concentration, requires vitamin B6 as a co-factor [7]. Thus, administration of vitamin B6 antagonist impaired S1P lyase activity, which consequently led to the defect of lymphocyte trafficking caused by inappropriate S1P gradient [7]. Similar effect was noted in 2-acetyl-4-tetrahydroxybutylimidazole (THI), a component of caramel food colorant III used in food products. THI inhibits S1P lyase and thus, like treatment with vitamin B6 antagonist, prevents normal lymphocyte trafficking [7]. These findings led to the use of THI for the treatment of immune diseases [30–32].

4. S1P Regulates Innate and Acquired Phases of Intestinal IgA Responses

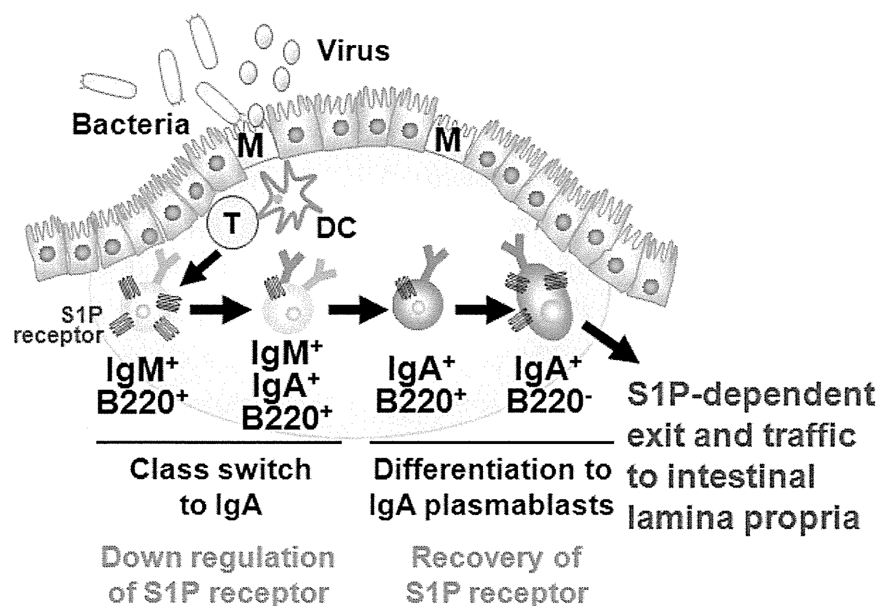
IgA is the most frequently observed antibody isotype in the intestinal compartments and provides the first line of defense against pathogenic microorganisms invading through mucosal tissues. Therefore, the induction of appropriate IgA responses is a logical strategy for the development of oral vaccines [33]. Since IgA antibody is one of the major arms of the mucosal immune system in the digestive tract, which covers a large surface area, the intestinal IgA is originated from several induction sites including Peyer's patches (PPs), isolated lymphoid follicles, and the peritoneal cavity [34].

A well characterized gut-associated lymphoid tissue (GALT) is PPs. PPs act as induction sites for the initiation of IgA responses against T-cell-dependent antigens [35]. PPs are covered with a specialized epithelium known as follicle associated epithelium (FAE) containing antigen-sampling M cells, which are responsible for the uptake and transport of antigens from the intestinal lumen to antigen-presenting cells such as dendritic cells (DCs) (Figure 2) [36]. Then, DCs capture antigens from the M cells, process and present them to T cells. It has been shown that the formation of PP DC-T cell clusters provide both cellular and molecular environment for the generation of IgA committed B cells in PPs [34]. In this pathway, some of the activated T cells differentiate into follicular helper T cells to help the antibody class switching of B cells in the germinal centers [34]. Because of the unique cytokine environment (e.g., TGF-β, IL-4, and IL-21) and continuous stimulation by commensal bacteria in the intestine, PPs have been shown to equip with efficient molecular and cellular environment for the spontaneous and continuous B cell class switching from IgM to IgA [34,35]. After class switching to IgA, B cells further differentiate into IgA plasmablasts and then migrate out from the PPs for their subsequent trafficking to the intestinal lamina propria, where they terminally differentiate into plasma cells producing dimeric (or polymeric) forms of IgA. This process mainly contributes to the development of T cell-dependent antigen-specific immune responses. Thus, the PP-mediated induction pathway is considered to be a major arm of the acquired IgA response [34].

Our investigation provided new evidence that S1P regulated the B cell trafficking in the PPs for the intestinal IgA production [37]. We initially found that S1P1 expression in B cells changes during differentiation in the PPs (Figure 2) [37]. High levels of S1P1 expression were detected in IgM$^+$ naive B cells, and expression was down-regulated when B cells started class switching to IgA. The low expressions of S1P1 allowed newly class-switched IgA$^+$ B cells to retain in the PPs for the sufficient differentiation into the IgA$^+$ plasmablasts. S1P1 expression was restored on the IgA$^+$ plasmablasts, resulting in their emigration from the PPs. Mice treated with FTY720, an immunosuppressant inducing S1P1 downregulation [38], show selective accumulation of IgA$^+$ plasmablasts in the PPs, leading to the disturbance of continuous delivery of IgA committed B cells from the PPs to the lamina propria of intestine. Consequently, the decrease of same population in the intestinal lamina propria was noted,

which associated with the reduction of intestinal antigen-specific IgA responses against orally immunized protein antigen [37].

Figure 2. Sequential changes in S1P1 expression during B-cell differentiation in Peyer's patches. Dendritic cells (DC) take the antigens transported by M cells from intestinal lumen and present them to T cells for their activation. Through the interaction with T cells and DCs, IgM^+ naive B cells show class-switch from IgM to IgA. During this process, S1P1 is expressed at high levels in IgM^+ naive B cells and downregulated on B cells class-switching from IgM to IgA and subsequently recovered on IgA^+ $B220^-$ plasmablasts, resulting in their emigration from the Peyer's patches and traffic into the intestinal lamina propria.



In the IgA production pathway in the gut, peritoneal B cells are an additional source of intestinal IgA [39]. A number of peritoneal B cells belong to a unique B-cell subset, termed as B1 cells, which produces antibodies against T-cell-independent antigens such as lipids and polysaccharides. Because these T-cell-independent antigens are conserved in various microorganisms, B1-cell-derived antibodies undiscriminatingly react to commensal and pathogenic bacteria and prevent their attachment and invasion into the host. This reaction is opposite to antibody responses against protein antigen mediated by PP B cells, which show rigid specificity against microorganisms. Therefore, it has been considered that B1-cell-derived IgA is categorized as to be innate-type antibodies that recognize a wide range of microorganisms in the intestine [39].

Trafficking of peritoneal B1 cells into the intestine requires S1P-mediated signaling [40]. Like B cells in the PPs, peritoneal B1 cells identically expressed S1P1. Thus, trafficking of peritoneal B cells into the intestine and consequent production of intestinal IgA are diminished by treatment with FTY720, mainly because of the inhibition of B1 cell emigration from the parathymic lymph nodes, which drain to the peritoneal cavity [40]. This impaired trafficking in FTY720-treated mice was associated with the decreased IgA responses against phosphorylcholine (a T-cell-independent antigen) induced by oral immunization with heat-killed *Streptococcal pneumoniae* [40].

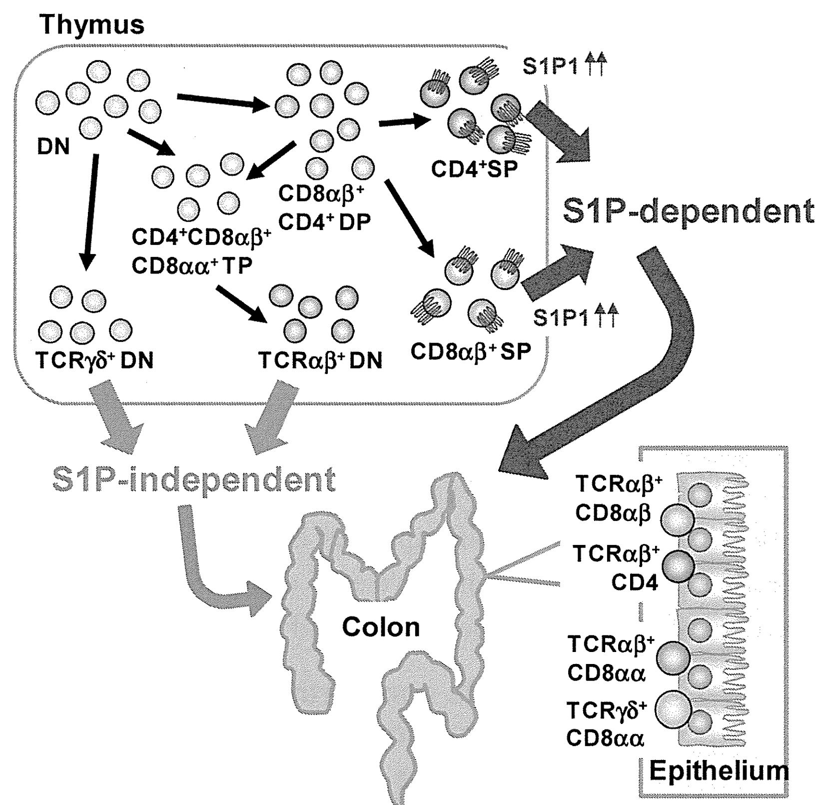
We also found that S1P-mediated regulation of peritoneal B-cell trafficking requires crosstalk with stromal cells in the peritoneal cavity [41]. This interaction mediated by adhesion molecules (e.g., ICAM-1 and VCAM-1) on stromal cells and the expression is regulated by NFκB-inducing kinase (NIK). Therefore, NIK-mutant aly/aly mice show decreased sensitivity to FTY720 in the regulation of peritoneal B-cell trafficking due to the impaired expression of adhesion molecules although peritoneal B1 cells in aly/aly mice expressed comparable levels of S1P1.

5. Distinct S1P Dependency of Trafficking of Intraepithelial T-Lymphocytes in the Gut

Large numbers of lymphocytes are also present in the intestinal epithelium and called as intraepithelial lymphocytes (IELs) [42]. IELs are mostly T cells, but unlike in conventional T cells observed in the systemic compartments (e.g., spleen) which predominantly express the αβ T-cell receptor (αβTCR), in the IEL subset there is an abundance of T cells expressing the γδ T cell receptor (γδTCR) in addition to $\alpha\beta TCR^{+}$ T cells [42]. αβTCR recognizes peptide antigen presented via major histocompatibility complex (MHC) molecules, whereas γδTCR recognizes non-classical MHC molecules such as MHC class I chain-related proteins (MIC) A and B (MICA/B) in human and Rae-1 in mouse [43]. Unlike MHC molecules that act as ligand by presenting peptide antigen, non-classical MHC molecules act as a ligand by itself and the expression was induced by stress (e.g., infection, tumors, or chemical treatment) [44]. Thus, it is considered that αβTCR is involved in acquired immunity through the activation by specific presentation of antigenic peptides, whereas γδTCR is involved in innate immunity by the ligation of non-classical MHC molecules [42]. A distinctive pattern of CD8 expression has also been noted in IELs. Conventional $\alpha\beta TCR^{+}$ T cells express CD8 as a heterodimer of α and β (CD8αβ). In contrast, some IELs uniquely express CD8 as a homodimer (CD8αα) [42]. A previous study identified a unique precursor of CD8αα IELs in the thymus [45]. In the thymus, $CD4^{-}$ $CD8^{-}$ double-negative thymocytes differentiate into $CD4^{+}$ $CD8^{+}$ double-positive thymocytes and then further differentiate into single-positive thymocytes expressing either CD4 or CD8. $CD8\alpha\beta^{+}$ IELs are derived mainly from $CD8^{+}$ single-positive thymocytes expressing αβTCR. $CD8\alpha\alpha^{+}$ IELs, however, originate from double-negative thymocytes expressing either αβTCR or γδTCR that have themselves differentiated from unique $CD4^{+}$ $CD8\alpha\alpha^{+}$ $CD8\alpha\beta^{+}$ triple-positive thymocytes (Figure 3) [45].

