**Table 1.** Primers Used in This Study

| Symbol | Gene Name | Assay ID |
|---|---|---|
| Housekeeping genes | | |
| *Gapdh* | glyceraldehyde-3-phosphate dehydrogenase | Hs99999905_m1 |
| *Actb* | actin, beta | Hs99999903_m1 |
| *Ppi* | peptidylprolyl isomerase A | Hs99999904_m1 |
| *Rpl4* | ribosomal protein L4 | Hs03044647_g1 |
| *Tfrc* | transferrin receptor | Hs00174608_m1 |
| *Gusb* | glucuronidase, beta | Hs99999908_m1 |
| *Hprt1* | hypoxanthine phosphoribosyltransferase | Hs99999909_m1 |
| Target genes | | |
| *F2* | coagulation factor II (prothrombin) | Hs01011995_g1 |
| *F7* | coagulation factor VII | Hs00173398_m1 |
| *F9* | coagulation factor IX | Hs00609168_m1 |
| *F10* | coagulation factor X | Hs00173450_m1 |
| *Prosc* | protein C | Hs00165584_m1 |
| *Pros1* | protein S, alpha | Hs00165590_m1 |
| *F8* | coagulation factor VIII | Hs00240767_m1 |

*Expression of Coagulation Factor Genes*

Human-specific coagulation-related gene expression levels were assessed on human hepatocyte-repopulated uPA/SCID mouse livers. The genes analyzed were: vitamin K-dependent coagulation factors (prothrombin, factor VII, and factor X) and anticoagulation factors (protein C and protein S), in addition to factor VIII. Raw expression levels of all vitamin K-dependent coagulation and anticoagulation factor genes showed a positive correlation with the repopulation ratios (Fig. 2). The correlation coefficient between the gene expression levels and the repopulation ratios were 0.78, 0.74, 0.80, 0.80, and 0.82 in *F2*, *F7*, *F10*, *Prosc*, and *Pros1*, respectively. The raw gene expression levels of all but *F8* were beyond the levels of the normal human liver samples (defined as 1.0) as the repopulation ratios increased. In marked contrast, *F8* gene expression levels were less than 40% of normal human liver tissues even though the repopulation ratios reached approximately 100%. The low levels of *F8* gene expression failed to show a significant correlation with the repopulation ratios ($R = 0.66$).

In order to evaluate the gene expression levels per human hepatocytes, the gene expression levels were normalized by *ACTB* gene expression levels in each sample (Fig. 3). As a result, normalized gene expression values of all the analyzed coagulation-related factor genes showed constant expression levels regardless of the repopulation ratios, demonstrating that the human hepatocytes in the uPA/SCID livers stably express coagulation-related factor genes throughout the repopulation stages.

## DISCUSSION

Propagation of primary human hepatocytes that possess hepatocyte-specific functionalities including blood clotting factor production has been one of the major paradigms in liver regenerative medicine (18,24). In the present study, we transplanted primary human hepatocytes to the liver of uPA/SCID mice and succeeded in propagating the human hepatocytes in the mouse livers. We then investigated mRNA expression levels of human-specific vitamin K-dependent coagulation factors (prothrombin, factor VII, and factor X), anticoagulation factors (protein C and protein S), and factor VIII of the propagated hepatocytes at various stages of propagation. The results showed that mRNA expression levels per human hepatocyte of all the analyzed genes were maintained through the propagation stage, indicating that the uPA/SCID in-mouse hepatocyte propagation system is a viable method to propagate hepatocytes that are intact in coagulation factor productions.

Coagulation factors are produced mainly by hepatocytes, and the long-term synthesis of these factors from primary human hepatocytes in vitro have been recently achieved by plating cells inside a 3D collagen gel matrix together with hormonally enriched culture medium under chemically defined conditions (6), indicating the pivotal role of the extracellular environment for coagulation factor production. However, the current procedure for the culture of primary hepatocytes appears to be difficult to support extensive cell proliferation (19), still remaining the problem of donor cell shortage unresolved. It is true that isolated hepatocytes could obtain proliferating ability and long-term survival in vitro by immortalization (26,34,35) or by selective culture of small hepatocyte population (27), but there is no report for studying the gene expression and production of coagulation factors including factor IX in these cell types. On the other hand, embryonic stem (ES) cells and

induced pluripotent stem (iPS) cells have been intensively investigated as an attractive cell source for liver regenerative medicine, and differentiation technologies of these stem cells into hepatocyte-like cells have been improved (12). In these circumstances, Basma et al. (3) recently succeeded in differentiating human ES cells into hepatocytes-like cells maintaining the ability of human factor VII production; however, the expression of coagulation factors other than factor VII were undocumented. In contrast to these in vitro cell culture systems, there are several in vivo hepatocyate propagating systems, in which transplanted hepatocytes can efficiently proliferate in mouse livers, such as uPA/SCID mice (29) or fumarylacetoacetate hydrolase$^{-/-}$/recombination activating gene$^{-/-}$/gamma chain of the interleukin-2 receptor$^{-/-}$ (Fah$^{-/-}$/Rag2$^{-/-}$/Il2rg$^{-/-}$) mice (2).

In our previous series of experiments, we found that human hepatocytes that were transplanted into the liver of uPA/SCID mouse perform active cell proliferation leading to a nearly total repopulation of the liver (17, 29,30,36) and confirmed that those proliferated hepatocytes maintained their ability to produce and secrete biologically functional human factor IX (30). In addition to human hepatocytes, we also reported that primary canine hepatocytes could proliferate in uPA/SCID mouse liver while retaining functions for canine factor IX production

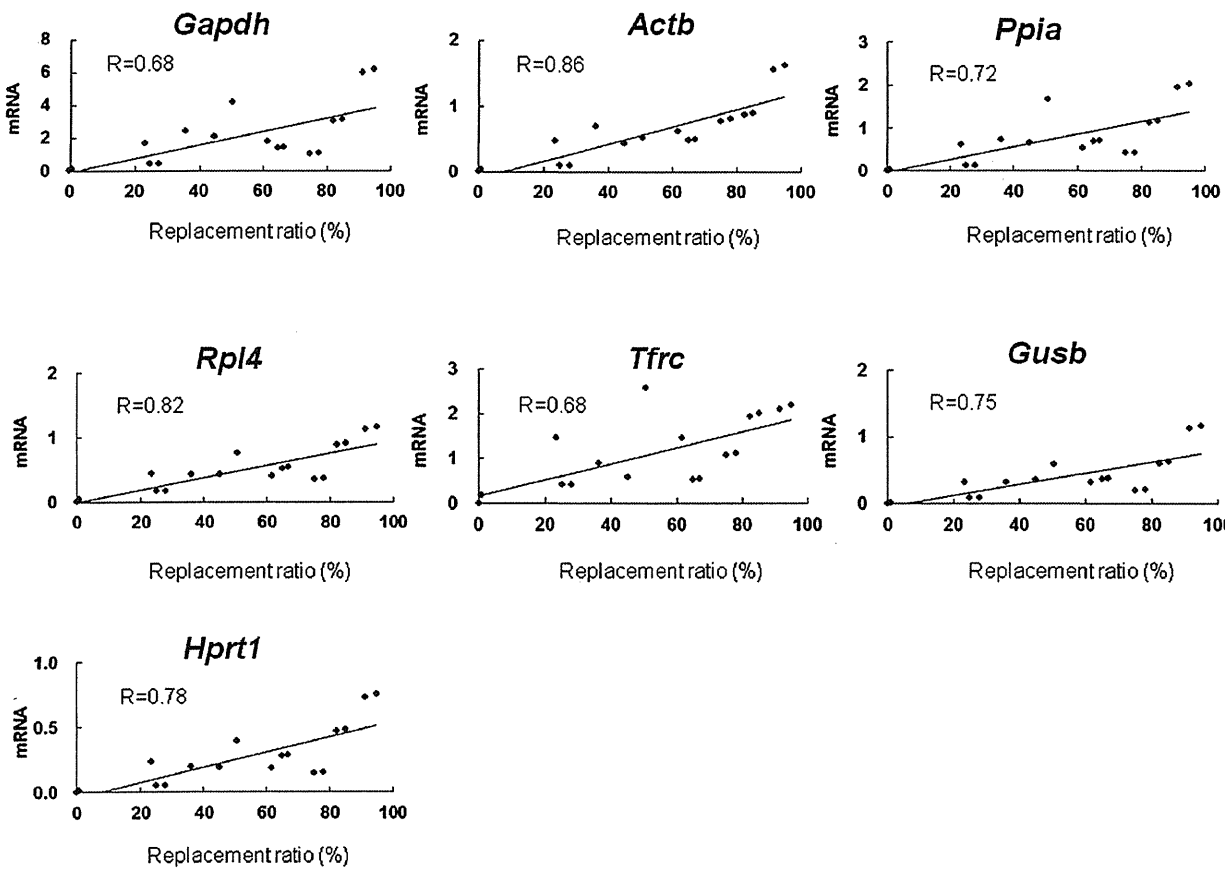


**Figure 1.** The raw gene expression levels of human housekeeping genes in the human hepatocytes repopulated in human hepatocytes were transplanted into urokinase-type plasminogen activator transgenic sever combined immunodeficient uPA/SCID mouse livers. Isolated uPA/SCID mouse livers ($n$ = 18), and the livers were excised at various points of repopulation ratios determined by blood human albumin levels and human-specific cytokeratins 8 and 18 (hCK8/18) immunohistochemistry on the liver sections. The repopulation ratio ranged from 0% to 98% (0–20%, two mice; 21–40%, four mice; 41–60%, four mice; 61–80%, four mice; and 81–100%, four mice). Gene expression levels of commonly used seven housekeeping genes [glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), β-actin (*Actb*), peptidylprolyl isomerase A (*Ppia*), ribosomal protein L4 (*Rpl4*), transferrin receptor (*Tfrc*), β-glucuronidase (*Gusb*), and hypoxanthine phosphoribosyltransferase (*Hprt1*)] in human–chimeric mouse liver samples were quantified by real-time PCR with human-specific primers and expressed as relative values against the control normal human liver tissue (defined as 1.0). The correlation coefficient of each gene was expressed as an $R$ value.

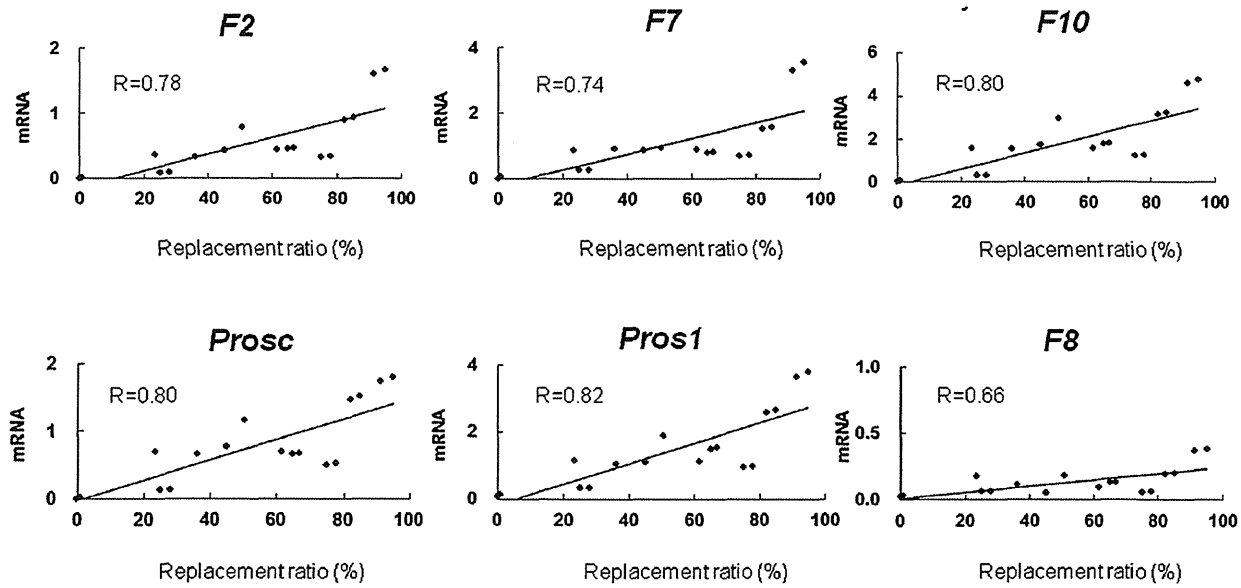


**Figure 2.** The raw gene expression levels of human coagulation and anticoagulation factors in the human hepatocytes repopulated in the uPA/SCID mouse livers. Isolated human hepatocytes were transplanted into uPA/SCID mouse livers ($n = 18$), and the livers were excised at various points of repopulation ratios determined by blood human albumin levels and hCK8/18 immunohistochemistry on the liver sections. The repopulation ratio ranged from 0% to 98% (0–20%, two mice; 21–40%, four mice; 41–60%, four mice; 61–80%, four mice; and 81–100%, four mice). Gene expression levels of coagulation factors (prothrombin, *F2*; factor VII, *F7*; factor X, *F10*; and factor VIII, *F8*) and anticoagulation factors (protein C, *Prosc*; protein S, *Pros1*) in the human–chimeric mouse liver samples were quantified by real-time PCR with human-specific primers and expressed as relative values against the control normal human liver tissue (defined as 1.0). The correlation coefficient of each gene was expressed as an *R* value.

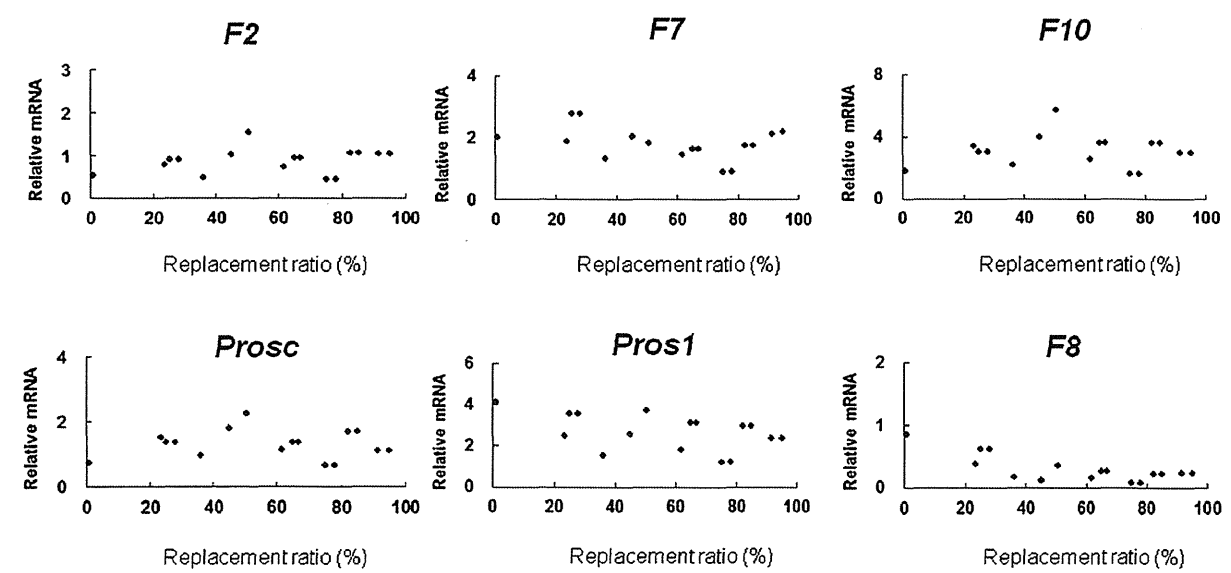


**Figure 3.** The relative gene expressions levels of human coagulation and anticoagulation factors in the human hepatocytes repopulated in the uPA/SCID mouse livers. The raw gene expression levels of human coagulation and anticoagulation factors show in Figure 2 were normalized with *Actb* gene expression levels in each human–chimeric mouse liver samples and plotted the repopulation ratios.

