Mini Review

Transient blocking of Lnk-mediated pathways as a potential approach to promote engrafting ability of hematopoietic progenitor cells

Hitoshi Takizawa[1)], Kiyoshi Takatsu[1)] and Satoshi Takaki[1,2,*)]

[1)]Division of Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, University of Tokyo, Tokyo, Japan

[2)]Department of Community Health and Medicine, Research Institute International Medicine of Japan, Tokyo, Japan

Lnk, an intracellular adaptor protein functions as a negative regulator of B lymphopoiesis, megakaryopoiesis and erythropoiesis by modulating signals through c-Kit, c-Mpl and Epo-R, respectively. In recent studies, we and other groups have shown that *Lnk*-/- mice display an increased number and enhanced repopulating capability of hematopoietic stem or progenitor cells (HSC/Ps), which is due to their hyperresponsiveness to thrombopoietin. Moreover, we have demonstrated that Src homology-2 (SH2) domain of Lnk is essential for the inhibitory function and that Lnk mutants with a point mutation in the SH2 domain potently act as dominant-negative mutant (DN-Lnk). Forced expression of DN-Lnk in HSC/Ps enhanced their repopulating capability in lethally irradiated recipient mice. Remarkably, transient expression of DN-Lnk also facilitated engraftment of HSC/Ps in immunodeficient animal under nonmyeloablative condition, leading to full reconstitution of lymphoid lineage cells. Transwell migration assay revealed that an interaction between HSC/Ps and vascular cell adhesion molecule-1 (VCAM-1) was augmented by Lnk deficiency or expression of DN-Lnk. These results suggest that Lnk modulates cell mobility of HSC/Ps in addition to their expansion. Lnk-mediated pathway therefore could become a potential target for enhancing both integrin-mediated engraftment and cytokine-dependent expansion of HSC/Ps.

Rec.10/10/2006, Acc.11/17/2006, pp59-64

* Correspondence should be addressed to:
Satoshi Takaki, Department of Community Health and Medicine, Research Institute International Medicine of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan. Phone: +81-3-3202-7181, Fax: +81-3-3208-5421, e-mail: stakaki@ri.imcj.go.jp

Key words adaptor protein, dominant-negative, hematopoietic stem cells, gene therapy, bone marrow transplantation

Introduction

Hematopoietic stem cells (HSCs) have the self-renewal activity and give rise to all lineages of blood system throughout the lifetime of an individual. The unique biological properties of HSCs have already been utilized extensively for therapeutic strategy to cure hematologic malignancies or genetic diseases by bone

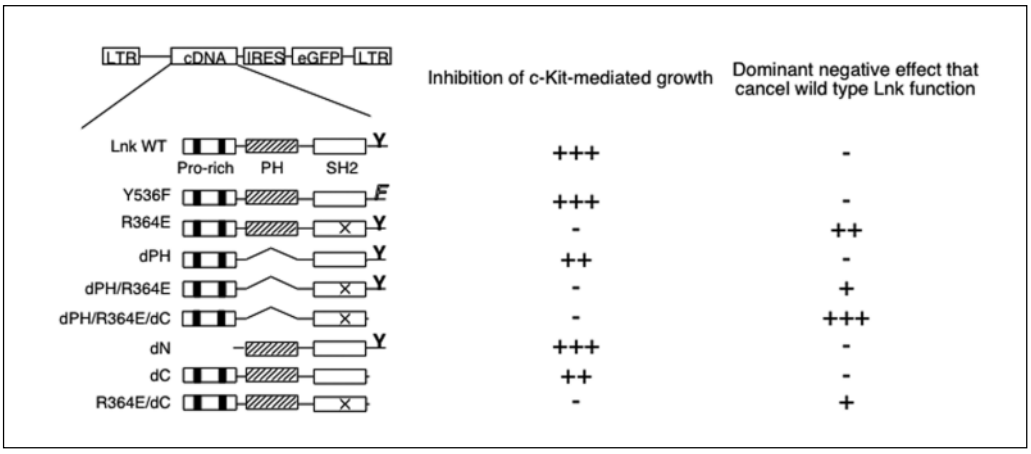

Fig.1 Functional domains of Lnk in c-Kit-mediated cell growth and screening of dominant-negative Lnk mutants

