The bHLH gene *Hes1* is essential for expansion of early T cell precursors

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Mice mutant for the bHLH gene *Hes1*, which is known to keep cells in a proliferative state, mostly lack thymus. Transfer of *Hes1*-null fetal liver cells into *RAG2*-null host mice normally reconstitutes B cells but fails to generate mature T cells in the thymus. In the reconstituted thymus, T cell differentiation is arrested at the CD4−CD8− double negative (DN) stage. Both the initial T cell receptor (TCR)-independent and the subsequent TCR-dependent selective expansion during the DN stage are severely affected. Thus, *Hes1* is essential for the earliest thymocyte expansion in a cell-autonomous manner.

[Key Words: bHLH; *Hes1*; T cell; thymocyte; thymus]

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Hes1 is critical for thymocyte expansion in a cell-autonomous manner

Because Hes1 is expressed in both stromal cells and thymocytes, the thymic phenotypes of Hes1-null mice could be due to abnormality of stromal cells and/or thymocytes. To determine whether Hes1-null thymocytes are intrinsically affected, we made bone marrow chimerae using RAG2-null mutant mice, which have no mature T or B lymphocytes (Shinkai et al. 1992). Fetal liver cells from Hes1-null or wild-type donor embryos were injected intravenously into irradiated RAG2-null mice of C57BL/6 background to allow differentiation of donor progenitors in the environment of normal thymic stromal cells. Lymphoid cells [Thy1+] of the donor origin were distinguished by Ly5.2 expression from those of possible host origin that are negative for Ly5.2 (Fig. 3B). This analysis could also help determine more precisely the stage at which T cell development is impaired in the absence of Hes1.

When reconstituted with wild-type or Hes1-null fetal liver cells, the RAG2-null hosts had a comparable number of mature B cells in the spleen [IgM+, B220+, Fig. 3C,D], indicating that reconstitution was successful and that B cells develop normally in the absence of Hes1. In contrast, the absolute number of thymocytes of the Hes1-null origin was by far less than that of the wild-type origin in the reconstituted RAG2-null thymus (Fig. 3A). When 1 × 10^6 and 1 × 10^7 fetal liver cells of wild-type origin were injected, an average of 1.6 × 10^6 [n = 13] and 1.0 × 10^7 [n = 6] thymocytes, respectively, was re-
covered. In contrast, when $1 \times 10^6$ Hes1-null cells were injected, only $1.7 \times 10^5$ thymocytes were recovered ($n = 9$); and even when $1 \times 10^7$ Hes1-null cells were injected, only $4.1 \times 10^5$ thymocytes were recovered ($n = 8$), indicating that the thymocyte number of the Hes1-null origin was 200- to 1000-fold less than that of wild-type origin. The thymocyte number of the Hes1-null origin was still 10- to 20-fold less than that of the nonreconstituted RAG2-null host mice ($3.2 \times 10^6$ cells; $n = 10$) [Fig. 3A]. These results demonstrate that thymocytes are intrinsically affected by Hes1 mutation and that Hes1 is critical for expansion of thymocytes.

**Thymocyte expansion at the DN stage is impaired by Hes1 mutation**

To determine the stage at which T cell development is impaired in the absence of Hes1, thymocytes of the donor origin were analyzed further with various surface markers. Whereas both $\alpha\beta$ and $\gamma\delta$ T cells were generated from wild-type donors, neither mature T cells were detected in the thymus reconstituted with Hes1-null donor cells [Fig. 4A]. Furthermore, the vast majority of thymocytes of Hes1-null origin were negative for expression of the surface antigens CD4 and CD8 [Fig. 4B] but highly expressed the early marker heat-stable antigen (HSA) in the virtual absence of CD3 [Fig. 4C]. Thus, in the absence of Hes1, T cell development is arrested at the CD3-DN stage. Essentially the same abnormality was observed irrespective of the number of the injected cells ($1 \times 10^6$ or $1 \times 10^7$, Fig. 4A–C).

