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Original article

The Role of an LMO2/TAL1(SCL)/GATA complex in definitive hematopoiesis

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Abstract

The basic helix-loop-helix transcription factor Tal1(Scl), the LIM-finger protein Lmo 2, and the GATA-finger proteins Gata1/2/3 have important roles in hematopoiesis. Genetic disruption of Tal1(Scl) and Lmo2 individually results in defective yolk sac erythropoiesis and definitive hematopoiesis. These transcription factors physically interact with each other to form an Lmo2/Tal1/Gata1/2/3 transcription factor complex. In order to know whether these transcription factors form a complex to play critical roles in hematopoiesis, mutant Lmo2s were made to specifically disrupt the interaction between Lmo2 and Gata1/2/3 by introducing a mutation in the LIM 2 domain of Lmo2. Overexpression of these mutant Lmo2s in mice severely impaired fetal liver hematopoiesis and thymus development, indicating its role as a master complex for the development of the entire hematopoietic system. In this complex, Lmo2 functions as an adapter molecule and cell-autonomously determines the fate of each blood cell to either erythroid or lymphoid cell by recruiting Gata1/2/3 at different stage of hematopoietic differentiation.

Key words: transcription factor complex, hematopoiesis, LIM domain, protein-protein interaction, chromosomal translocation

Introduction

In mammalian development, the specification of blood cells is controlled by intrinsic and humoral factors. Hematopoiesis in the mouse embryo initiates in the yolk sac at about E7.5 and produces a burst of primitive nucleated erythrocytes (yolk sac erythropoiesis/primitive hematopoiesis). The first stem cell activity, which can produce all types of terminally differentiated blood cells including lymphopoiesis, appears in the aorta-gonad-mesonephros (AGM) region at about E10.5 (definitive hematopoiesis) (Cumano et al., 1996; Medvinsky and Dzierzak, 1996). Histological findings of liver of the 4 lines of transgenic mouse at P0. Fetal liver becomes the next main site of blood cell production at about E 12.5 until, eventually, bone marrow hematopoiesis starts. The regulation of hematopoiesis by transcription factors is the most important intrinsic mechanism (Krause, 2002; Shivdasani and Orkin, 1996). The erythroid, myeloid, and lymphoid lineages of blood cells are differentiated from a pluripotent hematopoietic stem cell, and their fates are partly determined by the composition of transcripts in each cell (Hu et al., 1997).

A number of transcription factors that play pivotal roles in embryonic hematopoiesis have been identified in the study of phenotypes of gene knock-out mice. Among them, the basic helix-loop-helix protein Tal1(Scl) and LIM-only protein Lmo2 are individually necessary in yolk sac erythropoiesis and definitive hematopoiesis for the development of the entire blood cell system (Porcher et al., 1996; Robb et al., 1995; Shivdasani et al., 1995; Warren et al., 1994; Yamada et al., 1998). Both *Tal1(Scl)* and *Lmo2* are initially identified from the breakpoint of chromosomal translocation associated with T-cell acute lymphoblastic leukemia, and the inactivation of each gene results in embryonic death at around E 10.5 due to a lack of yolk sac erythropoiesis. The expression of both proteins is observed in yolk sac blood islands at E7.5 (Silver and Palis, 1997) and later in endothelium of major

arteries in the AGM region at E10.5 (Elefanty et al., 1999; Yamada et al., 2000) and fetal liver at E12.5. Thus, the anatomical distribution of Tal1(Scl) and Lmo2 is compatible with their crucial roles at each stage of embryonic hematopoiesis.

The zinc-finger proteins, Gata1, Gata2, and Gata3 constitute a hematopoietic subgroup of GATA family transcription factors (Patient and McGhee, 2002; Weiss and Orkin, 1995). Loss of Gata1 function in mice leads to a lack of yolk sac erythropoiesis and blocks erythroid and megakaryocytic cell maturation (Pevny et al., 1991). Its expression is highly restricted to hematopoietic cells, specifically to the erythroid, megakaryocytic, mast, and eosinophilic cell lineages (Martin et al., 1990; Romeo et al., 1990). Gata2 is considered to have a broader and earlier function in hematopoiesis compared with Gata1 (Tsai et al., 1994): it is necessary for the proliferation and survival of early hematopoietic progenitors. Gata 3 plays roles at different stages of hematopoiesis. Gene disruption of Gata3 results in a decrease of fetal liver hematopoiesis (Pandolfi et al., 1995) and a failure of T-cell lineage development (Ting et al., 1996) It is also shown to be required for the terminal differentiation of helper T cells to the Th2 subtype (Ranganath and Murphy, 2001; Zheng and Flavell, 1997). In mouse embryogenesis, the expression pattern of Gata3 suggests its role in the initiation of definitive hematopoiesis in the Aorta-Gonad-Mesonephros (AGM) region in addition to its definite role in T-cell development (Manaia et al., 2000).

A similar phenotype (a profound defect in hematopoiesis) observed in the gene disruption of these hematopoietic transcription factors (Tal1, Lmo2, Gata1, Gata2, and Gata3) in mouse embryos is partly explained by the fact that these transcription factors physically bind to each other to form a transcription factor complex and the hypothesis that it is actually a master transcription factor complex for each stage of hematopoiesis (Wadman et al., 1997b). In this complex, LIM-only protein Lmo2 can act as a bridging molecule between Tal1(Scl) and Gata1/2/3 (one of the Gata1, Gata2, and Gata3), and the possible composition of the complex could be Tal1-Lmo2-Ldb1-Ldb1-Lmo2-Gata1/2/3, which can recognize the bipartite DNA site composed of E-box and GATA (Wadman et al., 1997b). In this complex, Ldb1 is a LIM domain binding protein, which can interact with Lmo2 and seems to function as a homodimer in the complex (Jurata et al., 1996). The synergic action of Tal 1(Scl), Lmo2, and Gata1 was demonstrated in *Xenopus* primitive hematopoiesis (Mead et al., 2001) and *Zebrafish* (Gering et al., 2003). In addition, all members of the transcription factor complex were isolated from the *c-kit* promoter (Lecuyer et al., 2002; Vitelli et al., 2000). Although these results strongly suggest the role of this transcription factor complex at each step of hematopoiesis, there is still no direct evidence that it plays a role in hematopoiesis as a complex.

In this study, in order to clarify the function of this transcription factor complex as a master complex for the initiation of hematopoiesis *in vivo*, we tried to disrupt the complex by introducing mutations in LIM domains (protein-binding domain) in a bridging molecule, Lmo2. Lmo2 is a small molecule comprising two tandem LIM domains (LIM1 and LIM2) that constitute a protein-protein interaction surface. It was shown that the LIM1 domain of Lmo2 is used for interaction with Tal1(Scl) and Ldb1 and that the LIM2 domain is used for interaction with Gata1/2/3. In addition, the interaction of Lmo2 with Gata1 is much weaker than that with Tal1(Scl) and Ldb 1. Overexpression of LIM2 mutants, which specifically disrupt the interaction between Lmo2 and Gata1/2/3, resulted in a severe defect in fetal liver hematopoiesis and the normal development of the thymus in mice. Our results showed that the transcription factor complex composed of Tal1(Scl), Lmo2, and Gata1/2/3 is needed for the very early stage of hematopoietic differentiation and gave us a unique model of blood cell fate specification by gradual transition of the component of the transcription factor complex.

