殖を示さない Lnk 強発現トランスフェクタント細胞に SH2 変異 Lnk を導入したところ、c-Kit 依存性増殖能が回復し SH2 変異 Lnk がドミナントネガティブ(DN)変異体として働きうることがわかった。SH2 変異に加えて、PH ドメイン欠損、C 末端領域欠損を組み合わせることにより、より効率の良い DN-Lnk 変異体として作用することがわかった。

得られた DN-Lnk 変異体をレトロウィルスベクターを用いてマウス骨髄造血前駆細胞に感染導入した後、放射線照射したマウスへ移植した。Lnk 変異体を導入した造血前駆細胞を移植した群では、コントロール移植群に比し GFP 陽性血球細胞の割合が明らかに増加しており、特に B 細胞及び顆粒球系細胞の産生亢進が観察された。

レトロウィルスベクターでは導入細胞の悪性転換が懸念されている。この使用を回避できないかどうか、プラスミドの一過性発現系による効果を検討した。DN-Lnk を一過性発現させた骨髄細胞はコントロール細胞に比べ、致死量放射線を照射したマウスで高い造血能を示した。さらに正常細胞がなかなか生着しない条件で骨髄非破壊的前処置を施した免疫不全マウスにも生着し、免疫系を再構築することが確認できた。

D. 考察

本研究で同定・開発した DN-Lnk 変異体を用いることで造血前駆細胞に内因性に発現する Lnk の機能を阻害することが可能であり、造血系再構築能を亢進させることができることを骨髄移植モデルで示した。Lnk 変異体導入群における高い GFP 陽性率は、6ヶ月を超える長期間にわたって観察され、造血幹細胞に対しても効果があると考えられる。

DN-Lnk はその一過性発現によっても効果を発揮することが確認された。造血幹細胞及び前駆細胞の移植後早期の生着を亢進させることも考えられる。一過性発現による遺伝子導入では、染色体へのベクターの組み込みによる外来遺伝子の長期発現や異所性発現あるいは内因性遺伝子の破壊や制御異常等などの副作用の危惧は非常に少なく、造血幹細胞の機能制御に向け大変有用な方法と思われる

より安全で効率の良いドミナントネガティブ Lnk 変異体のデリバリー法の検討とともに作用機構の解明が肝要である。また、Lnk 依存性抑制経路を阻害する新たな阻害法の開発、標的分子の開拓を推進する意義は大きいと思われる。

E. 結論

Lnk による増殖抑制には SH2 ドメインが必須であり、SH2 変異に加えて、PH ドメイン欠損、C 末端領域欠損を組み合わせることで効率のよい DN-Lnk 変異体を作製することができた。この DN-Lnk 変異体により造血前駆細胞に内因性に発現する Lnk の機能を阻害することが可能であり、造血能を亢進させることをマウス骨髄移植モデルで確認した。近年、様々な分子の遺伝子導入を用いて造血幹細胞の増幅が試みられているが、本成果は腫瘍化を誘発する危険性が極めて低い分子及び使用法を確立した点で大きなインパクトがある。さらに骨髄への遊走、生着、骨髄微小環境内での増殖・分化における制御機構を検討するモデル系としての使用も考えられ、造血幹細胞の機能解明の面からも大変有用である。

F. 研究危険情報

特記事項なし

G. 研究発表

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H. 知的所有権の取得状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

特記なし

Developmental Cell (in press)

Quantification of self-renewal capacity in single hematopoietic stem cells from normal and Lnk-deficient mice

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Summary

Despite being a hallmark of hematopoietic stem cells (HSCs), HSC self-renewal has never been quantitatively assessed. Establishment of a clonal and quantitative assay for HSC function permitted demonstration that adult mouse HSCs are significantly heterogeneous in degree of multilineage repopulation and that higher repopulating potential reflects higher self-renewal activity. An HSC with high repopulating potential could regenerate approximately 1,000 HSCs whereas the repopulating activity of regenerated HSCs on average was significantly reduced, indicating extensive but limited self-renewal capacity in HSCs. Comparisons of wild-type mice with mutant mice deficient in the signal adaptor molecule Lnk showed that not only HSC numbers but also the self-renewal capacity of some HSCs are markedly increased when Lnk function is lost. Lnk appears to control HSC numbers by negatively regulating HSC self-renewal signalling.

