tors, i.e., Brn-1, Brn-2, Brn-4, and Oct-6, in addition to the widely expressed Oct-1 factor, are present in neural stem or progenitor cells. Moreover, our analyses revealed that, except for Oct-1, all of these factors were able to bind to the SRR2 sequence together with Sox-2 in gel shift DNA binding assays (data not shown). Therefore, we examined the potential involvement of these octamer factors and the Sox-2 protein in SRR2 activity. To this end, we introduced a luciferase reporter gene bearing wild-type SRR2 together with expression vectors of octamer factors and Sox-2 by transient transfection into COS cells which were devoid of endogenous Sox-2 protein. As shown in Fig. 7C, increasing the amount of the *Sox-2* expression vector boosted the level of transcription especially when a certain amount of octamer factor expression vectors were cotransfected. Indeed, except for Oct-1, all other octamer factors present in the brain showed activities equivalent to or even higher than that of Oct-3/4. The observed transcriptional activation is SRR2 dependent, because no elevation of transcriptional level was detected when the experiments were done with the reporter gene which lacks SRR2 (data not shown). Thus, these results indicate that all of these octamer factors except for Oct-1 have the potential to contribute to SRR2 activity in the developing brain, although we cannot eliminate the possibility that a novel neural stem or progenitor cell-specific octamer factor or another type of transcription factor(s) that contributes to SRR2 activity in the brain remains to be identified.

DISCUSSION

The *Sox-2* gene is known to be expressed in neural stem or progenitor cells as well as in ES cells (4, 6, 56, 57). In the present study, we have demonstrated that two *Sox-2* regulatory regions, SRR1 and SRR2, which were previously identified based on their activities in pluripotent ES cells, also function in neural stem or progenitor cell populations. There is a precedent for a regulatory region which supports gene expression in different tissues through the same DNA core element. Indeed, it has been demonstrated that the *mafK* gene encoding one of the small Maf proteins possesses an enhancer that functions in both cardiac muscle and hematopoietic cells and that distinct sets of GATA transcription factors are involved in each tissue to activate the enhancer (18). Thus, the transcriptional regulatory mechanism described in the present study is not restricted to stem cells but may operate in many aspects of development.

About the regulatory element involved in neural *Sox-2* expression, Zappone et al. (57) have previously reported the identification of the specific region acting as an enhancer in the developing telencephalon. Now we know that SRR1 activity in ES cells and the telencephalon-specific enhancer activity which Zappone et al. identified are defined by a single regulatory region, i.e., SRR1. Moreover, as with SRR2, SRR1 exerts its activity in ES cells and neural stem or progenitor cells in a similar manner by utilizing the common core sequence in which the octamer-like sequence also plays a central role (S. Nicolis, personal communication).

The activity of SRR2 in the developing brain has been examined for the first time in this study. We found that SRR2 was able to function in neural stem or progenitor cell population by experiments using neurospheres and also by neural differentiation of ES cells. The in utero electroporation analyses further corroborate and extend the results obtained with the in vitro culture systems. Indeed, these analyses clearly demonstrate that SRR2 is able to display its activity in neural stem or progenitor cells in the developing brains of mouse embryos. This system also allowed us to perform clonogenic analyses for demonstrating that at least a portion of cells in which SRR2 functions in the developing brain are multipotent neural stem cells. Zappone et al. (57) had previously shown that deletion of a DNA region carrying SRR1 resulted in the loss of the expression of the β-*geo* reporter gene, which was integrated into the *Sox-2* gene locus by homologous recombination in the telencephalic portion of developing brain. These results may cast doubt on the function of SRR2 in this portion of brain. However, this doubt should be banished. Because of the vector design, Zappone et al. deleted SRR2 as well as SRR1 during integration of the reporter gene in the *Sox-2* locus. This happened because SRR2, which is located in close proximity to the coding region of the gene, was not identified when they had done the analyses. Therefore, the loss of reporter gene expression in the telencephalon which Zappone et al. demonstrated is the consequence of the loss of both SRR1 and SRR2. Moreover, it should be noted that transgenic analyses revealed that, like SRR1, SRR2 functioned as a telencephalon-specific regulatory region (S.M. and A.O., unpublished data). Thus, we assume that both of them play at least a certain role in supporting the high level of *Sox-2* gene expression in the developing brain, although we do not know at present whether either one of them plays a more prominent role than the other.

Sox-2 is not the sole protein which is expressed in both ES and neural stem cells. Recent microarray technology revealed the significant similarity between these stem cells at the transcriptional level (36), although significant genetic dissimilarities have also been documented (8). One prominent characteristic shared by ES cells and neural stem cells is the ability to propagate in tissue culture systems without significantly losing multipotent properties, although neural stem cells would not grow indefinitely in vitro (22, 53). On the other hand, in the case of most other types of somatic stem cells, significant spontaneous differentiation occurs during the expansion of cells in vitro, and hematopoietic stem cells are one of the typical examples (3, 37). Therefore, it is possible to speculate that similar genetic regulatory networks operating in ES cells and neural stem cells are involved in sustaining the common biological property of these stem cells. Alternatively, as discussed by Ramalho-Santos et al. (36), this global overlap in expressed genes is simply due to the result of conversion of embryonic ectodermal cells to neural cells by a default mechanism (14). In any event, it is tempting to speculate that these commonly expressed genes also possess regulatory regions which are similar to SRR1 or SRR2 in sequences and/or element organizations.

Detailed analyses for characterizing the SRR2 core sequence revealed that the same or at least overlapping sequences were involved in its activity in ES cells and in the developing brain. For ES cells, the fact that SRR2 activity is mostly defined by the Oct-3/4–Sox-2 complex has previously been demonstrated (48). Although Sox-2 is present in the

developing brain, Oct-3/4 is essentially not present in this tissue. Therefore, we assume that similar protein complexes, such as the Brn-1–Sox-2 complex, contribute to SRR2 activities in the developing brain. Indeed, we have demonstrated that POU III class octamer factors present in brain, such as Brn-1 and Brn-2, show the potential to augment SRR2 activity together with that of Sox-2 in heterologous cells. We think that this Sox-2-mediated gene regulation with the aid of certain octamer factors in neural stem cells is particularly interesting. In fact, a large amount of data underscores the importance of the Oct-3/4–Sox-2 complex for maintaining the pluripotent state of ES cells by controlling the expression of many genes in ES cells (2, 4, 29, 47, 48, 56). Therefore, the presence of transcriptional regulation by similar protein complexes in neural stem cells indicates that these complexes also play a crucial role in preventing neural stem or progenitor cells from differentiating into postmitotic cells. It is noteworthy that essentially the same set of octamer factors are also shown to be involved in supporting the expression of genes for nestin and brain fatty acid binding protein, which are, like Sox-2, preferentially expressed in neural stem or progenitor cells in the brain (17). Thus, these results indicate that the requirement of these octamer factors is the general mechanism for restrictive gene expression in neural stem or progenitor cell populations. However, these octamer factors should not be thought sufficient to produce a stem or progenitor cell-specific expression profile in the developing brain; rather, they require some other factors' functions since these octamer factors by themselves are not exclusively expressed in neural stem or progenitor cells but are also expressed in subsets of differentiated cells (for details, see references 23 and 42). In this context, we assume that the specific activity of SRR2 in the stem or progenitor population is specified mainly by the Sox-2 protein by itself.

As described above, we have characterized the regulatory elements which support specific gene expression in these two distinct stem cells. Moreover, we have demonstrated that, at least for SRR2, transcription is activated by a common core sequence in ES and neural stem or progenitor cells. We hope that the data presented here may lead to the unraveling of the broader aspect of a common regulatory network which defines the nature of the stem cell state of ES cells and neural stem cells and possibly stem cells in general.

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Impaired Lymphopoiesis and Altered B Cell Subpopulations in Mice Overexpressing Lnk Adaptor Protein

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Lnk is an adaptor protein expressed primarily in lymphocytes and hemopoietic precursor cells. Marked expansion of B lineage cells occurs in $lnk^{-/-}$ mice, indicating that Lnk regulates B cell production by negatively controlling pro-B cell expansion. In addition, $lnk^{-/-}$ hemopoietic precursors have an advantage in repopulating the hemopoietic system of irradiated host animals. In this study, we show that Lnk overexpression results in impaired expansion of lymphoid precursor cells and altered mature B cell subpopulations. The representation of both B lineage and T lineage cells was reduced in transgenic mice overexpressing Lnk under the control of a lymphocyte-specific expression vector. Whereas the overall number of B and T cells was correlated with Lnk protein expression levels, marginal zone B cells in spleen and B1 cells in the peritoneal cavity were relatively resistant to Lnk overexpression. The C-terminal tyrosine residue, conserved among Lnk family adaptor proteins, was dispensable for the negative regulatory roles of Lnk in lymphocyte development. Our results illuminate the novel negative regulatory mechanism mediated by the Lnk adaptor protein in controlling lymphocyte production and function. *The Journal of Immunology*, **2003, 170: 703–710.**

B cells are continuously generated from hemopoietic progenitors in the fetal liver and in adult bone marrow (BM).[4] Multiple sequentially developing B cell precursor populations can be characterized based on the expression of various surface markers (1). Immature B cells generated in the BM emigrate into the peripheral immune system and give rise to a heterogeneous peripheral B cell population, consisting of recirculating cells located in follicles in the spleen and lymph nodes, and nonrecirculating cells mainly enriched in the splenic marginal zone (MZ). The majority of cells are the follicular (FO) B cells, with marginal zone (MZ) B cells representing 5–10% of the splenic B cells in an adult mouse (2, 3). There exists another self-renewing B cell subset, B1 cells, which predominates in the peritoneal and pleural cavities (4). MZ B cells and B1 cells produce natural Abs and provide a first line of defense against Ags. FO B cells are involved in thymus-dependent (TD) Ab responses, in which memory and plasma cells are generated (5, 6).

Lymphocyte differentiation is a series of finely regulated processes whereby the coordinate regulation of cell proliferation, differentiation, and death directs the development of functional cells. Through these processes, lymphocytes reactive against self-Ags are eliminated from the developing repertoire, and sufficient numbers of functional lymphocytes are produced to guarantee the prompt and effective elaboration of immune responses. Cell-to-cell contact and soluble growth factors play important roles in the regulation of developmental processes. Self-Ags presented on stromal cells trigger Ag receptors, signals from which help to determine the fate of lymphoid precursors (7). Various growth factors, such as stem cell factor (SCF) and IL-7, assist in regulating lymphoid precursor expansion by binding to c-Kit and the IL-7R, respectively. (8)

Binding of extracellular ligands to these polypeptide receptors initiates a cascade of events through the activation of intracellular protein kinases (9, 10). The phosphorylation events catalyzed by these kinases both modulate the catalytic activity of effector enzymes and mediate protein-protein interactions that juxtapose critical signal transduction elements. Although the details of how signaling molecules are activated or recruited to receptors remain incompletely elucidated, studies in recent years have defined an array of adaptor proteins that integrate and regulate multiple signaling events (11–13). Adaptor proteins lack kinase, phosphatase, or transcriptional domains, and instead consist of multiple binding sites mediating protein-protein or protein-lipid interactions, such as Src homology (SH) 2, SH3, or pleckstrin homology (PH) domains.

Lnk is an adaptor protein expressed mainly in lymphocytes (14, 15). Together with adaptor molecule containing PH and SH2 domains (APS) and SH2-B, Lnk is part of an adaptor protein family, whose members share the presence of a homologous N-terminal domain with putative proline-rich protein interaction motifs, followed by PH and SH2 domains, and a conserved C-terminal tyrosine phosphorylation site (16–18). SH2 domains of the Lnk family proteins, whose binding specificity remains unknown, are

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[4] Abbreviations used in this paper: BM, bone marrow; MZ, marginal zone; FO, follicular; TD, thymus-dependent; SCF, stem cell factor; SH, Src homology; PH, pleckstrin homology; KLH, keyhole limpet hemocyanin; TNP, trinitrophenyl; APS, adaptor molecule containing PH and SH2 domains; *hGH*, human growth hormone; HSA, heat stable Ag.

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approximately the same size and share over 90% similarity. Lnk regulates B cell production by negatively controlling pro-B cell expansion. Mutant mice lacking the *lnk* gene show enhanced B cell production (16, 19). This B cell overproduction is due to the hypersensitivity of B cell precursors to SCF, a c-Kit ligand (16). The absence of Lnk confers upon immature BM cells an enhanced ability to support B lymphopoiesis in adoptively transferred host animals, even in a competitive environment, such as the nonirradiated *RAG2*$^{-/-}$ host (16). In addition, the numbers of hemopoietic progenitors in the bone marrow increase in *lnk*-deficient mice (20). Competitive repopulation assays in irradiated host animals demonstrate that the ability of hemopoietic progenitors to generate various lineages of hemopoietic cells is greatly enhanced by the absence of Lnk.

In this study, we used a transgenic approach to define critical aspects of Lnk function in more detail. Lymphocyte production was impaired in a dose-dependent manner upon overexpression of Lnk in lymphoid cells. In addition to its importance in lymphopoiesis at the early developmental stages, Lnk also plays a role in peripheral maturing B cells. In transgenic mouse spleens, skewed B cell subpopulations and abnormalities in B cell morphology and cell cycle status were observed. Our results illuminate the novel negative regulatory mechanism mediated by the Lnk adaptor protein in controlling lymphocyte production and function.

