Long-term culture of hepatic progenitors derived from mouse Dlk$^+$ hepatoblasts

Naoki Tanimizu[1,2], Hiroki Saito[3], Keith Mostov[2] and Atsushi Miyajima[1,3,4,*]

[1]Stem Cell Regulation, Kanagawa Academy of Science and Technology (KAST), Teikyo University Biotechnology Research Center, 907 Nogawa, Kawasaki, Kanagawa 216-0001, Japan
[2]Department of Anatomy, Department of Biochemistry and Biophysics, University of California San Francisco, 600 16th street, San Francisco, CA 94143, USA
[3]Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan
[4]Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Kawaguchi, 332-0012, Japan
*Author for correspondence (e-mail: miyajima@ims.u-tokyo.ac.jp)

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Summary

We previously demonstrated that hepatoblasts can be isolated from mouse fetal liver based on the expression of delta-like leucine zipper kinase (Dlk), also known as Pref-1. Each Dlk$^+$ hepatoblast forms a colony containing both albumin$^+$ hepatocytes and cytokeratin 19$^+$ (CK19) cholangiocytic cells on either type IV collagen or laminin. Here we show that extracellular matrices (ECMs) significantly affect the growth of Dlk$^+$ cells. Dlk$^+$ cells vigorously proliferated on type IV collagen-coated dishes in the presence of EGF and HGF during the first 5 days, but their proliferative capability declined thereafter. Dlk$^+$ cells also proliferated on laminin-coated plates and some colonies continued to expand even beyond one month after plating. These hepatic progenitor cells proliferating on laminin (HPPL) efficiently proliferated even after replating. Moreover, they were induced to differentiate into hepatocytes and cholangiocytes by overlaying Engelbreth-Holm-Swarm sarcoma (EHS) gel and by embedding in type I collagen gel, respectively. HPPL acquired the metabolic functions of accumulating polysaccharides and detoxifying ammonium ions after hepatic differentiation. Surprisingly, HPPL expressed pancreatic genes such as Pdx1 when dexamethasone was depleted from the culture medium. Therefore, the long-term culture of hepatoblasts on laminin produces multi-potential hepatic progenitors, which possess a strong proliferative capability, differentiate into both hepatocytes and cholangiocytes, and potentially give rise to pancreatic cells.

Key words: Dlk, Laminin, Type IV collagen, Hepatocyte, Cholangiocyte

Introduction

Liver stem/progenitor cells are the origin of two endodermal components of the liver, hepatocytes and cholangiocytes. Liver progenitors can be defined as a group of cells that include dormant hepatic stem cells, bipotential hepatic progenitors, such as hepatoblasts and oval cells, and committed immature cells, fetal hepatocytes. During development, hepatoblasts originate from the foregut endoderm and differentiate into hepatocytes and cholangiocytes (Shiojiri, 1984; Zaret, 2000). In adult injured liver, in which the proliferation of mature hepatocytes is blocked, oval cells emerge from the canal of Hering in the regenerating liver and repair the damaged tissue (Fausto and Campbell, 2003; Shiojiri et al., 1991). Hepatoblasts and oval cells have been isolated or enriched by using a cell sorter and shown to proliferate clonally and differentiate into both hepatocytes and cholangiocytes (Kubota and Reid, 2000; Minguet et al., 2003; Suzuki et al., 2002; Suzuki et al., 2000; Tanimizu et al., 2003). In addition, establishment of cell lines, which were highly proliferative and showed bi-directional differentiation potential, has contributed to the understanding of the nature of hepatic progenitors (Lazaro et al., 2003; Rogler, 1997; Spagnoli et al., 1998; Strick-Marchand and Weiss, 2002).

Some cytokines are known to affect the growth and differentiation of embryonic hepatic progenitors directly. Hepatocyte growth factor (HGF) is critical for the proliferation of hepatic stem cells and hepatoblasts, which is consistent with the expression of c-Met in these cells (Suzuki et al., 2002; Tanimizu et al., 2003), whereas oncostatin M (OSM) promotes the differentiation of fetal hepatocytes (Ito et al., 2000; Kamiya et al., 1999; Matsui et al., 2002; Minguet et al., 2003; Suzuki et al., 2003). In addition to cytokines, cell-cell contacts and extracellular matrices (ECMs) also affect the survival, proliferation and differentiation of hepatic progenitors. In fact, we previously showed that hepatic differentiation is induced by plating fetal hepatocytes at a high density (Kojima et al., 2000) and also that Engelbreth-Holm-Swarm sarcoma (EHS) gel, a mixture of ECMs, promotes hepatic differentiation induced by OSM (Kamiya et al., 2002). However, it remains unknown which ECM component controls the growth and differentiation of hepatic progenitors, though laminin, and type I and IV collagen weakly induce the hepatic differentiation of hepatic stem cells (Suzuki et al., 2003).

The liver and pancreas are closely related organs, as the liver primordium and the ventral pancreas are adjacent to each other in the ventral foregut endoderm at the onset of their

organogenesis. In addition, the transdifferentiation between hepatic and pancreatic cells has been shown in vivo and in vitro (Meivar-Levy and Ferber, 2003; Shen et al., 2003). The transition from pancreas to liver has been addressed by using mouse organ cultures and a pancreatic cell line (Deutsch et al., 2001; Tosh et al., 2002), whereas the mechanism underlying the transition from hepatic cells to pancreatic ones is still poorly understood. Thus, establishing an in vitro culture system of immature hepatic and/or pancreatic cells will provide a means for studying this mechanism.

We previously described the isolation of hepatoblasts from fetal liver based on the expression of Dlk, a type I membrane protein, using a specific monoclonal antibody and a cell sorter (Tanimizu et al., 2003). Dlk^+ cells efficiently proliferated and exhibited bi-directional differentiation capability in the presence of EGF and HGF in vitro. However, it remained unclear how ECM components affect the growth and differentiation of Dlk^+ hepatoblasts. In this paper, we show that some Dlk^+ hepatoblasts continuously proliferate on dishes coated with laminin and that the cells expanded on laminin differentiate into both hepatocytes and cholangiocytes under different conditions. Furthermore, we demonstrate that they also express pancreatic genes under specific culture conditions. Thus, multipotential endodermal progenitors can be reproducibly generated from Dlk^+ cells by long-term culture on laminin.

Materials and Methods

Mice and ECMs

C57BL/6CrSlc mice were purchased from Nippon SLC (Shizuoka, Japan). Engelbreth-Holm-Swarm sarcoma (EHS) gel and EHS-laminin were purchased from Becton Dickinson (BD) (Bedford, MA) and type IV collagen was obtained from Nitta gelatin (Osaka, Japan).

