Functional Domains of Runx1 Are Differentially Required for CD4 Repression, TCRβ Expression, and CD4/8 Double-Negative to CD4/8 Double-Positive Transition in Thymocyte Development

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Functional Domains of Runx1 Are Differentially Required for CD4 Repression, TCRβ Expression, and CD4/8 Double-Negative to CD4/8 Double-Positive Transition in Thymocyte Development

Masahito Kawazu,* Takashi Asai,* Motoshi Ichikawa,* Go Yamamoto,* Toshiki Saito,* Susumu Goyama,* Kinuko Mitani,† Kohei Miyazono,‡ Shigeru Chiba,* Seishi Ogawa,* Mineo Kurokawa,‡ and Hisamaru Hirai*‡

Runx1 (AML1) has multiple functions in thymocyte development, including CD4 repression in immature thymocytes, expression of TCRβ, and efficient β-selection. To determine the functional domains of Runx1 important for thymocyte development, we cultured Runx1-deficient murine fetal liver (FL) cells on OP9-Delta-like 1 murine stromal cells, which express Delta-like 1 and support thymocyte development in vitro, and introduced Runx1 or C-terminal-deletion mutants of Runx1 into the FL cells by retrovirus infection. In this system, Runx1-deficient FL cells failed to follow normal thymocyte development, whereas the introduction of Runx1 into the cells was sufficient to produce thymocyte development that was indistinguishable from that in wild-type FL cells. In contrast, Runx1 mutants that lacked the activation domain necessary for initiating gene transcription did not fully restore thymocyte differentiation, in that it neither repressed CD4 expression nor promoted the CD4/8 double-negative to CD4/8 double-positive transition. Although the C-terminal VWRPY motif-deficient mutant of Runx1, which cannot interact with the transcriptional corepressor Transducin-like enhancer of split (TLE), promoted the double-negative to double-positive transition, it did not efficiently repress CD4 expression. These results suggest that the activation domain is essential for Runx1 to establish thymocyte development and that Runx1 has both TLE-dependent and TLE-independent functions in thymocyte development. The Journal of Immunology, 2005, 174: 3526–3533.
the C-terminal VWRPY motif, which mediates the interaction with Transducin-like enhancer of split (TLE), a transcriptional corepressor (22, 23) (see Fig. 3A), and a domain which represses p21 transcription through the interaction with mammalian Sim3 isoform A corepressor (24) (not shown in Fig. 3A) are also known. Runx1 activates the transcription of different genes by interacting with different cofactors in various types of cells (25). To elucidate the mechanism by which Runx1 exerts various functions, the contributions of each domain to a particular function of Runx1 have been evaluated. Okuda et al. (26) examined the ability of full-length and mutant Runx1 genes to rescue the hematopoietic defect in Runx1-deficient embryonic stem cells through a knock-in approach and demonstrated that the activation domain, but not the VWRPY motif, is indispensable for definitive hematopoiesis. No alterations in thymocyte subpopulations were detected in mice in which the VWRPY motif of Runx1 is genetically disrupted, although they have a significantly small thymus (27). In their study, the roles of the activation domain during thymocyte development were not assessed, due to a profound defect in hematopoiesis in the absence of the activation domain of Runx1. Therefore, the roles of functional domains of Runx1 in thymocyte development have not yet been adequately clarified.

Although fetal thymus organ culture (FTOC) has been conventionally used for in vitro studies on thymocyte development (28), it is difficult to achieve high gene-transduction efficiency and to obtain a sufficient number of cells for analyses with FTOC. We used an in vitro culture system in which fetal liver (FL) cells from wild-type mouse embryos follow normal thymocyte development on a layer of OP9-Delta-like 1 (DL1) murine stromal cells expressing a Notch ligand, DL1, on their surface (29, 30). In this system, FL cells from Runx1-deficient embryos exhibited defective thymocyte development, which was successfully restored by the reintroduction of full-length Runx1 by retroviral infection. We also introduced several forms of Runx1 mutants into the Runx1-deficient FL cells and evaluated their ability to restore thymocyte development, which revealed distinct functions of Runx1 domains during thymocyte development.

