

# Suppression of Homeobox Transcription Factor VentX Promotes Expansion of Human Hematopoietic Stem/Multipotent Progenitor Cells

Received for publication, May 17, 2012, and in revised form, July 11, 2012. Published, JBC Papers in Press, July 12, 2012, DOI 10.1074/jbc.M112.383018

Hong Gao<sup>#1</sup>, Xiaoming Wu<sup>#1</sup>, Yan Sun<sup>§</sup>, Shuanhu Zhou<sup>¶</sup>, Leslie E. Silberstein<sup>§</sup>, and Zhenglun Zhu<sup>#2</sup>

From the <sup>#</sup>Gastroenterology Division, Department of Medicine, and <sup>¶</sup>Department of Orthopedic Surgery, Brigham and Women's Hospital and the <sup>§</sup>Department of Pathology, Boston Children's Hospital, Boston, Massachusetts 02115

**Background:** Difficulty in obtaining sufficient amounts of human hematopoietic stem/multipotent progenitor cells (HSC/MPPs) is a major hurdle that limits their application.

**Results:** Down-regulation of VentX, a hematopoietic homeobox transcription factor, allows multilineage expansion of hematopoietic cells *ex vivo* and *in vivo* in SCID/NOD $\gamma$ 2<sup>null</sup> mice.

**Conclusion:** VentX is a novel regulator of human HSC/MPP expansion.

**Significance:** VentX represents a novel target for mechanistic exploration and clinical application of human HSC/MPPs.

Mechanisms that regulate proliferation and expansion of human hematopoietic stem/multipotent progenitor cells (HSC/MPPs) are targets of intensive investigations. Several cell intrinsic factors and signaling pathways have been implicated in the proliferation and differentiation of human HSC/MPPs. Nevertheless, expansion of human HSC/MPPs for clinical application remains a critical challenge. VentX is a human homeobox transcription factor that was recently identified as an anti-proliferation and pro-differentiation factor in human hematopoietic cells. Here, we report that VentX expression is up-regulated during ontogenesis of human hematopoietic cells. Strikingly, suppression of VentX expression led to significant expansion of HSC/MPPs *ex vivo* and a 20-fold increase in engraftment potential in the NOD/SCID/IL2R $\gamma$ 2<sup>null</sup> mouse model. VentX suppression helped preserve the HSC/MPP pools and promote clonogenicity of hematopoietic progenitor cells. Mechanistically, we show that VentX regulates critical cell cycle regulators and Wnt downstream genes previously implicated in HSC/MPP proliferation and expansion.

diseases. The molecular mechanisms underlying HSC/MPP proliferation and differentiation have been explored extensively for effective expansion of human HSC/MPPs, which is required for clinical application of HSC/MPP transplantation-based therapy (1, 2).

Over the past decades, several cell extrinsic signaling pathways and cell intrinsic factors of early embryogenesis have been shown to play important roles in the proliferation and differentiation of HSC/MPPs (3–7). In particular, using mouse models, it has been shown that murine HSCs can be expanded by >100-fold by either enhanced Wnt signaling or overexpression of the homeobox protein HoxB4 (5, 6). In comparison, clinically significant expansion of human HSC/MPPs remained a fundamental challenge. Despite a recent advance in HSC/MPP expansion by cytokine mixtures (8), neither enhanced Wnt signaling nor overexpression of HoxB4 or BMI1 was able to expand human HSC/MPPs by >2–3-fold (9–11). As such, safe and efficacious expansion of human HSC/MPPs remains a bottleneck that limits the clinical application of HSC/MPP-based therapy.