S1P has been involved in the regulation of cell trafficking of different subsets of IELs originated from thymus. We found that each type of IEL shows a different dependency on S1P in its trafficking from the thymus to the intestine, especially in the colon (Figure 3) [46]. When mice were treated with FTY720, decreased numbers of $CD8\alpha\beta^{+}$ IELs were observed. In contrast, the numbers of $CD8\alpha\alpha^{+}$ IELs were barely affected. These data suggest that, in the colonic epithelium, $CD8\alpha\beta^{+}$ IELs are S1P dependent and $CD8\alpha\alpha^{+}$ IELs are S1P independent. Consistent with this finding, $CD8^{+}$ single-positive thymocytes—the precursors of $CD8\alpha\beta^{+}$ IELs—express high levels of S1P1 [8], whereas no S1P1 expression has been noted on double-negative thymocytes, the precursors of $CD8\alpha\alpha^{+}$ IELs [46]. These findings suggest that S1P1 expression was different in different subsets of thymic precursors of IELs and provide versatile immunological pathways in the intestine.

Figure 3. Distinct dependency on S1P in T-cell trafficking into the colonic epithelium. In the thymus, $CD4^-$ $CD8^-$ double-negative (DN) thymocytes differentiate into $CD4^+$ $CD8^+$ double-positive (DP) thymocytes and then into single-positive (SP) thymocytes expressing either CD4 or CD8 and αβTCR. These SP thymocytes express high levels of S1P1 and migrate out from the thymus and into the colon in an S1P-dependent manner. DN thymocytes express TCRαβ or TCRγδ. DN thymocytes expressing TCRαβ are derived from $CD4^+$ $CD8\alpha\alpha^+$ $CD8\alpha\beta^+$ triple-positive (TP) thymocytes differentiated from DN or DP thymocytes. Little or no S1P1 expression is noted in the DN thymocytes expressing TCRαβ or TCRγδ, so traffic to the colonic epithelium proceeds in an S1P-independent manner.

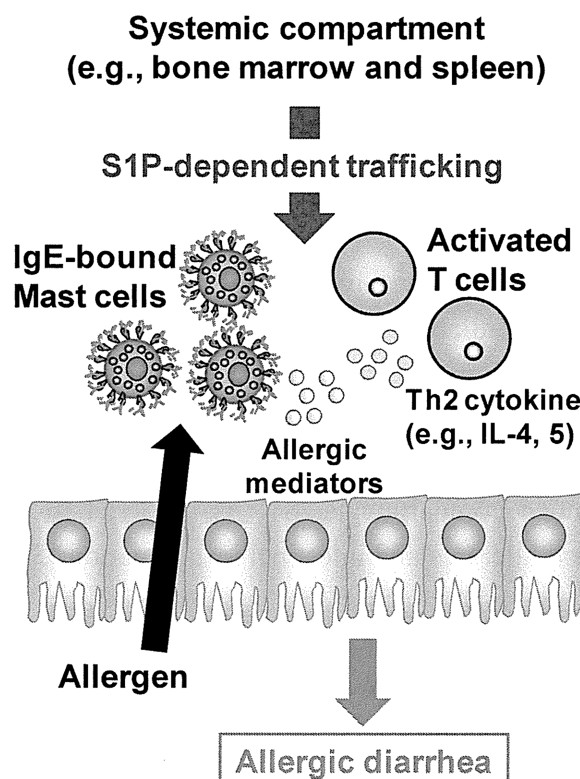


6. S1P-Mediated Regulation in the Development of Intestinal Immune Diseases

Accumulating evidence has revealed the pivotal role of S1P in the development of inflammatory diseases such as autoimmune type 1 diabetes, rheumatoid arthritis, and multiple sclerosis [5]. FTY720 prevents the egress of autoreactive lymphocytes from the lymph nodes into the peripheral circulation and subsequent across the blood–brain barrier into the central nerve system and thus has recently been approved as an oral therapy for multiple sclerosis [47]. In addition to being involved in these immune diseases at the systemic immune compartments, S1P is involved in the development of intestinal immune diseases including food allergies and intestinal inflammation [5]. The number of patients with food allergies has increased not only in children but also in adults; the development of effective preventive and therapeutic strategies for food allergies is therefore required to improve patients' quality of life. Using the ovalbumin-induced murine food-allergy model developed by our group [48], we examined the molecular and cellular mechanisms underlying the development of food allergies and found that, in allergic mice, activated T cells migrate into the colon, where they produced high

amounts of Th2 cytokines such as IL-4 and IL-5 [48]. We demonstrated that the trafficking of pathogenic T cells from the systemic compartments into the colon was mediated by S1P (Figure 4) [49]. Indeed, activated T cells in the colon of allergic mice expressed S1P1 and their infiltration into the colon and subsequent production of Th2 cytokines (e.g., IL-4 and IL-5) were inhibited by the treatment with FTY720 [49]. In addition, the infiltration of mast cells, effector cells in the development of food allergy, into the colon was also prevented in the FYT720-treated mice [49]. As a mechanism of FTY720-mediated inhibition of mast cell infiltration, it was likely that FTY720 directly and indirectly prevented the mast cell infiltration into the colon. Direct effect of FTY720 was predicted by results that mast cells expressed S1P1 and their *in vitro* migration was inhibited by FTY720 [49]. Indirect effect is mediated by activated T cells producing Th2 cytokines which enhanced the proliferation and recruitment of mast cells [50]. Thus, inhibition of activated T cell trafficking into the colon by FTY720 resulted in the reduced recruitment and/or proliferation of mast cells. Taken together, involvement of S1P in the trafficking of both pathogenic T cells and mast cells is a potential target for prevention and treatment of food allergies.

Figure 4. S1P mediates intestinal allergy by regulating pathogenic T and mast cell infiltration into the colon. In murine food allergy model, systemically sensitized T cells migrate into the colon upon the oral challenge with same allergen. This trafficking is mediated by S1P and thus treatment with FTY720 resulted in the inhibition of activated T cell trafficking into the colon. In the colon, these activated T cells produced high amounts of Th2 cytokines such as IL-4 and IL-5 for promotion of mast cell recruitment and proliferation. In addition, mast cell itself expresses S1P1. Therefore, FTY720 treatment directly and indirectly (Th2 cytokine from activated T cells) decreases the numbers of mast cells in the colon. These effects lead to the inhibition of allergic diarrhea.



Similarly, several lines of evidence have demonstrated that the FTY720 treatment prevents the development of intestinal inflammation [51–53]. For example, in a spontaneous colitis model in interleukin-10-deficient mice, administration of FTY720 suppressed the infiltration of pathogenic T cells producing interferon-γ [51]. Infiltration of the colon by pathogenic T cells was also inhibited by treatment with FTY720 in both a dextran sulfate sodium (DSS)-induced colitis model and a T-cell transfer model in mice [52,53]. Although S1P regulates the activation of several inflammatory cells via modulation of the signaling of certain innate receptors such as toll-like receptors, TNF receptor, and protease-activated receptor 1, and S1P itself is produced by activated inflammatory cells [4], collectively these findings suggest that S1P–S1P1 axis participates mainly in the development of intestinal immune diseases at the stage of pathogenic cell trafficking into the colon.

7. Conclusion

It is clear from past and current studies that S1P plays an important role in the regulation of the immune system of the gut in both healthy and disease states. In general, S1P is derived from sphingomyelin and is produced mainly by platelets, erythrocytes, and endothelial cells in the body. However, in the intestine, it is likely that epithelial cells contribute most to the production of S1P. Most importantly, S1P produced by epithelial cells seems to originate from dietary sphingolipids, especially sphingomyelin. Thus, elucidation of the complex networks established by dietary lipids will create a new era in nutrition-based mucosal immunology and should provide a new strategy against intestinal immune diseases.

Acknowledgements

Original works described in this review were supported by grants from the Ministry of Education, Sports, Science, and Technology, Japan (Grant-in Aid for Young Scientists (A) [22689015 to J.K.], Grants-in-Aid for Scientific Research on Innovative Areas [23116506 to J.K.], for Scientific Research (S) [23229004 to H.K.], and for Scientific Research on Priority Areas [19059003 to H.K.]); the Ministry of Health and Welfare of Japan (J.K. and H.K.); the Global Center of Excellence program of the Center of Education and Research for Advanced Genome-based Medicine (to H.K.); the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (to J.K.); and the Yakult Bio-Science Foundation (to J.K.).

Conflict of Interest

The authors declare no conflict of interest.

References

1. Kiyono, H.; Kunisawa, J.; McGhee, J.R.; Mestecky, J. The Mucosal Immune System. In *Fundamental Immunology*; Paul, W.E., Ed.; Lippincott-Raven: Philadelphia, PA, USA, 2008; Volume 6, pp. 983–1030.
2. Maslowski, K.M.; Mackay, C.R. Diet, gut microbiota and immune responses. *Nat. Immunol.* **2011**, *12*, 5–9.

3. Margioris, A.N. Fatty acids and postprandial inflammation. *Curr. Opin. Clin. Nutr. Metab. Care* **2009**, *12*, 129–137.
4. Chi, H. Sphingosine-1-phosphate and immune regulation: Trafficking and beyond. *Trends Pharmacol. Sci.* **2011**, *32*, 16–24.
5. Rivera, J.; Proia, R.L.; Olivera, A. The alliance of sphingosine-1-phosphate and its receptors in immunity. *Nat. Rev. Immunol.* **2008**, *8*, 753–763.
6. Pappu, R.; Schwab, S.R.; Cornelissen, I.; Pereira, J.P.; Regard, J.B.; Xu, Y.; Camerer, E.; Zheng, Y.W.; Huang, Y.; Cyster, J.G.; *et al.* Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science* **2007**, *316*, 295–298.
7. Schwab, S.R.; Pereira, J.P.; Matloubian, M.; Xu, Y.; Huang, Y.; Cyster, J.G. Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science* **2005**, *309*, 1735–1739.
8. Matloubian, M.; Lo, C.G.; Cinamon, G.; Lesneski, M.J.; Xu, Y.; Brinkmann, V.; Allende, M.L.; Proia, R.L.; Cyster, J.G. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* **2004**, *427*, 355–360.
9. Michaud, J.; Im, D.S.; Hla, T. Inhibitory role of sphingosine 1-phosphate receptor 2 in macrophage recruitment during inflammation. *J. Immunol.* **2010**, *184*, 1475–1483.
10. Maeda, Y.; Matsuyuki, H.; Shimano, K.; Kataoka, H.; Sugahara, K.; Chiba, K. Migration of CD4 T cells and dendritic cells toward sphingosine 1-phosphate (S1P) is mediated by different receptor subtypes: S1P regulates the functions of murine mature dendritic cells via S1P receptor type 3. *J. Immunol.* **2007**, *178*, 3437–3446.
11. Walzer, T.; Chiossone, L.; Chaix, J.; Calver, A.; Carozzo, C.; Garrigue-Antar, L.; Jacques, Y.; Baratin, M.; Tomasello, E.; Vivier, E. Natural killer cell trafficking *in vivo* requires a dedicated sphingosine 1-phosphate receptor. *Nat. Immunol.* **2007**, *8*, 1337–1344.
12. Liu, G.; Yang, K.; Burns, S.; Shrestha, S.; Chi, H. The S1P1–mTOR axis directs the reciprocal differentiation of Th1 and Treg cells. *Nat. Immunol.* **2010**, *11*, 1047–1056.
13. Wang, W.; Huang, M.C.; Goetzl, E.J. Type 1 sphingosine 1-phosphate G protein-coupled receptor (S1P1) mediation of enhanced IL-4 generation by CD4 T cells from S1P1 transgenic mice. *J. Immunol.* **2007**, *178*, 4885–4890.
14. Huang, M.C.; Watson, S.R.; Liao, J.J.; Goetzl, E.J. Th17 augmentation in OTII TCR plus T cell-selective type 1 sphingosine 1-phosphate receptor double transgenic mice. *J. Immunol.* **2007**, *178*, 6806–6813.
15. Oskeritzian, C.A.; Price, M.M.; Hait, N.C.; Kapitonov, D.; Falanga, Y.T.; Morales, J.K.; Ryan, J.J.; Milstien, S.; Spiegel, S. Essential roles of sphingosine-1-phosphate receptor 2 in human mast cell activation, anaphylaxis, and pulmonary edema. *J. Exp. Med.* **2010**, *207*, 465–474.
16. Hughes, J.E.; Srinivasan, S.; Lynch, K.R.; Proia, R.L.; Ferdek, P.; Hedrick, C.C. Sphingosine-1-phosphate induces an antiinflammatory phenotype in macrophages. *Circ. Res.* **2008**, *102*, 950–958.
17. Bouhours, D.; Bouhours, J.F. Developmental changes of monohexosylceramide and free ceramide in the large intestine of the rat. *J. Biochem.* **1985**, *98*, 1359–1366.