Materials and Methods
Preparation of cDNAs of Runx1 mutants and gene transduction
cDNAs of C-terminal deletion mutants of Runx1, Δ447, Δ372, Δ320, and Δ291, with a NotI site on their 5′ terminus and an XhoI site on their 3′ terminus, were PCR amplified from template murine Runx1 cDNA (a gift from M. Satake, Tohoku University, Sendai, Japan) using TaKaRa LA taq (Takara Bio) with the following sets of primers: a sense oligonucleotide for all constructs, 5′-AAAAAGCCCGCGATCGATCATCGATGATCCCGGT-3′; antisense oligonucleotides: Δ477, 5′-TTTTCTCGAAGTCGGCTCCTCTCATGGCCCGCGCCG-3′; Δ372, 5′-TTTTCTCGAAGTCGGCTCCTCTCATGGCCCGCGCCG-3′; Δ320, 5′-TTTTCTCGAAGTCGGCTCCTCTCATGGCCCGCGCCG-3′; Δ291, 5′-TTTTCTCGAAGTCGGCTCCTCTCATGGCCCGCGCCG-3′; 3′-end of the XhoI restriction site of pCDNSam (a gift from H. Nakao, Tokyo University, Tokyo, Japan) retrovirus vector (31). Nucleotide sequences of these mutant plasmids were confirmed using the ABI Ready Reaction Dye Terminator Cycle Sequencing kit and ABI3100 semiautomated sequencers (Applied Biosystems). To obtain retrovirus-producing cells, pMSP4 packaging cells (a gift from Wakenaga Pharmaceutical) were transfected with these retrovirus plasmids, followed by single cell sorting for GFP with a FACS Vantage (BD Biosciences). To characterize cells transduced with retrovirus plasmids, GFP-positive cells were gated and analyzed.

Cell preparation and genotyping
Embryos at 14.5 days postcoitus (E14.5) were obtained by mating Runx1<sup>−/−</sup> mice (female) and Runx1<sup>+/+<sup>male</sup></sup> Lck-Cre transgenic (tg) mice (male), both of which had been backcrossed for nine generations to C57BL/6. Lck-Cre tg mice were kindly provided by J. Takeda (Osaka University, Osaka, Japan) (32). FLs were dissected from the E14.5 embryos and then subjected to single cell suspension by pipetting. An aliquot of the FL cell suspension was subjected to DNA extraction followed by genotyping using PCR with primers f2 (5′-ACAAAAACCTAGTTGTCACAGGAGAACAAGT3′), f120 (5′-CCCTGAGAACAGGAGAGTTTCTCA-3′), and r1 (5′-GTCTACTCTTCTCCTGACAGGAAACAAA3′), in which flocked and flocked-out (or deleted) alleles were amplified as 280-bp (f120-r1) and 220-bp (f2-r1) PCR fragments, respectively.

Culture of FL cells on OP9-DL1 stromal cells
FL cells were cultured on OP9-DL1 cells (generous gifts from J.C. Zúñiga-Pflücker, University of Toronto, Toronto, Canada) (29) according to the original descriptions with minor modifications. In brief, mononuclear cells were separated from a single cell suspension of E14.5 embryos of C57BL/6 mice by centrifugation on a Ficoll-Hypaque (Axis-Shield; Lymphoprep) gradient. A total of 5 × 10<sup>4</sup> mononuclear cells, without further purification of hematopoietic progenitor cells, was cultured on confluent OP9-DL1 cells in flat-bottom 24-well culture plates with 500 µl of MEM (Invitrogen Life Technologies) supplemented with 20% FCS, penicillin/streptomycin, and 5 ng/ml recombinant human (rh) IL-7 (R&D Systems). After 5 days of culture, 5 × 10<sup>4</sup> cells were passed onto newly prepared OP9-DL1 cells in the presence of 5 ng/ml IL-7, and retrovirus infection was performed using polybrene (final concentration 8 µg/ml), followed by another 5 days of culture. A total of 1 × 10<sup>5</sup> cells were again passed onto newly prepared OP9-DL1 cells and cultured for another 5 days, but in IL-7-free culture medium.