(Left column) Schematic representation of retroviral vector includes IRES between cDNA encoding Lnk mutants and eGFP. (Middle column) Effect of indicated Lnk mutants on c-Kit-mediated growth of MC9 cells. (Right column) Dominant negative effect of indicated Lnk mutants on c-Kit-mediated growth inhibition of MC9 transfectant overexpressing Lnk (MC9-Lnk).

marrow transplantation for decades. However, since the number of available HSCs is limited (e.g. cord blood trans-plantation), *ex vivo* or *in vivo* expansion of HSC by genetic manipulation has been studied intensively[1].

Lnk, together with APS (adaptor molecule containing PH and SH2 domains) and SH2-B, forms an adaptor protein family whose members share a N-terminal homologous domain followed by pleckstrin homology (PH) domain, SH2 domain and a highly conserved tyrosine phosphorylation site at a C-terminus. Because of their structural similarity, nomenclature of this family genes has been recently approved by the HUGO Gene Nomenclature Committee as SH2B adaptor protein 1 (SH2B1) for SH2-B, SH2B2 for APS and SH2B3 for Lnk. Lnk is an intracellular adaptor protein mainly expressed on lymphocyte, megakaryocyte and hematopoietic stem or progenitor cells (HSC/Ps)[2,3]. In previous reports, we and others have demonstrated that *Lnk*-deficient mice show overproduction of lymphocytes and megakaryocytes due to augmentation of growth signals through c-Kit and c-Mpl, respectively[2,4]. In addition, the mice show increased number and enhanced repopulating ability of HSC/Ps, which are caused by their hypersensitivity to thrombopoietin (TPO) in the absence of Lnk[2,3,5]. Despite of these phenotypes, any obvious tumor formations have not been observed in *Lnk*-deficient mice on C57BL6/J background. Inhibition of Lnk-mediated pathway(s) therefore could be a potentially useful approach for *in vivo* or *in vitro* amplification of HSC/Ps without causing malignant transformation.

This mini-review focuses on establishment of a new approach to enhance the engrafting ability of HSC/Ps by blocking Lnk-mediated pathways. First, we identified functional domain of Lnk and generated dominant-negative Lnk mutant (DN-Lnk) that potently inhibits endogenous Lnk function. Second, we showed the effectiveness of DN-Lnk expression on multilineage reconstitution by HSC/Ps after transplantation and its efficacy for treatment of immunodeficient mice. Lastly, we will discuss the regulatory mechanism underlying the high repopulating ability of *Lnk*-deficient HSC/Ps.

Functional domains of Lnk in c-Kit-mediated proliferation

To determine the functional domains of Lnk, various Lnk mutants were constructed and influence of these mutants to c-Kit mediated cell growth was studied (Fig.1). Mast cell line MC9 were retrovirally transduced with Lnk mutants and then c-Kit-mediated growth of transduced cells was compared with that of non-transduced. MC9 cells expressing wild-type Lnk failed to proliferate, suggesting efficient inhibition of c-Kit-mediated cell growth by Lnk. Either deletion of N-terminal domain (dN) or mutation of a conserved tyrosine phosphorylation site (Y536F) showed a similar inhibitory effect compared to wild-type Lnk, while deletion of PH domain (dPH) or C-terminal tail (dC) partly reduced the inhibitory effect. Importantly, a point mutation in SH2 domain (R364E) completely abolished the growth inhibition (Fig.1). These results indicate that SH2 domain of Lnk has a

