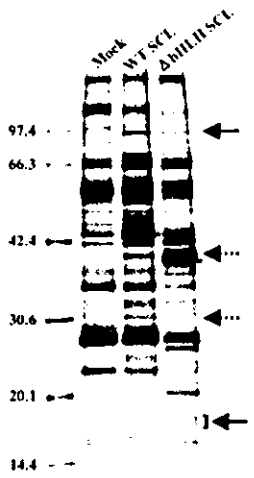

Figure 1. Proteins co-precipitated with WTSCL or ΔbHLH SCL. WT SCL and ΔbHLH SCL are indicated by asterisks. The bands indicated by arrows are reproducibly precipitated.

interact with the N- or C-terminal region of SCL, and thus, it is speculated that ΔbHLH SCL functions against WT SCL through the competition for binding to these proteins. To further explore the underlying biochemical mechanisms, we have sequenced some of these coprecipitated proteins, which we hope will be reported in the near future.

We disagree with the comment by the questioners that we should be able to show the rescue of the effect of ΔbHLH SCL with WT SCL. This is not an appropriate experiment to show the dominant-negative effect of ΔbHLH SCL.

Although the biochemical mechanisms need to be further disclosed, clear are our findings on the distinct biologic functions of WT SCL and ΔbHLH SCL on the commitment fate determination of hematopoietic stem cells. We hope that our ongoing study will give a clear answer to the mechanisms for how ΔbHLH SCL functions in a dominant-negative fashion against WT SCL.

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To the editor:

Potential curability of newly diagnosed acute promyelocytic leukemia without use of chemotherapy: the example of liposomal all-*trans* retinoic acid

Several years ago we reported that liposomal all-*trans* retinoic acid (L-ATRA) used alone might cure some patients with untreated acute promyelocytic leukemia (APL).[1,2] The median follow-up was 1.5 years from complete remission (CR) date. Because the risk of relapse does not decrease appreciably until considerably later,[3] we herein update the study. The L-ATRA dose was 90 mg/m^2 every other day until CR, after which this dose was given 3 times weekly for 9 months. Using a sensitivity level of 10 (−4), polymerase chain reaction (PCR) testing for the promyelocytic leukemia/retinoic acid receptor{alpha} (PML-RAR) (fusion protein) was done every 3 months for 2 years from CR date. If positive, the test was repeated 2 to 4 weeks later. If again positive ("molecular relapse"), patients received 12 mg/m^2 idarubicin 3 times daily every 4 to 5 weeks for 3 courses. Thirty-four patients, median age 49 years, median white blood cell count (WBC) 2000/μL, were treated: 8 were high risk (WBC count > 10 000/μL) using Sanz et al's system.[4] The CR rate was 26 of 34, but only 3 of 8 in high-risk patients. Of the 34 patients, 10 (29%, 95% CI 15%-47%) remain alive in first CR at a median of 4.5 years (range, 3.0-5.5 years) from CR date despite never receiving other anti-APL therapy. In each case, the most recent PCR test is negative, having been obtained a median of 3.2 years (range, 1.4-5.5 years) years from CR date. The remaining 16 patients who entered CR received idarubicin; in one of these, the reports of the relevant PCRs were later changed to negative and in another only a single PCR test was positive. Thus, 14 patients "required" idarubicin: 8 had molecular relapse (at a median of 9 months from CR date), 2 failed to achieve molecular CR despite 6 months of L-ATRA monotherapy, and 4 had simultaneous clinical and molecular relapse (at a median of 17 months from CR date). The 10 patients who never received treatment other than L-ATRA each had a presenting WBC count less than 10 000/μL, as did 13 of the 14 who required idarubicin. The 2 groups also had similar distributions of initial WBC count, platelet count, and type of PML-RAR (isoform and age). The PCR status at CR was of no discriminatory value since all patients but one were PCR positive at CR, with 24 of 26 becoming negative by 3 months. Of the 10 patients whose first indication of failure was molecular, 9 received idarubicin in (hematologic) CR. Of those, 6 remain in hematologic CR, while 3 had hematologic relapse, which occurred within 1 year of molecular failure.

The immediate significance of our results is limited. L-ATRA is unavailable commercially. The 3 of 8 CR rate in high-risk patients seems extraordinarily low. Furthermore, while possibly sparing two fifths (ie, 10 of 26) of low-risk patients the need for chemotherapy, L-ATRA was not free of toxicity[1,2] and required 3 intravenous infusions weekly. Nonetheless, the observation that patients can be potentially cured of APL without use of chemotherapy should encourage further attempts in the same direction as, for example, in our current trial using ATRA and arsenic trioxide.[5]

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[1]

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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.**

Runx1 (also called *AML1*, *Pebpa2b*, or *Cbfa2*) encodes a member of a family of runt transcription factors that was first identified in humans as a gene that is disrupted in t(8;21) acute myeloid leukemia (1). Homozygous disruption of *Runx1* in mice revealed that Runx1 plays an essential role in definitive hematopoiesis (2, 3). Furthermore, it has been suggested from the very beginning of its cloning that Runx1 also plays roles during thymocyte development (4–6). Runx1, together with other cofactors, binds to the enhancers of *TCRα* (7), β (8), γ (9), and δ (10) and activates transcription of these genes. Runx1 is expressed during thymocyte development as demonstrated by Northern blotting, as well as in situ hybridization of mRNA (11, 12). It is mainly expressed in cortical thymocytes (13), and quantitative real-time PCR of reverse-transcribed RNA revealed that Runx1 mRNA is abundant in CD4/CD8 double-negative (DN)[3] thymocytes (14). When Runx1 was overexpressed in thymocytes using a transgenic system, it was shown to induce CD8 single-positive (SP) thymocyte differentiation (15) and to inhibit the differentiation of Th2 effector T cells (16). Recently, we found that T cell-specific disruption of *Runx1* in mice using the *Cre-loxP* recombinase system results in a profound defect in the DN to CD4/8 double-positive (DP) transition,[4] and others also demonstrated that Runx1 actively represses *CD4* expression in DN thymocytes (14). Together, these findings confirm that Runx1 plays an essential role in early thymocyte development. In view of its functions in T cell development, it is noteworthy that the *Runx1* gene is disrupted in t(4;21)(q28;q22) found in T cell acute lymphoblastic leukemia (17, 18).

Runx1 has several distinct domains of defined biochemical functions. The Runt domain mediates both binding to DNA and dimerization with core-binding factor β subunit (4), whereas the activation domain interacts with transcriptional coactivators to upregulate transcription of the target genes (19, 20). Toward the C terminus of the activation domain lies an inhibitory domain that counteracts the effect of the activation domain (21). Furthermore,

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[3] Abbreviations used in this paper: DN, double-negative; SP, single-positive; DP, double-positive; TLE, Transducin-like enhancer of split; FTOC, fetal thymus organ culture; FL, fetal liver; tg, transgenic; rh, recombinant human; cko, conditionally knocked out; ctrl, control.

[4] T. Asai, T. Yamagata, T. Saito, M. Ichikawa, S. Seo, G. Yamamoto, K. Maki, K. Mitani, H. Oda, S. Chiba, et al. Runx1 is required for integrity of the pre-T cell receptor complex and Lck kinase activity in early thymocyte development. *Submitted for publication.*

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the C-terminal VWRPY motif, which mediates the interaction with Transducin-like enhancer of split (TLE), a transcriptional corepressor (22, 23) (see Fig. 3*A*), and a domain which represses *p21* transcription through the interaction with mammalian Sim3 isoform A corepressor (24) (not shown in Fig. 3*A*) 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 hemopoietic 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 *Not*I site on their 5′ terminus and an *Xho*I 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′-AAAAGCGGCCGCATCGATACCATGCGTATCCCC GT-3′; antisense oligonucleotides: *Δ477*, 5′-TTTTCTCGAGTCAGGC CTCCTCCAGGCGCGCGGG-3′; *Δ372*, 5′-TTTTCTCGAGTCAGCCGG TCTGGAAGGGCCCGGC-3′; *Δ320*, 5′-TTTTCTCGAGTCAGCGCGGG TCGGAGATGGACG-3′; and *Δ291*, 5′-TTTTCTCGAGTCAAAGTT CTGCAGAGAGGCTGG-3′. Each PCR product was digested with *Not*I and *Xho*I and cloned into the *Not*I-*Xho*I site, 5′ upstream of internal ribosomal entry site-*GFP* of the *pGCDNsam* (a gift from H. Nakauchi, 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, ψMP34 packaging cells (a gift from Wakunaga Pharmaceutical) were transfected with these retrovirus plasmids, followed by single cell sorting for GFP with a FACSVantage (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*$^{+/-}$ mice (female) and *Runx1*$^{floxed/+}$, *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′-ACAAAACCTAGGTGTAC CAGGAGAACAAGT-3′), *f120* (5′-CCCTGAAGACAGGAGAAGTTT CCA-3′), and *r1* (5′-GTCTACTCCTTGCCTCAGAAAACAAAAAC-3′), in which floxed and floxed-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 5×10^4 mononuclear cells, without further purification of hemopoietic 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, 5×10^4 cells were passed onto newly prepared OP9-DL1 cells in the presence of 5 ng/ml rhIL-7, and retrovirus infection was performed using polybrene (final concentration 8 μg/ml), followed by another 5 days of culture. A total of 1×10^5 cells were again passed onto newly prepared OP9-DL1 cells and cultured for another 5 days, but in rhIL-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 FACSCalibur (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 CD3ε (500A2), CD4 (RM4-5), CD8a (53-6.7), CD24 (M1/69), CD25 (PC61), CD44 (IM7), CD45.2 (104), CD45R/B220 (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 hemopoietic progenitor cells (CD24^{low}, Lin$^-$, Sca-1^{high}, CD117/c-Kit$^+$) (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. 1*A*). After 15 days of culture on OP9-DL1 cells, a considerable number of FL-derived cells became CD4$^+$CD8$^+$ (Fig. 1*B*) and were thought to correspond to CD4/8 DP thymocytes. These CD4$^+$CD8$^+$ cells also expressed TCRβ at a level comparable with that in DP thymocytes in adult thymus (Fig. 1*C*), 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$^+$CD8$^-$ or CD4$^-$CD8$^+$) cells were also observed, but they expressed only intermediate levels of TCRβ on their cell surface (Fig. 1*C*), 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$^-$CD8$^-$ cells, which were considered to be reminiscent of CD4/CD8 DN thymocytes. DN thymocytes differentiate through the maturation sequences DN1 (CD44$^+$CD25$^-$), DN2 (CD44$^+$CD25$^+$), DN3 (CD44^{low}CD25$^-$), and DN4 (CD44$^-$CD25$^-$) (33), and each DN fraction was detected in FL-derived CD4$^-$CD8$^-$ cells cultured on OP9-DL1 cells