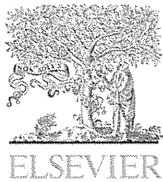
18. Gustafsson, B.E.; Karlsson, K.A.; Larson, G.; Midtvedt, T.; Stromberg, N.; Teneberg, S.; Thurin, J. Glycosphingolipid patterns of the gastrointestinal tract and feces of germ-free and conventional rats. *J. Biol. Chem.* **1986**, *261*, 15294–15300.
19. Vesper, H.; Schmelz, E.M.; Nikolova-Karakashian, M.N.; Dillehay, D.L.; Lynch, D.V.; Merrill, A.H., Jr. Sphingolipids in food and the emerging importance of sphingolipids to nutrition. *J. Nutr.* **1999**, *129*, 1239–1250.
20. Nilsson, A. Metabolism of sphingomyelin in the intestinal tract of the rat. *Biochim. Biophys. Acta* **1968**, *164*, 575–584.
21. Schmelz, E.M.; Crall, K.J.; Larocque, R.; Dillehay, D.L.; Merrill, A.H., Jr. Uptake and metabolism of sphingolipids in isolated intestinal loops of mice. *J. Nutr.* **1994**, *124*, 702–712.
22. Nilsson, A.; Duan, R.D. Alkaline sphingomyelinases and ceramidases of the gastrointestinal tract. *Chem. Phys. Lipids* **1999**, *102*, 97–105.
23. Tani, M.; Ito, M.; Igarashi, Y. Ceramide/sphingosine/sphingosine 1-phosphate metabolism on the cell surface and in the extracellular space. *Cell. Signal.* **2007**, *19*, 229–237.
24. Duan, R.D.; Nilsson, A. Metabolism of sphingolipids in the gut and its relation to inflammation and cancer development. *Prog. Lipid Res.* **2009**, *48*, 62–72.
25. Mazzei, J.C.; Zhou, H.; Brayfield, B.P.; Hontecillas, R.; Bassaganya-Riera, J.; Schmelz, E.M. Suppression of intestinal inflammation and inflammation-driven colon cancer in mice by dietary sphingomyelin: Importance of peroxisome proliferator-activated receptor gamma expression. *J. Nutr. Biochem.* **2011**, *22*, 1160–1171.
26. Fischbeck, A.; Leucht, K.; Frey-Wagner, I.; Bentz, S.; Pesch, T.; Kellermeier, S.; Krebs, M.; Fried, M.; Rogler, G.; Hausmann, M.; *et al.* Sphingomyelin induces cathepsin D-mediated apoptosis in intestinal epithelial cells and increases inflammation in DSS colitis. *Gut* **2011**, *60*, 55–65.
27. Sjoqvist, U.; Hertervig, E.; Nilsson, A.; Duan, R.D.; Ost, A.; Tribukait, B.; Lofberg, R. Chronic colitis is associated with a reduction of mucosal alkaline sphingomyelinase activity. *Inflamm. Bowel Dis.* **2002**, *8*, 258–263.
28. Maines, L.W.; Fitzpatrick, L.R.; French, K.J.; Zhuang, Y.; Xia, Z.; Keller, S.N.; Upson, J.J.; Smith, C.D. Suppression of ulcerative colitis in mice by orally available inhibitors of sphingosine kinase. *Dig. Dis. Sci.* **2008**, *53*, 997–1012.
29. Nyberg, L.; Duan, R.D.; Nilsson, A. A mutual inhibitory effect on absorption of sphingomyelin and cholesterol. *J. Nutr. Biochem.* **2000**, *11*, 244–249.
30. Allende, M.L.; Bektas, M.; Lee, B.G.; Bonifacino, E.; Kang, J.; Tuymetova, G.; Chen, W.; Saba, J.D.; Proia, R.L. Sphingosine-1-phosphate lyase deficiency produces a pro-inflammatory response while impairing neutrophil trafficking. *J. Biol. Chem.* **2011**, *286*, 7348–7358.
31. Bagdanoff, J.T.; Donoviel, M.S.; Nouraldeen, A.; Tarver, J.; Fu, Q.; Carlsen, M.; Jessop, T.C.; Zhang, H.; Hazelwood, J.; Nguyen, H.; *et al.* Inhibition of sphingosine-1-phosphate lyase for the treatment of autoimmune disorders. *J. Med. Chem.* **2009**, *52*, 3941–3953.
32. Bandhuvula, P.; Honbo, N.; Wang, G.Y.; Jin, Z.Q.; Fyrst, H.; Zhang, M.; Borowsky, A.D.; Dillard, L.; Karliner, J.S.; Saba, J.D. S1p lyase: A novel therapeutic target for ischemia-reperfusion injury of the heart. *Am. J. Physiol. Heart Circ. Physiol.* **2011**, *300*, H1753–H1761.

33. Kunisawa, J.; McGhee, J.; Kiyono, H. Mucosal S-IgA Enhancement: Development of Safe and Effective Mucosal Adjuvants and Mucosal Antigen Delivery Vehicles. In *Mucosal Immune Defense: Immunoglobulin A*; Kaetzel, C., Ed.; Kluwer Academic/Plenum Publishers: New York, NY, USA, 2007; pp. 346–389.
34. Fagarasan, S.; Kawamoto, S.; Kanagawa, O.; Suzuki, K. Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis. *Annu. Rev. Immunol.* **2010**, *28*, 243–273.
35. Kunisawa, J.; Nochi, T.; Kiyono, H. Immunological commonalities and distinctions between airway and digestive immunity. *Trends Immunol.* **2008**, *29*, 505–513.
36. Neutra, M.R.; Mantis, N.J.; Kraehenbuhl, J.P. Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nat. Immunol.* **2001**, *2*, 1004–1009.
37. Gohda, M.; Kunisawa, J.; Miura, F.; Kagiyama, Y.; Kurashima, Y.; Higuchi, M.; Ishikawa, I.; Ogahara, I.; Kiyono, H. Sphingosine 1-phosphate regulates the egress of IgA plasmablasts from Peyer's patches for intestinal IgA responses. *J. Immunol.* **2008**, *180*, 5335–5343.
38. Graler, M.H.; Goetzl, E.J. The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB J.* **2004**, *18*, 551–553.
39. Kunisawa, J.; Kiyono, H. A marvel of mucosal T cells and secretory antibodies for the creation of first lines of defense. *Cell Mol. Life Sci.* **2005**, *62*, 1308–1321.
40. Kunisawa, J.; Kurashima, Y.; Gohda, M.; Higuchi, M.; Ishikawa, I.; Miura, F.; Ogahara, I.; Kiyono, H. Sphingosine 1-phosphate regulates peritoneal B-cell trafficking for subsequent intestinal IgA production. *Blood* **2007**, *109*, 3749–3756.
41. Kunisawa, J.; Gohda, M.; Kurashima, Y.; Ishikawa, I.; Higuchi, M.; Kiyono, H. Sphingosine 1-phosphate-dependent trafficking of peritoneal B cells requires functional NFκB-inducing kinase in stromal cells. *Blood* **2008**, *111*, 4646–4652.
42. Kunisawa, J.; Takahashi, I.; Kiyono, H. Intraepithelial lymphocytes: Their shared and divergent immunological behaviors in the small and large intestine. *Immunol. Rev.* **2007**, *215*, 136–153.
43. Vivier, E.; Tomasello, E.; Paul, P. Lymphocyte activation via NKG2D: Towards a new paradigm in immune recognition? *Curr. Opin. Immunol.* **2002**, *14*, 306–311.
44. Natarajan, K.; Dimasi, N.; Wang, J.; Mariuzza, R.A.; Margulies, D.H. Structure and function of natural killer cell receptors: Multiple molecular solutions to self, nonself discrimination. *Annu. Rev. Immunol.* **2002**, *20*, 853–885.
45. Gangadharan, D.; Lambolez, F.; Attinger, A.; Wang-Zhu, Y.; Sullivan, B.A.; Cheroutre, H. Identification of pre- and postselection TCR$\alpha\beta^+$ intraepithelial lymphocyte precursors in the thymus. *Immunity* **2006**, *25*, 631–641.
46. Kunisawa, J.; Kurashima, Y.; Higuchi, M.; Gohda, M.; Ishikawa, I.; Ogahara, I.; Kim, N.; Shimizu, M.; Kiyono, H. Sphingosine 1-phosphate dependence in the regulation of lymphocyte trafficking to the gut epithelium. *J. Exp. Med.* **2007**, *204*, 2335–2348.
47. Sharma, S.; Mathur, A.G.; Pradhan, S.; Singh, D.B.; Gupta, S. Fingolimod (FTY720): First approved oral therapy for multiple sclerosis. *J. Pharmacol. Pharmacother.* **2011**, *2*, 49–51.
48. Kweon, M.N.; Yamamoto, M.; Kajiki, M.; Takahashi, I.; Kiyono, H. Systemically derived large intestinal CD4$^+$ Th2 cells play a central role in STAT6-mediated allergic diarrhea. *J. Clin. Invest.* **2000**, *106*, 199–206.

49. Kurashima, Y.; Kunisawa, J.; Higuchi, M.; Gohda, M.; Ishikawa, I.; Takayama, N.; Shimizu, M.; Kiyono, H. Sphingosine 1-phosphate-mediated trafficking of pathogenic Th2 and mast cells for the control of food allergy. *J. Immunol.* **2007**, *179*, 1577–1585.
50. Gurish, M.F.; Boyce, J.A. Mast cell growth, differentiation, and death. *Clin. Rev. Allergy Immunol.* **2002**, *22*, 107–118.
51. Mizushima, T.; Ito, T.; Kishi, D.; Kai, Y.; Tamagawa, H.; Nezu, R.; Kiyono, H.; Matsuda, H. Therapeutic effects of a new lymphocyte homing reagent FTY720 in interleukin-10 gene-deficient mice with colitis. *Inflamm. Bowel Dis.* **2004**, *10*, 182–192.
52. Fujii, R.; Kanai, T.; Nemoto, Y.; Makita, S.; Oshima, S.; Okamoto, R.; Tsuchiya, K.; Totsuka, T.; Watanabe, M. FTY720 suppresses $CD4^+$ $CD44^{high}$ $CD62L^-$ effector memory T cell-mediated colitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2006**, *291*, G267–G274.
53. Deguchi, Y.; Andoh, A.; Yagi, Y.; Bamba, S.; Inatomi, O.; Tsujikawa, T.; Fujiyama, Y. The S1P receptor modulator FTY720 prevents the development of experimental colitis in mice. *Oncol. Rep.* **2006**, *16*, 699–703.

© 2012 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/).