Running title

Clonal analysis of normal and Lnk-deficient HSCs

Introduction

Stem cells have been heralded as limitless sources for tissue or organ regeneration because of their self-renewal capacity. However, self-renewal capacity has never been quantified for any type of stem cell, including hematopoietic stem cells (HSCs). Long-term multilineage repopulating activity detectable by transplantation experiments is the most reliable HSC marker. Based on competitive repopulation (Micklem et al., 1972), repopulating units (RUs) (Harrison et al., 1993) and competitive repopulating units (CRUs) (Szilvassy et al., 1990) have been used to express stem cell activity quantitatively (Fig. 1). RUs indicate the amount of repopulating activity and CRUs the number of stem cells; these two units thus complement one another. Given both RU and CRU values, the mean activity of stem cells (MAS) can be calculated (MAS = RU / CRU). We have proposed use of MAS in comparison of stem cell qualities (Ema and Nakauchi, 2000).

Evidence for self-renewal of HSCs has been provided by retroviral marking studies in which HSC clones tagged with proviral integration sites were transplanted into secondary recipients (Dick et al., 1985; Keller et al., 1985; Keller and Snodgrass, 1990; Lemischka et al., 1986). A high degree of HSC purification enabled successful long-term reconstitution with single HSCs (Ema et al., 2000; Osawa et al., 1996; Takano et al., 2004). After transplantation of single $CD34^{-/low}$c-$Kit^{+}Sca\text{-}1^{+}$lineage marker^{-} ($CD34^{-}$KSL) cells, we observed emergence of donor-derived $CD34^{-}$KSL cells in the recipients' bone marrow (BM), indicating self-renewal and expansion of the originally transplanted single $CD34^{-}$KSL cells. However, when these cells were sorted and transplanted into secondary recipients, the reconstitution capacity of the $CD34^{-}$KSL cells appeared significantly diminished (unpublished data, Nakauchi 1998). These data imply that while HSCs do self-renew in BM of primary recipients, their capacity to self-renew declines.

To examine this possibility we needed to develop a clonal and quantitative assay for self-renewal of HSCs (Fig. 1). First, single-cell-reconstituted mice were created. BM cells of these mice then were analyzed for RU and CRU produced by single donor cells. This let us know the number of HSCs regenerated from single HSCs and let us use the MAS of these regenerated HSCs to evaluate their quality.

During our study, we encountered an interesting mutant mouse lacking a signal adaptor molecule, Lnk. Lnk shares with APS and SH2-B a pleckstrin homology domain, a Src homology 2 domain, and potential tyrosine phosphorylation sites. It belongs to a family of adaptor proteins implicated in integration and regulation of multiple signalling events (Ahmed and Pillay, 2003; Huang et al., 1995; Li et al., 2000; Takaki et al., 1997;

Yokouchi et al., 1997). *Lnk* is expressed in a variety of hematopoietic cell lineages, including $CD34^{-}KSL$ cells and $CD34^{+}KSL$ cells (Supplemental Fig. S1).
Lnk null-mutation mice accumulate myeloid and B-lymphoid precursor cells in adult BM and spleen; this is ascribed to hypersensitivity to c-Kit ligand (KL) (Takaki et al., 2000; Velazquez et al., 2002). Lnk may act as a negative regulator in the c-Kit signalling pathway (Takaki et al., 2002). Unlike BM cells of various mutant mice with a deficiency of a particular molecule and severely disturbed early hematopoiesis (Bjornsson et al., 2003; Kimura et al., 1998; Lessard and Sauvageau, 2003; Mikkola et al., 2003; Miller et al., 1996; Ohta et al., 2002; Okuda et al., 2000; Park et al., 2003; Tsai et al., 1994; Warren et al., 1994), BM cells of Lnk-deficient mice are competitively superior in hematopoietic repopulation to those of wild-type mice (Takaki et al., 2002). However, it was unclear whether the characteristics and numbers of HSCs are altered in Lnk-deficient mice. We therefore analyzed HSCs from wild-type mice as well as Lnk-deficient mice and quantitatively compared their self-renewal activity.

