

NF- κ B activates multiple hematopoietic stem cell (HSC) regulatory genes and promotes HSC self-renewal

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Hematopoietic stem cell (HSC) self-renewal and differentiation are influenced through multiple pathways, including homeobox transcription factors, signaling through β -catenin and Notch-1, telomerase, and p27. How these multiple pathways interact and are orchestrated is currently unknown. We now report that NF- κ B, the regulatory and DNA-binding subunit of the trimeric transcription factor NF- κ B, plays a central, integrating role in several of these HSC pathways. NF- κ B is preferentially expressed in HSC-enriched bone marrow subpopulations, and NF- κ B mRNA rapidly declines with HSC differentiation. Overexpression of NF- κ B in primitive hematopoietic cells activates the transcription of multiple HOXB4 paralogs, as well as Notch-1, LEF-1, and telomerase RNA. HSCs overexpressing NF- κ B are biased toward primitive hematopoiesis *in vitro* and show strikingly increased *in vivo* repopulating abilities after single or sequential bone marrow transplantation. Thus, NF- κ B is a potent cellular regulator of HSC self-renewal.

HSCs produce myeloid and lymphoid precursors throughout the lifetime of the organism. To sustain hematopoiesis over months and years, hematopoietic stem cells (HSCs) have the unique ability to balance committed differentiation with HSC self-renewal. The self-renewal process, in which HSCs generate one or two HSC daughter cells per division, is thus central for maintaining HSC pool size throughout life. At the level of the HSCs themselves, several genetic regulatory programs have been identified to play important roles in self-renewal decisions, including HOXB4, Notch1, Bmi-1, and the β -catenin pathway (1–4). Determining whether, and how, these subprograms are coordinated at the molecular level is a central issue in understanding HSC biology. How these diverse intracellular biochemical pathways are integrated remains largely obscure (5).

The Homeobox gene family member HOXB4 was the first transcription factor demonstrated to promote the expansion of HSCs both *in vivo* and *in vitro* while still retaining the HSCs' ability to differentiate into normal mature lymphoid and myeloid cells (1). Recently, we demonstrated that the trimeric transcription factor NF- κ B activates the HOXB4 promoter in cooperation with upstream/ubiquitous stimulating factor 1 and 2 (USF1/2) in normal and malignant hematopoietic cells (6, 7). Studies from other laboratories suggested that NF- κ B is also a potent inducer for numerous other genes whose function likely influences stem cell (SC) function, including HOXB7, JunB, p27, CDK1, MDR1, and TGF β R-II (8–13). Such a broad role for NF- κ B in early tissue development was also intimated by studies in *Drosophila* in which a homologue of NF- κ B was shown to regulate dorsal–ventral patterning (14). Thus, we reasoned that NF- κ B might act as a master gene among the network regulating HSC self-renewal and differentiation.

Materials and Methods

Mice. Female and male B6.SJL-Ptprc^aPep3^b/BoyJ (SJL, CD45.1⁺) and C57BL/6J (B6, CD45.2⁺) mice at 8–16 weeks of age were used as donors/primary recipients and secondary recipients for the bone marrow transplantation (BMT) experiments. The mice were pur-

chased from The Jackson Laboratory and maintained in the animal facilities of the University of Pennsylvania with sterile water or with the water supplemented with neomycin sulfate and polymyxin B (Sigma) within 3–4 weeks after BMT.

NF- κ B cDNA, MigR1 Retroviral Vector, and Preparation of Retroviruses. cDNA for NF- κ B was amplified from human normal bone marrow (BM) cell RNA by using Pfu DNA polymerase (Stratagene) (7). MigR1 retroviral vector was obtained from Warren Pear (Department of Pathology and Laboratory Medicine, University of Pennsylvania). NF- κ B cDNA was directionally cloned into MigR1 vector by EcoRI and XhoI sites to form plasmid MigR1-NF- κ B. For the preparation of retroviruses, the ecotropic packaging cells EcoPack2–293 (Clontech) were transiently transfected by MigR1 or MigR1-NF- κ B plasmid with Superfect (Qiagen). Between 48 and 72 h after transfection, the retroviral particle-containing supernatants were collected and filtered through 0.45- μ m filters (Millipore) stored at -80°C before use.

Primitive BM Cell Isolation, Retroviral Transfection, and Primary BM Transplantation. Primitive mouse BM cells were isolated either by phenotype-sorting of Lin^{-lo} Sca-1⁺ c-Kit⁺⁺ (LSK) BM cells or by pretreating the donor mice with 5-fluorouracil (5-FU, Amersham Pharmacia and Upjohn) at a concentration of 150 mg/kg for 4 days before BM cell harvesting. Then, 2×10^6 5-FU-treated BM cells per ml were first preincubated in 15% FBS/DMEM (GIBCO/BRL) supplemented with 6 ng of IL-3, 10 ng of IL-6, and 100 ng of stem cell factor (SCF) per ml (R & D Systems) overnight. One milliliter of viral supernatant ($\approx 1 \times 10^6$ viruses per ml) was added to these cytokine-stimulated cells the next morning, along with 4 μ g/ml Polybrene (Sigma), before the spin infection was done as described by Pui (15). The infection was repeated once 24 h later. Six to 8 h after the second infection, the nonadherent cells were harvested and washed in PBS once. Then, 2×10^5 live nonadherent cells were injected into the tail vein of one syngeneic recipient that had received a split 10-Gy whole-body γ -ray irradiation. Six to 12 weeks after primary BMT, tissue samples were collected from the recipient mice and analyzed.

