FPs with excitation/emission spectra in the red or far-red wavelengths have several attractive features such as low background auto-fluorescence and unique spectra distinct from other available green FPs [9,10], allowing multi-colour marking and imaging *in vivo* experiments to be performed. However, marking of cells with such red FPs has been hampered by nonviability or toxicity of the proteins as a result of the obligate tetramer formation that aggregates as spots in the perinuclear/Golgi region [25]. The drawback may be overcome by the use of red FP-based monomeric or tandem dimeric fluorescent proteins, such as Cherry, DsRed-monomer, and Tomato [12–15], although they are to be tested in transplant experiments with respect to their capability of marking HSCs. From this point of view, huKO is one of the few RFPs to mark HSCs efficiently without any apparent cytotoxicity.

The potential of huKO as a red tracer for HSCs was likely to be accentuated by the gene transfer system used in the present study, which allowed high and stable expression of the transgene in serially transplanted mice. Of importance was that KSL cells were isolated from nonconditioned mice and cultured in serum-free medium supplemented with the minimal essential cytokines in a relatively short period of time, so that they underwent the cell division while maintaining their maturity [24]. In addition, the vector pseudotyped with VSV-G envelope protein, allowing concentration into virus pellets by centrifugation, made it possible to remove serum from the virus supernatant and increase the virus titer. Because the GCDNsap vector also allowed long-term expression of the transgene in murine HSCs comparable to the DΔNsap (data not shown), it was unclear whether the binding site of NFAT in the LTR was indispensable for long-term expression of the transgene in murine HSCs.

Because the huKO is easily and clearly distinct from other green FPs without the need for specialized devices, the true red FP is available as one of a repertoire of fluorescent dyes for multicolor imaging of hematopoietic cells. In combination with the gene transfer method described in the present study, huKO may provide a powerful tool for detailed analyses of mechanisms underlying hematopoietic stemness.

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Prospero-Related Homeobox 1 and Liver Receptor Homolog 1 Coordinately Regulate Long-Term Proliferation of Murine Fetal Hepatoblasts

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During early to late-fetal liver development, bipotential hepatoblasts proliferate and differentiate into hepatocytes and cholangiocytes. The prospero-related homeobox 1 gene (*Prox1*) is expressed in hepatoblasts, and the inactivation of *Prox1* causes defective early liver development, in particular, faulty migration of fetal hepatoblasts. Prox1 binds to another hepatocyte-enriched transcription factor, liver receptor homolog 1 (Lrh1), and suppresses its transcriptional activity. However, the molecular mechanism by which Prox1 and Lrh1 regulate the characteristics of fetal hepatic cells remains unknown. We investigated the contribution of Prox1 and Lrh1 in early liver development. Embryonic day 13 liver-derived CD45$^-$Ter119$^-$Dlk$^+$ cells were purified as fetal hepatic stem/progenitor cells, and formation of colonies derived from single cells was detected under low-density culture conditions. We found that overexpression of Prox1 using retrovirus infection induced migration and proliferation of fetal hepatic stem/progenitor cells. In contrast, overexpression of Lrh1 suppressed colony formation. Prox1 induced the long-term proliferation of fetal hepatic stem/progenitor cells, which exhibited both high proliferative activity and bipotency for differentiation. Prox1 up-regulated expression of cyclins D2, E1, and E2, whereas it suppressed expression of p16^{ink4a}, the cdk inhibitor. In addition, overexpression of Prox1 significantly inhibited the proximal promoter activity of p16^{ink4a}. *Conclusion:* These results suggested that Prox1 and Lrh1 coordinately regulate development of hepatic stem/progenitor cells and that Prox1 induces fetal hepatocytic proliferation through the suppression of the promoter activity of p16^{ink4a}. (HEPATOLOGY 2008;48:252-264.)

Liver development comprises multiple stages and is influenced by hormonal factors as well as by intercellular and matrix cellular interactions. Stimulating factors from both cardiac mesoderm and septum transversum are important for the beginning of liver development.[1] This process begins on embryonic day (E) 8.0 in the mouse with proliferation of undifferentiated endodermal cells of the ventral foregut and their migration into the septum transversum.[2,3] Using an embryonic tissue organ culture system, fibroblast growth factor

Abbreviations: cDNA, complementary DNA; CK19, cytokeratin 19; DMEM, Dulbecco's minimum essential medium; E13HPC, E13 liver-derived hepatic progenitor cell; EGF, epidermal growth factor; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; G6Pase, glucose-6-phosphatase; GFP, green fluorescing protein; HGF, hepatocyte growth factor; IgG, immunoglobulin G; IRES, internal ribosomal entry site; KO, Kusabira-Orange; Lrh1, liver receptor homolog 1; MEM, minimum essential medium; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; Prox1, prospero-related homeobox 1; RT, reverse transcription; SD, standard deviation.

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(FGF) signaling from the cardiac mesoderm and bone morphogenetic proteins from septum transversum mesenchyme cells was shown to be necessary and sufficient to induce hepatocyte specification in the liver bud.[4,5] After the specification process, the interaction between hematopoietic cells and hepatocytes regulates liver development in the mid- to late-fetal stage. Oncostatin M, an interleukin-6 family cytokine, in the presence of glucocorticoid hormones promotes maturation of fetal hepatocytes derived from E14.5 livers *in vitro*.[6] Oncostatin M induces expression of several metabolic enzymes such as glucose-6-phosphatase and tyrosine aminotransferase in cultured fetal hepatocytes. The stimulation of extracellular matrices also regulates maturation of fetal hepatocytes.[7]

In addition to soluble factors, several transcription factors also are required for liver development and mature liver functions. The gene Prospero-related homeobox 1 (*Prox1*) was cloned by exploiting its homology to the *Drosophila melanogaster* gene prospero.[8] Gene targeting of Prox1 causes alternations of the lens and lymphatic vasculature and leads to embryonic lethality.[9,10] During liver development, Prox1 was localized in the hepatic primordium and dorsal pancreatic bud at E9.0, and expression of *Prox1* was detected in albumin-positive hepatocytes at E10.5.[11-13] Loss of Prox1 leads to formation of a smaller liver with clustered hepatocytes because the hepatic cord cannot migrate into the septum transversum without Prox1.[14] Subsequently, after FGF-induced and bone morphogenetic proteins–induced hepatocyte specification in the liver bud, Prox1 regulates the next step of liver development, migration of the liver bud and liver enlargement. Liver receptor homolog 1 (Lrh1), a member of the fushi tarazu factor 1 subfamily of nuclear receptor transcription factors, has been reported to play a role in bile acid biosynthesis and reverse cholesterol transport.[15] In the liver, Lrh1 regulates several hepatocyte functional genes such as cytochrome P450 7a1 and the transcriptional repressor small heterodimer partner.[16,17] Prox1 has been shown to interact directly with Lrh1 in a yeast two-hybrid screening system. Prox1 also suppresses Lrh1-mediated transcriptional activation of the cytochrome P450 7a1 and small heterodimer partner promoters.[18,19]

In the developing liver, both hepatocytes and bile ductal cells reportedly differentiate from a common cell.[20] Transplantation experiments using fetal rat liver cells indicated that these cells could reconstitute both hepatocyte and bile duct structures. This suggests that the developing liver contains bi-potent hepatic stem or progenitor cells. To identify fetal hepatic stem/progenitor cells, *in vitro* clonal analyses were used for the isolation and characterization of candidate cells. Using monoclonal antibodies and fluorescence-activated cell sorting (FACS), our group and others reported the prospective isolation of liver stem cells, with capacity for self-renewal and bipotent differentiation, from mouse mid-fetal livers.[21-23] A single $CD29^{+}CD49^{+}Ter119^{-}CD45^{-}c\text{-}kit^{-/low}$ cell or Dlk^{+} cell derived from fetal mouse mid-gestational liver can form a colony containing both albumin-positive hepatocytes and cytokeratin 19 (CK19)-positive bile ductal cells *in vitro* colony assay system, suggesting that these cells have the characters of hepatic stem/progenitor cells (active proliferative capability and bipotency for hepatic and bile-epithelial differentiation). However, the molecular mechanisms regulating proliferation and differentiation of hepatic stem/progenitor cells remain unknown.

In this study, we used a single cell–derived colony culture system to investigate whether Prox1 regulates development of hepatic stem/progenitor cells. Overexpression of Prox1 induced migration and proliferation of hepatic stem/progenitor cells. In contrast, overexpression of Lrh1 inhibited hepatic stem/progenitor cell colony formation. Prox1 supported long-term growth of hepatic stem/progenitor cells, and expression of $p16^{ink4a}$, the cdk inhibitor, was suppressed by Prox1 through regulation of the $p16^{ink4a}$ promoter activity. These results suggested that Prox1 and Lrh1 coordinately regulate development of hepatic stem/progenitor cells.

Materials and Methods

Materials. All embryonic hepatocyte culture experiments were performed with E13.5 C57BL6Cr mice (Nihon SLC, Shizuoka, Japan). All animals were treated under the guidelines of the Institute of Medical Science, University of Tokyo. Minimum essential medium (MEM) nonessential amino acid solution and Insulin-Transferin-Selenium X were purchased from Gibco-BRL (Rockville, MD). Fetal bovine serum (FBS) was purchased from Tissue Culture Biologicals (Tulare, CA). Dulbecco's modified Eagle medium (DMEM), dexamethasone, penicillin/streptomycin/L-glutamine (100×), and nicotinamide were purchased from Sigma (St. Louis, MO). Collagenase was purchased from Yakult (Tokyo, Japan). Matrigel, a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm mouse sarcoma, was purchased from BD Biosciences (Bedford, MA). Hepatocyte growth factor (HGF) and epidermal growth factor (EGF) were purchased from Peprotech (Rocky Hill, NJ). Anti-Prox1 antibody was purchased from Upstate (Charlottesville, VA). Anti-actin antibody was purchased from Sigma.

