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Roles for c-Myc in self-renewal of hematopoietic stem cells

Yusuke Satoh¹, Itaru Matsumura¹, Hirokazu Tanaka¹, Sachiko Ezoe¹, Hiroyuki Sugahara¹, Masao Mizuki¹, Hirohiko Shibayama¹, Eri Ishiko¹, Jun Ishiko¹, Koichi Nakajima², and Yuzuru Kanakura¹.

From¹Department of Hematology and Oncology, Osaka University Graduate School of Medicine, 2-2, Yamada-oka, Suita, Osaka 565-0871, Japan; ²Department of Immunology, Osaka City University Graduate School of Medicine

Running title: Roles for c-Myc in self-renewal of HSCs

Address correspondence to Itaru Matsumura, Department of Hematology and Oncology, Osaka University Graduate School of Medicine, 2-2, Yamada-oka, Suita, Osaka 565-0871, Japan. TEL: +81-6-6879-3871; FAX: +81-6-6879-3879; E-mail: matumura@bldon.med.osaka-u.ac.jp
SUMMARY

Notch and HOXB4 have been reported to expand hematopoietic stem cells (HSCs) in vitro. However, their critical effector molecules remain undetermined. We found the expression of c-myc, cyclin D2, cyclin D3, cyclin E, and E2F1 was induced or enhanced during Notch1- or HOXB4-induced self-renewal of murine HSCs. Since c-Myc can act as a primary regulator of G1/S transition, we examined whether c-Myc alone can induce self-renewal of HSCs. In culture with SCF, FLT3 ligand, and IL-6, a 4-hydroxytamoxifen (4-HT)-inducible form of c-Myc (Myc/ERT) enabled murine Lin- Sca-1+ HSCs to proliferate with the surface phenotype compatible with HSCs for more than 28 days. c-Myc activated by 4-HT augmented telomerase activities and increased the number of CFU-Mix about 2-fold in colony assays. Also, in reconstitution assays, HSCs expanded by c-Myc could reconstitute hematopoiesis for more than 6 months. As for the mechanism of c-myc induction by Notch1, we found that activated forms of Notch1 (NotchIC) and its downstream effector RBP-J (RBP-VP16) can activate the c-myc promoter through the element between -195 bp and -161 bp by inducing the DNA-binding complex. Together, these results suggest that c-Myc can support self-renewal of HSCs as a downstream mediator of Notch and HOXB4.
INTRODUCTION

Hematopoietic stem cells (HSCs) are characterized by two distinct abilities: self-renewal ability and pluripotentiality. With these activities, HSCs supply all lineages of hematopoietic cells throughout their life. In a murine experimental model, only a single HSC with the CD34^{low/-} c-Kit^+ Sca-1^+ phenotype can reconstitute whole hematopoiesis in vivo (1, 2). To maintain homeostasis of hematopoiesis and protect exhaustion of HSC population, most of the HSCs are kept quiescent and only a limited number of cells enter the cell cycle to supply mature blood cells (3). As external regulatory factors, various cytokines such as stem cell factor (SCF), Flt3 ligand (FL), thrombopoietin (TPO), IL-3, and IL-6 promote the growth of HSCs (4-6). The combination of SCF, FL, TPO and IL-6, in particular, was reported to efficiently induce the in vitro cell division with characteristics of self-renewal in HSCs (7). In contrast, TGF-β is known to inhibit the growth of HSCs (8, 9). Besides these cytokines, the direct interactions with stromal cells and extracellular matrix in the bone marrow microenvironment also influence the fates of HSCs (10, 11). As one mechanism responsible for this effect, Bernstein et al. showed that the activation of Notch transmembrane receptors expressed on HSCs by their ligand (Jagged 1 or Jagged2) on stromal cells promotes the self-renewal of HSCs (12).

Furthermore, accumulated evidence indicated that cell cycle state of HSCs is regulated by intrinsic transcription factors such as c-Myb and GATA-2. c-Myb promotes the growth of HSCs, and c-Myb-deficient mice die at embryonic day 15.5 (E15.5) due to the defect of definitive hematopoiesis (13). Similarly, GATA-2^{-/-} mice are embryonic lethal around E11.5 because of the defect in the development and/or maintenance of HSCs (14), whereas functional roles of GATA-2 in the growth of HSCs are still controversial (15, 16). Also, HOXB4, a member of Hox family of transcription factors, was reported to induce the marked in vitro expansion of HSCs (17, 18).
During the last decade, a number of cell cycle regulatory molecules such as cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs) have been identified and characterized in various types of cells. As for the roles of these molecules in primitive hematopoietic cells, p21WAF1 and p27Kip1 keep the quiescence of HSCs and of progenitor cells, respectively, thereby governing their pool sizes (19, 20). Meanwhile, inactivation and/or deletion of p16INK4A and p15INK4B genes are supposed to contribute to leukemogenesis in a substantial proportion of ALL cases (21, 22). These results imply that appropriate cell cycle control, particularly at the early stage of stem/progenitor cells, is required for maintaining normal hematopoiesis.