Competitive Long-Term Lymphomyeloid Repopulation Assay. For secondary BMT, pooled BM samples from vector or NF- κ B primary recipients were sorted out into pure transduced cells by GFP-activated cell sorting. Then, the vector- or NF- κ B-transduced cells were prepared into three or four dosage levels by serial dilutions with PBS. Next, each dosage of GFP⁺ cells was mixed with a fixed amount of compromised (via BMT) competing B6 BM cells

Abbreviations: BM, bone marrow; BMT, bone marrow transplantation; ChIP, chromatin immunoprecipitation; CRU, competitive repopulating units; HSC, hematopoietic stem cell; SC, stem cell; SCF, stem cell factor.

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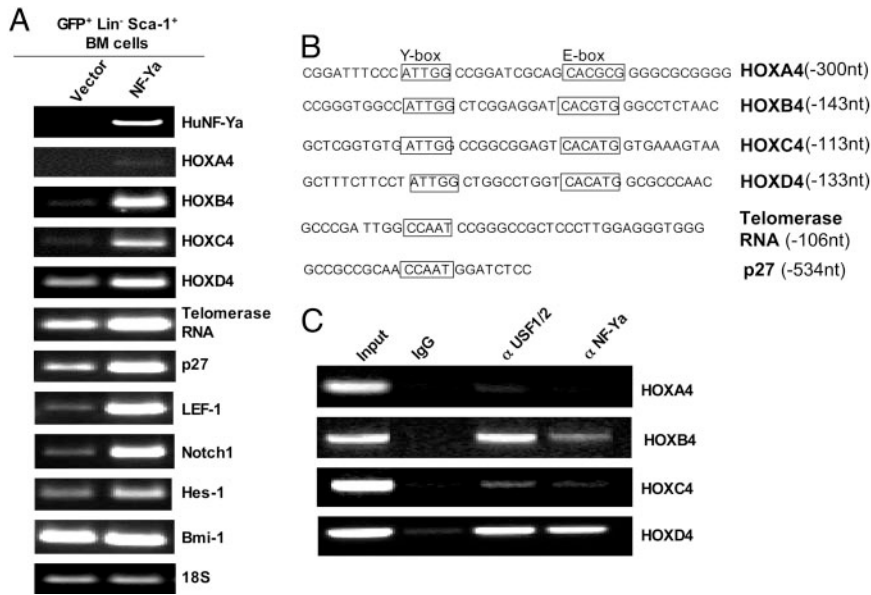


Fig. 1. NF-Ya overexpression induces the expression of HOX and other HSC regulatory genes in primitive hematopoietic compartment. (A) GFP⁺Lin⁻Sca-1⁺ cells were isolated by FACS from vector- or NF-Ya-transduced BM cells 10 weeks after primary transplantation. RNA was extracted from the same amount of vector- or NF-Ya-transduced cells (1×10^5), and mRNA for the indicated genes was measured by RT-PCR reactions. (B) Four promoter regions of murine HOXA4 paralogs are aligned via the conserved Y-box-E box structure. The CCAAT box-containing sequences identified within the promoters of p27 and telomerase RNA gene are shown at the bottom (10, 26). The numbers in bracket indicate the distances of shown sequences from the presumed transcription start site. (C) The cobinding of NF-Y and USF1/2 to the endogenous HOXB4, HOXD4, and HOXC4 promoters within leukemia cell line K562, as detected by ChIP assay.

before being infused into the 10-Gy γ -ray-irradiated B6 mice (CD45.2⁺). The secondary recipients were killed 12–16 weeks later for collection of hematopoietic tissues.

Flow Cytometry. Freshly harvested hematopoietic tissue cells were suspended in 1% FCS/PBS (GIBCO/BRL), with or without 0.1% NaN₃ (Sigma). The nucleated cells were then stained by fluorescence-labeled antibodies (Pharmingen). The flow cytometry data were collected by using a FACScan or FACSCalibur machine (Becton Dickinson) and analyzed by using FLOWJO or CELLQUEST software.

Western Blotting. Nuclear extracts were prepared as described in ref. 7. Ten micrograms of lysate was loaded onto each lane of SDS/12% PAGE for separation and then blotted to Immobilon-P transfer membranes (Millipore). The membrane was sequentially stained with a rabbit polyclonal anti-NF-Ya IgG (Rockland, Gilbertsville, PA), secondary antibodies (Santa Cruz Biotechnology), and ECL reagents (Amersham Pharmacia Biosciences) before the signals were developed on X-OMAT film (Kodak).