Culture of Dlk^+ cells

Mouse fetal hepatic cells were obtained according to the method of Kamiya et al. (Kamiya et al., 1999). The method used to isolate Dlk^+ hepatoblasts from E14.5 livers was described previously (Tanimizu et al., 2003) using hamster anti-mouse Dlk monoclonal antibody (mAb) (provided by Kirin Brewery Co. Ltd.) (Kaneta et al., 2000). Dlk^+ cells were resuspended in DMEM/F12 (Sigma, St Louis, MO) containing 10% FBS (Gibco BRL, Gaithersburg, MD), 1× insulin/transferrin/selenium (ITS) (Gibco BRL), 10 mM nicotinamide (Wako, Osaka, Japan), 0.1 μM dexamethasone (Sigma) and 5 mM L-glutamine (Gibco BRL). Cells were plated at 50 cells/cm^2 and 200 cells/cm^2, on six-well plates coated with either type IV collagen or laminin, respectively, and were incubated with 20 ng/ml of hepatocyte growth factor (HGF) (R&D, Minneapolis, MN) and epidermal growth factor (EGF) (PeproTech Ltd., London, UK). After 5, 14 and 21 days of culture, large colonies containing more than 100 cells were enumerated. Student's *t*-test was performed on Microsoft excel. After 21 days of culture, each large colony was placed within a cloning ring and dissolved in Trizol reagent (Invitrogen, Carlsbad, CA) to prepare total RNA.

Immunocytochemistry

To examine the expression of Dlk, cells cultured for 5 days were fixed in PBS containing 4% paraformaldehyde and permeabilized with methanol. They were incubated with hamster anti-Dlk mAb. Signals were visualized by incubation with biotinylated anti-hamster IgG (Vector) and FITC-conjugated streptavidin (Pharmingen, Peapack, NJ). To evaluate bidirectional differentiation of Dlk^+ cells, the colonies on the plates after 5 days of culture were incubated with 2 μg/ml goat anti-mouse albumin antibody (Bethyl Laboratory, Montgomery, TX) and rabbit anti-mouse CK19 serum diluted 1000-fold (Tanimizu et al., 2003) after fixation with paraformaldehyde and permeabilization with methanol. Signals were visualized using Cy3-conjugated anti-goat IgG and FITC-conjugated anti-rabbit IgG (Rockland, Gilbertsville, PA) under a Nikon Eclipse E800 fluorescence microscope. Cells that strongly expressed albumin were visualized as orange instead of red in our experiments.

Subconfluent HPPL were detached from plates with trypsin/EDTA. They were mounted on glass slides by cytospinning and then incubated with anti-CK19 serum and anti-mouse albumin antibody. Signals were visualized using Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 555 anti-goat IgG (Molecular Probes) under a fluorescence microscope.

Flow cytometry

To examine the expression of Dlk, cultured cells were detached from plates with trypsin/EDTA (Gibco BRL) and fixed in PBS containing 4% paraformaldehyde. After a wash with PBS, the cells were incubated with anti-Dlk mAb and subsequently incubated with FITC-conjugated anti-hamster IgG (Vector). To determine the expression of Ki67, cultured cells were fixed and permeabilized, and then incubated with mouse anti-human Ki67 mAb (Pharmingen, Peapack, NJ) followed by the incubation with FITC-conjugated anti-mouse IgG (KPL). To detect the expression of integrin β1 (CD29) and integrin α6 (CD49f), cultured cells were incubated with FITC-conjugated anti-CD29 and PE-conjugated anti-CD49f mAbs (Pharmingen). After a wash with PBS, the expression of Dlk, Ki67 or CD29 and CD49f was examined using FACScaliber (BD).

Differentiation of HPPL into three different lineages

E14.5 Dlk^+ cells were kept on laminin-coated dishes for 2 months by supplying fresh medium every 4 days. After 2 months, the cells were dissociated from plates with trypsin/EDTA and passaged onto new plates coated with laminin every 4 days. Cells grown under these conditions were named HPPL (hepatic progenitor cells proliferating on laminin). After replating four to six times, HPPL were treated with various conditions to induce multi-directional differentiation. In order to assure that different culture conditions definitely induce differentiation but do not promote proliferation of a certain cell population, confluent HPPL were stimulated to differentiate by changing the medium.

For the induction of hepatic differentiation, HPPL were plated at a density of 2×10^5 cells/well on six-well plates coated with laminin. When the cells became confluent, the medium was changed to 2 ml fresh DMEM/F12 supplemented with 20 ng/ml oncostatin M (OSM). After 5 days of incubation, 300 μl EHS gel mixed with six volumes of DMEM/F12 was added to each well according to a published method (Kamiya et al., 2002). After an additional 5 days of incubation, cells were dissolved in Trizol reagent and used for gene expression analysis. The intracellular accumulation of polysaccharide was examined with PAS staining solution (Muto Pure Chemical, Tokyo, Japan) according to the standard protocol after removal of the EHS gel and fixing the cells in 4% paraformaldehyde. Activity of ammonia detoxification was also examined. After treatment with OSM and EHS gel, the layer of EHS gel was removed by a wash with PBS. Then, 2 ml fresh DMEM containing 2 mM NH_4Cl was added to the cells. After 24 and 48 hours of incubation, the concentration of ammonium ions was measured by the modified indo-phenol method using a commercial kit, Ammonia-test Wako (Wako).

For the induction of cholangiocytic differentiation, HPPL were cultured in collagen gel. To make collagen gel plates, 1 ml collagen gel solution, consisting of 800 μl Collagen type I-A (Nitta gelatin),

Table 1. Oligonucleotides used in RT-PCR

Gene name		Sequence
Albumin	Sense	5′-CATGACACCATGCCTGCTGAT-3′
	Antisense	5′-GTGGATCCCTGGTGGAAAGGC-3′
Glucose-6-phosphatase (G6Pase)	Sense	5′-GAATTACCAAGACTCCCAGG-3′
	Antisense	5′-TGAGACAATACTTCCGGAGG-3′
Carbamoylphosphate synthetase (CPS)	Sense	5′-ACTGAGAGATGCTGACCCTA-3′
	Antisense	5′-CCTGGAAATTGGTGAGGAGA-3′
Glutamine synthetase (GS)	Sense	5′-CCAGGGTGAGAAAGTCCAAGC-3′
	Antisense	5′-GTTCGTCGCCTGTTTCGTTGAG-3′
CK7	Sense	5′-GATGACCTCCGCAACACC-3′
	Antisense	5′-TCCAGCAGCTTGCGGTAG-3′
CK19	Sense	5′-GTCCTACAGATTGACAATGC-3′
	Antisense	5′-CACGCTCTGGATCTGTGACAG-3′
Connexin 32 (Cx32)	Sense	5′-CTATCTGGGTTTGCCATAAG-3′
	Antisense	5′-TCTTTACCTCTTCCAGGTGA-3′
Cx43	Sense	5′-GTGATGAACAGTCTGCCTTT-3′
	Antisense	5′-TGATGAAGATGGTTTTCTCC-3′
Integrin β4	Sense	5′-GACCTATGAAGAAGGTGCTC-3′
	Antisense	5′-GCTCAGATGCGTGCCATAG-3′
Transforming growth factor (TGF) α	Sense	5′-ACACGAACCTCAAAGGGTTG-3′
	Antisense	5′-CAGGGACTTTCTTGCCTGAG-3′
p57	Sense	5′-GGGACTTCAACTTCCAGCAG-3′
	Antisense	5′-CTCAGAGACCGGCTCAGTTC-3′
Pdx1	Sense	5′-TTACAAGCTCGCTGGGATCA-3′
	Antisense	5′-GTCCCGCTACTACGTTTCTT-3′
Insulin	Sense	5′-CTGTTGGTGCACTTCCTAC-3′
	Antisense	5′-GTTGCAGTAGTTCTCCAGCTG-3′
Glucagon	Sense	5′-CGACTACAGCAAATACCTCG-3′
	Antisense	5′-CAGCCAGTTGATGAAGTCC-3′
Lipase	Sense	5′–CTGCAAGAGTATGTCACGC-3′
	Antisense	5′-CACAGTGGTTGATGCCATG-3′
Somatostatin	Sense	5′-CTGCATCGTCCTGGCTTG-3′
	Antisense	5′-CAGGATGTGAATGTCTTCCAG-3′
GAPDH	Sense	5′-ACCACAGTCCATGCCATCAC-3′
	Antisense	5′-TCCACCACCCTGTTGCTGTA-3′