c-Myc belongs to a family of transcription factors containing basic, helix-loop-helix and leucine zipper domains. The expression of c-Myc is absent in quiescent cells, and is rapidly induced by growth factors in normal tissues, while it is continuously overexpressed in a variety of neoplasms (23-25). In addition, c-Myc is highly expressed in various tissues during embryogenesis, and c-myc−/− mice die at E10.5, indicating that c-Myc is essential for normal embryonic development (26). c-Myc, in conjunction with its heterodimer partner Max, regulates gene transcription through the element called E box (CACGTG) (27, 28). c-Myc induces a number of target genes involved in G1/S transition such as E2Fs, CDC25A, CDK2, CDK4, Cul1, Id2, and Rb (29-35). Also, c-Myc activates cyclin E/CDK2 complexes alone or in combination with Ras-mediated signals (36-38). With these activities, c-Myc works at several points during the cell cycle. In fact, fibroblasts isolated from c-myc-deficient rats revealed a markedly prolonged cell doubling time accompanied by the drastic reduction of CDK4/6 and CDK2 activities (32, 33). Furthermore, ectopic expression of c-Myc in quiescent cells under some conditions is sufficient for inducing S phase entry (39). These lines of evidence indicates that c-Myc plays a central role in G1/S transition as an upstream regulator of cell cycle regulatory molecules (40).
Once HSCs are induced to enter cell cycle under certain conditions, HSCs are obliged to select either self-renewal or differentiation during cell division. In this process, Notch signals and HOXB4 promote self-renewal of HSCs rather than differentiation as described above. Therefore, it is speculated that, there must be a set of genes induced by Notch signals or HOXB4 contributing to self-renewal of HSCs. However, at present, their critical target molecules remain to be determined. Moreover, it remains unknown how this process is regulated by cell cycle regulatory molecules.

To clarify the roles of cell cycle regulatory molecules in the cell division of HSCs, in this study, we investigated the changes of their expression during Notch- or HOXB4-induced self-renewal of HSCs. We found that c-Myc was transcriptionally induced by Notch and HOXB4. In addition, we found its ectopic expression induced the growth of HSCs without disrupting their biologic properties in terms of surface phenotypes, colony forming activities and reconstituting activities. Together, these results suggest c-Myc plays a major role in self-renewal of HSCs as an effector molecule of Notch signals and HOXB4.
EXPERIMENTAL PROCEDURES

Reagents and antibodies—Recombinant human (h) interleukin-6 (hIL-6), murine (m) IL-3, and murine stem cell factor (mSCF) were provided by Kirin Brewery Company (Tokyo, Japan). Human flt3-ligand (hFL) was purchased from PEPROTECH (London, UK). Fluorescein isothiocyanate (FITC)-conjugated rat IgG1 and biotinylated rat IgG2b were purchased from IMMUNOTECH (Marseilles, France). Biotinylated anti-lineage antibodies (Abs) against Gr-1 (RB6-8C5), B220 (RA3-6B2), CD3 (145-2C11), Mac1 (M1/70), and Ter119 (TER119); a fluorescence-labeled anti-Sca-1 Ab (D7), an allophycocyanin (APC)-labeled anti-c-Kit Ab (2B8), an FITC-conjugated anti-Ly5.1 Ab (A20), a biotinylated anti-Ly5.2 Ab (104), and streptavidin-phycoerythrin (PE) were purchased from PharMingen (San Diego, CA). An FITC-conjugated anti-CD34 Ab was kindly provided by Dr. T. Hirano (Osaka University, Osaka, Japan).

Plasmid constructs—An expression vector encoding murine activated Notch 1 (NotchIC) (pSG5mNotchIC) was provided by Dr. G.W. Bornkamm (Institute for Clinical Molecular Biology, Munich, Germany); Notch1/ERT (rNERTneo) by Dr. U. Just (Institute for Clinical Molecular Biology, Munich, Germany); Flag-tagged RBP-VP16 (pSG5Flag-RBP-VP16) by Dr. E. Manet (Unité de Virologie Humaine, Lyon, France); HOXB4 (pMSCVneoHOXB4) by Dr. R.K. Humphries (British Columbia Cancer Agency, Vancouver, Canada). A retrovirus expression vector for Myc/ERT was generated by subcloning its cDNA into pMSCVneo (BD Biosciences Clontech, Palo Alto, CA).

Flow cytometry—DNA content of cultured cells was examined by staining with propidium iodide (PI) and analyzed on FACSort (Beckton Dickinson, Oxnard, CA). Cell cycle analysis was performed with a program Modfit LT2.0 (Beckton Dickinson). Surface phenotypes of cultured cells were examined with FACSCalibur (Beckton Dickinson).