RT-PCR. Total RNAs from 1×10^5 phenotypically sorted primary HSCs and their progeny cells, or 1×10^6 purified GFP⁺ cells sorted out of pooled BM samples from recipients, were isolated by using TRIZOL (Invitrogen) and quantified by using a DU640B spectrophotometer (Beckman Coulter). Fifty nanograms of RNA was used as the template in each PCR reaction to measure the expression levels of NF-Y and its target genes with a one-step RT-PCR kit (Qiagen), as evaluated by at least three different cycle numbers. The primers used were as follows. Mouse *NF-Ya* primers: sense 5'-TGGAGCCTCTGATTGGGTTTCG-3' and antisense 5'-TCCTCCTGAGTTGACCATTCC-3'; *NF-Yb* primers: sense 5'-AGCAAGCGAAAGGTGTCATCAG-3' and antisense 5'-TGAATGCCTCTCCGTGAGC-3'; *NF-Yc* primers: sense 5'-CCACACAGAGGATAACAAGCGTC-3' and antisense 5'-TGATACAGGCTGGGCTAATCGG-3'; *HOXB4* primers: sense 5'-GGAGTTTCACTACAACCGCTACCTG-3' and antisense 5'-CTACCCCTTCTCTGTGTTTATTC-3'; *JunB* primers: sense 5'-AAACTCTGAAACACCTTGG-3' and antisense 5'-ACAAAACCGTCCGCAAAGCC-3'; *p27* primers: sense 5'-CTCTGCTCCATTTGACTGTCTGTG-3' and antisense 5'-CCAGGGGCTTATGATTCTGAAAG-3'; human *NF-Ya* primers: sense 5'-GGCACCATTCTCCAGCAAGTTAC-3' and

antisense 5'-AAATCGTCCACCTTCACCACG-3'; mouse *HOXA4* primers: sense 5'-CCCAAGTTCCTCCTTTCG-3' and antisense 5'-ATTCCTTCTCCAGTTCCAAGAC-3'; *HOXC4* primers: sense 5'-GCCAGCAAGCAACCCATAGTCTAC-3' and antisense 5'-TCGGTGGTCTTCTTCCATTTC-3'; *HOXD4* primers: 5'-GCCTACACCAGACAGCAAGTCC-3' and antisense 5'-AAGGTCGTCAGGTCCGTATGG-3'; *LEF-1* primers: sense 5'-GCCGACATCAAGTCATCTTTGG-3' and antisense 5'-GGGTAGAAGGTGGGGATTTTCAG-3'; *Bmi-1* primers: sense 5'-AATGTGTGCTCTGTGTGGAGGG-3' and antisense 5'-TTGCTGTGGGCATCGTAAG-3'; telomerase RNA primers: sense 5'-GCTGTGGGTTCTGGTCTTTTGTTC-3' and 5'-CGTTTGTTTTTGAGGCTCGGG-3'.

In Vitro LSK Cell Culture and CFC Assay. The LSK BM cells were infected *in vitro* by MigR1 or MigR1-NF-Ya retroviruses by following the protocol of Varnum-Finney *et al.* (16). Then, 1×10^3 transduced cells were plated into 1 ml of 0.9% methylcellulose culture medium (StemCell Technologies, Vancouver) with 50 ng/ml SCF and 10 ng/ml IL-3 and IL-6 in triplicate (17). After 7–8 days of cultivation at 37°C, the primary colonies were counted (>25 cells per colony). The cells retrieved from sample primary and secondary colonies were stained with Hema 3 (Fisher Scientific) for morphology examination.

Cell-Cycling Analysis and Annexin V Staining. The cell-cycle analysis was performed by first staining the sorted GFP⁺-transduced cells with Sca-1-APC and Lin-PerCP, then fixing by 70% ethanol treatment and staining with 1 mg/ml propidium iodide plus 100 units/ml RNase A (Sigma) for 1 h. For Annexin V-Cy5 (Pharmingen) staining, the sorted GFP⁺ cells were costained with Sca-1-APC and Lin-PE. The data were collected on the FACSCalibur machine.

Chromatin Immunoprecipitation (ChIP) Assay. The ChIP analysis of HOXA4 paralog promoters was performed as described in ref. 7. The primers were as follows. HOXA4 promoter: sense 5'-GGACAGCAGCATCCATCACG-3' and antisense 5'-AGACGCCGCCACAAAGTTC-3'; HOXC4 promoter: sense 5'-TTGTCTGCTGCGGGATGTGC-3' and antisense 5'-TCTGCTCATAAA-GCCCTCTAC-3'; and HOXD4 promoter: sense 5'-GGGAT-TACCTGAGGGGAATG-3' and antisense 5'-CTCGTGTGTT-GTCTTTTTTCTCCTTC-3'.