100 μl 10× DMEM and 100 μl of 200 mM HEPES buffer containing 2.2% sodium bicarbonate and 0.05 N NaOH, was added to each well of six-well plates and was incubated at 37°C for 30 minutes. HPPL (1×10^5 cells) resuspended in 1 ml DMEM/F12 were mixed with 1 ml of the collagen gel solution and plated onto the basal layer of collagen. After 30 minutes of incubation at 37°C, 2 ml DMEM supplemented with 20 ng/ml of HGF was added to the well. To harvest the cells from the collagen gel, the gel was cut into cubes of 3 mm with a knife and mixed with 1/50 volume of 1% type I-A collagenase (Sigma) solution. After 30 minutes of incubation at 37°C with gentle shaking, the cells were collected by centrifugation and dissolved in Trizol reagent to prepare total RNA. To examine the protein expression of CK19, collagen gels were treated with collagenase and then the cells were fixed in 4% paraformaldehyde. After blocking, the cells were incubated with anti-CK19 serum and then incubated with Alexa Fluor 488 anti-rabbit IgG. (Molecular Probes). The samples were examined under a Zeiss Confocal Microscope.

For the induction of pancreatic differentiation, HPPL were plated at a density of 2×10^5 cells/well on six-well plates and incubated for 2 days until confluent. Then, dexamethasone and ITS were depleted from the medium and 20 ng/ml HGF and 2 μM retinoic acid were added. After an additional 5 days of incubation, the cells were dissolved in Trizol reagent and used for preparation of total RNA.

Microarray analysis

Total RNA samples were isolated from E14.5 Dlk^+ cells and HPPL. The first strand was synthesized with Superscript II reverse transcriptase (Invitrogen) in the presence of [^{35}S]dCTP (Amersham). Uptake of ^{35}S into cDNA was measured with a liquid scintillation counter. The membranes spotted with mouse gene fragments were incubated with hybridization buffer containing the cDNA and tet-1 probe labeled with [^{35}S]dCTP at 68°C for 12 hours. As an internal control, the ratio of ^{35}S counts between the tet-1 probe and each cDNA sample was adjusted to 1:2000. After washing and drying the membranes, they were exposed to an imaging plate for 3 days and then signals on the plate were detected with FLA-3000. The levels of gene expression were quantified using VersArray Analyzer program.

Reverse transcription polymerase chain reaction (RT-PCR)

cDNA was synthesized from 1 μg total RNA prepared from cell lysates. One-thirtieth of the cDNA was used for PCR. The thermal cycle (denaturation at 94°C for 30 seconds, annealing at the temperature set for each pair of primers for 30 seconds and extension at 72°C for 1 minute) was used to amplify each DNA fragment. The primers used are listed in Table 1.

Results

Hepatic and cholangiocytic cells arise from a single Dlk^+ cell

In order to examine the hepatic and cholangiocytic differentiation potential of Dlk^+ cells, they were plated on type IV collagen and laminin at densities of 50 and 200 cells/cm^2, respectively. In this culture condition, each Dlk^+ cell clonally proliferated and formed a colony (Tanimizu et al., 2003). After 5 days of culture, various sized colonies that mostly contained both albumin$^+$ cells and CK19$^+$ cells were formed on plates coated with either type IV collagen or laminin (Fig. 1A,B and Table 2). At this time point, Dlk expression disappeared from most of the cells regardless of whether they are cultured on

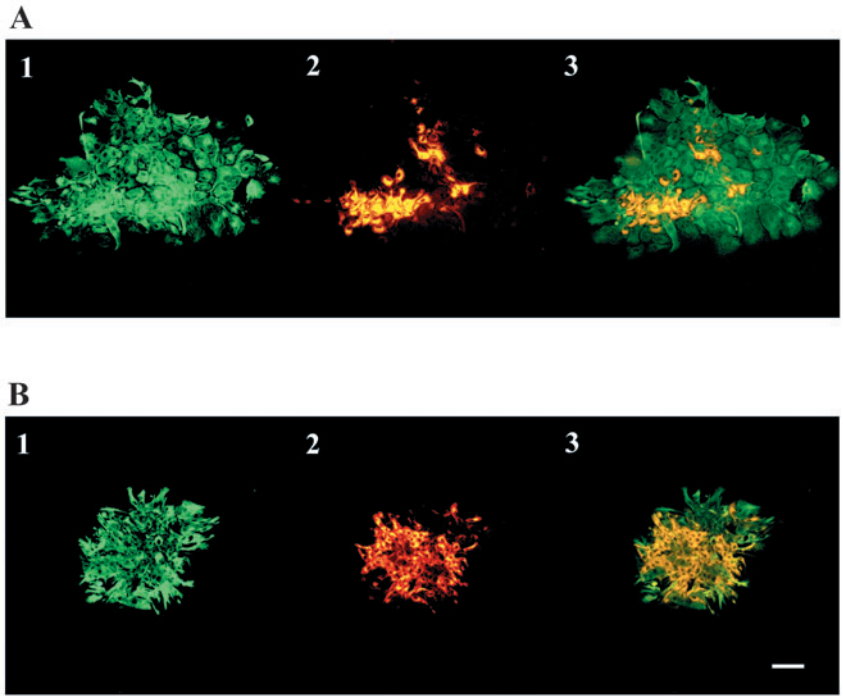

Fig. 1. Bi-directional differentiation of E14.5 Dlk$^+$ cells. (A,B) Typical colonies formed from single Dlk$^+$ cells on type IV collagen (A) or laminin (B) after 5 days of culture contain both CK19$^+$ and albumin$^+$ cells. Colonies were stained with anti-CK19 antibody and FITC-conjugated anti-rabbit IgG (green in panels 1), and anti-albumin antibody and Cy3-conjugated anti-goat IgG (red in panels 2). Panels 3 show the merged images of panels 1 and 2. Bar, 100 μm.

type IV collagen (Fig. 2A, panel 2) or on laminin (Fig. 2B, panel 2). FACS analysis further confirmed that Dlk expression was significantly downregulated: only 0.3% of the cells expressed Dlk after 5 days of culture (Fig. 2C, panel 2). These colonies were maintained for an additional 16 days and then ten colonies each from type IV collagen-coated and laminin-coated dishes were picked for gene expression analysis (Table 3). RT-PCR analysis showed that hepatic differentiation markers, carbamoyl phosphate synthetase (CPS) and glucose-6-phosphatase (G6Pase) were expressed in several colonies, in addition to albumin, glutamine synthetase (GS) and connexin 32 (Cx32). The expression of cholangiocyte markers, CK7, Cx43, and integrin β4 in addition to CK19 were also detected. Taken together, although the expression of CPS and G6Pase was more frequently found in colonies formed on laminin, each Dlk$^+$ cell basically differentiated into hepatic and cholangiocytic lineage cells on either ECM.