Cell Preparation From the Mid-fetal Livers and Adult Livers. Single-cell suspensions of liver cells were

prepared from E13.5 fetal mice. Minced embryonic liver tissues were dissociated with liver digest medium (0.05% collagenase solution).[6] Cells were washed several times by phosphate-buffered saline (PBS) with 3% FBS (staining medium). Liver cells were incubated at 4°C for 1 hour with fluorescein isothiocyanate–conjugated anti-Dlk antibody (Preadipocyte factor-1; Medical and Biological Laboratories, Nagoya, Japan) and allophycocyanin-conjugated anti-CD45 and anti-Ter119 antibodies (Pharmingen, San Jose, CA). After washing with staining medium, labeled cells were analyzed and separated using a MoFlo fluorescence-activated cell sorter (DAKO, Glostrup, Denmark). Residual erythrocytes, debris, and doublets were excluded by forward scatter and side scatter. Dlk is a known hepatoblast surface marker antigen.[21] To gate out hematopoietic cells in the fetal livers, sorting gates were set for the $CD45^-Ter119^-Dlk^+$ subpopulation. Sorted cells were plated and cultured on type I collagen (Nitta Gelatin, Tokyo, Japan) coated dishes.

Adult hepatocyte isolation was performed following a two-step collagenase digestion according to the protocol established by Seglen.[24] The parenchymal cell (mature hepatocyte) fraction was separated from nonparenchymal cells by low-speed centrifugation (50*g*, 1 minute). Dead cells were removed by centrifugation in 50% Percoll solution (GE Healthcare UK, Buckinghamshire, UK). Hepatocytes were washed with PBS and collected.

Generation of Retroviruses. The retroviral vector pGCDNsam, with a long terminal repeat derived from MSCV, has intact splice donor and splice acceptor sequences for generation of subgenomic messenger RNA.[25] The complementary DNA (cDNA) of Prox1 or Lrh1 was subcloned into an upstream sequence of an internal ribosomal entry site (IRES) in a pGCDNsam vector. This vector has the sequence of enhanced green fluorescent protein (GFP) or Kusabira-Orange (KO) downstream from the IRES.[26] Infected cells thus can be detected using fluorescence-activated cell sorting (FACS) or a fluorescent microscope. Retroviruses were generated as previously described.[27] Virus titers were determined by infection of NIH3T3 cells.

In Vitro *Colony Assays.* $CD45^-Ter119^-Dlk^+$ cells isolated by FACS were cultured at low density (50 cells per cm^2 on 35-mm dishes). Our standard culture medium is a 1:1 mixture of DMEM and F-12 (Sigma) with 10% FBS, 1 × Insulin-Transferin-Selenium X, 10 mM nicotinamide, 10^{-7} M dexamethasone, 5 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 1× penicillin/streptomycin/L-glutamine, and 1× nonessential amino acid solution. Cells were incubated in 1:1 mixture of standard culture medium and the conditioned medium derived from E14.5 hepatocytes. After 14 to 16 hours, cells were stimulated by the addition of hepatocyte growth factor (HGF) (40 ng/mL) and epidermal growth factor (EGF) (20 ng/mL), and were infected with retrovirus (multiplicity of infection 100) in the presence of 1 μg/mL protamine sulfate. After 24 hours of incubation, the cells were washed and cultured in a 1:1 mixture of standard culture medium and conditioned medium supplemented with HGF and EGF. Colonies derived from a single $CD45^-Ter119^-Dlk^+$ cell (colony cell count > 10 cells) were assessed.

Establishment of Long-Term Proliferative Hepatic Progenitor Cells. After 3 weeks of colony culture, most mock-infected cells died. A few Prox1-overexpressing colonies continued to grow. Cloning rings were placed around them, and they were subcultured into individual wells of a 48-well culture dish in a 1:1 mixture of standard culture medium and conditioned medium supplemented with HGF and EGF. The expanded cells were transferred to large culture dishes and used for experiments. To induce hepatic maturation, cells were washed with PBS twice, and culture media were changed to hepatic culture medium (DMEM with 10% FBS, 1× penicillin/streptomycin/L-glutamine, 10^{-7} M dexamethasone and 1× nonessential amino acid solution) with 20% matrigel.[7]

Migration Assays. For wound-closure assays, 4×10^5 fetal hepatic stem/progenitor cells were cultured on six-well plates in hepatic culture medium for 4 hours. Fetal hepatic stem/progenitor cells were infected with retroviruses (incubation, 24 hours), and culture medium was replaced. Epithelial monolayer sheets more than 90% confluent had formed after 48 hours of infections. Wounds 600 to 700 μm broad were generated by scraping cell monolayers with 200-μm pipet tips. Wounded cells were cultured in hepatic culture medium supplemented with HGF, as a scattering factor. After 14 and 20 hours, images were collected with a fluorescent microscope.

Immunocytochemistry. Fetal hepatic stem/progenitor cells were cultured at low density (50 cells per cm^2 on 35-mm dishes) for 10 days and fixed with 3.7% formaldehyde in PBS for 10 minutes. After three washing steps with PBS, cells were permeabilized using methanol. Then they were incubated with a rabbit anti-mouse proliferating cell nuclear antigen (PCNA) antibody (Santa Cruz Biotech., Santa Cruz, CA) in 10% goat serum/PBS. As negative control, cells were incubated with a rabbit immunoglobulin G (IgG) fraction in 10% goat serum/PBS. Cells were washed with PBS and were incubated with an Alexa546-conjugated goat anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA). Expression of PCNA was observed under a fluorescent microscope. For double staining of albumin and CK19, cells were fixed with 4%

paraformaldehyde in PBS for 10 minutes. After washing as described, cells were permeabilized using methanol and stained with a rabbit anti-mouse CK19 antibody[21] and a goat anti-mouse albumin antibody (Bethyl Laboratories, Montgomery, TX) in 5% donkey serum/PBS. As a negative control, cells were incubated with rabbit and goat IgG fractions in 5% donkey serum/PBS. Cells then were washed and incubated with an Alexa350-conjugated donkey anti-goat IgG antibody and an Alexa555-conjugated donkey anti-rabbit IgG antibody (Invitrogen).

Western Blot Analyses. Detergent lysates of mock and Prox1-expressing fetal hepatic stem/progenitor cells were prepared using radioimmunoprecipitation assay buffer (0.1% sodium dodecyl sulfate, 1% Trition X-100, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl, 1 mM sodium vanadate, 1 mM ethylenediaminetetra-acetic acid, and protein inhibitors). Protocols of sosdium dodecyl sulfate polyacrylamide gel electrophoresis and western blots were described previously.[6]

Messenger RNA Detection of Reverse Transcription Polymerase Chain Reaction. Total RNA was extracted from fetal hepatic stem/progenitor cells using Trizol (Invitrogen). First-strand cDNA was synthesized using the Primescript 1st strand cDNA synthesis kit (TAKARA, Otsu, Japan) and was used as a template for polymerase chain reaction (PCR) amplification. The cDNA samples were normalized by number of glyceraldehyde 3-phosphate dehydrogenase copies using quantitative PCR with the TaqMan probe (Applied Biosystems, Foster City, CA). Equal numbers of copies were applied as PCR templates. Primer sequences are listed in Supplementary Table 1. The primers were annealed at 55°C for 30 seconds, and amplification was repeated for 27 to 40 cycles. Amplified products were separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. Gel pictures of reverse transcription (RT)-PCR were analyzed and quantified using ImageJ (http://rsb.info.nih.gov/ij/).

Construction of the Mouse $p16^{ink4a}$ Promoter Plasmids and Expression Plasmids for Prox1 and Lrh1. The −1172 and −586/+81 fragments from the transcription start site of the mouse $p16^{ink4a}$ promoter[28] were amplified by PCR and cloned into the luciferase reporter vector pGL3basic (Promega, Madison, WI). The cDNAs of Prox1 and Lrh1 were subcloned into the expression vector pCAG-IP (pCAGGS with IRES-puromycin resistance gene).[29,30] All plasmids were confirmed by nucleotide sequencing.

Transient Transfection Luciferase Assay. The transformation and analyses of promoter activities using luciferase plasmids were described previously.[31] Briefly, HuH2.2 cells were cultured in DMEM containing 10% FBS and 1× penicillin/streptomycin/glutamine. The cells were seeded into 24-well tissue culture plates, grown to 90% to 95% confluency and transfected with the pGL3basic reporter plasmid using LipofectAMINE LTX (Invitrogen). The pCAG-IP mock vector or pCAG-IP-Prox1 vector was also co-transfected. As an internal control, the plasmid pRL-TK (Promega) containing the Renilla luciferase gene was transfected. Cells were cultured in medium for 48 hours and then lysed with passive lysis buffer (Promega). Luciferase activity was measured according to the technical manual for the Dual-Luciferase Reporter Assay System (Promega).

Statistics. We used the Microsoft Excel program to calculate standard deviations (SD) and statistically significant differences between samples using the two-tailed Student t test.

Results

Prox1 Regulates Migration and Proliferation of Fetal Hepatic Stem/Progenitor Cells. To examine whether Prox1 directly regulates functions of hepatic stem/progenitor cells, a retroviral overexpression system was established in the clonal culture of fetal hepatic stem/progenitor cells. Sorted $CD45^{-}Ter119^{-}Dlk^{+}$ cells were inoculated into collagen I–coated dishes (Fig. 1A and B). Prox1 and Lrh1 were expressed in primary sorted hepatoblasts derived from E13.5 fetal livers (Fig. 1C). We constructed a retrovirus vector expressing both Prox1 and enhanced green fluorescing protein (GFP) using the IRES sequence, and production of Prox1 was detected in fetal hepatoblasts infected with this retrovirus (Fig. 1C, D).