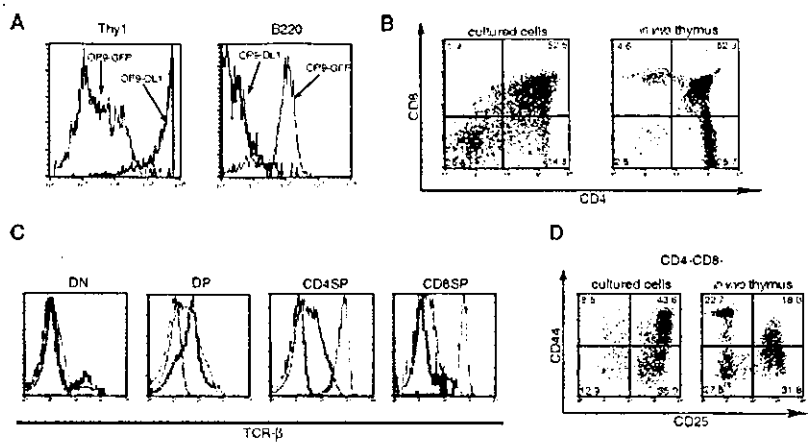

FIGURE 1. FACS analysis of wild-type C57BL/6 FL cells cultured on a stromal layer of OP9 cells that express DL1 (OP9-DL1). *A*, Expression levels of B220 and Thy1 on day 15 in FL cells cultured on an OP9-DL1 layer (thick line) and in FL cells cultured on a control OP9 layer (OP9-GFP; thin line) are shown. Cells were stained with anti-B220 PerCP and anti-Thy1.2 FITC. *B*, CD4/8 expression profile of FL cells cultured on OP9-DL1 for 15 days. The percentage of cells in each quadrant is indicated. *C*, FL cells cultured on OP9-DL1 for 15 days were stained with anti-CD4 PE, anti-CD8 PerCP, and anti-TCRβ allophycocyanin. Expression levels of TCRβ (filled histograms) in each subpopulation, as determined by CD4 and CD8 expression, are shown with the isotype control (blue lines) and expression levels of TCRβ in a corresponding population of adult thymocytes (red lines). *D*, Cells cultured on OP9-DL1 for 15 days were stained with anti-CD4 FITC, anti-CD44 PE, anti-CD8 PerCP, and anti-CD25 allophycocyanin. $CD4^-CD8^-$ cells were gated and their CD25/CD44 expression profile was analyzed. The percentage of cells in each quadrant is indicated.

by staining with CD25 and CD44, although the proportion of cells at the DN2 stage was prominent (Fig. 1*D*).

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: $Runx1^{floxed/-}$, *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 ($CD4^{int}CD8^-$) in addition to $CD4^-CD8^-$ cells (Fig. 2*A*). The $CD4^{int}CD8^-$ 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 that fact that only a small proportion of ctrl cells progressed to the $CD4^+CD8^+$ cells after 10 days of culture (Fig. 2*A*). Indeed, TCRβ and CD5, whose expression levels rise as thymocytes maturate, were up-regulated in $CD4^+CD8^+$ ctrl cells, but not in $CD4^+CD8^-$ cko cells (Fig. 2*B*). In addition, CD24, whose expression level diminishes as thymocytes maturate, is down-regulated in $CD4^+CD8^+$ ctrl cells, but not in $CD4^+CD8^-$ cko cells (Fig. 2*B*). Furthermore, the expression profile of CD44 and CD25 was comparable with that of $CD4^-CD8^-$ cells (Fig. 2*C*). The extent of Cre-mediated depletion of the floxed *Runx1* allele was greater in $CD4^{int}CD8^-$ cells than in the $CD4^-CD8^-$ cells (Fig. 2*D*), 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. 2*A*). 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.[4]

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. 2*E*). 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. 3*B*, *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. 3*B*, *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. 3*A*) 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

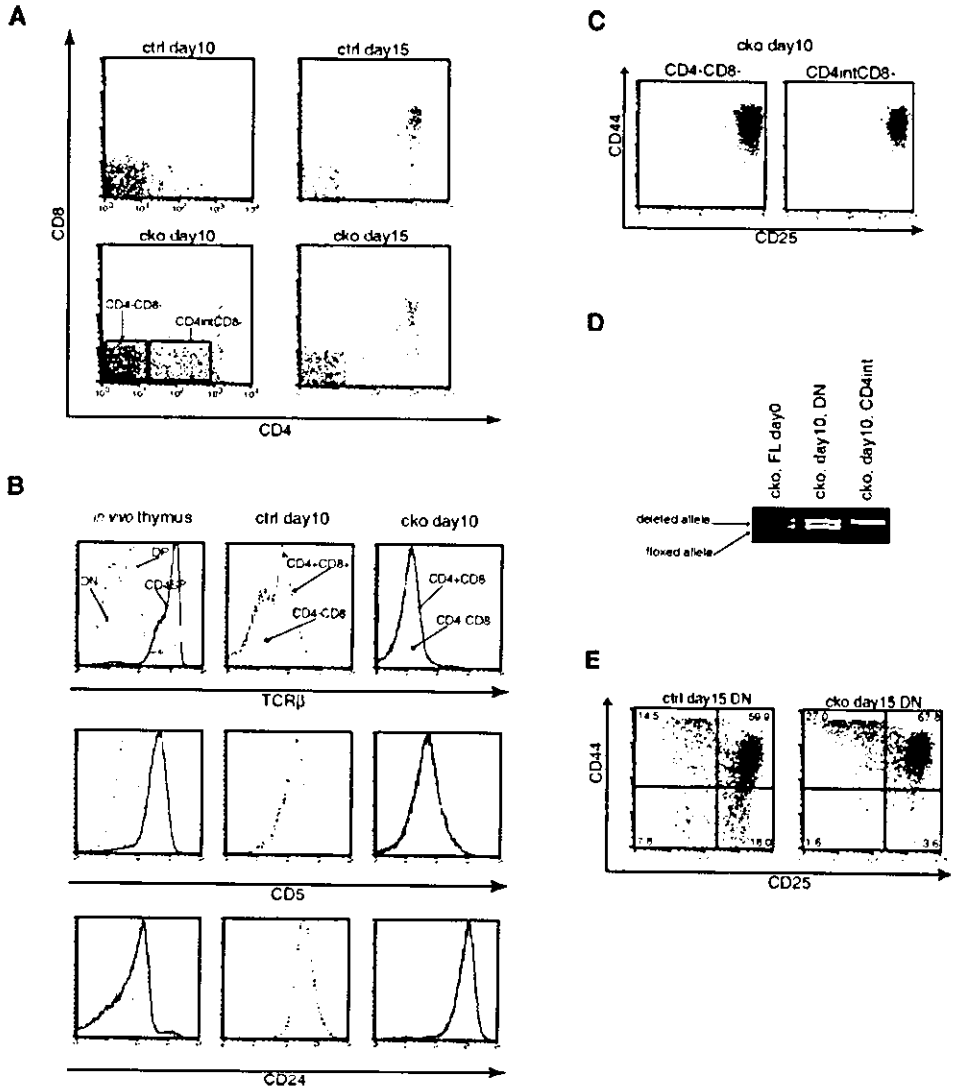

FIGURE 2. FACS analysis of cko FL cells and ctrl FL cells cultured on OP9-DL1. *A*, CD4/CD8 expression profiles of each type of FL-derived cell on days 10 and 15. *B*, Expression levels of TCRβ, CD5, and CD24 in $CD4^+CD8^-$ cko cells (solid lines) and $CD4^+CD8^+$ ctrl cells (dotted lines) cultured 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 $CD4^{int}CD8^-$ 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.

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. 3*C*).

Δ*447*-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 Δ*372*-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 Δ*320*- or Δ*291*-transduced control FL cells were diminished compared with mock-infected ctrl FL cells (Fig. 3*B*, *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*-transduced *Runx1*$^{floxed/floxed}$, *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. 3*D*). 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,[4] we examined expression of intracellular TCRβ in DN