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)
analyses—Semiquantitative RT-PCR analyses were performed as previously reported (41). Briefly, total cellular RNA was extracted from cultured cells (about 10^5 cells) and reverse transcribed into cDNA with oligo(dT) primers (Pharmacia, Piscataway, NJ) using SuperScript II reverse transcriptase (Gibco BRL, Gaithersburg MD). PCR was performed in a total volume of 30 µl using 1 µl of the cDNA product as a template. The primer sets to amplify c-myc, cyclin D1, cyclin D2, cyclin D3, cyclin E, E2F1 and β-actin are as follows: c-myc, 5'-TCACCAGCAACAATACGCGC-3' and 5'-
CAGGATGTAGGCGTGCTT-3'; cyclin D1, 5'-
AGGCAGATGAGAACAAACGAG-3' and 5'-CAGGCTGACTCCACAGAGG-3';
cyclin D2, 5'-AAGGAGAGCAGCTGTCCTGAC-3' and 5'-
GAACGCTGCAGCTGGTAC-3'; cyclin D3, GCCTCCACCAGGAAAGCG-3'
and CCAGGAGTCTGTCGCAATCC-3'; cyclin E, 5'-
CCCAGCAGTAAAGGCGAGAg-3' and 5'-CAGCTTCTGGAGCAGACTCAGTG-3';
E2F1, 5'-GACTGTGACTTTGGGACC-3' and 5'-TGTCACCTTCATTCCCC-3';
β-actin, 5'-CATCCTATGACAGCAACGGC-3' and 5'-
ACGCAGCTCAGTAACAGTC-3'. The samples were denatured at 94°C for 10 min, followed by 20 to 35 cycles of amplification (94°C, 30 sec for denaturation; 56°C, 30 sec for annealing; 72°C, 30 sec for extension). At first, we adjusted the amount of cDNA products among several samples based on the amount of β-actin PCR products. Then, an equal amount of cDNA products were subjected to the PCR. The amount of each mRNA was evaluated after 29~35 cycles of PCR, during which PCR products were exponentially amplified in all of the samples. The PCR products were electrophoresed on agarose gels and their amount was evaluated by staining with SYBR GREEN I (BioWhittaker Molecular Applications, Rockland, ME).

Luciferase assays—To construct reporter genes containing various fragments of murine c-myc promoter, we performed PCR and subcloned PCR products into the
luciferase plasmid. Luciferase assays were performed with a Dual-Luciferase Reporter System (Promega, Madison, WI) as previously described (42). In short, 293T cells (2 × 10^5 cells) cultured in DMEM containing 10% FBS were seeded in a 60-mm dish and transfected with the appropriate effector gene (2 µg) and reporter gene (2 µg) together with 5 ng of pRL-CMV-Rluc, an expression vector of renilla luciferase, by the calcium phosphate coprecipitation method. After 12 h, the cells were washed, and serum-deprived for 24 h. Then, the cells were lysed and subjected to the measurement of the firefly and renilla luciferase activities on a luminometer LB96P (Berthold Japan, Tokyo, Japan). The relative firefly luciferase activities were calculated by normalizing transfection efficiency according to the renilla luciferase activities. The experiments were performed in triplicate and similar results were obtained from at least 3 independent experiments.

**Electrophoretic mobility shift assay (EMSA)**—EMSA was performed as previously described (42). Seven types of double-stranded oligonucleotides were used as probes or competitors, and their sequences are shown in the figure legend.

**Assays for telomerase activities**—Telomerase activities were measured by the stretch PCR method with a TeloChaser system (TOYOBO, Osaka, Japan) according to the manufacture’s instructions.

**Isolation of murine bone marrow hematopoietic stem/progenitor cells**—We isolated murine bone marrow hematopoietic stem/progenitor cells as previously reported (41). Briefly, bone marrow cells were harvested from 8- to 10-week-old Ly5.2 mice pretreated with 150 mg/kg of 5-fluoruracil (5-FU) for 4 days. Mononuclear cells were isolated by density gradient centrifugation. Then, Lin (CD3, B220, Ter119, Mac1 and Gr-1)^−^ Sca-1^+^ cells were collected using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). At this step, we confirmed that more than 95% of the separated cells were Lin^−^ Sca-1^+^ cells by flow cytometric analysis.
Retrovirus transfection into murine bone marrow cells—At first, we prepared conditioned media containing high-titer virus particles using Plat-E cells as described previously (41). The isolated Lin- Sca-1+ cells were cultured in DMEM supplemented with 10% FBS in the presence of mIL-3 (10 ng/ml), mSCF (50 ng/ml), hIL-6 (50 ng/ml) and hFL (30 ng/ml) for 72 h. Then, the cultured cells were cultured with conditioned media containing high-titer retrovirus in the presence of polybrene (8 μg/ml). After 24 h, the cells were washed and cultured in DMEM supplemented with 10% FBS containing the same cytokines and 1 mg/ml G418 for 72 h. After the selection with G418, retrovirus-infected cells were cultured under the conditions as indicated.

Colony assays—Retrovirus-infected cells (1 × 10⁵ cells/35-mm dish) were cultured in the methylcellulose media containing human erythropoietin (hEPO) (3 U/ml), mIL-3 (10 ng/ml), hIL-6 (10 ng/ml), and mSCF (50 ng/ml) (Stem Cell Technologies, Vancouver, BC, Canada). G418 selection was continued during methylcellulose cultures. The number of colonies was counted after 12 days.

Transplantation assays—Ly5.2 mice were lethally irradiated (950 rads) 24-h before the transplantation. For transplantation assays, we prepared Myc/ERT-transduced bone marrow cells from congenic C57BL/6 (B6-Ly5.1) mice. Myc/ERT-transduced Ly5.1 cells (4 × 10⁶ cells) were injected intravenously in combination with 1 × 10⁵ of normal bone marrow cells with Ly5.2 phenotype.