VentX is a unique human homeobox protein that is expressed restrictedly in human hematopoietic cells in a highly regulated manner. Initially cloned as a human homolog of the vertebrate Vent family of homeobox transcription factors, the function of VentX in cell proliferation and differentiation is being appreciated through reverse genetic modeling of the molecular mechanisms underlying dorsoventral axis formation during early vertebrate embryogenesis (12, 13). As an human homolog of the vertebrate Xom/Vent2 homeobox genes, VentX was found to be a novel lymphoid enhancer factor/T-cell factor-associated antagonist of the canonical Wnt signaling and plays an important role in normal and malignant hematopoiesis (14–16). Our recent investigations showed that, in addition to antagonizing canonical Wnt signaling, VentX is also an activator of the p53/p21 and p16<sup>ink4a</sup>/Rb pathways and is able to induce senescence phenotypes in tumor cells (17).

Several lines of evidence suggest the potential involvement of VentX in controlling proliferation and differentiation of HSC/

Hematopoietic stem cells (HSCs)<sup>3</sup> give rise to multipotent progenitor cells (MPPs) and eventually mature cells of all lineages of blood and immune systems. HSC/MPPs play essential role in tissue homeostasis, regeneration, and host defense. HSC/MPP transplantation has shown great promise in the reconstitution of hematopoietic systems and treatment of malignancies. HSC/MPP-based gene therapy has revealed potentials for correcting hereditary metabolic and autoimmune

<sup>1</sup> Both authors contributed equally to this work.

<sup>2</sup> Supported by National Institutes of Health Grant 1R03HD064617, the Department of Defense, the American Cancer Society, and a research fund from Brigham and Women's Hospital. To whom correspondence should be addressed. Tel.: 617-732-5467; Fax: 617-730-5807; E-mail: zzhu@partners.org.

<sup>3</sup> The abbreviations used are: HSC, hematopoietic stem cell; MPP, multipotent progenitor cell; CFC, colony-forming cell; LTCIC, long-term culture-initiating cell; PE, phycoerythrin; BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming unit-erythroid; CFU-GM, colony-forming unit-granulocyte/macrophage; SR1, StemRegenin 1.

## Regulation of HSC Expansion by VentX

MPPs. First, VentX expression is up-regulated during both myeloid and lymphoid ontogenesis and during early hematopoiesis (15, 16, 18). Second, VentX controls signaling pathways and cell cycle regulators that have been previously implicated in the proliferation and differentiation of hematopoietic cells (15–17). Third, VentX has been shown to control differentiation of primary human hematopoietic cells (16, 18).

To further define a potential role of VentX in the proliferation and differentiation of human HSC/MPPs, we examined VentX expression during early hematopoiesis and explored the function of VentX in the expansion of CD34<sup>+</sup> HSC/MPPs through gain-of-function and loss-of-function approaches *ex vivo* and *in vivo* in the NOD/SCID $\gamma$ 2<sup>null</sup> mouse model. We found that VentX inhibition helped preserve the stem cell pool of HSC/MPPs. Strikingly, we observed that knockdown of VentX in bone marrow CD34<sup>+</sup> HSC/MPPs resulted in a 20-fold increase in engraftment potential and multilineage development of hematopoietic cells in the NOD/SCID $\gamma$ 2<sup>null</sup> mouse model. We found that VentX regulates the expression of several cell cycle regulators and Wnt downstream genes implicated in HSC/MPP proliferation and differentiation. Our data therefore reveal VentX as a novel regulator of HSC/MPP proliferation and differentiation and a potential druggable target for clinical application of HSC/MPP-based therapy.

### EXPERIMENTAL PROCEDURES

**CD34<sup>+</sup> Cell Isolation**—Human bone marrow samples were obtained from discarded femoral head tissue after hip replacement surgery at Brigham and Women's Hospital, with Institutional Review Board approval. After Ficoll separation of mononuclear cells, the CD34<sup>+</sup> cells were enriched using a magnetically activated cell sorting CD34<sup>+</sup> progenitor kit (Miltenyi Biotec). The purity of the bone marrow CD34<sup>+</sup> cells was >95% as assessed by flow cytometry. Flow cytometry sorting of different subpopulations of CD34<sup>+</sup> cells (common myeloid progenitors, megakaryocyte/erythroid progenitors, and granulocyte/macrophage progenitors) was as described previously (11).