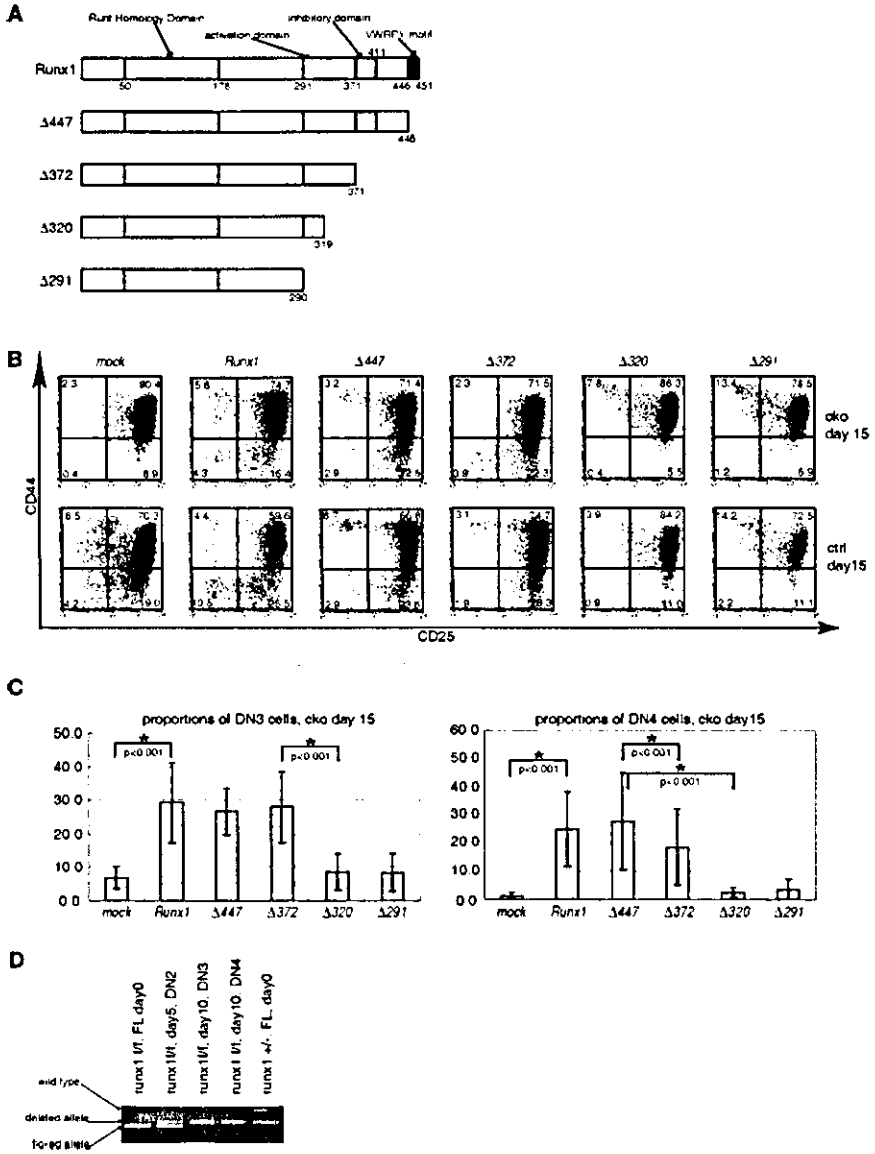

FIGURE 3. Development of DN3 and DN4 cells in FL-derived cells, which were transduced with the genes for Runx1 or its C-terminal deletion mutants. *A*, Construction of Runx1 and C-terminal deletion mutants. Numbers indicate the positions of amino acid residues from the N terminus. *B*, CD25/CD44 expression 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-CD44 PE, anti-CD3 PerCP, anti-CD4 PerCP, 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 ($CD44^{low}CD25^{+}$) 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-CD3ε 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.

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. 3*B*, *bottom*), the percentage of *Runx1*-transduced ctrl DN cells expressing intracellular TCRβ was increased compared with the

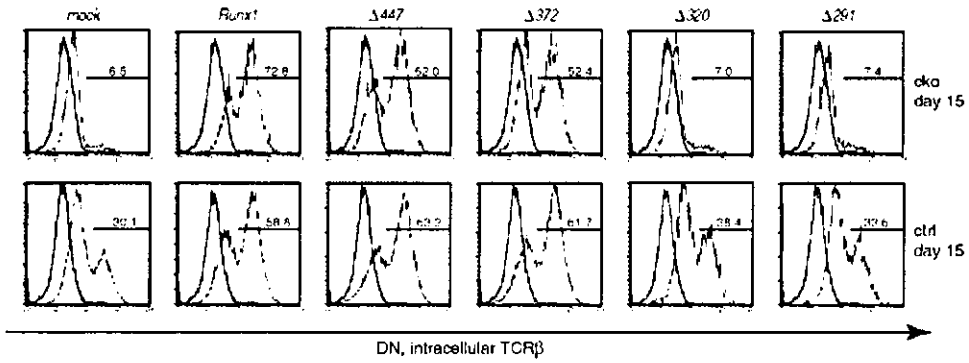

FIGURE 4. Expression levels of intracellular TCRβ in the $CD4^{-}CD8^{-}$ subset among cko (*top panels*) and ctrl (*bottom panels*) FL-derived cells on day 15. Transduced Runx1 mutants are shown above. Cells were stained with anti-CD4 PE, anti-CD8 PerCP, and anti-TCRβ allophycocyanin. GFP-positive, 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.

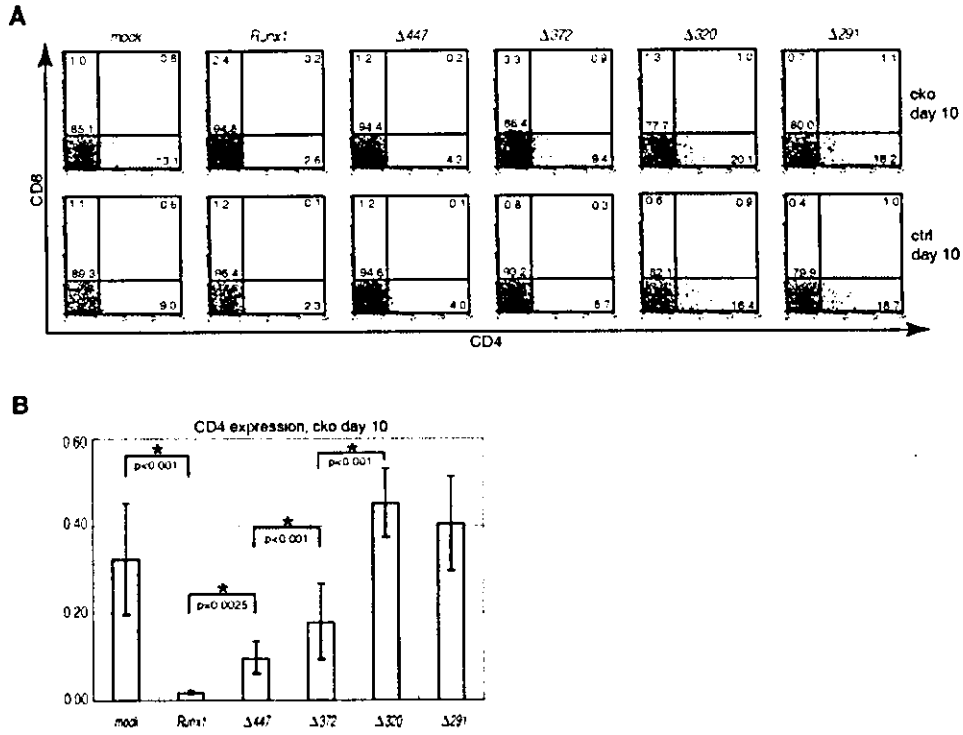

FIGURE 5. CD4 repression in the FL-derived cells transduced with Runx1 or its C-terminal deletion mutants. *A*, $CD4^{-}CD8$ expression profiles of cko (*top panels*) and ctrl (*bottom panels*) FL cells on day 10 of culture. Transduced Runx1 mutants are shown above. Cells were stained with anti-CD4 PE, 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 $CD4^{int}$ 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. 5*A*, the $CD4^{int}CD8^{-}$ subsets in day 10 culture of cko FL cells disappeared upon the reintroduction of *Runx1* (Fig. 5*A*), 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 $CD4^{int}CD8^{-}$ 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 $CD4^{int}$ 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. 2*D*), the increase in the $CD4^{int}$ 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 $CD4^{int}CD8^{-}$ cells, which were barely detected in mock-infected ctrl FL-derived cells (Fig. 5*A*, *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 DN stage at which 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. 3*D*, *lane 3*). *Lck-Cre* tg mice harbor a transgenic gene encoding Cre recombinase driven by the $p56^{lck}$ 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 $p56^{lck}$ proximal promoter at the DN2 and DN3 stages when Vβ gene rearrangement and subsequent β-selection occurs. However, even if the same $p56^{lck}$ 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 corepressor 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/OP9-DL1 coculture 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

The authors have no financial conflict of interest

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Extra Views

Runx1/AML-1 Ranks as a Master Regulator of Adult Hematopoiesis

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ABSTRACT

Runx1 (AML-1) is a critical gene involved in human leukemogenesis, originally identified at the 21q22 breakpoint of the leukemic translocations of t(8;21)(q21;q22), and is thought to be involved in as much as 25% of human leukemia. It encodes a transcription factor that has close homology to a *Drosophila* protein, runt, and is found to play essential roles in regulation of hematopoietic systems. Really a gene disruption experiment unequivocally shows that Runx1 is absolutely required for the establishment of definitive or adult-type hematopoiesis. Moreover, accumulated evidence from a number of in vitro studies and findings in patients with familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML) strongly suggests that it also commits to the control of hematopoietic system in adult life, although the in depth analysis of its roles in adult hematopoiesis has been largely hampered by premature lethality of *Runx1*-null animals. Recently we have developed conditional knockout mice in which *Runx1* is disrupted specifically in hematopoietic compartments after birth and dissected its roles in adult hematopoiesis. Notably, in these mice, maturation of megakaryocytes and development of both T and B lymphocytes were severely impaired, whereas hematopoietic progenitors were maintained or even expanded with apparently normal myeloid and erythroid differentiation in the periphery and bone marrow. Our findings clearly demonstrated differential requirement of *Runx1* in stem cell development and in its maintenance together with multi-modal functions of this transcription factor that are critically required for maturation of megakaryocytes and lymphocyte development, also providing a novel insight into how deeply and meticulously Runx1 is involved in regulation of mammalian hematopoiesis.

Mammalian hematopoietic development is believed to arise from two distinct cellular origins. In mice, the primitive erythroid cells that appears around day 7.5 postcoitus (E7.5) in the yolk sac is the first hematopoiesis thus far detected, known as primitive hematopoiesis, and is exclusively composed of large and nucleated erythrocytes.[1] On the other hand, the second wave of hematopoiesis, or definitive hematopoiesis, is heralded later in the ventral region of the aorta-gonad-mesonephros (AGM) region in E10.5 and consists of enucleated erythrocytes, myeloid cells and lymphoid progenitors, which ensures following expansion of hematopoietic stem cells (HSCs) and blood production in the fetal liver around E12.5.[2] These are finely regulated processes in which a bunch of genes are expressed in a well-coordinated manner and the growing lines of evidence suggest that these regulations are mediated by a number of hematopoietic transcription factors.

Runx1, also known as *AML-1*, *CBFA2* or *PEBP2αB* is a transcription factor first isolated from t(8;21)(q21;q22) and later in t(3;21)(q26;q22), t(12;21)(p13;q22), and t(16;21)(q24;q21) translocations found in human leukemia in which the aberrant fusion genes, *AML-1/ETO* and *AML-1/Evi1*, *TEL/AML-1*, and *AML-1/MTG16*, were generated, respectively.[3] It has high homology to the *Drosophila* segmentation gene, *runt*, and also has two mammalian homologues, Runx2 (AML-3) and Runx3 (AML-2), collectively called the *Runx* family transcription factors. It is shown that Runx1 dimerizes with the common β-subunit, CBFβ, to bind to its target sequences known as PEBP2 sequences and regulates a variety of hematopoietic lineage-specific genes, including interleukin-3, granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor receptor, neutrophil elastase, granzyme B, myeloperoxidase, neutrophil defensin, and subunits of the T-cell and B-cell antigen receptor, in cooperation with other transcription factors.[3] Runx1 is absolutely required for mouse embryogenesis and hematopoiesis, since *Runx1*-null embryos die at midgestation by E12.5 with massive hemorrhage in the central nervous system and complete effacement of definitive hematopoiesis in the fetal liver, though primitive erythropoiesis is preserved.[4,5] In fact it has been demonstrated using

Runx1-LacZ knock-in embryos that Runx1 is essential for development of HSCs from the endothelial cells in the embryonic AGM region,[6] where definitive hematopoiesis specifically arises from the ventral endothelial linings that express Runx1.