Statistical analysis—Statistical analysis was performed with Student’s t-test.
RESULTS

The effects of Notch1 and HOXB4 on the expression of cell cycle regulatory molecules in murine Lin−Sca-1+ cells—First, we analyzed how self-renewal is regulated by cell cycle regulatory molecules in HSCs. For this purpose, we introduced a 4-HT-inducible form of Notch1 (N/ERT, a chimeric molecule consisting of the intracellular domain of Notch1 fused to the estrogen receptor) and HOXB4 into murine Lin− Sca-1+ cells, since both molecules were reported to induce self-renewal in HSCs (17, 43). In the absence of 4-HT, N/ERT-transduced cells could not keep proliferating for 14 days under any cytokine combinations (i.e., SCF alone, SCF+FL or SCF+FL+IL-6) (Figure 1A, upper panel). In contrast, 4-HT-treated cells kept growing under the same cytokine combinations during 14 days. Similarly, HOXB4-transduced cells could proliferate for up to 14 days, whereas mock (an empty vector)-transduced cells could not (Figure 1A, lower panel). In DNA content analysis, the 4-HT treatment increased the proportion of the cells in S-G2/M phase from 11.6% to 22.2% in N/ERT-transduced cells under the culture with SCF (Figure 1B, left panel). Also, HOXB4 augmented the proportion of the cells in S-G2/M phase from 12.4% to 26.5% under the culture with SCF+FL+IL-6 (Figure 1B, right panel).

Next, we examined the effect of Notch1 and HOXB4 on the expression of cell cycle regulatory molecules by semiquantitative RT-PCR analysis (Figure 2). With or without the 4-HT treatment, total cellular RNA was extracted from N/ERT-transfected cells after a 7-day culture with SCF. Also, HOXB4-transfected cells were subjected to RT-PCR analysis after 7-day culture with SCF. At first, we adjusted the amount of cDNAs according to the amounts of the β-actin PCR products (Figure 2, bottom panel). Then, we evaluated the expression levels of c-myc, cyclin D1, cyclin D2, cyclin D3, cyclin E, and E2F1. After 29-35 cycles of PCR, all of these PCR products were amplified exponentially. As shown in Figure 2, the 4-HT treatment markedly induced or
enhanced the expression of c-myc, cyclin D2, cyclin D3, cyclin E, and E2F1, whereas it could not induce the expression of cyclin D1. Similarly, we found that HOXB4 enhanced the expression of c-myc, cyclin D2, cyclin D3, cyclin E, and E2F1 but not of cyclin D1.

c-Myc enhances and supports the growth of murine Lin-Sca-1+ cells—c-Myc is a primary regulator of G1/S transition, and its ectopic expression can drive quiescent cells to S phase in fibroblasts (as described in the Introduction). Therefore, we examined the effect of c-Myc on the growth and biologic properties of HSCs. We retrovirally transduced a 4-HT-inducible form of Myc (Myc/ERT) to murine Lin-Sca-1+ cells. When treated with 4-HT, Myc/ERT-transduced cells proliferated for more than 21 days under the culture with SCF, SCF+FL or SCF+FL+IL-6 (Figure 3A). In contrast, 4-HT-untreated cells died within 21 days under the same cytokine combinations. In DNA content analysis, the 4-HT addition prominently increased the cells in S-G2/M phase in Myc/ERT-transduced cells after 7 days (SCF, from 11.7% to 49.3%; SCF+FL, from 11.9% to 52.2%; SCF+FL+IL-6, from 12.2% to 53.2%) (Figure 3B). Next, we examined the effects of c-Myc on the expression profile of cell cycle regulatory molecules. With or without the 4-HT treatment, we isolated total cellular RNA from Myc/ERT-transfected cells after a 7-day culture with SCF and performed semiquantitative RT-PCR analysis. In Myc/ERT-transduced cells, 4-HT-activated c-Myc induced or enhanced the expression of cyclin D1, cyclin D2, cyclin D3, cyclin E and E2F1 mRNA (Figure 3C), suggesting a possibility that c-Myc would be a primary regulator of Notch- or HOXB4-mediated cell growth among various cell cycle regulatory molecules.

Characterization of the cells expanded by Myc/ERT using in vitro assays—The 4-HT treatment was able to keep Myc/ERT-transduced cells viable even after a 28-day culture with SCF or SCF+FL+IL-6 (data not shown). To investigate the biologic
properties of these cells, we initially examined their surface phenotypes by FACS. Before the addition of 4-HT, 77.2% of the Myc/ERT-transduced cells were Sca-1+c-Kit+, and 94.7 (64.9 + 29.8)% were Lin- (including CD34-positive and negative cells). After 28-day culture with SCF, more than 50% of the cultured cells still revealed immature surface phenotypes: Sca-1+c-Kit+ cells, 56.6%; Lin- cells, 63.4 (24.5 + 38.9)% (Figure 4A). Similar results were also observed after a 28-day culture with SCF+FL+IL-6: Sca-1+c-Kit+ cells, 47.7%; Lin- cells, 81.3 (32.0 + 49.3)%. Next, we analyzed the effects of c-Myc on the colony forming ability of HSCs. After the 3-day selection with G418, Myc/ERT-transduced Sca-1+Lin- cells were seeded into methylcellulose media (containing IL-3, IL-6, SCF and EPO) and cultured with or without 4-HT for 12 days. As shown in Figure 4B, 4-HT-activated Myc/ERT increased the number of CFU-Mix with a statistically significant difference p<0.05. Also, Myc/ERT significantly increased BFU-E (p<0.05) and CFU-GM (p<0.01). We also analyzed changes of telomerase activity in the Myc/ERT-transduced cells. Quiescent HSCs have been reported to exhibit a low level of telomerase activity, which is upregulated in response to cytokine stimulation (44, 45), while it declines during the maturation process (46). After a 7-day culture in the presence of SCF with or without the 4-HT treatment, we isolated cell extracts from Myc/ERT-transduced cells and evaluated telomerase activity by the stretch PCR method. As observed in a positive control, Hela, the 4-HT treatment induced telomerase activity in Myc/ERT-transduced cells, of which specificity was confirmed by its heat instability. In contrast, Myc/ERT-transduced cells cultured without 4-HT did not have telomerase activity (Figure 4C). Together, these results suggest that c-Myc-induced cell growth does not spoil the immature characteristics of HSCs.