After a total of 6 days of culture, we analyzed single-cell–derived colonies that were infected with retrovirus, as shown by GFP expression. In mock virus–infected cultures, compact epithelial colonies formed. In contrast, Prox1 induced changes in size and morphology of colonies; larger and looser, scattered colonies were formed, suggesting that Prox1 regulated proliferation and migration of fetal hepatic stem/progenitor cells (Fig. 2A, B). We analyzed the effect of Prox1 on cell proliferation by counting colonies consisting of more than 10 cells. Both mock and Prox1-infected cells formed a lot of single-cell–derived colonies. However, the size of colonies differed between mock and Prox1-expressing cultures. Under our culture conditions, 3 to 7 colonies consisting of over 100 cells (large colonies) were detected per 500 hepatoblasts infected with mock retroviral vector. Large colonies derived from Prox1-expressing hepatic stem/progenitor cells were significantly increased in number, versus those derived from mock-infected cells ($P = 0.019$) (Fig. 2C).

To determine whether overexpression of Prox1 directly induced cell mobilization, we used wound closure

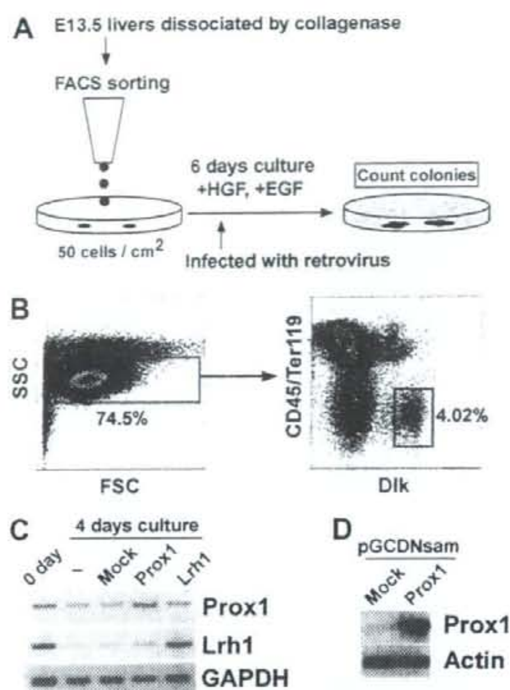

Fig. 1. *In vitro* colony assay system for fetal hepatic stem/progenitor cells. (A) E13.5 embryonic livers were dissected and dissociated by collagenase. After staining with an anti-Dlk antibody and anti-CD45 and anti-Ter119 antibodies, fetal hepatic stem/progenitor cells were sorted using FACS. Cells were inoculated at low density (50 cells/cm^2) and were cultured in the presence of HGF and EGF. (B) CD45$^-$Ter119$^-$Dlk$^+$ cells were sorted as fetal hepatic stem/progenitor cells. (C) Expression of Prox1 and Lrh1 in fetal hepatic stem/progenitor cells. Fetal hepatic stem/progenitor cells derived from E13.5 livers were sorted using FACS and cultured at low density for 4 days in the infection with no virus (−), mock virus (mock), Prox1-overexpressing virus (Prox1), and Lrh1-overexpressing virus (Lrh1). Total RNAs were purified from fresh sorted cells (0 day) and cultured cells. Expression of Prox1 and Lrh1 was detected using RT-PCR. (D) Infection of pGCDNsam-Prox1-IRES-GFP induced production of Prox1 protein in fetal hepatic stem/progenitor culture. Dlk$^+$ cells were infected with retroviruses and cultured for 4 days.

assays. Fetal hepatic stem/progenitor cells were cultured and infected with mock and Prox1-expression retroviruses. After a total of 3 days of culture, epithelial monolayer sheets had formed. Wounds were generated by scraping the cell monolayers, and culture was continued for 20 hours. Figure 2D shows that wounds persisted after 20 hours in mock-infected culture. Overexpression of Prox1 induced cell migration after 14 hours, and wounds had almost recovered within 20 hours. Our data clearly indicate that overexpression of Prox1 induced both hepatic proliferation and migration of hepatic stem/progenitor cells isolated from the E13.5 mouse liver.

Lrh1 Inhibits Proliferation of Fetal Hepatic Stem/Progenitor Cells. The transcriptional activity of Lrh1 is suppressed by the interaction with Prox1.[18,19] Expression of Lrh1 was detected in primary fetal hepatoblasts and significantly decreased in our culture system (Fig. 1C). Then, the effect of overexpression of Lrh1 on cell proliferation was examined. Sorted CD45$^-$Ter119$^-$Dlk$^+$ cells were inoculated and infected with pGCDNsam-Lrh1-IRES-enhanced GFP, a retrovirus capable of inducing overexpression of Lrh1. Expression of Lrh1 in fetal hepatoblasts infected with retroviruses was examined using RT-PCR (Fig. 1C). Figure 3A shows the morphology of colonies derived from mock and Lrh1-expressing cells. Colonies were compact in both, but colony size was decreased by Lrh1 overexpression. For quantitation of the Lrh1 effect, the colonies were counted and sized. Colonies derived from Lrh1-expressing cells were significantly fewer and smaller than those derived from mock-expressing controls. Numbers of medium-sized (50-99 cells) colonies in Lrh1-expressing culture decreased (Fig. 3B, $P <$ 0.01). In addition, large colonies disappeared with overexpression of Lrh1. These results indicate that Lrh1 and Prox1, when expressed to excess in our culture system, have opposing effects; Lrh1 inhibits proliferation of fetal hepatic stem/progenitor cells.

Prox1 Regulates Long-term Proliferation of Fetal Hepatic Stem/Progenitor Cells. As shown, Prox1 induced proliferation of fetal hepatic stem/progenitor cells in our 6-day colony assay system. When the culture was continued for a total of 10 days, proliferation of most cells stopped in mock-infected cultures. By contrast, in Prox1-overexpressing cultures, several colonies appeared proliferative. To evaluate the growth capacity of colonies in long-term cultures, expression of PCNA was analyzed. Figure 4A shows representative data on PCNA staining in mock and Prox1-infected colonies. Colonies with many PCNA-positive cells were detected in Prox1-expressing culture, whereas almost all mock-infected colonies had no PCNA-positive cells. We counted PCNA-positive colonies in 10-day colony cultures (Fig. 4B). PCNA-positive colonies could barely be detected in mock-expressing cultures derived from 500 fetal hepatic stem/progenitor cells. In contrast, 10 to 13 PCNA-positive colonies were found in Prox1-overexpressing culture. We inferred that long-term proliferation might be induced by overexpression of Prox1 in fetal hepatic stem/progenitor cells. Indeed, after 3 weeks of culture, no mock-infected colonies continued to proliferate, and none could be subcultured. However, a few Prox1-overexpressing colonies (1-2 colonies per 4000-8000 cells) continued to grow and could be subcultured into new collagen type I-coated dishes. Cells derived from subcultured colonies efficiently proliferated even af-

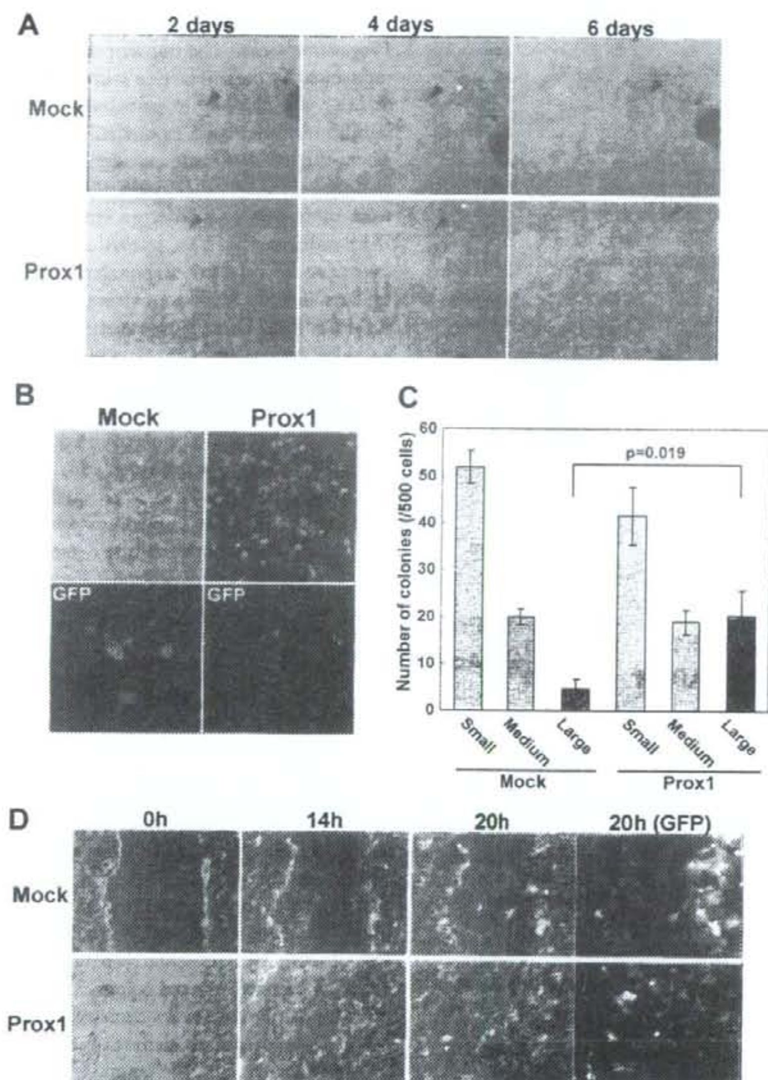

Fig. 2. Proliferation and migration of fetal hepatic stem/progenitor cells induced by Prox1. (A) Formation of a colony derived from a sorted fetal hepatic stem/progenitor cell. Marks in the substrate (shown by asterisks) serve to identify the field. Mock-infected and Prox1-overexpressing cells (shown by arrowheads) continued to grow and formed colonies. (B) Representative fluorescent view of colonies derived from single $CD45^-Ter119^-Dlk^+$ cells. Mock retrovirus-infected cells formed a compact epithelial colony (left panels). In contrast, Prox1-overexpressing cells formed a loose, scattering colony (right panels). Infection was indicated by GFP expression. (C) Numbers of GFP-positive colonies per 500 cells in fetal hepatic stem/progenitor cell culture system. Small colonies were 10 to 49 cells in number. Medium-sized colonies were formed by 50 to 99 cells in number. Large colonies were more than 100 cells in number. Results are represented as mean colony count ± SD (triplicate samples). (D) Photomicrographs of monolayers of Dlk^+ cells infected with mock and Prox1-expressing retroviruses. Wounds were generated by scraping epithelial cell sheets, and cells were cultured for 20 hours. Wound recoveries induced by cell migration were analyzed. Infection of retroviruses was detected by GFP expression.