On the other hand, genetic analysis of Runx1 functions in adult life is largely limited because of the embryonic lethality of the homozygously gene-targeted mice, and our knowledge about its in vivo functions in adult hematopoiesis has mostly come from the analysis of murine models of *Runx1*-involving translocations, although there exist a large body of in vitro studies that argue its postnatal functions. Among these, the most intensively studied is *AML-1-ETO* generated in t(8;21)(q21;q22) translocation. Since this aberrant Runx1 (AML-1) fusion protein seems to have a dominant-negative effect on Runx1 functions and its knock-in mice recapitulate a *Runx1*-null phenotype, several mice models of t(8;21)(q21;q22) have been developed using inducible or retrovirus-mediated expression of the fusion protein in bone marrow in order to clarify its leukemogenic functions in vivo.[7-9] The common features of these mouse models are expanded hematopoietic progenitor pools and increased self-renewal capacity of stem cells with varying degrees of abnormalities in differentiation. While these observations provide important clues to the understanding of leukemogenic mechanism through AML-1-ETO and also of physiological Runx1 functions, it cannot be determined to what extent we are able to ascribe these phenotypes to loss of Runx1 functions.

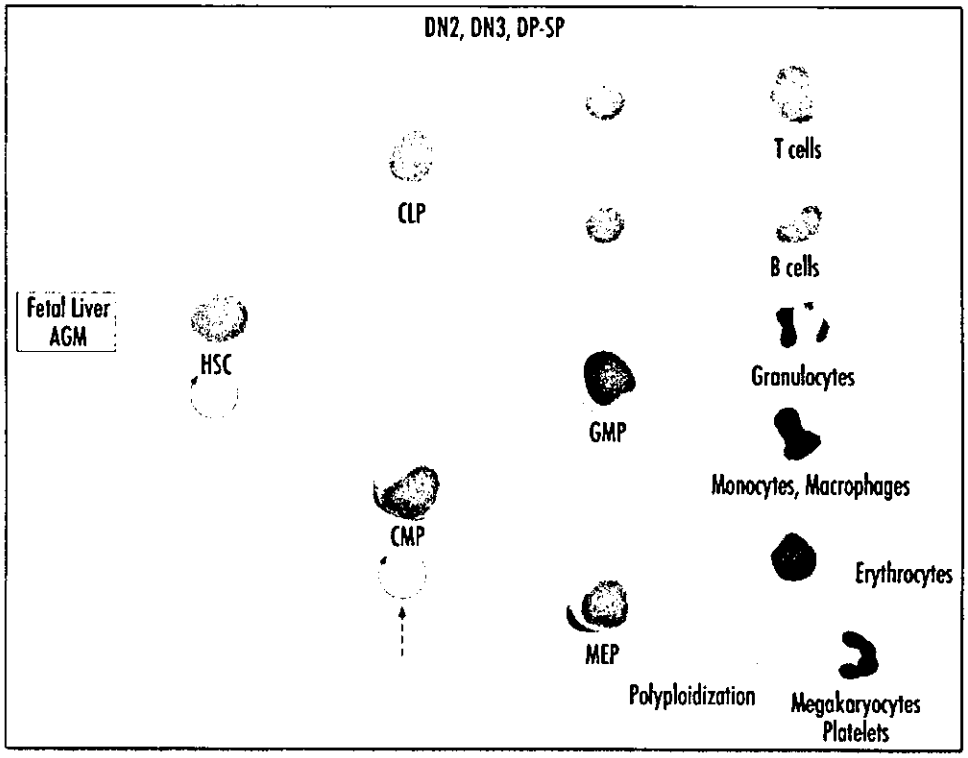

Figure 1. Function of Runx1 in adult hematopoietic maintenance. Red arrows: Runx1 is required. Runx1 is required for development of definitive hematopoiesis at the embryonic stage, several steps in T cell development, early B cell development, and polyploidization of the megakaryocytes, but not for maintenance of early hematopoietic progenitors. Runx1 also negatively regulates the number of myeloid progenitors (broken arrow).

In the article recently published, we analyzed the in vivo role of Runx1 in adult hematopoiesis using the conditional gene targeting system.[10] We generated mice in which exon 5 of the *Runx1* locus was flanked by two *loxP* sites and bred them with mice expressing an interferon-inducible Cre recombinase. With this approach, *Runx1* could be successfully disrupted in the hematopoietic compartments in the adult animals. Although the absence of *Runx1* during developmental stages results in total loss of definitive hematopoiesis and HSC generation, in our conditional knockout mice hematopoietic progenitors were maintained with normal myeloid as well as erythroid development despite complete lack of Runx1, demonstrating that Runx1 is not absolutely required for the maintenance of established adult HSCs per se. It was further supported by transplantation experiments of *Runx1*-null bone marrow cells, in which *Runx1*-null hematopoietic progenitors could fully repopulate recipients' bone marrow for at least three months. On the other hand, however, there exist severe defects about *Runx1*-null hematopoietic progenitors in producing platelets and mature lymphocytes.

A number of transcription factors, including c-Myb, GATA-1, GATA-2, SCL, and LMO-2, also participate in the regulation of the committed hematopoietic progenitors and are indispensable either for the development of embryonic hematopoiesis or for the expansion of HSCs, but their precise roles in maintenance of adult HSCs were largely unknown. However, of particular note is a recent report that SCL, a transcription factor, indispensable for the development of primitive erythropoiesis at the embryonic stage, is not essential for the maintenance of adult hematopoietic, although it is still required for erythroid and megakaryocytic differentiation of the committed progenitor cells.[11] In addition, Kunisato et al[12] demonstrated that SCL does not affect long term repopulating capacity of HSCs but direct their commitment to myeloid lineage using retrovirus-mediated gene transfer of a dominant-negative SCL mutant into HSCs. These findings on *SCL*-null mice are comparable to those on our *Runx1*-null animals, where Runx1 is required specifically for embryonic development of definitive hematopoiesis and regulation of lymphoid differentiation and megakaryocytic maturation, but is also dispensable for the maintenance of adult HSCs. Both examples may represent the functional multi-modality of hematopoietic transcription factors that participated in exquisite regulation of development and maintenance of hematopoietic systems. In our *Runx1*-null mice, there was the increased number of hematopoietic progenitor cells as well as the augmented replating capacity of these progenitors from the *Runx1*-knockout bone marrow. Because *Runx1* is mutated in FPD/AML[13] as well as some sporadic MDS cases,[14-16] it is intriguing in view of the leukemogenic role of Runx1 deficiency that the expansion of hematopoietic progenitors and increased self-renewal capacity have also been observed in the mouse models of t(8;21) (q21;q22)-carrying leukemias, indicating that the size of progenitor cell pool seems to be negatively regulated by Runx1 function, which may be overridden by AML-1-ETO. Although it is not clear whether the increased progenitor pool reflects differentiation block of downstream cell lineages or unregulated cell cycling or apoptosis of the progenitors, it may well be possible that the expanded progenitor pool also

contribute to leukemia development by, for example, increasing the chance of additional mutations. It should also be noted that neither *Runx1*-null mice nor murine models of t(8;21)(q21;q22) develop spontaneous leukemia, indicating additional mutations are required for development of full-blown leukemia.

As already mentioned, the *Runx1*-null mice show greatly reduced platelet counts with severely impaired maturation of megakaryocytes as evident from their smaller cell size, hypoploidy, and abnormalities in ultrastructure. Although the precise mechanism of this phenotype is still unclear, it is worth noting that the similar megakaryocytic maturation arrest at the polyploidization step is also observed in *Fli-1* knockout mice and *GATA-1* knockdown mice.[17-19] Since Runx1 and GATA-1 are known to physically interact with each other in megakaryocytes and promote megakaryocyte-specific gene expression,[20] it may be speculated that Runx1 regulates megakaryocytic maturation in cooperation with GATA-1 through affecting megakaryocyte-specific gene expression.

Mature lymphocyte production is also severely defective in both T and B lineages and competitively transplanted *Runx1*-null HSCs could not contribute to recipients' T or B populations in spite of the normal number of common lymphoid progenitors. *Runx1* is expressed in the developing thymocytes and in B cells, and is known to regulate the expression of T cell and B cell-specific genes. A previous study using T-cell specific *Runx1* knockout mice revealed the essential role of Runx1 in T cell development, in which Runx1 was shown to be required for development of double negative ($CD4^-CD8^-$) thymocytes in transition from $CD44^-CD25^+$ (DN3) to $CD44^-CD25^-$ (DN4) phenotypes.[21] However, our bone marrow reconstitution experiment using Runx1-null hematopoietic stem cells also demonstrated that Runx1 expression is absolutely required for more early stages of T cell development in transition from $CD44^+CD25^+$ (DN2) to DN3 double negative thymocytes. On the other hand, no direct evidence has been reported about the exact biological role of Runx1 in B cell development. Runx1-null bone marrow cells could not repopulate the peripheral T and B lymphocyte population of sublethally irradiated recipients while CLP fraction of the conditional knockout animals is not decreased. Therefore, our data revealed the previously unknown role of Runx1 in B cell development. The precise regulation of Runx1 in B cell development should be analyzed by further investigation, and B-cell lineage-specific targeting of the Runx1 gene is under way to clarify the physiological role of Runx1 in the committed B cells.

In conclusion, from the analysis of conditional Runx1-knockout mice, it became clear that Runx1 is not required for the maintenance of HSC functions in adult mice, but is indispensable for maturation and differentiation of various blood components including T and B lymphocytes, as well as megakaryocytes (Fig. 1). Our data not only recapitulate the human diseases FPD/AML and MDS related to Runx1, but also demonstrate that Runx1 is still a multi-role regulator in maintenance of the lineage-committed cells in adult hematopoiesis, although for HSCs, it is essential only once at the embryonic developmental stage.