(30). At least, this in-mouse hepatocyte propagating system is only an available and promising procedure for proliferating factor IX-producing hepatocytes at the present time. In this regard, it is important to clarify gene expression of other coagulation factors as well as factor IX in propagated human hepatocyte in uPA/SCID mouse liver, because to establish a method for hepatocytes proliferation while retaining ability for coagulation factor production is indispensable for clinical cell therapy toward coagulation disorders.

We first assessed the expression levels of seven commonly analyzed HKGs for human genome in the human hepatocyte-repopulated uPA/SCID mouse livers. The gene expression levels of all seven HKGs increased in parallel to the increase of the repopulation ratio with a high correlation coefficient, providing direct evidence that the transplanted human hepatocytes progressively proliferated in uPA/SCID mouse livers (Fig. 1). Interestingly, as for six out of seven HKGs analyzed, the gene expression levels of samples with more than 80% repopulation ratios surpassed the levels of normal human livers (arbitrarily defied as 1.0). It was reported that the upregulation of certain HKGs, especially *Gapdh*, was closely associated with the events of DNA synthesis and cell division (1). Such HKG upregulation profiles associated with the hepatocyte proliferation were also observed in our previous works where hepatocyte proliferation in mouse livers was induced through the mode of compensatory regeneration (32) or direct hyperplasia (28). These findings suggest that human hepatocytes progress their cell cycling events in the uPA/SCID livers while upregulating their structural proteins necessary for cell proliferation. Since HKGs are used as internal reference gene(s) in gene expression analyses, it is essential to identify appropriate HKGs that are stably expressed during examined pathological process. During the human hepatocyte repopulation process in the uPA/SCID livers, we found that the expression levels of *Actb* showed the highest correlation coefficient ($R = 0.86$) with the repopulation ratios among seven HKGs analyzed. Therefore, we concluded that the use of *Actb* for normalization of gene expression levels was appropriate for obtaining accurate gene expression values.

To elucidate gene expression levels of coagulation and anticoagulation factors in propagating human hepatocytes in the liver of uPA/SCID mice was the main objective in this study. Using human-specific PCR primers, we found that raw expression levels of all genes increased in parallel to the increase of repopulation ratios with a high correlation coefficient. As the repopulation ratios increased higher than 80%, the expression levels of all genes except *F8* surpassed the levels of normal human livers (Fig. 2). We also investigated the gene expression levels per human hepatocyte by normalizing the expression levels of each gene by the levels of *Actb*. Results clearly showed that the gene expression levels of all analyzed coagulation and anticoagulation factors were stably maintained throughout the in-mouse repopulation process (Fig. 3). Overall, the present study combined with our previous investigation (30) demonstrated that human primary hepatocytes were able to proliferate in the liver of uPA/SCID mice while retaining the cellular machinery for expressing *F2*, *F7*, *F9*, *F10*, *Prosc*, and *Pros1*. Since human hepatocyte propagated in uPA/SCID mice are able to be isolated and purified by cell-sorting technology (37), the present in-mouse propagated human hepatocytes is a feasible candidate cell source for a future therapeutic use toward coagulation factor deficiencies. Furthermore, it was reported that efficient gene transduction into proliferated human hepatocytes in uPA/SCID mice was possible by using retroviral vector system (9), indicating the capability of obtaining hepatocytes that were genetically modified in vivo for the purpose to achieve higher expression levels of target proteins (15).

In the present study, gene expression levels of *F8* were remarkably low compared with those of other factors assessed. Even the liver samples showing the repopulation ratio more than 80% demonstrated only less than 40% of the control normal human liver tissues in human *F8* gene expression (Fig. 2). uPA/SCID mice have a characteristic to allow their own hepatocytes to be replaced by repopulated human hepatocytes, but all other intravital environment as well as nonparenchymal liver cells remain predominantly host origin. As a result, it is likely that various signal cross-talks between repopulated human hepatocytes and host cells or humoral factors might become dysfunctional. For example, although hepatocytes require growth hormone (GH) for their active cell proliferation, human hepatocytes transplanted into mouse livers failed to be the target for such growth advantage because rodent GH is unable to bind to human GH receptors (17). We have indeed established that this functional deficiency could be fully recovered by administration of human GH to the human hepatocyte-repopulated uPA/SCID mice (17). The fact that human *F8* gene expression level failed to increase may be explained by some mechanism similar to this type of interspecies incompatibility. An alternative possibility is that nonparenchymal cells, but hepatocytes, are the main responsive cells for human factor VIII productions. Although the liver has been shown to be the major site of factor VIII production as evidenced by previous liver transplantation clinical experiences (10,13,33), the precise type of liver cells contributing to factor VIII production has not been fully identified (4,6,8,16). In either the case, the present in-mouse hepatocyte propagation system using uPA/SCID mouse might be a valuable tool

for the elucidation of cellular mechanism of factor VIII synthesis and production.

In conclusion, the present study provides encouraging evidence that uPA/SCID mouse system supported the active proliferation of human hepatocytes while maintaining cellular machinery to produce vitamin K-dependent coagulation (prothrombin, factor VII, and factor X) and anticoagulation factors (protein C and protein S) in addition to factor VIII. The current work thus can serve as a basis to create a hepatocyte propagation system to prepare sufficient amount of cells for the therapeutic purposes for deficiencies of these factors as well as for the research purpose to investigate the hepatocyte-specific production mechanisms of coagulation factors.

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## REFERENCE

1. Asahina, K., Sato, H., Yamasaki, C.; Kataoka, M.; Shiokawa, M.; Katayama, S.; Tateno, C.; Yoshizato, K. Pleiotrophin/heparin-binding growth-associated molecule as a mitogen of rat hepatocytes and its role in regeneration and development of liver. Am. J. Pathol. 160:2191–2205; 2002.
2. Azuma, H.; Paulk, N.; Ranade, A., Dorrell, C., Al-Dhalimy, M.; Ellis, E., Strom, S.; Kay, M. A., Finegold, M.; Grompe, M. Robust expansion of human hepatocytes in $Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$ mice. Nat. Biotechnol. 25:903–910; 2007
3. Basma, H., Soto-Gutierrez, A.; Yannam, G. R.; Liu, L.; Ito, R.; Yamamoto, T.; Ellis, E.; Carson, S. D.; Sato, S.; Chen, Y., Muirhead, D.; Navarro-Alvarez, N.; Wong, R. J., Roy-Chowdhury, J.; Platt, J. L., Mercer, D. F.; Miller, J. D., Strom, S. C.; Kobayashi, N., Fox, I. J. Differentiation and transplantation of human embryonic stem cell-derived hepatocytes. Gastroenterology 136:990–999; 2009.
4. Biron-Andreani, C.; Bezat-Bouchahda, C.; Raulet, E.; Pichard-Garcia, L.; Fabre, J. M.; Saric, J.; Baulieux, J., Schved, J. F.; Maurel, P. Secretion of functional plasma haemostasis proteins in long-term primary cultures of human hepatocytes. Br. J. Haematol. 125:638–646; 2004.
5. Bolton-Maggs, P. H., Pasi, K. J. Haemophilias A and B. Lancet 361:1801–1809; 2003.
6. Boost, K. A., Auth, M. K.; Woitaschek, D.; Kim, H. S.; Hilgard, P.; Nadalin, S.; Blaheta, R. A. Long-term production of major coagulation factors and inhibitors by primary human hepatocytes in vitro: Perspectives for clinical application. Liver Int. 27:832–844; 2007
7. Dhawan, A.; Mitry, R. R., Hughes, R. D., Lehec, S.; Terry, C.; Bansal, S., Arya, R., Wade, J. J.; Verma, A., Heaton, N. D.; Rela, M.; Mieli-Vergani, G. Hepatocyte transplantation for inherited factor VII deficiency. Transplantation 78:1812–1814; 2004.
8. Do, H.; Healey, J. F.; Waller, E. K., Lollar, P Expression of factor VIII by murine liver sinusoidal endothelial cells. J. Biol. Chem. 274:19587–19592; 1999.
9. Emoto, K., Tateno, C.; Hino, H.; Amano, H., Imaoka, Y., Asahina, K.; Asahara, T.; Yoshizato, K. Efficient in vivo xenogeneic retroviral vector-mediated gene transduction into human hepatocytes. Hum. Gene Ther. 16:1168–1174; 2005.
10. Gordon, F. H.; Mistry, P K.; Sabin, C. A., Lee, C. A. Outcome of orthotopic liver transplantation in patients with haemophilia. Gut 42:744–749; 1998.
11. Hamatani, K.; Matsuda, Y.; Araki, R.; Itoh, M.; Abe, M. Cloning and chromosomal mapping of the mouse DNA-dependent protein kinase gene. Immunogenetics 45:1–5; 1996.
12. Iwamuro, M., Komaki, T., Kubota, Y., Seita, M.; Kawamoto, H., Yuasa, T.; Shahid, J. M.; Hassan, R. A.; Hassan, W. A.; Nakaji, S.; Nishikawa, Y.; Kondo, E., Yamamoto, K., Fox, I. J., Kobayashi, N. Hepatic differentiation of mouse iPS cells in vitro. Cell Transplant. 19: 841–847; 2010.
13. Ko, S.; Tanaka, I., Kanehiro, H.; Kanokogi, H.; Ori, J.; Shima, M.; Yoshioka, A.; Giles, A., Nakajima, Y. Preclinical experiment of auxiliary partial orthotopic liver transplantation as a curative treatment for hemophilia. Liver Transpl. 11:579–584; 2005.
14. Ko, S.; Tanaka, I., Kanokogi, H.; Kanehiro, H.; Okayama, J.; Ori, J., Shima, M.; Yoshioka, A., Giles, A.; Nakajima, Y. Efficacy of auxiliary partial orthotopic liver transplantation for cure of hemophilia in a canine hemophilia A model. Transplant. Proc. 37:1131–1133; 2005.
15. Kuge, H., Ohashi, K.; Yokoyama, T.; Kanehiro, H.; Hisanaga, M.; Koyama, F.; Bumgardner, G. L., Kosai, K.; Nakajima, Y. Genetic modification of hepatocytes towards hepatocyte transplantation and liver tissue engineering. Cell Transplant. 15:1–12; 2006.
16. Kumaran, V.; Benten, D.; Follenzi, A.; Joseph, B.; Sarkar, R., Gupta, S. Transplantation of endothelial cells corrects the phenotype in hemophilia A mice. J. Thromb. Haemost. 3:2022–2031; 2005.
17. Masumoto, N.; Tateno, C.; Tachibana, A.; Utoh, R.; Morikawa, Y.; Shimada, T.; Momisako, H.; Itamoto, T.; Asahara, T.; Yoshizato, K. GH enhances proliferation of human hepatocytes grafted into immunodeficient mice with damaged liver. J. Endocrinol. 194:529–537; 2007
18. Ohashi, K. Liver tissue engineering: The future of liver therapeutics. Hepatol. Res. 38:S76–S87; 2008.
19. Ohashi, K., Park, F.; Kay, M. A. Hepatocyte transplantation: Clinical and experimental application. J. Mol. Med. 79:617–630; 2001.
20. Ohashi, K., Tatsumi, K.; Utoh, R.; Takagi, S., Shima, M., Okano, T. Engineering liver tissues under the kidney capsule site provides therapeutic effects to hemophilia B mice. Cell Transplant. 19:807–813; 2010.
21. Ohashi, K., Waugh, J. M., Dake, M. D.; Yokoyama, T.; Kuge, H.; Nakajima, Y.; Yamanouchi, M.; Naka, H., Yoshioka, A.; Kay, M. A. Liver tissue engineering at extrahepatic sites in mice as a potential new therapy for genetic liver diseases. Hepatology 41:132–140; 2005.
22. Ohashi, K.; Yokoyama, T., Yamato, M.; Kuge, H.; Kanehiro, H., Tsutsumi, M., Amanuma, T.; Iwata, H.,

