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A new allele of *Lyl1* confirms its important role in hematopoietic stem cell function

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Abstract

Lyl1 codes for a bHLH protein that is an important regulator of hematopoietic stem cell function. An existing mutant allele of *Lyl1* features a lacZ gene inserted in-frame into the fourth exon, leaving behind the N-terminus and the DNA-binding basic region, resulting in a translated chimeric protein. Here, we have generated a null allele, which allowed us to examine residual function of the N-terminus in the absence of a bHLH region. The new *Lyl1*^{-/-} mouse model exhibited a reduced ability to generate lymphoid lineages and a somewhat more severe hematopoietic repopulation defect when transplanting purified hematopoietic stem cells. Our data show that in the absence of the HLH but presence of the N-terminus, residual function of the *Lyl1* is detectable but relatively minor. The new model may be of use for studies of *Lyl1* in which a null allele is required, or for which presence of the LacZ may complicate the combined use of additional mouse models bearing the lacZ marker.

Keywords

Hematopoietic Stem Cells; *Lyl1*; Knockout

Introduction

Hematopoietic stem cells (HSCs) have enormous self-renewal and differentiation potential, and give rise to all the mature blood lineages through a hierarchy of progenitors. Regulation of hematopoiesis is a finely controlled process that involves a number of transcription factors, which when deregulated, can give rise to hematopoietic malignancies. One class of proteins that have been extensively studied in relation to hematopoiesis are bHLH transcription factors. bHLH transcription factors act as intrinsic regulators of a myriad of developmental processes, including lymphoid specification, and involves E-box factors and Id (inhibitory) bHLH proteins among others (Kee, 2009).

The Lymphoblastic leukemia 1 gene (*Lyl1*) is a class II bHLH transcription factor and was originally identified at the breakpoint of a chromosomal translocation, resulting in T cell acute lymphoblastic leukemia (Mellentin *et al.*, 1989). Class II bHLH transcription factors regulate transcription by binding to target gene sequences as heterodimers with E-proteins, in association with Gata1 and Gata2. They recognize an E box domain (CANNTG) to either activate or repress transcription (Goldfarb and Lewandowska, 1995; Hofmann and Cole,

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1996; Hsu *et al.*, 1994; Wadman *et al.*, 1997). Both *Lyl1* and its paralog, *Scl* (aka *Tall1*) have been shown to form heterodimers with E2A (Miyamoto *et al.*, 1996).

A mutant *Lyl1* allele generated previously has revealed the role of *Lyl1* in hematopoiesis. Specifically, after bone marrow transplantation, a reduced capacity to repopulate the blood and bone marrow was noted, including lymphoid and myeloid lineages (Capron *et al.*, 2006). Furthermore, our lab has recently shown that *Lyl1* plays a critical role in adult HSC maintenance and survival and that it shares some level of functional redundancy with its paralog, *Scl* (Souroullas *et al.*, 2009). The *Lyl1* knockout mouse model that was used in these studies was generated by inserting a β -galactosidase/Neomycin cassette in frame into the fourth exon of *Lyl1*, replacing part of the protein-coding sequence (Capron *et al.*, 2006), allowing expression of *Lyl1* to be tracked (Giroux *et al.*, 2007). This *Lyl1* allele will hereafter be referred to as *Lyl1^{lacZ}*. The helix-loop-helix (HLH) region, thought to be critical for function of all bHLH-type proteins, was deleted in this allele, but the DNA-binding basic region of the bHLH remain intact, as well as the N-terminus.

The N-terminus of *Lyl1* is highly conserved evolutionarily, suggesting a functional role in the protein. Indeed, we have recently shown that within a proline-rich region of the N-terminus there is a PEST motif, which is responsible for stability of *Lyl1* protein, regulating degradation via the proteasome (Lukov and Goodell, 2010). Therefore, retention of the basic and N-terminal regions of the protein in the *Lyl1^{lacZ}* mouse model might result in a hypomorphic allele.

Thus, we generated a *Lyl1*-null allele in which the entire coding region was deleted. This allowed us to examine any residual function of *Lyl1* associated with retention of the N-terminus (in the *Lyl1^{lacZ}* allele), and would also serve as a reagent for future studies of the function *Lyl1*, in which the potentially confounding N-terminus and LacZ marker remained.