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The transcriptionally active form of AML1 is required for hematopoietic rescue of the *AML1*-deficient embryonic para-aortic splanchnopleural (P-Sp) region

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Acute myelogenous leukemia 1 (AML1; runt-related transcription factor 1 [Runx1]) is a member of Runx transcription factors and is essential for definitive hematopoiesis. Although AML1 possesses several subdomains of defined biochemical functions, the physiologic relevance of each subdomain to hematopoietic development has been poorly understood. Recently, the consequence of carboxy-terminal truncation in AML1 was analyzed by the hematopoietic rescue assay of *AML1*-deficient mouse embryonic stem cells using the gene knock-in approach. Nonetheless, a role for specific internal domains, as well as for mutations found in a human disease, of AML1 remains to be elucidated. In this study, we established an experimental system to efficiently evaluate the hematopoietic potential of AML1 using a coculture system of the murine embryonic para-aortic splanchnopleural (P-Sp) region with a stromal cell line, OP9. In this system, the hematopoietic defect of *AML1*-deficient P-Sp can be rescued by expressing AML1 with retroviral infection. By analysis of AML1 mutants, we demonstrated that the hematopoietic potential of AML1 was closely related to its transcriptional activity. Furthermore, we showed that other Runx transcription factors, Runx2/AML3 or Runx3/AML2, could rescue the hematopoietic defect of *AML1*-deficient P-Sp. Thus, this experimental system will become a valuable tool to analyze the physiologic function and domain contribution of Runx proteins in hematopoiesis. (Blood. 2004;104:3558-3564)

Introduction

Acute myelogenous leukemia 1 (AML1)/runt-related transcription factor 1 (Runx1) belongs to a family of transcriptional regulators called Runx, which contain a conserved 128–amino acid Runt domain responsible for sequence-specific DNA binding.[1] Runx proteins make heterodimeric complexes with a partner protein, CBFβ/PEBP2β (core-binding factor β/polyomavirus enhancer–binding protein 2β),[2-4] and this association is essential for its biologic activity.[5-7] There are 3 known mammalian Runx family members: AML1/Runx1, Runx2/AML3, and Runx3/AML2. Typically, Runx functions as a transcriptional activator of target gene expression. Under some conditions, however, it can repress the transcription of specific genes.

AML1 was originally identified on chromosome 21 as the gene that is disrupted in the (8;21)(q22;q22) translocation, which is one of the most frequent chromosome abnormalities associated with human AML.[8,9] Subsequently, AML1 was shown to be one of the most frequent targets of leukemia-associated gene aberrations.[10,11] Moreover, somatic point mutations of the *AML1* gene were also demonstrated in patients with AML and myelodysplastic syndrome (MDS).[12-14] In addition to a role in leukemic transformation, gene-targeting studies in mice have demonstrated that AML1 is essential for early development of definitive hematopoiesis. *AML1*-deficient embryos develop through the yolk sac stage but die around 12 to 13 days of gestation following complete block of fetal liver hematopoiesis.[15,16]

AML1 includes at least 3 alternative splicing forms: AML1a, AML1b, and AML1c.[17] In AML1b and AML1c, the carboxy (C)–terminal to the Runt domain lies in a region that contains sequences of defined biochemical functions, which are absent in AML1a. Several functional domains have been identified in the C-terminal half, such as *trans*-activation domain,[18,19] *trans*-repression domain,[20] and VWRPY motif.[21-23]

During vertebrate embryogenesis, hematopoietic development consists of 2 distinct waves of discrete cellular components known as primitive and definitive hematopoiesis.[24] In mice, the first wave of primitive hematopoiesis, which consists predominantly of a large and nucleated erythroid cell, emerges in the yolk sac at 7.5 embryonic days after coitus (dpc). Then, primitive hematopoiesis begins to be replaced around 9.5 dpc by definitive hematopoiesis, generally described as the second wave. Progenitors for definitive hematopoiesis originate from para-aortic splanchnopleural (P-Sp) region at 7.5 to 9.5 dpc,[25,26] and long-term repopulating hematopoietic stem cells (LTR-HSCs) that can reconstitute adult mice appear in the aorta-gonad-mesonephros (AGM) at 10.5 to 11.5 dpc.[27,28] These cells subsequently colonize the fetal liver, where they expand and differentiate. Active sites for definitive hematopoiesis

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are transferred to bone marrow and spleen prior to birth and function throughout life within these organs. Recent studies have shown that AML1 is expressed in the hematopoietic cell clusters within the P-Sp/AGM region and *AML1*-deficient embryos are devoid of these hematopoietic clusters.[29-32]

The hematopoietic defect of *AML1*-deficient mice could be replicated in vitro by several culture systems, including the P-Sp/AGM culture[33,34] and the embryonic stem (ES) cell culture.[35] In these systems, hematopoietic cells are generated in wild-type cultures but not in *AML1*-deficient cultures. Recently, a gene knock-in approach was used to demonstrate rescue in vivo of hematopoiesis from the *AML1*-deficient ES cells.[35,36] This hematopoietic rescue requires the *trans*-activation domain of AML1 but not the C-terminal *trans*-repression subdomain. However, no report has elucidated roles for specific internal domains or disease-related mutations of AML1 in hematopoiesis.

In the present study, we used a coculture system of cells derived from the P-Sp region with a layer of a stromal cell line, OP9, in which hematopoietic cell development of various lineages is efficiently induced.[37] The cultured P-Sp–derived cells show a significant colony-forming activity in semisolid culture with appropriate cytokines, as well as distinct surface expression of hematopoietic markers. In this culture system, *AML1*-deficient P-Sp–derived cells failed to show any hematopoietic activity. This defect was efficiently rescued by reactivating AML1 by retroviral-mediated expression. Using this system, we then examined a hematopoietic potential of a series of AML1 mutants and demonstrated that the hematopoietic rescue of *AML1*-deficient P-Sp regions require transcriptionally active forms of AML1. We also showed that enforced expression of other Runx transcription factors, Runx2/AML3 or Runx3/AML2, could rescue the hematopoietic defect of *AML1*-deficient P-Sp regions. These results provide evidence that transcriptional activity of AML1 is essential for hematopoietic development from P-Sp regions. In addition, this coculture system makes a useful method to determine functional consequences of AML1 on its hematopoietic potential.

Materials and methods

Mice and embryos

AML1-deficient mice were generated as described previously[38] and were crossed onto the C57BL/6 background. To generate embryos, timed matings were set up between $AML1^{+/-}$ males and $AML1^{+/-}$ females. The time at midday (12:00) was taken to be 0.5 dpc for the plugged mice.

In vitro P-Sp culture

P-Sp culture was performed as described previously[39] with a minor modification. In brief, isolated P-Sp regions of 9.5 dpc embryos were dissociated by incubation with 250 U/mL dispase (Godo Shusei, Tokyo, Japan) for 20 minutes and cell dissociation buffer (Gibco BRL, Carlsbad, CA) for 20 minutes at 37°C, washed once in phosphate-buffered saline (PBS), followed by vigorous pipetting. Approximately 5×10^4 P-Sp–derived cells were suspended in 300 μL serum-free StemPro media (Life Technologies, Gaithersburg, MD) supplemented with 50 ng/mL stem cell factor (SCF), 5 ng/mL interleukin (IL3; gifts from Kirin Brewery, Takasaki, Japan), and 10 ng/mL murine oncostatin M (R&D Systems, Minneapolis, MN). Single-cell suspensions were seeded on preplated OP-9 stromal cells in the 24-well plate, followed by incubation at 37°C.

Plasmid construction

The cDNAs of human AML1a, AML1b, and various AML1 mutants were subcloned as *Eco*RI-*Eco*RI fragments into the retrovirus vector pMY/internal ribosomal entry site–enhanced green fluorescent protein (IRES-EGFP; pMY/IG).[40] C-terminal deletion mutants of AML1b, AML1b-R139G, AML1b-S249/266A, and AML1b-K24/43R were constructed as described previously.[13,41-43] For construction of AML1bΔ(205-332), we deleted the *Pvu*II-*Bst*PI fragment from AML1b, filled the resultant plasmid with a Klenow fragment, and religated it. AML1bΔ(181-210) was created by polymerase chain reaction (PCR) with the insertion of a *Bgl*II restriction site to join the fragments. Flag-tagged human Runx2/AML3 cDNA was inserted into the *Bam*HI and the *Eco*RI restriction sites of pMYs/IRES-EGFP (pMYs/IG).[40] Flag-tagged human Runx3/AML2 cDNA was inserted into the *Sac*II and the *Xho*I sites of the same vector.

Retroviral transduction

Plat-E packaging cells[44] (2×10^6) were transiently transfected with 3 μg of AML1, Runx2/AML3, Runx3/AML2, or AML1 mutants; mixed with 9 μL of FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN); followed by incubation at 37°C. Supernatant containing retrovirus was collected 48 hours after transfection and used immediately for infection. Retroviral transduction to the cells derived from *AML1*-deficient P-Sp regions was performed as described previously with minor modification.[45] In brief, the viral supernatant was added to the P-Sp culture together with 10 μg/mL Polybrene (Sigma, St Louis, MO). After 72 hours of incubation, virus-containing medium was replaced by standard culture medium. The cells were incubated for another 10 days and processed for analysis. To confirm the expression of Runx proteins, NIH3T3 cells were also infected with the same viral supernatants. The number of retrovirus-infected cells was evaluated by the expression of green fluorescent protein (GFP).

Colony-forming cell (CFC) assay

The nonadherent or semiadherent cells rescued from *AML1*-deficient P-Sp regions were used for CFC assay. Cells (6×10^4) were plated into MethoCult3434 medium (StemCell Technologies, Vancouver, BC, Canada) and cultured in a 5% CO_2 incubator at 37°C. Colony types were determined at day 7 by morphologic appearance and by Wright-Giemsa staining of each colony.