Yang, J.; Okano, T., Nakajima, Y. Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. Nat. Med. 13:880–885; 2007.
23. Ostrowska, A.; Bode, D. C., Pruss, J.; Bilir, B.; Smith, G. D.; Zeisloft, S. Investigation of functional and morphological integrity of freshly isolated and cryopreserved human hepatocytes. Cell Tissue Bank. 1:55–68; 2000.
24. Pipe, S. W., High, K. A., Ohashi, K.; Ural, A. U., Lillicrap, D. Progress in the molecular biology of inherited bleeding disorders. Haemophilia 14:130–137; 2008.
25. Rosen, E. D., Cornelissen, I.; Liang, Z., Zollman, A., Casad, M.; Roahrig, J., Suckow, M.; Castellino, F. J. In utero transplantation of wild-type fetal liver cells rescues factor X-deficient mice from fatal neonatal bleeding diatheses. J. Thromb. Haemost. 1:19–27; 2003.
26. Shahid, J. M., Iwamuro, M.; Sasamoto, H., Kubota, Y., Seita, M.; Kawamoto, H.; Nakaji, S., Noguchi, H.; Yamamoto, K.; Kobayashi, N. Establishment of an immortalized porcine liver cell line JSNK-1 with retroviral transduction of SV40T. Cell Transplant. 19:849–856; 2010.
27. Shibata, C.; Mizuguchi, T.; Kikkawa, Y., Nobuoka, T.; Oshima, H., Kawasaki, H.; Kawamoto, M.; Katsuramaki, T.; Mitaka, T.; Hirata, K. Liver repopulation and long-term function of rat small hepatocyte transplantation as an alternative cell source for hepatocyte transplantation. Liver Transpl. 12:78–87; 2006.
28. Takagi, S.; Ohashi, K., Utoh, R.; Tatsumi, K.; Shima, M., Okano, T. Suitable reference genes for the analysis of direct hyperplasia in mice. Biochem. Biophys. Res. Commun. 377:1259–1264; 2008.
29. Tateno, C.; Yoshizane, Y.; Saito, N.; Kataoka, M.; Utoh, R.; Yamasaki, C., Tachibana, A.; Soeno, Y.; Asahina, K.; Hino, H.; Asahara, T., Yokoi, T., Furukawa, T.; Yoshizato, K. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am. J. Pathol. 165:901–912; 2004.
30. Tatsumi, K., Ohashi, K.; Kataoka, M.; Tateno, C., Shibata, M., Naka, H.; Shima, M., Hisanaga, M.; Kanehiro, H.; Okano, T., Yoshizato, K.; Nakajima, Y., Yoshioka, A. Successful in vivo propagation of factor IX-producing hepatocytes in mice: Potential for cell-based therapy in haemophilia B. Thromb. Haemost. 99:883–891; 2008.
31. Tatsumi, K.; Ohashi, K.; Shima, M., Nakajima, Y., Okano, T., Yoshioka, A. Therapeutic effects of hepatocyte transplantation on hemophilia B. Transplantation 86:167–170; 2008.
32. Tatsumi, K., Ohashi, K., Taminishi, S., Okano, T.; Yoshioka, A., Shima, M. Reference gene selection for real-time RT-PCR in regenerating mouse livers. Biochem. Biophys. Res. Commun. 374:106–110; 2008.
33. Tatsumi, K.; Ohashi, K.; Taminishi, S.; Takagi, S., Utoh, R., Yoshioka, A.; Shima, M.; Okano, T. Effects on coagulation factor production following primary hepatomitogen-induced direct hyperplasia. World J. Gastroenterol. 15: 5307–5315; 2009.
34. Tsuruga, Y.; Kiyono, T.; Matsushita, M.; Takahashi, T.; Kasai, H.; Matsumoto, S.; Todo, S. Establishment of immortalized human hepatocytes by introduction of HPV16 E6/E7 and hTERT as cell sources for liver cell-based therapy. Cell Transplant. 17:1083–1094; 2008.
35. Tsuruga, Y.; Kiyono, T.; Matsushita, M.; Takahashi, T.; Kasai, N., Matsumoto, S.; Todo, S. Effect of intrasplenic transplantation of immortalized human hepatocytes in the treatment of acetaminophen-induced acute liver failure SCID mice. Transplant. Proc. 40:617–619; 2008.
36. Utoh, R., Tateno, C.; Yamasaki, C.; Hiraga, N.; Kataoka, M.; Shimada, T.; Chayama, K., Yoshizato, K. Susceptibility of chimeric mice with livers repopulated by serially subcultured human hepatocytes to hepatitis B virus. Hepatology 47:435–446; 2008.
37. Yoshitsugu, H., Nishimura, M.; Tateno, C., Kataoka, M., Takahashi, E.; Soeno, Y., Yoshizato, K., Yokoi, T., Naito, S. Evaluation of human CYP1A2 and CYP3A4 mRNA expression in hepatocytes from chimeric mice with humanized liver. Drug Metab. Pharmacokinet. 21:465–474; 2006.

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# Hepatocyte Is a Sole Cell Type Responsible for the Production of Coagulation Factor IX In Vivo

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Coagulation factor IX (FIX) is synthesized by hepatocytes, and the lack of this protein causes hemophilia B. Liver nonparenchymal cells, including liver sinusoidal endothelial cells (LSECs) and extrahepatic cells in the body, are scarcely shown to have an ability to synthesize and secrete FIX. The present study investigated the existence of cells responsible for synthesizing FIX other than hepatocytes in mice using gene expression analyses and FIX-specific clotting assays. Among the several organs investigated, including liver, lung, spleen, kidney, brain, intestine, and tongue, FIX mRNA expressions were observed only in the liver. From the liver, hepatocytes and LSECs were isolated. FIX mRNA expression and FIX protein secretion were observed exclusively in the hepatocytes. Furthermore, the clotting activity of FIX secreted from the cultured hepatocytes was found to be dependent on the concentration of vitamin $K_2$. These findings indicated that the hepatocyte is the only cell type that biochemically produces functional FIX in vivo. This highlights the importance of hepatocytes or cells that are fully differentiated toward the hepatic lineage for possible application for regenerative medicine and for targeting gene delivery to establish new cell-based treatments for hemophilia B.

Key words: Factor IX; Hemophilia B; Hepatocyte; Nonparenchymal cell

## INTRODUCTION

Coagulation factor IX (FIX) is one of the vitamin K-dependent serine proteases essential for blood coagulation. The lack of this protein causes hemophilia B, a recessive X-chromosome-linked congenital bleeding disorder (3,13). Patients having this inherited disorder can suffer from unpredictable, recurrent, spontaneous bleeding in various areas, including soft tissues, major joints, and occasionally in internal organs. Standard treatment for hemophilia B is either on-demand or prophylactic therapy with plasma-derived or recombinant human FIX concentrate. However, commercially available FIX concentrates are expensive, and this type of treatment requires lifelong frequent intravenous infusion, which can give a significant impact on economic resources as well as the quality of life for the patient. Under these circumstances, curable therapeutic options that are free from FIX concentrates have been desired and investigated. FIX is mainly produced in the liver, and so liver-directed gene therapy (15) and liver transplantation (14) have been attempted as new types of therapies for hemophilia B. Considering the fact that only 1% elevation in biologically active FIX levels in plasma can attenuate the bleeding diathesis of hemophilia B (3), the symptom of hemophilia B could be attenuated by cell-based therapy using FIX-producing cells.

FIX has been reported to be synthesized by mature hepatocytes in the liver (4). Based on this report, our laboratory transplanted hepatocytes isolated from wild-type mice to hemophilia B mice (20,26). Hepatocyte transplantation to either the liver or under the kidney capsule sites of hemophilia B mice led to 1–3% increase in plasma FIX activity level in the recipient mice (20,26). Apart from FIX, hepactocytes produce many forms of coagulation factors including fibrinogen, factor VII, and factor VIII (4). Our group has previously reported the therapeutic potential of hepatocyte-based therapy for factor VIII deficiency using a mice model (21). At present, however, the hepatocytes used in clinical hepatocyte transplantation are isolated from the discarded liver pieces

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that are unable to be used for liver transplantation due to their marginal quality. This severely limits the number of viable donor hepatocytes (22).

In this study, to further develop cell-based therapies for hemophilia B, we investigated the existence of cells, which can synthesize FIX, other than hepatocytes in mice using gene expression analyses and FIX-specific clotting assays. More specifically, nonparenchymal cells in the liver, particularly liver sinusoidal endothelial cells (LSECs), and extrahepatic organs were isolated from mice to detect and assess the function of biologically active FIX. Therefore, if alternate cell types or organs could produce functionally active FIX other than hepatocytes, obstacles regarding donor cell shortage are resolved.

## MATERIALS AND METHODS

### *Animals*

Female wild-type FVB/N mice (CLEA Japan, Tokyo, Japan) were used at 8–12 weeks of age. Experimental protocols were developed in accordance with the guidelines outlined by the Institutional Animal Care and Use Committee at Tokyo Women's University.

### *Isolation of Primary Hepatocytes and Liver Sinusoidal Endothelial Cells (LSECs)*

Mouse primary hepatocytes and liver sinusoidal cells were isolated from the livers of FVB/N wild-type mice using an in situ collagenase perfusion method (1). Isolated hepatocytes were separated from nonparenchymal cells by three rounds of low-speed centrifugation at 50×*g*, followed by Percoll (Percoll™, American Biosciences, Uppsala, Sweden) isodensity centrifugation. Hepatocytes with viabilities of >90%, as quantified by a trypan blue dye exclusion test, were used in this study. Supernatants after the low-speed centrifugations were used for LSECs isolation. Supernatants containing nonparenchymal cell fractions were filtered through a 40-μm filter and blocked with purified rat antimouse CD16/CD32 antibody (BD Biosciences, San Jose, CA). LSEC fraction was enriched by a magnetic cell sorting instrument (Miltenyi Biotech, Gladbach, Germany) with a magnetic antibody targeted for mouse CD146 (Miltenyi Biotech). Each fraction of isolated cells was partly snap-frozen for subsequent gene expression analyses, and the remaining cells were seeded in type I collagen-coated six-well culture dishes (Iwaki, Tokyo) at a density of $7.5\times10^5$ cells per well for both hepatocytes and LSECs. After the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) containing 10% fetal bovine serum with supplements (L-glutamine, HEPES buffer, penicillin, and streptomycin) for 8 h in humidified culture chamber at 37°C, floating cells were removed, and the culture medium was replaced with hepatocyte culture medium lacking serum. This medium was composed of DMEM supplemented with 20 mmol/L HEPES, $10^{-7}$ mol/L dexamethasone (Sigma), 0.5 μg/ml insulin (Wako, Tokyo, Japan), 30 μg/ml L-proline (Wako), 10 mM nicotinamide (Kanto Chemicals, Tokyo, Japan), 10 ng/ml epidermal growth factor (PeproTech, Rocky Hill, NJ), 0.2 mmol/L ascorbic acid-2 phosphate (Wako), 1% dimethyl sulfoxide (DMSO; Sigma), 100 IU/ml penicillin, and 100 μg/ml streptomycin. Vitamin $K_2$ (chemical name: menaquinone; Kaytwo N, Eisai, Tokyo) was also added to the culture medium at final concentrations of 0.1, 1, and 10 μg/ml. After 16-h cell culture, the media were collected for factor IX activity assay.

### *RNA Isolation and cDNA Synthesis*

The liver, lung, spleen, kidney, brain, intestine, and tongue were collected from four FVB/N mice. Total RNA was extracted from each organ or isolated liver cell samples using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. To eliminate genomic DNA contamination, the extracted total RNA was treated with DNase I (Qiagen). Aliquots of total RNA samples were diluted, and the concentration and purity of each sample was measured at wavelengths of 260 nm (A260) and 280 nm (A280) using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). The A260/A280 ratios for each of the total RNA samples were between 1.9 and 2.1. The total RNA (1 μg) was reverse-transcribed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA), as described by the manufacturer's instructions.

### *Real-Time PCR for FIX Expression Levels*

Quantitative real-time PCR analysis was performed using a PRISM 7300 Sequence Detector with a universal PCR master mix according to the specifications of the manufacturer (Applied Biosystems). TaqMan probes and primers for mouse FIX (Mm01308427_m1) and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm99999915_g1) were chosen from TaqMan Gene Expression Assay (Applied Biosystems). The integrity and purity of the extracted organ samples were evaluated by assessing the expression levels of genes that were known to be specifically expressed in the respective organs: albumin (*Alb*) (Mm00802090_m1) for the liver, NK2 homeobox 1 (*Nkx2-1*) (Mm00447558_m1) for the lung, spleen tyrosine kinase (*Syk*) (Mm01333032_m1) for the spleen, nephrosis 1, congenital, Finnish type (nephrin) (*Nphs1*) (Mm00497828_m1) for the kidney, sex-determining region Y-box 11 (*Sox11*) (Mm01281943_s1) for the brain, and defensin-related sequence cryptdin peptide (*Defcr-rs1*) (Mm00655850_m1) for the intestine. All real-time PCR analyses were performed in duplicate using 96-well optical reaction plates (Applied Biosystems), and the following

cycling conditions were used: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. For quantifying FIX and GAPDH gene expressions, cDNAs derived from pooled normal mouse livers were used to prepare standard reference curves.

*Measurement of FIX Activity in the Cultured Medium*

FIX clotting activities of cultured medium were measured based on one-stage clotting assay with human FIX-deficient plasma and ThromboCheck APTT-SLA (Sysmex, Kobe, Japan) using a KC4 Delta Coagulometer (Trinity Biotech, Co Wicklow, Ireland). Briefly, 50 μl of one tenth of the diluted samples, together with the same amount of FIX-deficient plasma and APTT-SLA, was incubated at 37°C for 4 min, followed by the addition of 50 μl of 0.02 mol/L $CaCl_2$. The activities were calculated from the clotting time based on the standard values of pooled plasma collected from FVB/N mice.

*Statistical Analysis*

The significant levels of comparisons between two groups were performed by Student's *t* test. Differences between three or more groups were tested using ANOVA. If ANOVA showed significant differences, the significances were evaluated by the Tukey's HSD test. The level of significance was set at $p<0.05$.

## RESULTS

*Validation of the Extraction of Organ Samples*

The integrity and purity of the extracted organ samples were validated by assessing their specific gene expressions by real-time PCR ($n$=4). As demonstrated in Figure 1, *Alb*, *Nkx2-1*, *Syk*, *Nphs1*, *Sox11*, and *Defcr-rs1* were highly expressed in the liver, lung, spleen, kidney, brain, and intestine, respectively. This indicated that the organ samples were appropriately extracted and processed.

*FIX Gene Expression in Liver and Extrahepatic Mouse Organs*

Mouse FIX mRNA expression levels in several organs, including liver, lung, spleen, kidney, brain, intestine, and tongue, were evaluated by real-time PCR ($n$=4). FIX mRNA expression was exclusively detected in the liver, with the expression in other extrahepatic organs being undetectable (Fig. 2).