Results and Discussion

We generated a knockout mouse by replacing the coding region of *Lyl1* with a lox-P-flanked Neo cassette. Correctly targeted ES cell clones were confirmed by Southern blot using both 3' and 5' external probes that can distinguish the wild-type (WT) and targeted alleles due to engineered restriction enzyme sites into the targeting cassette (Figure 1a). Southern blot of the ES cell clones identified a number of correctly targeted clones (Figure 1b), one of which was injected into the blastocyst of 129 AB2.2 mice. Chimeric mice were generated, which were then backcrossed to the C57Bl/6 strain for ten generations. Initial genotyping of mice was carried out by Southern blotting using mouse tail genomic DNA (Figure 1c). Routine genotyping of the *Lyl1* targeted locus was carried out by PCR as shown in Figure 1d and 1e. The new mouse model is referred to as the *Lyl1^{Mg}* allele.

Homozygous *Lyl1^{Mg/Mg}* mice were viable and fertile, with no obvious developmental defects. Once the mice reached adulthood, we analyzed their complete blood counts in the peripheral blood at steady state and noticed no difference in the number of myeloid or lymphoid cells between the knockout strains and WT mice (Table 1). There was a defect however in the number of platelets which was more pronounced in the *Lyl1^{Mg/Mg}* mice ($p < 0.01$) compared to WT mice.

We also analyzed the percentage of HSCs using the Hoechst dye efflux properties of HSCs (SP) in addition to cell surface markers ckit⁺, Sca-1⁺ and lineage – (SP^{KSL}). There may be a small decrease in SP^{KSL} cell numbers (Figure 2), but a larger number of mice would have to be analyzed to determine if this is statistically significant. Under steady-state hematopoiesis therefore, loss of *Lyl1* does not seem to have a major impact on the hematopoietic system, or its loss is compensated for by its paralog, *Scl/Tall1*.

We next studied the effect that loss of *Lyl1* has on HSC function under more stressful conditions. We performed experiments with *Lyl1*^{MG} mice in comparison with both WT mice and *Lyl1*^{lacZ} mice in order to determine whether the N-terminus of *Lyl1* plays a significant role in HSC maintenance (Figure 3a). We first investigated the repopulation potential of the knockout HSCs in a competitive bone marrow transplantation assay as shown in figure 3b. In this competitive repopulation assay, we test the ability of a donor population of cells to compete against WT cells in repopulating a lethally irradiated recipient. Donor and competitor cells were distinguished using the CD45 allelic system.

Results show that both knockout models have a repopulation defect, giving rise to about 10% hematopoietic engraftment, compared to about 50% of the WT control group (Figure 3c and e). Lineage analysis of the donor-derived cells shows that both mouse models generate relatively more myeloid than lymphoid cells (Figure 3d). There was, however, a subtle but consistent difference in the transplantation phenotype at the earliest time point (Figure 3c), where bone marrow from the *Lyl1*^{MG} null allele exhibited a slightly lower engraftment than the *Lyl1*^{lacZ} allele. This was a consistent, but statistically insignificant difference that appeared in multiple experiments, but was not apparent at later time points.

In order to further investigate the engraftment defect in more detail, we performed competitive transplantation assays using purified HSCs, which were isolated using the side population (SP) method for Hoechst dye efflux (Goodell *et al.*, 1996), in addition to staining for KSL (cKit+, Sca-1+, lineage-). Analysis of the peripheral blood over time revealed that the *Lyl1*^{LacZ} knock-in HSCs initially engrafted as well as the WT HSCs, however, the *Lyl1*^{MG} null HSCs had negligible engraftment as early as 4 weeks post transplant (Figure 4a and c). Lineage analysis from donor-derived cells showed the same skewing towards the myeloid compartment in both models (Figure 4b), indicating a significant defect in lymphoid development in the absence of functional *Lyl1*. At subsequent time points, engraftment from both mouse models was diminished to minimal levels. Together, these data suggest that the *Lyl1*^{lacZ} allele has some residual activity in supporting short-term HSC function, whereas when *Lyl1* is completely absent, short- and long-term HSC activity is severely compromised.