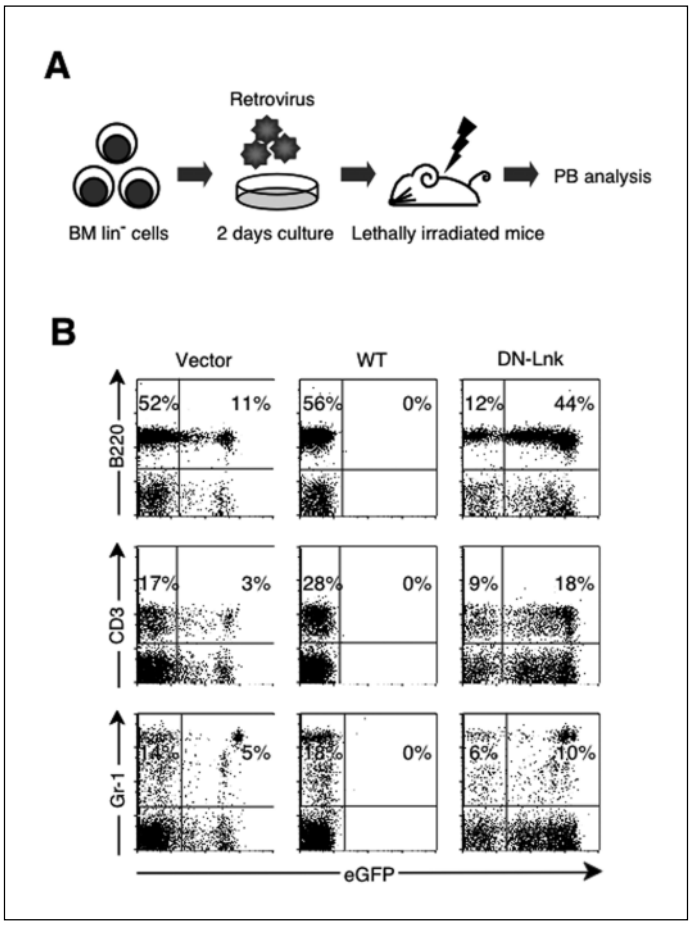

Fig.2 Expression of DN-Lnk in HSC/Ps facilitates blood cell repopulation

(A) Bone marrow transplantation model. Retrovirally transduced Lin⁻ cells were transferred into lethally irradianted mice. Peripheral blood (PB) harvested from transplanted mice was analyzed by flowcytometry.

(B) Contribution of transduced cells to B-, T- and myeloid lineage in PB. Flow cytometric analysis of PB cells from mice 17 weeks after transplantation are shown.

crucial role for inhibiting c-Kit-mediated cell growth and that PH domain and C-terminal tail of Lnk, not Y536 residue within the region, partly contributes to the efficient inhibition.

Screening for dominant negative mutant of Lnk

We next investigated whether non-inhibitory mutants, identified in experiments described above, could exert dominant-negative (DN) effect. Using MC9 transfectant overexpressing Lnk (MC9-Lnk) that hardly proliferates in response to SCF[2], we screened the DN Lnk mutants that could block wild-type Lnk function and cancel the growth inhibition. MC9-Lnk cells expressing R364E outgrew over non-transduced cells, indicating that this mutant acted as a DN mutant. Of generated Lnk mutants, Lnk mutant (dPH/R364E/dC) carrying combined deletion of PH domain and C-terminal tail with R364E mutation exhibited the most strong DN effect (Fig.1). These results suggest that mutation of SH2 domain is critical for DN effect and deletion of PH domain and C-terminal tail supports effective blocking of Lnk function.