Results

Mutation in LIM domains of LMO2 leads to disruption of protein-protein interaction

T cell oncoprotein Tal1(Scl), Lmo2, GATA-finger type transcription factor Gata1, Gata2, and Gata3 play critical roles in hematopoiesis individually. In addition, they physically bind to each other to form a transcription factor complex. The putative form of the complex is Tal 1-Lmo 2-Ldb 1-Ldb 1-Lmo 2-Gata 1 / 2 / 3 (Wadman et al., 1997a). In this complex, Lmo2

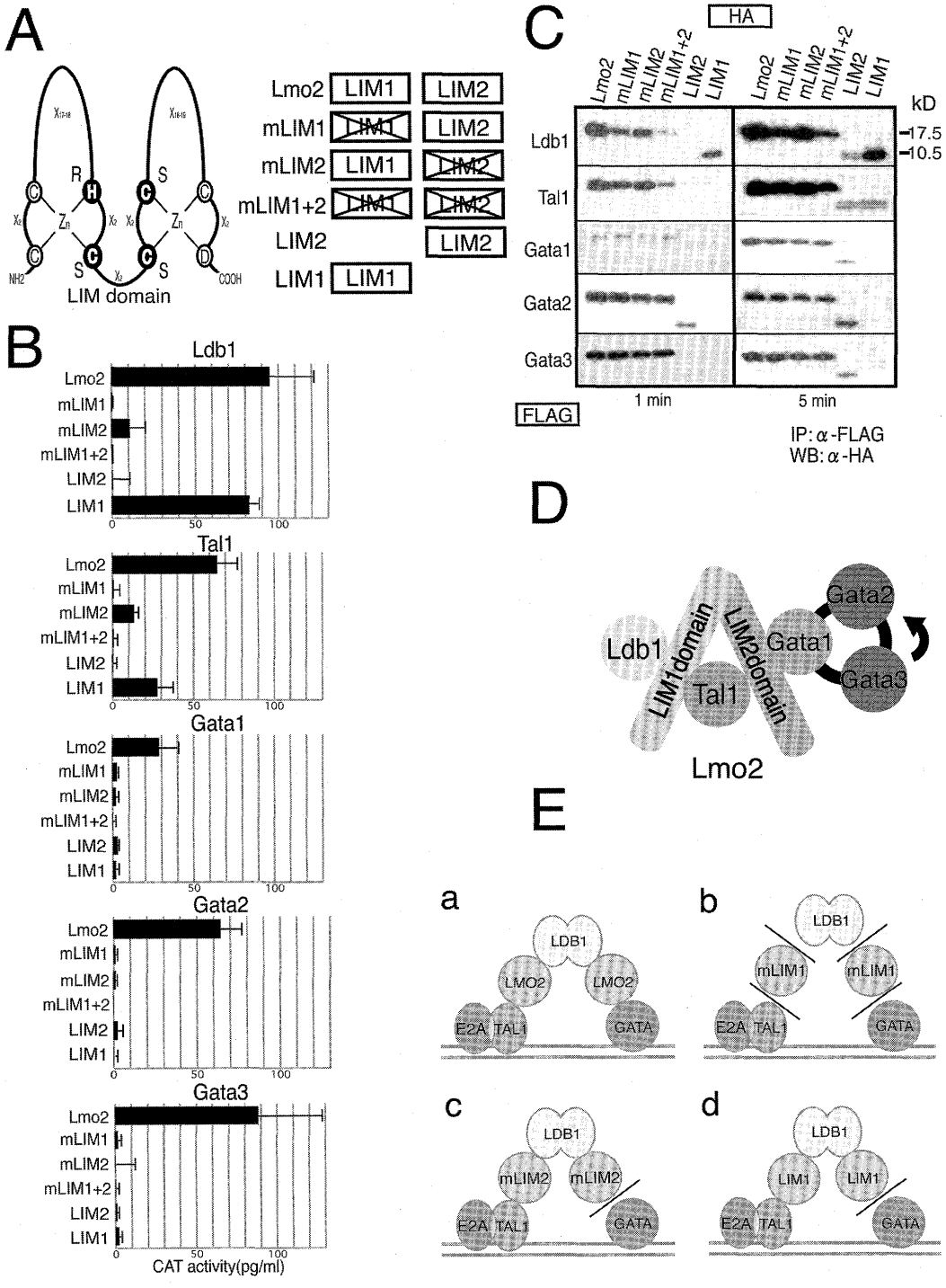
binds directly to Tal1, Ldb1, and Gata1/2/3, functioning as a bridging molecule between Tal1 and Gata1/2/3. Lmo2 is a member of the LIM only protein and composed of two tandemly repeated cysteine rich LIM domains from N-terminal, LIM1 and LIM2. Although the LIM domain is a zinc-binding finger-like structure, which is similar to the DNA-binding GATA finger, no direct LIM-nucleic acid interaction has been reported. In order to clarify the role of the LIM domain as a protein-protein interaction motif and, more importantly, to get the Lmo2 mutants, which specifically disrupt the protein-protein interaction in the putative complex, mutations and deletions were made in the LIM domains. As shown in Fig. 1A, five Lmo2 mutant constructs were made; in mutant LIM1 (mLIM1), four amino acids interacting with zinc in the LIM1 domain were replaced with other amino acids (51H to R, 54C to S, 57C to S, and 60C to S). Mutant LIM2 (mLIM2) has a similar replacement in the LIM2 domain (116H to R, 119C to S, 122C to S, and 125C to S). The mutant LIM1+2 (mLIM1+2) has the same mutations in both LIM1 and LIM2. Deletion mutants lacking the LIM1 domain (LIM2/delLIM1) and lacking LIM2 domain (LIM1/delLIM2) were also made.

Protein interaction between each mutant Lmo2 (mLIM1, mLIM2, mLIM1+2, LIM2, and LIM1) and Lmo2 partners in the putative complex (Ldb1, Tal1, and Gata1/2/3) was first studied in a yeast two-hybrid system and secondly in a mammalian two-hybrid system. The interaction between Lmo2 and Ldb1 was the only positive one in the yeast two-hybrid system, and other interactions were not compatible in this system. YRG-2 yeast strains, which were transformed using the Gal 4 activation domain-Ldb1 fusion plasmid (pAD-GAL4-Ldb1), the Gal4 DNA-binding domain-wild-type / mutant Lmo 2 s fusion plasmids (pBD-GAL 4-Lmo 2 s), and the β-galactosidase/HIS3 reporter plasmid, were cultured on yeast-selective agar plates. Ldb1 and Lmo2 bound to each other and led to growth on selective plate and blue colonies. Weaker interaction (light-blue colonies) was seen between Ldb1 and mLIM2; however, no interaction was detected between Ldb1 and mLIM1/mLIM1+2 (Data not shown). This result in yeast indicates that the LIM domain in Lmo2 is indeed the protein-protein interaction motif, and the LIM1 domain is chiefly used in the interaction between Ldb1 and Lmo 2, which is compatible with the previous report (Jurata et al., 1996). Secondly, five fusion construct plasmids of the activation domain and Ldb1, Tal1(Scl), Gata1, Gata2, and Gata3 (pVP16-Ldb1, pVP16-Tal1, pVP16-Gata 1, pVP16-Gata2, and pVP16-Gata3) and six fusion construct plasmids of the GAL4-DNA-binding domain and wild-type/mutant Lmo2s (pM-Lmo2, pM-mLIM1, pM-mLIM2, pM-mLIM 1+2, pM-LIM2, and pM-LIM1) were made. Each pair of pVP16 fusion constructs and the pM fusion construct were co-transfected with a CAT (chloramphenicol acetyltransferase) reporter plasmid into 10T1/2 cells, and CAT activity was measured. Figure 1B shows the results of this mammalian two-hybrid screening. In the interaction between Ldb1 and mutant Lmo2s, mLIM2 and LIM1 bound to Ldb1; however, the interaction of mLIM2 with Ldb1 was weaker than that between wild-type Lmo2 and Ldb1. The interaction of Tal1 and mutant Lmo2s showed a similar pattern; mLIM2 and LIM1 had interaction with Tal1, indicating that the LIM1 domain is the main motif that mediates the protein interaction of Lmo2 with Ldb1 and Tal1. On the other hand, the interactions between wild-type/mutant Lmo2s and Gata1/2/3 showed distinctive patterns. The binding of Gata1 to wild-type Lmo2 was relatively weaker than those of other partners of Lmo2, and none of the five Lmo2 mutants (mLIM1, mLIM2, mLIM1+2, LIM2, and LIM1) showed remarkable binding activity to Gata1/2/3. This strongly suggests that the LIM2 domain is also involved in the interaction between Lmo2 and Gata1/2/3.

The LIM2 domain in LMO2 is important for the interaction between LMO2 and GATA1/2/3

In order to clarify the protein interaction properties of five Lmo2 mutants more extensively, the third method, immunoprecipitation combined with Western blot, was applied. FLAG-tagged Ldb1, Tal1, and Gata1/2/3 were cloned into the expression vector pEF-BOS, and HA-tagged wild-type/mutant Lmo2s were also cloned into the same vector. A pair of FLAG-tagged and HA-tagged fusion constructs was co-transfected to 293F cells (Invitrogen, CA). Cell lysate immunoprecipitated using anti-FLAG M2 affinity gel (Sigma-Aldrich, Inc., MO) was applied to Western blot with an anti-HA antibody (Roche Diagnostics Corporation, Germany). As shown in Fig. 1C,

wild-type Lmo2 was co-immunoprecipitated with Ldb1, Tal1, and Gata1/2/3. Most interestingly, LIM1, which lacked the LIM2 domain, was co-immunoprecipitated with Ldb1 and Tal1 but not with Gata1/2/3. Although this method was not completely quantitative, the amount of immunoprecipitated mLIM2 protein was larger than that of the mLIM1 protein in the case of co-transfection with Ldb 1 and Tal1. In the case of co-transfection with Gata 1 / 2 / 3, more mLIM 1 protein was immunoprecipitated than mLIM2. Taken all these data into consideration, in the bridging molecule Lmo2, the LIM1 domain is mainly used for the interaction with Ldb1 and Tal1, the LIM2 domain for Gata1/2/3 (Fig. 1D).