Results

RU and CRU in bone marrow

We first attempted quantitative comparison of HSC activities in BM of wild-type C56BL/6 (B6) mice and of Lnk-deficient B6 mice (Takaki et al., 2002) in terms of RU. One RU is defined as the amount of repopulating activity in 10^5 BM cells from wild-type mice, based on competitive repopulation assays (Harrison et al., 1993). Lnk-deficient mice had approximately 63 RU per 10^5 BM cells (Table 1). We then estimated the number of HSCs by measuring the number of long-term marrow repopulating cells as CRU. Limiting-dilution analysis revealed that 10^5 BM cells from wild-type mice had 3.2 CRU on average (Fig. 2 and Table 1), consistent with our previous estimation (Sudo et al., 2000). In contrast, Lnk-deficient mice of the same age had 52.6 CRU in 10^5 BM cells (Table 1 and Fig. 2). Since total numbers of BM cells did not differ significantly between these two kinds of mice, the absolute number of HSCs in Lnk-deficient BM was clearly increased over 16-fold as compared with that in wild-type BM. The average RU per individual HSC, or mean activity of stem cells (MAS) (Ema and Nakauchi, 2000) in wild-type BM was calculated as approximately 0.3 RU/cell (Table 1). The MAS value in Lnk-deficient BM was 1.2 RU/cell. Lnk-deficient HSCs thus have, on average, approximately fourfold greater repopulating activity than do wild-type HSCs.

Phenotypically defined HSC populations

We next attempted to estimate the HSC pool size, based on HSC surface phenotypes and Hoechst 33342 dye efflux capacity. More than 10 times as many $CD34^-KSL$ cells as well as lineage $marker^-$side-population (Lin^-SP) cells (Goodell et al., 1996) were found in BM of 8-week-old Lnk-deficient mice as in BM of age-matched wild-type mice (Fig. 3A). Interestingly, numbers of both $CD34^-KSL$ cells and Lin^-SP cells in BM from femora and tibiae of Lnk-deficient mice started to increase exponentially at around 4 weeks of age (Fig. 3B).

Cell cycle analysis of HSCs

Cell cycle analysis was performed on 5,000 or more $CD34^-KSL$ cells isolated from 8-week-old wild-type or Lnk-deficient mouse BM, stained with propidium iodide (PI). More than 98% of the cells were in the G_0/G_1 phase in both cases (Supplemental Fig. S2A). To compare the turnover rate of Lnk-deficient HSCs with that of wild-type HSCs, mice were fed water containing bromodeoxyuridine (BrdU) for 2 weeks,

"(+)-5-bromo-2'-deoxyuridine" *is your designation later. Consistency?*

and BrdU incorporation by $CD34^+KSL$ cells or $CD34^-KSL$ cells was analyzed (Supplemental Fig. S2B). Over 95% of the $CD34^+KSL$ cells isolated from either wild-type or Lnk-deficient mice took up BrdU by 2 weeks. Consistent with previous observations (Sudo et al., 2000), 76.3 % of the $CD34^-KSL$ cells from wild-type mice were labelled with BrdU by 2 weeks. In contrast, 94.6% of those cells from Lnk-deficient mice were labelled. These data suggest that Lnk-deficient HSCs enter the cell cycle more frequently than do wild-type HSCs. However, the cell cycling status at any one time remains unchanged in $CD34^-KSL$ cells, presumably because of their discontinuous cycling.