Flow cytometry
Cells were collected from culture plates, suspended in PBS, and then incubated with mAbs for 30 min on ice. If necessary, this was followed by additional incubation with the secondary reagents for another 30 min on ice. After being washed with PBS, cells were analyzed by flow cytometry using a FACS Calibur (BD Biosciences) equipped with CellQuest software. All mAbs and fluorochromes used in flow cytometry were purchased from BD Pharmingen: FITC, PE, PerCP, PerCP-Cy5.5, allophycocyanin, or Biotin-conjugated CD3e (500A2), CD4 (RM4-5), CD8a (53-6.7), CD24 (M1/69), CD25 (PC61), CD44 (IM7), CD45.2 (104), CD45RB/B20 (RA3-6B2), CD90.2 (Thy1.2: 52-2.1), or TCRβ (H57-597). Intracellular anti-TCRβ allophycocyanin staining was performed using a BD Cytofix/Cytoperm kit (BD Pharmingen) in accordance with the manufacturer’s instructions.

Results
Normal FL cells can differentiate into DN and DP thymocytes on OP9-DL1 cells
The ontogenic profiles of nonpurified FL cells on OP9-DL1 cells were essentially similar to those of purified FL cells for hematopoietic progenitor cells (CD24<sup>low</sup>, Lin<sup>−</sup>, Sca-1<sup>high</sup>, CD117<sup>cl−</sup>Kit<sup>−</sup>) (29). Most of the FL cells from wild-type C57BL/6 mouse embryos cultured on OP9-DL1 cells expressed Thy1 without the distinct expression of B220, whereas FL cells cultured on parental OP9 cells did not show a high expression level of Thy1 but had apparently committed to B lymphocytes, as manifested by B220 expression (Fig. 1A). After 15 days of culture on OP9-DL1 cells, a considerable number of FL-derived cells became CD4<sup>+</sup>CD8<sup>+</sup> (Fig. 1B) and were thought to correspond to CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. These CD4<sup>+</sup>CD8<sup>+</sup> cells also expressed TCRβ at a level comparable with that in DP thymocytes in adult thymus (Fig. 1C), indicating that the FL cells cultured on OP9-DL1 cells in vitro can follow the normal development of DP thymocytes in the thymus. A small number of SP (i.e., CD4<sup>+</sup>CD<sup>−</sup> or CD8<sup>+</sup>CD<sup>−</sup>) cells were also observed, but they expressed only intermediate levels of TCRβ on their cell surface (Fig. 1C), suggesting that these cells were not as fully mature as the CD4 SP cells or the CD8 SP cells in the thymus. Another prevalent population in the normal FL cell culture on OP9-DL1 cells was CD4<sup>+</sup>CD<sup>−</sup> cells, which were considered to be reminiscent of CD4<sup>+</sup>CD8<sup>−</sup> DN thymocytes. DN thymocytes differentiate through the maturation sequences DN1 (CD4<sup>+</sup>CD25<sup>+</sup>), DN2 (CD4<sup>+</sup>CD25<sup>−</sup>), DN3 (CD4<sup>+</sup>CD25<sup>+</sup>), and DN4 (CD4<sup>+</sup>CD25<sup>−</sup>) (33), and each DN fraction was detected in FL-derived CD4<sup>+</sup>CD8<sup>−</sup> cells cultured on OP9-DL1 cells.
by staining with CD25 and CD44, although the proportion of cells at the DN2 stage was prominent (Fig. 1D).