Flow cytometry analysis

Flow cytometry analysis was performed in a FACScalibur with the Cellquest program (Becton Dickinson, San Jose, CA) after addition of propidium iodide to exclude dead cells. For surface staining, cell suspensions collected from the P-Sp cultures were incubated on ice for 30 minutes in the presence of various mixtures of labeled monoclonal antibodies. The monoclonal antibodies used were phycoerythrin (PE)–conjugated anti–granulocyte 1 (anti-Gr1), anti–macrophage antigen 1 (anti-Mac1), anti–stem cell antigen 1 (anti-Sca1), allophycocyanin (APC)–conjugated anti-CD45, anti–c-Kit, and biotin-conjugated anti-CD34. Biotinylated antibodies were then counterstained with PE- or APC-conjugated streptavidin. Isotype-matched antibodies conjugated with the appropriate fluorochrome were used as negative controls.

Western blot analysis

Retrovirus-infected NIH3T3 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer.[41] Whole-cell lysates containing 100 μg of proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA). The membrane was blocked with 10% skim milk, treated with anti-AML1 (PC284L; Oncogene, Cambridge, MA) or anti-Flag (M2; Sigma), washed, and reacted with the rabbit anti–immunoglobulin G (anti-IgG) antibody coupled to horseradish peroxidase. The blot was visualized using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ).

Results

Retroviral expression of AML1 rescues hematopoiesis by *AML1*-deficient P-Sp region

AML1-deficient mice die in midgestation as a result of a complete block in fetal liver hematopoiesis, indicating the strict in vivo requirement of AML1 in definitive hematopoiesis. Consistently, the primary culture system of the P-Sp region has demonstrated that the failure of hematopoiesis in the fetal liver is preceded by a hematopoietic defect in the P-Sp region, from which the development of hematopoietic cells was never detected in *AML1*-deficient embryos.[34,45] When the cells isolated from wild-type P-Sp regions at 9.5 dpc were cocultured with OP9 stromal cells, small and round-shaped nonadherent cells were produced in 5 days (Figure 1A). These cells were thought to represent a hematopoietic cell population of various lineages because they expressed hematopoietic cell surface markers and generated hematopoietic cell colonies when plated into a semisolid culture (Figure 2; data not shown). In contrast, the cells from *AML1*-deficient P-Sp regions failed to develop any hematopoietic cells (Figure 1B), which coincides with the notion that AML1 is a prerequisite for hematopoietic cell production in the P-Sp region. Thus, AML1-dependent hematopoiesis could be recapitulated in vitro, and we went on further to examine whether reactivation of a transcriptionally active form of AML1 can rescue this hematopoietic defect. First, by packaging the pMY/IG-AML1b in Plat-E cells, we generated the AML1b-IRES-GFP retrovirus that expresses AML1b and GFP. Then we infected the cells derived from the *AML1*-deficient P-Sp region with the AML1b-IRES-GFP retrovirus and cultured for an additional 10 days. Interestingly, AML1b-infected cultures generated numerous small and round cells with a nonadherent property (Figure 1C). In contrast, cultures infected with the empty vector (control) produced no such cells (Figure 1D). These nonadherent cells were morphologically indistinguishable from the hematopoietic cells generated from the wild-type P-Sp cells and proliferated continuously for more than 30 days. These results suggest that lack of P-Sp hematopoiesis can be complemented by retrovirus-mediated reactivation of AML1 in this culture system.

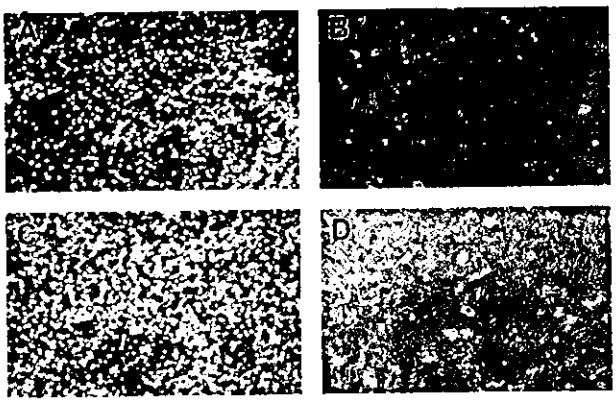

Figure 1. Retroviral expression of AML1b rescues hematopoiesis from *AML1*-deficient P-Sp regions. Photographs were taken with a Nikon Eclipse TE2000-U (Nikon Sankei, Tokyo, Japan) at a magnification of × 100 after 5 days of culture (A-B) and 14 days of culture (C-D). (A) Hematopoietic cells emerged at day 5 from wild-type P-Sp regions. (B) No hematopoietic cells were observed in the culture of *AML1*-deficient P-Sp regions, showing only the background of OP9 cells. (C) AML1b-transduced P-Sp regions from an *AML1*-deficient embryo generated numerous round, nonadherent, or semiadherent cells. (D) A control culture infected with mock virus failed to generate any hematopoietic cells.

Rescued cells retain the features of hematopoietic cells

In the previous study, Mukouyama et al[45] described that the retroviral transfer of AML1 into the *AML1*-deficient P-Sp region gave rise to the production of small and round cells in the culture with an appropriate combination of cytokines. However, under their experimental condition in which no stromal cell layer was employed, the recovered cells showed neither CFC activity nor expression of hematopoietic cell surface markers, such as CD45 and c-Kit, which indicates that the rescue of the hematopoietic defect is incomplete, if it occurs at all. To determine whether the nonadherent cells recovered under our experimental condition retain the features of hematopoietic cells, we examined CFC activity and surface markers of those cells, both of which are distinctly detected in wild-type P-Sp–derived cells in our coculture system. On the 10th day of culture, the nonadherent cells were collected and seeded into a semisolid medium. As shown in Figure 3, these cells generated a number of mixed, granulocyte/macrophage, and erythroid colonies, indicating that the recovered cells should contain various types of CFCs, possibly including definitive lineages. In addition, the flow cytometric analysis revealed that the rescued cells expressed hematopoietic cell surface markers, such as a marker of hematopoietic cells, CD45; myeloid markers Gr1/Mac1; and markers of hematopoietic progenitors c-Kit, Sca1, and CD34 (Figure 2). Their expression profiles were nearly identical to those of hematopoietic cells generated from the wild-type P-Sp cells (Figure 2).

Thus, in contrast to the previous report,[45] we found that the nonadherent cells derived from *AML1*-deficient P-Sp regions in our culture system retained the features of hematopoietic cells that are indistinguishable from those of wild-type P-Sp–derived cells. It is, thus, likely that seeding onto OP9 stromal cells may provide a more favorable environment for production of hematopoietic cells from P-Sp regions and/or the expansion of P-Sp–derived hematopoietic cells.

Hematopoietic potential of AML1 mutants

Using this experimental system, we then analyzed the hematopoietic potential of various AML1 mutants (Figure 4). We generated retroviruses that express a variety of AML1 mutants, including serial C-terminal truncation, deletion of functional domains, and substitution of specific residues. NIH3T3 cells were infected with these viruses and the titer of the retroviruses was evaluated by flow cytometric measurement of GFP-positive NIH3T3 cells (Figure 5A). Coincidently, retrovirus-mediated expression of each mutant in infected cells was confirmed by Western blotting (Figure 5B).

Among those mutants, we first used a series of C-terminal deletion mutants including AML1a and examined their hematopoietic potential by delivering them into the *AML1*-deficient P-Sp region in our coculture system. AML1bΔ444 and AML1bΔ397, which possess the *trans*-activation subdomain, retained the ability to rescue the hematopoietic defect of the *AML1*-deficient P-Sp region (Figure 6A-B). In contrast, AML1bΔ335, AML1bΔ288, and AML1a, which lack the *trans*-activation domain, failed to produce any hematopoietic cells (Figure 6C-E). In addition, AML1bΔ(205-332), which retains the C-terminal region but lacks a half of the activation domain, has also lost the hematopoietic potential (Figure 6G). Thus, consistent with the observation in the previous report,[35] in vitro hematopoietic rescue requires the *trans*-activation domain of AML1, whereas the C-terminal repression domain including VWRPY motif is dispensable for this function.

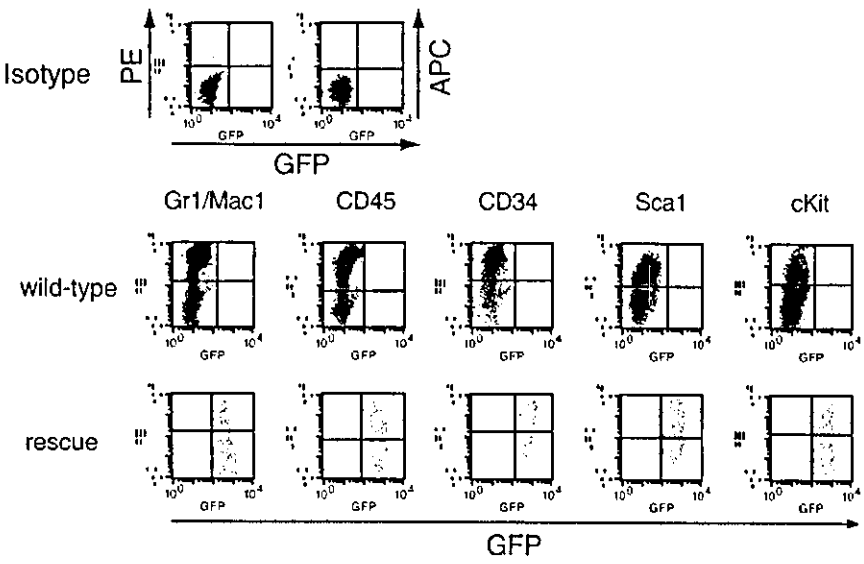

Figure 2. Expression of the hematopoietic markers on the rescued cells from *AML1*-deficient P-Sp culture. Flow cytometric profiles of the cells stained with antibodies against Gr1/Mac1, CD45, CD34, Sca1, and c-Kit. Note that the rescued cells from *AML1*-deficient P-Sp regions are GFP-positive and express various hematopoietic cell surface markers. Flow cytometric profiles of the rescued cells are similar to those of hematopoietic cells in wild-type P-Sp culture. GFP intensity (marking retrovirally transduced cells) is plotted on the x-axis and intensity of counterstaining of hematopoietic surface markers is plotted on the y-axis. Isotype-matched control staining of the hematopoietic cells from wild-type P-Sp regions is also shown.