Overexpression of Lmo2 mutants, which have mutations in the LIM2 domain, results in a fetal liver hematopoiesis defect and thymus disorganization

In the search for Lmo2 mutants, which can specifically disrupt the protein-protein interaction in the putative transcription factor complex for hematopoiesis, the protein interaction properties of each mutant Lmo2 have been revealed. Among five Lmo2 mutants, LIM1(delLIM2) had a distinctive and interesting pattern of protein interaction; it can bind to Ldb1 and Tal1 but not to Gata1/2/3. mLIM2 showed a similar pattern of interaction, but less typically than LIM1. The interactions between mLIM1 and Ldb1, Tal1, and Gata1/2/3 were largely decreased; however, weak interactions between them remained. In order to disrupt the Tal1-Lmo2-Ldb 1-Ldb 1-Lmo 2-Gata 1 / 2 / 3 transcription factor complex *in vivo* by overexpressing dominant negative Lmo2 mutants and making aberrant complexes in a competitive manner, we made four lines of transgenic mice ubiquitously expressing wild-type Lmo2, mLIM1, mLIM2, and LIM1 driven by a promoter of the human elongation factor gene. In the case of overexpression of mLIM2 and LIM 1, they might sequester Ldb1 and Tal1(Scl) from the physiological complex (Fig. 1E-c, d). On the other hand, overexpression of mLIM1 might have little effect on hematopoiesis in TG mice because the interaction between mLIM1 and all five Lmo2 partners decreased significantly (Fig. 1E-b). Four kinds of DNA fragments were injected in a fertilized egg in exactly the same manner. In each line of transgenic mice, the copy numbers of each transgene were estimated using Southern-blot with Sac I-digested genomic DNA, and the ones that showed the highest copy number were used as the founders of each transgenic line. As shown in Fig. 2A, we obtained an mLIM1 transgenic mouse (TG), which had a high copy number in this procedure. Wild-type Lmo2 TG mice had the second-highest number of copies, and the number of copies of LIM1 TG born alive was the lowest of the four TG mice. Every four lines of TG embryos expressed transgene-specific mRNA (Fig. 2B).

Four lines of TG were crossed with wild-type C57BL/6 mice to obtain germ-line trans-

Fig. 1

A. Structure of LIM domain and mutations made in the LIM domains of Lmo2. The LIM domain is a double zinc-finger structure. The amino acids (C, H, and D) interacting with zinc are indicated by circles. Residues shown in reverse type were mutated (H into R and C into S) to disrupt the zinc-finger structure. Lmo2 is composed of two tandemly repeated LIM domains (LIM1 and LIM2). mLIM1 has this mutation in its LIM1 domain, mLIM2, in the LIM2 domain, and mLIM1+2, in both LIM1 and LIM2. Deletion mutants of Lmo2, which were lacking the LIM 1 domain (LIM2) or LIM2 domain (LIM1), were also made.

B. Mammalian two-hybrid assay of the interaction between wild-type/mutant Lmo2s and Ldb1, Tal1(Scl), and Gata1/2/3. 10T1/2 cells were co-transfected with a pair of Gal4 DNA-binding domain fusion and Gal4 DNA-activation domain fusion plasmids as indicated. The intensity of the protein-protein interaction between two proteins was estimated as CAT activity measured using the CAT ELISA Kit (Roche Diagnostics Corp.). In this assay, Ldb1 and Tal1 can bind to mLIM2 and LIM1, indicating that the LIM1 domain of Lmo2 is the interacting surface for Ldb1 and Tal1. On the other hand, none of the 5 Lmo2 mutants can bind to Gata1/2/3, indicating that the LIM2 domain is also needed for their interaction. The interaction between Lmo2 and Gata1 is weaker than that between Lmo2 and each of the other Lmo2 partners.

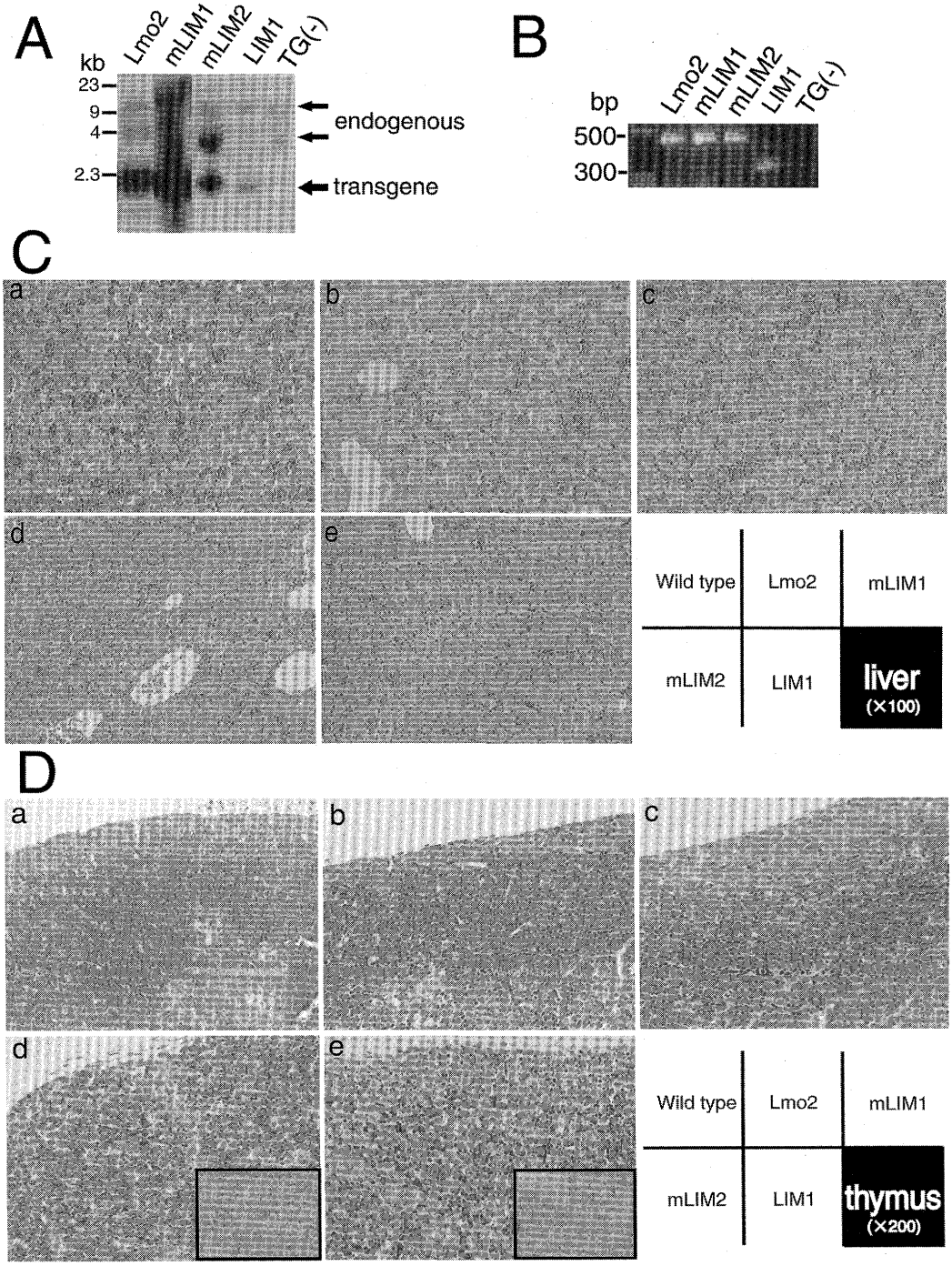
C. Protein-protein interaction profile of the wild-type/mutant Lmo2s using Western blot coupled with immunoprecipitation. 293F cells were co-transfected with a pair of expression vectors (pEF-BOS) coding the HA-tagged wild-type/mutant Lmo2s and FLAG-tagged Lmo2 partners (Ldb1, Tal1, and Gata1/2/3). The cell lysate was immunoprecipitated with anti-FLAG monoclonal antibody, and immunoprecipitates were analyzed by Western blotting with HRP-conjugated anti-HA antibody. Blotting results were shown after two different exposure times (1 minute and 5 minutes). Note that LIM1 can bind to Ldb1 and Tal1 but not to Gata1/2/3. mLIM2 keeps its interaction with Ldb1 and Tal1 ; however, its interaction with Gata1/2/3 becomes slightly weaker.