Long-term culture of Dlk$^+$ hepatoblasts

Although the colonies on either ECM contained both albumin$^+$ cells and CK19$^+$ cells, Dlk$^+$ cells formed more colonies (with more than 100 cells) on type IV collagen than on laminin 5 days after plating (Fig. 3A,B). Thus, type IV collagen supports growth of hepatoblasts more effectively at least in the short term when compared with laminin. In spite of the vigorous growth of Dlk$^+$ cells on type IV collagen during the first 5 days of culture, the proliferation declined on type IV collagen as shown by the reduction in the number of large colonies that were present on the 5th day after plating, and many cells had died by day 21 (Fig. 3A). In contrast, some of the medium-sized colonies on laminin observed on the 5th day continued to expand and became large colonies by day 14 (red in Fig. 3B) and by day 21 (yellow in Fig. 3B). Even at day 21, about 20% of the cells on laminin proliferated as shown by Ki67 expression (Fig. 3C, panel 3), whereas most cells on collagen did not express Ki67 (Fig. 3C, panel 2). Furthermore, cells cultured on laminin grew continuously for more than one month (Fig. 3D) and proliferated efficiently even after replating, indicating that those cells maintained high proliferation potential as long as they were cultured on laminin-coated dishes. We named these cells HPPL (hepatic progenitor cells proliferating on laminin). Consistent with the fact that HPPL were established on laminin, integrin α6β1, a receptor for laminin, was expressed on their surfaces as shown by FACS analysis (Fig. 4A, panel 2). In contrast to hepatoblasts, HPPL did not express Dlk (Fig. 4B, panel 2).

To compare HPPL with E14.5 Dlk$^+$ hepatoblasts, we examined their gene expression patterns by using a microarray technique. We first checked the expression pattern of hepatic and cholangiocyte markers. The data showed that HPPL expressed a hepatic lineage marker, albumin, but not hepatic differentiation markers, such as CPS, tyrosine aminotransferase (TAT), and tryptophan oxygenase (TO) in a manner similar to hepatoblasts. However, in contrast to hepatoblasts, HPPL expressed a cholangiocyte marker, CK19. The expression of CK19 and albumin were further examined by immunocytochemical analysis that showed most HPPL expressed both CK19 and albumin (Fig. 4C). We also found several genes differentially expressed in HPPL and hepatoblasts and confirmed their expression by RT-PCR. Consistent with the continuous growth capability of HPPL,

Table 2. Classification of colonies derived from single E14.5 Dlk$^+$ cells according to colony size and the expression of albumin and CK19

Matrix	Colony size†	Bipotential	Unipotent albumin	Unipotent CK19
Type IV collagen	Small	70	2	24
	Medium	60	0	16
	Large	20	0	8
Laminin	Small	100	26	13
	Medium	52	1	0
	Large	8	0	0

E14.5 Dlk$^+$ cells were plated at a density of 50 cells/cm^2 and 200 cells/cm^2, on type IV collagen- and laminin-coated dishes, respectively. In each case, 200 colonies were separated into nine groups based on colony size and the expression of albumin and cytokeratin 19 (CK19). The numbers represent colony counts. After 5 days incubation with EGF and HGF, cells were fixed in 4% paraformaldehyde and used for double immunostaining.

†Colonies containing less than 50 cells, between 50 and 100 cells and over 100 cells are defined as small, medium and large, respectively.

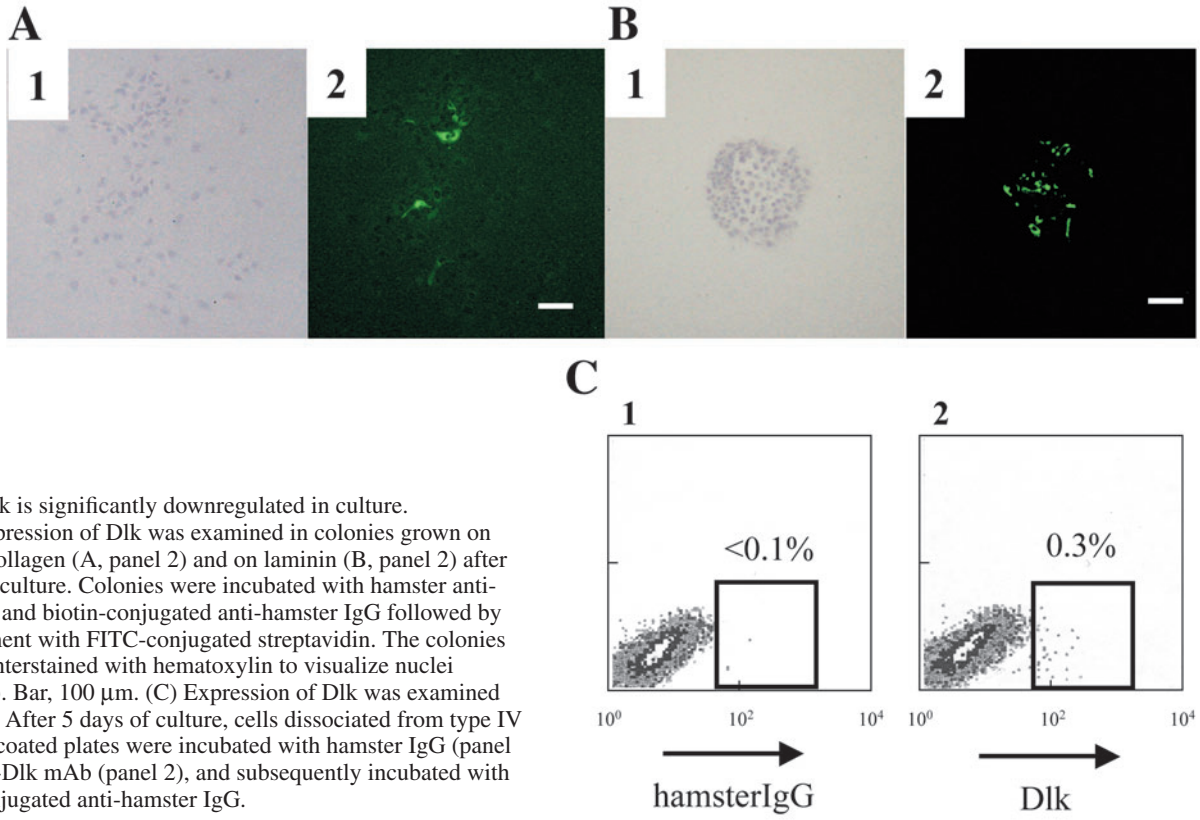

Fig. 2. Dlk is significantly downregulated in culture. (A,B) Expression of Dlk was examined in colonies grown on type IV collagen (A, panel 2) and on laminin (B, panel 2) after 5 days of culture. Colonies were incubated with hamster anti-Dlk mAb and biotin-conjugated anti-hamster IgG followed by the treatment with FITC-conjugated streptavidin. The colonies were counterstained with hematoxylin to visualize nuclei (panels 1). Bar, 100 μm. (C) Expression of Dlk was examined by FACS. After 5 days of culture, cells dissociated from type IV collagen-coated plates were incubated with hamster IgG (panel 1) or anti-Dlk mAb (panel 2), and subsequently incubated with FITC-conjugated anti-hamster IgG.

transforming growth factor α (TGFα), a possible autocrine growth factor, and p57, a negative regulator for cell cycle progression, were upregulated and downregulated in HPPL, respectively when compared to expression in Dlk$^+$ hepatoblasts (Fig. 4D).