*FIX Gene Expression in Fractions of Isolated Liver Cells*

Liver cells were isolated by a collagenase perfusion method from the livers of FVB/N mice. The hepatocyte fraction was purified by Percoll isodensity centrifugation, and LSECs fraction was condensed by magnetic

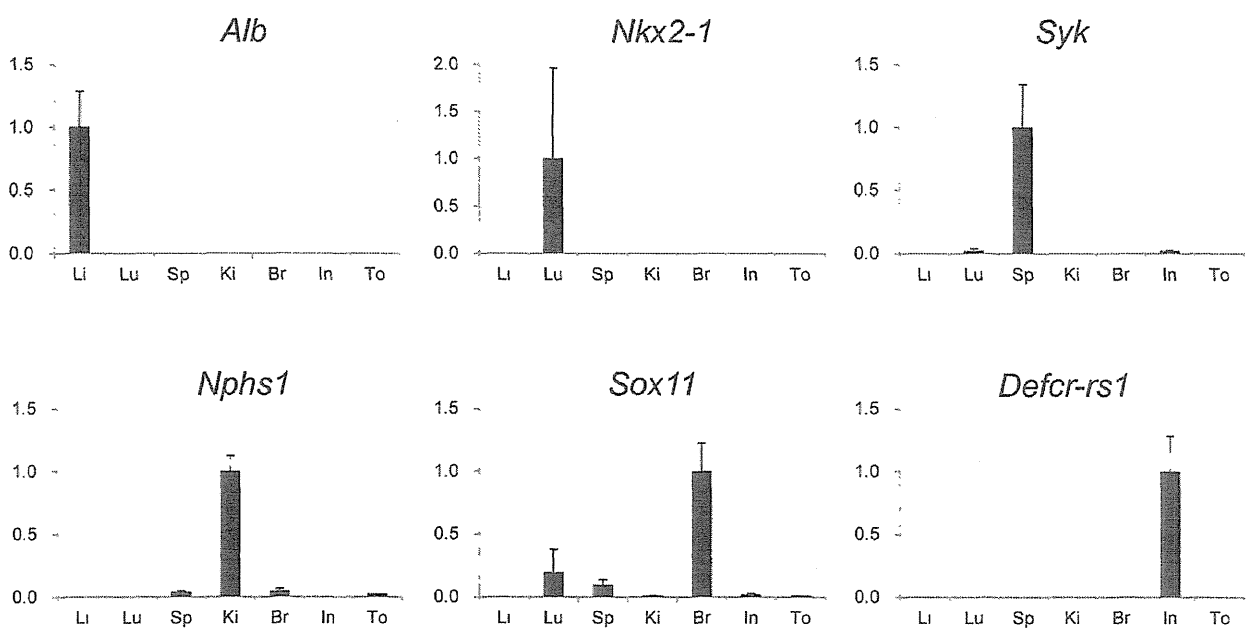


**Figure 1.** Validation of the extraction of organ samples. Using extracted organ samples, the gene expression levels of albumin (*Alb*), NK2 homeobox 1 (*Nkx2-1*), spleen tyrosine kinase (*Syk*), nephrosis 1, congenital, Finnish type (nephrin) (*Nphs1*), sex-determining region Y-box 11 (*Sox11*), and defensin-related sequence cryptdin peptide (*Defcr-rs1*) were evaluated by real-time PCR ($n$=3). Data were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels and analyzed by Δ–ΔCt method. Values were graphed as comparative ratios to the levels in their specific organs and were represented as mean ± standard error of the mean (SEM). Li, liver; Lu, lung; Sp, spleen; Ki, kidney; Br, brain; In, Intestine; To, tongue.

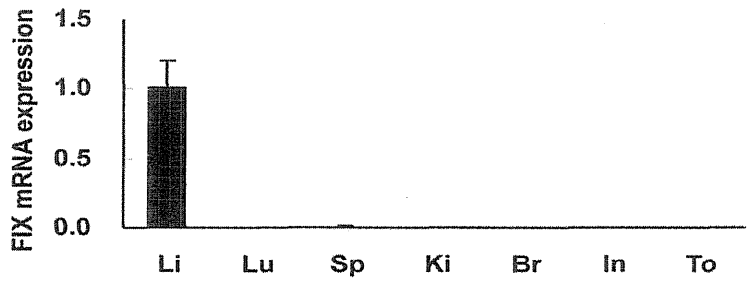


**Figure 2.** Coagulation factor IX (FIX) gene expression in mouse organs. Mouse FIX mRNA expression levels in several mouse organs were determined by real-time PCR ($n$=4). Data were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels and graphed as a comparative ratio to the liver. Values were represented as mean±SEM. Li, liver; Lu, lung; Sp, spleen; Ki, kidney; Br, brain; In, Intestine; To, tongue.

cell sorting. As shown in Figure 3, the former cell fraction showed a cuboidal, organelle-rich, and binucleate cell morphology, which is commonly observed in cultured hepatocytes. On the other hand, the latter cell fraction showed the typical cell morphology of endothelial cells, indicating that these cells were almost corresponding to LSECs. To obtain 1 μg of total RNA to evaluate FIX mRNA expression by real-time RT-PCR, $2.8\times10^4$ hepatocytes and $2\times10^6$ LSECs were needed. The ratios of FIX to GAPDH were 0.79±0.1 in hepatocytes and 0.11±0.06 in LSECs. Since nearly 70 times more LSECs were necessary to obtain the same amount of total RNA from the hepatocytes, the FIX expression levels per cell were recalculated to be about 0.79 and 0.0016 (0.11/70) in hepatocytes and LSECs, respectively.

Furthermore, the number of hepatocytes constituting the whole liver is known to be approximately three times more than that of LSECs. Considering this, the contribution of hepatocytes to produce FIX expression in the liver should be far greater. Upon the recalculation of the contribution ratio of both cell types to FIX mRNA expression in the liver, the ratios of hepatocytes and LSECs were 99.93% and 0.07%, respectively (Fig. 4). Therefore, FIX mRNA expression was observed exclusively in the hepatocyte fraction, and FIX expression in the LSEC fraction was below 1% of the hepatocyte fraction. This result clearly indicated that hepatocyte was the sole cell type responsible for FIX production in the liver.

*Measurement of FIX Activity in the Culture Medium of Cultured Liver Cells*

To demonstrate whether cultured hepatocytes could secrete biologically active FIX, FIX clotting activities in the culture medium of hepatocytes and LSECs were measured (Fig. 5). Basal clotting activities were observed in the culture media collected from hepatocytes incubated in the absence of vitamin $K_2$ (0.2±0.03%). In the presence of increasing doses of vitamin $K_2$ at 0.1, 1, and

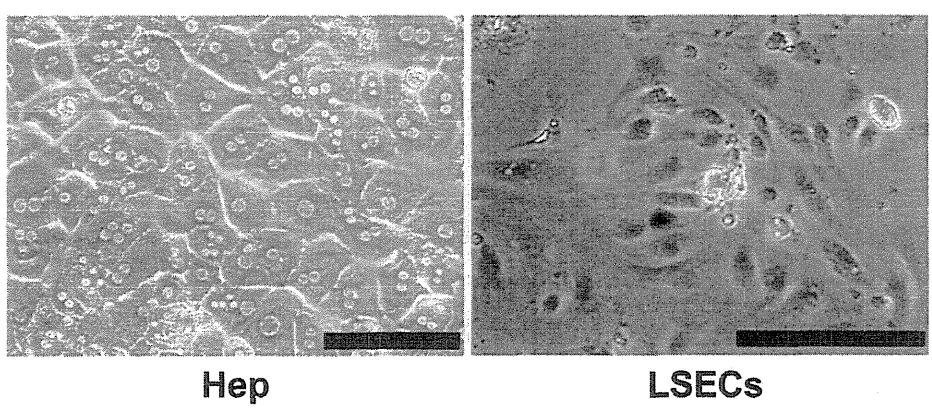


**Figure 3.** Cell morphology of isolated cell fractions. Liver cells were isolated from the livers of FVB/N mice by a collagenase perfusion method. Hepatocyte (Hep) fraction was purified by Percoll isodensity centrifugation, and liver sinusoidal endothelial cell (LSEC) fraction was condensed by magnetic cell sorting. Isolated cells were seeded on type I collagen-coated six-well culture dishes at a density of $7.5\times10^5$ cells per well and were cultured for 48 h. Scale bars: 100 μm.

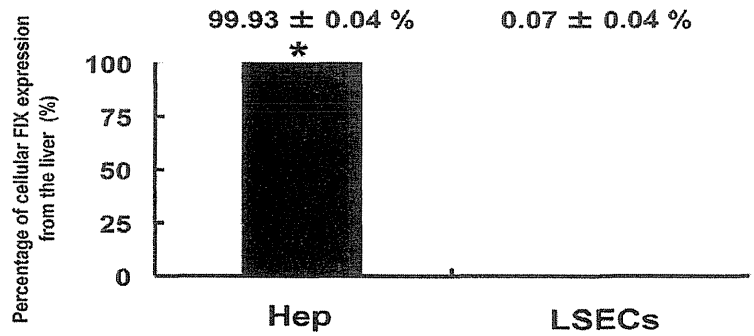


**Figure 4.** FIX gene expression in fractions of isolated liver cells. Hepatocytes (Hep) and liver sinusoidal endothelial cells (LSECs) were isolated from mouse livers by collagenase perfusion. Mouse FIX mRNA expression levels in each cell fraction were determined by real-time PCR ($n$=6, respectively). Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels and graphed as a comparative ratio to the liver. Values were represented as mean±SEM. Asterisks indicate significant differences.

10 mg/ml, clotting activities were detected to be similarly increased in a dose-dependent manner at 0.28±0.04%, 0.59±0.04%, and 0.96±0.02%, respectively). In marked contrast, there was no measurable FIX activity in the medium harvested from LSECs.

## DISCUSSION

The present study was conducted to precisely investigate alternate cell type(s) capable of producing biologically active FIX in vivo. Our results showed that the liver was the only organ that expressed FIX mRNA among the organs and tissues tested in vivo, which was comparable to our previous experimental report using mouse organs from a different strain (C57Bl/6) (10). Furthermore, FIX mRNA expression and FIX production were observed exclusively in isolated hepatocytes, but not in isolated LSECs. This indicated that the hepatocyte was the only cell type that produced FIX in vivo.

The findings obtained in this study elucidated that circulating FIX levels were maintained only by hepatocytes. However, a possible physiological mechanism that maintains a specific concentration of FIX in plasma (approximately 5,000 ng/ml) remains unknown. We have previously elucidated that FIX mRNA expression levels in hepatocytes are significantly suppressed when hepatocytes are in a proliferating state such as liver regeneration induced by partial hepatectomy (27) or primary mitogen (28) in mouse models. FIX mRNA expressions in cultured hepatocytes were also observed to be decreased depending on the number of days in culture (unpublished

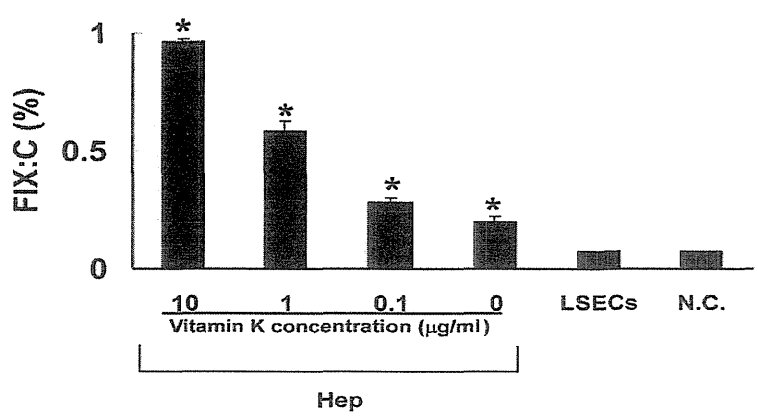


**Figure 5.** Measurement of FIX activity in the culture medium of cultured liver cells. Isolated hepatocytes and liver sinusoidal endothelial cells were cultured for 16 h in serum-free culture media. Vitamin $K_2$ was added to the media at increasing concentrations from 0.1 to 10 μg/ml. FIX clotting activity in the media was measured by a one-stage clotting assay ($n$=6, respectively). Data were expressed as the percentage of normal mouse plasma. Values were represented as mean±SEM. Hep, hepatocytes; LSECs, liver sinusoidal endothelial cells; N.C., cell-free control media. Asterisks indicate significant differences versus N.C.

data). Elucidating the mechanism of FIX production in hepatocytes would allow the mechanism of FIX regulation to be well understood.

Several functions of hepatocytes have been reported to have a dependency on their located zone in the liver (7). However, FIX production in hepatocytes was found to be uniformly observed in the liver regardless of their location (25), indicating that no cell sorting was required in preparing hepatocytes for cell-based therapy toward hemophilia B. To develop hepatocyte-based therapy for hemophilia B, preparing enough numbers of hepatocytes, preserving their ability to produce functional FIX, is essential. At present, however, the number of donor livers remains severely restricted, and even if they are available, these livers are frequently of marginal quality (22). Furthermore, conventional procedures for the culture of primary hepatocytes are found to be unable to support extensive cell proliferation (18). To solve this problem, several approaches have been investigated such as (1) hepatocyte propagation systems in mice (25), (2) the technical refinement of hepatocyte cryopreservation for the establishment of hepatocyte bank (24), (3) cell therapy using fetal hepatocytes or hepatocyte progenitor cells (6), and (4) hepatic differentiation from several types of stem cells such as embryonic stem (ES) cells (9), induced pluripotent stem (iPS) cells (10), and mesenchymal stem cells (5).

Considering that cell-based therapy using allogenic cells requires long-term immunosuppression to achieve therapeutic cell engraftment, the safest and most feasible way is applying hemophilia B patients' own cells that will be genetically modified to produce FIX. Ex vivo gene modification of hepatocyte using viral vector has been investigated (12,19). In fact, cell therapies using ex vivo genetically modified hepatocytes have been applied for several types of liver diseases (2,16). This type of therapy is unable to be theoretically limited to hepatocytes, and other cell types that are easily harvested from the patients, such as mesenchymal stem cells derived from bone marrow (5,17) or adipose tissue, and iPS cells (30) could be viable candidate cells. However, FIX protein is known to gain its full functionality after several steps of posttranslational modification such as γ-carboxylation by γ-glutamyl-carboxylase and the propeptide cleavage by furin/paired amino acid-cleaving enzyme (PACE) (11), which are physiologically functioned in hepatocytes. Especially, γ-carboxylation is critical for FIX to obtain a binding ability to calcium ions and phospholipid surface, and this reaction requires the presence of vitamin K and its related internal enzymes including γ-glutamyl-carboxylase and vitamin K epoxide reductase. Actually, this study demonstrated that higher FIX activity was obtained in culture media in the presence of vitamin K compared with the absence of vitamin K (Fig. 5). Therefore, upon the collection of cells in preparing cell-based therapy, the cells should be carefully verified to retain an ability to perform FIX posttranslational modification and vitamin K-related enzymes for efficient FIX protein production. Recently, the generation of modified FIX gene, named triple FIX, that can bear the significant amount of FIX protein in plasma has been reported (8,29), and naturally occurring FIX mutation that demonstrates extraordinary hyper-FIX activity in plasma in spite of its normal antigen levels, leading to X-linked thrombophilia, has been identified (FIX Padua) (23). By using these mutated hyperactive FIX genes as a transduction gene to hemophilic cells, higher therapeutic efficacy could be achieved with a fewer amount of cells.

In conclusion, coagulation FIX is physiologically synthesized only by hepatocytes. Therefore, mature hepatocytes or cells that are fully differentiated to hepatic lineage could be the most appropriate cell source for cell-based therapy in hemophilia B.