**Phenotypes of Runx1 conditionally knocked out (cko) FL cells cultured on OP9-DL1 cells**

Using this FL/OP9-DL1 coculture system, FL cells from Runx1-targeted (cko: Runx1floxed−/−, Lck-Cre tg) mice were tested for their capacity to differentiate into DP thymocytes. Whereas 10 days of culture of the control (ctrl: Runx1+/−, Lck-Cre tg) FL cells on OP9-DL1 cells exclusively produced CD4+CD8− cells, a similar culture of cko FL cells generated a population that showed an intermediate expression level of CD4 without CD8 (CD4intCD8−) in addition to CD4+CD8− cells (Fig. 2A). The CD4intCD8− subset in the cko FL cell culture is thought to be as immature as the CD4−CD8+ subset because it is quite unlikely that so many cko cells can differentiate beyond DP stage, due to the fact that only a small proportion of ctrl cells progressed to the CD4+CD8− cells after 10 days of culture (Fig. 2A). Indeed, TCRβ and CD5, whose expression levels rise as thymocytes mature, were up-regulated in CD4+CD8− ctrl cells, but not in CD4+CD8− cko cells (Fig. 2B). In addition, CD24, whose expression level diminishes as thymocytes mature, is down-regulated in CD4+CD8+ ctrl cells, but not in CD4+CD8− cko cells (Fig. 2B). Furthermore, the expression profile of CD44 and CD25 was comparable with that of CD4+CD8− cells (Fig. 2C). The extent of Cre-mediated depletion of the floxed Runx1 allele was greater in CD4intCD8− cells than in the CD4−CD8− cells (Fig. 2D), which is consistent with the fact that Runx1 actively represses CD4 expression in DN thymocytes (14). After 15 days of culture, the ctrl FL cells cultured on OP9-DL1 cells consisted mainly of CD4+CD8+ and CD4−CD8− cells, corresponding to DP and DN thymocytes in the thymus, respectively (Fig. 2A). In contrast, cko FL cells cultured for 15 days contained mainly CD4+CD8− cells, and only a small fraction were CD4+CD8− cells. The CD4+CD8− cells from the ctrl FL cell culture showed higher expression levels of TCRβ than did CD4−CD8− cells, whereas expression of TCRβ on CD4+CD8− cells derived from cko FL cells was as low as that on CD4−CD8− cells (data not shown), indicating the impaired maturation of CD4+CD8− cells derived from cko FL cells on day 15. These observations are consistent with our unpublished finding in Runx1 cko mouse, in which TCRβ expression on DP and CD4+ SP thymocytes was significantly reduced.

The DN (CD4−CD8+) population in the ctrl FL-derived cells appeared to contain four subsets of DN1 to DN4 on day 15 of culture (Fig. 2E). In contrast, the CD4−CD8+ population observed in the cko FL cell culture mainly consisted of DN1 and DN2 cells, indicating differentiation arrested at the DN2−3 transition. Thus, on OP9-DL1 cells, ctrl FL cells produced both DN and DP cells in almost the same manner as FL cells from wild-type C57BL/6 mice, whereas thymocyte development from cko FL cells was significantly impaired at the DN2−3 transition and showed the premature expression of CD4.

**Runx1 gene transduction can restore the impaired differentiation of Runx1-deficient FL cells**

To confirm that the impaired maturation of cko FL-derived cells was caused by a lack of Runx1, we examined whether the reintroduction of Runx1 could rescue the block in the DN2−3 transition found in cko FL-derived cells. The cko FL-derived cells transduced with Runx1 by retrovirus infection showed a significant increase in DN3 cells accompanied by the appearance of DN4 cells, which was not seen in mock-infected cells (Fig. 3B, top panel). These results demonstrated that Runx1 is essential for the DN2−3 transition during thymocyte development. Remarkably, when Runx1 was introduced, the control FL cells generated more DN3 and DN4 cells than did mock-infected ctrl FL cells (Fig. 3B, bottom panel), suggesting that an increased dosage of Runx1 may also affect thymocyte development.