HPPL differentiate into multi-lineage cells

We cultured HPPL in three different conditions to test the differentiation potential of HPPL. In order to clarify that each culture condition affects the differentiation of HPPL but does not promote the growth of a certain cell population, HPPL were grown for 3 days after plating to reach confluence and then treated under different conditions.

First, in order to examine the hepatic differentiation potential, we cultured HPPL with OSM, which induces hepatic differentiation in vitro (Kamiya et al., 1999). By day 5 of the incubation with OSM, hepatic differentiation was induced slightly as shown by weak expression of TAT (Fig. 5A, panel 1). As the differentiation of fetal hepatic cells is further promoted by EHS gel (Kamiya et al., 2002), HPPL treated with OSM for 5 days were overlaid with EHS gel and maintained for an additional 5 days. Under these conditions, the expression of TAT and CPS was significantly induced (Fig. 5A, panel 1),

Table 3. Expression of hepatocyte and cholangiocyte marker genes in colonies

Type IV collagen											Laminin									
1	2	3	4	5	6	7	8	9	10	Colony number	1	2	3	4	5	6	7	8	9	10
+	+	+	+	+	+	+	–	+	+	*Albumin*	+	+	+	+	+	+	+	+	+	+
+	–	+	+	+	–	+	–	–	+	*G6Pase*	+	+	–	+	–	+	–	+	+	–
+	+	–	–	–	–	–	–	–	–	*CPS*	–	+	+	+	+	+	–	+	+	–
–	–	–	–	–	–	–	–	–	–	*TAT*	–	–	–	–	–	–	–	–	–	–
+	+	+	+	+	+	+	+	+	+	*GS*		+	+	+	+	+	+	+	+	+
+																				
–	–	+	–	+	–	–	–	–	+	*Cx32*	–	+	+	+	–	–	–	+	+	+
–	+	–	+	+	+	–	–	–	+	*CK7*	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	–	–	+	–	+	*CK19*	+	+	+	+	+	+	+	+	+	+
–	–	–	+	+	+	–	–	–	–	*Integrin β4*	–	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	*Cx43*	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	*HPRT*	+	+	+	+	+	+	+	+	+	+

E14.5 Dlk$^+$ cells were incubated for 21 days. Ten colonies each were selected from among the colonies formed on type IV collagen and laminin. Total RNA was extracted from each colony and used for cDNA synthesis. PCR product was separated on a 1.5% agarose gel and visualized by staining in ethidium bromide. +, PCR product detected; –, PCR product not detected.