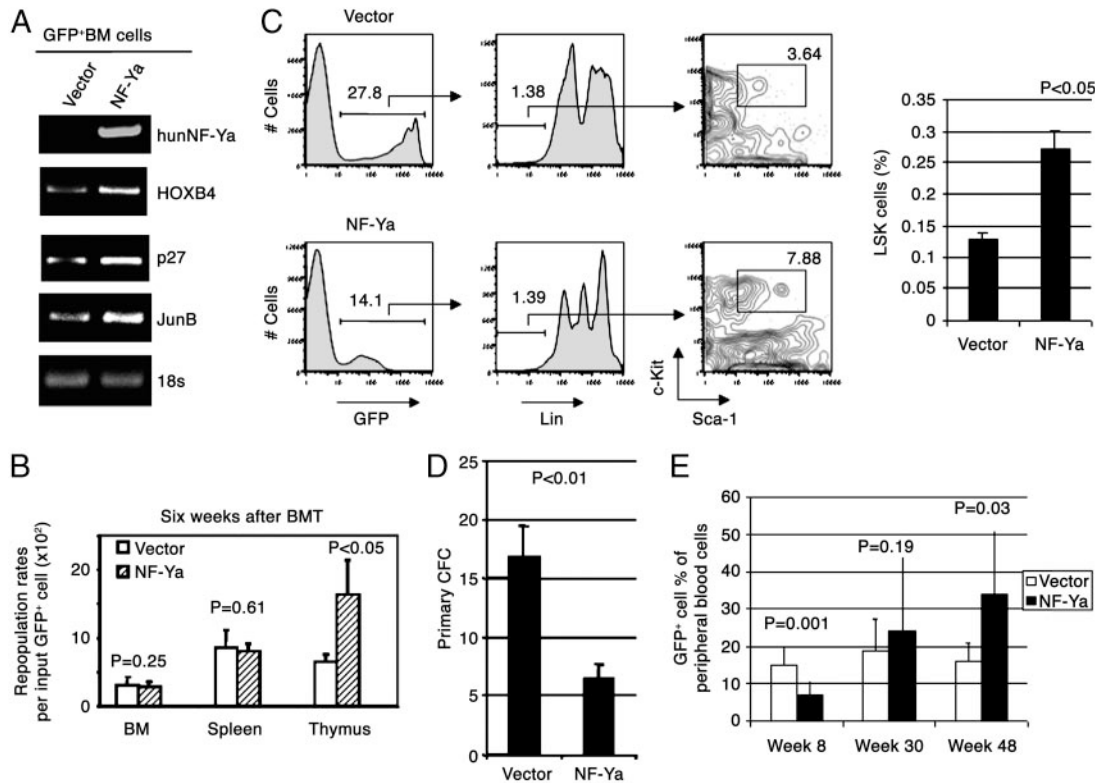


Fig. 2. Effect of NF-Ya overexpression on primary hematopoietic reconstitution. (A) The sustained expression of transduced NF-Ya cDNA as well as NF-Y target genes within the transduced BM cells. (B) Regenerated cellularity per infused vector- or NF-Ya-transduced cells into BM, spleen, and thymus in recipients. (C) BM cells were collected from the primary recipients 10 weeks after transplantation and simultaneously stained with a phycoerythrin-labeled lineage antibody mixture (Mac-1, Gr-1, Ter119, B220, and CD3), PerCP-labeled Sca-1 antibody, and allophycocyanin-labeled c-Kit antibody. The transduced cell compartments were selected by GFP⁺ signal, from which the gated lineage^{-/lo} cells were further plotted against Sca-1 and c-Kit signal intensities. The percentages of LSK cells within the vector- or NF-Ya-transduced BM cells are expressed as the mean \pm SD ($n = 4$) (Right). (D) GFP⁺ cells were sorted from the pooled BM cell samples at 10 weeks post-BMT ($n = 4$). Shown is the average day-7 colony-forming unit production per 1×10^3 inoculated GFP⁺ BM cells (\pm SD), in the presence of 50 ng/ml SCF and 10 ng/ml IL-3 and IL-6. (E) The GFP⁺ cell percentages within the peripheral white blood cells of recipients ($n = 5$), measured at 8, 30, and 48 weeks after primary transplantation of vector (open bars) or NF-Ya (filled bars) retrovirus-infected BM cells. The data shown derive from five experiments with similar results.

Results

Enhanced NF-Ya Activity Induces the Expression of Multiple HOX4 Paralogs as Well as Other HSC Regulatory Genes *in Vivo*.

Functional NF-Y levels are regulated by alterations in the cellular concentration of its NF-Ya subunit, with NF-Yb and NF-Yc being constitutively expressed. The *NF-Ya* gene encodes two alternatively spliced transcripts, with the longer mRNA producing an isoform containing 29 additional amino acids (18). We have recently found that the shorter transcript of NF-Ya is highly expressed in immature c-kit⁺ BM cells, but its concentration declines rapidly with hematopoietic differentiation, resulting in a >50-fold loss of NF-Ya occupancy on the *HOXB4* promoter with terminal myelopoiesis (7). To determine whether NF-Ya might have a broader role in HSC biology beyond that of regulating *HOXB4* expression, we used retroviral infection with the MSCV-vector MigR1 encoding the short-form NF-Ya cDNA to overexpress NF-Ya protein \approx 3-fold in mouse primitive hematopoietic cells (15). As predicted from our recent studies (6, 7), NF-Ya overexpression induced the expression of *HOXB4* in HSCs-enriched Lin⁻Sca-1⁺ population *in vivo* (Fig. 1A). In parallel, NF-Ya overexpression increased the expression of the *HOX4* paralogs *HOXC4* and *HOXD4*, and to a lesser extent *HOXA4* (Fig. 1A). We previously found that NF-Y cooperates with USF1/2 to activate *HOXB4* by interacting biochemically with USF1/2 on their juxtaposed DNA-binding sites, an inverted CCAAT box (Y box), and an E-box (7). This same Y-box-E-box structure, with 10 bp separating the DNA-binding sites, is conserved in the promoters of *HOXC4* and *HOXD4* in both human and

mouse, but their separation is increased 1 bp in the *HOXA4* promoter (Fig. 1B). Thus, the ability for NF-Y and USF1/2 to interact and costabilize their binding to DNA may identify a class of genes being regulated by their precise contextual interaction and activation. Indeed, we confirmed that NF-Ya and USF1/2 occupy the endogenous *HOXD4* and *HOXC4* promoters as measured by ChIP assay in leukemia K562 cells (Fig. 1C), as previously demonstrated for the *HOXB4* promoter (7). In addition to the *HOX4* genes, NF-Ya overexpression in HSCs/early progenitor-enriched compartment also induced the expression of several additional genes believed to influence HSC self-renewal and differentiation, including *p27* (19), telomerase RNA (20), β -catenin partner *LEF-1* (2), and *Notch1* and Notch1 target gene *Hes-1* (4).