We next sought to determine the functional domains of Runx1 that are involved in thymocyte development. For this purpose, we generated a series of C-terminal deletion mutants of Runx1 (Fig. 3A) and transduced them into cko FL cells by retrovirus infection. Infection efficiencies were ~80% as assessed by GFP positivity and were almost constant for all of the constructs (data not shown). Δ447 lacks the C-terminal VWRPY motif, which is required for interaction with TLE (22, 23), whereas Δ372 lacks the inhibitory domain that impedes transcriptional activity mediated by the activation domain of Runx1 (21). The Δ320 mutant lacks a part of the
activation domain, and Δ291, which completely lacks the activation domain, shows less potent transcriptional activity than does Δ320 (21). The proportions of DN3 and DN4 cells on day 15 of culture were calculated for cko FL-derived cells infected with each mutant (Fig. 3C).

Δ47-transduced cko FL cells produced DN3 and DN4 cells in numbers comparable with full-length Runx1-transduced cko FL cells. Therefore, the VWRPY motif is not necessary for the function of Runx1 in the DN2–3 transition. Although Δ372, which lacks the inhibitory domain, can rescue the DN2–3 transition as efficiently as full-length Runx1, rescue of the DN3–4 transition was still marginally impaired. Despite the fact that the transcriptional activity of Runx1 is derepressed in the absence of the inhibitory domain (21), the differentiation of Δ372-transduced cko FL cells is not promoted compared with that of Runx1-transduced cko FL cells in this culture system, suggesting that the elevated transcriptional activity does not affect Runx1-dependent thymocyte development.

In contrast, both Δ320 and Δ291, which lack part of and the entire activation domain, respectively, failed to restore either the DN2–3 or DN3–4 transition. Thus, the activation domain is required for the function of Runx1 in the DN2–3 and DN3–4 transitions. Interestingly, the DN3 and DN4 subsets of Δ320- or Δ291-transduced control FL cells were diminished compared with mock-infected ctrl FL cells (Fig. 3B, bottom panels), which raises the possibility that both Δ320 and Δ291 suppress the function of endogenous Runx1 in the DN2–3 and DN3–4 transitions in a dominant-negative manner. The suppressive effects of Δ320 and Δ291 were confirmed in three independent experiments (proportions of DN3 cells, p = 0.031 for mock vs Δ320 and p = 0.016 for mock vs Δ291; proportions of DN4 cells, p = 0.028 for mock vs Δ320 and p = 0.029 for mock vs Δ291).

To determine the efficiency of Cre-mediated gene deletion in this culture system, genotyping of the Runx1 alleles was performed for each stage of DN cells. DN3 and DN4 cells were obtained from day 10 culture of Runx1-deficient Lck-Cre tg FL cells. The whole culture on day 5 was used to genotype DN2 cells, because almost all of the cells were at the DN2 stage on day 5. Genomic DNA was extracted from each DN subpopulation and used as a template for genotyping. Only the floxed allele was detected in the FL cells on day 0, whereas both the floxed and deleted alleles were detected in day 5 DN2 cells. In contrast, only the deleted allele was detected from the DN3 and DN4 subsets derived from Runx1-transduced FL cells (Fig. 3D). These results indicated that Cre-mediated gene deletion was only partially achieved in the DN2 cells, but was complete at the DN3 stage in this culture system.

Because our unpublished observation using Runx1 cko mice revealed decreased TCRβ expression in Runx1-deficient DN3 thymocytes,7 we examined expression of intracellular TCRβ in DN