**Lentiviral Vector Construct and Transduction of Bone Marrow CD34<sup>+</sup> Cells**—The lentiviral vector pHAGE-CMV-eGFPW, which expresses a shRNA targeting VentX, was used for the transduction of bone marrow CD34<sup>+</sup> cells. The lentiviral vector contains an internal ribosome entry site that allows simultaneous expression of GFP monitoring the transduction rate or for cell sorting. The sequence of VentX shRNA was identical to that of the VentX siRNA described previously (15). A non-effective sequence targeting GFP was used as a control. Lentiviral packaging was carried out in the Dana-Farber/Harvard Cancer Center Vector Core Facility, and viral supernatants were stored at –70 °C in aliquots until used. The CD34<sup>+</sup> cells were first cultured in Iscove's modified Dulbecco's medium containing FBS (10%), stem cell factor (100 ng/ml), Flt3 ligand (100 ng/ml), thrombopoietin (100 ng/ml), IL-3 (10 ng/ml), and IL-6 (10 ng/ml) (PeproTech) for 2 days and then transduced with a multiplicity of infection of 5 in the presence of 4  $\mu$ g/ml Polybrene. GFP-positive cells were sorted using a FACS Aria high-speed sorter (BD Biosciences) at 24 h post-transduction at

the Dana-Farber Cancer Institute Flow Cytometry Core Facility and used for all *ex vivo* experiments.

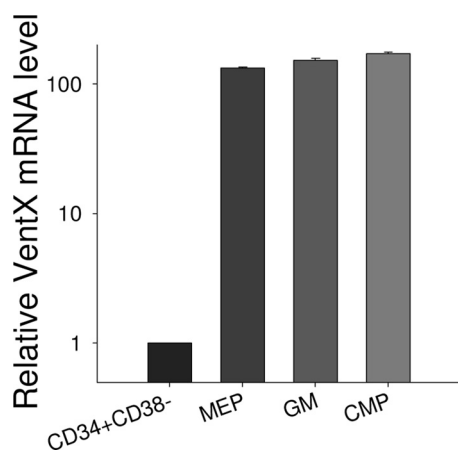
**Overexpression of VentX in Bone Marrow CD34<sup>+</sup> Cells**—Human bone marrow CD34<sup>+</sup> cells were transfected with pCS2-GFP or pCS2-GFP-VentX plasmid using a Nucleofector kit for human CD34<sup>+</sup> cells (Lonza) as described above. GFP-positive cells were sorted using a FACS Aria high-speed sorter at 24 h post-transfection and used for subsequent experiments.

**Colony-forming Cell (CFC) and Long-term Culture-initiating Cell (LTCIC) Assays**—Assays for *in vitro* CFCs were performed by plating sorted GFP-positive CD34<sup>+</sup> cells ( $1 \times 10^4$ /dish) in triplicates in complete methylcellulose medium (MethoCult GF H4434, STEMCELL Technologies Inc.), which contained a mixture of recombinant human cytokines (stem cell factor, IL-3, GM-CSF, and erythropoietin). Colonies were counted after 14 days of incubation at 37 °C and classified according to standard criteria. For the LTCIC assay, transduced GFP-positive cells were sorted on M2-10B4 murine fibroblast cells in limiting dilutions from 30 to 900 cells/well in 96-well plates. Cultures were weekly fed with new medium. After 5 weeks of culture, all cells from the wells were transferred to a 35-mm Petri dish in complete methylcellulose medium as described above. After an additional 2 weeks of culture, wells were scored as positive or negative to yield the LTCIC frequency.