Single cell reconstitution

As in wild-type mice, transplantation of 100 or more $CD34^+KSL$ cells isolated from Lnk-deficient mice resulted in little reconstitution (data not shown), indicative of enrichment of HSCs in the $CD34^-$ fraction among KSL BM cells of the mutant mice. Because discrepancy between numbers of $CD34^-KSL$ cells and actual numbers of HSCs are discrepant in aged normal mice (Sudo et al., 2000), $CD34^-KSL$ cells in Lnk-deficient mice were subjected to competitive repopulation. Single such cells isolated from wild-type or Lnk-deficient mice were transplanted into a total of 168 lethally irradiated mice, along with $2x10^5$ competitor BM cells. Four months after transplantation, multilineage reconstitution with more than 1% test donor-chimerism in myeloid, B-lymphoid, and T-lymphoid lineages was observed in 24 of 94 (26%) mice and in 17 of 76 (22%) mice transplanted with single $CD34^-KSL$ cells from wild-type and Lnk-deficient mice, respectively (Fig. 4). Of note is that the engraftment rates of $CD34^-KSL$ cells were similar in these groups. Moreover, RU values varied widely among HSCs of both wild-type and Lnk-deficient mice. RU values in mice which received wild-type cells ranged from 0.05 to 3.26 RU per cell, showing that HSCs are extremely heterogeneous in myeloid- and lymphoid lineage reconstituting activity (Fig 4). RU values in mice which received Lnk-deficient cells ranged from 0.06 to 14.4. Lnk-deficient HSCs showed a wider range of RU and therefore appeared more heterogeneous in terms of repopulating ability. The average of RU/cell in repopulating cells, that is MAS by definition, from Lnk-deficient mice was significantly greater than that from wild-type mice ($p<0.05$) (Fig. 4). Consistent with data for unfractionated BM cells (Table 1), the MAS in Lnk-deficient mice appeared to be 4.3-fold higher than that in wild-type mice in this clonal analysis. These data indicate that in this mutant mouse strain, not only the absolute number of HSCs is increased, but also the repopulating activity of an individual HSC. This increased RU in Lnk-deficient HSCs is partly attributed to enhanced B-lymphoid lineage expansion, but as shown in Figure 4,

the contribution of T-lymphoid and myeloid RU is evident, implying that HSCs also are under the direct influence of Lnk deficiency.

Self-renewal capacity of single HSCs

Numbers of RU and CRU regenerated from single $CD34^-KSL$ cells in BM of the recipient mice were estimated by secondary transplantation as illustrated in Fig.1. Six mice reconstituted with single wild-type cells and two mice reconstituted with single Lnk-deficient cells were successfully analyzed, as shown in Table 2. With wild-type HSCs, each CRU having a RU/cell in a range of 0.27 to 3.26 gave rise to different numbers of CRUs in a range of 350 to 976. As demonstrated in Supplemental Fig. S3, RU/cell and the number of secondary CRUs regenerated were positively correlated ($p<0.05$). The numbers of RU/BM did not increase as much as did those of CRU/BM in these cases whereas these two different measurements exhibited linear correlation ($p<0.005$).

I don't understand the use of "whereas" here.

Interestingly, the MAS levels remained in a low and narrow range (0.03-0.09 RU/cell), suggesting that RU/cell is determined by the total number of regenerated CRUs with relatively constant MAS. These data indicate that RU/cell can be one of the parameters of self-renewal capacity in HSCs.

On the other hand, with Lnk-deficient mice each CRU produced 963 or 3,030 CRUs, and the RU increased from 7.62 to 385 or from 3.38 to 872. The MAS levels remained at relatively higher levels (0.29 and 0.40). These data show that Lnk-deficient HSCs, particularly ones with high RUs, can maintain higher levels of self-renewal activity than can wild-type HSCs.

Lnk acts as a negative regulator in presumptive self-renewal signalling

Since Lnk has been shown to be associated with c-Kit (Takaki et al., 2002), we examined the effect of KL on in vitro self-renewal of HSCs.

"KL" has been introduced before as an abbreviation. Of course that was a while ago! Would this phrase work? "...associated with c-Kit and its ligand KL (Takaki et al., *2002), we..."*

Varying concentrations of KL were tested on single $CD34^-KSL$ cells in serum-free culture. The frequency of division and the survival rate of Lnk-deficient $CD34^-KSL$ cells did not significantly differ from those of wild-type $CD34^-KSL$ cells (Supplemental

Fig. S4). Using an in vitro self-renewal assay (Ema et al., 2000), we further examined whether KL can modulate c-Kit signaling to induce self-renewal division of Lnk-deficient $CD34^-KSL$ cells. As in wild-type HSCs, KL was necessary for in vitro survival of Lnk-deficient HSCs, but its signal alone was insufficient to promote their self-renewal (data not shown).