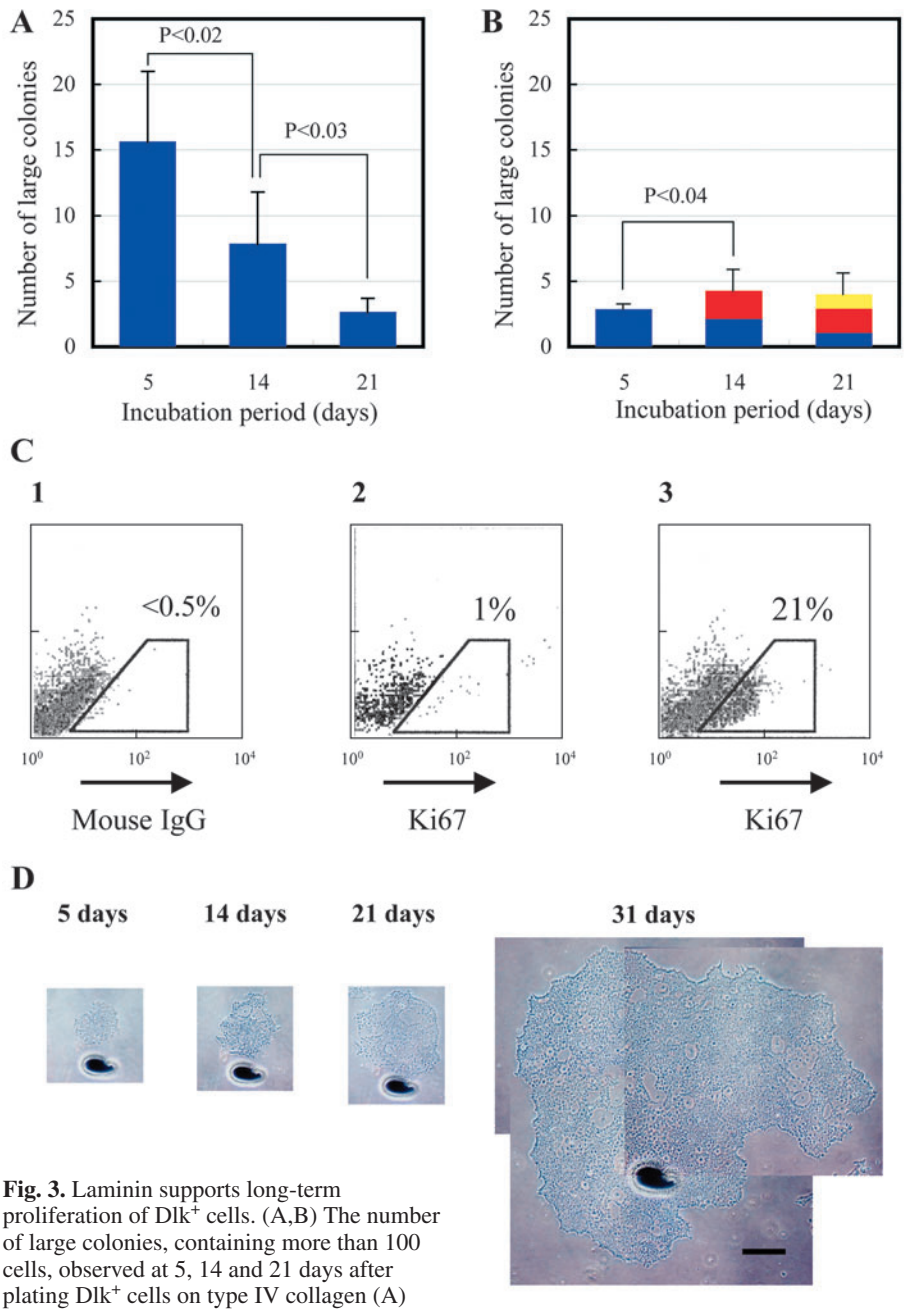

Fig. 3. Laminin supports long-term proliferation of Dlk$^+$ cells. (A,B) The number of large colonies, containing more than 100 cells, observed at 5, 14 and 21 days after plating Dlk$^+$ cells on type IV collagen (A) and laminin (B). While on type IV collagen, the number of large colonies decreased during long-term culture (A), some medium-sized colonies continued to expand on laminin after 5 days and became large colonies at 14 days (red portion of bar) and at 21 days (yellow portion of bar) (B). Large colonies were marked at the 5th day after plating and their sizes were checked at days 14 and 21. The colonies that contained more than 100 cells on day 14 and 21 were also marked. Data shown are the number of colonies formed from 1000 Dlk$^+$ cells. The culture was repeated independently four times, and average values of colony number are shown. Error bars represent standard deviation for the total number of large colonies at each time point. Student's *t*-test was performed and *P* values are shown in A and B. (C) Expression of Ki67 after 21 days of culture. About 20% of the cells on laminin expressed Ki67 (panel 3), whereas most of the cells on type IV collagen did not (panel 2). Cultured cells were dissociated from plates after 21 days of culture, fixed in 4% paraformaldehyde, and permeabilized in methanol. After incubation with mouse IgG (panel 1) or mouse anti-human Ki67 mAb (panels 2 and 3) followed by treatment with FITC-conjugated anti-mouse IgG, the expression of Ki67 was examined by using FACScaliber. (D) The colony derived from a single Dlk$^+$ cell grew continuously on laminin. The same colony was photographed at 5, 14, 21 and 31 days after plating. Bar, 200 μm.

although they were also expressed without pretreatment with OSM (data not shown). In addition, EHS gel induced the formation of clusters of cells with a round nucleus and granulated cytoplasm, characteristics of mature hepatocytes (Fig. 5A, panel 3), whereas such cells were not observed before induction of hepatic differentiation (panel 2). To evaluate the metabolic functions of HPPL, we examined the accumulation of polysaccharides such as glycogen using PAS staining and found that HPPL significantly accumulated intracellular polysaccharides upon treatment with OSM and EHS gel (Fig. 5B, panel 2) when compared with the control cells (panel 1). We also examined the cellular activity of ammonia detoxification before and after hepatic differentiation. The addition of EHS gel conferred the ability to eliminate ammonia quickly from the culture medium to HPPL (Fig. 5C).

Next, to induce cholangiocytic characteristics, HPPL were cultured in type I collagen gel. Cholangiocyte markers, CK7, CK19, and integrin β4, were detected in HPPL before the induction of cholangiocyte differentiation as shown by microarray analysis, and the level of their expression was not promoted even in collagen gel (Fig. 5D). On the other hand, in collagen gel, HPPL showed tube-like structures, which were immunostained with anti-CK19 antibody (Fig. 5E). The morphological change in HPPL under these culture conditions suggested that they have the potential to differentiate into cholangiocytes.

Finally, we tried to induce the pancreatic differentiation of HPPL as it is postulated that hepatoblasts and pancreatic progenitors are derived from the same endodermal stem cells (Deutsch et al., 2001) and transdifferentiation from hepatocytes to pancreatic cells and vice versa has been reported. In the transdifferentiation processes, dexamethasone promotes the transition from a pancreatic to a hepatic lineage (Tosh et al., 2002). In addition, retinoic acid is known to play an important role in pancreatic differentiation (Kumar and Melton, 2003). Therefore, to induce pancreatic differentiation, we depleted dexamethasone from the culture medium and added retinoic acid when the HPPL became confluent. After 5 days of

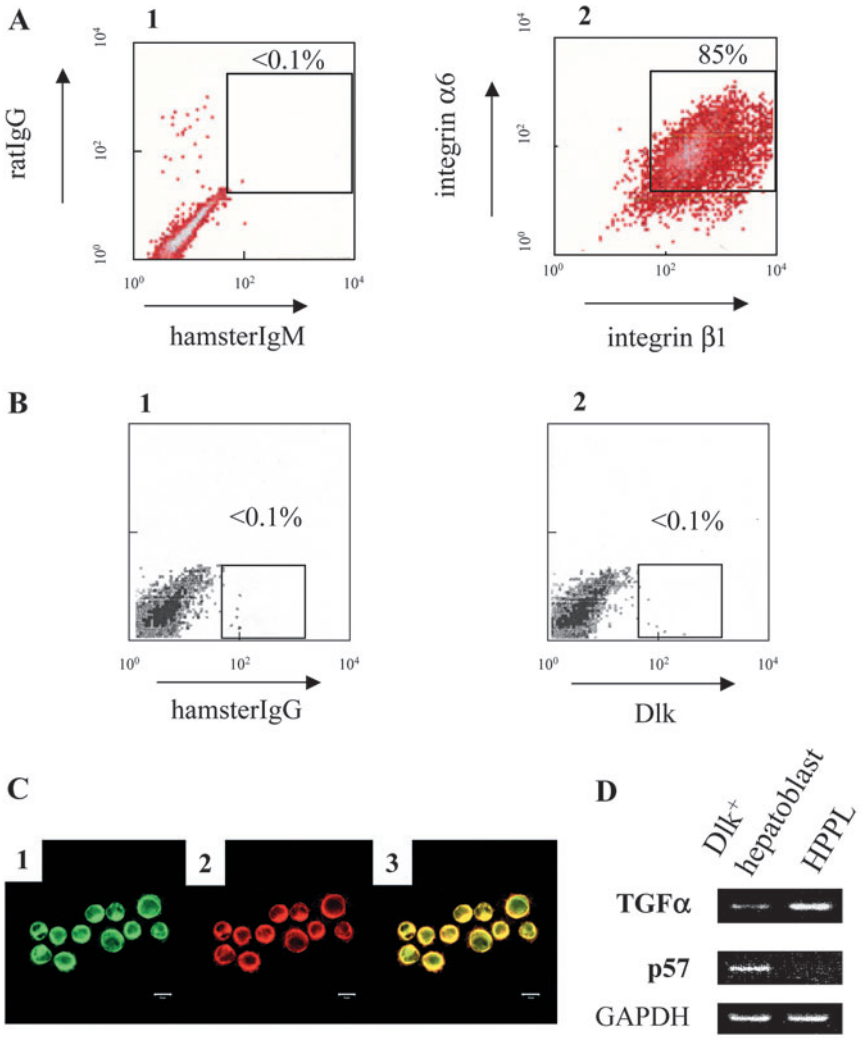

Fig. 4. Characteristics of HPPL. (A) HPPL strongly expressed a laminin receptor, integrin α6β1. Expression of integrin α6 and β1 was analyzed by FACScaliber using FITC-conjugated anti-CD29 (integrinβ1) and PE-conjugated anti-CD49f (integrin α6) mAbs (panel 2). As a negative control, HPPL were incubated with FITC-conjugated hamster IgM and PE-conjugated rat IgG (panel 1). (B) HPPL did not express Dlk. HPPL were incubated with anti-Dlk mAb followed by FITC-conjugated anti-hamster IgG (panel 2). As a negative control, HPPL were incubated with hamster IgG and FITC-conjugated anti-hamster IgG (panel 1). (C) HPPL expressed both CK19 and albumin. HPPL detached form plates were mounted on glass slides by cytospinning and incubated with anti-CK19 serum and anti-albumin antibody. The expression of CK19 and albumin were visualized with Alexa 488 anti-rabbit IgG (green in panel 1) and Alexa 555 anti-goat IgG (red in panel 2), respectively. Panel 3 is a merged image of panels 1 and 2. Bar, 10 μm. (D) TGFα and p57 were upregulated and downregulated, respectively, in HPPL when compared with expression in E14.5 Dlk^+ hepatoblasts. Gene expression was examined by RT-PCR. The thermal cycle was repeated 30 times for TGFα and GAPDH, and 35 times for p57.