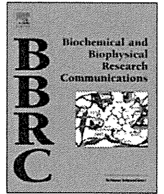
Biochemical and Biophysical Research Communications 419 (2012) 477–481



Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc



Embryonic stem cell differentiation system for evaluating gene functions involved in physiological megakaryocytic differentiation

Yoshiaki Okada [a,*,1], Masaaki Yonekura [a,1], Miho Watanabe [b], Tomoko Nakai [a], Atsuko Wakimura [a], Mikiko Shimizu [a], Yuko Kamikawa [a], Mie Kitayama [a], Kenji Kitajima [c], William C. Aird [d], Takefumi Doi [a]

[a] Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[b] Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan
[c] Stem Cell Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan
[d] Center for Vascular Biology Research and Division of Molecular and Vascular Medicine, Beth Israel Deaconess Medical Center, Boston, MA, USA

ARTICLE INFO

Article history:
Received 16 January 2012
Available online 10 February 2012

Keywords:
Megakaryocyte
Platelet
Embryonic stem cells
Differentiation
Platelet factor 4
Glycoprotein IIb

ABSTRACT

Megakaryocytic differentiation is accompanied by marked morphological changes induced by endomitosis and proplatelet formation. Molecular mechanisms underlying this unique cell differentiation process have been investigated by gain/loss-of-function studies using leukemic cell lines. However, these cell lines cannot completely mimic physiological megakaryocytic differentiation, including the morphological changes, and sometimes lead to contradictory results between cell lines. The goal of this study was to establish a novel cell differentiation system that completely mimics physiological megakaryocytic differentiation for analyzing gene function. To that end, we used homologous recombination to prepare an embryonic stem (ES) cell line containing a GFP-transgene driven by the PF4 promoter at the Hprt locus. Differentiation of these cells resulted in megakaryocytes and proplatelets, suggesting physiological megakaryocytic differentiation. However, the number of GFP-expressing cells was low (1.7% GFP^+ cells among $CD41^+$ cells). Insertion of full-length or small core β-globin insulators on either side of the transgene significantly increased the number of GFP-expressing cells (~60% GFP^+ cells among $CD41^+$ cells), and GFP-expression was specifically observed in megakaryocytic cells. Similar results were obtained with other ES cells containing a GPIIb-GFP transgene. Altogether, we have succeeded in efficiently expressing exogenous genes specifically in differentiating megakaryocytes and in establishing a novel ES cell differentiation system for analyzing gene function involved in physiological megakaryocytic differentiation.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Megakaryocytic differentiation is accompanied by marked morphological changes, induced by endomitosis and proplatelet formation. To understand the molecular mechanisms underlying megakaryocytic differentiation, gene functions have been investigated by gain/loss-of-function studies, both in vivo and in vitro. In vivo studies using transgenic and knockout mice are a powerful tool for investigating gene functions involved in physiological megakaryocytic differentiation. However, due to the low number of megakaryocytes in vivo (0.4% of the total nucleated cells within bone marrow [1]) and the non-synchronized differentiation of these cells, it is technically difficult to investigate gene functions at any given differentiation stage. In addition, the generation of mutant mice is a costly and time-consuming process.

On the other hand, cell lines with megakaryocytic features, such as HEL [2,3], UT-7/GM [4], K562 [5], and Meg01 [6,7] cells, have been widely used in in vitro studies. Because these cell lines are synchronically differentiated into a megakaryocytic lineage by treatment with differentiation stimulators, such as 12-O-tetradecanoylphorbol-13-acetate (TPA) and thrombopoietin (TPO), these cells can be used for gain/loss-of-function studies by overexpression of a gene of interest, or by reducing its expression by RNA interference. However, because these leukemic cells do not express all physiological megakaryocytic markers and cannot completely recapitulate morphological changes during megakaryocytic differentiation [8,9], studies using these cells sometimes lead to controversial results. For example, an investigation of TPO/Mpl signaling revealed cell line-specific responses that differ from those observed in primary cells [10].

An in vitro embryonic stem (ES) cell differentiation system completely mimics megakaryocytic differentiation, including polyploidization, proplatelet formation, and functional platelet

* Corresponding author. Address: Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 8164.
E-mail address: okadabos@phs.osaka-u.ac.jp (Y. Okada).
[1] These authors equally contributed to this work.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2012.02.021

production [11,12]. However, this system has seldom been used for the study of gene function in megakaryocytic differentiation, due to the technical difficulties involved in expressing exogenous genes specifically in megakaryocytic cells. Although a previous report succeeded in gain-of-function studies using a retrovirus [12], this method has a potential drawback in that insertion of the transgene into the ES cell genome may disrupt endogenous genes.

In this study, we generated a mouse ES cell line containing a transgene with insulators in the Hprt locus and succeeded in expressing the exogenous gene specifically in differentiating megakaryocytes. This system promises to be a powerful and convenient tool for investigating the gene functions underlying physiological megakaryocytic differentiation.

2. Materials and methods

2.1. Preparation of targeting vectors

Preparation of the plasmid PF4-GFP, which encodes the PF4 promoter and the AcGFP gene, was described previously [13]. To generate the targeting vectors, including β-globin insulators (pMP8II-Ins and pMP8II-Core), full-length and core insulator fragments were purified from pJC13-1 and pNI-CD [14,15], respectively, and cloned into the Hprt-targeting vector pMP8II [16]. To generate pMP8II-Ins-PF4-GFP and pMP8II-Core-PF4-GFP, PF4-AcGFP was digested with *Afl*II and blunt-ended with T4 DNA polymerase. The resulting DNA was digested with *Mlu*I. A fragment encoding the PF4 promoter coupled to GFP was purified and cloned between the *Mlu*I and *Pme*I sites of pMP8II-Ins and pMP8II-Core.

To generate the plasmid GPIIb-AcGFP, a 0.8-kb region (−813 to +32) of the human glycoprotein IIb (GPIIb) gene promoter was amplified by PCR. The resulting promoter fragment was digested with *Xho*I and *Sac*II, and cloned between the *Xho*I and *Sac*II sites of pAcGFP1-1 (Takara, Siga, Japan) to generate GPIIb-GFP. The promoter sequence was verified by DNA sequencing.

To generate pMP8II-GPIIb-GFP, pMP8II-Ins-GPIIb-GFP, and pMP8II-Core-GPIIb-GFP, GPIIb-AcGFP was digested with *Afl*II and blunt-ended with T4 DNA polymerase. The resulting DNA was digested with *Mlu*I. A fragment containing the GPIIb promoter coupled to GFP was purified and cloned between the *Mlu*I and *Pme*I sites of pMP8II, pMP8II-Ins, and pMP8II-Core.

2.2. Generation and differentiation of Hprt-targeted ES cells

Generation and differentiation of Hprt-targeted ES cells were described previously [13]. In brief, undifferentiated Hprt-deficient ES cells (BK4 cells) were electroporated with 25 μg of *Sal*I-linearized targeting vectors. Homologous recombinants were selected in a medium containing HAT (SIGMA, St. Louis, MO) for approximately 10 days, at which time individual colonies were picked for expansion and verification of the desired recombination. At least two independent ES cell lines were established for each construct. Established ES cell lines were differentiated into megakaryocytes on OP9 stromal cells by culturing for 9–10 days in a medium containing 10 ng/mL TPO.

2.3. Flow cytometry

ES cell lines containing each transgene, with or without the insulator, and a non-recombined control ES cell line were differentiated into megakaryocytic cells for 10 days. The resulting cells were harvested by treating with 0.25% trypsin and then incubated on the plate for 30 min to remove the OP9 cells. The cells (1×10^6) floating in the supernatant were collected and incubated with phycoerythrin-conjugated rat anti-mouse CD41 antibody (BD Pharmingen, Franklin Lakes, NJ) or phycoerythrin-conjugated control rat IgG (BD Pharmingen) in phosphate-buffered saline containing 0.5% BSA, at 4 °C for 30 min. The cells were washed 3 times and analyzed by a flow cytometer.

3. Results

3.1. Establishment of an ES cell line that efficiently expresses the transgene during megakaryocytic differentiation

The goal of this study was to establish an ES cell line that efficiently expresses the transgene during the process of differentiation into a megakaryocytic lineage. We have previously reported the generation of a mouse ES cell line (PF4-GFP), which includes a single copy of a transgene encoding the rat PF4 promoter linked to the GFP gene, in the Hprt locus [13] (Fig. 1A). To investigate whether this cell line efficiently expresses GFP during its differentiation into the megakaryocytic lineage, PF4-GFP cells were differentiated. Culture of the cells along with OP9 cells and TPO resulted in cell differentiation into megakaryocytes and proplatelets, via hematopoietic progenitor cells, suggesting physiological megakaryocytic differentiation (Fig. 1B). Analysis of the differentiated cells by fluorescence microscopy, however, revealed only a small number of GFP-expressing cells (Fig. 2A).

Consistent with this observation, FACS analysis showed only 1.7% GFP^+ cells among the $CD41^+$ megakaryocytic cells, although GFP was specifically expressed in the megakaryocytic cells (Fig. 2B). This result indicated that the Hprt locus-targeted ES cells can mimic physiological megakaryocytic differentiation and that the transgene, driven by the PF4 promoter, can express an exogenous gene specifically in megakaryocytic cells, even though gene

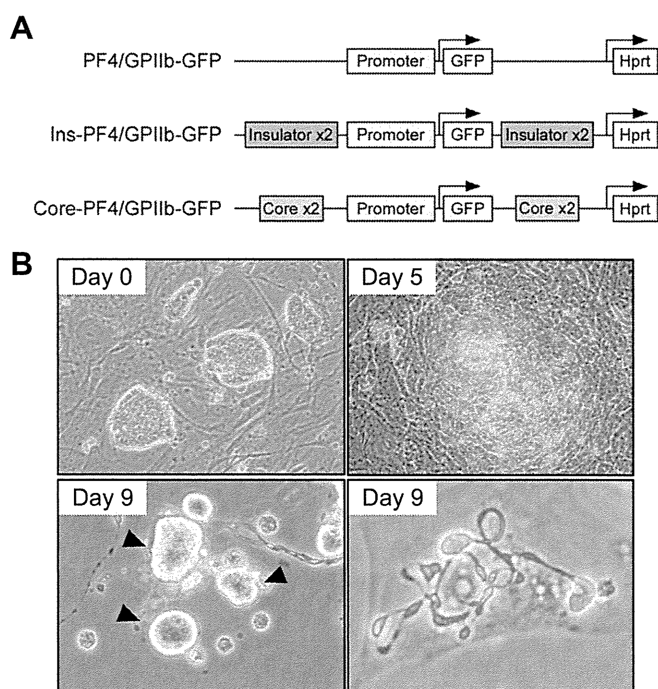


Fig. 1. Differentiation of targeted ES cells into a megakaryocytic lineage. (A) Schematic representation of the Hprt-targeted alleles. A single-copy transgene encoding the PF4 or GPIIb promoter coupled with the GFP gene was inserted into the Hprt locus of mouse ES cells by homologous recombination. "Insulator" and "Core" indicate the full length (1.2 kb) or core (0.25 kb) chicken β-globin insulator fragments, respectively. (B) Undifferentiated ES cells (day 0), differentiated mesodermal cell colonies (day 5), and megakaryocytes and proplatelets (day 9). Arrow heads indicate the large megakaryocytes.

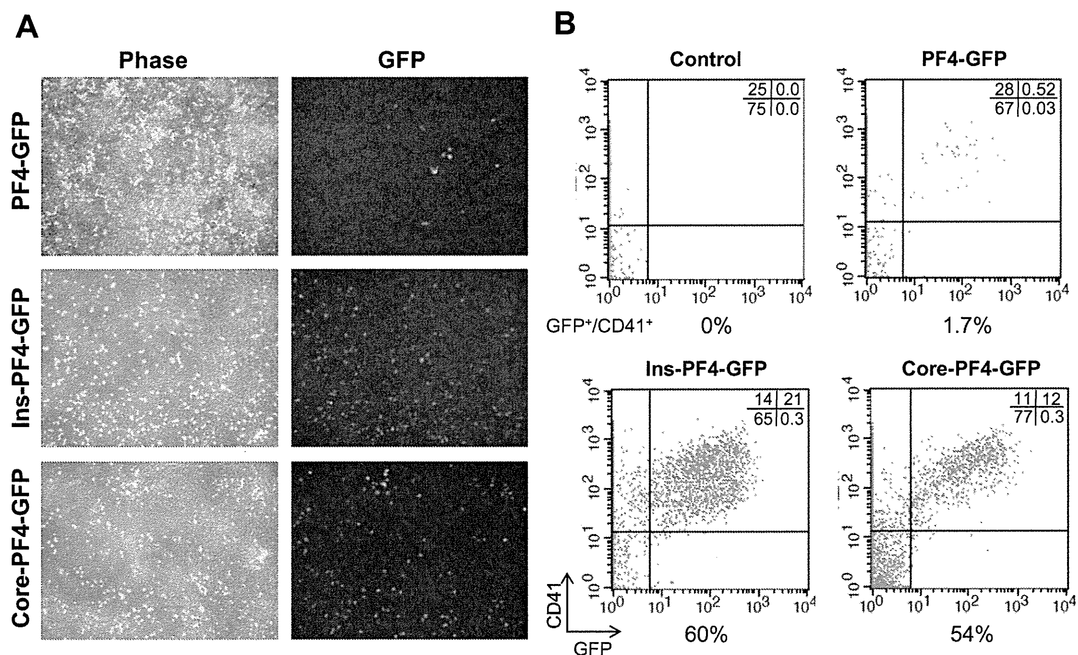


Fig. 2. Efficiency of GFP expression in megakaryocytic cells derived from PF4-GFP targeted cells. (A) GFP-expressing cells differentiated from PF4-GFP, Ins-PF4-GFP, and Core-PF4-GFP cells at day 9. (B) Flow cytometric analyses at day 10 of GFP- and CD41-expressing cells differentiated from non-targeted ES cells (Control), PF4-GFP, Ins-PF4-GFP, and Core-PF4-GFP cells.

expression from the PF4-GFP transgene was not detected in most of these differentiated ES cells.