Materials and Methods

Mice

All mice were bred and maintained at the animal facility of the Institute of Medical Science (University of Tokyo, Tokyo, Japan) under specific pathogen-free conditions. The *Nco*I-*Eco*RI cDNA fragment encompassing the entire coding region of the mouse Lnk cDNA was subcloned into the *Bam*HI site of pcDNA3 (Stratagene, La Jolla, CA), a eukaryotic expression vector driven by the CMV enhancer and promoter, resulting in pcDNA3-Lnk as previously described (20). The *Bam*HI fragment containing the Lnk cDNA was subcloned into the *Bam*HI cloning site of the p1026x vector that consists of the murine *lck* proximal promoter, Ig intronic H chain enhancer Eμ, and a human growth hormone (*hGH*) gene cassette (21). A substitutional mutation at the C-terminal tyrosine residue to phenylalanine (Y536F) was introduced into the Lnk cDNA by PCR-based site-directed mutagenesis, and confirmed by DNA sequencing (20). The resulting mutated cDNA was also inserted into the p1026x vector. The *lnk* transgenes, purified as *Not*I fragments, were injected into C57BL/6J mouse zygote pronuclei as previously described (22). Transgenic founders were detected by hybridization of genomic tail DNA with a *hGH* probe or PCR, and stable mouse lines were generated by backcrossing founders with C57BL/6J mice.

Western blotting

Single cell suspensions were prepared from lymphoid organs of 6- to 8-wk-old mice. Cells were lysed with lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_4, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg ml aprotinin), and the lysates were clarified by centrifugation. Total lysates derived from 4×10^6, 2×10^6, or 1×10^6 thymocytes were separated on 8% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed with anti-Lnk-C-terminal Abs (15). Bound Abs were detected using HRP-conjugated secondary Abs via chemiluminescence.

Flow cytometry

Single cell suspensions were prepared from lymphoid organs of 6- to 8-wk-old mice, and cells were stained using predetermined optimal concentrations of the respective Abs. The stained cells were then analyzed on a FACScan or FACSCalibur instrument (BD Biosciences, San Jose, CA). The following mAbs were used: PE-conjugated anti-CD43 (S7), biotin-conjugated anti-BP-1/Ly-51, FITC-coupled anti-heat stable Ag (anti-HSA, J11d), FITC-anti-CD8 (53-6.7), FITC- or PE-anti-CD4 (RM4-5), biotin-anti-CD25 (PC61), PE-anti-CD44 (IM7), biotin-anti-CD23 (B3B4), FITC-anti-CD21 CD35 (7G6), PE- or biotin-anti-CD3ε (145-2C11), biotin-anti-TER-119, biotin-anti-Gr-1 (RB6-8C5), (all purchased from BD PharMingen, San Diego, CA); FITC-, PE-, or biotin-anti-B220 (RA3-6B2), PE-anti-IgM $F(ab')_2$, FITC-anti-Mac-1 (M1/70), (obtained from Caltag Laboratories, Burlingame, CA), and biotin-anti-IgD (CS15, a gift from Dr. K. Miyake, University of Tokyo). PE-streptavidin (Ancell, Bayport, MN), Tri-color-conjugated streptavidin (Caltag Laboratories) or allophycocyanin-conjugated streptavidin (BD PharMingen) were used for biotin-coupled Ab staining. In some staining, 2 μg/ml 7-amino-actinomycin D (Sigma-Aldrich) were used to gate out dead cells. For DNA staining, splenocytes were stained with FITC-anti-CD21/CD35 and PE-anti-IgM $F(ab')_2$ and $CD21^-IgM^+$ T1 or $CD21^{high}IgM^{high}$ T2 and MZ B cells were purified using FACSVantage (BD Biosciences). Cells were then fixed in ethanol and stained in 20 μg/ml propidium iodide, 0.5 mg/ml RNase H, and 0.2% Tween 20 at room temperature for 60 min. Stained cells were then analyzed on a FACSCalibur instrument.

Serology

Serum concentrations of each Ig isotype were determined by isotype-specific ELISA as described previously (23). To examine the Ab production against TI-2 Ags, mice were i.p. injected with 100 μg of trinitrophenyl (TNP)-Ficoll in saline and were bled 10 days after the injection. To examine the response against TD Ags, mice were immunized i.p. with 100 μg of keyhole limpet hemocyanin (KLH) in a 1:1 homogenate with CFA (Difco, Detroit, MI), and were bled on day 12. Serum serial dilutions were analyzed for TNP- or KLH-specific Ig isotypes by ELISA using dinitrophenyl-coupled BSA (cross-reacts with anti-TNP Abs) or KLH as the capture reagent.

Proliferation assay

Splenic B cells were purified using a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) after incubation with biotin-conjugated anti-CD43 and streptavidin-coupled microbeads. Resulting purified B cells (1×10^5) were cultivated in 200 μl of medium in 96-well plates. Cells were stimulated with various concentrations of anti-IgM $F(ab')_2$ (The Jackson Laboratory, Bar Harbor, ME), anti-CD40 mAb (BD PharMingen), IL-4 (PeproTech, London, U.K.), or LPS. Cells were pulse-labeled with [^{3}H]thymidine (0.2 μCi per well) during the last 16 h of the 72-h culture period, and incorporated [^{3}H]thymidine was measured using a Matrix 96 direct beta counter (Packard Instrument, Meriden, CT).

Results

Generation of transgenic mice overexpressing Lnk at various levels

In mice lacking the *lnk* gene, an accumulation of B lineage cells caused by overproduction of pro-B cells was observed (16). To characterize further the importance of Lnk, and to reveal its roles in lymphopoiesis and lymphocyte function, we generated transgenic mice that overexpress Lnk under the control of the *lck* proximal promoter in combination with the Eμ enhancer (Fig. 1*A*). The promoter drives expression of the inserted cDNA in T and B lineage cells from their early developmental stages (21). Seven lines of transgenic mice were obtained from independent founders, and five lines that overexpress Lnk at various levels were further analyzed. Lnk protein expression levels in thymocytes from each transgenic line were measured by immunoblotting a 2-fold serial dilution of the lysates using anti-Lnk Ab in combination with densitometric quantification (Fig. 1*B*). Transgene expression in peripheral T or B cells was well correlated with that in thymocytes in low or medium expressers (data not shown). Severe reduction of peripheral B cells and altered distribution of mature B cell fractions in a high expressing line (see below) made it difficult to directly measure the Lnk protein expression in B cells. In contrast, T cell development assessed on the basis of CD4 and CD8 expression was unaffected in the transgenic lines (see below). Thus, expression levels in the thymus were used to compare the Lnk levels in each transgenic line. The Lnk no. 4 line expressed Lnk at the highest level, 23-fold over endogenous Lnk protein levels in normal C57BL/6 thymocytes. The Lnk no. 99 line expressed Lnk at the lowest level, 2.5-fold greater than the endogenous level.

Perturbed B lymphopoiesis in BM by Lnk overexpression

B cell development in each transgenic mouse line was analyzed. The highest expresser, Lnk no. 4, showed severe reduction of B lineage cells as shown in Fig. 2. The BM contained very few

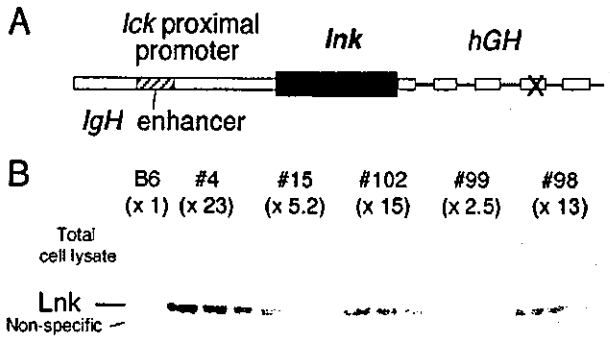

FIGURE 1. *A*, The p1026x-*lnk* transgene construct. The full-length cDNA fragment encoding the Lnk protein was inserted downstream of the *lck* proximal promoter and the Eμ enhancer, followed by the *hGH* mini gene cassette. The *hGH* gene carries a mutation in the fourth exon (indicated by an "X") so a functional hGH protein is not expressed. *B*, Lnk protein expression in thymocytes obtained from five independent transgenic mouse lines (Lnk numbers 4, 15, 102, 99, and 98) and control C57BL/6 (B6) mice. Serial dilutions (2-fold) of total cell lysates were separated by SDS-PAGE, and subjected to immunoblotting using anti-Lnk Abs. Lnk expression levels were determined by densitometry, and the fold expression over endogenous Lnk protein levels in nontransgenic C57BL/6 animals is indicated in parentheses under the transgenic line name. Amounts of lysates loaded in each lane can be estimated from nonspecific bands cross-reacted with our anti-Lnk Abs.

$B220^+$ cells, many of which were $CD43^+$ pro-B cells. Most of the pro-B cells were HSA^- and $BP\text{-}1^-$ (Hardy et al.'s fraction A; Ref. 1), indicating that B cell development was blocked at a very early stage. Other transgenic lines showed mild reduction of B lineage cells that correlated in a dose-dependent manner with the Lnk expression level (Fig. 3). Interestingly, the correlation between reduction of B lineage cells and Lnk expression levels was not a linear correlation. Instead, the reduction correlated with the logarithmic value of Lnk expression levels. The reduction of pre-B and immature B cells was more severe than that of pro-B cells. This may suggest the existence of Lnk-dependent regulation in the transition from pro-B to pre-B cell stages, in addition to the known role for Lnk in pro-B cell expansion (16). However, our pro-B cell discrimination based on the method of Hardy and colleagues (24) contains non-B lineage cells, especially in Fraction A ($B220^+$ $CD43^+HSA^-BP\text{-}1^-$). Thus, an alternative possibility could be that those non-B lineage cells in the pro-B cell fraction not expressing the transgene were maintained at relatively normal levels, leading to an underestimation of the pro-B cell reduction. As expected, no cell number reduction was observed in myeloid cells, which should not express the transgenes.

Impaired expansion of T precursors by Lnk overexpression

We next examined consequences of Lnk overexpression in T lineage cell development. Total thymocyte numbers declined as Lnk expression in thymocytes increased (Fig. 3). However, thymocyte development, evaluated on the basis of CD4 and CD8 expression, was grossly normal (Fig. 2), except for a slight reduction of $CD4^+CD8^-$ and $CD4^-CD8^+$ mature thymocytes. The reduction in number was slightly more severe in $CD4^-CD8^+$ thymocytes than that of $CD4^+CD8^-$ mature thymocytes in Lnk transgenic lines. Within the $CD4^-CD8^-$ immature thymocyte compartment, a proportion of $CD44^-CD25^+$ or $CD44^-CD25^-$ cells was significantly reduced in transgenic mice compared with normal littermates (Fig. 2). This led to the overrepresentation of $CD44^+CD25^+$ cells indicating inefficient transition of $CD44^+CD25^+$ to $CD44^-CD25^+$ cells or impaired expansion of $CD44^-CD25^+$ cells.

Consequence of Lnk overexpression in peripheral lymphocytes

The total cell number in the spleen was also reduced as more Lnk transgenes were expressed. Only a residual number of B cells remained in the spleen of the highest expresser, Lnk no. 4 (Fig. 2). T cell numbers also declined as Lnk expression increased, although B cell reduction was more prominent than that of T cells (Fig. 3). We then examined splenic B cell subfractions by staining for CD21, CD23, and IgM. This analysis demonstrated that the $CD21^+CD23^-IgM^{high}$ MZ B cells were relatively maintained in

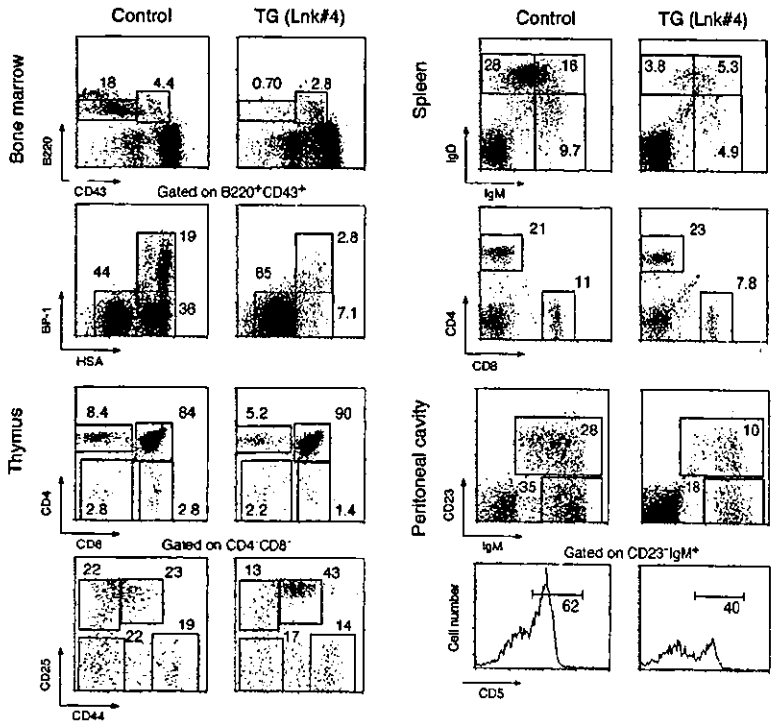

FIGURE 2. Representative multicolor fluorescence plots showing severe impairment of B cell development and mild reduction of T lineage cells in transgenic mice highly expressing Lnk protein (Lnk no. 4). Cell suspensions prepared from 6- to 8-wk-old animals were stained with combinations of labeled Abs and analyzed by flow cytometry. BM: expression of B220/CD45R and CD43 on total BM cells, and expression of BP1/Ly-51 and HSA/CD24 on $B220^+CD43^+$ pro-B cells. Spleen: total splenocytes were stained for surface IgM and IgD, or for CD4 and CD8. Thymus: expression of CD4 and CD8 on total thymocytes, and expression of CD25 and CD44 on $CD4^-CD8^-$ double-negative thymocytes. Peritoneal cavity cells: expression of IgM and CD23 on lymphocytes in peritoneal cavity, and expression of CD5 on $CD23^-IgM^+$ B1 cells. Numbers represent the percentages of cells in each box or area.