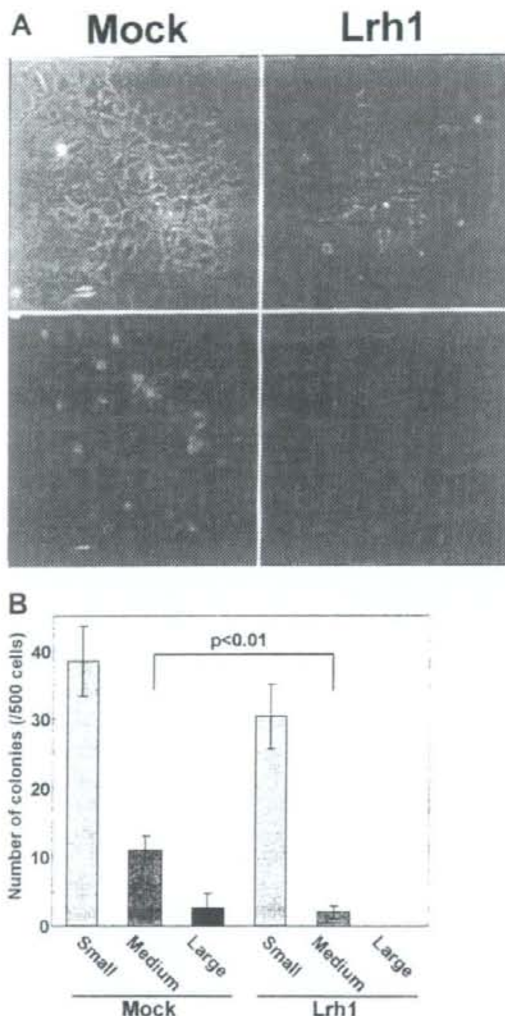

Fig. 3. Proliferation of fetal hepatic stem/progenitor cells inhibited by Lrh1. (A) Representative fluorescent view of colonies derived from single cells infected by mock and Lrh1-expressing retroviruses. Smaller colonies were formed with the overexpression of Lrh1 (right panels). Infection was indicated by GFP expression. (B) Number of GFP-positive colonies per 500 cells in fetal hepatic stem/progenitor cell culture system. Small colonies were 10-49 cells in number. Medium-sized colonies were 50-99 cells in number. Large colonies were more than 100 cells in number. Results are represented as mean colony count ± SD (triplicate samples).

ter several replatings, indicating that those cells maintained high proliferation potential. We named these cells E13HPCs (E13 liver-derived Hepatic Progenitor Cells). Representative views of two established E13HPC lines are shown in Fig. 4C. Both E13HPC lines formed epithelial monolayer sheets like those seen with fetal hepatic primary culture.[6] Expression of several hepatic genes was analyzed in E13HPC using RT-PCR (Fig. 4D). Both E13HPC lines expressed albumin and CK19, hepatocyte and cholangiocyte markers, respectively. In contrast, messenger RNA of the mature hepatocyte functional genes, glucose 6 phosphatase and tyrosine aminotransferase, was weakly or barely detected. Matrigel, an extracellular matrix produced from Engelbreth-Holm-Swarm mouse sarcomas, induces maturation of E14.5 liver-derived fetal hepatic cells *in vitro*.[7] The effect of matrigel on maturation was analyzed using cultured E13HPCs. Stimulation with matrigel for 48 hours significantly induced expression of both glucose-6-phosphatase and tyrosine aminotransferase (Fig. 4D). CK19 expression was decreased by exposure to matrigel. We infer that E13HPCs might be capable of both hepatocytic and cholangiocytic differentiation and that matrigel selectively induces differentiation of E13HPCs into mature hepatocytes.

Lrh1 Inhibits Prox1-Induced Continuous Growth of E13HPCs. To elucidate whether E13HPCs have features of multipotent progenitors, single-cell cultures of E13HPC were analyzed. E13HPCs were established from cells infected with pGCDNsam Prox1-IRES-GFP retrovirus, and single GFP^+ E13HPCs were sorted into individual wells of collagen type I–coated 96-well plates (Fig. 5A). After 14 days of culture, many large colonies had formed from individual single cells. We analyzed expression of hepatic marker genes in these colonies using RT-PCR. All colonies derived from single E13HPCs expressed both albumin and CK19 (Fig. 5B). In addition, we immunocytochemically assessed expression of albumin and CK19 in single-cell–derived colonies. Figure 5C shows that an individual E13HPC-derived colony contained $albumin^+$ and $CK19^+$ cells. These results suggest that E13HPCs maintained both high proliferative activity and bipotency for hepatocytic and cholangiocytic differentiation.

We analyzed whether Lrh1 inhibited proliferation of E13HPCs that continuously expressed Prox1. E13HPCs were infected with pGCDNsam Lrh1-IRES-KO retrovirus (Fig. 6A). Because individual GFP^+KO^+ cells expressed both Prox1 and Lrh1, these cells were sorted and individual cells were inoculated into single wells of 96-well plates (Fig. 6B, left panel). After 14 days, medium-sized colonies (50-100 cells) and large colonies (more than 100 cells) were counted. Over 20 colonies per 96 cells were formed in mock-infected culture, whereas only a very few colonies were detected in Lrh1-overexpressing culture (Fig. 6B, right panel). We also tested the dependency of Lrh1 expression levels on proliferation of E13HPCs. E13HPCs were infected with Lrh1-expressing retroviruses and sorted by Lrh1 and KO expression levels, using three sorting gates, KO^{high}, KO^{mid}, and KO^-. The

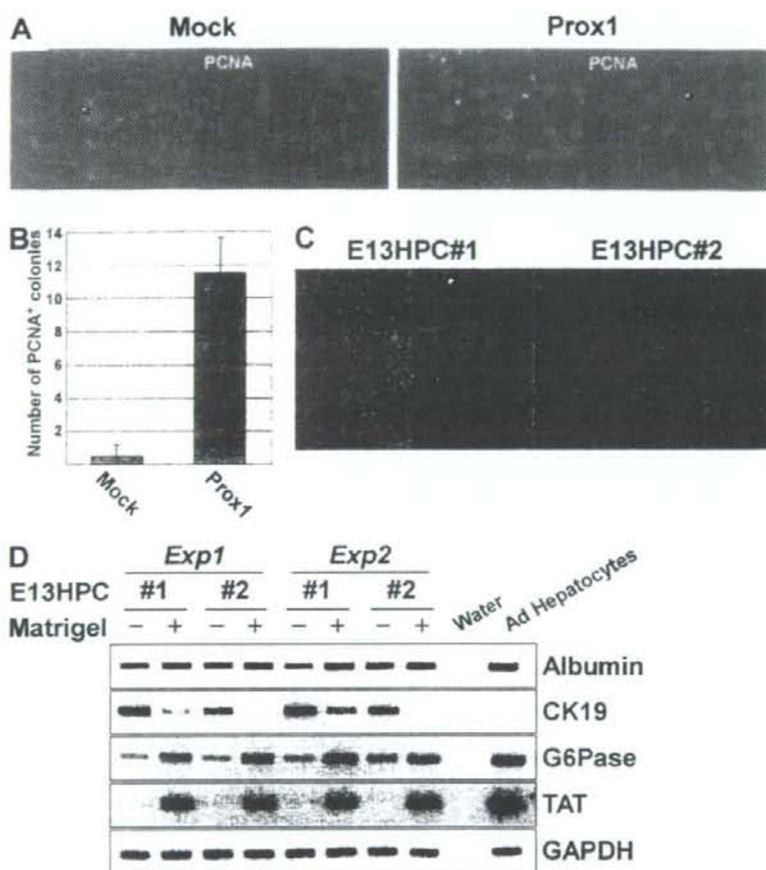

Fig. 4. Long-term proliferation of fetal hepatic stem/progenitor cells regulated by Prox1. (A) Ten-day cultured colonies derived from CD45$^-$Ter119$^-$Dlk$^+$ cells were fixed with formaldehyde and stained with a rabbit anti-PCNA antibody and an Alexa546-conjugated anti-rabbit IgG antibody. Representative fluorescent views are shown in mock (left panel) and Prox1-overexpressed (right panel) colonies. (B) Quantitation of PCNA-positive colonies per 500 cells in 10 days of culture shown in A. Results are represented as mean colony count ± SD of (duplicate samples). (C) After 3 weeks, continued growth of a few colonies was detected in Prox1-overexpressing culture. These colonies were subcultured using cloning rings. Phase-contrast photomicrographs show E13 liver-derived hepatic progenitor cells (E13HPCs) of clones 1 and 2. (D) Expression of mature liver marker genes in E13HPCs. Afterward, 2×10^5 cells were plated into individual wells of a six-well culture dish and were cultured for 3 days. Cells were continuously cultured in standard growth medium with EGF and HGF or stimulated by 20% Engelbreth-Holm-Swarm gel (Matrigel) for another 2 days. Expression of liver genes was detected using RT-PCR. Samples were normalized by copy numbers of glyceraldehyde 3-phosphate dehydrogenase using real-time PCR, with equal copies applied as templates. The results of two experiments are shown. Water, no template; Ad Hepatocytes, cDNA derived from purified adult hepatocytes.

expression level of Lrh1 regulated colony formation by E13HPCs in a dose-dependent manner (Fig. 6C). We infer that Lrh1 inhibited Prox1-induced continuous growth of E13HPCs.