3.2. Improvement of the GFP-expression efficiency by using β-globin insulators

In addition to GFP being expressed in a small number of differentiated cells, GFP-expressing cells had a tendency to gather in one place on the culture plate. From these observations, we hypothesized that transgenes in most cells are suppressed by epigenetic control, although a small number of ES cells seemed to be able to escape from this suppression and form colonies consisting of GFP-expressing megakaryocytic cells. To investigate this hypothesis, we prepared another transgene that included 2 duplicated 1.2-kb β-globin insulators flanking the PF4-GFP construct (Fig. 1A). This transgene was inserted into the mouse ES cells by homologous recombination, and it resulted in the generation of Ins-PF4-GFP cells. These cells were differentiated into a megakaryocytic lineage, and GFP-expression in the differentiated cells was analyzed.

Analysis by fluorescence microscopy showed a significant increase in GFP-expressing cells (Fig. 2A). Consistent with this, FACS analysis also demonstrated a significant increase in GFP-expressing cells (60% GFP^+ cells among $CD41^+$ cells), as compared to the PF4-GFP cells (Fig. 2B). In addition, GFP was exclusively expressed in the $CD41^+$ cells. These results demonstrated that β-globin insulators removed epigenetic suppression and strongly enhanced transgene expression.

3.3. β-globin core insulators are functionally comparable to full-length insulators

The core insulator (0.25 kb) is reported to be a functional sequence within the β-globin insulator [15]. Usage of the core insulator instead of the full-length insulator was thought to facilitate preparation of the targeting vector. To investigate whether a core insulator also improved transgene expression, we prepared another transgene, using 2 duplicated core insulators on either side of the PF4-GFP (Fig. 1A). This transgene was inserted into mouse ES cells by homologous recombination, and it resulted in the generation of Core-PF4-GFP cells. These cells were differentiated into a megakaryocytic lineage and GFP expression was again analyzed.

Fluorescence microscopy and FACS analysis showed a significant increase in GFP-expressing cells (54% GFP^+ cells among $CD41^+$ cells) (Fig. 2A) and platelet lineage-specific GFP-expression (Fig. 3B). The ratio of the GFP-expressing cells among the $CD41^+$ cells was comparable to that observed with Ins-PF4GFP cells. These results demonstrated that β-globin core insulators removed epigenetic suppression and promoted transgene expression as well as full-length insulators.

3.4. Insulators also improve GFP-expression from the GPIIb promoter-GFP transgene

To investigate the other megakaryocytic promoter, we generated three more ES cell lines, GPIIb-GFP, Ins-GPIIb-GFP, and Core-GPIIb-GFP, that contained a GPIIb-GFP transgene with or without insulators (Fig. 1A). All these ES cells were differentiated into a platelet lineage, and GFP expression was analyzed by fluorescent microscopy and FACS.

Consistent with the findings from the PF4-GFP cells, GFP expression from the GPIIb-GFP transgene without insulators was very low (4.6% GFP^+ cells among $CD41^+$ cells), although GFP-expression was observed predominantly in megakaryocytic cells (Fig. 3). On the other hand, insertion of full-length or core insulators on either side of the GPIIb-GFP transgene significantly increased the number of GFP-expressing cells (43% or 42% GFP^+ cells among $CD41^+$ cells, respectively). The full-length and core insulators improved the efficiency of GFP-expression in megakaryocytic cells equally (Fig. 3).

Comparison of GFP expression between Core-PF4-GFP and Core-GPIIb-GFP cells demonstrated a similar time-dependent increase in GFP-expressing cells and GFP expression levels, although the PF4 promoter tended to be activated at a later stage than the GPIIb promoter (Fig. 4). Together, these results indicate that suppression of

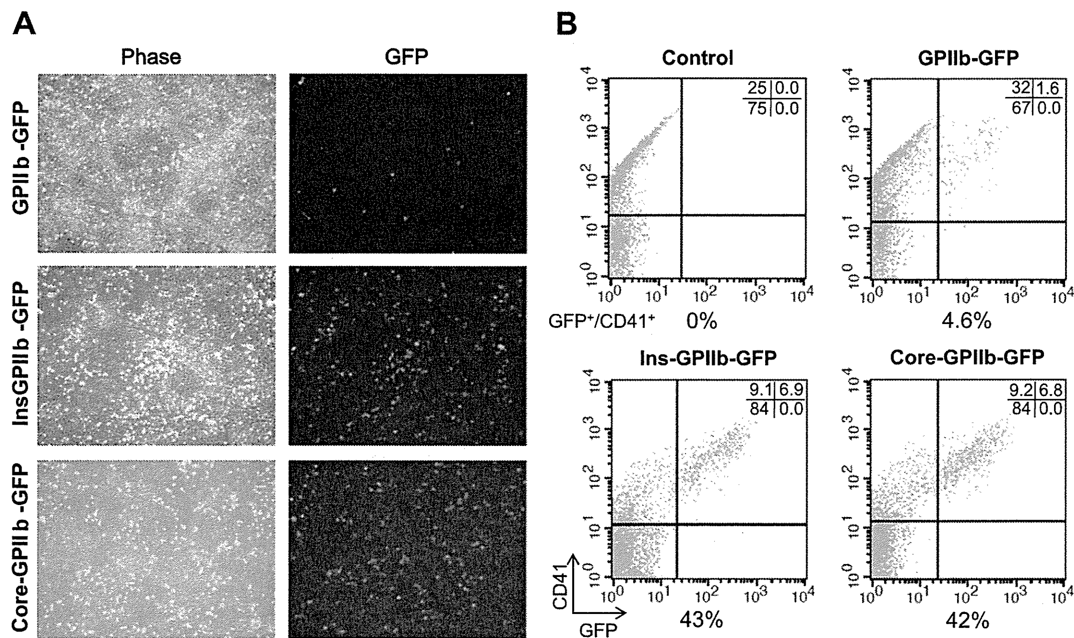


Fig. 3. Efficiency of GFP expression in megakaryocytic cells derived from GPIIb-GFP targeted cells. (A) GFP-expressing cells differentiated from GPIIb-GFP, Ins- GPIIb-GFP, and Core-GPIIb-GFP cells at day 9. (B) Flow cytometric analyses of GFP- and CD41-expressing cells differentiated from non-targeted ES cells (Control), GPIIb-GFP, Ins- GPIIb-GFP, and Core- GPIIb-GFP cells at day 10.

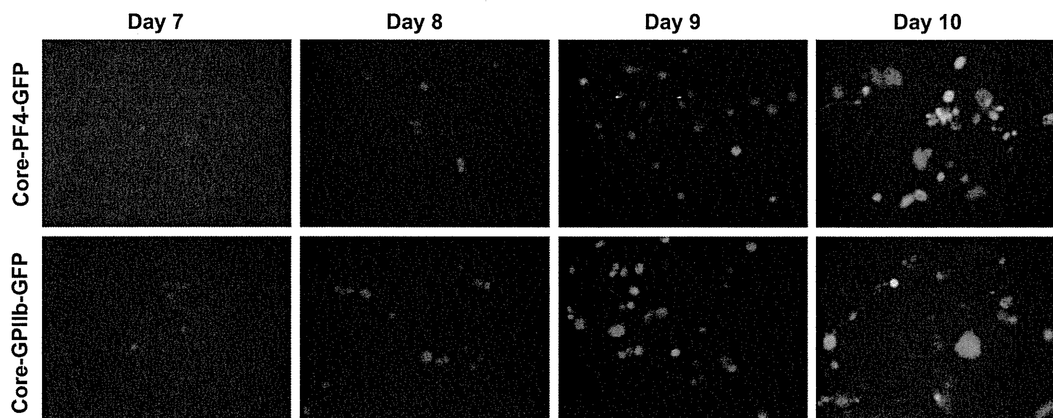


Fig. 4. Comparison of GFP-expression patterns between Core-PF4-GFP and Core-GPIIb-GFP cells. Core-PF4-GFP and Core-GPIIb-GFP cells were differentiated into a megakaryocytic lineage and GFP-expressing cells were compared between day 7 and 10.

GFP expression from the GPIIb-GFP was removed by using the insulators, and suggested the possibility to use the GPIIb promoter for expressing genes in the earlier stage of megakaryocytic differentiation.

4. Discussion

In this study, we have succeeded in efficiently and specifically expressing exogenous genes in differentiating megakaryocytes, and have established a novel ES cell differentiation system for analyzing gene function involved in physiological megakaryocytic differentiation.

Analysis of gene function involved in megakaryocytic differentiation has been technically difficult because of the low number of megakaryocytes in vivo, and the lack of cell lines that fully mimic physiological megakaryocytic differentiation in vitro. Pendaries et al. have suggested that results from in vitro studies using megakaryocytic cell lines are at times contradictory, and that such results should be interpreted with caution [17]. This cell type-specific result could be caused by differences in megakaryocytic features between cell lines. These cell lines are typically established from different leukemic cells that are similar to megakaryocytic cells at specific differentiation stages. These cell lines express different patterns of megakaryocytic markers and show differences in gene expression and morphological changes under treatment with various differentiation stimulators, such as TPA and TPO. Thus, our previous study indicated that RUNX1 suppressed PF4 gene expression in TPO-treated UT-7 cells [18], while another group demonstrated PF4 gene activation by RUNX1 in HEL cells treated with TPA [19].

To overcome this problem, we attempted to establish an ES cell differentiation system with which gene functions can be evaluated during physiological megakaryocytic differentiation by gain/loss-of-function studies. To express the transgene without disrupting expressions of endogenous genes, a single copy of the PF4-GFP or GPIIb-GFP transgenes was inserted into the Hprt locus of mouse

ES cells by homologous recombination. Because the Hprt gene is one of the major housekeeping genes, the Hprt gene has been believed to not be affected by epigenetic suppression induced by chromatin condensation. Unexpectedly, our results indicated that both PF4 and GPIIb promoter activity was suppressed by epigenetic control, which could be undone by insulators. To our knowledge, this is the first study demonstrating transgene suppression at the Hprt locus. However, we have previously reported that endothelial cell-specific gene promoters, such as Robo4 and vWF promoters, are strongly activated without insulators [20–23], suggesting that this suppression at the Hprt locus does not occur with all transgenes. These results suggested that use of promoters with insulator function can avoid epigenetic suppression.

Although insulators efficiently improve transgene expression, it is difficult to prepare a large targeting vector containing full-length insulators. To resolve this problem, we tested shorter core insulators, and demonstrated that the core insulator has functionality that is comparable to that of the full-length insulator for improving expression from the PF4-GFP and GpIIb-GFP transgenes.

Thus, in conclusion, we have succeeded in establishing a novel Hprt-targeted ES cell differentiation system for evaluating gene functions during physiological megakaryocytic differentiation. This system, using a convenient Hprt-targeting vector, may also be a powerful tool for generating genetically modified platelets for therapeutic studies.

Disclosure of conflicts of interest

The authors have no conflict of interest.

Acknowledgments

We thank Dr. Sarah Bronson and Dr. Gary Felsenfeld for gifting BK4 cells and β-globin insulators. This work was supported by JSPS KAKENHI, MEXT KAKENHI, the Takeda Science Foundation, and the Mitsubishi Pharma Research Foundation.