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## REFERENCES

1. Berry, M. N.; Friend, D. S. High-yield preparation of isolated rat liver parenchymal cells: A biochemical and fine structural study. J. Cell Biol. 43:506–520; 1969.
2. Birraux, J.; Menzel, O.; Wildhaber, B.; Jond, C.; Nguyen, T. H.; Chardot, C. A step toward liver gene therapy: Efficient correction of the genetic defect of hepatocytes isolated from a patient with Crigler-Najjar syndrome type 1 with lentiviral vectors. Transplantation 87:1006–1012; 2009.
3. Bolton-Maggs, P. H.; Pasi, K. J. Haemophilias A and B. Lancet 361:1801–1809; 2003.
4. Boost, K. A.; Auth, M. K.; Woitaschek, D.; Kim, H. S.; Hilgard, P.; Nadalin, S.; Blaheta, R. A. Long-term production of major coagulation factors and inhibitors by primary human hepatocytes in vitro: Perspectives for clinical application. Liver Int. 27:832–844; 2007.
5. Coutu, D. L.; Cuerquis, J.; El Ayoubi, R.; Forner, K. A.; Roy, R.; Francois, M.; Griffith, M.; Lillicrap, D.; Yousefi, A. M.; Blostein, M. D.; Galipeau, J. Hierarchical scaffold design for mesenchymal stem cell-based gene therapy of hemophilia B. Biomaterials 32:295–305; 2011.
6. Fiegel, H. C.; Lange, C.; Kneser, U.; Lambrecht, W.; Zander, A. R.; Rogiers, X.; Kluth, D. Fetal and adult liver stem cells for liver regeneration and tissue engineering. J. Cell. Mol. Med. 10:577–587; 2006.
7. Goto, Y.; Ohashi, K.; Utoh, R.; Yamamoto, M.; Okano, T. Hepatocyte transplantation through the hepatic vein: A new

route of cell transplantation to the liver. Cell Transplant. 20:1259–1270; 2011.
8. Kao, C. Y.; Lin, C. N.; Yu, I. S.; Tao, M. H.; Wu, H. L.; Shi, G. Y.; Yang, Y. L.; Kao, J. T.; Lin, S. W. FIX-Triple, a gain-of-function factor IX variant, improves haemostasis in mouse models without increased risk of thrombosis. Thromb. Haemost. 104:355–365; 2010.
9. Kasuda, S.; Kubo, A.; Sakurai, Y.; Irion, S.; Ohashi, K.; Tatsumi, K.; Nakajima, Y.; Saito, Y.; Hatake, K.; Pipe, S. W.; Shima, M.; Yoshioka, A. Establishment of embryonic stem cells secreting human factor VIII for cell-based treatment of hemophilia A. J. Thromb. Haemost. 6:1352–1359; 2008.
10. Kasuda, S.; Tatsumi, K.; Sakurai, Y.; Kato, J.; Taminishi, S.; Takeda, T.; Ohashi, K.; Okano, T.; Hatake, K.; Shima, M. Expression of coagulation factors from murine iPS-derived liver cells. Blood Coagul. Fibrinolysis 22:271–279; 2011.
11. Kaufman, R. J. Post-translational modifications required for coagulation factor secretion and function. Thromb. Haemost. 79:1068–1079; 1998.
12. Kuge, H.; Ohashi, K.; Yokoyama, T.; Kanehiro, H.; Hisanaga, M.; Koyama, F.; Bumgardner, G. L.; Kosai, K.; Nakajima, Y. Genetic modification of hepatocytes towards hepatocyte transplantation and liver tissue engineering. Cell Transplant. 15:1–12; 2006.
13. Kurachi, K.; Davie, E. W. Isolation and characterization of a cDNA coding for human factor IX. Proc. Natl. Acad. Sci. USA 79:6461–6464; 1982.
14. Merion, R. M.; Delius, R. E.; Campbell, Jr., D. A.; Turcotte, J. G. Orthotopic liver transplantation totally corrects factor IX deficiency in hemophilia B. Surgery 104:929–931; 1988.
15. Nathwani, A. C.; Davidoff, A. M.; Tuddenham, E. G. Prospects for gene therapy of haemophilia. Haemophilia 10:309–318; 2004.
16. Nguyen, T. H.; Mainot, S.; Lainas, P.; Groyer-Picard, M. T.; Franco, D.; Dagher, I.; Weber, A. Ex vivo liver-directed gene therapy for the treatment of metabolic diseases: Advances in hepatocyte transplantation and retroviral vectors. Curr. Gene Ther. 9:136–149; 2009.
17. Oh, T.; Peister, A.; Ohashi, K.; Park, F. Transplantation of murine bone marrow stromal cells under the kidney capsule to secrete coagulation factor VIII. Cell Transplant. 15:637–645; 2006.
18. Ohashi, K.; Park, F.; Kay, M. A. Hepatocyte transplantation: Clinical and experimental application. J. Mol. Med. 79:617–630; 2001.
19. Ohashi, K.; Park, F.; Schwall, R.; Kay, M. Efficient gene transduction to cultured hepatocytes by HIV-1 derived lentiviral vector. Transplant. Proc. 34:1431–1433; 2002.
20. Ohashi, K.; Tatsumi, K.; Utoh, R.; Takagi, S.; Shima, M.; Okano, T. Engineering liver tissues under the kidney capsule site provides therapeutic effects to hemophilia B mice. Cell Transplant. 19:807–813; 2010.
21. Ohashi, K.; Waugh, J. M.; Dake, M. D.; Yokoyama, T.; Kuge, H.; Nakajima, Y.; Yamanouchi, M.; Naka, H.; Yoshioka, A.; Kay, M. A. Liver tissue engineering at extrahepatic sites in mice as a potential new therapy for genetic liver diseases. Hepatology 41:132–140; 2005.
22. Ostrowska, A.; Bode, D. C.; Pruss, J.; Bilir, B.; Smith, G. D.; Zeisloft, S. Investigation of functional and morphological integrity of freshly isolated and cryopreserved human hepatocytes. Cell Tissue Bank. 1:55–68; 2000.
23. Simioni, P.; Tormene, D.; Tognin, G.; Gavasso, S.; Bulato, C.; Iacobelli, N. P.; Finn, J. D.; Spiezia, L.; Radu, C.; Arruda, V. R. X-linked thrombophilia with a mutant factor IX (factor IX Padua). N. Engl. J. Med. 361:1671–1675; 2009.
24. Stephenne, X.; Najimi, M.; Sokal, E. M. Hepatocyte cryopreservation: Is it time to change the strategy? World J. Gastroenterol. 16:1–14; 2010.
25. Tatsumi, K.; Ohashi, K.; Kataoka, M.; Tateno, C.; Shibata, M.; Naka, H.; Shima, M.; Hisanaga, M.; Kanehiro, H.; Okano, T.; Yoshizato, K.; Nakajima, Y.; Yoshioka, A. Successful in vivo propagation of factor IX-producing hepatocytes in mice: Potential for cell-based therapy in haemophilia B. Thromb. Haemost. 99:883–891; 2008.
26. Tatsumi, K.; Ohashi, K.; Shima, M.; Nakajima, Y.; Okano, T.; Yoshioka, A. Therapeutic effects of hepatocyte transplantation on hemophilia B. Transplantation 86:167–170; 2008.
27. Tatsumi, K.; Ohashi, K.; Taminishi, S.; Sakurai, Y.; Ogiwara, K.; Yoshioka, A.; Okano, T.; Shima, M. Regulation of coagulation factors during liver regeneration in mice: Mechanism of factor VIII elevation in plasma. Thromb. Res. 128:54–61; 2011.
28. Tatsumi, K.; Ohashi, K.; Taminishi, S.; Takagi, S.; Utoh, R.; Yoshioka, A.; Shima, M.; Okano, T. Effects on coagulation factor production following primary hepatomitogen-induced direct hyperplasia. World J. Gastroenterol. 15: 5307–5315; 2009.
29. Wu, Y. M.; Kao, C. Y.; Huang, Y. J.; Yu, I. S.; Lee, H. S.; Lai, H. S.; Lee, P. H.; Lin, C. N.; Lin, S. W. Genetic modification of donor hepatocytes improves therapeutic efficacy for hemophilia B in mice. Cell Transplant. 19:1169–1180; 2010.
30. Xu, D.; Alipio, Z.; Fink, L. M.; Adcock, D. M.; Yang, J.; Ward, D. C.; Ma, Y. Phenotypic correction of murine hemophilia A using an iPS cell-based therapy. Proc. Natl. Acad. Sci. USA 106:808–813; 2009.

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# The non-invasive cell surface modification of hepatocytes with PEG-lipid derivatives

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ABSTRACT

Hepatocyte-based therapies are promising regenerative approaches for liver diseases. In this study, we sought to develop a versatile method to modify the surface of hepatocytes by immobilizing synthetic polymers around the cells. The surface of murine primary hepatocytes was modified using poly(ethylene glycol)-phospholipids conjugate bearing FITC (FITC-PEG-lipid) in suspension. Hepatocyte function was assessed *in vitro* by examining cell viability, plating efficiency, protein production, metabolizing activity, hepatocyte-specific gene expressions, and cytochrome P450 induction. The engraftment of the PEG-lipid modified cells was studied following transplantation to both the liver or alternate ectopic sites. Among the types of phospholipids analyzed in our study, 1,2-dimyristoil-*sn*-glycerol-3-phosphatidylethanolamine (DMPE) was found to be uniformly anchored to the hepatocyte cell membrane (>99% of hepatocytes). Cell surface modification using FITC-PEG-DMPE did not result in any loss of *in vitro* functional parameters nor affect the engraftment potential *in vivo* by the modified cells. This modification was also successfully performed on dispersed hepatocytes and engineered hepatocyte sheets. In all, the ability to modify the surface of isolated hepatocytes with functional proteins, instead of FITC as shown in our proof-of-concept study, has the potential to move hepatocyte-based cell therapy another step forward as a viable therapeutic application.



## 1. Introduction

Hepatocyte-based therapies have attracted increased attention as an alternate treatment modality for liver transplantation to ameliorate many forms of liver diseases, including acute/chronic liver failure and liver-based metabolic disorders. Freshly isolated, cryopreserved, or cultured hepatocytes have been transplanted exclusively into the liver via cell infusion directly into portal circulation [1]. In many cases of acute liver failure, hepatocyte transplantation has been successfully adopted as a temporary measure to bridge the patient until liver transplantation can be performed [1]. In various inherited liver-based metabolic disorders, phenotypic corrections had been reported in experimental and clinical hepatocyte transplantation [2–4]. Recent tissue engineering technologies has also enabled hepatocytes to be engrafted at ectopic sites allowing functional liver systems to be formed and provide therapeutic efficacy in the treatment of several disease models [5,6].

There are a number of potential advantages of hepatocyte-based therapy, if sufficient levels of cell engraftment and survival can be provided. For both hepatocyte transplantation and liver tissue engineering, there is a general consensus that improved engraftment of hepatocytes *in vivo* is crucial for any beneficial enhancement in therapeutic efficacy [7]. However, recent investigators have revealed that intra-portal transplanted hepatocytes were associated with instant blood-mediated inflammatory reactions (IBMIR) and innate immunity resulting in a marked loss of transplanted cells during the engraftment process [8,9]. Since these problems are likely initiated by interactions between the cellular surface and soluble factors in the blood stream, a conceivable strategy to overcome these problems would be to modify the cell surface of the hepatocyte, especially using non-toxic compounds.

Poly(ethylene glycol)-phospholipid complexes (PEG-lipids) have been widely used for the surface modification of chemicals to improve biocompatibility and prolong the half-life of the chemicals in blood circulation *in vivo* [10]. Furthermore, studies have demonstrated that derivatives of the PEG-lipids can be used to modify cellular surface [11]. Recently, Iwata *et al.* [11,12] developed technologies to immobilize bioactive substances, such enzymes

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[13], low-molecular weight drugs [14], and even living cells on the surface of pancreatic islets using PEG-lipids [15]. The PEG-lipids provided an ultra-thin coating on the cell surface of pancreatic islet cells, and demonstrated the suppression of IBMIR in two islet transplantation studies [16,17].

In the present study, we created polymers in which PEG was conjugated with 3 different types of phospholipids and investigated their effectiveness to modify surfaces of primary hepatocytes *in vitro*. Molecular and cell biological analyses were performed to validate the phenotypes of the modified hepatocytes, their ability to engraft in the liver or under the kidney capsule, and lastly, to assess their ability to form hepatocyte sheets. These studies were designed to determine whether surface modifications using PEG on isolated hepatocytes would have enhanced therapeutic efficacy and merit for continued use in the development of new protocols for hepatocyte-based therapies.

## 2. Materials and methods

### 2.1. Materials

α-*N*-Hydroxysuccinimidyl-ω-tert- butoxycarbonyl poly(ethylene glycol) (NHS-PEG-Boc, Mw: 5000) was purchased from Nektar Therapeutics (San Carlos, CA). 1,2-dipalmitoyl-*sn*-glycerol-3-phosphatidylethanolamine (DPPE, Mw: 692), 1,2-dimyristoyl-*sn*-glycerol-3-phosphatidylethanolamine (DMPE, Mw: 636), and 1,2-distearoyl-*sn*-glycerol-3-phosphatidylethanolamine (DSPE, Mw: 748) were purchased from NOF Corporation (Tokyo, Japan).3,30-Dithiodipropionic acid and dithiothreitol (DTT) were purchased from Wako Pure Chemical (Osaka, Japan). Fluorescein isothiocyanate (FITC, Mw: 389) was purchased from Dojindo Laboratories (Kumamoto, Japan). Hanks' balanced salt solution (HBSS) and phosphate-buffered saline (PBS) were purchased from Invitrogen Co. (Carlsbad, CA). Dulbecco's modified Eagle medium (DMEM) were purchased from Sigma–Aldrich (Seelze, Germany).

### 2.2. Synthesis of FITC-labeled PEG-lipids

The synthesis of PEG-lipid was performed using a previously described method [11,18]. In brief, NHS-PEG-Boc (185 mg), triethylamine (3 μL), and one of the following [DPPE (21 mg), DMPE (18 mg), or DSPE (24 mg)], were dissolved in 3 mL dichloromethane and stirred for 4 days at room temperature. Subsequently, 99% trifluoroacetic acid (1 mL) was added to the resulting solution for 30 min at 4 °C to remove the Boc group. The crude products were purified by re-precipitation into diethyl ether. $NH_2$-PEG-lipid ($NH_2$-PEG-DMPE, $NH_2$-PEG-DPPE, and $NH_2$-PEG-DSPE) was obtained as a white solid after extraction with chloroform, evaporation, and a freeze-dried process. The yield from our reactions was 80%, 74%, and 75%, respectively. FITC-labeling of PEG-lipids (20 mg) was performed by reacting $NH_2$-PEG-lipids with FITC (6 mg) in acetone for 12 h. FITC-PEG-lipids were purified by gel permeation chromatography (Sephadex G-25) to separate FITC-PEG-lipids from non-conjugated FITC. Most of the FITC-PEG-lipids were detected by thin layer chromatography (TLC, silica gel, chloroform/methanol = 4/1, v/v).