NF-Ya Transduction Selectively Expands HSC-Enriched LSK Cells *in Vivo*.

Because overexpression of NF-Ya in HSCs up-regulated the expression of several genes that have been implicated in HSC proliferation, we asked whether NF-Ya overexpression affected the proliferative capacity of HSCs *in vivo*, as measured after SC transplantation. To test this, enriched mouse HSCs were infected with MigR1-NF-Ya or control MigR1 retroviruses and transplanted into lethally irradiated syngeneic mice without preselection. Eight weeks after BMT, persistent expression of the transduced NF-Ya was confirmed in GFP⁺ BM cells but not in vector-transduced cells. The mRNA levels of three NF-Ya target genes (*HOXB4*, *p27*, and *JunB*) were also measured and confirmed to be up-regulated in these cells as well (Fig. 2A).

The regeneration kinetics of GFP⁺ cells contributing to recov-

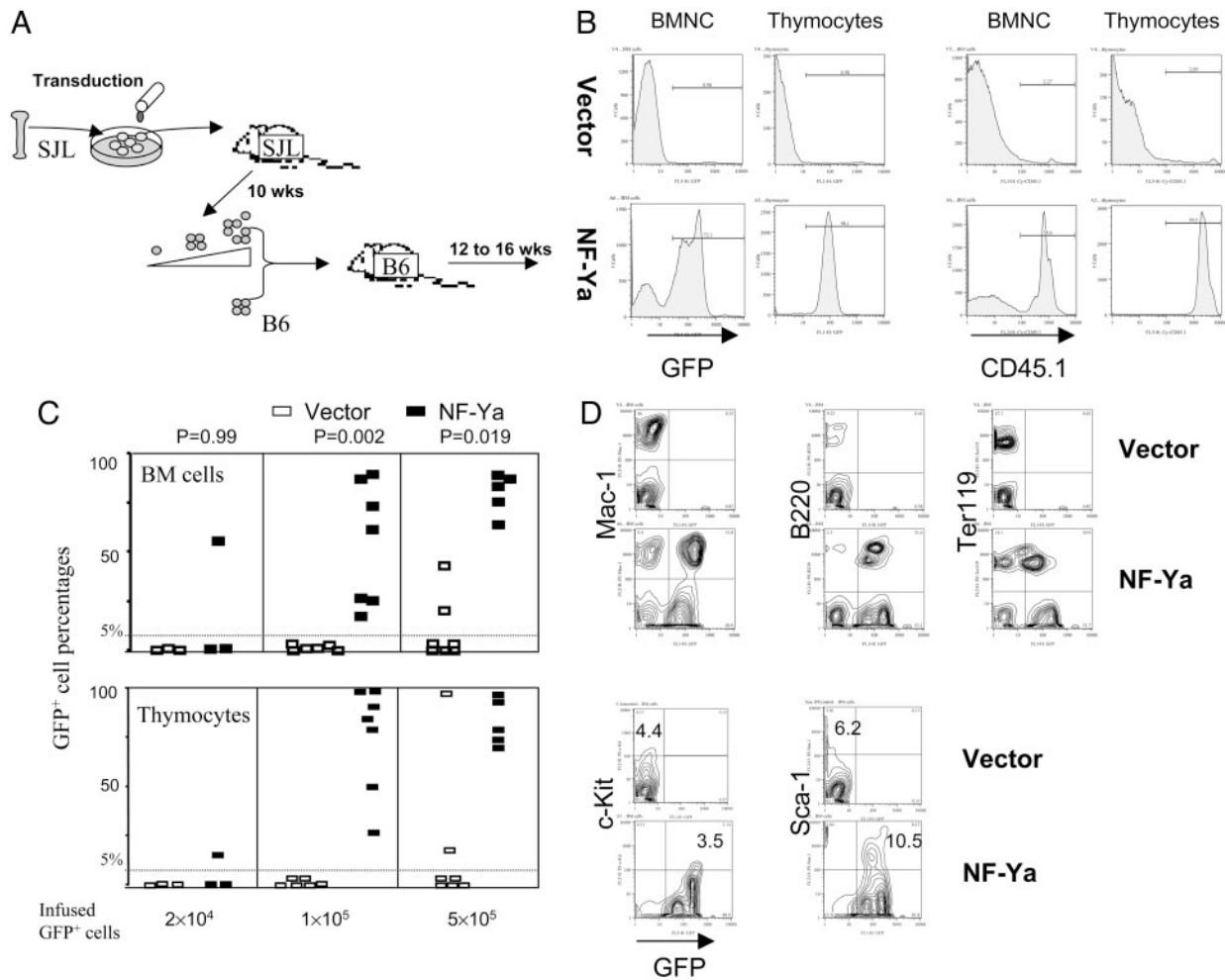


Fig. 3. Enhancement of HSC self-renewal by NF-Ya overexpression. (A) The Diagram for CRU measurement experiment. (B) 1×10^5 vector- or NF-Ya-GFP⁺/CD45.1⁺ cells, sorted from pooled BM ($n = 4$), were mixed with 2×10^5 CD45.2⁺ BM cells before being infused into lethally irradiated CD45.2⁺ recipients. The relative contribution of the vector- or NF-Ya-transduced cells to the regeneration of BMMCs and thymocytes of recipients was evaluated by the percentage of GFP⁺ and CD45.1⁺ cells in the BM or thymus at week 12 ($n = 4$). The diagrams show a representative sample of flow cytometry analysis. (C) Data summary of two CRU experiments about the relative contributions of the vector-transduced (□) or NF-Ya-transduced (■) cells to BMNC or thymocyte regeneration in individual recipients at 16 weeks post-BMT. (D) The regeneration of myeloid (Mac-1), B lymphoid (B220), and erythroid (Ter119) BM cell in the secondary recipient at 16 weeks after receiving 1×10^5 NF-Ya-transduced cells. Regeneration of c-Kit⁺ or Sca-1⁺ BM cells is shown in Lower. The numbers in up quadrants show the percentages of c-Kit⁺ or Sca-1⁺ cells within GFP⁻ and GFP⁺ subpopulations.