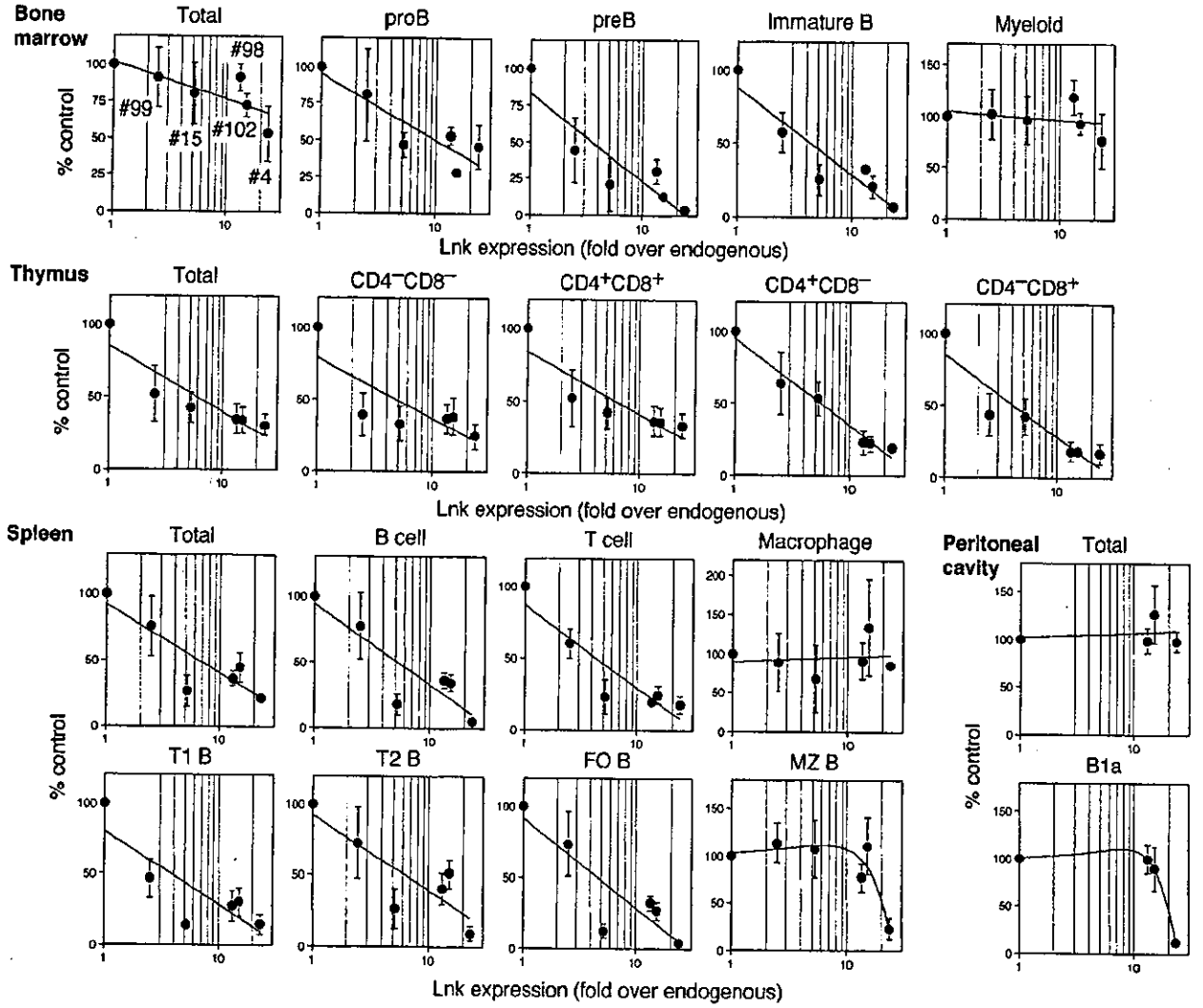

FIGURE 3. Dose-dependent reduction of lymphoid cells in p1026x-*lnk* transgenic mice. Cell suspensions prepared from 6- to 8-wk-old animals were analyzed by flow cytometry; the absolute cell numbers within each cell fraction were calculated and shown as percentage of those in littermate control mice. The relative amount of Lnk protein expressed in thymocytes from each transgenic line was assessed by immunoblotting and densitometry. Shown are means ± SD for four to six animals of each transgenic line. BM: pro-B ($B220^+CD43^+$), pre-B ($B220^+CD43^-IgM^-$), immature B ($B220^{low}IgM^+$), and myeloid cells ($B220^-CD43^+$). Spleen: B ($B220^+$), T ($CD3^+$), macrophages ($Mac\text{-}1^+$), transitional T1 B ($IgM^+CD23^-CD21^-$), transitional T2 B ($IgM^{high}CD23^+CD21^{high}$), FO B ($IgM^+CD23^+CD21^{low}$), and MZ B ($IgM^+CD23^-CD21^{high}$). Thymus: $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$. Peritoneal cavity cells: B1a ($IgM^+CD23^-CD5^+$). B1 cells in the peritoneal cavity and MZ B cells are relatively resistant to Lnk overexpression.

most transgenic lines, except in the most potent expresser, Lnk no. 4. In contrast, all other peripheral B cell fractions in spleen, transitional T1 and T2 cells, and FO B cells decreased in a dose-dependent manner (Fig. 3). $CD23^-IgM^{high}CD5^+$ B1a and $CD23^-IgM^{high}CD5^-$ B1b cells in the peritoneal cavity were also relatively maintained in most transgenic lines, except in Lnk no. 4, whereas B2 cells and T cells in the cavity were reduced (Fig. 3 and data not shown).

Next, we examined whether Lnk overexpression affects the function of peripheral mature B cells. Lnk no. 98 moderately expresses Lnk protein (13-fold greater expression than endogenous Lnk in thymocytes) and was able to produce substantial numbers of splenic lymphocytes (~40% of normal C57BL/6 control mice). Histological and immunohistochemical analysis demonstrated that splenic architecture was grossly maintained in these transgenic mice, although the white pulp regions were reduced (data not shown). However, $CD21^+CD23^-IgM^{high}$ MZ B cells occupied a major compartment of splenic B cell populations (Fig. 4*A*). CD23 expression levels on $CD23^+IgM^{low}$ FO B cells in transgenic mice were slightly higher than those observed on normal FO B cells. $CD21^-CD23^-IgM^+$ T1 B cells showed slightly decreased IgM levels. In addition, T1, as well as $CD21^{high}CD23^+IgM^{high}$ T2, cells in transgenic mice were larger in size. In contrast, cells from other B lineage fractions, pro-B and pre-B cells, immature B cells in the BM, and FO and MZ B cells in the spleen were all similar in size to those of normal C57BL/6 mice (Fig. 4*B*). Interestingly, larger transgenic T1 cells were not actively proliferating (Fig. 4*C*). The cycling fraction of T1 B cells was severely reduced as assessed via DNA content analysis. In addition, expression levels of B cell activation markers, such as MHC class II and CD86 or CD25, on T1 cells were unchanged (data not shown). Thus, Lnk overexpression in peripheral splenic B cells compromised B cell maturation and proliferation, especially in the T1 fraction.

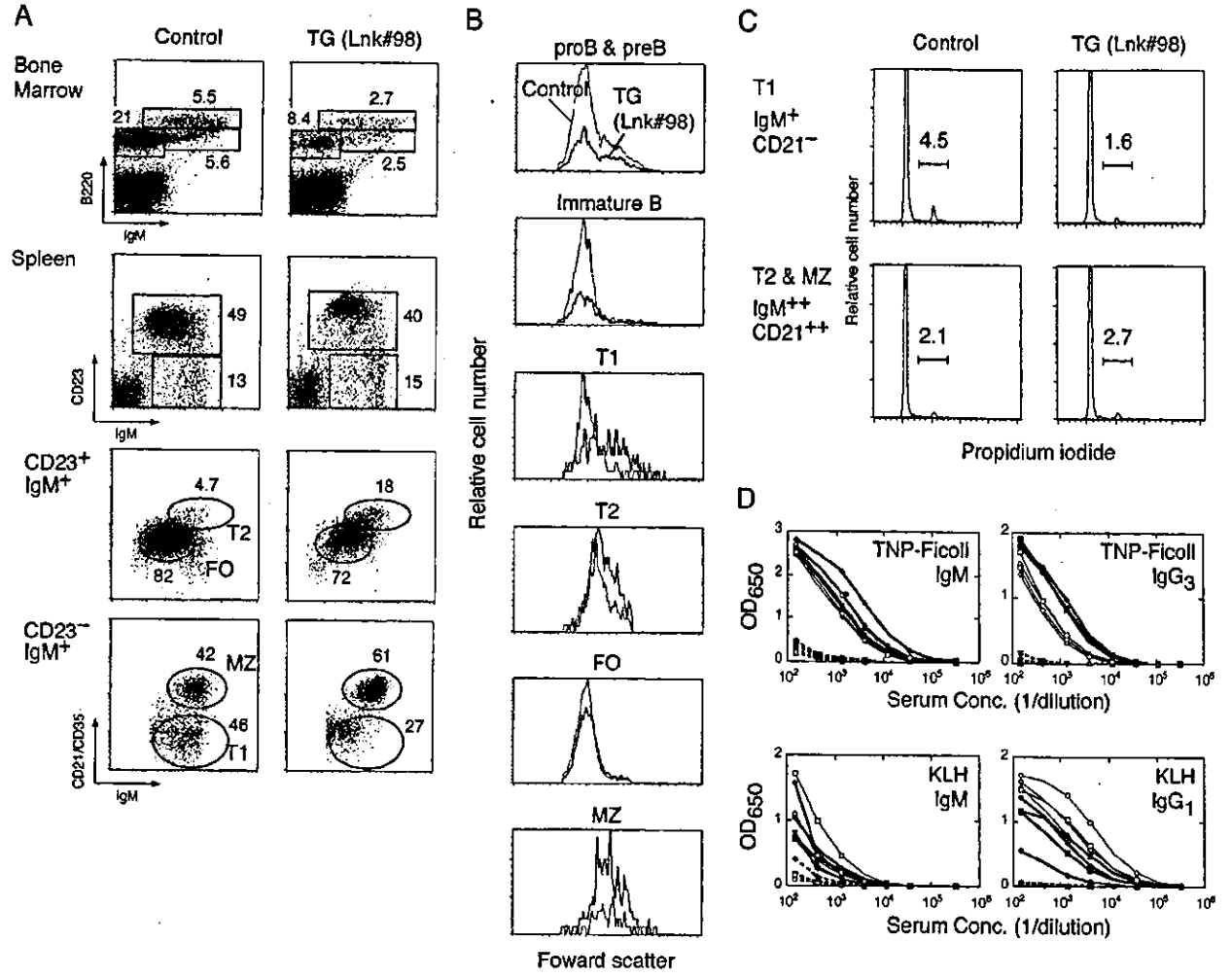

FIGURE 4. *A*, Representative multicolor fluorescence plots showing skewed splenic B cell compartments and increased cell size of transitional B cells in transgenic mice overexpressing Lnk at a moderate level (Lnk no. 98). BM: expression of B220 and IgM on total BM cells. Spleen: expression of CD23 and IgM on total splenocytes, and expression of CD21/CD35 and IgM on $CD23^+$ or $CD23^-$ splenic B cells. Numbers represent the percentages of cells in each box or area. *B*, Enlarged cell size of transitional T1 and T2 B cells in transgenic mice. Splenic B cells were fractionated electrically based on expression of IgM, CD23, and CD21/CD35. Forward scatter of transitional T1 B ($IgM^+CD23^-CD21^-$), transitional T2 B ($IgM^{high}CD23^+CD21^{high}$), FO B ($IgM^+CD23^+CD21^{low}$), and MZ B ($IgM^+CD23^-CD21^{high}$) were compared between control C57BL/6 (thin lines) and Lnk no. 98 mice (bold lines). *C*, Decreased cycling B cells in transgenic mice. T1 ($CD21^-IgM^+$), or T2 and MZ ($CD21^{high}IgM^+$) B cells were purified by cell sorting, fixed, and stained with propidium iodide. Numbers represent the percentages of cycling cells that fall into the indicated range. *D*, Response to TI-2 Ag, TNP-Ficoll (*upper panels*) and TD Ag, KLH (*lower panels*). Serial dilutions of serum obtained before immunization (dashed lines) or after immunization (solid lines) from control C57BL 6 mice (thin lines, open symbols) or Lnk no. 98 transgenic mice (bold lines, filled symbols) were analyzed for TNP- or KLH-specific Ig isotypes by ELISA.