### 2.3. Animals

As hepatocyte donors, transgenic mice (12–13 weeks of age) expressing human alpha-1 antitrypsin (hA1AT) driven by the hepatocyte-specific promoter (hA1AT-FVB/N, H-2$^q$; kindly provided by Dr. Bumgardner, Ohio State University, Columbus, Ohio) were used to collect hepatocytes [19] for transplantation into recipient wild-type female FVB/N mice (10–12 weeks of age: CLEA Japan, Tokyo, Japan). The FVB/N mice are syngenic to the hA1AT-FVB/N mice.

### 2.4. Hepatocyte isolation and hepatocyte sheet creation

Primary hepatocytes were isolated from the livers of hA1AT-FVB/N mice using an *in situ* collagenase perfusion method as previously described [5,20,21]. Isolated hepatocytes were separated from non-parenchymal cells by three rounds of low-speed centrifugation at 50 × *g*, followed by Percoll (Percoll™, American Biosciences, Uppsala, Sweden) isodensity centrifugation. In the present studies, experiments were conducted only when the hepatocyte viabilities exceeded 90% by trypan blue exclusion tests.

The engineering of hepatocyte sheets were performed using temperature-responsive polymer [poly(*N*-isopropylacrylamide)]-coated (PIPAAm) dishes as described previously [20,21]. Briefly, isolated hA1AT hepatocytes were plated on the PIPAAm dishes (UpCell, 35 mm, CellSeed, Tokyo, Japan) at a density of 8 × $10^5$ cells per dish. Cell culture was performed at 37 °C. Three days later, the culture temperature was lowered to 20 °C for 15 min to allow the cultured hepatocytes to spontaneously detach from the culture dish surface for harvesting as a uniformly connected tissue sheet.

### 2.5. Interaction of PEG-lipids with hepatocytes in suspension and hepatocyte sheet

Isolated hepatocytes were resuspended with the synthesized polymers. FITC-PEG-DPPE (50 μg), FITC-PEG-DMPE (50 μg), FITC-PEG-DSPE (50 μg), or vehicle solution (HBSS) was added to 1 x $10^6$ hepatocytes in suspension, and incubated for 30 min with gentle agitation at 4 °C. These hepatocytes were then washed twice with HBSS and collected by centrifugation (50 × g, 5 min at 4 °C). The surface of the harvested hepatocytes sheets were modified by incubating with FITC-PEG-DMPE (50 μg) for 30 min at 4 °C, and followed by two wash steps with HBSS in the culture dishes.

### 2.6. Incorporation efficiency of three types of PEG-lipids into the surface of hepatocytes

The surface modified or non-modified hepatocytes were observed by a confocal fluorescence microscopy (FLUOVIEW FV500, Olympus, Tokyo). A series of cross-sections was placed in a horizontal direction by 1.5 mm to obtain horizontal sectional images of FITC-PEG-lipid-modified cells. The ratio of the hepatocytes with FITC signals on the cell surface to all hepatocytes was determined by Gallios Flow Cytometer (Beckman Coulter, Brea, CA). Modified or non-modified hepatocyte sheets were also observed by confocal laser scanning microscopy (FLUOVIEW FV10i, Olympus, Tokyo), and the obtained images were analyzed and processed using Volocity software (Improvision, Coventry, UK).

### 2.7. Cell viability and plating efficiency of surface modified hepatocyte

The cell viabilities of isolated hepatocytes with either surface modified or non-modified were determined by trypan blue exclusion test immediately after the completion of surface modification steps. These cells were resuspended in DMEM supplemented with $10^{-7}$ M dexamethasone (Sigma), 0.5 μg/mL insulin (Wako Pure Chemicals, Tokyo, Japan), 30 μg/mL L-proline (ICN Biomedicals, Aurora, OH), 10 mM nicotinamide (Kanto Chemicals, Tokyo, Japan), 10 ng/mL epidermal growth factor (Invitrogen, Carlsbad, CA), 0.2 mM ascorbic acid-2 phosphate, 1% dimethyl sulfoxide (Wako), 10% fetal bovine serum (Japan Bioserum, Japan), 100 IU/mL penicillin, and 100 μg/mL streptomycin, and were cultured on Type I Collagen-coated 6 well plate (AGC Techno Glass, Chiba, Japan) at a density of 0.8 × $10^4$/cm$^2$ in 37 °C. Eighteen hours after plating, plating efficiency was determined by counting attached and non-attached hepatocytes in 10 randomly-selected 20x fields per dish, and expressed as a percentage of attached cells per total cells.

### 2.8. Protein secretion ability of modified or non-modified hepatocytes

To assess the ability of surface modified hepatocytes to secrete proteins, culture medium was replenished at 24 h and collected 24 h later (at 48 h). The amount of mouse albumin and hA1AT in the medium were quantified by specific ELISA. Albumin ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX) was used to measure albumin levels. To measure hA1AT, anti-human α1-antitrypsin IgG (DiaSorin, Stillwater, MN) and goat anti α1-anti-trypsin HRP conjugated (Fitzgerald Industries International, Concord, MA) were used as described previously [5,20,22].

### 2.9. Metabolic activity of modified or non-modified hepatocytes

To assess the metabolic activity of cultured hepatocytes with either surface modified or non-modified, ammonia and lidocaine metabolism was assessed. To determine ammonia metabolism, ammonium chloride (Wako Pure Chemical Industries) was inoculated into the culture medium (final concentration = 1 mM) for 6 h, and the residual amount of ammonia in the culture medium was assessed by an Ammonia-Test-Wako kit (Wako Pure Chemical). To determine lidocaine metabolism, the culture medium was replenished with the addition of lidocaine (1 mg; AstraZeneca, Osaka, Japan) per well and maintained for 8 h. The residual amount of lidocaine in the culture medium was assessed by enzyme immunoassay at SRL (Tokyo). The amount of metabolized ammonia and lidocaine was calculated by subtracting the values of experimental groups from the non-hepatocyte control groups.

### 2.10. Hepatocyte-specific gene expression analyses of modified or non-modified hepatocytes

Cultured hepatocytes with either surface modified or non-modified were harvested at 48 h of culture protocol. Total RNA was extracted from each organ or isolated liver cell samples using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Genomic DNA contamination was eliminated by treating the extracted total RNA with DNase I (Qiagen). The DNase-treated total RNA (1 μg) was reverse-transcribed using High Capacity RNA-to-cDNA Master Mix (Applied

Biosystems, Foster City, CA) as described by the manufacturer's instructions. Quantitative real-time PCR analysis was performed using a PRISM 7300 Sequence Detector with a universal PCR master mix according to the specifications of the manufacturer (Applied Biosystems). The following hepatocyte-specific genes were evaluated using TaqMan probes and primers (Applied Biosystems) under cycling conditions of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C; *Alb* (Mm00802090_m1), *F7* (Mm00487329_m1), *F9* (Mm01308427_m1), *Serpinc1* (Mm00446573_m1), *Otc* (Mm00493267_m1), *Abcc2* (MRP2) (Mm00496899_m1), *Abcb11* (BSEP) (Mm00445168_m1), *Cyp3a11* (Mm00731567_m1), *Cyp1a2* (Mm00487224_m1), and *Nr1h4* (FXR) (Mm00436419_m1). For accurate quantification of gene expressions, we validated that *Ppia* (Mm02342430_g1) and *Hprt* (Mm03024075_m1) were the most stable housekeeping genes by geNorm algorism [23], and the geometric mean of these two housekeeping genes would be the most appropriate for normalization with the target genes. The cDNAs derived from pooled normal mouse livers were used to prepare standard reference curves.

### 2.11. Cytochrome P450 induction of modified or non-modified hepatocytes

To assess the cytochrome P450 induction of cultured hepatocytes, phenobarbital (Sigma) [an inducer of Cyp2b10] dissolved in DMSO were inoculated into the culture medium (final concentration = 1 mM) at 48 h after cell seeding. Twenty hours later, hepatocytes were harvested for the gene expression analysis of *Nr1i3* (CAR) (Mm00437986_m1) and *Cyp2b10* (Mm00456591_m1) by real-time PCR as described above.

### 2.12. Hepatocyte transplantation and liver tissue engineering experiment

Isolated hA1AT hepatocytes with either surface modified or non-modified were transplanted into the liver or under the kidney capsules of recipient FVB/N mice. For cell transplantation to the liver, a total of 1.5 x $10^6$ cells were infused using a 27-G needle as previously described [21,24]. For liver tissue engineering under the kidney capsules, isolated hepatocytes were resuspended with serum-free DMEM with an equal volume of Engelbreth-Holm-Swarm (E HS)-matrix (Matrigel, BD Biosciences, Bedford, MA) to a final ratio of 1.5 x $10^6$ hepatocytes per 100 μl. A total of 1.2 x $10^6$ hepatocytes were transplanted under the unilateral kidney capsule space of the recipient mice [5,22]. Recipient serum samples were periodically collected to measure serum hA1AT concentrations, which provide an index of engraftment by the transplanted hepatocytes. In addition, engrafted hepatocytes were visualized by hA1AT immunostaining using the frozen sections of the liver and kidney from the recipient mice. Goat anti-hAAT antibody (1:100, Bethyl Laboratories, Montgomery, TX, USA) and Alexa-fluor-594 (Molecular Probes, Eugene, OR) were used as a detecting antibody and a secondary reagent, respectively.

### 2.13. Statistical analysis

All of the values calculated were provided as a mean ± SEM. Statistical differences in the values were determined by repeated measures ANOVA with a post-hoc test. A probability value of $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. PEG-lipid-based ultra-thin coating of primary hepatocytes

We first attempted to find an optimal PEG-lipid for efficient surface modification of hepatocyte and ultra-thin coating. In this experiment, we examined three kinds of PEG-lipids with different alkyl chain lengths, since the hydrophobicity of PEG-lipids can impact the interaction with the cell membrane [25]. We chose to examine DMPE, DPPE, or DSPE by reacting the hydrophobic domain with hetero-bifunctional PEG followed by FITC-labeling (FITC-PEG-lipid). After incubation of the FITC-PEG-lipids with primary hepatocytes to anchor the lipids part to a lipid bilayer of cell membrane (Fig. 1A), FITC-PEG-DMPE exhibited the strongest fluorescence intensity compared to the other two types of FITC-PEG-lipids (Fig. 1B–E). Confocal microscopy revealed that the fluorescence signals were uniformly localized on the cellular surface with no detectable signals within the cytoplasm, specifically for DMPE (Fig. 1C) and to a lesser extent for DPPE (Fig. 1D). The DSPE label was not detectable (Fig. 1E). Flow cytometry analysis demonstrated that nearly all of the primary hepatocytes (99.79 ± 0.14%) were coated with FITC-PEG-DMPE (Fig. 1G), and this highly efficient surface modification focused our subsequent studies to be conducted with FITC-PEG-DMPE.

### 3.2. Influence of PEG-lipid-based ultra-thin coating on hepatocyte viability in vitro

To determine the influence of PEG-lipid-based surface modification on hepatocyte *in vitro*, we first assessed cellular viability by trypan blue exclusion test after the reaction with FITC-PEG-DMPE. As a result, no difference in the viability was observed between hepatocytes exposed to a FITC-PEG-DMPE solution and HBSS control (88.1 ± 2.8% vs 90.7 ± 2.5%, $P = 1.295$). Next, we investigated the influence of DMPE modification on attachment efficiency to the culture plates. Hepatocytes either exposed to a FITC-PEG-DMPE solution or HBSS were plated on type I collagen-coated dish, and attachment efficiency was determined 18 h later. Hepatocytes in both groups showed favorable attachment with no intergroup differences in attachment efficiencies (HBSS; 62.4 ± 2.8% vs DMPE; 61.0 ± 3.1%, $P = 0.617$). These *in vitro* experimental data indicated that the ultra-thin coating procedure using FITC-PEG-DMPE was not associated with any observable negative effects on cell viability and adhesive ability to the culture surface.

### 3.3. Influence of PEG-lipid-based ultra-thin coating on hepatocyte functionality in culture

Since our initial experiments showed that PEG-lipid-based surface-modified hepatocytes are amenable for primary culturing, we further investigated hepatocyte-specific functionalities by measuring protein production and chemical metabolism. As demonstrated in Table 1, there were no statistical difference in the production of albumin and hA1AT between the FITC-PEG-DMPE and HBSS groups. Similarly, the metabolism of both ammonia and lidocaine were similar in the control (HBSS) and DMPE-modified hepatocytes (Table 1). These data provide evidence that ultra-thin coated hepatocytes using FITC-PEG-DMPE did not hamper their ability to produce protein and metabolize chemicals *in vitro*.

### 3.4. Hepatocyte-specific gene expressions in culture

Messenger RNA (mRNA) expression levels were examined on 10 different hepatocyte-specific genes by real-time PCR. The analyzed genes were categorized as (1) protein/enzyme productions (*Alb, F7, F9, Serpinc1, and Otc*), (2) biliary transporters (*Abcc2* and *Abcb11*), (3) drug-metabolizing enzymes (*Cyp3a11, Cyp1a2*), and (4) nuclear receptors (*Nr1h4*). The expression levels of each amplified gene were normalized to the geometric mean of two housekeeping genes (*Ppia* and *Hprt*). As shown in Table 2, there was no significant difference between groups for all of the analyzed genes, except for a significant increase detected in the *Alb* levels.

Subsequently, we assessed the gene expression levels of nuclear receptor *Nr1i3* and the drug-metabolizing enzyme *Cyp2b10* prior to and after the exposure with phenobarbital. We determined that the surface-modified hepatocytes had increased expression of *Nr1i3* and *Cyp2b10* in response to the phenobarbital exposure compared to the non-modified (HBSS) hepatocytes (Fig. 2).