Regulation of p16^{ink4a} Expression by Prox1 Through a Proximal Promoter Region. To analyze molecular mechanisms regulating Prox1-induced proliferation of fetal hepatic stem/progenitor cells, expression of cell cycle molecules was examined using RT-PCR. CD45$^-$Ter119$^-$Dlk$^+$ cells were sorted and cultured for 5 days after infection with mock and Prox1-expressing retroviruses. Total RNA was purified from these cells, and cDNAs were synthesized. Expression of cyclins D2, E1, and E2 were induced by overexpression of Prox1, suggesting that the increases in these cyclins may correlate to induction of proliferation (Fig. 7A). In addition, expression of cyclin inhibitors was analyzed (Fig. 7B). Consistent with results in other cells,[9] Prox1 up-regulated expression of p57^{kip2}. Expression of Prox1 in fetal hepatic stem/progenitor culture did not alter expression of other

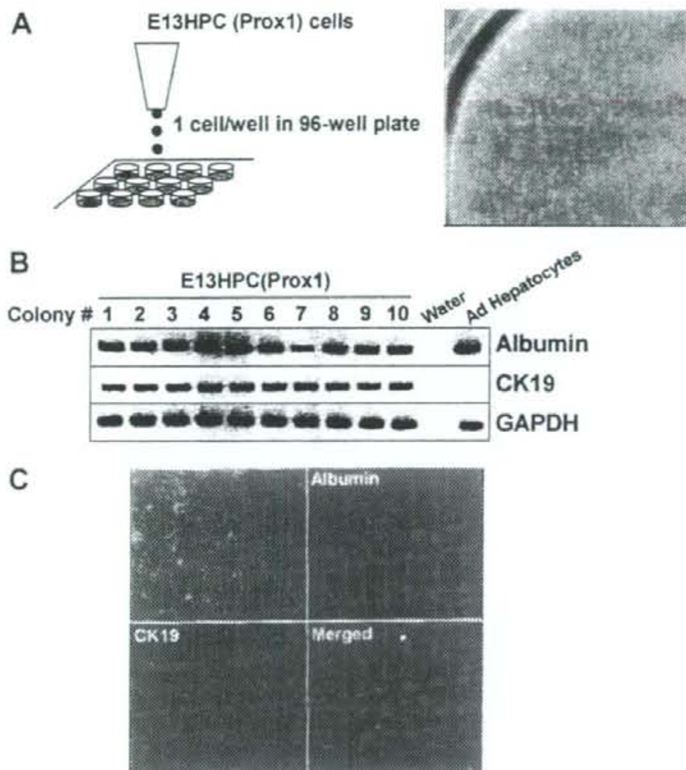

Fig. 5. Prox1-expressing E13HPCs manifest phenotypes of hepatic progenitor cells. (A) E13HPCs were sorted using FACS, and single-cell culture was performed on collagen type I–coated 96-well plates. A representative colony derived from E13HPCs in one well of a 96-well plate is shown in the right panel. (B) Clone-sorted E13HPCs cells were cultured for 14 days, and total RNAs were purified from 10 colonies. Expression of hepatic genes was detected in several colonies using RT-PCR. Water, no template; Ad Hepatocytes, cDNA derived from purified adult hepatocytes. (C) Clone-sorted E13HPC cells were cultured for 12 days and fixed with 4% paraformaldehyde. Cells were stained with anti-albumin and anti-CK19 antibodies. Signals were detected using Alexa350- and Alexa555-conjugated antibodies (for albumin and CK19, respectively).

cdk inhibitors, whereas it significantly suppressed expression of p16^{ink4a}. To elucidate whether Prox1 and Lrh1 regulates expression of p16^{ink4a} through its promoter, transcriptional activity of the p16^{ink4a}-proximal promoter region was analyzed using a luciferase assay system (Fig. 7C, D). Two p16^{ink4a} promoter-luciferase plasmids, the −1172 and −586/+81 fragments from a known transcription start site,[28] were constructed and analyzed by transient transfections. When Huh2.2 cells were transfected, the promoter activities of both −1172/+81 and −586/+81 fragments were almost 50-fold higher than that of the control pGL3basic vector lacking promoter sequence. To determine the effect of Prox1 on the promoter activity of p16^{ink4a}, Huh2.2 cells were co-transfected with the mock and Prox1-expression vectors. The activities of both −1172/+81 and −586/+81 fragments of p16^{ink4a} promoter were significantly suppressed by overexpression of Prox1 (Fig. 7C). In contrast, when cells were transfected with the Lrh1-expression vector, the activity of −1172/+81 fragment of p16^{ink4a} promoter was increased (Fig. 7D). In addition, overexpression of Prox1 induced expression of Lrh1 in fetal hepatoblasts (Fig. 1C and 7E), suggesting that expression and activation of Lrh1 was regulated by some feedback signals; Prox1 induced expression of Lrh1 and also suppressed the transcriptional activity of Lrh1. These results indicate that Prox1 inhibited expression of p16^{ink4a} through its proximal upstream region, and interaction of Prox1 and Lrh1 might be important for the regulation of the p16^{ink4a} promoter.

Ectopic expression of Prox1 in blood vascular endothelial cells up-regulates expression of the IIIc isoform of fibroblast growth factor (FGF) receptor (FGFR)-3, the major isoform of FGFR-3 expressed in lymphatic endothelial cells.[32] Consistently, overexpression of Prox1 in

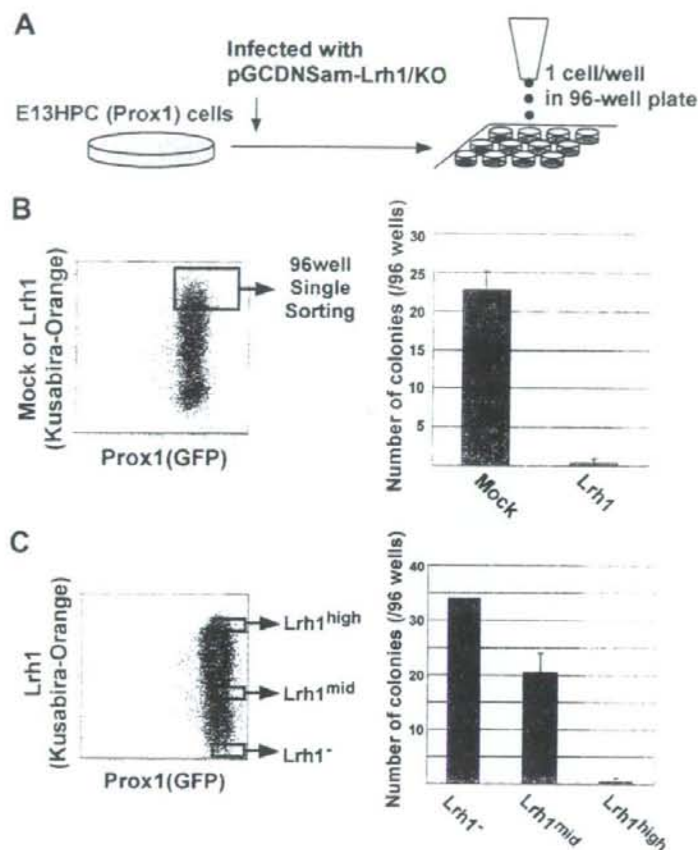

Fig. 6. Prox1-induced continuous growth of E13HPCs was inhibited by Lrh1. (A) E13HPCs were infected with pGCDNSam-IRES-KO or pGCDNSam-Lrh1-IRES-KO retroviruses. After 24 hours of incubation, cells were washed and cultured for 48 hours more. They were then sorted using FACS, and single-cell culture was performed using collagen type I–coated 96-well plates. (B) E13HPCs were infected by mock and Lrh1-overexpressing retroviruses containing IRES and KO genes. Infection by retroviruses was identified using FACS. Because E13HPCs had been established from Prox1-expressing cultured cell, the GFP signal was detected in almost all cells. Single GFP^+KO^+ E13HPC cells were sorted into individual wells of 96-well plates. (Right panel) Quantitation of single-cell–derived E13HPC colonies per 96 wells during 14 days of culture is shown in left panel. Colonies formed by over 50 cells were counted. Results are represented as mean colony count ± SD (triplicate samples). (C) E13HPCs were infected with Lrh1-expressing retroviruses, and cells were sorted using sorting gates responsive to the Lrh1-KO expression levels. Single GFP^+KO^{high}, GFP^+KO^{mid} and GFP^+KO^- E13HPC cells were sorted into individual wells of 96-well plates. (Right panel) Quantitation of single-cell-derived colonies per 96 wells after 12 days of culture is shown in left panel. Colonies formed by over 50 cells were counted. Results are represented as mean colony count ± SD (duplicate samples).

fetal hepatic stem/progenitor cells significantly induced expression of FGFR-3 but not of the HGF receptor *c-Met* (Fig. 7E). Recently, Prox1 suppressed expression of hepatic nuclear factor 4α in met murine hepatocyte line (MMH), a murine fetal hepatocyte cell line established from constitutive active c-Met transgenic mice.[33] In contrast, overexpression of Prox1 did not suppress hepatic nuclear factor 4α in our primary culture system, suggesting that functions of Prox1 are different between primary cells and immortal cell lines.