References

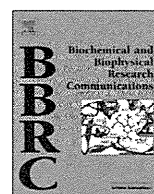
[1] R.F. Levine, Isolation and characterization of normal human megakaryocytes, Br. J. Haematol. 45 (1980) 487–497.
[2] M. Micallef, Y. Matsuo, K. Takayama, T. Ariyasu, T. Otani, J. Minowada, Differential platelet-associated factor gene expression by a panel of human cell lines with megakaryoblastic properties after treatment with phorbol myristate acetate, Hematol. Oncol. 12 (1994) 163–174.
[3] G. Zauli, A. Bassini, L. Catani, D. Gibellini, C. Celeghini, P. Borgatti, E. Caramelli, L. Guidotti, S. Capitani, PMA-induced megakaryocytic differentiation of HEL cells is accompanied by striking modifications of protein kinase C catalytic activity and isoform composition at the nuclear level, Br. J. Haematol. 92 (1996) 530–536.
[4] Y. Miura, N. Komatsu, T. Suda, Growth and differentiation of two human megakaryoblastic cell lines; CMK and UT-7, Prog. Clin. Biol. Res. 356 (1990) 259–270.
[5] J.A. Sutherland, A.R. Turner, P. Mannoni, L.E. McGann, J.M. Turc, Differentiation of K562 leukemia cells along erythroid, macrophage, and megakaryocyte lineages, J. Biol. Response Modif. 5 (1986) 250–262.
[6] S. Goto, M. Kobayashi, T. Murate, T. Hotta, M. Hagiwara, T. Hoshino, H. Hidaka, H. Saito, Thrombin-induced shape change in human megakaryoblastic leukemic cells, MEG-01, is mediated by protein kinase C, Thromb. Res. 68 (1992) 87–95.
[7] M. Ogura, Y. Morishima, R. Ohno, Y. Kato, N. Hirabayashi, H. Nagura, H. Saito, Establishment of a novel human megakaryoblastic leukemia cell line, MEG-01, with positive Philadelphia chromosome, Blood 66 (1985) 1384–1392.
[8] S. Baatout, Cell lines with potential for megakaryocytic differentiation: a review, Haematologia (Budap) 27 (1996) 161–183.
[9] H. Saito, Megakaryocytic cell lines, Baillieres Clin. Haematol. 10 (1997) 47–63.
[10] J.G. Drachman, P. Rojnuckarin, K. Kaushansky, Thrombopoietin signal transduction: studies from cell lines and primary cells, Methods 17 (1999) 238–249.
[11] T.T. Fujimoto, S. Kohata, H. Suzuki, H. Miyazaki, K. Fujimura, Production of functional platelets by differentiated embryonic stem (ES) cells in vitro, Blood 102 (2003) 4044–4051.
[12] K. Eto, R. Murphy, S.W. Kerrigan, A. Bertoni, H. Stuhlmann, T. Nakano, A.D. Leavitt, S.J. Shattil, Megakaryocytes derived from embryonic stem cells implicate CalDAG-GEFI in integrin signaling, Proc. Natl. Acad. Sci. USA 99 (2002) 12819–12824.
[13] Y. Okada, H. Nobori, M. Shimizu, M. Watanabe, M. Yonekura, T. Nakai, Y. Kamikawa, A. Wakimura, N. Funahashi, H. Naruse, A. Watanabe, D. Yamasaki, S. Fukada, K. Yasui, K. Matsumoto, T. Sato, K. Kitajima, T. Nakano, W.C. Aird, T. Doi, Multiple ETS family proteins regulate PF4 gene expression by binding to the same ETS binding site, PLoS One 6 (2011) e24837.
[14] J.H. Chung, M. Whiteley, G. Felsenfeld, A 5' element of the chicken beta-globin domain serves as an insulator in human erythroid cells and protects against position effect in Drosophila, Cell 74 (1993) 505–514.
[15] J.H. Chung, A.C. Bell, G. Felsenfeld, Characterization of the chicken beta-globin insulator, Proc. Natl. Acad. Sci. USA 94 (1997) 575–580.
[16] V. Evans, A. Hatzopoulos, W.C. Aird, H.B. Rayburn, R.D. Rosenberg, J.A. Kuivenhoven, Targeting the Hprt locus in mice reveals differential regulation of Tie2 gene expression in the endothelium, Physiol. Genomics 2 (2000) 67–75.
[17] C. Pendaries, S.P. Watson, J.C. Spalton, Methods for genetic modification of megakaryocytes and platelets, Platelets 18 (2007) 393–408.
[18] R. Nagai, E. Matsuura, Y. Hoshika, E. Nakata, H. Nagura, A. Watanabe, N. Komatsu, Y. Okada, T. Doi, RUNX1 suppression induces megakaryocytic differentiation of UT-7/GM cells, Biochem. Biophys. Res. Commun. 345 (2006) 78–84.
[19] K. Aneja, G. Jalagadugula, G. Mao, A. Singh, A.K. Rao, Mechanism of platelet factor 4 (PF4) deficiency with RUNX1 haplodeficiency: RUNX1 is a transcriptional regulator of PF4, J. Thromb. Haemost. 9 (2011) 383–391.
[20] Y. Okada, K. Yano, E. Jin, N. Funahashi, M. Kitayama, T. Doi, K. Spokes, D.L. Beeler, S.C. Shih, H. Okada, T.A. Danilov, E. Maynard, T. Minami, P. Oettgen, W.C. Aird, A three-kilobase fragment of the human Robo4 promoter directs cell type-specific expression in endothelium, Circ. Res. 100 (2007) 1712–1722.
[21] Y. Okada, E. Jin, V. Nikolova-Krstevski, K. Yano, J. Liu, D. Beeler, K. Spokes, M. Kitayama, N. Funahashi, T. Doi, L. Janes, T. Minami, P. Oettgen, W.C. Aird, A GABP-binding element in the Robo4 promoter is necessary for endothelial expression in vivo, Blood 112 (2008) 2336–2339.
[22] J. Liu, Y. Kanki, Y. Okada, E. Jin, K. Yano, S.C. Shih, T. Minami, W.C. Aird, A +220 GATA motif mediates basal but not endotoxin-repressible expression of the von Willebrand factor promoter in Hprt-targeted transgenic mice, J. Thromb. Haemost. 7 (2009) 1384–1392.
[23] J. Liu, L. Yuan, G. Molema, E. Regan, L. Janes, D. Beeler, K.C. Spokes, Y. Okada, T. Minami, P. Oettgen, W.C. Aird, Vascular bed-specific regulation of the von Willebrand factor promoter in the heart and skeletal muscle, Blood 117 (2011) 342–351.

Biochemical and Biophysical Research Communications 431 (2013) 336–341

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc



An induced pluripotent stem cell-mediated and integration-free factor VIII expression system

Yuwna Yakura [a,b,g], Chie Ishihara [b], Hajime Kurosaki [a], Yasuhiro Kazuki [a,c], Norio Komatsu [d], Yoshiaki Okada [e], Takefumi Doi [e], Hiroyuki Takeya [b,f], Mitsuo Oshimura [a,c,h,*]

[a] Department of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Graduate School of Medical Science, Tottori University, Tottori, Japan
[b] Division of Pathological Biochemistry, Department of Life Sciences, Tottori University School of Medicine, Tottori, Japan
[c] Chromosome Engineering Research Center, Tottori University, Tottori, Japan
[d] Department of Hematology, Juntendo University School of Medicine, Japan
[e] Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[f] Department of Applied Life Science, Faculty of Biotechnology and Life Science, Sojo University, Kumamoto, Japan
[g] JSPS Research Fellowships for Young Scientists, Tokyo, Japan
[h] JST, CREST, Tokyo, Japan

ARTICLE INFO

Article history:
Received 10 December 2012
Available online 3 January 2013

Keywords:
Human artificial chromosome
Induced pluripotent stem cells
Sendai virus vector
Integration-free
Hemophilia A
Factor VIII

ABSTRACT

Human artificial chromosome (HAC) has several advantages as a gene therapy vector, including stable episomal maintenance and the ability to carry large gene inserts. Induced pluripotent stem (iPS) cells also have a great potential for gene therapy, which can be generated from an individual's own tissues and contribute to any tissues when reintroduced. A Sendai virus (SeV) vector with reprogramming factors is a powerful tool for generating iPS cells because of the high infection efficiency without the risk of integration into host chromosomes. In this study, we developed an iPS cell-mediated and integration-free coagulation factor VIII (FVIII) expression system using non-integrating SeV- and HAC-vectors. Multiple human FVIII genes, which were under the control of the megakaryocyte-specific platelet factor-4 (PF4) promoter for development of a treatment for hemophilia A, were inserted into a HAC vector (PF4-FVIII-HAC). The PF4-FVIII-HAC was introduced into SeV vector-mediated iPS cells derived from a mouse model of hemophilia A. After *in vitro* differentiation of iPS cells with the PF4-FVIII-HAC into megakaryocytes/platelets, the PF4-FVIII-HAC resulted in expression of FVIII. This study has developed the iPS cell-mediated PF4-driven FVIII expression system using two non-integrating vectors; therefore, this system may be a promising tool for safer gene- and cell-therapy of hemophilia A.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

Hemophilia A is an X chromosome-linked hemorrhagic disorder caused by defects in the coagulation factor VIII (FVIII) gene [1]. Current treatment consists of factor replacement with plasma-derived or recombinant FVIII products. However, these therapies are limited by the risk of infectious disease, the need for frequent injections, and the high cost of treatment [2]. Alternatively, gene therapy is an attractive approach for the treatment of hemophilia A, because a relatively modest increase in FVIII levels results in a sufficient therapeutic effect and it may provide sustained levels of FVIII. Hence, many groups have previously developed various strategies for gene therapy of hemophilia [3–5]. However, the ultimate cure for hemophilia by gene therapy has not been achieved because of several issues with conventional vector systems as follows; (1) the limited packaging capacity of vector particles, (2) the risk of oncogene activation caused by insertional mutagenesis, (3) over-expression or silencing, and (4) immune responses to the viral capsid [6].

Human artificial chromosome (HAC) vectors show considerable promise for gene therapy applications because they are stably maintained independent of host chromosomes as a mini-chromosome, thus diminishing or eliminating the risk of insertional mutagenesis [7]. In addition, HACs have the capacity to deliver an extremely large genomic region, such as 5 Mb [8], and allow physiological regulation of the introduced gene in a manner similar to that of the native chromosome [9–11]. Therefore, the use of HACs as a vector for gene therapy can solve the problems of conventional vectors, and are expected to be used for future gene- and cell-therapy.

Induced pluripotent stem (iPS) cells also have a great potential for gene therapy, which can be generated from an individual's own

* Corresponding author. Address: Department of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Graduate School of Medical Science, 86 Nishi-cho, Yonago, Tottori 683-8503, Japan. Fax: +81 859 38 6210.
E-mail address: oshimura@grape.med.tottori-u.ac.jp (M. Oshimura).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.12.096

tissues and contribute to the specialized function of any tissue when reintroduced. However, a problem with iPS cell induction is integration of transgenes into host chromosomes, which includes the risk of oncogene activation [12]. Sendai virus (SeV) exists as a form of negative-sense single-stranded RNA in the cytoplasm of infected cells. Therefore, a SeV vector with reprogramming factors has been a solution for induction of integration-free iPS cells [13].

In the present study, we used the megakaryocyte-specific platelet factor-4 (PF4) promoter [14], and established a HAC vector containing multiple FVIII expression cassettes under the control of the PF4 promoter. We transferred the HAC vector into hemophilia A model mouse iPS (FVIII KO-iPS) cells induced by the SeV vector with reprogramming factors, and examined whether the HAC vector is able to induce FVIII expression in megakaryocytes. Although we must perform *in vivo* experiments to demonstrate the safety and efficiency of this strategy, the present findings suggest that this approach may be a promising strategy for safe gene- and cell-therapy of hemophilia A.

2. Materials and methods

2.1. Vector construction

We previously developed a P1 bacteriophage artificial chromosome (pPAC)-FVIII vector with FVIII under the control of the cytomegalovirus immediate early enhancer-chicken β-actin hybrid (CAG) promoter [15]. In this study, the CAG promoter was excised by *XhoI* and replaced by the human megakaryocyte-specific PF4 promoter [14]. Multiple tandem copies of the FVIII expression cassette were constructed using compatible restriction sites. The FVIII expression cassette was excised by *AscI* and *AvrII*, and cloned into the pPAC-PF4-FVIII vector digested by *AscI* and *NheI*. Using this strategy, we obtained pPAC-PF4-FVIII with two and four copies of the FVIII expression cassette.

2.2. Cell culture

Hypoxanthine phosphoribosyl transferase (HPRT)-deficient Chinese hamster ovary (CHO) cells (JCR B0218) containing the HAC vector were cultured at 37 °C with 5% CO_2 in Ham's F-12 nutrient mixture (Invitrogen) plus 10% fetal bovine serum (FBS) with 8 µg/ml blasticidin S (BS; Funakoshi). Mouse iPS cells (see Section 2.8) were maintained on mitomycin C (Sigma)-treated mouse embryonic fibroblasts (MEFs) at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (Wako) containing 20% FBS, 1 mM sodium pyruvate (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), and 1,000 U/ml leukemia inhibitory factor (Funakoshi).