Because DN cells are further divided into four distinct differentiation stages based on the profiles in the surface expression of CD44 and CD25 (Godfrey and Zlotnik 1993; Godfrey et al. 1993, 1994; Pénit et al. 1995), donor DN thymocytes were analyzed with these markers [Fig. 4D]. At the first stage (CD44$^+$CD25$^-$) DN cells are normally resting and the absolute number of Hes1-null DN thymocytes was, on an average, $1.3 \times 10^5$ cells ($n = 4$) and $0.6 \times 10^5$ cells ($n = 4$) when $1 \times 10^6$ and $1 \times 10^7$ cells, respectively, were injected. These numbers were comparable to those of wild-type DN cells ($1.2 \times 10^5$ cells; $n = 9$). However, when $1 \times 10^6$ Hes1-null cells were injected, at the subsequent stages the number of Hes1-null thymocytes was by far less than that of the wild-type cells, which are known to proliferate TCR independently first and then TCR dependently during the DN stages [Fig. 4D]. When $1 \times 10^7$ Hes1-null cells were injected, DN thymocytes increased at the CD44$^-$CD25$^+$ stage ($2.2 \times 10^5$ cells; $n = 4$), but the number was still much less than the wild type ($1.5 \times 10^6$ cells; $n = 9$) [Fig. 4D]. Thus, proper expansion of DN thymocytes was severely impaired in the absence of Hes1.

The spleen reconstituted with Hes1-null donors contained virtually no $\alpha\beta$ T cells, but interestingly it had some $\gamma\delta$ T cells [Fig. 4E]. Because the RAG2-null host mice did not have such T cells [Fig. 4F], they were derived from Hes1-null fetal liver cells. Thus, some $\gamma\delta$ T cells may develop normally in the absence of Hes1, although it remains to be determined whether they differentiate at the intrathymic or extrathymic environments.

Both TCR-independent and -dependent thymocyte expansion is affected by Hes1 mutation

DN cells normally undergo rearrangement of TCR$\beta$ lo-

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**Figure 2.** Flow cytometric analysis of fetal liver cells. Flow cytometric analysis was performed to determine the surface expression of TER-119 (A), Gr-1 (B), and Mac-1 (C). For B cell lineage analysis, fetal liver cells were cultured on PA6 stromal cells in the presence of IL-7 and examined for expression of IgM (D) and B220 (E). Cells were analyzed after incubation in the presence (shaded area) or absence (bold line only) of FITC-conjugated antibodies. No abnormality was detected in Hes1-null cells.
cus at the CD44−CD25+ stage, and only when the β locus is productively rearranged and mature into the last stage (CD44−CD25−) (Godfrey and Zlotnik 1993; Godfrey et al. 1993, 1994; Pe`nit et al. 1995). Because the thymocyte number at the CD44−CD25+ and CD44−CD25− stages was reduced significantly by Hes1 mutation, it is possible that TCR gene rearrangement is affected in Hes1-null thymocytes. We therefore examined rearrangement of TCR genes by amplifying the genomic DNA of the reconstituted thymi (Anderson et al. 1992; Itohara et al. 1993; Maki et al. 1996). Because in reconstituted mice Hes1-null thymocytes were, on an average, 200- to 1000-fold less in number than wild-type thymocytes, we used a 500-fold dilution of the wild-type DNA as a template to normalize the cell numbers. In the thymus and spleen of RAG2 mutant mice reconstituted with wild-type cells, TCR gene rearrangements clearly occurred (+/+ in Fig. 5A–H). In contrast, when RAG2 mutant mice were reconstituted with 1 × 10^6 Hes1-null cells, complete rearrangement was not detected at the TCRβ or β locus (−/− in Fig. 5A,E), although only a low level of D–J recombi-

Figure 3. Reconstitution of the lymphoid system of RAG2 mutant mice by transfer of wild-type and Hes1-null fetal liver cells. Either wild-type or Hes1-null fetal liver cells (1 × 10^6 or 1 × 10^7, indicated in parentheses above the panel) were transferred intravenously into RAG2 mutant mice to reconstitute the lymphoid system, and after 4–6 weeks of transfer, the lymphoid cells were examined. (A) The absolute numbers of total thymocytes. From the reconstituted thymuses, only Ly5.2+ cells were counted. The average thymocyte number with standard error is RAG2+/+; (3.2 ± 0.6) × 10^6 (n = 10); RAG2~/~/WT[1 × 10^6 cells injected]; (1.6 ± 0.3) × 10^6 (n = 13); RAG2+/~/WT[1 × 10^6 cells injected]; (1.0 ± 0.2) × 10^6 (n = 6); RAG2~/~/Hes1~/~[1 × 10^6 cells injected]; (1.7 ± 0.7) × 10^5 (n = 9); RAG2~/~/Hes1~/~[1 × 10^7 cells injected]; (4.1 ± 1.0) × 10^5 (n = 8). The number of Hes1-null thymocytes averaged 200- to 1000-fold less than that of wild-type thymocytes and 10- to 20-fold less than that of irradiated and nonreconstituted RAG2-null thymocytes. (B) Flow cytometric analysis of the reconstituted thymus with α-Ly5.2 and α-Thy1. The absolute number of total cells is indicated above each panel; the number of Ly5.2+Thy1+ cells is shown above the gated region. (C) The absolute numbers of B cells in the spleen. The B cell number of Hes1-null origin was normal. (D) Flow cytometric analysis of the reconstituted spleen with α-IgM and α-B220. B cells developed normally in the absence of Hes1.
were consistently and selectively detectable in the spleen but not in the thymus when RAG2 mutant mice were reconstituted with $1 \times 10^6$ Hes1-null fetal liver cells (−/− in Fig. 5G). Thus, gd T cells with restricted repertoire could develop normally in the absence of Hes1.