To elucidate the role of Lnk in self-renewal of HSCs, we next co-cultured 150 $CD34^-$ KSL cells with an OP-9 stromal cell line for 7 days in the absence of cytokines, and then transferred one-fifteenth of the culture into lethally irradiated mice. As a control, one-fifteenth of 150 $CD34^-KSL$ cells freshly isolated from wild-type or Lnk-deficient mice were transplanted into each of a group of lethally irradiated mice along with $2x10^5$ competitor cells. Multilineage reconstitution was observed 3 months after transplantation in 9 of 9 mice transplanted with wild-type cells and in 9 of 9 mice transplanted with Lnk-deficient cells (Fig. 5). After culture, only one of 9 mice that had received cultured wild-type cells showed reconstitution with test donor cells. In contrast, 9 of 9 mice that had received cultured Lnk-deficient cells were reconstituted. Since many $CD34^-KSL$ cells underwent at least one cell division during culture, not only survival but also self-renewal of HSCs led to the in vitro maintenance of stem cell activity in Lnk-deficient cells. These data together suggest that under certain in vivo and in vitro conditions the likelihood of self-renewal of HSCs is increased when Lnk is absent.

Discussion

Our newly established clonal assay revealed the great diversity of repopulating activity in HSCs. While all myeloid, B-lymphoid, and T-lymphoid lineages were reconstituted, the degrees of reconstitution in each lineage varied (Fig. 4). The proliferation capacity of HSCs is thus dissociated from their multilineage differentiation capacity. This heterogeneity of HSCs likely results from their different levels of self-renewal capacity. Measurements of CRUs given by single HSCs, in fact, suggest that the greater the self-renewal capacity, the higher the repopulating activity (Table 2 and Supplemental Fig. S3). It seems unlikely that HSCs with low RU can regenerate themselves more than those with high RU, but regenerated CRUs can be assumed to maintain a high MAS level when HSCs have undergone only a limited number of divisions. Heterogeneity in self-renewal capacity by itself implies that HSC self-renewal is not an unlimited capability.

Serial transplantation of BM cells has long been known to lead to a decline of stem cell activity (Cudkowicz et al., 1964; Harrison and Astle, 1982; Hellman et al., 1978; Ogden and Micklem, 1976; Sminovitch et al., 1964). However, whether this is due to simple dilution or truly to exhaustion of stem cells by transplantation is still debated (Iscove and Nawa, 1997). This study clearly demonstrates a clonal expansion of stem cell activity and number (RUs and CRUs) after transplantation, supporting the notion of extensive self-renewal capacity of HSCs (Iscove and Nawa, 1997; Pawliuk et al., 1996). One might interpret these findings as indicating unlimited self-renewal capacity of HSCs when either RU or CRU is examined (Iscove and Nawa, 1997). However, when MAS is taken into account, it is clear that the repopulating activity of regenerated stem cells were significantly reduced versus that in originally transplanted stem cells and that in normal BM cells (Table 2). These data would explain why BM cells cannot be serially transplanted in mice more than 4 to 6 times. This study and the binomial model-based study by Harrison and his colleagues (Harrison et al., 1990) together support the generation-age model (Rosendaal et al., 1979) although lethally irradiated mice were used as recipients in these experiments. Molecular mechanisms responsible for this decline in repopulating ability remain largely unknown. Telomeres shorten in HSCs after transplantation (Allsopp et al., 2003a). A decline in RU seems positively correlated with telomere shortening (unpublished data, Miyoshi and Nakauchi 2004). It has been reported that over-expression of telomerase can prevent telomere shortening, yet it cannot prevent a decline in stem cell activity (Allsopp et al., 2003b). Thus, unless telomeres are critically short, telomere shortening itself does not seem to be a limiting factor for repopulating ability of HSCs. Homing