Efficient hematopoietic reconstitution by DN Lnk mutant-transduced HSC/Ps in irradiated mice

We next assessed whether dPH/R364E/dC can block the function of Lnk endogenously expressed in HSC/Ps and facilitates the repopulating ability in transplantation model. Lineage marker negative (Lin^-) bone marrow cells containing HSC/Ps was retrovirally transduced with dPH/R364E/dC expression vector for 48 hours and intravenously injected into lethally irradiated recipient mice. Percentage of $eGFP^+$ cells in peripheral blood (PB) cells from the transferred mice was analyzed at several time points after transfer (Fig.2A). Very few $eGFP^+$ cells expressing wild-type Lnk were detected in peripheral lymphocytes, while significant fractions of mature B ($B220^+$)-, T ($CD3^+$)- and myeloid lineage ($Gr\text{-}1^+$) cells were differentiated from $eGFP^+$ cells expressing vector alone. Mice transplanted with DN-Lnk expressing cells displayed significantly higher percentage of $eGFP^+$ cells in each lineage than those transplanted with vector expressing cells (Fig.2B). These results indicated that HSC/Ps expressing DN-Lnk gained enhanced repopulating ability through inhibi-

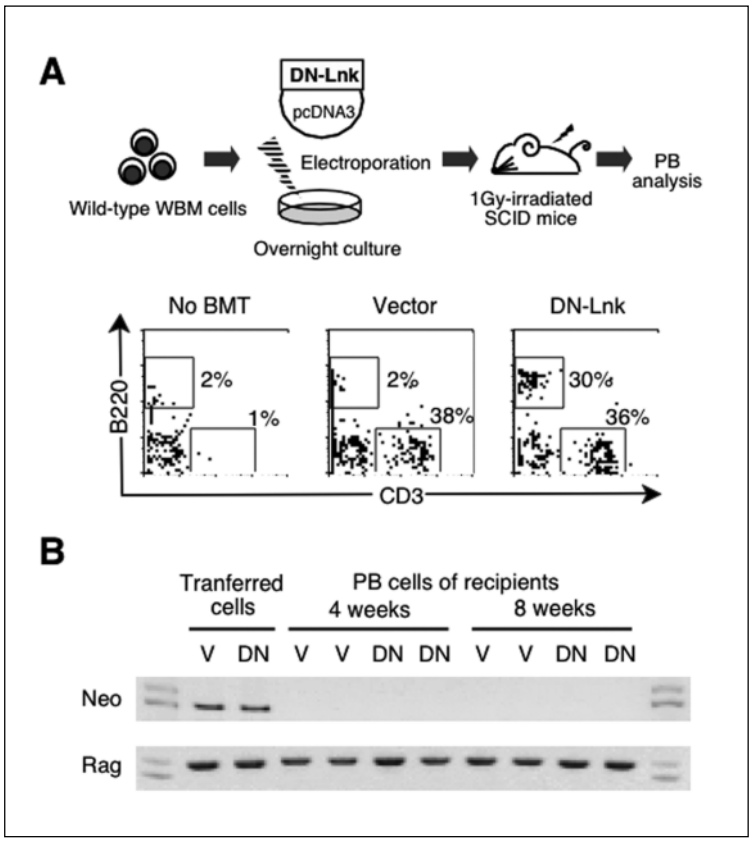

Fig.3 HSC/Ps transiently expressing DN-Lnk also showed efficient reconstitution

(A) Successful reconstitution of lymphoid compartment in SCID mice. Whole bone marrow cells from wild-type mice were transfected with control vector (middle) or DN-Lnk expressing vector (right) and transferred into SCID mice. PB cells obtained from transferred mice were analyzed 8 weeks after transplant.

(B) PCR analysis for plasmid vector in DNA isolated from PB cells of transplanted mice. Probes used are indicated to the left. Genomic DNA content in each sample was normalized by RAG gene. Each lane shows analysis of DNA from individual mouse. V: vector-transfected group, DN: DN-Lnk-transfected group.

tion of Lnk function.