**Unique and essential functions of Hes1 in T cell development**

In this study, we have demonstrated that early events of αβ and γδ T cell development in the thymus, such as cell expansion and TCR gene rearrangement, are severely impaired in Hes1-null mice. Particularly, Hes1 seems to be essential for cell expansion at the very early stages, as Hes1-null cells did not expand at the CD44+CD25+ stage in the reconstituted thymus when $1 \times 10^6$ fetal liver cells were injected. Homing of Hes1-null progenitors to the thymus appeared unaffected, as the number of Hes1-null thymocytes at the CD44+CD25− stage was comparable with that of wild-type cells. It is likely that Hes1-null thymocytes might remain as resting cells or be more...
susceptible to apoptosis. This defect of expansion might contribute to the lack of or a small-sized thymus in \(\text{Hes1}\) mutant mice, although it is possible that certain functional defects of the \(\text{Hes1}\)-null stromal cells additionally contribute to the thymic aplasia.

It has been shown that the selective proliferation of DN cells that express functional TCRs involves various transcription factors such as HMG-box factors LEF-1 and TCF-1 and zinc finger transcription factor GATA-3 (Ting et al. 1996; Okamura et al. 1998). However, none of these factors appears to be involved in cell expansion before TCR gene rearrangement. Thus, \(\text{Hes1}\) is unique in regulating TCR-independent expansion of thymocytes at the earliest stage. Interestingly, in the absence of IL-7 receptor, thymocyte expansion is severely disturbed before TCR gene rearrangement (Peschon et al. 1994; Candeias et al. 1997), suggesting that IL-7 is one of the major growth factors involved in initial TCR-independent thymocyte proliferation. It is therefore tempting to speculate that \(\text{Hes1}\) functions downstream of the IL-7 signaling pathway. In addition to TCR-independent expansion, \(\text{Hes1}\) seems to be involved in expansion at later stages, as—even though TCR gene rearrangement occurred in the thymus of \(\text{RAG2}\)-null mice reconstituted with as many as \(1 \times 10^7\) fetal liver cells—\(\text{Hes1}\)-null thymocytes were still confined exclusively to the DN fraction with negligible increase in cell number. In the developing nervous system, \(\text{Hes1}\) is known to keep cells in a proliferative state and down regulation of \(\text{Hes1}\) expression leads to transition to a nonproliferative differentiation stage (Kageyama and Nakanishi 1997). Present results have indicated that similarly in the thymus \(\text{Hes1}\) promotes the initial expansion of immigrant progenitor cells, which is essential for the extensive clonal diversification and selection to generate mature T cells.

\(\text{Hes1}\) is also known as a target gene of the Notch signaling (Jarriault et al. 1995; Nishimura et al. 1998). It has been shown that both Notch and \(\text{Hes1}\) regulate CD4 SP versus CD8 SP fate choice of T cell development (Robey et al. 1996; Kim and Siu 1998). Although this function could not be examined here, as T cell development was arrested much earlier by \(\text{Hes1}\) mutation, collectively these data suggest that \(\text{Hes1}\) might function at multiple steps of T cell development.

**Materials and methods**

**Hes1 expression in the thymus**

Northern blot analysis was done as described previously (Sasai et al. 1992). The thymic stroma devoid of thymocytes was prepared as reported (Ikuta et al. 1990), with some modifications: Fetal thymic lobes were cultured at 37°C for 6 days in 7% CO\(_2\) on filters in 12-well plates containing RPMI1640 supplemented with 10% fetal calf serum, 1.35 mM dGUO, and 50 µM β-mercaptoethanol.