Despite the reduced proliferation in the B cell compartment, in vitro proliferative responses of splenic B cells upon anti-IgM stimulation were only slightly impaired (data not shown). Ab production was also relatively well-maintained in *lnk*-transgenic mice. Reflecting the increased proportion of MZ B cells in the spleen and B1 cells in the peritoneal cavity, Ab production against a TI-2 Ag (TNP-Ficoll) was augmented in the *lnk*-transgenic mice compared with normal littermates. Sera of transgenic mice immunized with TNP-Ficoll contained a higher titer of anti-TNP Abs in both IgM and IgG3 subclasses (Fig. 4*D*). In contrast, IgM and IgG1 production against the TD Ag KLH was slightly compromised, which was consistent with the significant reduction of FO B cells in the spleen, and of T cells in the thymus and spleen. Interestingly, serum Ig levels of all subclasses were slightly increased in *lnk*-transgenic mice (data not shown). It seems that a strong signal through the B cell receptor could override the cell proliferation inhibition imposed by augmented expression of Lnk.

Negative regulation of lymphocyte development by Lnk is independent of its phosphorylation

Lnk contains a tyrosine phosphorylation motif involving Y536 at its C terminus that is conserved among all Lnk family adaptor proteins, including APS, SH2-B, and the Lnk-like protein in *Drosophila*. Y536 is the major site phosphorylated by c-Kit (20) and by various tyrosine kinases when simultaneously overexpressed in COS7 cells (S. Takaki, unpublished observation). We examined a potential role for the conserved tyrosine phosphorylation motif in the negative regulatory functions of Lnk in lymphocyte development. Y536 was substituted with a phenylalanine residue and the resulting mutant form of Lnk (Y536F) was inserted downstream of the *lck* proximal promoter and the Eμ enhancer, and expressed in lymphoid cells in transgenic mice. The Y536F transgenic mice expressed comparable amounts of Lnk protein with the mice expressing the wild-type Lnk, Lnk no. 4 (Fig. 5*B*). As in the case of

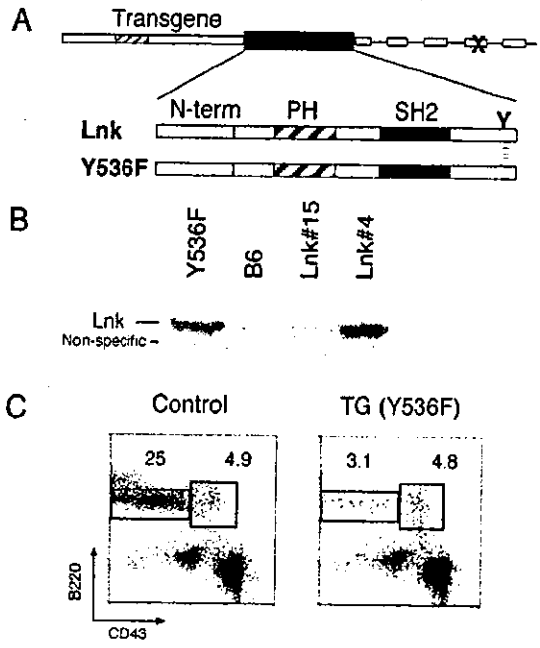

FIGURE 5. *A*, The transgene construct for the expression of Y536F mutant Lnk. The cDNA fragment encoding Lnk protein carrying a substitutional mutation of phenylalanine for tyrosine at position 536 (Y536F) was inserted downstream of the *lck* proximal promoter and the Eμ enhancer. *B*, Amounts of mutant Lnk protein in thymocytes obtained from Y536F transgenic mice were compared with those of Lnk protein in thymocytes from Lnk nos. 4, 15, and C57BL/6 (B6) mice. Equal amounts of total cell lysates were separated by SDS-PAGE, and subjected to immunoblotting using anti-Lnk Abs. *C*, Impairment of conventional B cell development in Y536F transgenic mice. Representative multicolor fluorescence plots showing expression of B220/CD45R and CD43 on total BM cells. Numbers represent the percentages of cells in each box.

wild-type Lnk overexpression, B cell development in Y536F transgenic mice was also inhibited at the pro-B cell stage. The BM contained very few $B220^+$ cells, which were mostly $CD43^+$ pro-B cells, similar to the BM of Lnk no. 4 transgenic mice (Figs. 5 and 1). The numbers of thymocytes and B1 cells in the peritoneal cavity were also decreased in the Y536F transgenic mice (data not shown). These results indicate that the Lnk C-terminal tyrosine phosphorylation site is dispensable for the negative regulatory effects of overexpressed Lnk in lymphocyte development.

Discussion

Lnk constrains lymphocyte production

Our results document a potent regulatory mechanism in lymphopoiesis mediated by the adaptor protein, Lnk. Lnk overexpression in lymphoid precursors resulted in both B and T cell reduction. Expansion of pro-B cells in bone marrow, and of pro-T cells in thymus was impaired as Lnk expression increased. We previously reported that mutant mice lacking the *lnk* gene showed enhanced B cell production due to the hypersensitivity of B cell precursors to SCF, a c-Kit ligand. However, the $lnk^{-/-}$ mice do not show any abnormality in T cell development or altered thymocyte expansion as seen in *lnk*-transgenic mice (16). B lineage cells express more Lnk than T lineage cells (15). Thus, different levels of endogenous Lnk expression in B lineage cells from those found in T lineage cells probably account for this discrepancy. In other words, Lnk could function in both B and T precursor cells, however, B precursors that express higher amounts of Lnk are more stringently controlled by Lnk-mediated regulatory signals. The physiological importance of this more stringent restriction of conventional B cell production by Lnk remains to be elucidated. In the C57BL/6 background, loss of the *lnk* gene alone does not cause autoimmunity, malignant transformation of B lineage cells, nor exhaustive loss of hemopoietic progenitor cells. Although it is possible that other Lnk family proteins might compensate for some Lnk function in *lnk*-deficient T cells, T lineage cells do not express APS (25). Moreover, despite the homology between Lnk and SH2-B, many studies have demonstrated positive effects of SH2-B on cell growth and differentiation (26–32), while Lnk functions as a negative regulator of lymphopoiesis as clearly shown in this study and in the analysis of the *lnk*-deficient mice. We recently reported that male and female gonad maturation is impaired in $SH2\text{-}B^{-/-}$ mice, while both B and T cell development are normal (33). Thus, in $lnk^{-/-}$ T lineage cells, compensation by other Lnk-family members does not appear likely. The generation of *lnk, SH2-B* double-deficient mice will help to elucidate the special regulatory characteristics of thymic progenitors.

Both B and T cell numbers were reduced as the amount of Lnk exponentially increased. This contrasts with that of the perturbed lymphocyte development by the increasing activity of many effector enzymes, such as $p56^{lck}$, by overexpression (34) or by the decreasing activity of $p56^{lck}$, Erk1, and *ras* by their dominant negative mutants (21, 35, 36). All these examples show a linear correlation between phenotype and protein expression levels. SH2-B forms a pentameric complex through homotypic association via its N-terminal domain (30). Because the N-terminal domains of Lnk-family adaptors are conserved, Lnk may also manifest its function as a multimeric complex. In transfected COS7 cells, Lnk indeed exists as a multimer (S. M. Kwon and S. Takaki, unpublished observation). Lnk multimerization could account for the semilogarithmic relation between lymphocyte reduction and Lnk expression levels. The stoichiometries between Lnk complexes, membrane receptors, and signaling molecules may also contribute to this unique correlation between phenotype and Lnk expression levels.

Lnk function in peripheral lymphocytes

Many unrelated transgenic and gene-targeted models show an enlarged splenic MZ compartment (3). In several cases, the enlarged MZ phenotype is linked to compromised peripheral B cell generation. For example, $IL\text{-}7^{-/-}$ (37) and conditional *Rag*-knockout mice (38) show reduced B lymphopoiesis at precursor levels and develop a larger MZ B subset. The peripheral B cells in these mice with impaired B lymphopoiesis show an activated phenotype: increased expression of CD25, class II, and CD86, and augmented entrance into the cell cycle (37, 38). These properties could result from compensatory mechanisms which allow enhanced generation of the mature B cell compartment. The peripheral phenotype observed in our *lnk*-transgenic mice was similar to that observed in $IL\text{-}7^{-/-}$ or conditional *Rag*-knockout mice in terms of increased MZ B cells accompanied by reduced B lymphopoiesis, but not fully consistent with the phenotype due to limited BM B lymphopoiesis. Immature splenic T1 and T2 B cells in *lnk*-transgenic mice were enlarged, but were not entered in the G_2/M phase of the cell cycle. These results imply that Lnk also constrains the proliferating ability of peripheral B cells, as well as BM B cell precursors. Apart from FO or MZ B cells, most B cells in $IL\text{-}7^{-/-}$ and conditional *Rag*-knockout mice are enlarged, express activation markers, and show an increased proportion of proliferating cells (37, 38). In contrast, in *lnk*-transgenic mice, enlarged cells were only seen in newly generated T1 and T2 cell compartments. Hence, maturation or compensatory proliferation of peripheral B cells might be perturbed by Lnk overload.

Another possibility is that the phenotype observed in peripheral B cells could be a consequence of unusual selection of B cells that are relatively resistant to Lnk overexpression. Cells which have an advantage in maturation or proliferation might selectively develop in the BM and expand into the periphery in *lnk*-transgenic mice, where they could preferentially differentiate into the B1 and MZ B cell compartments. As shown in recent gene disruption studies, signaling components of B cell receptors, such as tyrosine kinases and coreceptors, survival factors like B cell activating factor from the TNF family/B cell activating factor from the TNF family receptor, components controlling cell migration including protein tyrosin kinase 2 and Lsc (Lbc's second cousin, the murine homolog of human p115 Rho GEF), and transcription factors like, those of the NF-κB family, Aiolos, and recombination signal binding protein-J are all critical for MZ B cell development (3, 39). Cells that could expand carrying high amounts of Lnk might preferentially differentiate in B1 and MZ B cell compartments as a result of altered expression of molecules critical for MZ B cell development.

How does Lnk control lymphocyte proliferation?

Using a c-Kit$^+$ mast cell line, we previously demonstrated that Lnk is tyrosine-phosphorylated by c-Kit and interacts with phosphorylated c-Kit (20). Lnk specifically inhibited c-Kit-mediated signaling for cell growth by attenuating Gab2 (a family member of Gab1, Grb2-associated binder 1) phosphorylation and the subsequent activation of the mitogen-activated protein kinase pathway (20). However, previous studies demonstrated that a c-Kit signal is indispensable for T precursor expansion, whereas B cell generation occurs in the absence of c-Kit (40). Although the impaired expansion of CD4$^-$CD8$^-$ double-negative T precursors in *lnk*-transgenic mice could result from an inhibitory effect of overexpressed Lnk on c-Kit signaling, the observed impairment of B cell production strongly indicates that c-Kit is not the sole target for Lnk. Consistent with this idea, the enhanced hemopoietic ability by *lnk*-deficient hemopoietic precursors was not significantly normalized by attenuating c-Kit signals with the introduction of the heterozygous *W* mutation in the c-Kit locus (20). Flt3/Flk-2 may be involved in such a pathway because it has been shown to support proliferation and differentiation of hemopoietic progenitor cells, while disruption of Flt3/Flk-2 perturbed the production of various blood cell lineages (41). *W/Wflk2*$^{-/-}$ mice show impaired hemopoiesis with severe lymphocytopenia. However, injection of anti-Flt3/Flk-2 Abs into adult mice together with anti-c-Kit Abs does not inhibit B lymphopoiesis, while the treatment severely inhibits erythropoiesis and myelopoiesis (42). Injection of anti-Flt3/Flk-2 Abs into *lnk*$^{-/-}$ mice failed to normalize the B cell overproduction (S. Takaki, unpublished observation). These observations suggest that Flt3/Flk-2 signaling is not likely to be a target affected by Lnk overexpression. Further studies will be required to identify the molecular targets of Lnk and to understand how Lnk regulates the expansion of hemopoietic and lymphoid precursor cells in vivo.

It has been shown that Lnk associates with an actin binding protein, ABP-280 (43). It has also been shown that SH2-B, a member of the Lnk family adaptor proteins, is required for growth hormone induced actin reorganization and regulates cell motility (44, 45). These data suggest that Lnk could associate with an actin-containing complex and may control the actin cytoskeleton during cell division or migration. The relatively maintained splenic architecture in *lnk*-transgenic mice suggests that B cell migration during maturation was unperturbed. However, enlargement of T1 and T2 B cells in transgenic mice might reflect Lnk function in controlling the actin cytoskeleton. In line with the reduction in the fraction of cycling B cells, Lnk might regulate expansion of precursor and mature B cells by preventing both entry into the cell cycle and cell division, in part via effects on cytoskeletal remodeling involving actin.