We speculate that the phenotypic differences between the two alleles may be due to some residual activity of the retained N-terminus of *Lyl1* in the *Lyl1*^{lacZ} allele, which may allow the protein to retain some function independently of the bHLH. This is contrary to the prevailing view that the class II bHLH can only act as transcription factors via their interactions with E-proteins, through the bHLH domain (reviewed in (Kee, 2009)). The E-protein interactions are thought to be required to allow binding to the E-box sites in promoters and enhancers. Without the bHLH to interact with E-proteins and an E-box, class II bHLH proteins are thought to be devoid of function.

However, genome-wide maps of *Lyl1*-binding sites have indicated that *Lyl1* protein can be bound to promoters and enhancers that lack a classical E-box binding motif (Wilson *et al.*, 2010). Both *Lyl1* and its paralog *Scl* were seen to bind to sites that are also usually bound by ten other proteins in the same cell line, including *Runx1*, *Lmo2*, *Meis1*, *Sfp1*, *Fli1* and *Gfi1B*. It is possible that participation of *Lyl1* in these complexes is not completely dependent on the bHLH. Thus, the N-terminus, as present in the *Lyl1*^{lacZ} allele, could support some complex formation, allowing binding to some of the non-E-box sites. Because the *Lyl1*^{lacZ} allele retains some short-term HSC activity, unlike the *Lyl1*^{MG} allele, we might then hypothesize that the genes regulated by E-box and non-E-box *Lyl1*-binding sites may play slightly different roles in short-term and long-term HSC function. Full understanding of the mechanisms which account for the differences between the *Lyl1* alleles would require more investigation.

In summary, we describe here the generation of a new mouse model for studying *Ly11*. Loss of *Ly11* leads to a severe HSC defect, as shown in HSC repopulation assays, with both *Ly11* alleles, although the defect in the null allele appears somewhat more severe. *Ly11* is also expressed outside the hematopoietic system (Chan *et al.*, 2007; Giroux *et al.*, 2007; Gray *et al.*, 2004), so these two alleles may also be of use to investigate the role of *Ly11* in other tissues.

Materials and Methods

Generation of targeting construct

The *Ly11* targeting construct was generated using recombineering technology. In order to construct a targeting vector, we first retrieved approximately 15kb of genomic region encompassing *Ly11* from a BAC clone by gap repair. The *Ly11* BAC (bMQ-456-A17) was introduced into the SW102 cells as previously described (Lee *et al.*, 2001). Homology arms for BAC retrieval were cloned using the following primers: Upstream arm: Fwd: 5' - CAAGGGGCCCTGGTGTGAAGGTTTGAGA-3' (ApaI), Rev: 5' - CCTACCGCGGGCCAGGATTTTCTGTTCTCAG-3' (SacII); Downstream arm: Fwd: 5' - **ACTCAGATCTTCTCCCCATTTTGAG**-3' (BglII), Rev: 5' - **ACTCCTCGAGGGCCACCCCACTACT**-3' (XhoI). The underlined nucleotide sequences indicate restriction enzyme sites added for cloning into the pDTA-4B plasmid. Gap repair recombination was carried out as previously described (Lee *et al.*, 2001). The floxed neomycin resistance cassette flanked by the targeting homology arms was amplified from the pL452 plasmid using the following primers: (underlined=homology sequence) Fwd: 5' - **CACACGTTTGGCGCTGGGCTCCTGAGGGTTGGGGGTCTTCCAGTGCTGTAAGC** TTGTACCGGGCCCCCTCG -3' (HindIII), Rev: 5' - **GTATGTGAAATAAAGAAATAAAGAAAAACAAATGCAAGAGTGCCTGAGTCTTC** GTTGATA**TCCTCGAGGGACCTAATA**-3' (EcoRV). Nucleotides in bold indicate base modifications to insert restriction enzyme sites to create a good screening strategy by southern blot. The resulting targeting construct was linearized and electroporated into ES cells, which upon homologous recombination, replaced all four exons of *Ly11* with the Neo cassette.

Mice

The *Ly11^{lacZ}* allele has been described (Capron *et al.*, 2006). Mice carrying the *Ly11^{Mg}* allele were backcrossed to the C57Bl/6 background without excising the floxed neomycin cassette, and were housed in a specific-pathogen-free animal facility, AALAC-accredited, at Baylor College of Medicine (Houston, TX). The *Ly11^{Mg}* allele was created using 129 AB2.2 ES cells and generated chimeras. Chimeric mice were backcrossed to the C57Bl/6 background strain for ten generations.