incubation, the expression of Pdx1, a transcription factor involved in pancreatic organogenesis, was detected by RT-PCR (Fig. 6). Furthermore, the gene expression of pancreatic proteins such as insulin, glucagon, and lipase, was also promoted. Taken together, these results show that HPPL generated from Dlk^+ hepatoblasts, are able to differentiate into hepatocytes, cholangiocytes and pancreatic cells depending on the culture conditions.

Discussion

We previously described that a single Dlk^+ cell gives rise to $CK19^+$ cells and $albumin^+$ cells during 5 days of culture. In the present study, we further demonstrated that a single Dlk^+ cell was able to differentiate into hepatocytes and cholangiocytes, as shown by the expression of G6Pase and CPS and the expression of CK7, CK19 and integrin β4, when a Dlk^+ cell was allowed to proliferate clonally for 3 weeks. At this stage, the colonies contained three types of cells: $CK19^+albumin^-$, $CK19^+albumin^+$ and $CK19^-albumin^+$ cells. Although we have not been able to show the expression of other markers in each type of cell by immunostaining, $CK19^+albumin^-$ and $CK19^-albumin^+$ cells might represent committed cholangiocytes and hepatocytes, respectively. These results further support our previous finding that Dlk^+ cells are bipotential hepatoblasts. Here, we also found that the proliferation of hepatoblasts in vitro was significantly affected by ECM components used to coat the culture plates. Type IV collagen is suitable for the vigorous proliferation of hepatoblasts for the first 5 days after plating, whereas laminin supports the long-term proliferation of the progeny of Dlk^+ cells. It is possible that type IV collagen can neither support the long-term proliferation nor protect cultured cells from cell death, because many cells on type IV collagen died between day 5 and day 21 of culture. Type I collagen and fibronectin were also tested but they did not support the long-term proliferation of the progeny of Dlk^+ cells (data not shown).

We were able to generate HPPL reproducibly from the long-term culture of Dlk^+ cells on laminin. As compared with hepatoblasts, HPPL efficiently proliferate even after replating. It could be postulated that this continuous growth capability is attributed to pro-growth signals and/or anti-apoptotic signals. Microarray analysis showed that TGFα and p57 were upregulated and downregulated, respectively, in HPPL compared with Dlk^+ hepatoblasts. TGFα is known as a growth