Reconstitution assays using HSCs expanded by Myc/ERT—Next, we evaluated the in vivo function of Myc/ERT-expanded HSCs with reconstitution assays. After 12-day
cultures, 4-HT-treated or untreated Myc/ERT-transduced cells (4 \times 10^6 cells) with Ly5.1 phenotype were transfused into lethally irradiated Ly5.2 mice in combination with 1 \times 10^5 of Ly5.2+ normal bone marrow cells. When 4-HT-treated cells were transplanted, 11.3% of the peripheral blood cells were Ly5.1+ in the recipient mice after 4 weeks (Figure 4D, upper left panel). In contrast, Ly5.1+ cells were hardly detected in the mice transplanted with 4-HT-untreated cells after 4 weeks (Figure 4D, upper right panel). Furthermore, Ly5.1+ cells were still detected in 7.9% of the peripheral blood cells in the mice transplanted with 4-HT-treated cells even after 12 weeks (Figure 4D, lower left panel). In this Ly5.1+ fraction, 15% of the cells were Mac1/Gr-1+, 37.1% were CD4/8+, and 50.7% were B220+, indicating that HSCs expanded by Myc/ERT has an ability of multilinage reconstitution. Also, these cells were able to supply hematopoietic cells in the recipient mice for more than 6 months (data not shown). Collectively, these results suggested that ectopically introduced c-Myc can induce self-renewal of HSCs.

**The effects of Notch signaling and HOXB4 on c-myc promoter activities**—Since c-Myc was considered to be important for self-renewal of HSCs, we next tried to clarify the mechanism through which Notch1 and HOXB4 induce the c-myc mRNA expression. For this purpose, we performed luciferase assays with a reporter gene -1137/+516-Luc containing the murine c-myc promoter (numbered from the transcription initiation site) with NotchIC (an activated form of Notch1), RBP-VP16 (a transcriptionally active derivative of RBP-J) and HOXB4 as effector genes. In 293T cells, NotchIC, RBP-VP16 and HOXB4 activated -1137/+516-Luc by 2.9-fold, 6.9-
fold and 4.7-fold, respectively, while these effector genes hardly stimulated its backbone vector +141/+516-Luc (Figure 5A). Similar results were obtained from a murine fibroblast cell line NIH3T3 (data not shown). To determine which region is responsive to Notch in the c-myc promoter, we generated several reporter genes and performed luciferase assays with RBP-VP16 as an effector gene. As shown in Figure 5B, -195/+24-Luc was most responsive to RBP-VP16 (18-fold induction). Also, the similar level (16.1-fold) of activation was induced by RBP-VP16 in -195/-31-Luc. To further identify which element is responsive to RBP-VP16, we isolated nuclear extracts from 293T cells transfected with Flag-RBP-VP16 or a mock vector and performed EMSA with 7 overlapping probes spanning between -195 and -11 of the c-myc promoter (Figure 6A). As compared with DNA-binding patterns formed from mock-transfected cells, nuclear extracts isolated from Flag-RBP-VP16-transfected cells bound to the probes 1, 6 and 7 (Figure 6B, upper panel, lanes 2, 12 and 14, DNA-binding complexes are indicated by arrows), while a significant difference was not detected with the other probes. These binding complexes were abolished by non-labeled wild-type (WT) competitors (Figure 6B lower panel, lanes 3, 7 and 11) but not by mutated (MT) competitors (Figure 6B lower panel, lanes 4, 8 and 12), indicating that these complexes were formed in a sequence specific manner. To further characterize the role of these sites in Notch signals, we performed luciferase assays with reporter genes each containing the sequence of the probe 1 (-195/-161-Luc) and probes 6-7 (-70/-
11-Luc). As shown in Figure 6C, RBP-VP16 activated -195/-161-Luc by 4.1-fold, while it was hardly effective on -70/-11-Luc, implying that the protein that binds to the probe 1 would contribute to the activation of the c-myc promoter by Notch signaling. However, this complex reacted neither to the anti-Flag Ab nor the anti-RBP-J Ab (data not shown). Also, the probe 1 did not contain the typical RBP-J-binding sequence. These results suggest that RBP-VP16 would activate this element indirectly. In addition, since the reactivity of -195/-161-Luc was not as prominent as that of -195/-31-Luc, it was speculated that an additional element would be necessary for the c-myc promoter to achieve the maximal response to Notch signals.
DISCUSSION