Enhanced engraftment of HSC/Ps by transient inhibition of Lnk

Recent papers have reported that retrovirus-mediated gene transfer into HSC/Ps induces hematologic malignancies because of random integration of transgene into genome of target cells[6,7]. To avoid such side effects accompanied by retroviral transduction, we sought to test the effectiveness of transient expression of DN-Lnk on their engraftment using plasmid vector, which is scarcely integrated into host genome, in competitive repopulation assay. In mice receiving DN-Lnk-transfected HSC/Ps, chimerism of donor-derived cells in all lineage was higher and number of platelets was rapidly recovered compared with control group[8]. We next evaluated the efficacy of transient Lnk inhibition for treatment of severe combined immunodeficient (SCID) mice under nonmyeloablative condition. Bone marrow cells from wild-type animals were transfected with plasmid vector by electroporation, transplanted into SCID mice treated with a low dose of irradiation (1.0 Gly) (Fig.3A). A few control vector-transfected cells engrafted into the BM of recipient animals and gave rise to lymphoid lineage cells. In contrast, DN-Lnk-transfected cells fully reconstituted lymphoid compartment of SCID mice. Especially, rapid and robust production of B-lineage cells was observed from early time point (Fig.3A). Whether the employed plasmid vector was integrated into genome of transfected cells was examined by PCR amplifying neomycin-resistant gene. As expected, neomycin-resistant gene existing only in the plasmid vector was detected in transferred donor cells, however, it was not detected at all in DNA isolated from the reconstituted peripheral blood cells 4 or 8 weeks after transplantation (Fig.3B). These data suggested that transient inhibition of Lnk also could facilitate engraftment of HSC/Ps and early recovery of platelet production with no integration of transgene into genome.

Augmented interaction of HSC/Ps with VCAM-1 by *Lnk* deficiency

The successful multilineage reconstitution by transplanted HSC/Ps requires migration and attachment to a specific microenvironment in bone marrow, so-called niche, where they self-renew and differentiate. Short-term inhibition of Lnk by expressing DN-Lnk enhanced engrafting potential of HSC/Ps. This implies that blocking of Lnk-mediated pathway(s) might affect the migration or adhesion of HSC/Ps to niche shortly after trans-

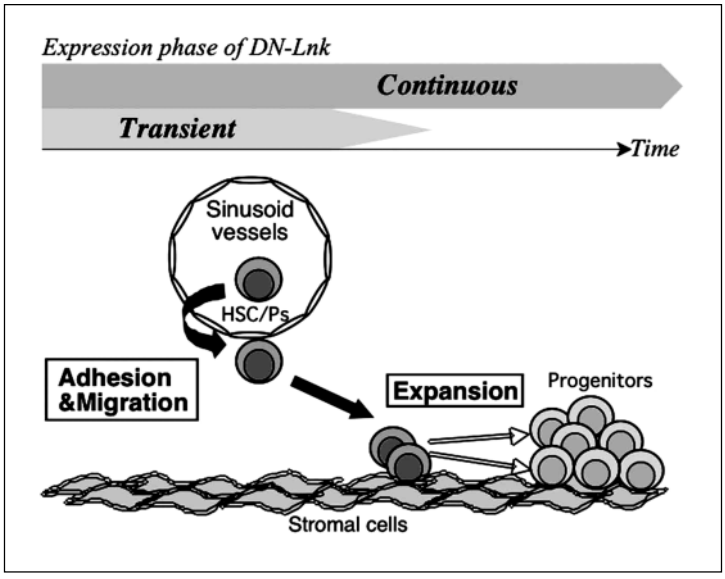

Fig.4 Timing of Lnk inhibition during engraftment of HSC/Ps

plantation in addition to their expansion (Fig.4). To address this possibility, we evaluated the migration responses of Lin^- c-Kit^+ $Sca1^+$ cells from *Lnk-/-* or wild-type mice against CXCL12, an essential chemokine for homing of HSC/Ps to BM[9] using transwell migration assay. CXCL12-induced migration through membrane was comparable between *Lnk-/-* and normal progenitors. Interestingly, *Lnk-/-* progenitors didn't migrate as efficiently as wild-type cells in the presence of BM cells other than Lin^- cells or VCAM-1, one of extracellular matrices associated with homing of HSC/Ps[8,10]. This implicated that Lnk plays a role in controlling cell motility of HSC/Ps by modulating an interaction between adhesion molecules like VCAM-1 on BM cells and integrins on HSC/Ps.