D. Summary of the roles of the LIM1 and LIM2 domain of Lmo2 in protein-protein interaction. The LIM1 domain of Lmo2 is mainly used for its interaction with Ldb1 and Tal1, forming the backbone of the basic transcription factor complex throughout the early stage of hematopoietic specification. The LIM2 domain, on the other hand, provides the interacting surface for Gata1/2/3 and recruits each Gata-type zinc-finger transcription factor at a different stage of blood cell differentiation.

E. Expected composition of the transcription factor complex composed of wild-type Lmo2 and mutant Lmo2s (mLIM 1, mLIM2, and LIM1). a. Physiological form of the transcription factor complex composed of E2A, Tal1, Lmo2, Ldb 1, and Gata. b. According to the results of the mammalian two-hybrid assay of protein-protein interaction, mLIM1 cannot bind efficiently with Tal1, Ldb1, or Gata1/2/3. Overexpression of mLIM1 might not affect the formation of a physiological complex. c, d. mLIM2 and LIM1 can interact with Tal1 and Ldb1 but not with Gata1/2/3. Overexpression of mLIM2 and LIM1 might inhibit the formation of a physiological complex in a competitive manner.

mission, and P0 mice were sacrificed soon after birth for microscopic analysis of the liver, bone marrow, and thymus. The presence of a transgene in the genomic DNA of each mouse was determined using Southern blot of tail DNA. All four lines of TG mice were fertile. In LIM1 TG mice, about half of the transgene-positive P0 mice were pale and smaller than their transgene-negative littermates, and they usually died soon after birth.

As shown in Fig. 2C-a, a number of blood cell islands, including colonies of erythroblasts, were seen in the liver sinusoids of wild-type P0 mice. In Lmo2 and mLIM1 TG mice, comparable numbers of blood cell islands were seen in the liver (Fig. 2C-b, c). However, marked decreases in the number of blood cells in the P0 liver were seen in mLIM2 and LIM1 TG mice (Fig. 2C-d, e). In addition to the decrease of P0 liver hematopoiesis, disorganization of thymic architecture and massive apoptotic death of thymocytes were ob-



served in the P0 thymi of mLIM 2 (this finding was seen in 4 mice out of 39 transgene-positive mice) and LIM1 TG mice (seen in 10 out of 31 mice) (Fig. 2 D-d, e). Apoptotic thymocytes were positively stained by direct immunoperoxidase detection of digoxigenin-labeled genomic DNA (Fig. 2D-d, e inset). P0 thymus of wild-type mice typically has well organized lamellar architecture (from outside to inside, sub-capsular zone, cortex and medulla) as shown in Fig. 2D-a. This architecture was well preserved in all P0 thymi of Lmo2 and mLIM1 TG mice analyzed.

Table 1 Hematopoietic progenitors in fetal liver (E12.5)

Mouse strain (n)	E	M	Mix	Total
Wild type (6)	1350±454	7050±1368	1525±487	9925±2120
Lmo2 (6)	877±372	3709±1354	934±379	5520±1461
mLIM1 (2)	965±558	7820±2645	1430±141	10215±1945
mLIM2 (6)	310±268	1724±702	573±360	2607±1139
LIM1 (5)	187±73	1073±731	288±126	1548±909

Abbreviations: n, numbers of embryos analyzed; E, erythroid colonies; M, myeloid colonies; Mix, erythro-myeloid mixed colonies. Wild type, C57BL/6 mouse strain.

The decrease of blood cells in the liver demonstrated in two lines of TG mice was quantitatively assessed using the method of hematopoietic colony assay of E12.5 (embryonic day 12.5) fetal liver. Male mice of Lmo2, mLIM1, mLIM2, and LIM1 TG lines were crossed with wild-type female C57BL/6 mice to get E12.5 embryos. A single-cell suspension of each six E12.5 fetal livers of Lmo2, mLIM1, mLIM2, and LIM1 TG was prepared, and 5×10^3 cells per mouse were plated in 1ml Methocult (StemCell Technologies, Canada). Hematopoietic (erythroid, myeloid, and mixed) colonies were counted after 7 days of incubation. Other parts of the embryos were used for genomic DNA analysis of the inheritance of the transgene. Table 1 shows the number of hematopoietic cell colonies (erythroid, myeloid, and mixed) per liver of transgene-positive E12.5 embryos. Among four TG mouse lines, only mLIM1 TG mice had an equivalent number of hematopoietic cell colonies per E12.5 fetal liver to that of wild-type C57BL/6 mice. A slight decrease in the number of colonies was seen in Lmo2 TG mice. The marked decrease in the number of both erythroid and myeloid colonies observed in mLIM2 and LIM1 TG mice was compatible with the defective liver hematopoiesis exhibited in P0 mice (Fig. 2C-a-e).

Because no transgenic mice with a high number of copies of mLIM2 and LIM1(delLIM 2) were born alive and defective liver hematopoiesis at the stage of P0 combined with thymic architecture disorganization was seen, irrespective of the relatively low number of copies of mLIM2, and, especially, LIM1 transgenes, we suspected that a high dose of mLIM2 and LIM1 proteins caused embryonic lethality in TG mice. In order to study the effect of the high dose of the transgenes, mLIM2 and LIM1, DNAs (human elongation factor promoter-mutant Lmo2s-polyA) were injected into fertilized eggs and transferred to the uterus. In the mLIM2 injection experiment, a total of 78 embryos were analyzed at E12.5. Among them, 15 embryos were transgene-positive (usually with a medium to high number of copies of the transgene), and 14 out of 15 transgene-positive embryos

Fig. 2

A. Copy number of the transgene coding Lmo2, mLIM1, mLIM2, and LIM1 in transgenic mice (TG) born alive. The Southern blot analysis of 10μg of SacI-digested genomic DNA hybridized with an Lmo2 cDNA probe is shown. Bands of transgene and endogenous Lmo2 are indicated. The mLIM1 TG mouse has the highest copy number and LIM1 TG mouse, the lowest.

B. The presence of transgene-derived mRNA in 4 lines of transgenic mouse strains. RNA was isolated from E12.5 embryos of 4 lines (Lmo2, mLIM1, mLIM2, and LIM1) of transgenic mice and transgene-negative littermate (TG(−)). Result of reverse transcriptase-dependent PCR using transgene-specific synthetic oligonucleotides (see Methods section) is shown.

C. Histological findings of liver of the 4 lines of transgenic mouse at P0. Livers were dissected out from 4 lines (Lmo 2, mLIM1, mLIM2, and LIM1) of P0 transgenic mice. Organs were fixed in 10% buffered formalin, and sections were stained with hematoxylin and eosin (H&E). a-e show the findings of the liver. At the P0 stage, a number of blood cell colonies can be seen in the liver sinusoid of a wild-type mouse (a) and mLIM1 TG mouse (c). Note the marked decrease in the number of blood cell colonies in the P0 liver of mLIM2 (d) and LIM1 TG mouse (e).

D. Histological findings of thymuses of the 4 lines of transgenic mouse at P0. A clear structure composed of three layers of thymocytes (subcapsular, cortex, and medulla) is seen in the P0 thymi of wild-type (a), Lmo2 TG (b), and mLIM1 TG (c) mice. Disorganization and disruption of the normal architecture of the P0 thymus are seen in mLIM 2 TG (d) and LIM1 TG (e) mice. A number of apoptotic cells are seen in those sections as detected by direct immunoperoxidase staining of digoxigenin-labeled genomic DNA (d, e inset).

Table 2 Correlation between the presence of transgene and macroscopic liver findings (loss of hemoglobinization) in transgenic founder E12.5 embryos.