The Runt domain of AML1 is essential for both DNA binding and heterodimerization with CBFβ, but its role in hematopoietic development has not yet been directly investigated. Therefore, we next examined the hematopoietic potential of AML1bΔRD, a deletion mutant that lacks the Runt domain and is defective for both DNA binding and heterodimerization with CBFβ. As shown in Figure 6F, AML1bΔRD could not rescue hematopoiesis from *AML1*-deficient P-Sp regions, indicating an essential role for the Runt domain in the hematopoietic potential of AML1. To elucidate more explicitly a role of DNA binding of AML1, we used AML1b-R139G, a mutant isolated from a patient with MDS, which harbors point mutation causing substitution of Arg139 in the Runt domain with Gly.[13] The DNA-binding ability is severely impaired in AML1b-R139G, whereas heterodimerization with CBFβ is spared. As shown in Figure 6I, AML1b-R139G also failed to show any hematopoietic potential. These results indicate that DNA binding of AML1 through the Runt domain is also indispensable for in vitro hematopoietic rescue of the *AML1*-deficient P-Sp region.

Among corepressors that are recruited by AML1 is mSin3A, which may contribute to AML1-mediated repression of gene transcription, as well as to intracellular stability of AML1.[20,46] Indeed, the AML1 mutant that cannot interact with mSin3A [AML1bΔ(181-210)] is defective for repression of the *p21* promoter in the in vitro transcription response assay. Posttranscriptional modification is also one of the important mechanisms that regulate AML1 function.[42,43] For example, transcriptional activity of AML1 is enhanced by extracellular signal-regulated kinase (ERK)–dependent phosphorylation on Ser249 and Ser266, whereas p300-mediated acetylation on Lys24 and Lys43 augments DNA binding of AML1. To clarify roles of these regulatory mechanisms in the hematopoietic potential of AML1, we used 3 types of AML1 mutants: AML1bΔ(181-210), AML1b-S249/266A, and AML1b-K24/43R. AML1bΔ(181-210) is an internal deletion mutant that lacks the binding domain for the mSin3A.[20] In AML1b-S249/266A, the 2 target serines for ERK-mediated phosphorylation were replaced with alanines, which results in lack of ERK-induced

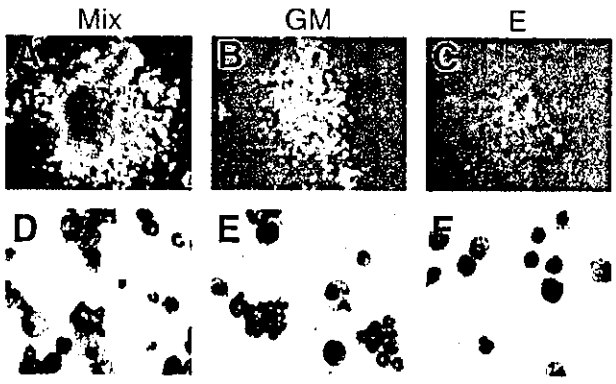

Figure 3. Colony formation of the rescued cells from *AML1*-deficient P-Sp. The hematopoietic defect of *AML1*-deficient P-Sp regions was rescued by retroviral expression of AML1b, and the rescued cells were plated into MethoCult3434 medium. The rescued cells generated various types of hematopoietic colonies including definitive origins. Representative hematopoietic colonies by 7 days of culture are shown. (A-C) Morphology of the colonies. (D-F) Cytospin preparation of corresponding cell populations. Cytospins were stained with Wright-Giemsa. Mix indicates mixed colony; GM, granulocyte/macrophage colony; and E, erythroid colony. Photographs were taken with a Nikon Eclipse TE2000-U (Nikon Sankei) at a magnification of × 100.

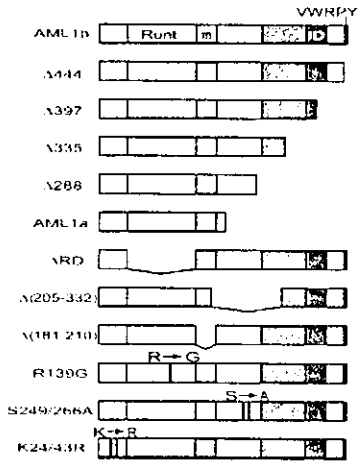

Figure 4. Structures of AML1 and its mutants. The structures of various AML1 mutants are presented schematically. Runt indicates the Runt domain; AD, *trans*-activation domain; ID, inhibitory domain; VWRPY, VWRPY motif; m, a binding region for mSin3A. R→G means a missense mutation at codon 139, which lead to a change of amino acid (R139G; single-letter amino acid code). S→A means a missense mutation at codon 249 and 266, which lead to changes of amino acids (S249A and S266A). K→R means a missense mutation at codon 24 and 43, which lead to changes of amino acid (K24R and K43R).

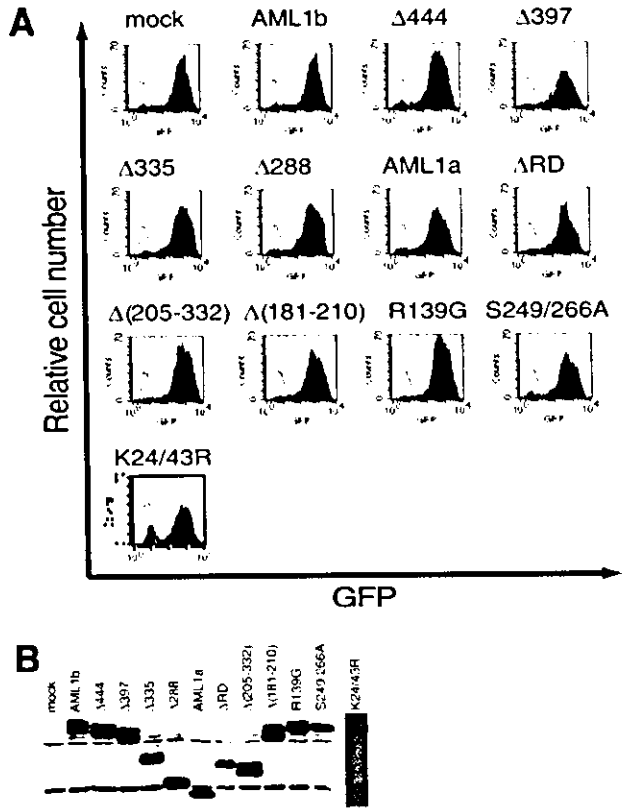

Figure 5. Infection efficiencies of retroviruses expressing AML1 or its mutants. (A) The efficiency of retrovirus-mediated gene transfer of AML1 or its mutants was estimated by infecting NIH3T3 cells. Retrovirus-infected cells were evaluated by the expression of GFP (shaded histograms). Also shown are the noninfected NIH3T3 cells (open histograms). All the retroviruses infected more than 80% of NIH3T3 cells. (B) Expression of AML1 or its mutant proteins in infected NIH3T3 cells. The expression is monitored by immunoblotting of whole-cell lysates with anti-AML1.

enhancement of the transcriptional activity. Finally, AML1b-K24/43R is an acetylation-defective mutant, in which the 2 lysine residues were substituted with arginines. Remarkably, all of these mutants retained the ability to rescue the hematopoietic defect in contrast to the mutants of the Runt domain (Figure 6H,J,K). The cells rescued by these AML1 mutants contained CFCs, expressed hematopoietic cell surface markers, and were morphologically indistinguishable from the rescued cells by wild-type AML1b (data not shown). From these findings, we concluded that the hematopoietic potential of AML1 does not require the interaction with mSin3A, ERK-dependent phosphorylation, or p300-mediated acetylation. Some posttranslational modifications, as well as repressor activities, of AML1 may not necessarily be required for early hematopoietic development. Given that all of these mutants retain a basal activity of gene transcription,[20,42,43] however, these results again argue a close correlation between the transcriptional activity of AML1 and its hematopoietic potential.

Runx2/AML3 and Runx3/AML2 have the capacity to rescue the hematopoietic defect of *AML1*-deficient P-Sp regions

In addition to AML1, there are 2 other known mammalian Runx transcription factors, Runx2/AML3 and Runx3/AML2. To determine whether these Runx proteins have the capacity to substitute for AML1 in hematopoiesis, we infected *AML1*-deficient P-Sp with retroviruses carrying Runx2/AML3 or Runx3/AML2. The infection efficiency and protein expression were assessed by the same method used for AML1 mutants (Figure 7A-B). Interestingly, enforced expression of either Runx2 or Runx3 in *AML1*-deficient P-Sp resulted in the generation of numerous hematopoietic cells (Figure 7C). There is no difference among the rescued hematopoietic cells by all 3 Runx proteins in terms of morphology, expression of surface markers, and CFC activity (data not shown). These results suggest redundant roles among Runx proteins in early hematopoietic development.

Discussion

The striking phenotype of *AML1*-deficient mice has demonstrated an essential role for AML1 in the formation of definitive hematopoiesis during development. However, domain contribution of AML1 in early hematopoietic development has not yet been fully elucidated. Here we described an assay for AML1 function based on the ability to rescue hematopoiesis from the *AML1*-deficient P-Sp regions. Using this system, we found that the hematopoietic potential of AML1 was closely related to its transcriptional activity. Among those mutants used in this study, AML1bΔ444, AML1bΔ397, and AML1bΔ(181-210) are transcriptionally active in a luciferase assay (Kurokawa et al[41]; data not shown). AML1b-S249/266A and AML1b-K24/43R also retain a basal activity of gene transcription. All of these transcriptionally active mutants of AML1 could confer hematopoietic activity on *AML1*-deficient P-Sp regions. On the contrary, other mutants that lose the transcriptional activation ability (Lutterbach et al,[20] Kurokawa et al[41]; data

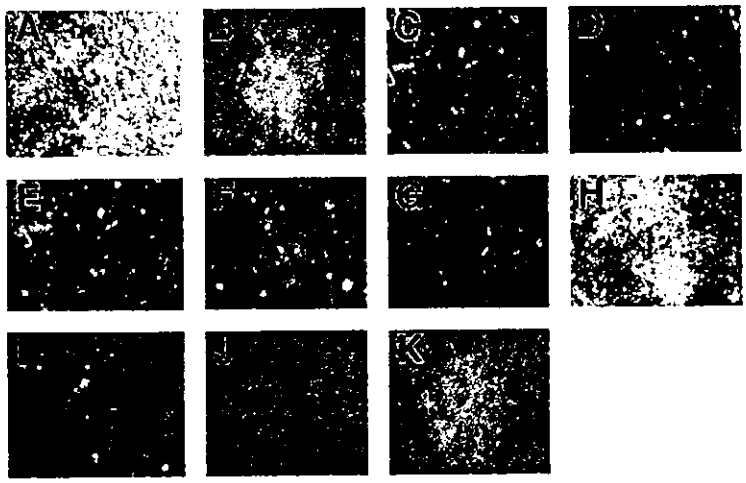

Figure 6. Hematopoietic potential of the AML1 mutants. Cells isolated from *AML1*-deficient P-Sp regions were infected with retrovirus containing the AML1 mutants. Each retrovirus contained (A) AML1bΔ444, (B) AML1bΔ397, (C) AML1bΔ335, (D) AML1bΔ288, (E) AML1a, (F) AML1bΔRD, (G) AML1bΔ(205-332), (H) AML1bΔ(181-210), (I) AML1b-R139G, (J) AML1b-S249/266A, or (K) AML1b-K24/43R. AML1bΔ444 (A), AML1bΔ397 (B), AML1bΔ(181-210) (H), AML1b-S249/266A (J), and AML1b-K24/43R (K) retain the ability to rescue the hematopoietic defect of *AML1*-deficient P-Sp regions, whereas other mutants do not. Shown are phase-contrast microscopic views of these cultures at 14 days. Photographs were taken with a Nikon Eclipse TE2000-U (Nikon Sankei) at a magnification of × 100.

