of EMT in the liver and the development of therapeutic strategies for liver fibrosis and the control of hepatic carcinoma, which are closely associated with EMT in the liver.

Author contribution

Takato Takenouchi and Hiroshi Kitani designed the experiments, collected data and wrote the manuscript. Miyako Yoshioka and Noriko Yamanaka conducted the animal experiments. All authors discussed the results and commented on the manuscript.

Funding

This work was supported by a research grant and a Grant-in-Aid from the Food Nanotechnology Project of the Ministry of Agriculture, Forestry, and Fisheries of Japan. The study was also supported by a research grant from The Takeda Science Foundation.

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Received 8 March 2010; accepted 11 March 2010

Published as Immediate Publication 18 May 2010, doi 10.1042/CBR20100001