2.3. Transient transfection

Human megakaryoblastic leukemia cell line UT-7/GM cells [16] were maintained in Iscove's modified Dulbecco's medium (Invitrogen) containing 10% FBS and 1 ng/ml granulocyte–macrophage colony-stimulating factor (GM-CSF; Sigma). Cells were washed twice in PBS and resuspended in K-PBS buffer (31 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, and 10 mM $MgCl_2$) to a final concentration of 2×10^6 cells per 100 µl. These cells were mixed with each pPAC-PF4-FVIII vector, exposed to an exponential discharge of 150 V from a 25 µF capacitor using a Gene Pulser apparatus (Bio-Rad), and then transferred into culture medium. For induction of megakaryocytes/platelets differentiation, UT-7/GM cells were cultured for 72 h with 10 ng/ml thrombopoietin (TPO; a gift from Kirin Brewery) in place of GM-CSF.

2.4. Construction of PF4-FVIII-HAC

Modified pPAC-PF4-FVIII and Cre-recombinase expression vectors (pBS185; Invitrogen) were co-transfected into CHO cells containing the 21HAC2 vector [8] using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h of culture in basic growth medium, cells were cultured in medium containing hypoxanthine–aminopterin–thymidine (HAT; Sigma). After 12 days of selection, HAT-resistant colonies were picked up and expanded for genomic PCR and fluorescence *in situ* hybridization (FISH) analyses.

2.5. Microcell-mediated chromosome transfer (MMCT)

MMCT was performed as described previously [17]. CHO cells containing PF4-FVIII-HAC were used as donor microcell hybrids. Briefly, mouse iPS cells were fused with microcells prepared from donor hybrid CHO cells, and then selected with BS (10 µg/ml). The transferred PF4-FVIII-HAC in each cell line was characterized by genomic PCR and FISH analyses.

2.6. Genomic PCR analyses

Genomic DNA was extracted from host cells containing PF4-FVIII-HAC using a Gentra Puregene Cell Kit (Qiagen), and PCR was performed using the following primers: FVIII-1 (sense, 5′-ggatcaccttttcaacatcg-3′; and antisense, 5′-tcttgaactgagggacactg-3′), FVIII-2 (sense, 5′-atacaacgctttctccccaa-3′; and antisense, 5′-gttcagtgtgttagtgtggc-3′), PF4 (sense, 5′-catatagttgtcagggaaggg-3′; and antisense, 5′-ggctgtttcttcattgtttcc-3′), and HPRT (sense, 5′-tggaggccataaacaagaagac-3′; and antisense, 5′-ccccttgacccagaaattcca-3′).

2.7. FISH analyses

FISH analyses were performed using either fixed metaphase or interphase spreads of each cell hybrid using digoxigenin-labeled (Roche) human Cot-1 DNA (Invitrogen) and biotin-labeled pPAC-PF4-FVIII DNA probes as described previously [17]. Chromosomal DNA was counterstained with DAPI (Sigma). Images were captured using an NIS-Elements system (Nikon) and Axio Imager-Z2 (Carl Zeiss).

2.8. Induction of iPS cells

Induction of iPS cells from hemophilia A model mouse embryonic fibroblasts (FVIII KO-MEFs; Jackson Laboratory, strain name: 129S4-F8tm1Kaz/J, stock number: 004424) was performed using a SeV vector system (Dnavec) as described previously [13]. Briefly, four SeV vectors containing Oct3/4, Klf4, c-MYC and Sox2 were used to infect FVIII KO-MEFs. At 6 days after infection, FVIII KO-MEFs were re-plated at 5×10^4 cells per 10-cm dish on MEF feeder cells. The next day, the medium was replaced with mouse iPS cell medium. At 24 days after transfection, mouse iPS-like colonies were selected and transferred onto feeder cells in 6-well plates.

2.9. Immunofluorescence staining

Immunofluorescence staining was performed using a primary anti-SeV polyclonal antibody (Medical and Biological laboratories) after fixation with 4% paraformaldehyde in PBS. A secondary anti-rabbit IgG antibody conjugated with Alexa Fluor 594 (Molecular Probes) was used, followed by analysis with a fluorescence microscope (ECLIPSE Ti-U, Nikon).

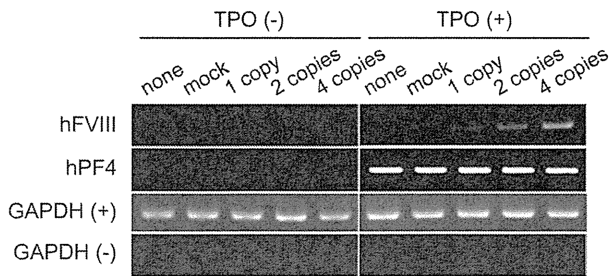


Fig. 1. UT-7/GM cells were transiently transfected with each of the pPAC-PF4-FVIII vectors (one, two and four copies) and cultured for 72 h with or without 10 ng/ml TPO. Total RNA was collected from these cells, and the levels of hPF4 and hFVIII mRNA were detected by RT-PCR. GAPDH was used as an internal control.

2.10. Differentiation of iPS cells

For differentiation into megakaryocytes/platelets, 1×10^4 iPS cells were seeded into each well of a 6-well plate containing confluent OP9 stromal cells (Riken BioResource Center) and cultured in α-MEM (Sigma) supplemented with 20% FBS and 1% L-glutamine [18]. After 5 days in culture, the cells were seeded onto a fresh OP9 layer in the same culture medium supplemented with 20 ng/ml human TPO. After 2 days of culture, the medium was changed every day. On day 12 of culture, FVIII expression was examined by RT-PCR.

2.11. RT-PCR

Total RNA was isolated from cells using Trizol (Invitrogen) and treated with a Turbo DNA-free kit (Ambion) to remove genomic DNA contamination. First-strand cDNA synthesis was performed using ReverTra Ace (Toyobo). After cDNA synthesis, cDNA was amplified by PCR using ExTaq (Takara). GAPDH and Nat1 were used as internal controls. PCR primers for amplification of each gene were as follows: Oct3/4 (SeV) (sense, 5′-cccgaaagagaaagcgaaccag-3′; and antisense, 5′-aatgtatcgaaggtgctcaa-3′), Sox2 (SeV) (sense, 5′-acaagagaaaaaacatgtatgg-3′; and antisense, 5′-atgcgctggttcacg cccgcgcccagg-3′), Klf4 (sense, 5′-acaagagaaaaaacatg tatgg-3′; and antisense, 5′-cgcgctggcagggccgctgctcgac-3′), c-MYC (sense, 5′-taac tgactagcaggcttgtcg-3′; and antisense, 5′-tccacatacagtcctggatgatgatg-3′), Oct4 (sense, 5′-tctttccaccaggcccccggctc-3′; and antisense, 5′-tgcgggcggacatggggagatcc-3′), Sox2 (endo) (sense, 5′-tagagctagactc cgggcgatga-3′; and antisense, 5′-ttgccttaaacaagaccacgaaa-3′), Nanog (sense, 5′-caggtgtttgagggtagctc-3′; and antisense, 5′-cggttcatcatgg-tacagtc-3′), Rex1 (sense, 5′-acgagtggcagtttcttcttggga-3′; and antisense, 5′-tatgactcacttccaggggggcact-3′), hFVIII (sense, 5′-tctgccac actaacacactg-3′; and antisense, 5′-gggtcttgatgccgtgaata-3′), hPF4 (sense, 5′-agcatgagctccgcagccgggttct-3′; and antisense, 5′-cttccat tcttcagcgtggctatca-3′), mPF4 (sense, 5′-gtagaactttatctttgggt-3′; and antisense, 5′-aattttcctcccattcttca-3′), GAPDH (sense, 5′-ctcact-caagattgtcagca-3′; and antisense, 5′-gagttgggatagggcctc-3′), and Nat1 (sense, 5′-attcttcgttgtcaagccgccaaagtggag-3′; and antisense, 5′-agttgtttgctgcggagttgtcatctcgtc-3′). Amplified DNA fragments

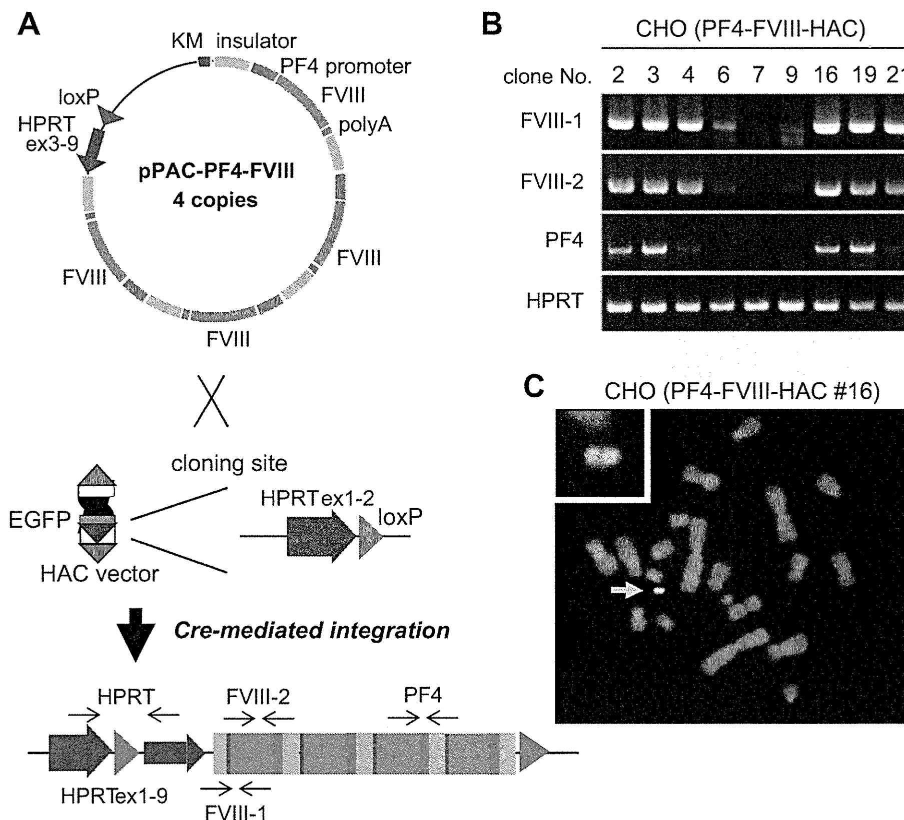


Fig. 2. (A) Schematic diagram of insertion of the FVIII expression cassette into the HAC vector. (B) Genomic PCR data of detecting PF4-FVIII-HAC in CHO cells. Each amplification site is mapped in (A, arrows). Representative data are shown. (C) FISH analysis of CHO cells. A digoxigenin-labeled human Cot 1 DNA probe (red) was used to detect the HAC vector. Biotin-labeled pPAC-PF4-FVIII (green) was used to detect the transgene on the HAC vector. Chromosomal DNA was counterstained with DAPI. The inset shows an enlarged image of the HAC vector (arrow). Representative data are shown.

were resolved by electrophoresis on a 2% agarose gel and visualized by ultraviolet fluorescence.

3. Results

3.1. Construction of the PF4-FVIII-HAC vector

To construct a HAC vector for gene expression of human FVIII (hFVIII) in megakaryocytes, the hFVIII gene was ligated to the human PF4 (hPF4) promoter. First, to demonstrate the ability of our construct to induce FVIII expression, human megakaryoblastic leukemia cell line UT-7/GM cells [16] was transiently transfected with each of the pPAC-PF4-FVIII vectors (one, two and four copies, see Section 2) (Fig. 1). Transfected cells were cultured with or without TPO, which has been shown to induce megakaryocytes/platelets differentiation of UT-7/GM cells [16], and differentiation into megakaryocytes/platelets was confirmed by detection of hPF4 mRNA expression (Fig. 1). As expected, hFVIII mRNA expression increased in a copy number-dependent manner.