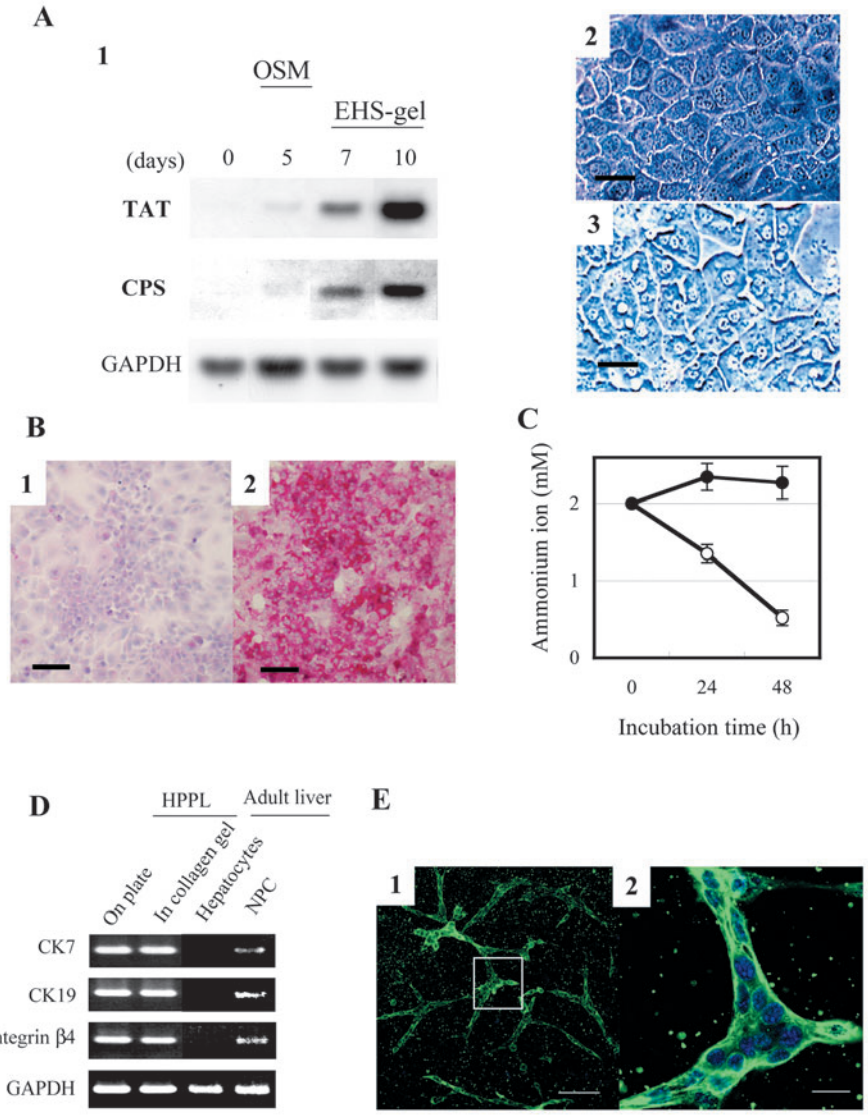

Fig. 5. Hepatic and cholangiocytic differentiation of HPPL. (A) Hepatic differentiation of HPPL was induced by OSM and EHS gel as shown by the expression of TAT and CPS (panel 1). The EHS gel treatment also induced the formation of cell clusters showing granulated cytosol and clear round nuclei (panel 3) when compared with the cells without OSM and EHS gel (panel 2). HPPL that became confluent were incubated with OSM for 5 days, and then overlaid with EHS gel for additional 5 days. OSM was not added to the medium during EHS-gel treatment. Numbers shown at the top of panel 1 represent days after addition of OSM. Bar, 25 μm. (B) Polysaccharide accumulation by HPPL. PAS staining showed that HPPL treated with OSM and EHS gel accumulated high levels of polysaccharide in their cytosol (red in panel 2) compared with HPPL before the induction of hepatic differentiation (panel 1). The nuclei were counter-stained with hematoxylin. Bar, 100 μm. (C) Clearance of ammonia from culture medium by HPPL. HPPL treated with OSM and EHS gel eliminated ammonium ions from the culture medium within 48 hours of incubation (open circles). By contrast, HPPL without hepatic differentiation failed to remove ammonium ions (filled circles). (D) Expression of cholangiocyte marker genes in HPPL before (lane 1) and after (lane 2) culture in collagen gel. HPPL were examined for the expression of CK7 and CK19, and integrin β4 by RT-PCR after 1 week of culture in type I collagen gel. Mature hepatocytes and non-parenchymal cells (NPC) containing cholangiocytes isolated from adult liver were also examined for the expression of these genes. The thermal cycle was repeated 30 times for GAPDH and 35 times for CK7, CK19 and integrin β4. (E) HPPL showed tube-like structures in collagen gel. After 5 days of incubation in type I collagen gel, the cells were stained with anti-CK19 antibody. Box in panel 1 was magnified and is shown in panel 2. Bar, 100 μm (panel 1); 20 μm (panel 2).

factor that promotes the proliferation of hepatocytes during liver regeneration. Although it has not been demonstrated that TGFα supports the growth of hepatic progenitors, it is a candidate driving the proliferation of HPPL in an autocrine manner. In addition, downregulation of p57, a cdk inhibitor that inhibits cell cycle progression, might result in the continuous growth of HPPL. In contrast, we did not find upregulation of anti-apoptotic genes such as Bcl-2 and Bcl-X. However, as HPPL were not exposed to apoptotic signals, upregulation of anti-apoptotic genes might not be necessary for HPPL to proliferate. Taken together, the continuous proliferative capability of HPPL is probably dependent on pro-growth signals rather than on anti-apoptotic factors.

HPPL are mostly $Dlk^{-}CK19^{+}albumin^{+}$, whereas hepatoblasts are $Dlk^{+}CK19^{-}albumin^{+}$, suggesting that HPPL do not exhibit exactly the same characteristics as hepatoblasts. However, HPPL possess not only a highly proliferative capability but also the potential to differentiate into hepatocytes and cholangiocytes. Although OSM weakly induced hepatic differentiation of HPPL, EHS gel significantly induced hepatic differentiation of HPPL as well as that of hepatoblasts. Furthermore, in type I collagen gel, HPPL formed tube-like structures that were stained with anti-CK19 antibody (Fig. 5E) in a manner similar to Dlk^{+} hepatoblasts (data not shown). These observations indicate that HPPL and Dlk^{+} hepatoblasts share a bi-directional differentiation potential elicited by external signals. Thus, HPPL provide a valuable means to study the mechanism of hepatic and cholangiocytic differentiation.

Interestingly, HPPL also differentiated into pancreatic cells. Consistent with the report that dexamethasone induces the transition from pancreas to liver (Tosh et al., 2002), depletion of dexamethasone from the culture significantly induced the expression of Pdx1 in HPPL. Furthermore, the addition of retinoic acid enhanced the expression of pancreatic genes, although it did not induce the expression of pancreatic genes in the presence of dexamethasone (data not shown). Two previous reports provide important insights into the mechanism of the transition of pancreatic cells into hepatocytes. First, the cells in the ventral foregut endoderm tend to become pancreatic cells whereas the signal from the cardiac mesoderm directs the hepatic

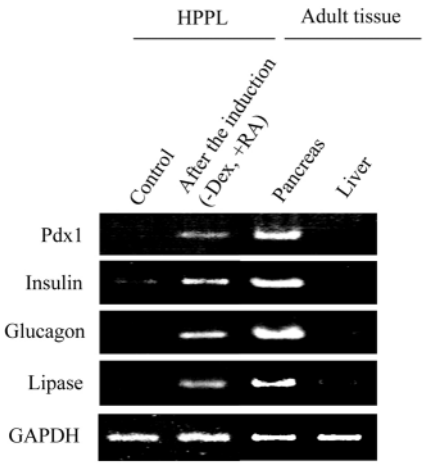

Fig. 6. Pancreatic differentiation of HPPL. The expression of Pdx1, insulin, glucagon, and lipase was examined by RT-PCR. HPPL before pancreatic differentiation (lane 1) and HPPL depleted of dexamethasone (Dex) and added with retinoic acid (RA) (lane 2) were compared. Adult pancreas (lane 3) and liver (lane 4) were also examined for the expression of these genes as positive and negative controls, respectively. After HPPL became confluent, dexamethasone and insulin/transferrin/selenium (ITS) were eliminated from the culture medium. Then, HPPL were incubated for an additional 5 days with 2 μM RA. Gene expression was examined by RT-PCR. The thermal cycle was repeated 30 times for lipase and GAPDH, 35 times for Pdx1 and insulin, and 40 times for glucagon.

fate (Deutsch et al., 2001). Second, C/EBPβ was shown to control the transition from pancreatic to hepatic cells (Tosh et al., 2002). However, the mechanism underlying the transition from hepatic to pancreatic cells remains unclear. Similar to HPPL, it was reported that H-CFUCs derived from $CD45^{-}TER119^{-}cKit^{-}cMet^{+}CD49^{+/low}$ fetal hepatic cells differentiate not only into two hepatic lineages but also into the pancreatic lineage (Suzuki et al., 2002). These hepatic progenitor cells with the ability to differentiate into pancreatic cells will provide a useful model to understand the mechanism underlying the lineage commitment of foregut endoderm and the transition from liver to pancreas.

As HPPL could be established on laminin but not on collagen or fibronectin, laminin is a critical factor to maintain the proliferation and multi-differentiation potential of HPPL. Because of the strong expression of integrin α6β1, a receptor for laminin, on HPPL, signals from laminin might be transmitted through integrin β1. However, as it was reported that the effect of laminin was not mediated through the integrin β1 subunit in some cases (Li et al., 2002), it remains unclear what signaling pathways are involved in the laminin-induced long-term proliferation of HPPL. In the liver, basement membranes including laminin are present around periportal biliary trees in addition to blood vessels. The hepatic portal area is considered the place where hepatic stem cells reside. Oval cells, the progeny of hepatic stem cells, emerge from the canal of Hering in the regenerating liver of rats treated with 2-acetylaminofluoren and subjected to partial hepatectomy. Interestingly, laminin is accumulated around oval cells whereas it is not found in the area where quiescent mature hepatocytes exist (Paku et al., 2001). It is thus tempting to speculate that laminin contributes to maintaining the hepatic stem cell population in the quiescent liver whereas it supports the proliferation of oval cells in the regenerating liver.

Our results indicate that the long-term culture of hepatoblasts on laminin produces HPPL that have high proliferative and multi-differentiation potentials. Although it remains unknown how laminin maintains HPPL in an immature state, laminin is an important ECM component for the long-term culture of hepatic progenitors. Furthermore, HPPL can be induced to differentiate into hepatocytes with metabolic functions. Thus, HPPL might be useful not only for studying the mechanisms underlying cell fate decision among hepatocytes, cholangiocytes and pancreatic cells, but also for expanding functional hepatocytes in vitro.

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