Perspectives

We identified a dominant-negative mutant of Lnk that blocks Lnk function endogenously expressed on HSC/Ps and established a novel and potential approach to facilitate the engraftment of HSC/Ps by transiently inhibiting Lnk-mediated pathway(s). We showed that retroviral transduction of DN-Lnk can enhance the repopulating capability of HSC/Ps, leading to an increased contribution of transduced cells in lymphoid and myeloid lineage cell production. However, recent studies have reported that retrovirus-mediated transduction into HSC/Ps may lead to unexpected side effects including hamatopoietic disorders or malignant transformations in human and mice due to integration of transgene into the cellular genome and long-term transgene expression[11,12]. Transient expression using plasmid vector has advantage to reduce those side effects because the transgene on plasmid is hardly integrated into cellular genome and declines as transfected cells divide. Short-term expression of DN-Lnk by plasmid vector could successfully augment engrafting potential of HSC/Ps and effectively reconstitute the immune system of immunodeficient animals under non-myeloablative condition. Thus, transient inhibition of Lnk-mediated pathway(s) could provide a useful therapeutic strategy for potentiating HSC/Ps for engraftment without any side effects followed by genetic modification.

We demonstrated that Lnk negatively regulated c-Kit-mediated proliferation and SH2 domain was crucial to the growth inhibition. It has been also shown that Lnk regulates TPO- as well as EPO-dependent growth signals, and the SH2 domain is important[4,13]. Data from transmigration assay indicates that Lnk controls cell motility of HSC/Ps possibly through integrin in addition to their cytokine-dependent expansion (Fig.4). This idea is supported by recent studies regarding APS and SH2-B, the other members of Lnk adaptor family, showing their roles in the regulation of actin cytoskeleton[14-16]. Especially, SH-2B interacts with Rac, small GTP protein, and enhances the growth hormone-induced actin rearrangement and cell motility[14,15]. Rac regulates actin cytoskeleton of HSC/Ps including migration, adhesion and cell cycle progression[17]. Thus, augmented interaction of HSC/Ps with adhesion molecule by blocking Lnk function might increase the chance for transplanted HSC/Ps to lodge in appropriate microenvironment in bone marrow by modulating actin cytoskeleton upon activation via integrins. It is likely that Lnk is a dual-functional adaptor not only controlling the expansion of HSC/Ps through cytokine-dependent pathway, but also modu-

lating their cell motility and/or integrin-mediated responses. The detailed molecular mechanisms by which Lnk modulates cell behavior on adhesion molecules should be addressed in future studies.

Acknowledgements

We would like to thank Drs. Chiyomi Kubo-Akashi, Ikuo Nobuhisa, Sang-Mo Kwon, Masanori Iseki, Toshio Kitamura and Tetsuya Taga for many collaborative works cited in this review. We are also grateful to Drs. Koji Eto, Hideo Ema, Jun Seita and Hiromitsu Nakauchi for helpful discussions. Hitoshi Takizawa is supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists. Our works were supported by Special Coordination Funds for Promoting Science and Technology, Grants-in-Aids from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government, and a grant from the Uehara Memorial Foundation.