is another factor that could influence repopulating ability of HSCs. However, it is uncertain whether homing is the only reason for poor repopulating activity in most HSCs regenerated in irradiated mice. Nonetheless, Lnk -/- mice may provide clues to the understanding of molecular mechanisms involved in HSC homing.
Previous studies showed increases of progenitor cell numbers in Lnk-deficient mice (Takaki et al., 2002; Velazquez et al., 2002). The present study demonstrated that the absolute number of HSCs is also increased. In an attempt to clarify the mechanisms of HSC expansion in this mutant mouse, we quantified the repopulating activity in HSCs. The MAS of Lnk-deficient HSCs appeared significantly greater than that of wild-type HSCs by population-type analysis as well as by clonal analysis (Table 1 and Fig. 4). Some Lnk-deficient HSCs regenerated a remarkably large number of HSCs after transplantation (Table 2). These results suggest that the proliferation of HSCs in irradiated recipient mice is enhanced by the lack of Lnk. Proliferative capacity should be directly associated with self-renewal events in HSCs. Of interest is that a single adaptor molecule, despite the existence of a variety of such molecules, can play a critical role in signal transduction responsible for determination of HSC fate.

Enhanced homing ability may explain higher repopulating ability in Lnk-deficient HSCs. If the expression levels of homing receptors are up-regulated in the HSC population of Lnk-deficient mice, HSCs with up-regulated receptors should show an advantage in competitive repopulation. Only Lnk-deficient HSCs, and not wild-type ones, may home to "incomplete niches" which express very low levels of cognate ligands. Alternatively, Lnk-deficient HSCs may proliferate more than do wild-type HSCs around niches with restricted mobilization ability.

Why "around"? Whose is the "mobilization activity", the Lnk-deficient or the wild-type HSCs', or even the niches'? This needs a bit of clarifying.

In single cell transplantation experiments in which an individual HSC was transplanted together with competitor cells, the engraftment rate of Lnk-deficient $CD34^-KSL$ cells did not differ from that of wild-type $CD34^-KSL$ cells. We at this moment interpret this result as indicating that Lnk-deficient HSCs do not have an obvious advantage over wild-type ones in homing to the stem cell niche in BM. Identification of specific homing receptors for HSCs, in combination with a solid assay for HSC homing, would be needed to evaluate these possibilities.

The frequency of cell cycling in HSCs from Lnk-deficient mice seemed greater than that from wild-type mice under physiological conditions (Supplemental Fig. S2). From the developmental point of view, the number of HSCs exponentially increased likely due to greater probability of self-renewal in addition to accelerated cycling in Lnk-deficient HSCs as compared with wild-type HSCs.

"...the exponential increase in HSC number with Lnk deficiency, as compared with wild-type, likely resulted from greater probability of self-renewal in addition to accelerated cycling."

We have not so far observed any differentiation block in Lnk-deficient HSCs, although it remains possible that reduced apoptosis of HSCs contributes to their accumulation in Lnk-deficient mice.

In consideration of the more or less limited capacity of adult HSCs as shown in this study, several researchers have attempted to enhance HSC self-renewal capacity by using gain-of-function or loss-of-function approaches (Calvi et al., 2003; Reya et al., 2003; Sauvageau et al., 1995; TeKippe et al., 2003; Varnum-Finney et al., 2000; Yuan et al., 2004; Zhang et al., 2003). In particular, overexpression of *HoxB4* and *Bmi-1* at the stem cell level has recently been reported to induce stem cell self-renewal in vitro (Antonchuk et al., 2002, Iwama et al. 2004). RT-PCR analysis did not detect differences between wild-type and Lnk-deficient HSCs in expression level of these genes (data not shown). Because Lnk is a signal adaptor protein, we may need to examine HSCs after stimulation with certain ligands that induce signalling via interaction with Lnk.

Even without full understanding, however, Lnk can perhaps be used as a tool to manipulate HSCs by using, for example, short interfering RNAs (McManus and Sharp, 2002) or dominant negative forms of Lnk. Nevertheless, quantitative evaluation of how self-renewal is attenuated at the clonal level by introduction of foreign genes or proteins into HSCs is important, because we should know how different from normal HSCs regenerated HSCs are and to what extent self-renewal is controllable in clinical settings.