er BM and spleen at 6 and 10 weeks were similar in mice transplanted with NF-Ya- and vector-transduced cells, whereas NF-Ya-transduced HSCs contributed to higher levels of thymocyte reconstitution (Fig. 2B). Although overall regeneration of NF-Ya-overexpressing BM cells was similar to the controls, the ratio of very primitive hematopoietic compartment to the less immature cells was perturbed. The percentage of primitive LSK cells comprising GFP⁺ BM at 10 weeks after BMT in the mice receiving NF-Ya-transduced BM was over twice that detected in mice transplanted with vector-transduced cells (Fig. 2C). In contrast, the percentage of mature Mac-1⁺/Gr-1⁺ myeloid cells was found to be identical between NF-Ya- and vector-transduced cells (data not shown). The ability of myeloid progenitors to differentiate into colonies of differentiated cells in semisolid medium was also assayed from GFP⁺ BM cells purified by FACS. As shown in Fig. 2D, the NF-Ya-transduced BM cells yielded approximately one-half the number of differentiated colonies as did vector-transduced cells, suggesting that NF-Ya overexpression partially inhibited myeloid differentiation.

Although colony-forming unit assays performed early on after BMT showed decreased numbers of NF-Ya-transduced progeni-

tors, the percentage of GFP⁺ blood cells deriving from NF-Ya-transduced HSCs rose over several weeks to months, surpassing the contribution from GFP⁺ cells in empty vector-transduced BMT recipients by week 30 and week 48 (Fig. 2E). These results raised the possibility that overexpression of NF-Ya might cause expansion of the primitive HSC pool, resulting in a gradually increased contribution of their progenies to all differentiated compartments after BMT.

NF-Ya Overexpression Promotes the Self-Renewal of the Competitive Repopulating Units (CRU) *in Vivo*. To test directly whether NF-Ya might specifically induce primitive HSC expansion, we measured the ability of NF-Ya-transduced BM cells 10 weeks post-BMT to repopulate secondary BMT recipients. In this CRU assay, GFP⁺ cells were isolated by FACS from primary recipients of NF-Ya- or control vector-transduced BM (CD45.1), and varying doses were mixed with a constant number of nontransduced competitive BM cells (CD45.2) and infused into lethally irradiated congenic mice (CD45.2) (Fig. 3A). After 12–16 weeks, the contributions of vector- or NF-Ya-transduced cells to the cellularities of BM nucleated cells or thymocytes (CD4⁺ and/or CD8⁺) were measured by both GFP