Genotyping

Genomic DNA was isolated from mouse tails and digested in DNA lysis buffer (Gerard Biotech). Primer sequences used for PCR amplification of the *Ly11* WT allele were: 5' - AAGCTGAGCAAGAACGAGGTGC-3' and 5' - TCTGCTCCAACCTTGATGGGTCTC-3', for the *lacZ* knockin allele: 5' - AGTGGGCGCCCTCTGTCCTCTA-3' and 5' - TAATTCGCGTCTGGCCTTCCTGTA-3', and for the null *Ly11^{Mg}* allele: 5' - CCTTCTTGACGAGTTCTTCTGAG-3', and 5' - CAAGAGTGCCTGAGTCTTCGTT-3'.

Complete Blood Counts

Peripheral blood was collected by retro-orbital bleeding and analyzed using the Hemavet 950FS analyzer (Drew Scientific, Dallas, TX)

Competitive bone marrow transplantation assays

Competitive bone marrow transplantation assays were performed by intravenous injection of admixed CD45.2 donor whole bone marrow cells with CD45.1 competitor bone marrow. Both *Lyl1* knockout mouse models carried the CD45.2 allele. Recipient C57Bl/6 mice had been lethally irradiated with a split dose of 10.5Gy, 3 hours apart. Sex- and age-matched C57Bl/6 mice were used as competitors for every experiment. The competitor cell dose was kept constant at 250,000 cells in all transplants. HSC transplants were carried out as described above, but instead of whole bone marrow, purified HSCs were transplanted. Cells were isolated using the side population (SP) method for Hoechst dye efflux (Goodell *et al.*, 1996), followed by KSL (cKit+, Sca1+, lineage) staining. Recipient mice received 20 sorted HSCs and 250,000 WT competitor cells. Stain and isolation of HSCs was carried out as previously described (Challen *et al.*, 2009).

Flow cytometry and lineage analysis

For peripheral blood analysis, mice were bled at 4, 8, 12 and 16 weeks post transplantation. Red blood cells were lysed and samples were stained with CD45.1-APC, CD45.2-FITC, CD4-pacific blue, CD8-pacific blue, B220-pacific blue, B220-PE-cy7, Mac1-PE-cy7 and Gr-1-PE-cy-7 antibodies (BD Pharmingen, eBiosciences). FACSARIA, LSRII and FACS-Scan flow cytometers were used for analysis and cell sorting.

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Figure 1. Targeting of the mouse *Lyl1* genomic locus

(a). Schematic representation of the *Lyl1* genomic locus. Map and restriction enzyme sites demonstrate the strategy used for targeting and screening ES clones. Thick lines indicate the DNA sequence used for homologous recombination, the black arrow indicates the PGK promoter that drives the neomycin resistance gene, and the grey arrows indicate the loxP sites. The diphtheria toxin gene (DTA) was used for negative selection. *Bam*HI and *Bgl*II sites were used for screening the 3' and 5' regions respectively. (b) ES cells electroporated with the targeting vector were screened by Southern blot. Correctly targeted clones were injected into blastocysts to generate chimeric mice. (c) Southern blot genotyping of *Lyl1* null mice using tail genomic DNA. (d) Schematic for PCR genotyping of the *Lyl1* null mice,

where two sets of primers are used that can recognize either the WT or targeted allele. (e)
Example of genotyping *Lyl1* null mice, showing WT, heterozygous (HET) and homozygous (KO) mice.

Figure 2. Phenotypic analysis of HSCs using the side population (SP) by Hoechst dye efflux and cell surface markers shows no significant difference between the two alleles

Whole bone marrow from WT and age-matched knockout mice was isolated and stained with the Hoechst dye in addition to ckit+, Sca-1+ and lineage markers (SP^{KSL}). Analysis by flow cytometry reveals that both knockout alleles have a small decrease in HSCs in their bone marrow as compared to the WT controls. However, due to the small pool size (n=3), the difference was not statistically significant.