**Histological analysis of mouse embryos**

Whole embryos were fixed with 4% paraformaldehyde in PBS at 4°C for 1 hr and incubated in 30% sucrose in PBS at 4°C over-

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**Figure 5.** Rearrangement of TCR genes. Either \(1 \times 10^6\) or \(1 \times 10^7\) fetal liver cells of wild-type (+/+) and \(\text{Hes1}\)-null origin (−/−) were transferred intravenously into \(\text{RAG2}\) mutant mice, and rearrangements of TCR genes were examined by PCR followed by Southern blot analysis with \(^{32}\)P-labeled oligonucleotide probes. In the thymus reconstituted with \(1 \times 10^6\) \(\text{Hes1}\)-null cells, no apparent rearrangement was detected in the TCR\(_\alpha\) and TCR\(_\beta\) loci \([A,E]\), although only a low level of \(D-J\) recombination could be detected \([G]\). Whereas TCR\(_\gamma\) gene rearrangement was not detected in the thymus reconstituted with \(1 \times 10^6\) \(\text{Hes1}\)-null cells, TCR\(_\gamma\)2 and TCR\(_\gamma\)4 genes \([\text{Maki et al. 1996}]\) were rearranged in the spleen in the absence of \(\text{Hes1}\) \([G]\). When \(1 \times 10^7\) \(\text{Hes1}\)-null cells were injected, TCR gene rearrangement was detected \([B,D,F,H]\). As an internal control for PCR, the \(\text{RAG2}\) gene was amplified. The \(\text{RAG2}\) signal was derived only from the donor cells.
night. Frozen sections were prepared at 16 μm thickness and subjected to hematoxylin–eosin (HE) staining and immunohistochemistry. For immunohistochemistry, sections were incubated with biotinylated anti-TCRβ (Pharmingen, H57-597) and anti-Thy1 antibody (Pharmingen, S-52) followed by visualization with avidin-labeled fluorescein (Vector).

Fetal liver cell analysis

Single cell suspensions from fetal livers of E17 wild-type or Hes1-null embryos were prepared and subjected to flow cytometric analysis (Becton-Dickinson). The antibodies used were as follows: anti-TER-119 (Pharmingen, TER-119), anti-Gr-1 (Pharmingen, RB6-8C5), and anti-Mac-1 (Pharmingen, M1/70). Cytosed fetal liver cells on PA6 stromal cells with IL-7 were subjected to immunostaining with anti-IgM (Pharmingen, R6-60.2), and anti-B220 antibodies (Pharmingen, RA3-6B2). Detailed culture conditions were described previously (Sudo et al. 1989; Rolink et al. 1993).

Reconstitution of RAG2 mutant mice

Fetal liver cells (1 × 10^6 to 10 × 10^6) from E13–E16 wild-type and Hes1-null embryos (Ly5.2+) were injected intravenously into 4 Gy irradiated RAG2 mutant mice (Ly5.2+). After 4–6 weeks, single cell suspensions were prepared from thymus and spleens of the reconstituted RAG2 mutant mice and subjected to flow cytometric analysis (Becton-Dickinson). Only Ly5.2+ cells (the donor origin) were analyzed. The antibodies used were as follows: anti-TCRβ (Pharmingen, H57-597), anti-γδ TCR (Pharmingen, GL-3), anti-CD4 (Pharmingen, RM4-5), anti-CD8 (Pharmingen, 53-6.7), anti-CD25 (Pharmingen, 7D4), anti-CD44 (Pharmingen, IM7), anti-CD24 (HSA) (Pharmingen, M1/69), anti-CD3 (Pharmingen, 145-2C11), anti-Thy1 (Pharmingen, 53-2.1), and anti-CD45.1 (Ly5.2) (Pharmingen, A20).

PCR analysis for TCR gene rearrangement

The genomic DNA extracted from the reconstituted thymuses was amplified by PCR and subjected to Southern blot analysis with 32P-labeled oligonucleotide probes as described previously (Anderson et al. 1992; Itohara et al. 1993; Maki et al. 1996). Because the numbers of Hes1-null thymocytes were 200- to 1000-fold less than those of wild-type thymocytes, we used a 500-fold dilution of the wild-type samples to normalize the cell numbers. As a PCR control, we amplified the RAG2 gene, which was derived only from donor cells. In each experiment, a similar intensity of the RAG2 band was detected, indicating that the number of donor cells was normalized.

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