FIGURE 2. FACS analysis of cko FL cells and ctrl FL cells cultured on OP9-DL1. A, CD4/CD8 expression profiles of each type of FL-derived cell on days 10 and 15. B, Expression levels of TCRβ, CD5, and CD24 in CD4+/CD8- cko cells (solid lines) and CD4+/CD8+ ctrl cells (dotted lines) cultured for 10 days were compared with those of CD4+ CD8- cells (gray shades without contour). Those for the indicated subsets of ctrl cells cultured for 10 days and thymocytes derived from adult thymus were also presented. C, CD25/CD44 expression profiles of CD4+ CD8- cells and CD4+/CD8+ cells among cko FL cells cultured for 10 days. D, Genotype of each subpopulation of cko FL-derived cells, which were sorted with a FACSVantage SE cell sorter (BD Biosciences) after being stained with anti-CD4 PE and anti-CD8 PerCP-CY5.5. Genomic DNA was extracted from sorted cells and electrophoresed after amplification by PCR. E, CD25/CD44 expression profiles of CD4+ CD8- cells on day 15.
cells in day 15 culture of FL cells. A significant proportion of Runx1-transduced cko DN cells expressed intracellular TCRβ, whereas TCRβ was barely detected in mock-infected cko DN cells (Fig. 4). Transduction of Δ447 or Δ372 restored intracellular TCRβ expression to a level comparable with that of full-length Runx1, whereas cko DN cells transduced with Δ320 or Δ291 did not express intracellular TCRβ. In accordance with the increase in the proportions of DN3 and DN4 cells among Runx1-transduced ctrl cells (Fig. 3B, bottom), the percentage of Runx1-transduced ctrl DN cells expressing intracellular TCRβ was increased compared with the

![FIGURE 3](image_url) Development of DN3 and DN4 cells in FL-derived cells, which were transduced with the genes for Runx1 or its C-terminal deletion mutants. A, Construction of Runx1 and C-terminal deletion mutants. Numbers indicate the positions of amino acid residues from the N terminus. B, CD25/CD44 expression profile of CD4/CD8 DN cells on day 15 are shown for cko FL-derived cells (top panels) and ctrl FL-derived cells (bottom panels) with transduced Runx1 mutants. Cells were stained with anti-CD4PE, anti-CD3PerCP, anti-CD4PerCP, anti-CD8 PerCP, and anti-CD25 allophycocyanin. GFP-positive and PerCP-negative cells were gated and analyzed for the CD25/CD44 expression profile. The percentage of cells in each quadrant is indicated. C, Proportions (%) of DN3 (CD44low/CD25+) and DN4 (CD44−/CD25−) cells on day 15 in nine independent experiments were averaged and are shown with ±1× SE. Asterisks indicate statistically significant differences, and p values were indicated. ANOVA and post hoc comparison (Fisher test) were performed using StatView software (SAS Institute). D, DN3 and DN4 thymocytes were sorted by a FACSVantage SE cell sorter (BD Biosciences) after being stained by anti-CD3 PE, anti-CD4 PE, anti-CD8 PE, anti-CD25 PerCP-CY5.5, and anti-CD44 allophycocyanin. Genomic DNA was extracted from the sorted cells and electrophoresed after PCR amplification.

![FIGURE 4](image_url) Expression levels of intracellular TCRβ in the CD4+CD8− subset among cko (top panels) and ctrl (bottom panels) FL-derived cells on day 15. Transduced Runx1 mutants are shown above. Cells were stained with anti-CD4 PE, anti-CD8 PerCP, and anti-TCRβ allophycocyanin. GFP-positive, PE-negative, and PerCP-negative cells were analyzed for TCRβ expression (filled histograms). Expression levels of intracellular TCRβ in splenic B cells are overlaid as negative controls (thick lines). The percentages of positive cells are indicated in each histogram.
FIGURE 5. CD4 repression in the FL-derived cells transduced with Runx1 or its C-terminal deletion mutants. A, CD4+CD8 expression profiles of cko (top panels) and ctrl (bottom panels) FL cells on day 10 of culture. Transduced Runx1 mutants are shown above. Cells were stained with anti-CD4 PE, anti-CD8 PerCP, and anti-CD45.2 allophycocyanin. GFP-positive and allophycocyanin-positive cells were analyzed for CD4/8 expression. The percentage of cells in each quadrant is indicated. B, Proportions (%) of CD4int cells among CD8-negative cells in nine independent experiments were averaged and are shown with ±1× SE. ANOVA and post hoc comparison (Fisher test) were performed using StatView software (SAS Institute). Asterisks indicate the statistically significant differences, and p values were indicated.

mock-infected cells (Fig. 4, bottom), and those TCRβ-expressing cells corresponded with CD44-negative (DN3 or DN4) cells (data not shown). Although it is yet to be determined whether decreased expression of TCRβ was the cause or the result of impaired thymocyte differentiation, the fact that the TCRβ gene has canonical binding sites for Runx1 within its enhancer region (34) and is transcriptionally up-regulated by Runx1 (8) supports the notion that Runx1 promotes thymocyte maturation at least partly by up-regulating TCRβ expression. Our results also indicate that the activation domain, but not the VWRPY motif, is critical for Runx1-mediated TCRβ up-regulation.