A mutant form of Lnk lacking the C-terminal tyrosine residue conserved among Lnk family proteins still efficiently inhibited lymphopoiesis in transgenic mice. This is consistent with observations obtained from transfection experiments using the c-Kit$^+$ mast cell line MC9 (20). Although Y536 of Lnk was the main c-Kit target phosphorylation site, SCF-dependent growth of MC9 cells was inhibited by Y536F as well as by wild-type Lnk. In contrast, APS inhibits Janus kinase 2- or platelet-derived growth factor receptor-mediated signaling in combination with c-Cbl, and the phosphorylation of the C-terminal tyrosine is essential for c-Cbl-binding and subsequent APS inhibitory effects (46, 47). This suggests that the Lnk inhibitory function on c-Kit and as yet unidentified signaling cascade(s) is accomplished by Lnk subdomains other than the C-terminal tyrosine and may involve a mechanism unique to Lnk. In contrast to the negative regulatory role of Lnk in lymphopoiesis, positive regulatory roles for SH2-B and APS in signaling via receptors for various cytokines and growth factors have been reported (26–32). Thus, despite the significant structural similarities between Lnk, APS, and SH2-B, their functions appear to be quite different from each other.

In summary, we used transgenic mice overproducing Lnk to demonstrate that this adaptor protein is a critical regulator of lymphocyte production. Expansion of lymphoid precursors was severely impaired in *lnk*-transgenic mice. The skewed peripheral B cell subpopulations, enlarged size of transitional T1 B cells, reduced cycling splenic B cells, and altered humoral immune responses in *lnk*-transgenic mice suggest a potential function for Lnk in peripheral lymphocytes. Thus our results precisely complement studies of *lnk*-deficient mice. In addition, they suggest that Lnk may perform previously undescribed functions in peripheral B cells and during T cell development. Taken together, these genetic studies in mice illuminate the novel negative regulatory mechanism by the Lnk adaptor protein in controlling lymphocyte production and function.

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Regulation of Hematopoietic Development in the Aorta-Gonad-Mesonephros Region Mediated by Lnk Adaptor Protein

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Development of hematopoietic cells in the aorta-gonad-mesonephros (AGM) region in the midgestation mouse embryo involves a multistep process, sequentially changing from endothelial cell-like cells, including hemangioblasts, into hematopoietic stem cells, progenitors, and/or lineage-committed cells. An adaptor molecule, Lnk, is known to negatively control the production of pro- and pre-B cells and hematopoietic progenitor cells in adult bone marrow. Here we show a role of Lnk in hematopoietic development in the AGM region. Lnk was predominantly expressed in the endothelial cells lining the dorsal aorta at embryonic day 11.5 (E11.5). Overexpression of Lnk in the primary culture of the AGM region at E11.5 suppressed the emergence of CD45$^+$ hematopoietic cells. Point mutation in the SH2 domain of Lnk, which abolishes the binding capability of Lnk to c-Kit upon stimulation with stem cell factor (SCF), led to loss of Lnk-dependent inhibition of hematopoietic cell development in AGM cultures, suggesting Lnk-mediated inhibition of the SCF/c-Kit signaling pathway. In cultured AGM cells from Lnk homozygous mutant mouse embryos, the number of emerged CD45$^+$ cells was 2.5-fold larger than that from heterozygous littermates. Furthermore, aorta cells of E11.5 Lnk homozygous mutant mice also showed enhanced hematopoietic colony-forming activity. Thus, Lnk is a negative regulator of hematopoiesis in the AGM region.

Hematopoietic stem cells are the source of all mature blood cells, erythrocytes, granulocytes, monocytes, platelets, and lymphocytes (13, 31). In early development in the mouse, hematopoiesis first arises from the blood islands of the yolk sac at embryonic day 7.5 (E7.5) and subsequently occurs in the para-aortic splanchnopleura region at E7.5 to 9.5, in parallel with the yolk sac, and in the aorta-gonad-mesonephros (AGM) region at E10.5 to 11.5 (4, 15, 16, 23). Hematopoietic stem cells are thought to migrate to the fetal liver and further emigrate to the spleen and bone marrow by the time of birth (6). Long-term repopulating hematopoietic stem cells, which express several marker proteins, emerge in the AGM region of the mouse at midgestation. In vitro differentiation of embryonic stem (ES) cells gives rise to a population of definitive hematopoietic cells via endothelial precursor cells (3, 7, 25, 27). These observations indicated the possibility that hematopoietic stem cells in early definitive hematopoiesis differentiate from the endothelial precursor cells and/or hemangioblasts, which are the precursors of both hematopoietic and endothelial cells.

Endothelial cells are known to differentiate into hematopoietic cells in the primary culture system of the AGM region at E10.5 to 11.5 (22). When the AGM region of the mouse embryo at E11.5 was cultured with cytokines such as stem cell factor (SCF), basic fibroblast growth factor (bFGF), and oncostatin M (OSM), the endothelial cell-like cells are first evident after a few days of culture, and then nonadherent cells including hematopoietic progenitors are detected and gradually increase in the culture (22). In the culture of the AGM region from mouse embryos lacking the transcription factor c-Myb and Runx1 (AML1, Cbfa2, and Pebp2αB), which are known to be important for hematopoiesis, endothelial cell-like cells are generated but not hematopoietic cells (20, 21). These results are in accord with the in vivo phenotype of mice lacking c-Myb or Runx1 and in vitro developmental experiments using Runx1-deficient ES cells (11). Moreover, introduction of cDNA for c-Myb or Runx1 into the cultured AGM from these mutant embryos partially restored the production of nonadherent cells (20, 21).

Lnk is composed of a number of functional regions, including the N-terminal region, which is likely to be required for multimerization, the pleckstrin homology domain, which is suggested to have a role in binding to phospholipids or other proteins, the Src homology 2 (SH2) domain, which is known to be critical for specific binding to a phosphotyrosine residue, and a Tyr phosphorylation motif that is phosphorylated in response to SCF. Lnk is thus suggested to have a role as an adaptor protein. It was originally reported that tyrosine-phosphorylated Lnk is bound to the SH2 domain of Grb2, phospholipase C γ-1, and phosphatidylinositol 3-kinase in activated T cells (8) and functions as a negative mediator of the T-cell receptor signaling pathway (12). However, development and activation of T cells are normal in Lnk-deficient mice. In contrast, these mice exhibit a significant increase in the number of pre-B cells in the spleen and pre- and pro-B cells in the bone marrow, indicating that Lnk has an important role in regulating B-cell development (40). In a more recent study on Lnk-

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deficient mice, Takaki et al. demonstrated that the mice display a significant increase in hematopoietic progenitor cells in the adult bone marrow (39).

Lnk has structural similarities to APS and SH2-B, which both contain the multimerization, pleckstrin homology, and SH2 domains, and these three proteins form a family of adaptor proteins (32, 40, 45). APS and SH2-B have been reported to associate with insulin receptor (1, 10, 18, 34), Trk family receptors (33, 36), platelet-derived growth factor receptor (35, 46), and tyrosine kinase Janus kinase (JAK) (37, 44). APS is tyrosine phosphorylated in response to B-cell antigen receptor stimulation (9). The existence of four SH2-B splice variants (α, β, γ, and δ) derived from the same gene has recently been reported (47).

In the present study, we observed the expression of Lnk in the AGM region of the E11.5 mouse embryo, in particular in the endothelium of the dorsal aorta. We also show that introduction of Lnk into the primary culture of the AGM region resulted in inhibition of the generation of hematopoietic cells. This inhibition was abolished by a defect in the binding of the SH2 domain of Lnk to the c-Kit cytoplasmic region. We further demonstrated that the number of the hematopoietic cells is increased in the AGM culture derived from homozygous Lnk mutant mice compared to heterozygotes. Our results reveal that the Lnk adaptor protein negatively regulates AGM hematopoiesis.

MATERIALS AND METHODS

Tissue culture. The AGM regions were excised at E11.5 from ICR or Lnk-targeted embryos and trypsinized. Cells were suspended in Dulbecco's modified Eagle's medium supplemented with 15% (vol/vol) fetal calf serum and cultured in gelatin-coated 12- or 24 well-plates in the presence of 100 ng of murine SCF (Pepro Tech Inc. Rocky Hill, N.J.), 1 ng of bFGF (R&D Systems, Minneapolis, Minn.), and 10 ng of OSM (R&D Systems) per ml.

Retrovirus infection of cells. cDNA was inserted into a pMY-IRES-EGFP vector. Plat-E cells for packaging ecotropic retrovirus were plated the night before transfection (19). Transient transfection of Plat-E cells with plasmid DNA was performed with Trans IT-293 reagent (Mirus, Madison, Wis.) according to the manufacturer's protocol. Cells were incubated for 48 h and the supernatant was collected and used for infection. The viral supernatant was added together with 1 μg of Polybrene per ml and cytokines as described above. After incubation for 9 h, the virus-containing medium was replaced with standard growth medium. Infected cells were confirmed by the fluorescence of green fluorescent protein (GFP).

Semisolid colony-forming assays. Nonadherent cells in the AGM culture and the trypsinized primary aorta cells of E11.5 embryos were suspended in minimal essential medium, alpha modification, containing 0.8% (wt/vol) methylcellulose, 30% fetal calf serum, 1% deionized bovine serum albumin, 100 μM 2-mercaptoethanol, 20 ng of murine interleukin-3 (IL-3)/ml, 100 ng of SCF/ml, and 4 U of erythropoietin (EPO)/ml. Cells were cultured in triplicate in 35-mm dishes at 37°C for 7 days. Individual colonies, GFP^+ cells, and all cells were scored by morphology.

Flow cytometry. After being washed in phosphate-buffered saline (PBS) containing 3% (vol/vol) fetal calf serum and 0.05% sodium azide, the nonadherent cells in the AGM culture were incubated for 30 min on ice with phycoerythrin-conjugated rat anti-mouse CD45 (30-F11) (Becton Dickinson, Lincoln, N.J.) and analyzed by FACSCalibur (Becton Dickinson). The percentage of the $CD45^+$ cells in the GFP^+ cells was determined.

Nonadherent cells were reacted with biotinylated anti-mouse Mac1 (M1/70), Gr1 (RB6-8C5), Ter119 (TER119), B220 (RA3-6B2), CD4 (RM4-5), and CD8 (53-6.7) (Becton Dickinson). After washing, cells were incubated with streptavidin-conjugated magnetic beads (Miltenyi Biotech). Lin^- cells ($Mac\text{-}1^-$ $Gr\text{-}1^-$ $Ter119^-$ $B220^-$ $CD4^-$ $CD8^-$) were separated with the MACS system (Mirus). Isolated cells were incubated with phycoerythrin-conjugated anti-mouse Sca-1 (E13-161.7) and allophycocyanin-conjugated anti-mouse c-Kit (2B8) (Becton Dickinson) and analyzed. The percentage of $c\text{-}Kit^+$ and $Sca\text{-}1^+$ cells in the GFP^+ cells was determined.

RT-PCR. Total RNAs were isolated from E9.5, 11.5, and 14.5 aorta, E14.5 fetal liver, and adult mouse muscle of adult mouse (negative control). cDNAs were synthesized with 5 μg of total RNAs as templates in 20 μl of the reaction mixture with Superscript II reverse transcriptase (Gibco-BRL, Rockville, Md.). PCRs were carried out with rTaq (Takara) with the following settings: 95°C for 3 min and 26 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 1 min. The primer sets used were as follows: 5′-CTCAAGGAGGTCGTATTGCGCTA-3′, 5′-TTCCAGTGGGATAGGAGAACGC-3′ (for Lnk); 5′-GAGACGACGACAGCGGTGGGTGCT-3′, 5′-GATGGGGTGGGTGTGGAAGTGACG-3′ (for APS); 5′-GAGGGGCCTCCAGCAGGGACA-3′, 5′-GCCTCTTCTGCCCCAGGATGT-3′ (for SH2-B); 5′-ACCACAGTCCATGCCATCAC-3′, 5′-TCCACCACCCTGTTGCTGTA-3′ (for glyceraldehyde-3-phosphate dehydrogenase).

Immunohistochemical staining. Mouse embryos at E11.5 were fixed in 2% (wt/vol) paraformaldehyde-PBS overnight at 4°C, equilibrated in 20% sucrose–PBS for 2 h at 4°C, quick-frozen in Tissue Tek, and stored at −70°C. Sections (5 μm) were washed with PBS and blocked in 3% fetal calf serum–PBS. Sections were then stained with control rabbit IgG, anti-Lnk (the C-terminal region of Lnk) (41), or anti-CD34 (RAM34) (Becton Dickinson) for 2 h at room temperature, followed by washing and treatment with a rhodamine-conjugated secondary antibody for 2 h. After washing, bisbenzimide H33258 fluorochrome trihydrochloride (Nacalai Tesque, Kyoto, Japan) was used to stain the nuclei.

Immunoblotting and coimmunoprecipitation analysis. Cultured AGM cells (6×10^5) were infected with mock or Flag-tagged Lnk mutant retroviruses and a c-Kit retrovirus. On the next day, cells were starved for 16 h and then treated with SCF (100 ng/ml) for 20 min. After 20 min, cells were dissolved with the lysis buffer (0.5% Nonidet-40, 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 5 μg of aprotinin/ml). Lysates were immunoprecipitated with anti-Flag antibody (M2; Sigma, St. Louis, Mo.) or anti-c-Kit antibody (M-14; Santa Cruz Biotechnology, Santa Cruz, Calif.). The immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-c-Kit antibody or anti-Flag antibody. Labeled proteins were detected with an enhanced chemiluminescence system (ECL, Amersham Bioscience Corp, Piscataway, N.J.).