EF-BOS-mLIM2 founders

	Loss of hemoglobinization in E12.5 fetal liver		
	Positive	Negative	Total
Transgene (+)	14	1	15
Transgene (−)	21	42	63
Total	35	43	78

$\chi^2=26$

EF-BOS-LIM1 founders

	Loss of hemoglobinization in E12.5 fetal liver		
	Positive	Negative	Total
Transgene (+)	13	4	17
Transgene (−)	24	41	65
Total	37	45	82

$\chi^2=13$

The liver phenotype is significantly correlated with the presence of the transgenes, mLIM2 and LIM1, because the chi-square (χ^2) values are larger than 7.88 (significant level 0.5%).

were slightly smaller than their littermates without the transgene, and, most importantly, had a pale liver (Table 2), which suggested a decreased production of red blood cells within the liver, as shown in Fig. 3A. Microscopic findings in serial sagital sections of these embryos consistently revealed underdeveloped heart and liver (Fig. 3B-b), a marked decrease of fetal liver hematopoiesis (Fig. 3 B-e), and a lack of well hemoglobinized definitive erythrocytes in the peripheral blood (Fig. 3B-k). In the LIM1 injection experiment, a total of 82 E12.5 embryos were studied. Seventeen embryos had the transgene, and some of them had many more copies of the transgene than those seen in P0 LIM1 TG mice. Interestingly, 13 of 17 transgene (LIM1/delLIM2)-positive embryos showed a very similar phenotype characterized by a small and pale liver (Table 2), as seen in the mLIM2 -injected one (Fig. 3A). They also showed decreased fetal liver blood cell production and definitive erythrocytes in the peripheral blood (Fig. 3B-c, f, i, l). As far as we examined, there were no other abnormalities besides those consistently seen in the hematopoietic and circulatory system in the all mLIM2 and LIM 1 (delLIM 2) transgene-positive E 12.5 embryos.

Lmo2 dissociation from the complex is needed for the terminal differentiation of erythroid cells

The effect of the presence of wild-type and five mutant Lmo2s (mLIM1, mLIM2, mLIM1+2, LIM2/delLIM1, and LIM1/delLIM2) on *in vitro* erythroid differentiation was studied using a MEL (murine erythroleukemia) cell erythroid differentiation system induced by dimethyl sulfoxide (DMSO). MEL-DS19 cells, which expressed wild-type and five mutant Lmo2s (mLIM1, mLIM2, mLIM1+2, LIM2/delLIM1, and LIM1/delLIM2) by retrovirus infection, were induced by 1.8% DMSO for 3 days. The proportions of hemoglobin-containing cells were estimated by staining with dianisidine. As shown in Fig. 4, about 35% of MEL-DS19 cells expressing only EGFP (enhanced green fluorescent protein) turned out to be dianisidine-positive cells after induction. Persistent expression of wild-type Lmo2 in MEL-DS19 cells inhibited the erythroid differentiation, which was compatible with the previous observation (Visvader et al., 1997). However, overexpression of mLIM1 and LIM2(delLIM1) had no effect on the differentiation of MEL-DS19 cells. Because the protein-protein interaction between mLIM1/LIM2 and five Lmo2 partners (Ldb1, Tal1, Gata1/2/3) was almost abolished in a mammalian two hybrid assay (Fig. 1B), the inhibitory effect of persistent Lmo2 expression on the differentiation of MEL-DS19 cells into red blood cells was dependent on its interaction with Lmo2 partners. Interestingly, overexpression of mLIM2 and LIM1(delLIM2), which kept its interaction with Ldb1 and Tal1 but not with Gata1/2/3, also inhibited the differentiation of MEL-DS19 cells.

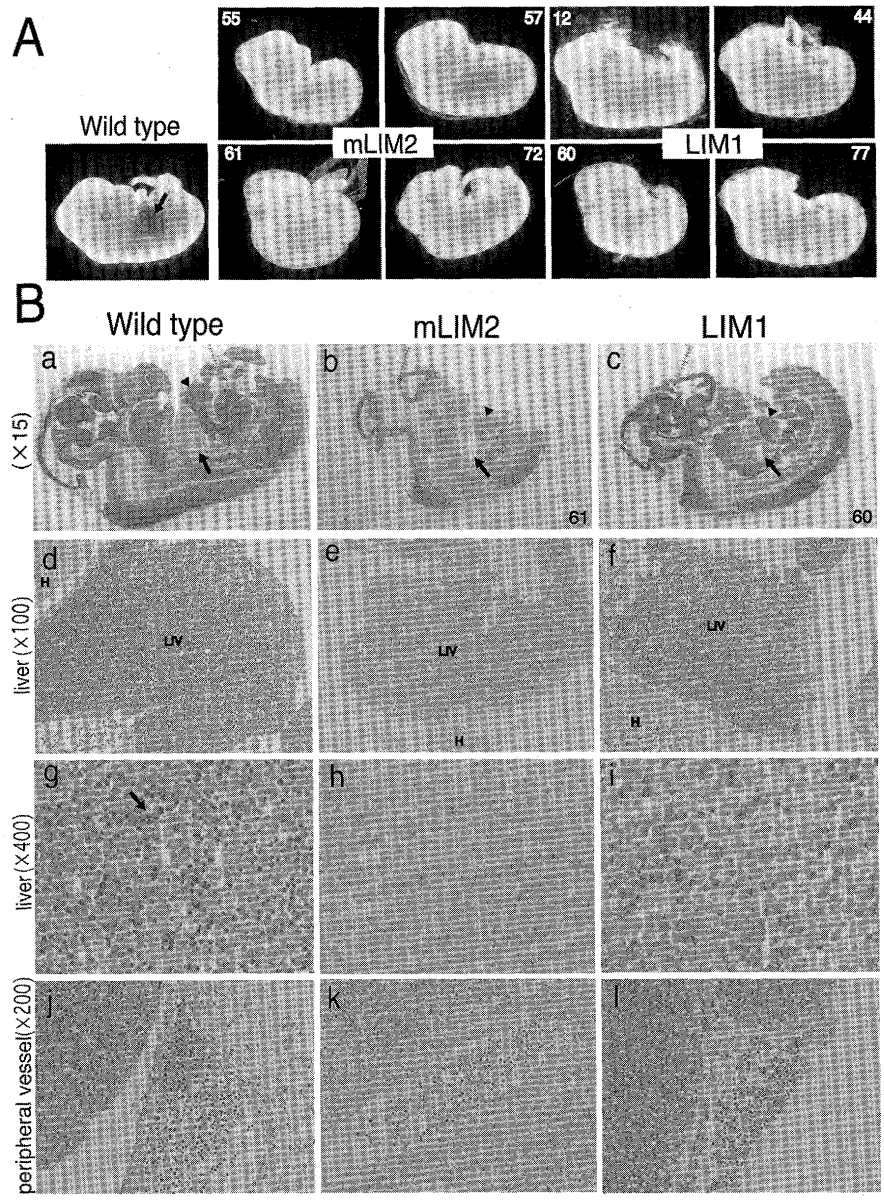


Fig. 3 Defective fetal liver (definitive) hematopoiesis appeared in E12.5 founder embryos, which have a high dose of the mLIM2 or LIM1(delLIM2) transgene. DNA fragments composed of the human elongation factor promoter-mLIM2/LIM1-human G-CSF poly-adenylation signal were injected into fertilized eggs, and they were transferred to the uterus. A total of 78 mLIM2-injected E12.5 and 82 LIM1-injected E12.5 embryos were analyzed at E12.5. Genotyping was done using Southern blot of yolk sac DNA, and 15 mLIM2 transgene-positive and 17 LIM1 transgene-positive embryos were obtained.

A. Macroscopic findings of E12.5 founders, which have higher copy numbers of the mLIM2 or LIM1 (delLIM2) transgene. Note the small and pale (less hemoglobinized) livers of transgenic mouse embryos as compared with the wild-type E12.5 embryo.

B. Sagital section of whole-mount E12.5 embryos and microscopic findings of mLIM2 and LIM1 (delLIM2) TG mouse embryos (liver(arrowhead), peripheral blood, heart (arrow)). mLIM2 and LIM1 embryos usually have small livers and hearts (b and c). The number of blood cell colonies in their fetal liver sinusoids is markedly decreased (e, f, h, and i) as compared with that of the wild-type embryo (d and g). Few well-hemoglobinized red blood cells (indicated by arrow in g) are seen in their liver (h and i). The decrease in the number of definitive erythrocytes, which have a smaller nucleus than primary erythrocytes, is also observed in their peripheral blood (k and l). Abbreviations; LIV, fetal liver; H, fetal heart.