Reference

1) Morrison SJ, Uchida N, Weissman IL: The biology of hematopoietic stem cells. Annu Rev Cell Dev Biol, 11: 35-71, 1995.
2) Takaki S, Morita H, Tezuka Y, Takatsu K: Enhanced hematopoiesis by hematopoietic progenitor cells lacking intracellular adaptor protein, Lnk. J Exp Med, 195: 151-160, 2002.
3) Ema H, Sudo K, Seita J, Matsubara A, Morita Y, Osawa M, Takatsu K, Takaki S, Nakauchi H: Quantification of self-renewal capacity in single hematopoietic stem cells from normal and Lnk-deficient mice. Dev Cell, 8: 907-914, 2005.
4) Tong W, Lodish HF: Lnk inhibits Tpo-mpl signaling and Tpo-mediated megakaryocytopoiesis. J Exp Med, 200: 569-580, 2004.
5) Buza-Vidas N, Antonchuk J, Qian H, Mansson R, Luc S, Zandi S, Anderson K, Takaki S, Nygren JM, Jensen CT, Jacobsen SE: Cytokines regulate postnatal hematopoietic stem cell expansion: opposing roles of thrombopoietin and LNK. Genes Dev, 20: 2018-2023, 2006.
6) Bunting KD, Galipeau J, Topham D, Benaim E, Sorrentino BP: Transduction of murine bone marrow cells with an MDR1 vector enables ex vivo stem cell expansion, but these expanded grafts cause a myeloproliferative syndrome in transplanted mice. Blood, 92: 2269-2279, 1998.
7) Baum C, Dullmann J, Li Z, Fehse B, Meyer J, Williams DA, von Kalle C: Side effects of retroviral gene transfer into hematopoietic stem cells. Blood, 101: 2099-2114, 2003.
8) Takizawa H, Kubo-Akashi C, Nobuhisa I, Kwon SM, Iseki M, Taga T, Takatsu K, Takaki S: Enhanced engraftment of hematopoietic stem/progenitor cells by the transient inhibition of an adaptor protein, Lnk. Blood, 107: 2968-2975, 2006.
9) Aiuti A, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos JC: The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J Exp Med, 185: 111-120, 1997.
10) Papayannopoulou T, Nakamoto B: Peripheralization of hemopoietic progenitors in primates treated with anti-VLA4 integrin. Proc Natl Acad Sci USA, 90: 9374-9378, 1993.
11) Li Z, Dullmann J, Schiedlmeier B, Schmidt M, von Kalle C, Meyer J, Forster M, Stocking C, Wahlers A, Frank O, Ostertag W, Kuhlcke K, Eckert HG, Fehse B, Baum C: Murine leukemia induced by retroviral gene marking. Science, 296: 497, 2002.
12) Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen JI, de Saint Basile G, Alexander I, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabesse E, Macintyre E, Sigaux F, Soulier J, Leiva LE, Wissler M, Prinz C, Rabbitts TH, Le Deist F, Fischer A, Cavazzana-Calvo M: LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science, 302: 415-419, 2003.
13) Tong W, Zhang J, Lodish HF: Lnk inhibits erythropoiesis and Epo-dependent JAK2 activation and downstream signaling pathways. Blood, 105: 4604-4612, 2005.
14) Herrington J, Diakonova M, Rui L, Gunter DR, Carter-Su C: SH2-B is required for growth hormone-induced actin reorganization. J Biol Chem, 275: 13126-13133, 2000.
15) Diakonova M, Gunter DR, Herrington J, Carter-Su C: SH2-Bbeta is a Rac-binding protein that regulates cell motility. J Biol Chem, 277: 10669-10677, 2002.
16) Kubo-Akashi C, Iseki M, Kwon SM, Takizawa H, Takatsu K, Takaki S: Roles of a conserved family of adaptor proteins, Lnk, SH2-B, and APS, for mast cell development, growth, and functions: APS-deficiency causes augmented degranulation and reduced actin assembly. Biochem Biophys Res Commun, 315: 356-362, 2004.
17) Gu Y, Filippi MD, Cancelas JA, Siefring JE, Williams EP, Jasti AC, Harris CE, Lee AW, Prabhakar R, Atkinson SJ, Kwiatkowski DJ, Williams DA: Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. Science, 302: 445-449, 2003.