With regard to the roles for Notch and HOXB4 in cell cycle regulation, Notch1 activation was reported to shorten the G1 phase of cell cycle, thereby delaying the differentiation of human HL-60 cells and primary CD34+ cells (47). Also, Capobianco et al. showed that intracellular domain of Notch directly activated cyclin D1 promoter in a human kidney cell line, 293T (48). In addition, Sauvageau et al. reported that overexpression of HOXB4 led to the induction of Jun-B and Fra-1 expression and subsequent up-regulated expression of cyclin D1 in Rat-1 fibroblast (49). To identify a critical target molecule in Notch- and HOXB4-induced self-renewal of HSCs, we analyzed their effects on the expression of cell cycle regulatory molecules, and found that both molecules up-regulated the expression of c-myc, cyclin D2, cyclin D3, cyclin E and E2F1. However, neither Notch 1 nor HOXB4 up-regulated the expression of cyclin D1, whereas its expression was up-regulated by these molecules in other cell types (as described above). These findings were consistent with the previous report that cyclin D1 was not detected in the proliferating normal hematopoietic stem/progenitor cells (50). Although cyclin D1 expression did not accompany the proliferation of normal hematopoietic stem/progenitor cells, our results suggest that G1/S transition in self-renewal of HSCs is not so unique, but rather common to other cell types. In addition, we found that Myc/ERT efficiently induced the expression of cyclin D2, cyclin D3, and cyclin E and promoted the growth of HSCs. In addition, previous reports suggested that c-Myc would transcriptionally repress the expression of p21WAF1, which plays a crucial role in the quiescence of HSCs (19, 51), and of p15INK4b, which is induced by TGF-β signaling (52, 53). Together, these results imply that the c-myc could be a master regulator of cell cycle in both Notch1- and HOXB4-induced self-renewal of HSCs.

In this study, promoter assays revealed that both Notch signaling and HOXB4
activated c-myc promoter. However, the element most responsive to RBP-VP16 in the c-myc promoter did not contain the putative RBP-J-binding sequence. Also, we confirmed the nuclear protein, which bound to this element, did not contain RBP-VP16 itself in EMSA with supershift assays, suggesting RBP-VP16 could activate the c-myc promoter indirectly. In order to understand the mechanism of Notch-induced self-renewal of HSCs, we are now trying to purify this molecule using nuclear extracts from RBP-VP16-transfected 293T cells.

In this study, reconstitution assays revealed that cells expanded by Myc/ERT could contribute to hematopoiesis for more than 6 months, indicating these cells still possess characteristics of HSCs in terms of long-term reconstitution. In addition, these cells developed Mac1/Gr-1+ cells, CD4/8+ cells, and B220+ cells, implying that the cells expanded by Myc/ERT has a multilineage reconstitution ability. When we compare the reconstitution efficiency of Myc/ERT-expanded cells with that of Notch1-expanded cells (43), Myc/ERT-expanded cells showed similar or a little more potent reconstitution abilities than Notch-expanded cells. [120-fold excess of Notch-1-expanded cells (i.e., transplantation of 120 × 10^5 of Notch1-expanded cells in combination with 1 × 10^5 of normal BM cells) contributed to hematopoiesis in about 23% of BM cells after transplantation vs. 40-fold excess of Myc/ERT-expanded cells (i.e., transplantation of 40 × 10^5 of Myc/ERT-expanded cells in combination with 1 × 10^5 of normal BM cells) participated in hematopoiesis in about 10% of the peripheral blood cells]. However, even if enough number (1 × 10^7) of Myc/ERT-expanded cells were transplanted, co-injection of normal supporting BM cells was required for radioprotection in the recipient mice (data not shown). These results indicate that the cells expanded by Myc/ERT lack some ability to fully or rapidly reconstitute hematopoiesis in vivo. The similar defect was observed in HSCs expanded by Notch1
Like Myc/ERT-expanded cells, Notch1-expanded cells alone could not reconstitute hematopoiesis in lethally irradiated mice, whereas these cells could participate in hematopoiesis for more than 6 months in combination with normal supporting BM cells. One possible explanation is that the *ex vivo* culture of HSCs or modulation of cell cycle by Notch1 or c-Myc might result in the reduced homing abilities after intravenous transplantation. For example, Szilvassy et al. showed that HSCs expanded by growth factors *in vitro* expressed little or no β1 integrin and this change was associated with a failure of radioprotection (54). Alternatively, HSCs induced to proliferate by Notch or c-Myc *in vitro* might not be able to rapidly undergo terminal differentiation after transplantation, which resulting in the failure to supply enough number of mature cells required for radioprotection.

In summary, our results indicate that c-Myc is, at least in part, capable of supporting self-renewal of HSCs as a downstream mediator of Notch and HOXB4. Further analyses on cell-cycle regulation would undoubtfully provide more informative findings to expand HSCs *in vitro.*
References

42. Matsumura, I., Kitamura, T., Wakao, H., Tanaka, H., Hashimoto, K., Albanese, C.,
Figure Legends

**Figure 1. Effects of Notch1 and HOXB4 on the growth of murine Lin\(^{−}\)Sca-1\(^{+}\)cells.** (A) Murine Lin\(^{−}\)Sca-1\(^{+}\)cells were infected with retrovirus each containing the indicated gene and cultured for up to 2 weeks. Total number of viable cells was counted by the trypan blue dye exclusion method at the times indicated. The results are shown as mean ± SD of triplicated cultures. (B) After 7-day cultures under the conditions indicated, DNA content of the cultured cells was examined by PI staining. The proportion of the S-G2/M phase is indicated.