Discussion

Like albumin and alpha-fetoprotein, Prox1 is an early marker of hepatoblasts in murine embryos. Using LacZ knock-in mice, expression of Prox1 was detected in early hepatic primordium and dorsal pancreatic bud at E9.0-9.5 and in hepatic bud at E10.0-10.5.[14] A low level of Lrh1 was detected using RT-PCR in early-fetal whole livers.[34] The increase in expression of Lrh1 during perinatal and postnatal liver development is very similar in ex-

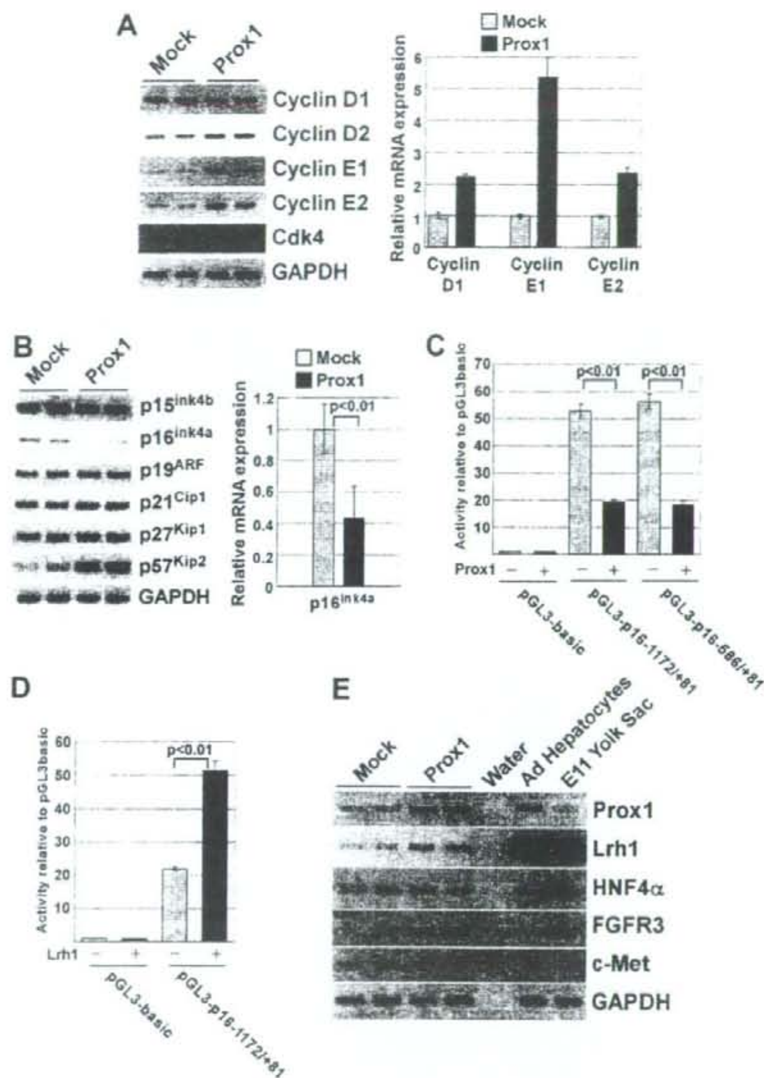

Fig. 7. Regulation of $p16^{ink4a}$ expression by Prox1 through a proximal promoter region. (A, B) (Left panels) Expression of cell cycle regulating molecules in fetal hepatic stem/progenitor cells. CD45 Ter119 Dlk^+ cells were sorted and infected with mock and Prox1-expression retroviruses. Duplicate samples were cultured for 5 days, and expression was analyzed using RT-PCR. Samples were normalized by copy numbers of glyceraldehyde 3-phosphate dehydrogenase, with equal copies applied as templates. (Right panels) Densitometries of expression of cell cycle regulating molecules. RT-PCR samples were electrophoresed and gel pictures were quantified using ImageJ. Results are represented as mean colony count ± SD (cyclin D2, E1, E2, $n = 2$; $p16^{ink4a}$, $n = 4$) (C, D) HuH2.2 cells were co-transfected with luciferase plasmids and expression vectors, as indicated. Promoter activities of the control vector, pGL3basic, co-transfected with mock, Prox1-expressing, and Lrh1-expressing vectors were set to 1.0. Each point is the mean ± SD (triplicate assays). (E) Expression of Prox1, Lrh1, hepatic nuclear factor 4α, FGFR3, and c-Met were examined using RT-PCR. Water, no template; Ad Hepatocytes, cDNA derived from purified adult hepatocytes. E11 Yolk Sac was used as positive control.

pression of Prox1.[13] We showed that Prox1 and Lrh1 have opposite effects on proliferation of fetal hepatic stem/progenitor cells (Figs. 2, 3). Prox1 directly interacts with Lrh1 and suppressed the transcriptional activity of Lrh1.[18,19] In keeping with these findings, overexpression of Lrh1 significantly suppressed sustainability in long-term culture of E13HPCs expressing Prox1 (Fig. 6). In addition, expression of Prox1 and Lrh1 was detected in sorted fetal hepatoblasts (Fig. 1C). After 4 days of culture, expression of Lrh1 was significantly suppressed, indicating that low-density expansion of hepatoblasts requires down-regulation of Lrh1, and that Prox1 might function in the Lrh1-independent manner. Previous results described that Prox1 interacted with and inhibited another transcription factor.[35] In contrast, expression of Lrh1 was slightly induced in Prox1-overexpressing cells (Fig. 1C, 7E). These results suggested that Prox1 induced proliferation of fetal hepatoblasts through both Lrh1-dependent and Lrh1-independent pathways. During the progress of liver development, expression of both Prox1 and Lrh1 synchronously increases, and the balance of Prox1 and Lrh1 regulates proliferation and maturation of hepatic development. A few $Prox1^+/CK19^+$ and $Prox1^+/CK7^+$ hepatic progenitor-like cells can be identified in liver after 2-acetyl-aminofluorene treatment with partial hepatectomy, when severe liver damage has led to proliferation of hepatic stem/progenitor cells, but not under normal conditions.[13] Expression of Prox1 thus appears important for proliferation of both early fetal hepatic stem/progenitor cells in normal liver development and adult hepatic progenitor-like cells in specifically damaged liver.

Prox1 also may be involved in tumorigenesis. Mutations and aberrant DNA methylation of *Prox1* occur in hematologic malignancies.[36] Hepatocellular carcinoma is a common solid tumor often induced by chronic infection with hepatitis B or C viruses. Shimoda et al. described that down-regulation of Prox1 expression in human hepatocellular carcinoma using small interfering RNA promotes proliferation of the hepatocellular carcinoma cell lines Hep3B and Huh7, and concluded that Prox1 might be a tumor suppressor.[37] In contrast, overexpression of Prox1 in blood vascular endothelial or other endothelial-type cells resulted in up-regulation of cell cycle progression genes, such as cyclins E1 and E2 and PCNA.[38] In addition, Fig. 7E shows that function of Prox1 was different between our primary hepatic culture and murine hepatic cell lines expressing constitutive active c-Met oncogene. These data, with our results in fetal hepatic stem/progenitor cells, suggest that Prox1 may activate proliferation in normal conditions but not in malignancy. Our data suggest that proliferation of fetal hepatic stem/progenitor cells is increased by Prox1 through suppression of $p16^{ink4a}$-expression (Fig. 7). Expression levels of $p16^{ink4a}$ and $p19^{ARF}$, shared with the same gene locus, are of significant importance in conferring stem cell potential. Overexpression of B cell-specific Moloney murine leukaemia virus integration site 1 (Bmi1) in fetal hepatic cells significantly induces proliferation and oncogenic features, as evidenced by morphology and continuous growth independent of contact inhibition.[39] Bmi1 down-regulates expression of both $p16^{ink4a}$ and $p19^{ARF}$, whereas inactivation of Ink4a/ARF gene locus largely rescues Bmi1-deficient phenotypes in the nervous and hematopoietic systems.[40-42] However, expression of $p19^{ARF}$ is not changed in fetal hepatic stem/progenitor cells infected with Prox1-expressing virus in our culture system (Fig. 7B). Prox1 can induce and establish long-term proliferative cells from fetal hepatic stem/progenitor culture, although efficiency is not high. Furthermore, when Prox1-induced long-term cultivated cells (E13HPCs) were transplanted into the non-obese diabetic/severe combined immunodeficiency mice, no malignancy was detected (Supplementary Fig. 1). These results indicate that another regulating molecule such as $p19^{ARF}$ might be of concern for high proliferation and oncogenesis in hepatocytes and that Prox1 might be useful for the induction of proliferation of fetal hepatic stem/progenitor cells without the risk of cancer formation. This study contributes not only to analyses of fetal liver development but also to progress toward safe clinical employment of hepatic stem/progenitor cells with active proliferative capability.