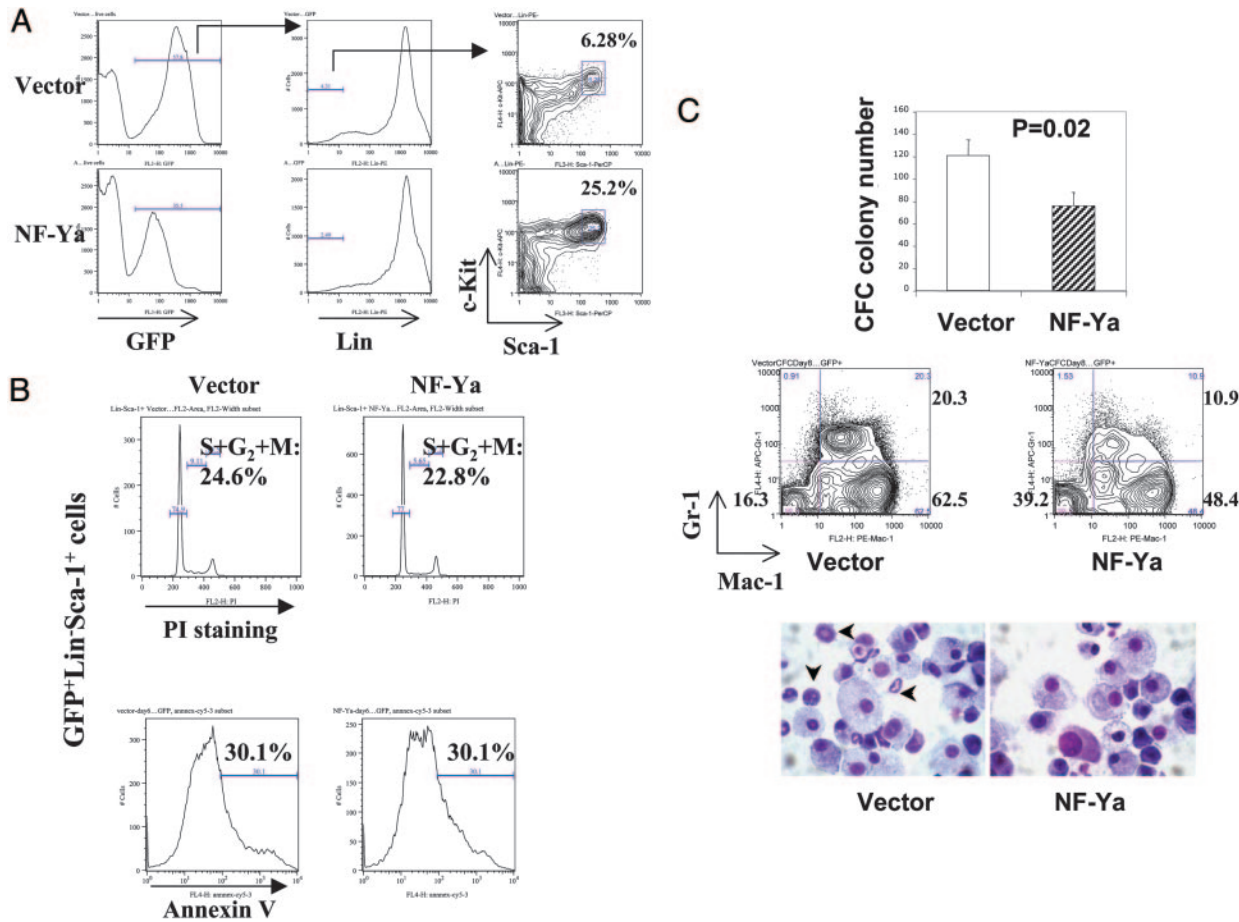


Fig. 4. Phenotype and proliferative potential of LSK cells overexpressing NF-Ya *in vitro*. LSK BMMCs were infected with MigR1-NF-Ya/GFP or with MigR1/GFP control and placed into liquid culture in 100 ng/ml SCF, 10 ng/ml IL-6, 6 ng/ml IL-3, and 50 ng/ml Tpo, in methylcellulose colony assays in the presence of 50 ng/ml SCF and 10 ng/ml IL-6 and IL-3, or in G-CSF (10 ng/ml) or M-CSF (10 ng/ml). (A) Flow cytograms of GFP⁺ cells recovered after 7 days of liquid culture and stained with Lin, c-Kit, and Sca-1 antibodies. (B) Cell-cycling and apoptosis status of Lin⁻Sca-1⁺ cells. (C) Methylcellulose colonies recovered per 10⁴ cells plated (Top). Colonies were plucked and analyzed by flow cytometry or stained with Wright–Giemsa (Middle and Bottom). The arrows indicate the mature granulocytes with ring-shaped nucleus.

and CD45.1 expression (Fig. 3B). As shown in Fig. 3C, when 2×10^4 GFP⁺ cells were mixed with 2×10^5 competitive cells, NF-Ya-transduced BM cells contributed substantially to the repopulation of one of three recipients (GFP⁺ cell percentage >5% in both tissues), whereas vector-transduced cells did not. When the number of infused GFP⁺ cells was raised to 1×10^5 cells, the NF-Ya-transduced cells successfully repopulated eight of eight recipients, whereas vector-transduced cells failed in six of six recipients. In fact, vector-transduced cells were able to repopulate in two of seven recipients when the infused cell dosage was raised to 5×10^5 cells. Moreover, NF-Ya-transduced BM cells generated multiple lineages of mature BM cells including myeloid cells (Mac-1⁺), B cells (B220⁺), and erythrocytes (Ter119⁺) as well as immature c-Kit⁺ and Sca-1⁺ cells (Fig. 3D). These data indicated that NF-Ya overexpression in HSCs increased the HSC activity of transduced BM cells.

NF-Ya Overexpression Supports LSK Cell Regeneration *in Vitro*. To test whether the stimulatory effect of NF-Ya could be demonstrated directly on cultured hematopoietic cells, isolated LSK cells were infected with MigR1-NF-Ya retroviruses and incubated for 7 days in SCF, IL-3, IL-6, and Tpo (21). Cell-surface flow cytometric analysis of cultured NF-Ya-infected cells showed a 4-fold higher percentage of LSK cells (Fig. 4A), whereas cell-cycle and apoptosis analysis with propidium iodide/DNA staining and Annexin V

staining of Lin⁻Sca-1⁺ cells (nearly all of which expressed c-Kit⁺ as well) showed no measurable difference from vector-transduced cells (Fig. 4B). *In vitro* colony-forming assays showed a lower density of more mature colony-forming unit cells in NF-Ya-transduced versus MigR1-infected LSK cells (Fig. 4C), suggesting that overexpression of NF-Ya down-modulates myeloid differentiation versus proliferation from early primitive cells. Flow-cytometric cell-surface phenotype analysis of cells recovered from NF-Ya-infected colonies confirmed the retention of more immature hematopoietic cells (Mac-1⁻/Gr-1⁻) (Fig. 4C Middle) and the production of less mature granulocytes (Fig. 4C Bottom). Interestingly, colony-forming unit frequencies from very late, committed progenitor cells induced by M-CSF or G-CSF alone showed no difference from NF-Ya overexpression (data not shown), supporting the notion that the myeloid differentiation-inhibiting effect imposed by NF-Ya overexpression targets the earlier stages of myeloid differentiation.