Luciferase assay. Elk activation was measured by the GAL-4 DNA-binding domain (DB)/Elk-1 fusion system according to the manufacturer's protocol (PathDetect in vitro signal transduction pathway trans-reporting system, Stratagene). Briefly, 293 cells (0.8×10^6) plated on 12-well plates were transfected with Elk-1 consisting of GAL-4 DB and Elk-1 (25 ng), pFR-Luc carrying the GAL-4 upstream activation sequence-fused luciferase gene (50 ng), pRL-CMV encoding the sea pansy luciferase gene (25 ng), c-Kit (67.5 ng, for SCF stimulation), and Lnk expression vectors (0.1, 0.3, 1, 3, or 10 ng) with Trans-IT 293 (Mirus). On the following day, cells were stimulated with SCF (20 ng/ml), bFGF (1 ng/ml), or IL-6/soluble IL-6 receptor (sIL-6R) (20 ng/ml) for 6 h and then solubilized. Luciferase activities in cell lysates were assessed with the Pikkagene dual luciferase assay system (Tokyo Ink Inc., Chuou-ku, Tokyo, Japan) and a MicroLumat LB96P luminometer (Berthold Technologies GmbH & Co. KG, Calmbacher, Bad Wild, Germany).

RESULTS

Expression and localization of Lnk in the AGM region. We first examined the expression of Lnk in fetal hematopoietic sites by RT-PCR (Fig. 1A). Transcripts for Lnk were detectable in the AGM region at E9.5, 11.5, and 14.5 and the fetal liver at E14.5, but not in the muscle of adult mice. We further analyzed the expression of Lnk protein in the AGM region at E11.5 by immunohistochemical staining of transverse sections (Fig. 1B). Lnk was present in the endothelial cells lining the dorsal aorta. These results indicate that Lnk is expressed in the AGM region at a stage of embryonic hematopoiesis. The expression pattern of Lnk overlapped with that of CD34 in the dorsal aorta, suggesting that Lnk might be involved in hematopoietic cell development from endothelial precursors.

Inhibition of hematopoietic differentiation in the AGM cultures by Lnk. Recent studies have shown that primary culture of the AGM region from mouse embryos at E11.5 can be used to examine the mechanism by which hematopoiesis of multi-

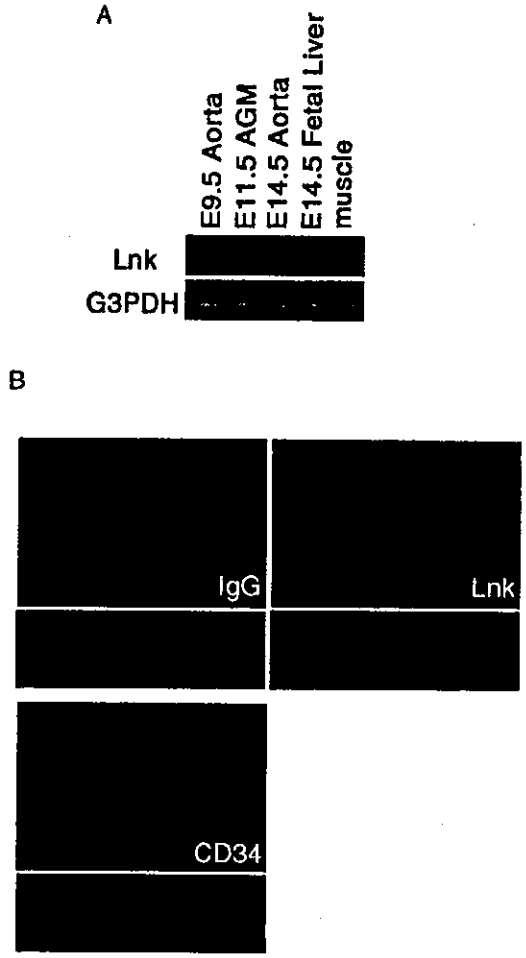


FIG. 1. Expression of Lnk in the AGM region. (A) Total RNAs were extracted from E9.5, 11.5, and 14.5 aorta, E14.5 fetal liver, and adult mouse muscle and then subjected to RT-PCR with specific primers for Lnk. (B) Tissue sections of E11.5 embryos were stained with control IgG antibody, anti-Lnk polyclonal antibody, and anti-CD34 monoclonal antibody. The lower panel shows a higher magnification view of endothelial cells. Endothelial cells lining the dorsal aorta are stained with anti-Lnk antibody. G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

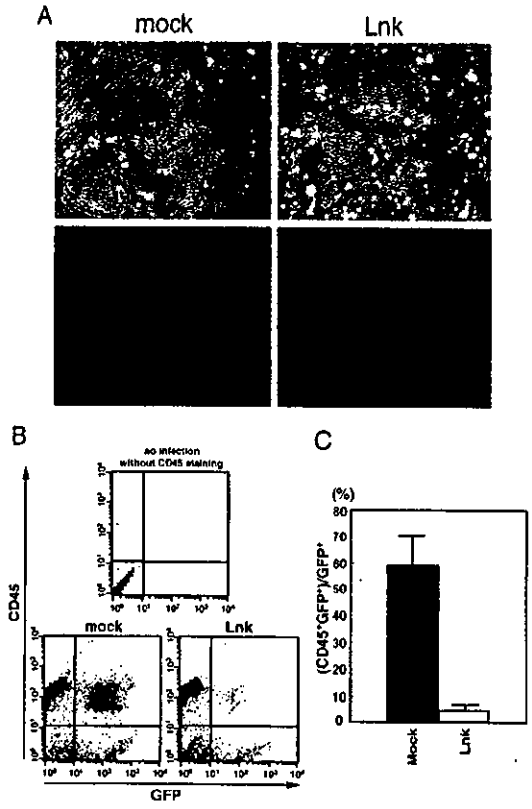


FIG. 2. Inhibition of hematopoietic differentiation by Lnk in cultured AGM cells. (A) E11.5 AGM cells were cultured with SCF (100 ng/ml), bFGF (1 ng/ml), and OSM (10 ng/ml). On day 2 of culture, cells were infected with GFP and/or Lnk retrovirus. On day 7 of culture, adherent cells in the vehicle- and Lnk-infected AGM cultures showed normal proliferation, and no significant difference in the number of the GFP^+ adherent cells between these cultures was observed. (B) On day 7 of culture, nonadherent cells were stained with anti-CD45 antibody, and 10^4 cells were analyzed by flow cytometry. Representative plots of four independent experiments are shown. (C) The percentage of the $CD45^+$ cells in the GFP^+ cells was determined. Error bars indicate the standard error of the mean ($n = 4$).

potential progenitors occurs in vitro (20–22, 28, 29). Addition of SCF, bFGF, and OSM to this culture system is required for the efficient expansion of hematopoietic progenitors from endothelial cell-like cells. To examine whether Lnk functions in the production of hematopoietic cells from the endothelial cell-like cells, we retrovirally overexpressed Lnk protein in the AGM culture. Lnk cDNA was ligated into a retroviral vector containing an internal ribosomal entry sequence (IRES) motif linked to the enhanced GFP (EGFP) to the track the transduced cells. Adherent cells in a 2-day AGM culture were infected with this retrovirus, and the infection was monitored by GFP expression.

Five days after infection, Lnk- and vehicle-infected adherent cells showed similar morphology and viability, as examined after removal of nonadherent cells by moderate pipetting (Fig. 2A). The expression of Lnk protein from the Lnk-IRES-GFP retrovirus in the infected cells was confirmed by immunoblotting (see Fig. 5). Expression of GFP in vehicle- and Lnk-infected cases was observed in the adherent cells, especially in the endothelial cell-like cells (Fig. 2A). These results indicate that retrovirally expressed Lnk has no influence on the growth or survival of the adherent cells in the AGM culture. Five days after infection, nonadherent cells in the AGM cultures were analyzed by flow cytometry with monoclonal antibodies against CD45, which is a marker of hematopoietic cells except for erythrocytes. As shown in Fig. 2B, overexpression of Lnk resulted in a dramatic reduction in CD45-positive cells. The percentage of $CD45^+$ GFP^+ cells to total GFP^+ cells in the Lnk-infected culture was only 4.3%, which was in marked contrast to that in the vehicle-infected culture (57.8%) (Fig. 2C). This result implies an inhibitory function of Lnk for hematopoietic differentiation in the AGM cultures.

Next, to further examine the effects of Lnk on AGM hematopoiesis, lineage marker-negative cells were isolated from the nonadherent cells in AGM cultures by negative selection and stained with c-Kit and Sca-1 antibodies. It has been reported

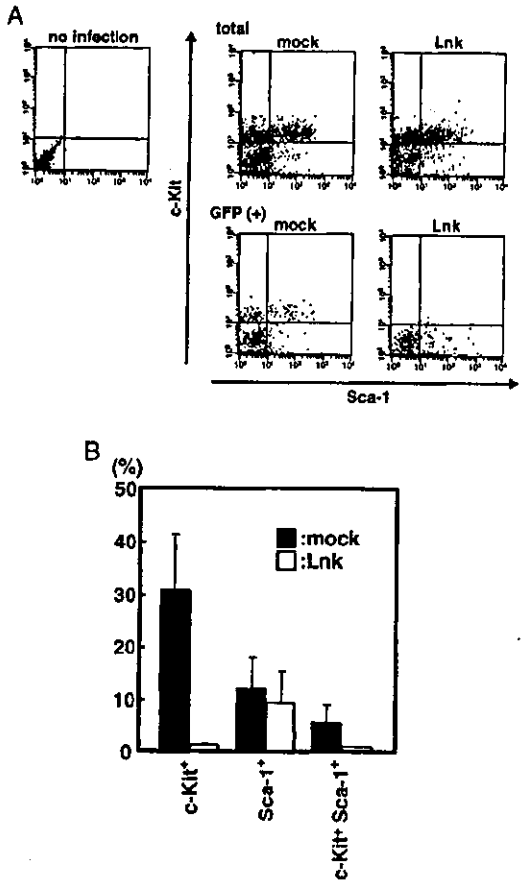


FIG. 3. Lnk-driven inhibition of the appearance of c-Kit on cultured AGM cells. (A) E11.5 AGM cells were cultured with SCF, bFGF, and OSM. On day 2 of culture, cells were infected with GFP or Lnk retrovirus. On day 7 of culture, Lin^- cells were separated from nonadherent cells with magnetic microbeads. Purified cells were stained with anti-c-Kit and Sca-1 antibodies and analyzed by flow cytometry. In each flow cytometric profile, 3×10^3 (total cells, upper three panels) or 5×10^2 (GFP^+ cells, lower two panels) events are recorded. (B) The percentage of c-Kit^+ and Sca-1^+ cells in the 5×10^2 GFP^+ cells was determined. Error bars indicate the standard error of the mean ($n = 5$).

that Lin^- c-Kit^+ Sca-1^+ cells contain most of the long-term multilineage reconstituting activities in the murine bone marrow. As shown in Fig. 3, the Lin^- c-Kit^+ Sca-1^+ GFP^+ cell fraction was markedly decreased in the Lnk-infected AGM cultures. The percentages of nonadherent cells that expressed c-Kit in the GFP-positive cells were 1.3% of Lnk-infected cells and 31.7% of vehicle-infected cells (Fig. 3B), suggesting that Lnk inhibits the appearance of c-Kit^+ cells.

Furthermore, the colony-forming activity of nonadherent cells from the AGM culture was assayed in methylcellulose medium containing SCF, IL-3, and EPO. The Lnk-expressing nonadherent cells derived from the AGM cultures were unable to form colonies containing granulocytes, macrophages, and erythrocytes (Table 1). These results indicate that Lnk has a role in the negative regulation of hematopoiesis in the AGM, at least in culture.

Essential role for the SH2 domain of Lnk in hematopoietic differentiation in AGM culture. To determine the region important for the inhibitory activity of Lnk, we constructed a series of point and deletion mutants of Lnk: a mutant (Y536F) with a substitution of Phe for Tyr^{536} which is phosphorylated in response to SCF stimulation (39); a mutant (R346E) with a substitution of Glu for the conserved Arg in the SH2 domain which is known to abolish binding to a phosphotyrosine-containing target; and mutants devoid of the N-terminal region (ΔN), the pleckstrin homology domain (ΔPH), and the C-terminal half (N-PH). As shown in Fig. 4, R364E and N-PH did not work as inhibitors of hematopoiesis. These data suggest that the SH2 domain of Lnk is essential for inhibition of hematopoiesis in the AGM culture.

It has been shown that Lnk is associated with the SCF receptor c-Kit (39). To examine the molecular mechanism of interaction between Lnk and c-Kit, we expressed wild-type Lnk, R364E mutant, and c-Kit proteins in the AGM culture. SCF stimulation induced the association between Lnk and c-Kit in the AGM culture, and the R364E substitution resulted in a loss of their interaction (Fig. 5). Thus, the cytoplasmic region of c-Kit likely conforms to the binding site of the SH2 domain of Lnk in the intermolecular interaction, which raises the possibility that hematopoiesis in the AGM culture was blocked by the inhibition of the c-Kit signal caused by the interaction of Lnk.