C-terminal VWRPY motif of Runx1 is necessary for CD4 repression

As shown in Fig. 5A, the CD4intCD8− subsets in day 10 culture of cko FL cells disappeared upon the reintroduction of Runx1 (Fig. 5A), which was again consistent with the established role of Runx1 in CD4 repression (14). This observation also demonstrates that the aberrant expression of CD4 observed in DN subsets of cko FL-derived cells can be ascribed to Runx1 depletion. To determine the domains of Runx1 that are relevant for CD4 repression, a series of C-terminal deletion mutants of Runx1 were transduced into cko or ctrl FL cells, and the proportion of CD4intCD8− cells was evaluated on day 10 of culture (Fig. 5). Whereas full-length Runx1 almost completely repressed aberrant CD4 expression, only partial repression was seen with Δ447 or Δ372 mutants. These results suggest that CD4 repression by Runx1 requires some C terminus-mediated interaction with other molecules such as TLE. The extent of CD4 repression by Δ447 is greater than that by Δ372, which might reflect the existence of an additional repression domain in the C terminus other than the VWRPY motif (23).

Δ320 and Δ291 each failed to repress CD4 expression, resulting in an increase in the CD4int population compared with the mock-infected cko FL cells. Because Runx1 depletion is incomplete in the DN subsets of cko FL-derived cells on day 10 (Fig. 2D), the increase in the CD4int population is probably due to a dominant-negative effect of Δ320 and Δ291 on remaining endogenous Runx1. This notion is supported by the observation that Δ320- or Δ291-transduced control FL cells produced a significant number of CD4intCD8− cells, which were barely detected in mock-infected ctrl FL-derived cells (Fig. 5A, bottom).

Discussion

In the current study, we demonstrated that Runx1 was important for thymocyte development using the FL/OP9-DL1 coculture system. This system is superior to conventional FTOC in that a sufficient number of cells for extensive analyses can be easily obtained, especially DN thymocytes. Another advantage of this system is the highly efficient transfer of the genes of interest. In this study, we were able to introduce various mutants of Runx1 by retroviral infection with an efficiency of ~80% (data not shown), which is higher than that obtained with FTOC. In contrast, terminal maturation of SP cells cannot be achieved in this culture system, which makes it difficult to analyze more mature stages of thymocytes.

The absolute need for Runx1 in thymocyte development in vivo has been unequivocally demonstrated using conditionally Runx1-targeted mice. When Runx1-deficient bone marrow cells are transplanted to lethally irradiated mice, the development of thymocytes is severely blocked at the DN2–3 transition (35), whereas the deletion of Runx1 in later stages of DN thymocytes using the Lck-Cre tg results in a profound defect in the DN3–4 transition.4 Together, these findings suggest that Runx1 is necessary for normal thymocyte development at multiple steps during the DN-DP transition. Despite the DN3–4 block in T lymphocyte-specific Runx1-targeted mice, thymocyte development of the cognate FL cells was arrested at the DN2–3 transition in this culture system. The difference in the developmental block occurs may be due to earlier Cre-mediated Runx1 deletion in vitro rather than in vivo. In the FL culture system, deletion of the floxed Runx1 allele occurs predominantly at the DN2–3 transition, which leaves few, if any, DN3 cells with an intact Runx1 allele (Fig. 3D, lane 3). Lck-Cre tg mice harbor a transgenic gene encoding Cre recombinase driven by the p56lck proximal promoter (32, 36). The Lck
encodes a lymphocyte-specific protein tyrosine kinase, which mediates β-chain-dependent signaling during β-selection, is associated with allelic exclusion of β locus (37), and is transcribed from two developmentally regulated, independently functioning promoters. The proximal promoter is used exclusively in thymocytes, but not in peripheral T lymphocytes (38), and CRE-mediated gene deletions are expected to be activated by p56Lck proximal promoter at the DN2 and DN3 stages when VB gene rearrangement and subsequent β-selection occurs. However, even if the same p56Lck proximal promoter is used, exact timing of gene expression differs depending on the transgenic mice lines, and different lines of Lck-Cre tg mice are used to target a gene at different developmental stages (39).