### 3.5. Engraftment of PEG-lipid-based ultra-thin coated hepatocytes after transplantation in vivo

To determine the feasibility of the PEG-based ultra-thin coated hepatocytes for cell-based therapies, we prepared surface modified hepatocytes using FITC-PEG-DMPE and immediately transplanted either into the liver through the portal vein or into an ectopic site under the kidney capsule. The engraftment level was determined by measuring the recipient serum hA1AT concentration after transplantation. There was a considerable amount of serum hA1AT levels regardless of the transplantation site (Fig. 3A–C). In order to

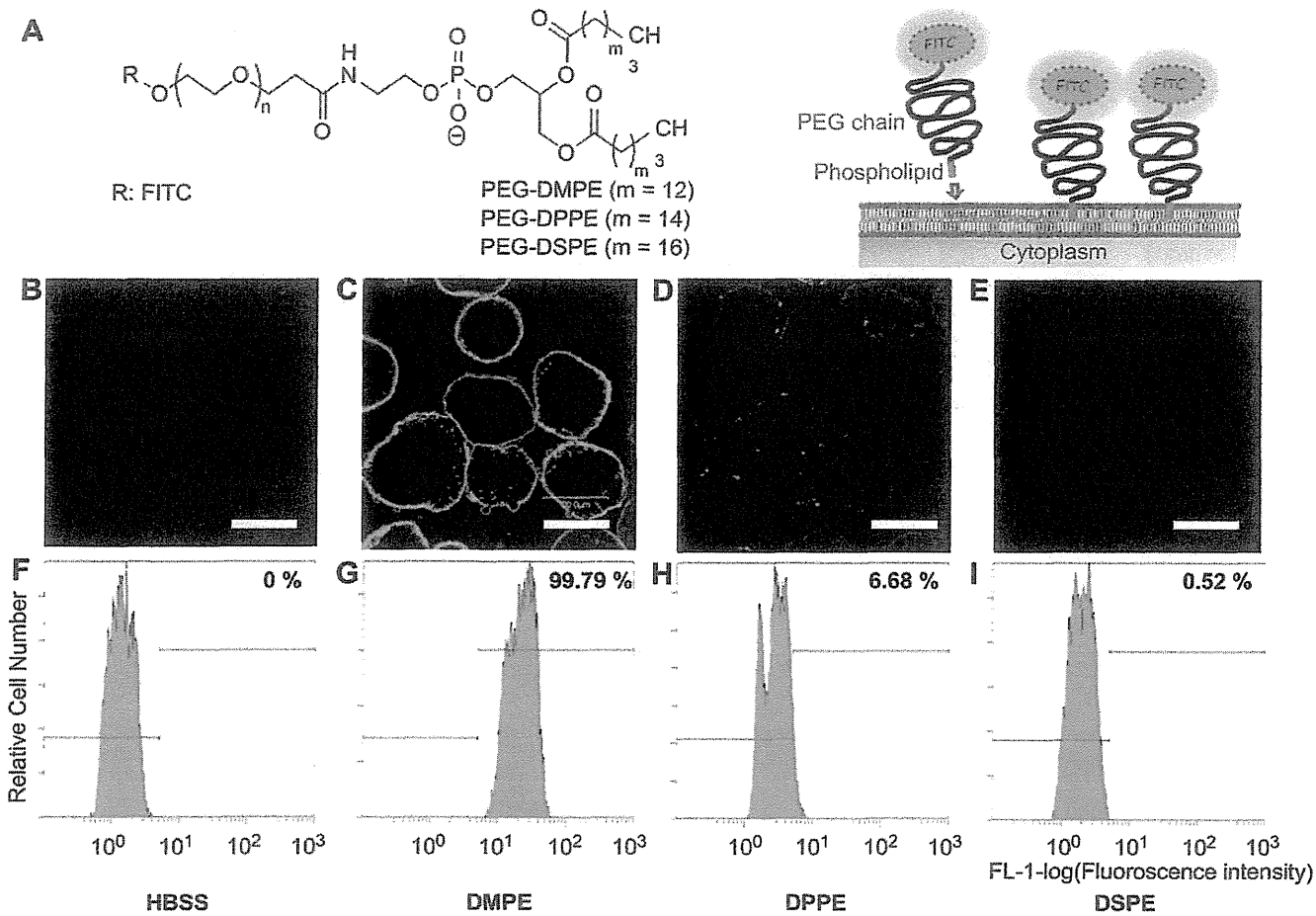


**Fig. 1.** Ultra-thin coating of the cell surface of primary hepatocytes using FITC-PEG-lipids. (A) Chemical structure of FITC-PEG-lipids (left) and schematic of the synthetic polymers on the hepatocyte surface (right). The PEG-lipid is comprised of hydrophobic alkyl chains that intercalate into the hepatocyte lipid bilayer membrane. (B—E) Representative confocal fluorescent microscopic images of mouse primary hepatocytes following incubation with the different PEG-lipids. Primary mouse hepatocytes mixed with either (B) HBSS as a negative control, (C) FITC-PEG-DMPE, (D) FITC-PEG-DPPE, or (E) FITC-PEG-DSPE. (F—I) Representative FACS scan demonstrating the percentage of positive surface-coated primary hepatocytes with control solutions (F; HBSS) or different polymers: (G) FITC-PEG-DMPE, (H) FITC-PEG-DPPE, or (I) FITC-PEG-DSPE. Values were obtained from 3 different donor mouse hepatocytes. Scale bars = 20 μm.

**Table 1**
In vitro comparison of the surface modified hepatocytes versus non-modified hepatocytes by protein production and drug metabolism. For the assessments of albumin and hA1AT production, primary mouse hepatocytes modified with either HBSS or FITC-PEG-DMPE were cultured at $8 \times 10^5$ hepatocytes in 6 well dishes coated with type I-collagen. At day 1, culture media was changed and the culture media was harvested 24 h later for assessment of albumin and hA1AT concentrations. For evaluating drug metabolism abilities of cultured hepatocytes, the culture media was replenished with the addition of $NH_4Cl$ (final conc. 1 mM) or 1 mg of lidocaine at 48 h of culture. Six hours after the addition of $NH_4Cl$, residual $NH_4Cl$ concentrations in the culture medium were measured and the metabolized $NH_4Cl$ doses were calculated. Eight hours after the addition of lidocaine, residual lidocaine concentrations in the culture medium were measured and the metabolized lidocaine doses were calculated. Data were represented as mean ± SEM. $n = 3$ each.

| Hepatocyte function | HBSS | DMPE | *P* value |
|---|---|---|---|
| Mouse albumin levels (ng/1 × $10^6$ of cells/24 h) | 5463 ± 875 | 5542 ± 1149 | 1.937 |
| hA1AT levels (ng/1 × $10^6$ of cells/24 h) | 2421 ± 671 | 4242 ± 1560 | 0.541 |
| Ammonia metabolic rate for 6 h (mg/1 × $10^6$ of cells) | 16.9 ± 0.3 | 18.7 ± 0.4 | 0.227 |
| Lidocaine metabolic rate for 8 h (mg/1 × $10^6$ of cells) | 75.0 ± 11.0 | 53.0 ± 15.0 | 0.947 |

**Table 2**
In vitro comparison of the surface modified hepatocytes versus non-modified hepatocytes by mRNA expression profiling of hepatocyte-specific genes. Primary mouse hepatocytes modified with either HBSS or FITC-PEG-DMPE were cultured at $8 \times 10^5$ hepatocytes in 6 well dishes coated with type I-collagen. At day 2, cells were harvested and their mRNA expression profile of hepatocyte-specific genes was analyzed by real-time PCR. The data for each hepatic gene was normalized to the geometric mean of *Ppia* and *Hprt* mRNA levels. Data were represented as mean ± SEM. $n = 3$ for each analyzed gene. Asterisk marks indicate $P < 0.05$.

| Gene Symbol | HBSS | DMPE | *P* value |
|---|---|---|---|
| *Alb* | 0.0170 ± 0.0001 | 0.0192 ± 0.0002 | 0.001* |
| *F7* | 0.0436 ± 0.0051 | 0.0231 ± 0.0054 | 0.376 |
| *F9* | 0.0252 ± 0.0043 | 0.0369 ± 0.0051 | 0.585 |
| *Serpinc1* | 0.0032 ± 0.0001 | 0.0031 ± 0.0002 | 1.665 |
| *Otc* | 0.0009 ± 0.0000 | 0.0016 ± 0.0001 | 0.132 |
| *Abcc2* | 0.2165 ± 0.0124 | 0.2789 ± 0.0048 | 0.137 |
| *Abcb11* | 0.0227 ± 0.0005 | 0.0342 ± 0.0027 | 0.141 |
| *Cyp3a11* | 0.0002 ± 0.0000 | 0.0003 ± 0.0000 | 0.154 |
| *Cyp1a2* | 0.0048 ± 0.0005 | 0.0070 ± 0.0003 | 0.096 |
| *Nr1h4* | 0.0336 ± 0.0029 | 0.0534 ± 0.0130 | 0.611 |

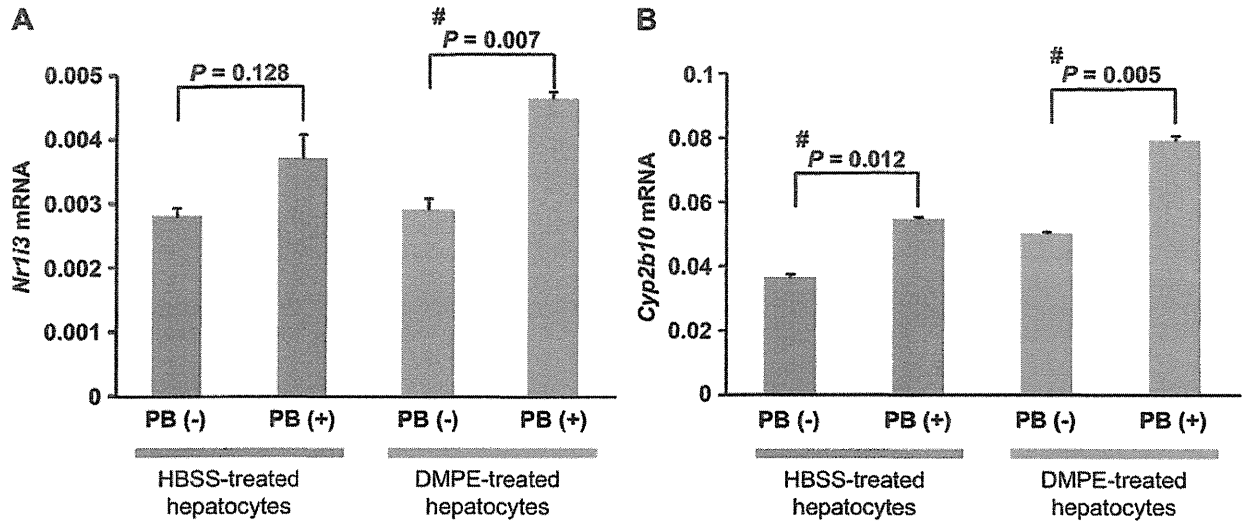


**Fig. 2.** *In vitro* comparison of the surface modified hepatocytes versus non-modified hepatocytes by measuring CYP gene induction with phenobarbital. Primary mouse hepatocytes modified with either HBSS (blue bars) or FITC-PEG-DMPE (green bars) were cultured at a density of $8 \times 10^5$ hepatocytes/well in 6 well dishes coated with type I-collagen. At day 2, phenobarbital was inoculated into the hepatocyte culture medium at a final concentration of 1 mM. Twenty hours later, hepatocytes were harvested to analyze gene expression of (A) *Nr1i3* (CAR) and (B) *Cyp2b10* by real-time PCR. The gene expression data was normalized to the geometric mean of *Ppia* and *Hprt* mRNA levels. $n = 3$ each for each group.

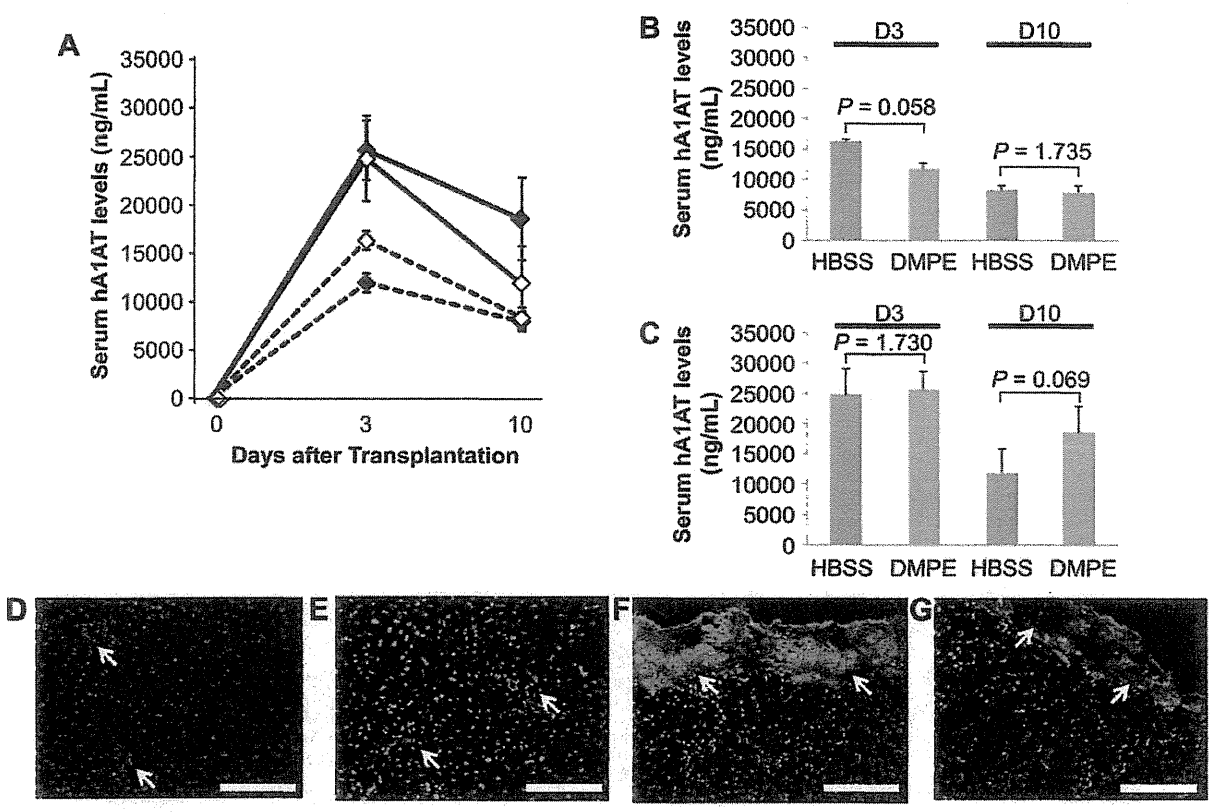


**Fig. 3.** In vivo comparison of the surface modified hepatocytes versus non-modified hepatocytes by determining engraftment efficiency after transplantation. (A) Primary hA1AT mouse hepatocytes modified with (filled symbol) or without FITC-PEG-DMPE (open symbol) were transplanted into wild-type FVB/N mice. HBSS was used as a vehicle control group (open symbol). Engraftment of transplanted hepatocytes was assessed by measuring recipient serum hA1AT levels at day 3 and 10. Dotted lines and straight lines indicate the recipient groups that were transplanted with cells via portal vein to liver and under kidney capsules, respectively. (B) Liver transplantation. Control (HBSS; blue bars) or FITC-PEG-DMPE (green bars) modified hepatocytes were resuspended in DMEM and transplanted into the liver through the portal vein using $1.5 \times 10^6$ cells/mouse. (C) Kidney capsule transplantation. Control (HBSS; blue bars) or FITC-PEG-DMPE modified hepatocytes (black bars) were transplanted under the left kidney capsule using $1.2 \times 10^6$ cells/mouse. Livers (D, E) and kidneys (F, G) were harvested 10 days after transplantation of control hepatocytes (D, F) or FITC-PEG-DMPE modified hepatocytes (E, G) for immunohistochemical staining of hA1AT.Arrows point to the engrafted hA1AT mouse hepatocytes. Scale bars = 100 μm.

engraft into the liver, hepatocytes need to translocate from the portal vein into the liver parenchyma through the invasion of sinusoidal plate. Immunohistochemical staining of liver samples taken 10 days after transplantation revealed that transplanted hepatocytes were capable of engraftment into the liver parenchyma (Fig. 3D, E). Immunohistochemistry also showed the engraftment of hepatocytes under the kidney capsules in both groups (Fig. 3F, G). These data taken together suggest that PEG-lipid-modified hepatocytes are able to be engrafted in the liver, as well as an ectopic site at identical efficiency to the naïve hepatocytes.