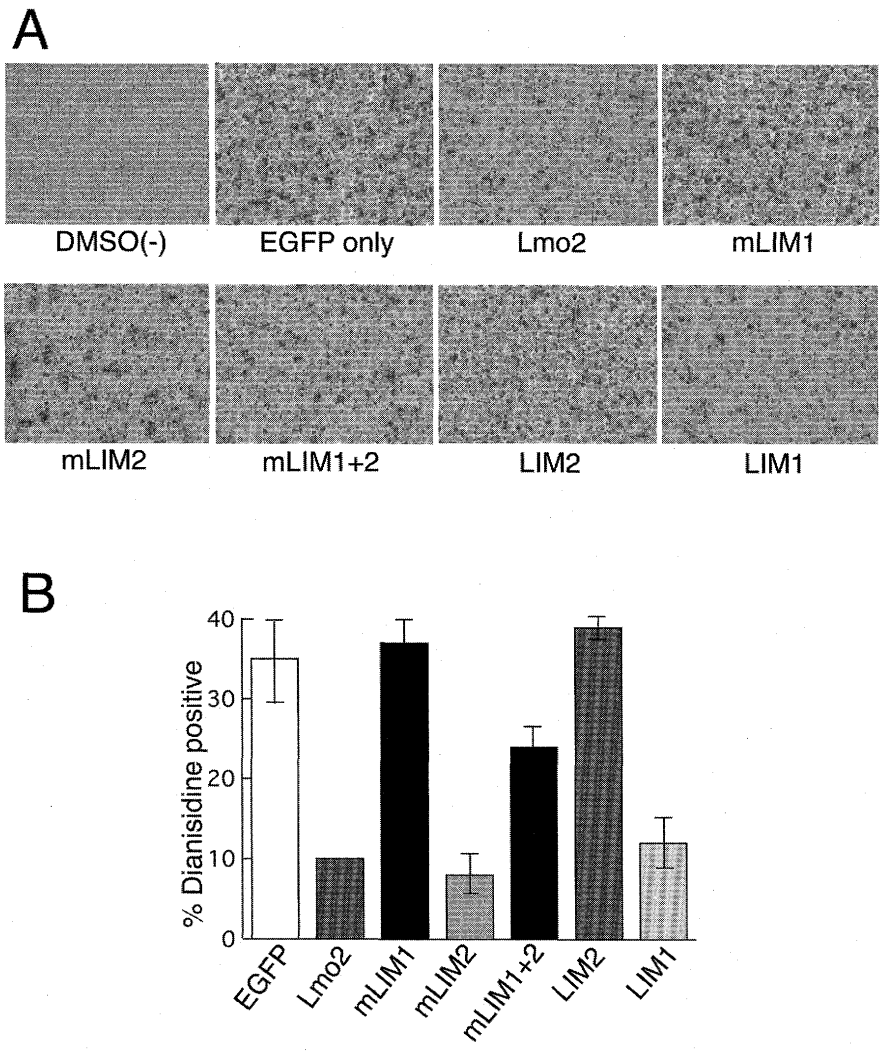


Fig. 4 Wild-type Lmo2, mLIM2 and LIM1 (delLIM2) block erythroid differentiation of MEL-DS19 cells.
A. Dianisidine staining (detecting haemoglobin) of retrovirus-infected MEL-DS19 cells prior to (DMSO(—)) and 72 hr (others) after induction with 1.8% DMSO. EGFP, wild-type Lmo2, and five mutant Lmo2s produced by retrovirus transfection are shown in the figure. Darkly stained cells are dianisidine positive.
B. Proportion of dianisidine-positive cells (100 cells are counted in each plate) in two independent DMSO induction experiments. Proteins expressed in MEL-DS19 cells are indicated in the figure.

Discussion

The whole hematopoietic system is constructed from the pluripotent hematopoietic stem cells, which have the capacity of self-renewal. The first production of hematopoietic stem cells themselves and the differentiation of hematopoietic stem cells and progenitors into various lineages involve complex interactions of transcription factors that regulate the cell-specific expression pattern of a number of downstream genes. From gene targeting studies, several transcription factors, which have a broad spectrum effect on the early stage of primitive and definitive hematopoiesis, have been identified. Tal1(Scl), Lmo2, Gata2, c-Myb, and AML1(Runx1) (Okuda et al., 1996) are individually necessary for the initiation of primitive and/or definitive hematopoiesis. Among these transcription factors, Tal1(Scl), Lmo2, and Gata2 can form a transcription factor complex using Lmo2 and Ldb1 as a bridging molecule (Fig. 1E-a). In addition, Lmo2 is able to bind other members of GATA-type zinc-finger proteins, Gata1 and Gata3 (Ono et al., 1998; Osada et al., 1995). Both transcription factors are considered to have more specific and restricted functions in definitive hematopoiesis. Gata1 is required for erythroid and megakaryocytic differentiation,

and Gata3, for lymphoid precursors and committed T cells.

Lmo2 as a bridging molecule between basic helix-loop-helix protein Tal1(Scl) and GATA-type zinc-finger protein Gata1/2/3

Lmo2 is a small molecule composed of two tandemly repeated LIM domains, LIM1 and LIM2. This cysteine-rich zinc finger-type motif functions as a protein-protein interaction surface. A protein interaction profile of mutant Lmo2s, which have a specific mutation or deletion in the LIM1 and/or LIM2 domain, revealed that the N-terminal LIM finger, LIM1, is mainly used for the interaction with Ldb1 and Tal1(Scl). Gata1, Gata2, and Gata3 chiefly use the C-terminal LIM finger, LIM2, for the interaction with Lmo2. It is intriguing all the hematopoiesis-related GATA-finger proteins, Gata1, Gata2, and Gata3, select LIM2 domain for their interactions because it is tempting to assume that Gata1, Gata2 and Gata3 are competing for the space in LIM2 domain. The complex composed of Tal1(Scl), Lmo2, and Ldb1 constitutes the steady backbone using the LIM1 domain in Lmo2 and might change the LIM2 partner, Gata1/2/3, according to the stage of differentiation of blood cells (Fig. 1D). It is reported that Gata1/2/3 have functional redundancy to some extent(Fujiwara et al., 2004; Takahashi et al., 2000). This structural property of the Tal1-Lmo2-Gata1/2/3 complex is compatible with the redundancy when they are functioning in the complex.

Lmo2/Tal1/Gata1/2/3 transcription factor complex as a master complex for definitive hematopoiesis

The mutant Lmo2 protein, which has a mutation destroying the zinc-finger structure of the LIM2 domain (mLIM2) and one that is lacking the whole LIM2 domain (LIM1), cannot bind strongly to Gata1/2/3, keeping its interaction with Tal1 and Ldb1. Ubiquitous over-expression of these two mutant proteins in transgenic mice almost abolished the fetal liver hematopoiesis. Overexpression of these two mutant proteins sequesters Tal1 and Ldb 1 from the physiological Tal1-Lmo2-Ldb1-Ldb 1-Lmo2-Gata1/2/3 complex to form the aberrant complex lacking Gata1/2/3 in a competitive manner. A relative decrease of the physiological complex is considered to be the direct cause of defective fetal liver hematopoiesis. These results strongly indicate that the transcription factor complex composed of Tal 1-Lmo 2-Ldb 1-Ldb 1-Lmo 2-Gata 1 / 2 / 3 is really a master complex for definitive haeamatopoiesis. The first definitive hematopoiesis in a mouse embryo begins in the wall of major arteries in the aorta-gonado-mesonephros (AGM) region (de Bruijin et al., 2002). It then progresses to the fetal liver. In both places, the development of blood cells is tightly linked with that of a vascular endothelial system. Hematopoietic stem cells and endothelial cells are assumed to be derived from the common precursor cells called hemangioblasts. Because Tal1, Lmo2, and Gata2 are expressed as both hematopoietic progenitors and endothelial cells (Dorfman et al., 1992; Elefanty et al., 1999; Yamada et al., 2000), the Tal1-Lmo2-Ldb1-Ldb1-Lmo2-Gata2 complex may first appear in the hemangioblasts and play a role in the specification of hematopoietic stem cells. Lmo2 and Gata3 are co-expressed with the hemopoietic clusters located in the aortic floor during fetal liver colonization (Manaia et al., 2000). Gata3 might be the earlier partner of Lmo2 in the cells located in intraembryonic hemogenic sites.