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Gene and cell therapy for relapsed leukemia after allo-stem cell transplantation

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TABLE OF CONTENTS

1. *Abstract*
2. *Introduction*
3. *Control of severe GvHD using the suicide gene*
4. *TK-DLI in the Tsukuba Hospital*
5. *Problems in TK-DLI*
6. *Conclusions*
7. *Acknowledgment*
8. *References*

1. ABSTRACT

To control severe GvHD while maintaining strong GvL effects in the context of allo-stem cell transplantation (allo-SCT), a phase I/II clinical trial of infusions of donor lymphocytes transduced with the herpes simplex virus thymidine kinase (TK-DLI) started at the Tsukuba University Hospital. To date, five (2 AML, 2 ALL, and 1 MDS) out of eight patients enrolled in the trial received approximately $7x10^7$ transduced cells per kilogram of body weight and four patients showed some clinical responses such inhibition of the leukemic cell proliferation or mitigation of lymph node swelling. Especially, one MDS patient achieved complete remission and has remained in CR for 2 years after the treatment. GvHD developed in two patients (1 acute and 1 chronic) and the acute (grade III) was successfully controlled by administration of ganciclovir without any immunosuppressive drugs. Since HSV-TK as a strong antigen induced CTLs against transduced cells in patients, however, TK-DLI is expected to provide a more effective adoptive immune cell therapy by performance just after allo-SCT where the patient's immune function is severely damaged.

2. INTRODUCTION

Transplantation of hematopoietic stem cells (HSCs) from HLA-matched related donors following both high-dose systemic chemotherapy and total-body irradiation (TBI) is the most effective treatment for patients with hematological malignancies (1-3). The initial rationale of allogeneic stem cell transplantation (allo-SCT) was based on a concept that SCT could provide patients with HSCs to reconstitute their bone marrow hematopoiesis that was devastatingly damaged by such intensive regimens. Recently, allo-SCT is referred to as immunotherapy for leukemia rather than solely a vehicle to delivery intensive therapy because donor lymphocytes transplanted with HSCs function as cytotoxic T lymphocytes (CTLs) against the patient's leukemic cells (4-6). Although only a few successful cases have demonstrated the existence of CTLs against leukemic cells (7), a strong graft-versus-leukemia (GvL) effect demonstrated by indirect evidence that the relapse rate increases if T cells are depleted from transplanted cells or in recipients of identical twin transplants, has made an infusion of donor lymphocytes (DLI) an standard treatment for patients with relapsed leukemia after allo-SCT, especially for those with relapse

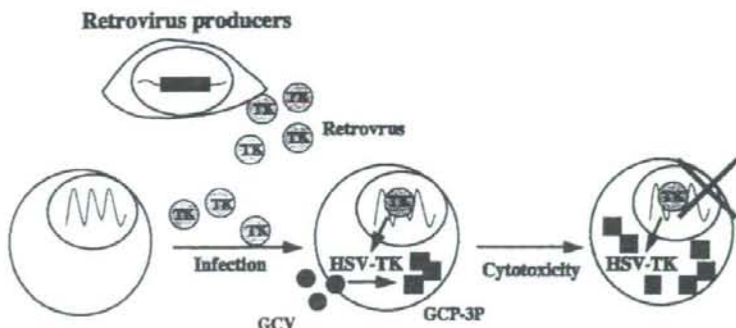

Figure 1. A strategy of TK-DLI using the HSV-TK/ GCV suicide system. Donor T lymphocytes are transduced with the HSV-TK gene using retroviral vectors and infused into a patient with relapsed leukemia. In a situation of no GCV, the cells are expected to function as CTLs against the patient's leukemic cells. In case of occurrence of severe GvHD, GCV is administrated into the patient to eradicate the transduced cells. GCV is phosphorylated in only the transduced cells and incorporated into the genomic DNA as a GCV-3P compound, resulting in inhibition of DNA chain elongation and apoptosis of the transduced cells.

of chronic myelogenous leukemia (CML) (8-12). Infusions of a large number of donor lymphocytes have proven to restore the full donor chimerism and produce long-term complete molecular genetic remissions in many CML relapsed cases because CML is more sensitive to DLI compared to other hematological malignancies. However, 50-60% of patients treated with DLI developed a possible adverse effect such as graft-versus-host disease (GvHD) resulting in a considerable amount of transplant-related mortality (TRM) (13). Although steroid (prednisolone) is used as first-line therapy against severe GvHD, the combination therapy with various immunosuppressive agents such as cyclosporine, anti-thymocyte globulin (ATG), and mycophenolate mefetil (MMF) is not frequently unsatisfactory in steroid-resistant GvHD (14). One of effective strategies to prevent the occurrence of severe GvHD while maximizing GvL effects is the escalating dose regimen (EDR) in which the number of donor cells infused into the patients increases in a step-wise manner until the disease responds to DLI or GvHD occurs (15). Indeed, the EDR can significantly reduce the rate of severe GvHD with equal GvL effects for CML patients. Since the strategy takes much time to acquire GvL effects, however, the ERD cannot be easily employed as a treatment for other acute leukemia with rapid progression. Another approach to decrease the risk of GvHD is to infuse specific effector lymphocytes that proliferate in response to leukemic cells into patients (16, 17). However, infusions of such cells show less significant GvL effects than do those of heterogenous lymphocytes because most of T cells exerting GvL effects recognize the patient's allo-antigens.

3. CONTROL OF SEVERE GvHD USING THE SUICIDE GENE

To overcome the problems, a strategy of genetic manipulation of donor lymphocytes using retroviral vectors expressing the herpes simplex virus thymidine kinase (HSV-TK) gene has been devised and tested in clinical trials (18-22). HSV-TK converts the prodrug ganciclovir (GCV) to its monophosphate imtermediate derivative that is further phosphorylated to di- and triphosphate (GCV-3P) compound by cellular kinases (23). The GCP-3P is incorporated into DNA during the cell division, resulting in inhibition of DNA chain elongation. In the trial, donor lymphocytes are transduced with the HSV-TK gene using retroviral vectors and infused into the patients (Figure 1). In a situation without GCV, the transduced lymphocytes are expected to function as CTLs against the patient's leukemic cells. If severe GvHD occurs, GCV is administrated into the patient to eradicate the transduced cells. In 1997, the Italian group reported successful cases of the gene therapy (TK-DLI) (18). They performed TK-DLI for 23 high-risk patients with hematological relapse after allo-SCT and reported clinical results of 17 patients who were alive more than 30 days after receiving the therapy. The number of transduced cells infused, although it varied among patients, was approximately $4x10^7$ per kilogram of body weight. Eleven patients (65%) experienced substantial clinical benefits, resulting in 6 complete remissions (2 CML, 1 AML, 2 NHL, and 1 multiple myeloma; 35%) and 5 partial responses (2 CML, 1 AML, 1 NHL, and 1 multiple myeloma; 29%). Four patients with GvHD (3 acute and 1 chronic) received GCV administrations, resulting in elimination of the transduced cells and control of severe GvHD.

Based on successful results of the clinical trial, they have extended the strategy to haplo-SCT for hematologic malignancies (24). Halpo-SCT is the last option for patients who lack an HLA-identical donor but it remarkably increases the rate of morbidity and mortality due to severe GvHD. Transplantation of hematopoietic progenitor cells ($CD34^+$ cells) obtained from haplo-identical donors followed by infusions of lymphocytes transduced with HSV-TK gene in an incremental manner (TK add-back) would help rapid immune recovery to protect from viral infection and relapse, and control severe GvHD by administration of GCV if it occurs. Eight patients with high-risk hematologic malignancies who underwent

Table 1. Clinical protocol of TK-DLI in Tsukuba university hospital

Title	Infusions of donor lymphocytes transduced with the herpes simplex virus thymidine kinase gene into patients with relapsed leukemia after allogeneic stem cell transplantation
Population	Patients >2years of age with relapsed hematologic malignancies after allo-SCT
Sample size	Five patients for 3 years
Treatment	Infusion of $1x10^8$ transduced cells per kilogram of body weight
	Infusion of GCV (5mg/kg) twice a day for 7 days at severe GvHD (grade III)
End Point	
1st	Safety, GvL effects, Control of GvHD by administration of GCV
2nd	Adverse effects, Overall survivals, relapse
3rd	Immunological study (CTLs against TK-expressing cells)

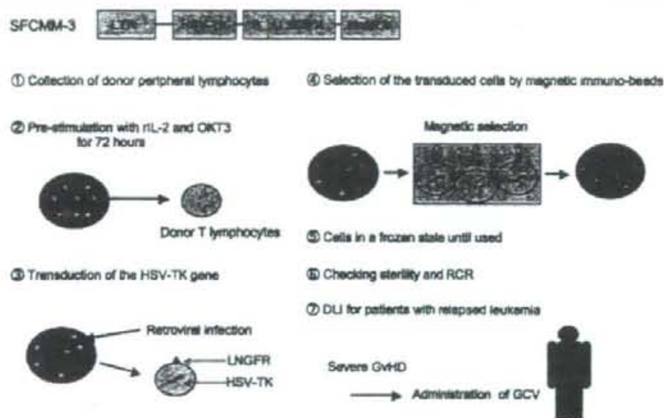

Figure 2. A structure of the retroviral vector SFCMM-3 and a Tsukuba TK-DLI protocol.

haplo-SCT were enrolled; three patients received $1x10^6$ and five did $1x10^7$ of transduced cells per kilogram of body weight. Although no immune reconstitution was observed in patients who received $1x10^6$, three out of 5 patients with infusions of $1x10^7$ recovered full immune reconstitution and showed significant reduction of the incidence of viral infection. Especially, two patients out of these three have been free from leukemic relapse. Regarding GvHD occurrence, one patient out of the three developed acute GvHD (grade II) that was quickly controlled by administration of GCV. Given that an effective dose to reconstitute the full immune function and prevent the relapse is $1x10^7$ per kilogram of body weight, a phase III multi-center trial in which patients undergoing halpo-SCT are infused with the similar dose of transduced cells several times in certain intervals is under way in Europe.