Figure 3. Functional analysis of hematopoietic stem cell activity of the *Lyl1^{Mag}* allele by bone marrow transplantation assays

(a) Schematic comparison of the two targeted alleles of *Lyl1*. The *Lyl1^{lacZ}* mouse model has a lacZ/Neo cassette inserted into the fourth exon of the gene, disrupting the HLH and C-terminus, whereas the *Lyl1^{Mag}* allele eliminates the entire coding region. (b) Schematic of the experimental design. Whole bone marrow cells from CD45.2 WT, *Lyl1^{lacZ/lacZ}* and *Lyl1^{Mag/Mag}* mice were isolated and 250,000 cells were transplanted into lethally irradiated recipients (n=6), along with equal number of competitor CD45.1 cells. (c) Engraftment assessed by flow cytometry at 4, 8, 12 and 16 weeks post transplantation using the CD45.2 marker, indicates a defect in both short- and long-term reconstitution in both knockout alleles (n=5). (d) Lineage contribution to peripheral blood from donor-derived cells at 16 weeks post transplant shows lineage skewing in both *Lyl1* knockout alleles, generating more myeloid cells at the expense of T and B cells (n=5). (e) The top row shows representative flow cytometry plots for each genotype, demonstrating that engraftment from the CD45.2+ cells was defective in both knockout mouse models compared to WT controls. CD45.2+ cells were then gated to show the lineage distribution of the engrafted cells. The bottom row of representative flow cytometry plots shows that mice lacking *Lyl1* generate more myeloid cells at the expense of the lymphoid compartment. The experiment was repeated three times with similar results.

Figure 4. Purified HSC transplantation assays

(a) One hundred purified HSCs from CD45.2 WT, *Lyl1^{lacZ/lacZ}* and *Lyl1^{Mg/Mg}* mice were transplanted into lethally irradiated recipients along with 250,000 whole bone marrow competitor cells. HSCs were purified using the side population (SP) in addition to staining for KSL. Engraftment analysis was carried out by flow cytometry at 4-week intervals post transplant and shows that the *Lyl1^{Mg}* allele is defective in reconstituting recipient mice as early as 4 weeks post transplant. Both knockout alleles have a severe long-term engraftment defect with almost zero engraftment compared to about 60% of the WT control ($n=5$, $p<0.05$). (b) Lineage distribution analysis of the CD45.2 donor-derived compartment shows the same defect as whole bone marrow transplants, with diminished B and T cell populations. (c) Representative flow cytometry plots of donor-derived cell engraftment for each genotype at 4 and 16 weeks post transplant. The experiment was repeated twice with five mice per group, showing similar results.

Table 1
Complete peripheral blood counts (CBC) in WT, *Ly11^{lacZ/lacZ}* and *Ly11^{Mg/Mg}* mice

Peripheral blood from mice was collected by retro-orbital bleeding. Analysis shows no major differences between the two *Ly11* alleles in the lymphoid, myeloid or red blood cells. Platelet counts on the other hand were lower in the *Ly11^{Mg/Mg}* mice as compared to both the *Ly11^{lacZ/lacZ}* and WT mice ($p < 0.01$). Values show the average $\pm$ SEM (n=6).

	WT	<i>Ly11^{Mg/Mg}</i>	<i>Ly11^{lacZ/lacZ}</i>
White Blood Cells, K/ μ l	8.20 $\pm$ 1.66	9.76 $\pm$ 0.48	8.25 $\pm$ 0.76
Red Blood Cells, M/ μ l	8.76 $\pm$ 0.59	7.80 $\pm$ 0.28	8.67 $\pm$ 0.26
Hemoglobin, g/dL	11.03 $\pm$ 0.67	10.70 $\pm$ 0.39	12.44 $\pm$ 0.22
Hematocrit, %	42.13 $\pm$ 0.50	47.55 $\pm$ 0.55	49.52 $\pm$ 0.99
MCV, fL	51.92 $\pm$ 0.50	47.55 $\pm$ 0.55	54.96 $\pm$ 2.68
MCH, Pg	13.98 $\pm$ 0.12	13.73 $\pm$ 0.08	14.54 $\pm$ 0.20
MCHC, g/dL	27.32 $\pm$ 0.38	28.88 $\pm$ 0.38	26.70 $\pm$ 1.43
Platelets, K/ μ L	1170.50 $\pm$ 51.25	602.75 $\pm$ 27.50	972.80 $\pm$ 47.00