### 3.6. PEG-based ultra-thin coating of hepatic tissue sheet

To determine whether the established PEG-based ultra-thin coating procedure could be applied to bioengineered tissues, hepatocyte sheets comprised of primary cultured hepatocytes (Fig. 4A) were conjugated with either FITC-PEG-DMPE or FITC-PEG-DPPE. HBSS was used as a control solution. Uniformly intense fluorescence signals were detected from the hepatocyte sheets reacted with FITC-PEG-DMPE (Fig. 4B). Confocal microscopy revealed that the fluorescence signals was uniformly localized on the hepatocytes and hepatocyte sheet surfaces on both sides, even on the side that faced the culture dish prior to cell sheet harvesting (Fig. 4C). In marked contrast, the hepatocyte sheets conjugated with FITC-PEG-DPPE showed little or no fluorescence signals, which is comparable to the hepatocytes treated with the control solution, HBSS (Fig. 4D, E).

## 4. Discussion

The present studies established a simple and efficient procedure to modify the surface of primary hepatocytes as well as hepatic tissue sheets using amphiphilic PEG-lipid (PEG-DMPE). The importance of our strategy is highlighted by the findings that the ultra-thin coating process did not negatively affect molecular and cellular function of the modified hepatocytes *in vitro*. Furthermore, *in vivo* transplantation experiments confirmed their favorable engraftment abilities. These results suggest the potential value of our present cell surface modification approach to advance hepatocyte-based applications *in vitro* and *in vivo*.

In general, the lipid bilayer provides the basic structure for all cell membrane. The major lipids in cell membrane are phosphoglycerides, sphingolipids, and sterols. Furthermore, there are several kinds of phosphoglycerides, such as phoshatidylethanolamine, phosphatidylserine, and phosphatidylcholine. The composition of these lipids in the cell membrane is different among cell types [26] or pathological condition of the cells [27]. In this study, among three types of lipid analyzed, DMPE demonstrated the strongest affinity for the cell surface of mouse hepatocytes, while the cell surface of hamster islet cells is known to be combined with DPPE and DMPE [11]. These differences of modification efficiency by PEG-lipids may arise from the diversity of lipid composition of cell membrane as mentioned above. As for the number of molecules that can attach on the surface of one cell, we have previously reported that approximately $1 \times 10^8$ molecules of PEG-lipid were incorporated onto one cell when human embryonic kidney cell line (HEK293) was used under the same protocol as described in the present study [11]. Although the number of attached molecules per hepatocytes was not directly measured in the present study, the qualitative fluorescent intensity observed on the cell surface suggests that similar number of molecules of PEG-lipid may have been conjugated on each hepatocyte.

Culture systems using primary hepatocytes has been an important platform for pharmacological and toxicological assessments [28], particularly in the study of cytochrome P450 enzymes (CYP isoforms) and their drug metabolizing abilities. Since hepatocytes in the conventional cell culture system are prone to lose

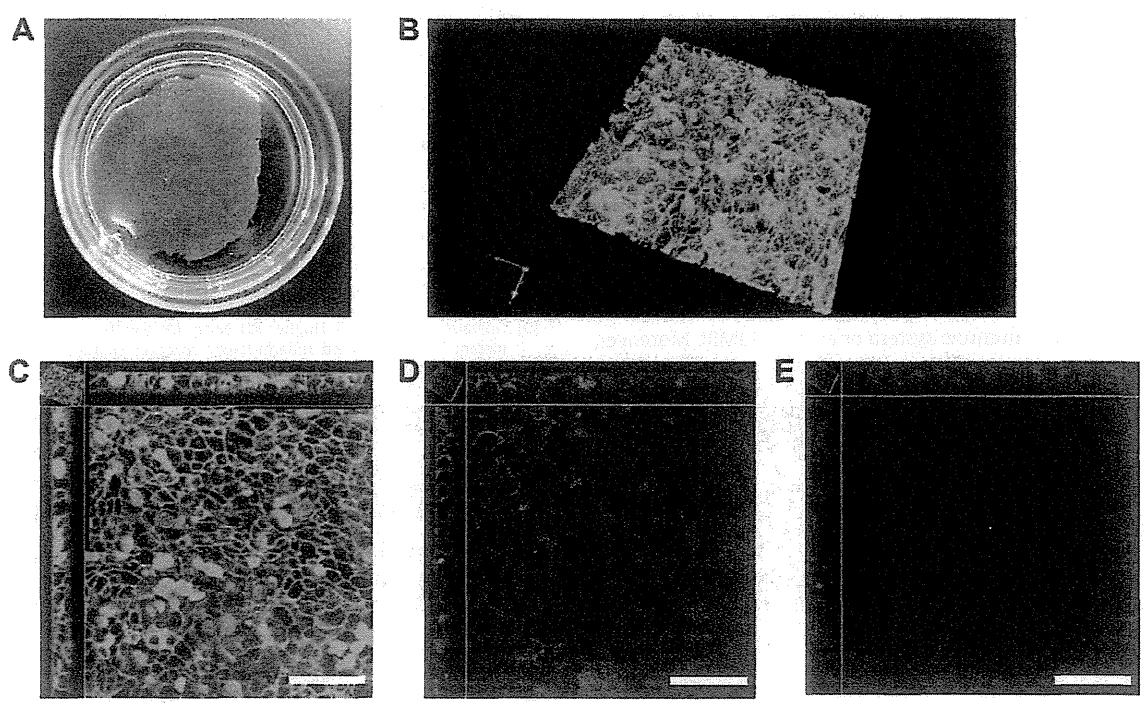


**Fig. 4.** Bioengineering hepatocyte sheets coated with an ultra-thin layer of PEG-lipids. (A) Gross morphology of the harvested hepatocyte sheet harvested using the temperature-sensitive PIPAAm culture dishes. (B—E) Confocal fluorescent microscopic image of the hepatocyte sheet. Harvested hepatocyte sheets were modified with either (B, C) FITC-PEG-DMPE or (D) FITC-PEG-DPPE. (E) HBSS was used as a negative control for the cell surface modification. (B) Representative 3D image of a surfaced-modified hepatocyte sheet with FITC-PEG-DMPE. (C—E) Horizontal and vertical views of surfaced-modified hepatocyte sheets. Scale bars = 100 μm.

normal functions within a few days after plating, it has been a point of interest to establish a methodology to modify hepatocytes in order to prolong their functional abilities [28–30]. In our study, we confirmed that PEG-lipid based modifications can efficiently coat the surface of primary hepatocytes without affecting normal drug metabolism, CYP expression levels and protein secretion. In the case for albumin, we determined that the secretion levels were comparable between the modified and non-modified hepatocytes even though the Alb mRNA levels in the modified cells were higher than those measured in the non-modified cells. At this time, we speculate that one possible reason for this apparent discordance between mRNA levels and protein secretion is due to a temporal lag between the molecular events between gene transcription, protein translation and ultimately, protein secretion from the cell.

The coating process described in our study could be applied to various basic and clinical settings because the replacement of FITC with other functional proteins, enzymes, or peptides is theoretically possible and may provide a method by which cells including primary hepatocytes can prolong their survivability and functionality. For instance, we have previously shown that pancreatic islets coated with PEG-lipids can be further coated with poly(vinyl alcohol) (PVA) that were anchored to maleimide groups [12]. By thiol/maleimide bonding, chemical agents, such as urokinase, were successfully anchored to the cellular surface without increasing the practical cell volume [31]. Biological activities of the immobilized urokinase on the islet surface was confirmed in intra-portal transplantation experiments *in vivo* [16]. Furthermore, heterotypic cells can be immobilized on the islet surface through interaction between biotin-PEG on islet surface and streptavidin immobilized on heterotypic cell surface [15]. Since it is important for hepatocytes to face other type of cells, such as endothelial cells or 3T3 cells, to express and retain hepatocyte-specific functions [32], the PEG-lipid-based immobilization of hepatocytes with heterotypic cells could lead to the development of a more effective hepatocyte culture system to study many types of biological functions.

Although hepatocytes have potential to engraft within the liver parenchyma when transplanted via portal vein, the efficiency of the engraftment has been extremely poor. This has greatly limited the therapeutic potential of hepatocyte-based therapies [7,29]. The limited engraftment efficiencies are attributed to the following responsible factors: 1) non-self recognition of the transplanted cells by the host immune system, 2) instant blood-mediated inflammatory reactions (IBMIR) [8,9], and 3) poor invasion into the hepatic cord though the sinusoidal endothelial wall and space of Disse [33]. Thus, a process in which we can camouflage the cell surface with functional proteins, such as immunosuppressive or anti-coagulant agents, would be extremely valuable by allowing these modified cells to evade the host immune system or activate IBMIR. Moreover, bioconjugation of functional moieties onto the cell surface may enhance the ability of these modified cells to translocate cells into the hepatic cord. Due to the ability to conjugate many kinds of biological agents to the cells in a simple protocol, we envision that this modification procedure may have the potential to be more superior to other reported methodologies for modifying hepatocytes, such as micro-encapsularization using alginate-polylysine hydorgel [34]. Detailed future experiments will need to be performed, however, to prove the feasibility of this approach on alleviating each of these aforementioned limitations to the current methodology.

## 5. Conclusions

In summary, the present study describes a proof-of-concept approach to modify the surface membrane of primary hepatocytes using PEG-lipid derivatives, in particular 1,2-dimyristoil -*sn*-glycerol-3-phosphatidylethanolamine (DMPE), without negatively affecting the *in vitro* functional parameters, molecular phenotypes, and their *in vivo* engraftment potential. This modification process was also successful in engineering hepatocyte sheets to produce ectopic liver tissues for therapeutic applications. The future potential in using out cell surface modification process to anchor PEG-lipid linked to therapeutically relevant functional proteins may serve as the foundation to advance hepatocyte-based therapy and drug discovery research.

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## References

[1] Fisher RA, Strom SC. Human hepatocyte transplantation: worldwide results. Transplantation 2006;82:441–9.
[2] Tatsumi K, Ohashi K, Shima M, Nakajima Y, Okano T, Yoshioka A. Therapeutic effects of hepatocyte transplantation on hemophilia B. Transplantation 2008; 86:167–70.
[3] Meyburg J, Das AM, Hoerster F, Lindner M, Kriegbaum H, Engelmann G, et al. One liver for four children: first clinical series of liver cell transplantation for severe neonatal urea cycle defects. Transplantation 2009;87:636–41.
[4] Dhawan A, Mitry RR, Hughes RD, Lehec S, Terry C, Bansal S, et al. Hepatocyte transplantation for inherited factor VII deficiency. Transplantation 2004;78: 1812–4.
[5] Ohashi K, Waugh JM, Dake MD, Yokoyama T, Kuge H, Nakajima Y, et al. Liver tissue engineering at extrahepatic sites in mice as a potential new therapy for genetic liver diseases. Hepatology 2005;41:132–40.
[6] Ohashi K, Tatsumi K, Utoh R, Takagi S, Shima M, Okano T. Engineering liver tissues under the kidney capsule site provides therapeutic effects to hemophilia B mice. Cell Transplant 2010;19:807–13.
[7] Puppi J, Strom SC, Hughes RD, Bansal S, Castell JV, Dagher I, et al. Improving the techniques for human hepatocyte transplantation: report from a consensus meeting in London. Cell Transplant. doi:10.3727/096368911X566208. Available from URL: http://www.ncbi.nlm.nih.gov/pubmed/21457616; 2011.
[8] Stephenne X, Vosters O, Najimi M, Beuneu C, Dung KN, Wijns W, et al. Tissue factor-dependent procoagulant activity of isolated human hepatocytes: relevance to liver cell transplantation. Liver Transpl 2007;13:599–606.
[9] Gustafson EK, Elgue G, Hughes RD, Mitry RR, Sanchez J, Haglund U, et al. The instant blood-mediated inflammatory reaction characterized in hepatocyte transplantation. Transplantation 2011;91:632–8.
[10] Zalipsky S. Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates. Bioconjug Chem 1995;6:150–65.
[11] Miura S, Teramura Y, Iwata H. Encapsulation of islets with ultra-thin polyion complex membrane through poly(ethylene glycol)-phospholipids anchored to cell membrane. Biomaterials 2006;27:5828–35.
[12] Teramura Y, Kaneda Y, Iwata H. Islet-encapsulation in ultra-thin layer-by-layer membranes of poly(vinyl alcohol) anchored to poly(ethylene glycol)-lipids in the cell membrane. Biomaterials 2007;28:4818–25.
[13] Chen H, Teramura Y, Iwata H. Co-immobilization of urokinase and thrombomodulin on islet surfaces by poly(ethylene glycol)-conjugated phospholipid. J Control Release 2011;150:229–34.
[14] Chen H, Teramura Y, Iwata H. Immobilization of anticoagulant-loaded liposomes on cell surfaces by DNA hybridization. Biomaterials; 2011.
[15] Teramura Y, Iwata H. Islet encapsulation with living cells for improvement of biocompatibility. Biomaterials 2009;30:2270–5.
[16] Teramura Y, Iwata H. Improvement of graft survival by surface modification with poly(ethylene glycol)-lipid and urokinase in intraportal islet transplantation. Transplantation 2011;91:271–8.
[17] Teramura Y, Iwata H. Surface modification of islets with PEG-lipid for improvement of graft survival in intraportal transplantation. Transplantation 2009;88:624–30.