Discussion

These results demonstrate that the trimeric transcription factor NF-Y activates the transcription of multiple genetic pathways involved in the maintenance and proliferation of HSCs. Although the way in which these gene programs are integrated remains unresolved, themes begin to emerge from the present findings. Within the HSC compartment, not only HOXB4 but also HOXC4 and HOXD4 are induced in response to the stimulating activity of

NF-Ya. As suggested by the conserved Y-box-E-box structure within the promoter regions of these HOX4 genes, and as directly demonstrated by ChIP assays, HOXB4, C4, and D4 are directly and cooperatively targeted via the synergistic action of NF-Y and USF1/2. From a physiologic perspective, the ability of NF-Y to activate multiple HOX4 paralogs might explain the discrepancy between *HOXB4* overexpression and knockout studies. Although *HOXB4* overexpression causes HSC expansion both *in vivo* and *in vitro* (22), *HOXB4* knockout mice show only minor defects in HSCs' lymphomyeloid repopulating ability (23). Given that all four HOX4 paralogs have now been shown to possess similar HSC-stimulatory activities (24, 25), the gene products (themselves NF-Y targets in HSCs) might compensate for the loss of *HOXB4* in the development or repopulation of HSCs (24, 25). Thus, NF-Ya may operate as a master transcription factor that regulates HSC activity through multiple parallel, reinforcing pathways. Direct promoter binding may also underlie the induction of telomerase RNA and p27 as well, given the functional CCAAT boxes identified within their promoters (10, 26), whereas the mechanisms by which NF-Ya induces the expression of Notch1, Hes-1, and LEF-1 may be more indirect.

It is very likely that NF-Ya plays a direct *in vivo* role in the normal up-regulation of HOX genes in HSC *in vivo* (27), via activation of the MAPK and PKA. TPO stimulation of HSCs activates MAPK, which we and others have shown leads to induction of *HOXB4* expression in both normal and K562 cells (6, 27). Although a pivotal role of NF-Ya in TPO-induced *HOXB4* activation is not yet known, MEK activation has itself been shown to up-regulate NF-Ya activity functioning on the promoter of other NF-Ya target genes such as GADD45 (28). In addition, it has recently been learned that NF-Ya's binding affinity for CCAAT sites is directly increased after phosphorylation by CDK2 (29) or by Sonic Hedgehog/PMP-mediated activation of PKA in hematopoietic cells (30). Further studies to determine the specific pathways whereby NF-Ya's activity is induced and regulated in hematopoietic cells will clearly increase our understanding of how cytokine and adhesive niche environmental cues are integrated into transcriptional mechanisms that sustain and amplify HSCs self-renewal *in vivo*.

The ability of NF-Ya to activate multiple HSC gene programs suggests that the mechanisms by which it induces HSC proliferation

are likely to be complex. Of interest, we found no effect of NF-Ya on HSC survival or cell cycling *per se*. Rather, the net effect of NF-Ya overexpression appears to be to shift the balance of HSC proliferation toward HSC self-renewal rather than differentiation. The effect of NF-Ya may be slightly different from that of overexpressing *HOXB4* alone, in that *HOXB4* overexpression has been suggested to stimulate HSCs to both proliferate and differentiate into more hematopoietic progenitors (1, 22). In this respect, the activation of cell-cycle inhibitor p27 by NF-Y may lead to somewhat modulated repopulation of the mature lineages, in agreement with the results showing that p27 is a critical factor restricting the pool size of progenitor cells derived from HSCs (19). Of note, the CRU-amplifying effect of NF-Ya overexpression is greater than its quantitative effect on increasing the number of LSK cells. This may reflect that true repopulating HSCs are only a primitive subset of LSK cells. On the other hand, similar to what has been observed in *HOXB4* overexpression studies, we have not detected any myeloproliferative or frankly leukemic syndromes in mice transplanted with NF-Ya-transduced HSCs, over a 1-year observation. Thus, overexpression of NF-Ya might be used to provide a global, physiologic approach to stimulating HSC expansion for clinical application.

In addition to effects on CRU expansion, HSCs overexpressing NF-Ya also give rise to increased thymocyte repopulation. We did not observe consistent increases in B cell repopulation primary transplant recipients, suggesting that NF-Y may also influence lymphoid differentiation at and beyond the level of the common lymphoid progenitor. Although we do not yet know the precise mechanism for this increased thymocyte production, one possible link is the enhanced expression of Notch1 induced by NF-Y, because signaling through Notch-1 is known to induce hematopoiesis toward T cell differentiation (15).

In summary, our results demonstrate that the activity of the trimeric transcription factor NF-Y, which is preferentially expressed within the HSC pool during hematopoiesis, activates and integrates multiple genetic pathways responsible for the regulated proliferation and differentiation of HSCs. NF-Y is thus an excellent candidate to be a key regulator in the genetic regulation of HSC self-renewal, and its biochemical manipulation may offer the opportunity for SC manipulation to direct proliferation versus differentiation *in vivo* and *in vitro*.

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