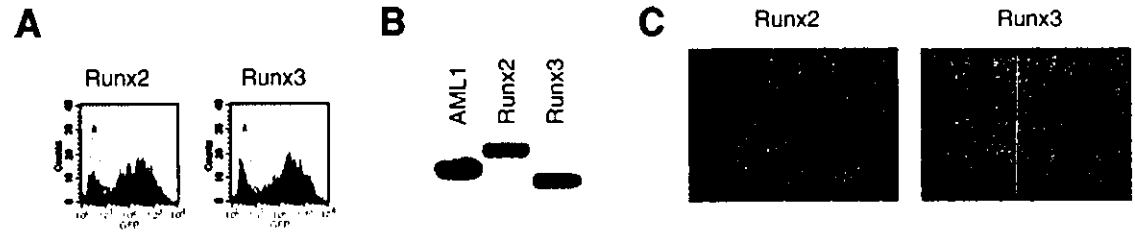

Figure 7. Runx2/AML3 and Runx3/AML2 have the capacity to rescue the hematopoietic defect of *AML1*-deficient P-Sp regions. (A) The efficiency of retrovirus-mediated gene transfer of Runx2/AML3 or Runx3/AML2 was estimated by infecting NIH3T3 cells. Retrovirus-infected cells were evaluated by the expression of GFP (shaded histograms). Also shown are the noninfected NIH3T3 cells (open histograms). (B) Expression of 3 Runx proteins (AML1, Runx2/AML3, and Runx3/AML2) in infected NIH3T3 cells. The expression is monitored by immunoblotting of whole-cell lysates with anti-Flag. (C) Both Runx2/AML3 and Runx3/AML2 have the capacity to rescue the hematopoietic defect of *AML1*-deficient P-Sp regions. Shown are phase-contrast microscopic views of these cultures at 14 days visualized using a Nikon Eclipse TE2000-U (Nikon Sankei) at a magnification of × 100.

not shown) did not rescue the hematopoietic defect. Previously, the C-terminal deletion mutants of AML1b were analyzed with the ES cell culture system. Among them, the mutants containing *trans*-activation subdomains retained the hematopoietic potential.[35] In the present study, we extended these analyses by examining various AML1 mutants and clearly demonstrated that the transcriptional activity of AML1 is essential for in vitro hematopoietic rescue of *AML1*-deficient P-Sp regions.

AML1 can also function as a transcriptional repressor depending on the target gene and the cellular context by recruiting corepressors, such as transduction-like enhancer of split (TLE) and mSin3A. Of these, TLE interacts with AML1 by recognizing its C-terminal VWRPY motif,[21-23] and mSin3A interacts mainly through the region between amino acids 181 and 210.[20] As shown in the current study, the deletion mutants of AML1 that do not interact with these corepressors retained the hematopoietic potential. Therefore, repressor activity of AML1 appears dispensable in early hematopoietic development. Recently, investigations using T-cell–specific *AML1*-deficient mice demonstrated that AML1 has critical functions during thymocyte development.[47] In addition, the *trans*-repression activity of AML1 was suggested to play a role in early thymocyte development.[36] Therefore, the function of AML1 as a transcriptional repressor should be important for appropriate T-cell differentiation. Because the culture system described here lacks the ability to support T-cell development, we are currently establishing another in vitro culture system to investigate the domain contribution of AML1 in T-cell development.

Although a genetic mutation of AML1 has been found in patients with hematologic malignancies,[12-14] the precise mechanisms of leukemogenesis caused by these mutations remain uncertain. Significant in this regard is our observation that AML1b-R139G, the AML1 mutant found in a MDS patient,[13] has lost the hematopoietic potential. This is the first direct evidence that the point mutation in the *AML1* gene, which was found in a patient with hematologic malignancy, leads to loss of its biologic activity in hematopoiesis. Taken together, this experimental system should contribute to further clarification of the molecular basis for leukemogenesis mediated by subtle mutations in the *AML1* gene.

Our study clearly demonstrated that both Runx2/AML3 and Runx3/AML2 have the capacity to rescue the hematopoietic defect of *AML1*-deficient P-Sp regions. Moreover, we also showed that "human" Runx proteins could substitute for the "murine" AML1 in early hematopoietic development because we used human cDNAs in this study. These results are consistent with the fact that the Runt and the *trans*-activation domains, which are essential for hematopoiesis, are highly conserved among members of mammalian Runx family. Thus, Runx-mediated hematopoietic activity depends on the evolutionarily conserved domains in Runx proteins.

In summary, we established an experimental culture system to efficiently examine the hematopoietic potential of Runx transcription factors. By analysis of the mutants, we precisely mapped the region responsible for the hematopoietic potential of AML1 and demonstrated that the transcriptional activity of AML1 is essential for early hematopoietic development. Furthermore, our results suggest a functional redundancy of mammalian Runx proteins in hematopoiesis.

Acknowledgments

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Leukemia-Related Transcription Factor TEL Is Negatively Regulated through Extracellular Signal-Regulated Kinase-Induced Phosphorylation

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TEL is an ETS family transcription factor that possesses multiple putative mitogen-activated protein kinase phosphorylation sites. We here describe the functional regulation of TEL via ERK pathways. Overexpressed TEL becomes phosphorylated in vivo by activated ERK. TEL is also directly phosphorylated in vitro by ERK. The inducible phosphorylation sites are Ser^{213} and Ser^{257}. TEL binds to a common docking domain in ERK. In vivo ERK-dependent phosphorylation reduces *trans*-repressional and DNA-binding abilities of TEL for ETS-binding sites. A mutant carrying substituted glutamates on both Ser^{213} and Ser^{257} functionally mimics hyperphosphorylated TEL and also shows a dominant-negative effect on TEL-induced transcriptional suppression. Losing DNA-binding affinity through phosphorylation but heterodimerizing with unmodified TEL could be an underlying mechanism. Moreover, the glutamate mutant dominantly interferes with TEL-induced erythroid differentiation in MEL cells and growth suppression in NIH 3T3 cells. Finally, endogenous TEL is dephosphorylated in parallel with ERK inactivation in differentiating MEL cells and is phosphorylated through ERK activation in Ras-transformed NIH 3T3 cells. These data indicate that TEL is a constituent downstream of ERK in signal transduction systems and is physiologically regulated by ERK in molecular and biological features.

TEL is a member of the ETS family transcription factors (7) that are essential for a variety of developmental processes and cellular responses to environmental stimuli. TEL shares with other ETS proteins an evolutionarily conserved ETS domain at the C terminus that is responsible for DNA binding to the ETS-binding consensus site (EBS) (26). TEL also contains an N-terminal domain that is referred to as the helix-loop-helix (HLH), or pointed, domain. The HLH domain in TEL has the unique property of inducing its stable homodimerization or heterodimerization with other ETS family members (9, 13, 18, 27). Being a transcriptional repressor, TEL is known to interact with the relevant cofactors mSin3A and N-CoR (34). By interacting with histone deacetylase-3 directly or indirectly, TEL is believed to mediate transcriptional repression of target genes such as *FLI-1* (21), *Id1* (R. Martinez and T. R. Golub, Abstr. 42nd Annu. Meet. Am. Soc. Hematol., abstr. 453a, 2000), and *stromelysin-1* (6).

Various 12p13 translocations involving the *TEL* gene and generating the *TEL*-related chimeric genes have been reported in many types of hematological malignancies. In some translocations, receptor-type or non-receptor-type tyrosine kinases are fused to the N-terminal portion of TEL and are thus activated by homodimerization through the HLH domain in the TEL moiety. Examples include platelet-derived growth factor receptor β in t(5;12)(q33;p13) (12), ABL in t(9;12)(q34;p13) (8), JAK2 in t(9;12)(p24;p13) (19), and Syk in t(9;12)(q22;p13) (16). In other translocations, transcription factors are structurally and functionally modified by fusion with the N- or C-terminal part of TEL. Examples include AML1 in t(12;21)(p13;q22) (5, 10, 11) and MN1 in t(12;22)(p13;q11) (3). Thus, perturbation of original functions of the partner genes could be a mechanism in causing leukemia in patients with such translocations. On the other hand, tumor-suppressive functions of TEL are suggested, because the expression of TEL in Ras-transformed NIH 3T3 cells inhibits cell growth in liquid and soft agar cultures (6, 32).

TEL is widely expressed throughout mouse embryonic development and in most human and mouse tissues. It is essential for mouse development, since its inactivation by homologous recombination results in embryonic lethality at E10.5 to E11.5 (35). The knockout embryos show defects in yolk sac angiogenesis and intraembryonic apoptosis of mesenchymal and neural cells, while they present normal yolk sac hematopoiesis. Analysis of chimeric mice with $TEL^{-/-}$ embryonic stem cells uncovered an essential role of TEL in establishing hematopoiesis of all lineages in neonatal bone marrow, although $TEL^{-/-}$ embryonic stem cells contributed to both primary and definitive fetal hematopoiesis (36). As for lineage-specific roles in hematopoietic systems, we have recently reported that TEL accelerates erythroid differentiation of mouse erythroleukemia (MEL) cells induced by hexamethylene bisacetamide (HMBA) or dimethyl sulfoxide (33).

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