Effect of Lnk on SCF, bFGF, and OSM signaling. We have previously shown that a stable transfectant of Lnk attenuated activation of mitogen-activated protein kinase as detected by phosphorylation of Erk upon stimulation with SCF (39). To evaluate the functional role of Lnk in SCF-, bFGF-, and OSM-induced signaling, we examined the effect of Lnk on the activation of Elk-1, one of the nuclear targets of Erk, reporter activity with 293 cells. 293 cells transfected with an Elk-1 reporter were used for this experiment. Since 293 cells do not express OSM receptors but express gp130, the signal transduc-

TABLE 1. Colony-forming activities in AGM-derived nonadherent cells in culture[a]

Cell source for colony assay	Infection	No. of colonies per 10^5 nonadherent cells				
		CFU-G	CFU-M	CFU-GM	CFU-E	CFU-Mix
GFP^+ cells	Mock	9.8 ± 5.3	58.0 ± 2.3	1.8 ± 1.0	0.0 ± 0.0	0.7 ± 0.3
	Lnk	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
GFP^{+-} cells	Mock	85.9 ± 7.2	605.3 ± 8.9	16.8 ± 2.3	11.3 ± 4.5	8.5 ± 3.3
	Lnk	109.0 ± 7.2	699.3 ± 10.0	20.3 ± 1.8	5.5 ± 6.1	5.5 ± 2.8

[a] Nonadherent cells expanded in the retrovirus-infected AGM cultures were inoculated in methylcellulose medium containing SCF (100 ng/ml), IL-3 (20 ng/ml), and EPO (4U/ml). Colonies were scored at 7 days of culture. Colony types: G, granulocyte; M, macrophage; GM, granulocyte and macrophage; E, erythrocyte; Mix, granulocyte, macrophage, erythrocyte, and megakaryocyte. Results represent the mean ± standard error of triplicate samples.

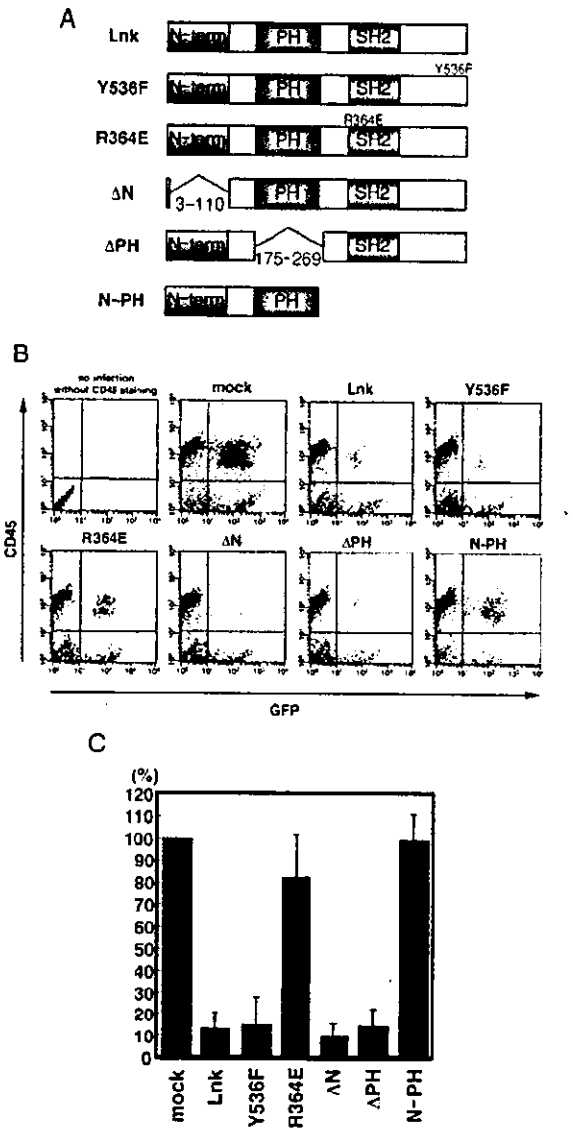


FIG. 4. Ability of various Lnk mutants to inhibit hematopoietic differentiation in cultured AGM cells. (A) The structures of the Lnk mutants are represented schematically. (B) E11.5 AGM cells were cultured with SCF (100 ng/ml), bFGF (1 ng/ml), and OSM (10 ng/ml). On day 2 of culture, cells were infected with retrovirus encoding GFP and/or Lnk mutants. On day 7 of culture, nonadherent cells were stained with anti-CD45 antibody, and 10^4 cells were analyzed by flow cytometry. (C) The percentages of $CD45^+$ cells among the Lnk mutant-expressing GFP^+ cells were determined. These data were normalized to the value in the mock culture which was set at 100%. Error bars indicate the standard error of the mean ($n = 4$).

ing receptor component of the OSM receptor complex, IL-6/sIL-6R fusion protein was used in place of OSM because IL-6/sIL-6R is known to stimulate gp130. It should be noted that IL-6/sIL-6R and OSM have similar biological effects on the AGM culture (42).

First, the 50% effective concentrations of the cytokines SCF, bFGF, and IL-6/sIL-6R for Elk-1 reporter activity were determined by dose-response studies: SCF, 20 ng/ml; bFGF, 1 ng/ml; and IL-6/sIL-6R, 20 ng/ml (data not shown). The effect of Lnk was examined in the AGM culture with either SCF, bFGF, or IL-6/sIL-6R at concentrations of 20, 1, and 20 ng/ml, respectively. As shown in Fig. 6, Lnk dose-dependently inhibited SCF-induced Elk-1 activation. In contrast, Lnk did not significantly affect bFGF- and IL-6/sIL-6R-induced Elk-1 activation (Fig. 6). These results suggest that Lnk selectively inhibited the SCF-induced Erk pathway activation.

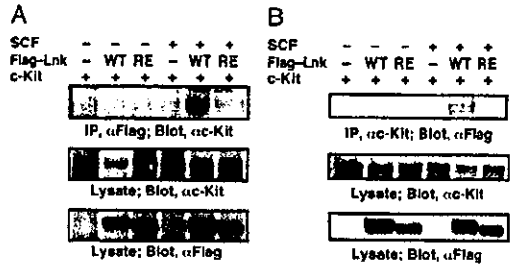


FIG. 5. Requirement of the SH2 domain of Lnk for interaction with c-Kit in cultured AGM cells. (A and B) Cultured AGM cells were infected with retroviruses encoding either Flag-tagged Lnk or Lnk-R364E together with a retrovirus encoding c-Kit. The cells were stimulated with SCF (100 ng/ml) for 20 min. Cell extracts were subjected to immunoprecipitation with anti-Flag antibody (A) and anti-c-Kit antibody (B). Precipitates or lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-c-Kit antibody (A) and anti-Flag antibody (B). The expression of Lnk and c-Kit was monitored.

Ability of Lnk family proteins, APS and SH2-B isoforms, to inhibit hematopoietic differentiation in cultured AGM cells. We examined the effects of the Lnk family proteins APS and SH2-B splicing variants on hematopoietic differentiation in cultured AGM cells. We first analyzed Lnk family protein expression in the AGM region at E11.5 by RT-PCR. As shown in Fig. 7A, transcripts for APS and a common part of the SH2-B splicing variants were observed in the AGM region at E11.5. Next, to investigate functional differences in the inhibitory activities of Lnk family proteins, we expressed Lnk, APS, and SH2-B isoforms (α, β, and δ) with retroviruses in AGM cultures (Fig. 7B). Overexpression of either APS or SH2-B isoforms could not inhibit the proliferation of the $CD45^+$ cells, indicating that the inhibitory effect of Lnk on the AGM-cultured cells was not generally applicable to other Lnk family proteins (Fig. 7C and D). Furthermore, we examined SCF-mediated interaction between Lnk family proteins and c-Kit in the AGM culture. Lnk bound strongly to c-Kit in the presence of SCF, but no other family members showed significant binding (Fig. 7E). Based on these observations, it seems that Lnk is highly effective in regulating hematopoiesis in AGM cells.

Role of Lnk in AGM hematopoiesis. In light of these results obtained from the Lnk overexpression experiments, we examined the effect of Lnk deficiency on embryonic hematopoiesis with AGM cells from E11.5 Lnk $^{+/-}$ and Lnk $^{-/-}$ littermates. As shown in Fig. 8A, a larger number of nonadherent cells were observed in Lnk $^{-/-}$ AGM cultures than in Lnk$^{+/-}$ cultures, while the expansion of adherent cells in Lnk $^{-/-}$ cultures was comparable to that in Lnk$^{+/-}$ cultures. Moreover, flow cytometric analysis showed that the expression of CD45 increased in nonadherent cells from Lnk $^{-/-}$ AGM cultures (Fig. 8B). The numbers of $CD45^+$ nonadherent cells in each well

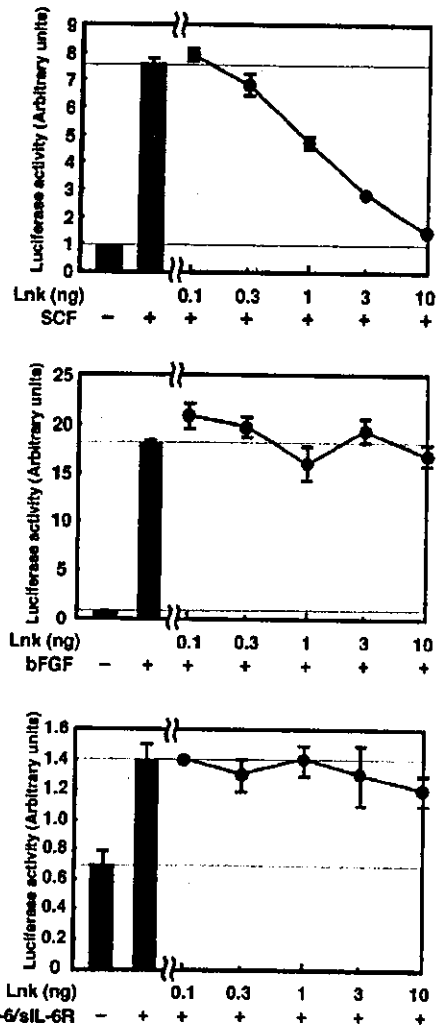


FIG. 6. Effect of Lnk on SCF-, bFGF-, and IL-6/sIL-6R-induced Elk-1 reporter activity. 293 cells were transfected with an Elk-1 reporter plasmid and the indicated amounts of Lnk plasmid. In the experiment with SCF, the c-Kit plasmid was also used. The cells were stimulated on the following day with either SCF (20 ng/ml), bFGF (1 ng/ml), or IL-6/sIL-6R (20 ng/ml) for 6 h, and luciferase activities in the cell lysates were measured.

and that in each embryo were both greater in Lnk $^{-/-}$ than in Lnk$^{+/-}$ mice (Fig. 8C).

Furthermore, the emergence of colony-forming activities of nonadherent cells was examined in the in vitro AGM culture. The frequency of nonadherent cells forming a colony in Lnk $^{-/-}$ mice was approximately twofold higher than that in Lnk$^{+/-}$ mice (Table 2). These results indicate that the AGM region of E11.5 Lnk$^{-/-}$ mice had a higher capacity to differentiate into hematopoietic cells than did that of Lnk$^{+/-}$ mice. To examine the ability of AGM cells with Lnk deficiency to form hematopoietic colonies, we performed the same experiments with the exception of omitting the 7-day culture. In contrast to the primary AGM cells of heterozygous littermates (Lnk$^{+/-}$), those of homozygous mice (Lnk$^{-/-}$) showed increased capability for colony formation (Table 3). The data suggest a negative regulatory role of Lnk in AGM hematopoiesis in vivo.

DISCUSSION

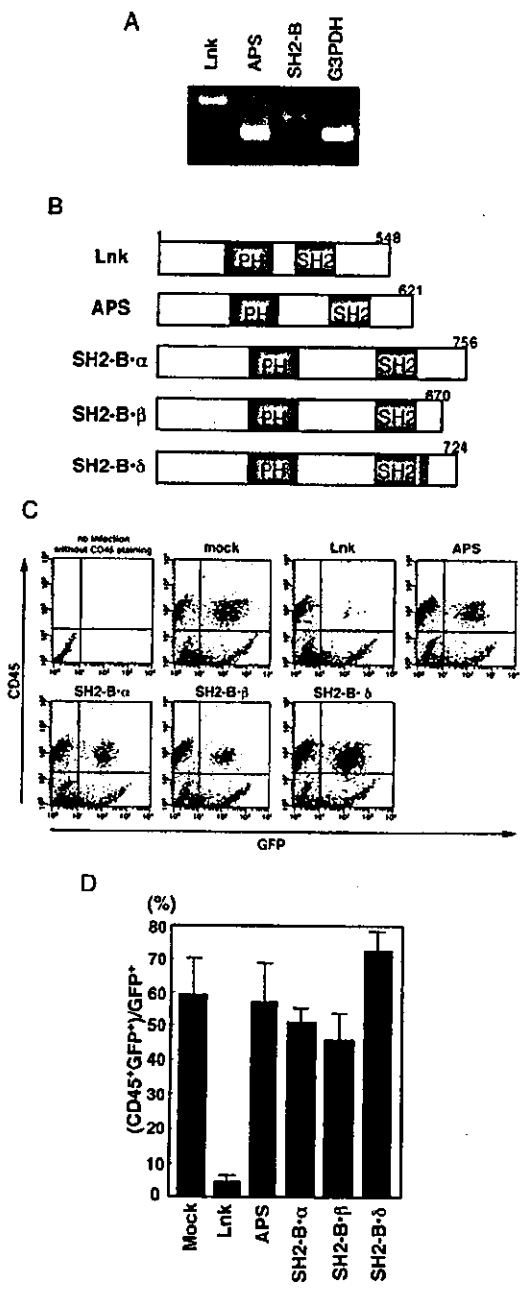
In the present study, Lnk was shown to function as a negative regulator of the development of hematopoiesis in the AGM region during mouse embryogenesis. We demonstrated that Lnk had inhibitory effects on hematopoietic differentiation in the AGM culture and colony-forming ability and that these inhibitory effects of Lnk were mediated by the binding of the Lnk SH2 domain to the phosphorylated c-Kit receptor.