Experimental Procedures

Purification of HSCs. CD34$^-$KSL cells were purified from mouse BM cells as previously described in detail (Ema et al., 2000; Osawa et al., 1996; Sudo et al., 2000). Hoechst 33342 staining was performed as described (Goodell et al., 1996). In brief, lineage-depleted cells at 10^6 cells per ml were stained with 5 μg/ml of Hoechst 33342 (Sigma) at 37°C for 90 min. After two washes, cells were subjected to antibody staining while continuously maintained at 4°C.

Competitive repopulation assay. Unfractionated BM or purified CD34$^-$KSL cells from 8-week-old C57BL/6 (B6)-Ly5.1 or Lnk-deficient B6-Ly5.2 mice were mixed with $2x10^5$ BM cells from 8-week-old B6-Ly5.1/Ly5.2 (F1) mice and transplanted into a group of mice irradiated at a dose of 9.5 Gy. B6-Ly5.2 and B6-Ly5.1 mice were used as recipients for cells from B6-Ly5.1 and B6-Ly5.2 donor mice, respectively. Peripheral blood cells of the recipient mice were taken between 3 and 4 months after transplantation and analyzed on a FACS for reconstitution in myeloid (Mac-1/Gr-1$^+$), B-lymphoid (B220$^+$), and T-lymphoid (CD4/CD8$^+$) lineages as previously described (Ema et al., 2000; Sudo et al., 2000).

For secondary transplantation, recipient mice were sacrificed between 4 and 5 months after transplantation. Three or four different numbers of BM cells reconstituted primarily with B6-Ly5.1 or B6-Ly5.2 test donor cells were mixed with $2x10^5$ BM cells from B6-Ly5.2 or B6-Ly5.1 mice and transplanted into at least 10 lethally irradiated B6-Ly5.2 or B6-Ly5.1 mice. F1-derived cells were excluded upon reconstitution analysis 3-4 months after transplantation.

RU, CRU, and MAS. The percentage of chimerism was calculated based on FACS data as follows: % chimerism = (% test donor cells) _ 100 / (% test donor cells + % competitor cells). When % chimerism was more than 1.0 for all myeloid, B-lymphoid, and T-lymphoid lineages, test donor cells were considered to contain at least one multilineage repopulating cell. The number of RU in test donor cells was calculated as follows: RU = 2 _ (% chimerism) / (100 - % chimerism)(Harrison et al., 1993) or 2 x (% test donor cells) / (% competitor cells), as $2x10^5$ competitor cells were used. The frequency of CRUs in test donor cells was estimated by limiting-dilution assay as previously described (Szilvassy et al., 1990). MAS has been defined as RU/CRU (RU/cell) (Ema and Nakauchi, 2000).

Cell cycle analysis. Cell cycle analysis was performed on 5,000 or more CD34$^-$KSL cells isolated from 8-week-old wild-type or Lnk-deficient mouse BM, stained with propidium iodide (PI) as previously described (Sudo et al., 2000). 5,000 cells or more were fixed with 70% ethanol in water, stained with 30 mg/ml of PI, and analyzed on a

FACSCaliur. The methods of BrdU labelling and analysis are described in Supplemental procedures.

Co-culture with OP-9 cells. 150 $CD34^-KSL$ cells from wild-type or Lnk-deficient mice were mixed with $3x10^6$ BM competitor cells. One-fifteenth of the aliquot (equivalent to 10 $CD34^-KSL$ cells and $2x10^5$ BM competitor cells) was transplanted into each of a cohort of lethally irradiated mice. In concurrent experiments, 150 $CD34^-$ KSL cells were directly sorted onto a monolayer of OP-9 stromal cell line (Nakano et al., 1996). Cells were incubated in 20% fetal calf serum in alpha-MEM at 37 °C with 5% CO_2 for 7 days. All adherent and non-adherent cells were collected and mixed with $3x10^6$ BM competitor cells. One-fifteenth of the aliquot was transplanted into each of a cohort of lethally irradiated mice. Recipient mice were analyzed 3 months after transplantation.

RT-PCR analysis.

PCR analyses for *Lnk* and other genes were performed on normalized cDNAs obtained from various hematopoietic cell populations in wild-type or Lnk-deficient mice as previously described (Osawa et al., 2000; Zhou et al., 2001).

Acknowledgments

The authors thank A. S. Knisely for critical reading of the manuscript. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology.

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