4. TK-DLI IN THE TSUKUBA HOSPITAL

In collaboration with Dr. Bordignon at the H. S. Raffaele Institute, we started a phase I/II clinical trial of TK-DLI for patients with relapsed leukemia after allo-SCT at the Tsukuba University Hospital in 2004 (Table 1). A retroviral vector used is the SFCMM-3 that expresses both HSV-TK and nerve growth factor receptor (NGFR) genes and a working process of transduction into peripheral mononuclear cells is shown in Figure 2 and 3. Peripheral mononuclear cells collected from donors by apheresis (CS3000plus; Baxter Corp, IL) are maintained in RPMI-1640 medium with 3% autologous serum in gas-permeable culture bags (GT-T610; Takara Bio, Japan) and pre-stimulated with a high dose of recombinant human interleukin-2 (600U/ml, Proleukin[R]; Chiron, CA) and anti-CD3 antibody (Orthoclone OKT3 Injection; Ortho, NJ) for 72 hours. For transduction, cells are suspended with the viral supernatant at $5x10^6$/ml, transferred into small bags with tolerance to centrifugation (Cryocyte Frysebeholder-50ml; Baxter), and then centrifuged at 2000*g* for 2 hours using the bucket-type centrifuge (MX301; TOMY, Japan). At 72 hours after two rounds of transduction, cells are stained with anti-NGFR antibody and magnetic immune-beads (DynaBeads M450, sheep anti-mouse IgG; Invitrogen, CA) to isolate transduced cells with Isolex 50 (Baxter). Isolated cells are cultured to expanded for additional 3 to 5 days and stocked in -150°C until used. In this trial, patients are supposed to receive transduced cells at $1x10^8$ per kilogram of body weight in a single infusion. To prepare such a large number of cells sterilely, we have developed the culture system allowing performance of all procedures from collection of donor mononuclear cells to infusion into patients in bags (Figure 3). In particular, the automatic cell manipulator, CyteMate (Nexell Therapeutics Inc.) enabled us to wash and concentrate a large number of cells in a relatively short time (one liter in a hour).

So far, nine transduction procedures have been done for eight enrolled patients and cells ranging from $4.4x10^9$ to $2.4x10^{10}$ was prepared (Table 2). The

Table 2. Patients enrolled in Tsukuba TK-DLI Trial

UPN	Diagnosis	Age, Sex	# of prep (/kg)	NGFR+	# of infused (/kg)	GvHD
1	MDS (RAEB)	42, M	$1.0x10^{10}$ ($1.8x10^{8}$)	93.5%		
2	ALL (Ph1+)	15, F	$4.6x10^{9}$ ($1.2x10^{8}$)	97.8%		
3	AML	60, M	$1.0x10^{10}$ ($2.3x10^{8}$)	97.2%	$3.8x10^{9}$ ($7.7x10^{7}$)	acute (grade III)
4[1]	ALL	20, M	$0.1x10^{9}$ ($0.2x10^{7}$)	37.7%		
5[1]	ALL		$4.4x10^{9}$ ($8.8x10^{7}$)	93.1%		
6	MDS (RAEB)	58, M	$2.4x10^{10}$ ($3.1x10^{8}$)	95.1%	$9.7x10^{8}$ ($9.5x10^{7}$)	chronic
7-1	ALL	14, M	$7.9x10^{9}$ ($2.6x10^{8}$)	94.9%	$2.0x10^{9}$ ($6.7x10^{7}$)	
-2					$5.0x10^{9}$ ($8.5x10^{7}$)	
8-1	AML	46, M	$1.8x10^{10}$ ($2.3x10^{8}$)	90.7%	$9.0x10^{9}$ ($8.5x10^{7}$)	
-2					$9.0x10^{9}$ ($8.5x10^{7}$)	
9-1	ALL	50, M	$7.2x10^{9}$ ($1.8x10^{8}$)	90.7%	$4.4x10^{9}$ ($8.6x10^{7}$)	
-2					$2.1x10^{9}$ ($4.1x10^{7}$)	

[1] an identical patient

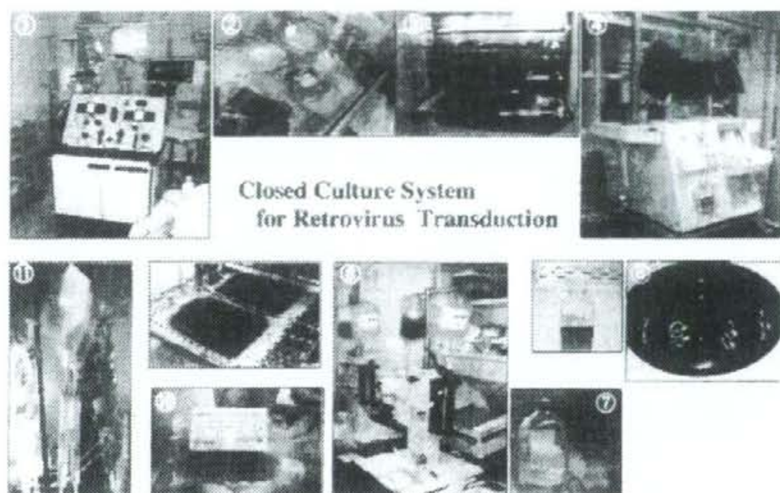

Figure 3. The closed culture system for transduction into peripheral lymphocytes. Collection of donor lymphocytes using CS3000plus (1), cell culture in gas-permeable culture bags (2, 3), cell washing using CyteMate (4), SFCMM-3 virus sup (5), transduction by spinoculation (6), anti-NGFR antibody (7), isolation of transduced cells using Isolex 50 (8), culture and collection of transduced cells (9, 10), and infusion into patients (11).

transduction efficiency was approximately 20% and the purity of NGFR-expressing cells isolated using Isolex 50 exceeded 90% in all the cases except for UPN 4. All samples cleared the safety tests including cell viability, sterility, existence of replication competent retrovirus (RCR), and sensitivity to GCV. Five out of 8 patients (2 AML, 2 ALL, and 1 MDS) were treated with TK-DLI and three (UPN 7, 8, and 9) among them received the infusions twice (Table 2). The number of cells infused, although it varied among patients, was approximately $8.7x10^{7}$ per kilogram of body weight. Four patients showed some clinical responses such as inhibition of leukemic cell proliferation, mitigation of lymph node swelling, and lowing the values of tumor markers. Especially, a MDS patient (UPN 6) achieved complete remission and has remained in CR for 2 years after the treatment. Regarding GvHD occurrence, a patient (UPN 3) developed the grade III GvHD due to severe liver dysfunction that was successfully controlled by administration of GCV without any immunosuppressive drugs. Another patient (UPN 6) showed chronic GvHD with precordial erythema that has been observed without any treatments. No adverse effects related to gene therapy have been observed.

5. PROBLEMS IN TK-DLI

Although TK-DLI proved to be clinically beneficial, it also has several critical problems. One of the major limitations of TK-DLI is considered to be the strong immunogenicity of the viral protein, HSV-TK. Two reports revealed that infusions of the transduced cells into immune-competent patients resulted in the development of an immune response to TK-derived epitoses (25, 26). Once cytotoxic T lymphocytes (CTLs) against cells expressing HSV-TK are developed in patients, the transduced cells infused would be eradicated from the patient body in no time at all. Indeed, HSV-TK-expressing cells in our patients without any GvL effects had a very short time to survive in the patient's peripheral blood, which was measured by quantitative PCR (TaqMan PCR).

Interestingly, the Italian group observed that patients who received infusions of transduced cells at the immuno-suppressed condition, e.g. soon after stem cell transplantation, caused less development on such an immune response. These results suggest that TK-DLI is the most suitable therapy in the context of allo-SCT from partially mismatched or unrelated donors, where the risk of severe GvHD is particularly high, and patients are profound immunodeficient.

Another problem is weaker immune response of cultured cells against allo-antigens compared with that of primary lymphocytes. In general, *in vitro* culture to manipulate donor lymphocytes genetically impairs their immune functions (27, 28), which may explain why a few patients developed severe GvHD despite infusions of a large number of donor cells in our trial. An improved culture condition could preserve the T-cell repertoire and their immune functions (29).

6. CONCLUSIONS

The clinical trial confirmed the safety and therapeutic effects of the suicide-gene transduced lymphocytes for relapsed leukemia after allo-SCT. Furthermore, acute GvHD could be controlled by administration of GCV without any immunosuppressive drugs. However, rapid disappearance of transduced lymphocytes was also observed in patients without any clinical benefits. Since the suicide gene derived from viruses elicits immune responses in patients as a strong antigen, it is likely that CTLs against HSV-TK eradicated transduced cells soon after infusions. While an approach to the problem is to use the suicide genes of human origin instead (29, 30), an alternative is to combine TK-DLI with allo-SCT in which the patient's immune function is severely damaged to impair T cell priming against foreign antigens (24).

With further modifications including vector constructs (31, 32), culture conditions (33), and the timing of infusions, the suicide-gene strategy would offer the safe and effective immune cell therapy for patients with hematologic malignancies.

7. ACKNOWLEDGMENT

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Abbreviations: allo-SCT: allogeneic stem cell transplantation; GvL: graft versus leukemia; GvHD: graft versus host disease; DLI: donor lymphocyte infusion; GCV: ganciclovir; HSCs: hematopoietic stem cells; TBI: total-body irradiation; CTLs: cytotoxic T lymphocytes; CML: chronic myelogenous leukemia; TRM: transplant-related mortality; ATG: anti-thymocyte globulin; EDR: escalating dose regimen; HSV-TK: herpes simplex virus thymidine kinase; NGFR: nerve growth factor receptor; rIL-2: recombinant human interleukin-2