Cocktail-party model of the gradual transition of the transcription factor complex for embryonic development of erythroid and lymphoid progenitors

The transcription factor complex composed of Tal1(Scl)/Lmo2/Gata has a critical role in the initiation of definitive hematopoiesis. Its role might be the specification of hematopoietic stem cells from hemangioblasts and/or the specification of erythroid/lymphoid progenitors from the hematopoietic stem cells (Mikkola et al., 2003). Because Lmo2 is down-regulated as the differentiation of these progenitors proceeds, this complex is considered to be formed transiently during the very early stage of hematopoietic differentiation. The hypothetical mechanism of the transcription factor complex transition during the progress of definitive hematopoietic differentiation is that the complex composed of Tal1/Lmo2/Gata3 first appears in the sub-aortic hemangioblasts in the AGM region. Gata3 is then replaced by Gata2 in hematopoietic stem cells. The commitment to the erythroid lineage occurs when Gata1 joins the complex (Fig. 5A). Alternatively, the complex composed of Tal1/Lmo2/Gata2 is formed in hematopoietic stem cells. Differentiation to red blood cells is

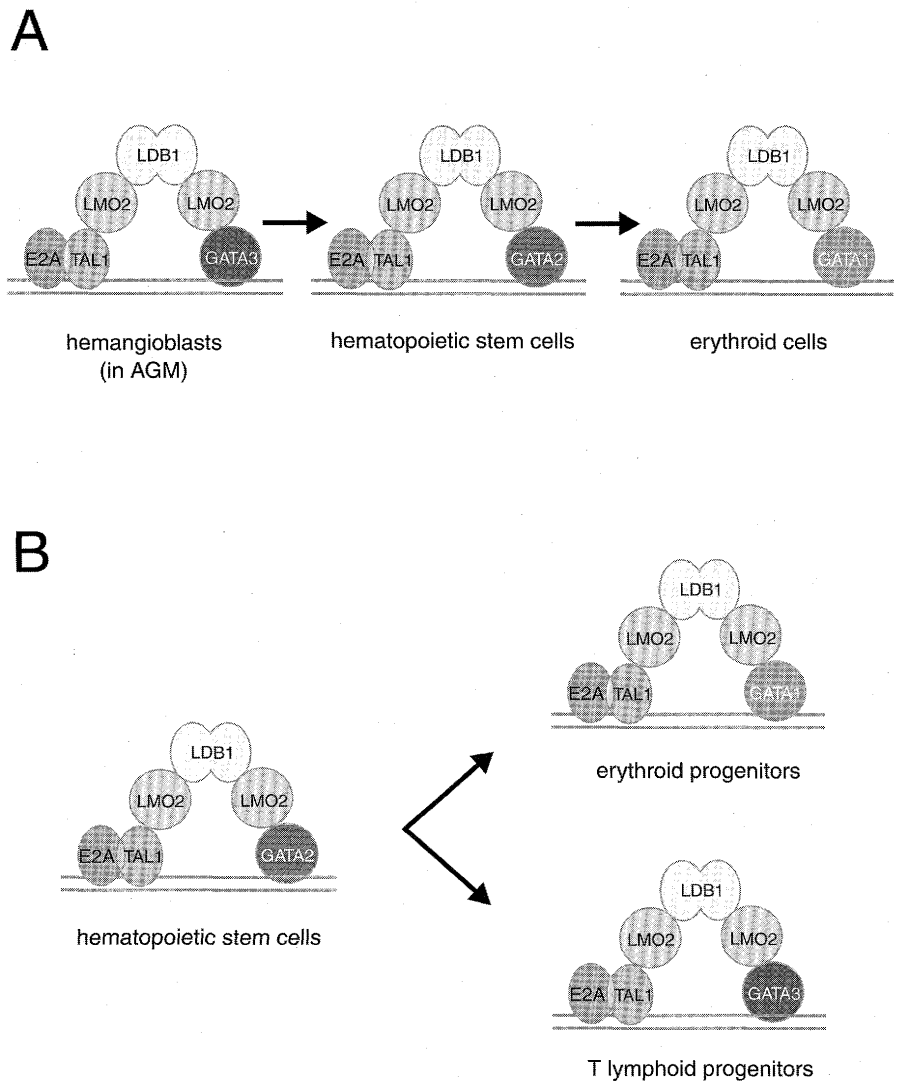


Fig. 5 Hypothetical mechanism of transcription factor complex transition during the progress of definitive hematopoietic differentiation.

A. The complex composed of Tal1/Lmo2/Gata3 appears first in the sub-aortic hemangioblasts in the AGM region. Gata3 is then replaced by Gata2 in hematopoietic stem cells. Commitment to erythroid lineage occurs when Gata1 joins the complex.

B. The complex composed of Tal1/Lmo2/Gata2 is formed in hematopoietic stem cells. Differentiation to red blood cells is selected when Gata1 joins the complex, and differentiation to T cells is selected when Gata3 joins the complex.

selected when Gata1 joins the complex, and differentiation to T cells is selected when Gata 3 joins the complex (Fig. 5B).

Lmo2 must leave the complex at a certain stage of red-cell and T-cell differentiation because persistent expression of Lmo2 in MEL cells as well as T cells inhibits the terminal differentiation (Larson et al., 1996; Larson et al., 1995) and eventually causes leukemia in the case of T cells (Hacein-Bey-Abina et al., 2003) In case of MEL cell differentiation to red cells, our result clearly shows it is due to the sequestration of Gata (probably Gata1) and/or Tal1. Physiologically, as erythroid progenitors differentiate, Lmo2 is down-regulated to make Gata1 and Tal1 free. Gata1 then forms a complex with FOG to activate different sets of downstream genes (Tsang et al., 1997), and Tal 1 might find other partners as well. Ectopic expression of Lmo2 blocks this smooth transition of the complex. Another possibility is that ectopic Lmo2 and/or LIM1 form aberrant transcription factor complexes such as Tal1-Lmo2/LIM1-Ldb1-Ldb1-Lmo2/LIM1-Tal1 which completely reset transcriptional mechanism (McCormack et al., 2003; Xu et al.,

2003).

Experimental Procedures

Plasmids and mutant constructs

A full-length murine Lmo2 cDNA was cloned into the XhoI-XbaI site of pBSpt (Stratagene, CA) to make pBSptXX-Lmo2. Site-directed mutagenesis was carried out in pBSptXX-Lmo2 with a QuikChange Site-Directed Mutagenesis Kit according to conditions recommended by the manufacturer (Stratagene, CA). Mutant LIM1 (mLIM1) had alterations in the LIM1 domain (HEDCLSCDLC to REDSLSSDLS) and mutant LIM2 (mLIM2) in the LIM2 domain (HLECFKCAAC to RLESFKSAAS). (Rincon-Limas et al., 2000) Mutant LIM1+2 (mLIM1+2) had both the LIM1 and LIM2 mutations. Deletion mutants of Lmo2 were made by making a new PstI site or BamHI with site-directed mutagenesis to cut out the PstI fragment containing the LIM1 domain to make a LIM2 or BamHI fragment containing the LIM2 domain to make a LIM1. The forward oligonucleotides used in site-directed mutagenesis were CAGTACTGGCGTGAGGATTCCCTCAG CTCTGACCTCTCTGGGTGT for mLIM1,AA AGTGTATCGCCTGGAGTCTTTCAAATCCG CCGCCTCTCAGAAG for mLIM2, AGGC-GCC TCTACTACAAGCTGCAGCGGAAATTGTGC AGGAGAGAC for LIM2, and GAGTGGACCA AGATCAATGGGATCCTCTAGGCCTCTAGA GCGGCC for LIM1.

Full-length cDNAs of human Tal1(Scl), murine Ldb1, and murine Gata1/2/3 were cloned into a multi-cloning site of the pCRII-TOPO vector using the TOPO TA Cloning system (Invitrogen, CA) and were used for further cloning.

Yeast two-hybrid screening

Yeast two-hybrid screening for protein-protein interaction was done with the HybriZAP-2.1 Two-Hybrid System (Stratagene, CA). The wild-type Lmo2 and three mutant Lmo2s (mLIM1, mLIM2, and mLIM1+2) were cloned in-frame into EcoRI-SalI sites of the GAL4 DNA-binding domain plasmid pBD-GAL4 using PCR amplification. The Ldb1, Tal1, Gata 1/2/3 cDNA sequences were cloned in-frame into EcoRI-SalI sites of the GAL4 DNA-activation domain plasmid pAD-GAL4. YRG-2 yeast strains carrying β-galactosidase/HIS3 reporter plasmid were transformed with a pair of each 40μg of pBD-GAL4-Lmo2s and pAD-GAL4-Ldb1 plasmid DNA and 2 mg of carrier DNA. Tranformed YRG-2 yeast strains were plated on -His dropout plates, and colonies on dropout plates, if any, were transferred to a nitrocellulose membrane for X-gal staining.