Cultured AGM cells derived from E11.5 mouse embryos retain many of the characteristics of in vivo hematopoiesis at this stage. In this culture system, endothelial cell-like cells, including hemangioblasts, are thought to expand and subsequently generate nonadherent hematopoietic progenitors (Lin$^-$ c-Kit$^+$ Sca-1$^+$) and then CD45$^+$ hematopoietic cells. Finally, these nonadherent cells display the morphology and markers of specific lineages. From our results, the introduction of a retrovirus encoding Lnk-IRES-GFP into endothelial cell-like cells decreased the number of cells expressing c-Kit and CD45. The proliferation of GFP$^+$ adherent cells in the AGM culture was unaffected by introduction of Lnk compared to that in the vehicle-infected cells (Fig. 2A), and expression of Flag-tagged Lnk in the AGM culture was confirmed by immunoblotting analysis (Fig. 5).

Recently, Ly-6A, a component of Sca-1 protein in mice, was shown to be expressed in the single cell layer lining the dorsal aorta at E11.5 (5), while c-Kit expression is found in the ventral wall of the dorsal aorta and hematopoietic cell clusters, which attach to the ventral wall of the dorsal aorta. The expression pattern of Runx1/AML-1 in the endothelial cells of the dorsal aorta at E11.5 shows similarity with that of c-Kit, while the clusters of CD45$^+$ cells are also present on the ventral side (26). Considering the expression patterns of these molecules, endothelial cells in the midgestation mouse aorta are suggested to have long-term reconstitution activities. c-Kit$^+$ cells are observed in all hematopoietic stem cells in the AGM region (38). We demonstrated that Lnk was expressed in the endothelial cells lining the dorsal aorta (Fig. 1B), and the introduction of Lnk significantly reduced the number of c-Kit-positive cells (Fig. 3), suggesting that Lnk inhibited the differentiation of endothelial cells (hemangioblasts) into hematopoietic progenitors and lineage-committed cells.

In the previous studies, AGM cultures from transcriptional factor c-Myb or Runx1 null embryos, which die at E15.5 and E12.5, respectively, have not produced hematopoietic cells (21, 22). In *op/op* mice with defective macrophage colony-stimulating factor, the expansion of hematopoietic progenitors and the reduction in the expression of endothelial markers were observed in the AGM culture (17). Mice deficient for Lnk are born normally and show no developmental abnormalities in appearance (38) but had an enhanced colony-forming ability of the primary AGM cells at E11.5, as well as that for nonadherent cells from cultured AGM cells (Tables 2 and 3) and exhibited an increase in the number of hematopoietic progenitors (Lin$^-$ c-Kit$^+$ Sca-1$^+$) in the adult bone marrow (39).

Our data raise a couple of possibilities for the precise physiological function of Lnk in AGM hematopoiesis: a modulation



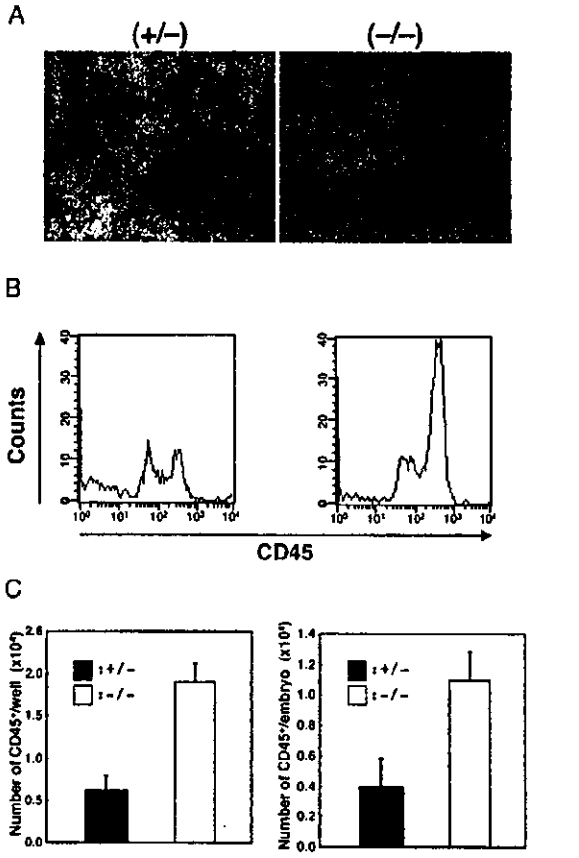


FIG. 8. Induction of hematopoietic differentiation in the $Lnk^{-/-}$ AGM culture. (A) Dynamic expansion of nonadherent cells from the $Lnk^{-/-}$ AGM culture. E11.5 AGM cells from $Lnk^{+/-}$ and $Lnk^{-/-}$ littermates were plated on gelatin-coated 24-well plates at a density of 1.5×10^5 per well with cytokines as described above. The proliferation rates of the adherent cells in the $Lnk^{-/-}$ and $Lnk^{+/-}$ cultures were comparable. After 7 days of culture, nonadherent cells were generated and counted. (B) Flow cytometric profiles of the nonadherent cells (10^4 cells) from the AGM culture stained with anti-CD45 antibody. (C) The numbers of $CD45^+$ nonadherent cells in each well (left) or each embryo (right) were determined by the percentages of $CD45^+$ cells in the nonadherent cells. Error bars indicate the standard error of the mean ($n = 3$).

FIG. 7. Ability of Lnk family proteins to inhibit hematopoietic differentiation in cultured AGM cells. (A) Expression of Lnk family proteins in the AGM region. Total RNAs were extracted from E11.5 aorta and subjected to RT-PCR with specific primers for each indicated molecule. (B) The structures of Lnk, APS, and SH2-B splicing variants (SH2-B α, β, and δ) are represented schematically. (C) E11.5 AGM cells were cultured with cytokines as described above. On day 2 of culture, cells were infected with retroviruses encoding GFP or Lnk family proteins. On day 7 of culture, nonadherent cells were stained with anti-CD45 antibody, and 10^4 cells were analyzed by flow cytometry. (D) The percentage of $CD45^+$ cells in the GFP^+ cells was determined. Error bars indicate the standard error of the mean ($n = 3$). (E) Flag-tagged Lnk family proteins were transfected into cultured AGM cells with a retrovirus carrying c-Kit. Cells were stimulated with SCF (100 ng/ml) for 20 min. Cell extracts were subjected to immunoprecipitation with anti-Flag antibody. Precipitates or lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-c-Kit antibody. The expression of Lnk and c-Kit was monitored.

TABLE 2. Colony-forming activities in AGM-derived nonadherent cell culture in Lnk-deficient mice[a]

Cell source for colony assay	No. of colonies per 2×10^5 nonadherent cells				
	CFU-G	CFU-M	CFU-GM	CFU-E	CFU-Mix
$Lnk^{+/-}$ cells	9.0 ± 2.6	51.3 ± 1.2	1.0 ± 0.0	0.7 ± 0.6	0.3 ± 0.6
$Lnk^{-/-}$ cells	21.3 ± 5.7	105.3 ± 8.1	1.7 ± 0.6	1.0 ± 1.0	0.3 ± 0.6

[a] Nonadherent cells expanded in the AGM cultures of Lnk-deficient mice were inoculated in methylcellulose medium containing SCF (100 ng/ml), IL-3 (20 ng/ml), and EPO (4 U/ml). Colonies were scored at 7 days of culture. Colony types: G, granulocyte; M, macrophage; GM, granulocyte and macrophage; E, erythrocyte; Mix, graneulocyte, macrophage, erythrocyte, and megakaryocyte. Results represent the mean ± standard error of triplicate samples.

of the timing of hematopoietic differentiation from the AGM regions by controlling SCF/c-Kit signaling and negative regulation of SCF/c-Kit signaling at the time of the movement of a site of definitive hematopoiesis from the AGM region to the fetal liver. Further studies regarding the molecular mechanisms of transcriptional regulation and these functions of Lnk should better clarify hematopoiesis during embryonic development.

We consider that Lnk acts through the c-Kit receptor to regulate the growth and differentiation of hematopoietic cells. Mukoyama et al. have shown that OSM is required for the development of hematopoietic progenitors in the AGM culture in the presence of SCF and bFGF (22). When cultured in serum-free medium with cytokine, E11.5 AGM cells were able to expand and differentiate into hematopoietic progenitor cells (data not shown). In serum-free culture, the nonadherent cells were sufficiently generated by the addition of only SCF, compared with those given SCF, bFGF, and OSM. This result indicates that the SCF/c-Kit signaling pathway is essential for the growth and differentiation of hematopoiesis in the AGM culture. Moreover, Lnk selectively inhibited the SCF signaling pathway, at least in 293 cells (Fig. 6). It is most likely that the overexpression of Lnk eventually led to the inhibition of the SCF/c-Kit signaling pathway in the AGM culture.

Lnk is an adaptor protein containing some functional regions or domains: the N-terminal putative multimerization region, the pleckstrin homology domain involved in interaction with phospholipids or other proteins, the SH2 domain capable of binding to phosphotyrosine residues, and the tyrosine 536 residue subjected to phosphorylation upon SCF stimulation (32, 39). As shown in the overexpression experiment with a series of Lnk mutants in the AGM culture, the SH2 domain of Lnk was a prerequisite for inhibition of the generation of $CD45^+$ nonadherent cells (Fig. 4). In addition, the SH2 domain of Lnk was involved in the interaction with c-Kit receptors (Fig. 5), suggesting that SCF-induced signaling might be inhibited by Lnk via the SH2 domain. SCF-induced tyrosine phosphorylation of c-Kit receptors generates the binding site for signal-transducing proteins, for example, Grb2, the p85 subunits of phosphatidylinositol 3-kinase, phospholipase C-γ1, and Src kinase, and consequently leads to proliferation, survival, calcium mobilization, cell migration, and differentiation (2, 14, 43). The mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways are known to be initiated by SCF stimulation. Expansion of nonadherent cells from AGM cultures was dose-dependently inhibited by the mitogen-activated protein kinase/ERK kinase inhibitor U0126 (I. Nobuhisa and T. Taga, unpublished data). A recent study has shown that a stable transformant of Lnk attenuates phosphorylation of Gab2 and activation of the mitogen-activated protein kinase pathway with an SCF-dependent mast cell line, MC9 (39). In present study, Lnk suppressed SCF-induced Erk activation (Fig. 6). Moreover, the SCF-induced phosphorylation levels of ERK and Akt (downstream of phosphatidylinositol 3-kinase) are reduced in $Gab2^{-/-}$ mast cells (24). These observations raise the possibility that Lnk blocks mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways by associating with c-Kit receptors.

Lnk is a member of the Lnk protein family including, for example, APS and SH2-B isoforms. Functional specificity is observed in Lnk family protein-deficient mice. Lnk-deficient mice exhibit a significant increase in the number of hematopoietic progenitors in the adult bone marrow (39, 40). In SH2-B-deficient mice, the numbers of follicles and sperms are both reduced compared with the wild type, resulting in small genital organs, ovaries, and testes (30). Among the Lnk family proteins, Lnk and APS have previously been reported to bind to c-Kit upon stimulation with SCF (9, 39, 46). Here we demonstrated that Lnk had inhibitory effects on AGM hematopoiesis but that APS and SH2-B did not, and, moreover, that a strong interaction of the SH2 domain of Lnk with c-Kit was observed in AGM culture. These results indicate that Lnk, but not APS or SH2-B, regulates AGM hematopoiesis.

c-Myb and Runx1 are known to play a positive regulatory role in the emergence of hematopoietic cells in the AGM culture (20, 21), whereas Lnk is revealed to function as a negative regulator in the present study. Since retrovirally expressed Lnk inhibited the appearance of GFP^+ $CD45^+$ cells in the AGM culture, the expression and/or transcriptional activity of c-Myb and Runx1 might be affected by Lnk. Negative regulators of cytokine signaling are most likely to modulate the amount or functional properties of transcription factors that are essential for AGM hematopoiesis.

TABLE 3. Colony-forming activities in primary E11.5 aorta cells in Lnk-deficient mice[a]

Cell source for colony assay	No. of colonies per 2×10^5 primary E11.5 aorta cells				
	CFU-G	CFU-M	CFU-GM	CFU-E	CFU-Mix
Lnk^{+-}	22.5 ± 4.7	107.3 ± 17.6	3.5 ± 2.1	2.3 ± 1.0	14.8 ± 3.0
Lnk^{--}	58.5 ± 13.8	175.5 ± 25.1	6.3 ± 3.0	2.5 ± 1.0	20.8 ± 3.6

[a] Primary E11.5 aorta cells from Lnk^{+-} and Lnk^{--} littermates were inoculated in methylcellulose medium containing SCF (100 ng/ml), IL-3 (20 ng/ml), and EPO (4 U/ml). Colonies were scored at 7 days of culture. Colony types: G, granulocyte; M, macrophage; GM, granulocyte and macrophage; E, erythrocyte; Mix, graneulocyte, macrophage, erythrocyte, and megakaryocyte. Results represent the mean ± standard error of quadruplicate samples.

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