**Figure 2. Effects of Notch1 and HOXB4 on the expression of cell cycle regulatory molecules.** Murine Lin\(^{−}\)Sca-1\(^{+}\)cells were infected with retrovirus each containing the indicated gene, cultured for 7 days and subjected to semiquantitative RT-PCR analyses on the expression of c-myc, cyclin D1, cyclin D2, cyclin D3, cyclin E and E2F1 mRNA. The PCR products were electrophoresed on agarose gels and visualized by staining with SYBR GREEN I.

**Figure 3. Effects of c-Myc on the growth of murine Lin\(^{−}\)Sca-1\(^{+}\)cells.** (A) Murine Lin\(^{−}\)Sca-1\(^{+}\)cells were infected with retrovirus containing Myc/ERT and cultured with or without 4-HT for up to 3 weeks. The total number of viable cells was counted by the trypan blue dye exclusion method at the times indicated. The results are shown as mean ± SD of triplicated cultures. (B) After 7-day cultures under the conditions indicated, DNA content of the Myc/ERT-transduced cells was examined by PI staining. The proportion of the S-G2/M phase is indicated. (C) After 7-day cultures with or without 4-HT, the Myc/ERT-transduced cells were subjected to semiquantitative RT-PCR analyses on the expression of cyclin D1, cyclin D2, cyclin D3, cyclin E and E2F1.
mRNA.

**Figure 4. Characterization of the cells expanded and maintained by c-Myc for 28 days.**

(A) The surface phenotype of Myc/ERT-transduced cells was examined by flow
cytometric analyses before and after the 28-day culture with 4-HT in the presence of
SCF or SCF+FL+IL-6. The anti-lineage Abs recognize Gr-1, B220, CD3e, Mac1 and
Ter119. (B) Myc/ERT-transduced murine Lin^-Sca-1^ cells were cultured with G418
for 3 days, and the living cells were seeded into methylcellulose media (containing IL-
3, IL-6, SCF and EPO). The number of the indicated colonies was counted after 12
days. The results are shown as mean ± SD of quintupled cultures. (C) After 7-day
cultures, the cultured cells were subjected to the stretch PCR method for the evaluation
of telomerase activities. Lane 1, Myc/ERT-transduced cells cultured without 4-HT;
lane 2, Myc/ERT-transduced cells cultured with 4-HT; lane 3, the heat-treated sample
of lane 2; lane 4, negative control (PCR was performed without any template.); lane 5,
Hela cells as a positive control. (D) Reconstitution assays using the cells expanded by
Myc/ERT. MycERT-transduced Ly5.1 cells (4 × 10^6 cells) cultured with (upper left
panel) or without (upper right panel) 4-HT were transplanted into lethally irradiated
mice in combination with 1 × 10^5 of normal bone marrow cells with Ly5.2 phenotype.
The contribution of Myc/ERT-transduced Ly5.1 cells in the peripheral blood of the
recipient mice was examined by flow cytometric analyses after 4 weeks (upper 2 panels)
and 12 weeks (lower 4 panels) from transplantation. The lineage distribution of Ly5.1
cells was examined with the indicated Abs. The relative frequency of the each fraction
in the Ly5.1^+ cells is shown in the parenthesis.

**Figure 5. The effects of Notch signaling and HOXB4 on the activity of the c-myc**
promoter. (A) 293T cells seeded in 60-mm dish were transfected with 2 µg of the appropriate effector genes and reporter gene together with 5 ng of pRL-CMV-Rluc by the calcium phosphate coprecipitation method. After 12 h, the cells were washed and cultured without serum for 24 h, and then subjected to luciferase assays. After normalizing transfection efficiencies according to the renilla luciferase activities, the relative firefly luciferase activities were calculated. The results are shown as the mean ± SD of triplicate cultures. (B) Structures of c-myc luciferase reporter genes are indicated in the left panel, and the effect of RBP-VP16 on the respective reporter gene is indicated as fold induction in the right panel.

Figure 6. Analysis of the responsive element to RBP-VP16 in the c-myc promoter. (A) EMSA was performed with 7 overlapping probes spanning between -195 bp and -11 bp in the c-myc promoter. The sequences of their probes are as follows: probe 1, 5’-ACTGGACOGCGGGCTGGAGGCTCCTCCTCCTCTTTTC-3’; probe 2, 5’-CTCCTCTTTTCGGGGCTCCACTAGCCCTCCTC-3’; probe 3, 5’-GCCCGCTCAGGTCGGCTGAAAGGAGGAGGAGGAGGC-3’; probe 4, 5’-AGGGCGGGGAAACGAGAGGAAGGAAAAATAGAG-3’; probe 5, 5’-AAAAATAGAGAGAGTGGGGAAGGGAGAAAGAGAG-3’; probe 6, 5’-AGAAAGAGAGGTTCTCTGGCTAAAACTCCCACC-3’; probe 7, 5’-CCCGCCACCACCCGCCTTATATCCGGGGGTCTGC-3’. (B) Nuclear extracts were isolated from 293 cells transfected with Flag-RBP-VP16 or a mock vector and subjected to EMSA. In competition assays, a 1000-fold molar excess of unlabeled wild-type (WT) or mutant (MT) competitor oligonucleotide was added to the binding mixture. (C) 293T cells were transfected with the indicated reporter gene together with Flag-RBP-VP16 as an effector gene. After 12h, the cells were washed and cultured without serum for 24h, and then subjected to luciferase assays. The results are shown as the mean ± SD of triplicate cultures.
Figure 1.