The HAC vector contained a HPRT exon1-2-loxP cloning site into which circular DNA can be inserted using the Cre-loxP system. Eight micrograms of the pPAC-PF4-FVIII vector (four copies) was co-transfected with 1 μg of the Cre-recombinase expression plasmid into CHO cells containing an empty HAC vector (Fig. 2A). Twenty-eight HAT-resistant colonies were expanded and screened by genomic PCR analyses with primers for the FVIII transgene, PF4 promoter and reconstructed HPRT gene (Fig. 2A, arrow). Thirteen out of the twenty-eight HAT-resistant clones contained an intact PF4-FVIII-HAC (Fig. 2B), and then three clones were randomly selected for FISH analyses. FISH analyses with a biotin-labeled FVIII cDNA probe and digoxigenin-labeled human Cot1 DNA probe revealed that the presence of PF4-FVIII-HAC resulted in neither insertion nor translocation to the host chromosomes in two of the three clones (Fig. 2C).

3.2. Production of FVIII KO-iPS cells with the PF4-FVIII-HAC vector

FVIII KO-iPS cells were induced from FVIII KO-MEFs by infection with a SeV vector carrying four reprogramming factors, i.e., Oct3/4, Klf4, c-MYC and Sox2. Infected MEFs gave rise to iPS cell-like colonies that were isolated and expanded to cell lines. Two clones displaying a 40,*XY* normal metaphase were used for the following experiments (Fig. 3A).

PF4-FVIII-HAC vector was transferred into the two FVIII KO-iPS clones by MMCT. Because the HAC vector contained a green fluorescence protein (GFP) marker gene (Fig. 2A), after BS selection, GFP-positive clones were selected and examined in the following experiments (Fig. 3B). Thirteen of the resulting GFP-positive clones

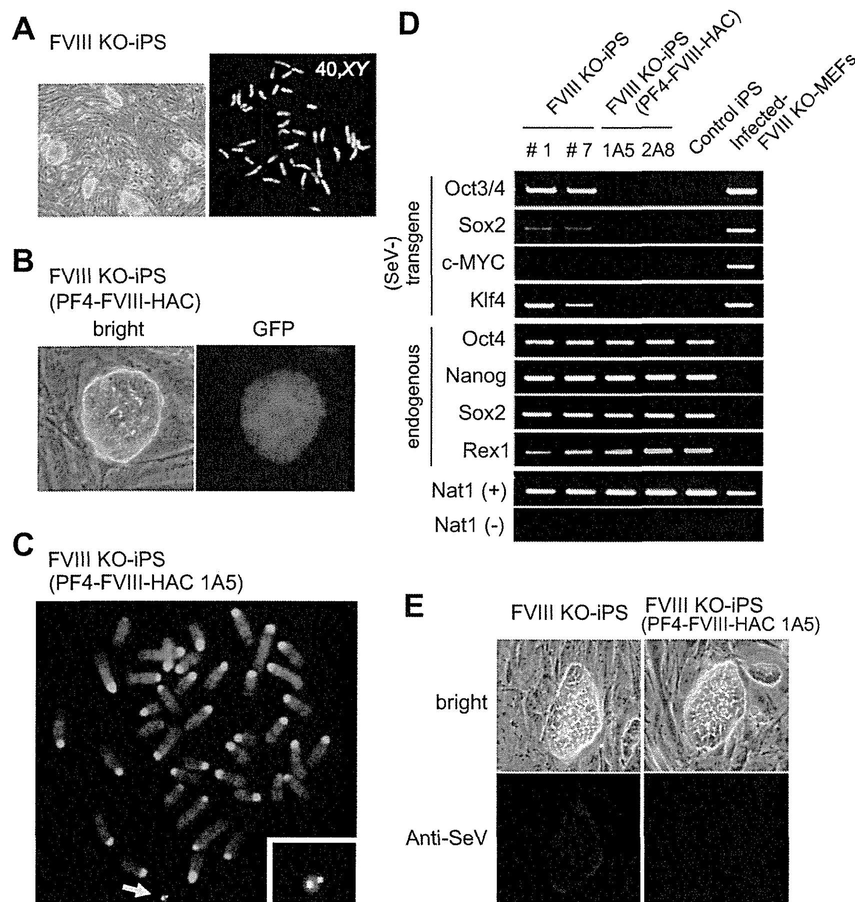


Fig. 3. Characterization of mouse iPS cells. (A) Morphology of FVIII KO-iPS clones (left panel). Q-banding metaphase are shown (right panel). (B) Morphology of FVIII KO-iPS (PF4-FVIII-HAC) cells. Bright field (left panel) and GFP fluorescence (right panel) microscopy images are shown. (C) FISH analysis of FVIII KO-iPS (PF4-FVIII-HAC) cells. A digoxigenin-labeled human Cot 1 DNA probe (red) was used to detect the HAC vector. Biotin-labeled pPAC-PF4-FVIII (green) was used to detect the transgene on the HAC vector. Chromosomal DNA was counterstained with DAPI. The inset shows an enlarged image of the HAC vector (arrow). Representative data are shown. (D) RT-PCR analyses of ES cell marker genes and four exogenous transcription factors. Nat1 was used as an internal control. Representative data are shown. (E) Immunostaining of parent FVIII KO-iPS cells and iPS cells containing PF4-FVIII-HAC. Bright field (top panel) and fluorescence (bottom panel) microscopy images are shown. Representative data are shown.

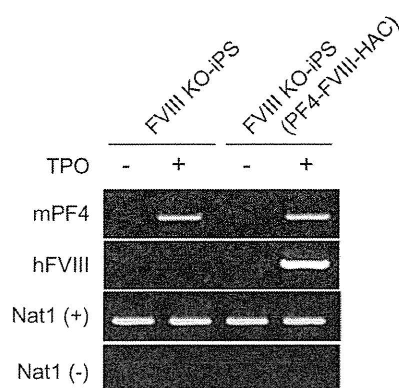


Fig. 4. *In vitro* differentiation of FVIII KO-iPS cells with or without PF4-FVIII-HAC. RT-PCR analyses of mPF4 and the hFVIII gene. Nat1 was used as an internal control.

were screened by genomic PCR analyses using primers to detect PF4-FVIII-HAC. Ten out of thirteen clones contained an intact PF4-FVIII-HAC (data not shown), and then FISH analyses showed that PF4-FVIII-HAC was present as an individual chromosome and normal karyotype in four clones (Fig. 3C). These four clones showed that the PF4-FVIII-HAC can be transferred to FVIII KO-iPS cells without affecting host chromosomes. Next, to test the stemness or undifferentiated state of these iPS cells, RT-PCR analyses using primers for embryonic stem (ES) cell-specific genes were performed. Endogenous Oct4, Nanog, Sox2 and Rex1 were expressed in two parent FVIII KO-iPS cell lines (#1 and #7) and all FVIII KO-iPS containing PF4-FVIII-HAC [FVIII KO-iPS (PF4-FVIII-HAC)] clones, comparable with that in control iPS cells (Fig. 3D). Exogenous SeV-transgenes, Oct3/4, Sox2, c-MYC and Klf4, were strongly or weakly expressed in two parent FVIII KO-iPS cell lines. However, expression of these genes was undetected in two FVIII KO-iPS (PF4-FVIII-HAC) clones (1A5 and 2A8) (Fig. 3D and E), suggesting that SeV-transgene expression was more or less decreased during cell division or after MMCT re-cloning. These results indicated that two clones (1A5 and 2A8) were SeV-free pluripotent stem cells and displayed a normal karyotype and independent PF4-FVIII-HAC vector.

3.3. Analyses of FVIII expression

We investigated whether *in vitro* megakaryocytes/platelets differentiation would result in FVIII expression. We cultured FVIII KO-iPS (PF4-FVIII-HAC) cells with TPO to induce differentiation, and then megakaryocytes/platelets differentiation was confirmed by detection of mPF4 mRNA expression (Fig. 4). FVIII expression was detected in megakaryocytes/platelets derived from FVIII KO-iPS (PF4-FVIII-HAC) cells, whereas undifferentiated cells showed no FVIII expression (Fig. 4). Taken together, these results show that PF4-FVIII-HAC vector accomplishes ectopic expression of FVIII in megakaryocytes/platelets derived from FVIII KO-iPS cells.

4. Discussion

Gene- and cell- therapy requires the development of an efficient and safe gene delivery system to be acceptable for clinical applications. Previously, both non-viral and viral vectors have been considered for the development of gene therapy of hemophilia in various animal models [3,19–21]. HAC vectors also support the correction of defective genes because expression from and transmission of these vectors are stable throughout many cell divisions [7,15,22]. In addition, because of their episomal nature, silencing of the introduced gene or oncogenesis resulting from integration into host chromosomes should be minimized. Another advantage of the HAC vector is transference into various cell types by MMCT methods without insertional mutagenesis. Nonetheless, the transfer rate of HAC via MMCT method is low, i.e., 10^5. Nevertheless, the MMCT frequency needs to improve. Moreover, in MMCT methods, it is possible to simultaneously transfer host chromosomes (e.g., CHO) along with the HAC. However, using FISH, karyotyping and RT-PCR, we have seldom observed co-transfer of host chromosomes during MMCT. Use of the HAC vector for FVIII expression is therefore expected to contribute to the development of new treatments for hemophilia A.

Integration of transgenes into host chromosomes is a major technical hurdle for clinical application. Although several methods for induction of iPS cells have been developed using adenoviruses [23] or plasmids [24], the risk of integration still remains while DNA vectors are used. In this study, we used a SeV vector, an RNA virus with no risk of altering host chromosomes, for iPS cell generation and successfully obtained exogenous transgene-free iPS cells. In addition, the HAC vector has a major advantage by being maintained independently of host chromosomes. Application of these methods to iPS cells combined with the HAC vector may be a powerful tool for safe gene- and cell- therapy.

In conclusion, we have developed HAC vectors containing multiple FVIII expression cassettes for gene therapy of hemophilia A, and transferred PF4-FVIII-HAC into SeV-free iPS cells, which is ectopically expressed in megakaryocytes/platelets. By combining integration-free iPS cells derived from patients [11,25,26], this HAC vector may be a promising strategy for novel and safe gene- and cell-therapy of hemophilia A. However, for such validation of the expression level or the therapeutic effect, *in vivo* experimental confirmation remains to be performed. Furthermore, several groups have reported that ectopic expression of the FVIII gene in megakaryocytes/platelets is therapeutic towards hemophilia A with inhibitory antibodies [27,28]; therefore, we need to verify the effect of inhibitory antibodies.

Funding

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (HT), the Japan Society for the Promotion of Science (YY) and the Japan Science and Technology Agency, CREST (MO).

Acknowledgments

We thank Sachiko Matsumoto for technical assistance.

References

[1] R.J. Kaufman, Advances toward gene therapy for hemophilia at the millennium, Hum. Gene Ther. 10 (1999) 2091–2107.
[2] K.A. High, Update on progress and hurdles in novel genetic therapies for hemophilia, Hematology Am. Soc. Hematol. Educ. Program (2007) 466–472.
[3] B.T. Kren, G.M. Unger, L. Sjeklocha, A.A. Trossen, V. Korman, B.M. Diethelm-Okita, M.T. Reding, C.J. Steer, Nanocapsule-delivered Sleeping Beauty mediates therapeutic Factor VIII expression in liver sinusoidal endothelial cells of hemophilia A mice, J. Clin. Invest. 119 (2009) 2086–2099.
[4] N.C. Hasbrouck, K.A. High, AAV-mediated gene transfer for the treatment of hemophilia B: problems and prospects, Gene Ther. 15 (2008) 870–875.
[5] D.W. Scott, J.N. Lozier, Gene therapy for haemophilia: prospects and challenges to prevent or reverse inhibitor formation, Br. J. Haematol. 156 (2012) 295–302.
[6] I. Petrus, M. Chuah, T. VandenDriessche, Gene therapy strategies for hemophilia: benefits versus risks, J. Gene Med. 12 (2010) 797–809.
[7] Y. Kazuki, M. Oshimura, Human artificial chromosomes for gene delivery and the development of animal models, Mol. Ther. 19 (2011) 1591–1601.
[8] Y. Kazuki, H. Hoshiya, M. Takiguchi, S. Abe, Y. Iida, M. Osaki, M. Katoh, M. Hiratsuka, Y. Shirayoshi, K. Hiramatsu, E. Ueno, N. Kajitani, T. Yoshino, K. Kazuki, C. Ishihara, S. Takehara, S. Tsuji, F. Ejima, A. Toyoda, Y. Sakaki, V. Larionov, N. Kouprina, M. Oshimura, Refined human artificial chromosome