The function of the VWRPY motif in hematopoiesis has been examined in embryonic stem cell culture (26) and in para-aortic splanchnopleural culture (40). Because Runx1 mutants that lack the VWRPY motif could fully restore hematopoiesis in Runx1-deficient cells in these two studies, the VWRPY motif does not seem to be necessary for hematopoiesis. On the contrary, because mice in which cDNA for the VWRPY-deficient Runx1 mutant had been homozygously Knocked-in to the Runx1 alleles exhibited a reduced number of thymocytes and deviant CD4 expression during thymocyte ontogeny (27), the VWRPY motif seems to play a role in thymocyte development, although the precise molecular mechanism is unclear. In the present study, although the VWRPY-deficient Runx1 mutant (Δ447) could restore not only maturation to the DN4 subset but also TCRβ expression in cko FL-derived thymocytes as efficiently as wild-type Runx1 (Fig. 4), it had only a limited capacity to repress aberrant CD4 expression (Fig. 5). These different requirements for the VWRPY motif indicate that Runx1 functions in both TLE-dependent and TLE-independent manners during early thymocyte development. In fact, the context-dependent need for interaction with a transcriptional coressesor has been reported for Runt and Groucho, Drosophila homologues of Runx and TLE, respectively (41). One possible explanation for TLE-dependent CD4 repression is that TLE actively converts Runx1 to a transcriptional repressor by recruiting histone deacetylase, as seen in Drosophila (41). Another possibility is that TLE displaces some coactivators from Runx1 under particular conditions, which prevents Runx1 from up-regulating CD4 expression.

A similar mechanism has been proposed for transcription by lymphoid enhancer binding protein 1/T cell factor, which is repressed until TLE is replaced by β-catenin (42). Further analyses are needed to clarify the role of the VWRPY motif in the regulation of CD4 transcription.

The introduction of Runx1 mutants into cko FL cells has shown that the activation domain makes a critical contribution to various functions of Runx1 in thymocyte development, including CD4 repression, the DN2–3 transition, and the expression of TCRβ. Significantly, Δ320 and Δ291, both of which lack the activation domain, dominantly suppress CD4 repression and the DN2–3 transition but do not interfere with TCRβ expression. This may be due to a higher affinity of Runx1 for the TCRβ enhancer compared with Δ320 and Δ291. Although this speculation is not supported by experimental evidence, a potential mechanism that accounts for this finding is that the interaction of Runx1 with other transcription factors may confer on Runx1 a higher affinity for specific gene promoters. Otherwise, Δ320 and Δ291 may retain a marginal potential to up-regulate TCRβ, which would prevent the total loss of TCRβ when they are forcibly expressed.

In conclusion, we have successfully reproduced the phenotype of Runx1-deficient thymocytes in vitro using the FL/O9-DL1 co-culture system and have evaluated the function of Runx1 and its mutants by retroviral gene transduction. The activation domain is essential for the function of Runx1 in CD4 repression, the DN2–3 transition, and the expression of TCRβ, whereas the VWRPY motif does not contribute to the DN2–3 transition or the expression of TCRβ, but it is partially involved in CD4 repression. Further studies are needed to understand how the VWRPY motif of Runx1 regulates CD4 transcription and how Runx1 functions at multiple steps in thymocyte development.

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Disclosures

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References