Mammalian two-hybrid screening

Mammalian two-hybrid screening for protein-protein interaction was done using the Mammalian MATCHMAKER Two-hybrid Assay Kit (CLONTECH, Germany) according to the manufacturer's instructions. Briefly, wild-type Lmo2 and 5 mutant Lmo2s (mLIM1, mLIM2, mLIM1+2, LIM2, and LIM1) were cloned into EcoRI-SalI sites of the Gal4 DNA-binding domain plasmid pM in-frame (pM-Lmo2s) to make fusion proteins using PCR. EcoRI-SalI fragments of pAD-Gal4-Tal1, Ldb1, Gata2, and Gata3 were subcloned into the same sites in the Gal4 DNA-activation domain plasmid pVP16 and blunt-ended BamHI-XbaI fragment of pAD-Gal4-Gata1 into the MluI-XbaI sites of pVP16 by blunt-end ligation. 10T1/2 cells were transfected by a calcium-phosphate method with a set of three plasmids, pM-, pVP16-, and a CAT reporter plasmid (pG5CAT), for each protein-protein interaction assay, and, after 48 hours, CAT activity in the cell lysate was measured using a CAT ELISA Kit (Roche Diagnostics Corp. Germany).

Immunoprecipitation

Expression plasmids for HA-tagged and FLAG-tagged proteins were made as follows. Two synthetic oligonucleotides, CTAGTACC ATGGACTACCCATACGACGTCCCAGACTA CGCTCAGGATCCT and CTAGAGGATCCTG AGCGTAGTCTGGGACGTCGTATGGGTAGT CCATGGAT, were annealed and cloned into the XbaI site of pEF-BOS (Mizushima and Nagata, 1990), yielding pEF-BOS-HA. Two synthetic oligonucleotides, CTAGTACCATGG ACTACAAGGACGACGACGACAAGGGATCC GCTAGCT and CTAGAGCTAGCGGATCCCT TGTCGTCGTCGTCCTTGTAGTCCATGGTA, were annealed and cloned into the XbaI site of pEF-BOS, yielding pEF-BOS-FLAG. Wild-type Lmo2 and 5 mutant Lmo2s (mLIM1, mLIM2, mLIM1+2, LIM2, and LIM1) were cloned into the pEF-BOS-HA vector subcloning the blunt-ended EcoRI-SalI fragment of pM-Lmo2s into BamHI-XbaI sites by blunt-end ligation. Subsequently, the NheI-XbaI fragment of pAD-

GAL4-Tal1, Ldb1, Gata2, and Gata3 and the BamHI-XbaI fragment of pAD-GAL4-Gata1 were subcloned into the same sites of pEF-BOS-FLAG. 293F cells were transfected with a pair of pEF-BOS-HA- and pEF-BOS-FLAG-plasmid using a Lipofectamine 2000 reagent (Invitrogen, CA). After 48 hours, cells were lysed in 1 ml of a low-stringency buffer (10ml Hepes pH7.6, 250mM NaCl, 5mM EDTA, 0.5% Nonidet P40) and immunoprecipitated with 40 μl of anti-FLAG M2 affinity gel (Sigma-Aldrich Co., MO). Immunoprecipitates (each 20 μl) were analyzed by immunoblotting with anti-HA antibody (Roche Diagnostics Corp., Germany).

Transgenic constructs and mice

Four transgenic mouse lines, which were ubiquitously over-expressing wild-type Lmo2, and three mutant Lmo2s (mLIM1, mLIM2, LIM1) were made. A blunt-ended XhoI-XbaI fragment of pBSptXX-Lmo2, -mLIM1, -mLIM 2, and -LIM1 was cloned into an XbaI site of pEF-BOS by blunt-end ligation, yielding pEF-BOS-Lmo2, -mLIM1, -mLIM2, and -LIM1. Purified 2.5-kb HindIII-PvuI fragments of pEF-BOS-Lmo2, -mLIM1, -mLIM2 or 2.3 kb fragment of pEF-BOS-LIM1, which were composed of promoter region of human elongation factor (EF-1α), wild type and mutant Lmo2 sequences and poly A signal from human G-CSF cDNA, were injected into 200 fertilized eggs of C57BL/6 x DBA F1 mice. Founders that had the highest number of copies of wild-type and mutant Lmo2s in Southern blot analysis of tail DNA were crossed with C57BL/6 partners to get germ-line transmission, and offspring were used for further analysis.

Reverse transcriptase-dependent PCR (RT-PCR)

The presence of transgene derived RNA in transgenic (TG) mice was analysed using reverse transcriptase-dependent PCR with a set of transgene-specific synthetic oligonucleotides, GATGAATTCTCCTCGGCCATCGAAA GGAAG and CAGGTGGGGACCCTCACTCTA. First-strand cDNA templates were synthesized from RNA of E12.5 embryos, P0 thymus, P0 liver, and P0 spleen.

Histological analysis

All samples were fixed in 10% (vol/vol) buffered formalin and embedded in paraffin. Sections were mounted on slides and stained with hematoxylin and eosin (H & E). Apoptotic cell detection in P0 thymi was performed by direct immunoperoxidase staining of digoxigenin-labeled genomic DNA using the ApopTaq Plus kit (Intergen, NY) on paraffin-embedded sections.

***In vitro* hematopoietic colony assay**

An *in vitro* hematopoietic colony assay from an E12.5 fetal liver was done according to the method described in (Okuda et al., 1996). Briefly, livers from E12.5 embryos were dispersed into single-cell suspensions, and 5×10^3 cells were resuspended in 1 ml of a Methocult solution (StemCell Technologies, Canada) and incubated under humidified conditions with 5% CO_2 for 7 days. Colonies that contained more than 50 cells were counted on day 7, and myeloid, erythroid, and mixed colonies were morphologically defined.

Retrovirus production and infection of MEL cells

Wild-type and five mutant Lmo2s were expressed in MEL (murine erythroleukemia) -DS19 cells (a gift from Dr. Tadashi Nagai) using the MSCV (Murine Stem Cell Virus) retroviral expression system (BD Bioscience Clontech, CA) according to the manufacturer's instructions with slight modifications. Briefly, the blunt-ended BglII-AflII fragment of pIRES 2-EGFP (BD Bioscience Clontech, CA) was first cloned into a BglII site in the multi-cloning site of pMSCV-neo (BD Bioscience Clontech, CA) using blunt-end ligation, yielding pMSCV-IRES-EGFP-neo. Secondly, XhoI-SacII fragments of pBSptXX-Lmo2, -mLIM1, -mLIM2, -mLIM1+2, -LIM2, and -LIM1 were cloned into the same site in the multi-cloning site of pMSCV-IRES-EGFP-neo, yielding pMSCV-Lmo2/mutant Lmo2s-IRES-EGFP-neo. A virus was produced by transfecting BDEcopack 2-293 cells with 7 kinds (empty, wild-type, and five mutants) of vector constructs using a Lipofectamine 2000 reagent (Invitrogen Corp., CA). A virus containing a supernatant virus was collected 48h after transfection. The retrovirus supernatant was pre-loaded onto a RetroNectin (Takara Biotech., Japan)-coated plate at 100μl/cm^2 and incubated for 5 hours at 37 C. Immediately after removal of the supernatant, MEL-DS19 cells in a growth medium were added onto the plates at a concentration of 2.5×10^4 cells/cm^2 and incubated for 3 days. The G418 selection was then carried out for 10 days at a concentration of 350μg/ml. Successful expression of wild-

type/mutant Lmo2 proteins in MEL-DS19 cells was monitored by the presence of EGFP (Enhanced Green Fluorescent Protein) translated from the bicistronic mRNA using flow cytometry.

In vitro differentiation of MEL cells to erythroid cells

In vitro differentiation of MEL-DS19 cells to erythroid cells was induced by adding 1.8% (vol/vol) DMSO into the growth medium and incubated for 3 days. Hemoglobin containing cells were detected by dianisidine staining using 2% (w/v) o-dianisidine/3% acetate/3% hydrogen peroxide (Nagai et al., 2003).

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