A

```
No. of Viable Cells (× 10^3 µL)

<table>
<thead>
<tr>
<th></th>
<th>SCF</th>
<th>SCF + FL</th>
<th>SCF + FL + IL-6</th>
</tr>
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<tbody>
<tr>
<td>Days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>14</td>
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- N/E R T 4-HT (-)
- N/E R T 4-HT (+)
```

B

```
DNA content

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<tr>
<th></th>
<th>SCF</th>
<th>SCF + FL + IL-6</th>
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</thead>
<tbody>
<tr>
<td>N/E R T 4-HT (-)</td>
<td>11.6%</td>
<td>12.4%</td>
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<tr>
<td>N/E R T 4-HT (+)</td>
<td>22.2%</td>
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</table>

- Mock
- HO X B 4
```
Figure 2.

<table>
<thead>
<tr>
<th>No. of PCR cycle</th>
<th>N/EER 4HT(-)</th>
<th>N/EER 4HT(+)</th>
<th>Mock</th>
<th>HO XB4</th>
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<tbody>
<tr>
<td>c-myc</td>
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<td></td>
</tr>
<tr>
<td>cyclin D1</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>cyclin D2</td>
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<tr>
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</tr>
<tr>
<td>E2F1</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>β-actin</td>
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</tr>
</tbody>
</table>
Figure 3.

A  Myc/ERT

<table>
<thead>
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<th>SCF</th>
<th>SCF + FL</th>
<th>SCF + FL + IL6</th>
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</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>1.4</td>
<td>1.6</td>
<td>1.8</td>
</tr>
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</table>

Days: 0, 7, 14, 21

A 4-HT(-)  A 4-HT(+)

B  Myc/ERT

<table>
<thead>
<tr>
<th>4-HT</th>
<th>SCF</th>
<th>SCF + FL</th>
<th>SCF + FL + IL6</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)</td>
<td>11.7%</td>
<td>11.9%</td>
<td>12.2%</td>
</tr>
<tr>
<td>(+)</td>
<td>49.3%</td>
<td>52.2%</td>
<td>53.2%</td>
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</table>

Relative Cell No.

DNA content

C  Myc/ERT

<table>
<thead>
<tr>
<th>No. of PCR cycle</th>
<th>4HT(-)</th>
<th>4HT(+)</th>
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<tbody>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

expression of genes:
cyclin D1
cyclin D2
cyclin D3
cyclin E
E2F1
β-actin
Figure 4.

A

Before the culture with 4-HT

SCF + FL
IL-3 + IL-6

2.7% e-Kit
20.0% Sca-1
3.5% Lineage
64.3% CD34

After the 28-day culture with 4-HT

SCF

34.3% e-Kit
21.0% Sca-1
24.5% CD34

SCF + FL + IL-6

10.4% e-Kit
15.7% Sca-1
32.0% CD34

B

MycERT

No. of Colonies

0 10 20

4-HT (-) (+)

p<0.05

p<0.01

CFU-Mix
BPU-E
CFU-GM

C

MycERT 4HT (-) 70°C 10 min
Negative control

HeLa

D

after 4 weeks

4HT (+)

LY5.2

87.8% 0.7%

0.2% 11.5%

4HT (-)

LY5.2

98.5% 0.5%

0.5% 6.1%

after 12 weeks

4HT (+)

LY5.2

91.4% 0.4%

0.3% 1.2%

4HT (+)

LY5.2

22.1% 1.2%

1.8% 5.1%

4HT (+)

MAC11/3-1

69.9% 0.4%

68.5% (52.9%)

CD48

51.8% 0.4%

4HT (+)

51.8% 3.9%

4HT (+)

51.8% 3.9%
Figure 5.

A

B

-1137/+516-Luc  +141/+516-Luc

<table>
<thead>
<tr>
<th>Luciferase</th>
<th>Fold Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
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</tr>
<tr>
<td>Notch IC</td>
<td>9.4</td>
</tr>
<tr>
<td>RBP-VP16</td>
<td>8.7</td>
</tr>
<tr>
<td>HOXB4</td>
<td>3.4</td>
</tr>
</tbody>
</table>

-1137 to -699
-195 to +516
+24 to +141
+516

-1137 to -699
-195 to +516
+24 to +516
+141 to +516

-699 to -195
-195 to +24
+24 to +141
+141 to +516

-195 to -31
-31 to JunB-MP

16.1
Figure 6.

A

B

probe RBP-VP16

1 2 3 4 5 6 7

(+) (+) (+) (+) (+) (+) (+)

C

probe 1 probe 2-5

-195-161 -160-71 -70-11

Luciferase Fold Induction

- 4.1

- 1.5

- 1.4

- 1.3

JunB-My

Roles for c-Myc in self-renewal of hematopoietic stem cells
Yusuke Satoh, Itaru Matsumura, Hirokazu Tanaka, Sachiko Ezoe, Hiroyuki Sugahara, Masao Mizuki, Hirohiko Shibayama, Eri Ishiko, Jun Ishiko, Koichi Nakajima and Yuzuru Kanakura

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