**FACS Analysis**—Phenotypic analysis of the CD34<sup>+</sup> cells and cells of different lineages was performed using flow cytometry after immunolabeling of cells with fluorescent dye-conjugated antibodies. Phycoerythrin (PE)-conjugated anti-human CD34 and CD45 and PE/Cy5-conjugated anti-human CD38 antibodies were obtained from eBioscience. The anti-cyclin D<sub>1</sub> primary antibody was purchased from Cell Signaling for intracellular staining. PE-conjugated rabbit anti-mouse IgG antibody was used for anti-cyclin D<sub>1</sub> secondary antibody staining. Staining of the cells was performed for 30 min on ice. Mouse bone marrow cells were blocked with anti-Fc antibody for 15 min at 4 °C to avoid nonspecific binding. FACS analyses were performed on a FACScan (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Inc.).

**Transplantation of NSG Mice**—8-week-old NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ mice (commonly known as NOD scid gamma, or NSG) were purchased from The Jackson Laboratory and maintained under specific pathogen-free conditions. Before transplantations, mice were sublethally irradiated with 100 rads.  $1 \times 10^6$  CD34<sup>+</sup> cells were transduced with VentX shRNA or GFP shRNA lentivirus. At 24 h post-transduction, all cells ( $1 \times 10^6$ , with ~20% GFP-positive cells) or positively transduced cells ( $2 \times 10^4$ , with all GFP-positive cells) were injected into recipient mice. Human CD45<sup>+</sup> cells from the bone marrow of recipient mice were analyzed. For secondary transplantation, the bone marrow of chimeric primary recipient mice was injected into secondary recipient NSG mice without further purification of human cells.

**RT-PCR**—Total RNA was isolated by the TRIzol method, and the same amount of RNA was used for first-strand cDNA synthesis with a SuperScript first-strand synthesis system (Invitrogen) according to the manufacturer's protocol. The VentX mRNA level was determined as described previously (16). We performed real-time PCR with SYBR Green on a LightCycler®



**FIGURE 1. Up-regulation of VentX expression during hematopoiesis.** The primitive CD34<sup>+</sup>CD38<sup>-</sup> cells and different subpopulations of CD34<sup>+</sup> progenitor cells were sorted as described under "Experimental Procedures," and the mRNA level of VentX was determined by real-time PCR. Results represent one of two independent experiments. *MEP*, megakaryocyte/erythroid progenitors; *GM*, granulocyte/macrophage progenitors; *CMP*, common myeloid progenitors.

480 real-time PCR system (Roche Applied Science). The following primer sets were used for amplification: p21, 5'-AAAC-TTTGGAGTCCCTCAC-3' and 5'-AAAGGCTCAACACT-GAGACG-3'; p16, 5'-CTTCCCCACTACCGTAAAT-3' and 5'-TGCTCACTCCAGAAACTCC-3'; *c-myc*, 5'-CAGCTG-CTTAGACGCTGGATT-3' and 5'-GTAGAAATACGGCTG-CACCGA-3'; and cyclin D<sub>1</sub>, 5'-GTTTCGTGGCCTCTAAG-ATG-3' and 5'-TTGTTACCAGGAGCAGC-3'.

## RESULTS

**VentX Expression Is Regulated during Hematopoiesis—**VentX is a homeobox transcription factor expressed in all lineages of hematopoietic cells. Our previous studies showed that VentX is a lymphoid enhancer factor/T-cell factor-associated Wnt repressor (15). Given the broad implications of Wnt signaling in hematopoiesis, we further explored the potential involvement of VentX in hematopoiesis by defining the expression pattern of VentX during early hematopoietic development. Through FACS analysis of different subpopulations of CD34<sup>+</sup> cells, followed by quantitative PCR analysis, we found that VentX expression was regulated during hematopoiesis (Fig. 1). VentX expression was negligible in primitive CD34<sup>+</sup>CD38<sup>-</sup> cells. Consistent with the findings of Rawat *et al.* (18), VentX expression levels increased significantly in progenitor cells of myeloid lineage (Fig. 1). There was no significant difference in VentX expression among different subgroups of myeloid lineages (Fig. 1).

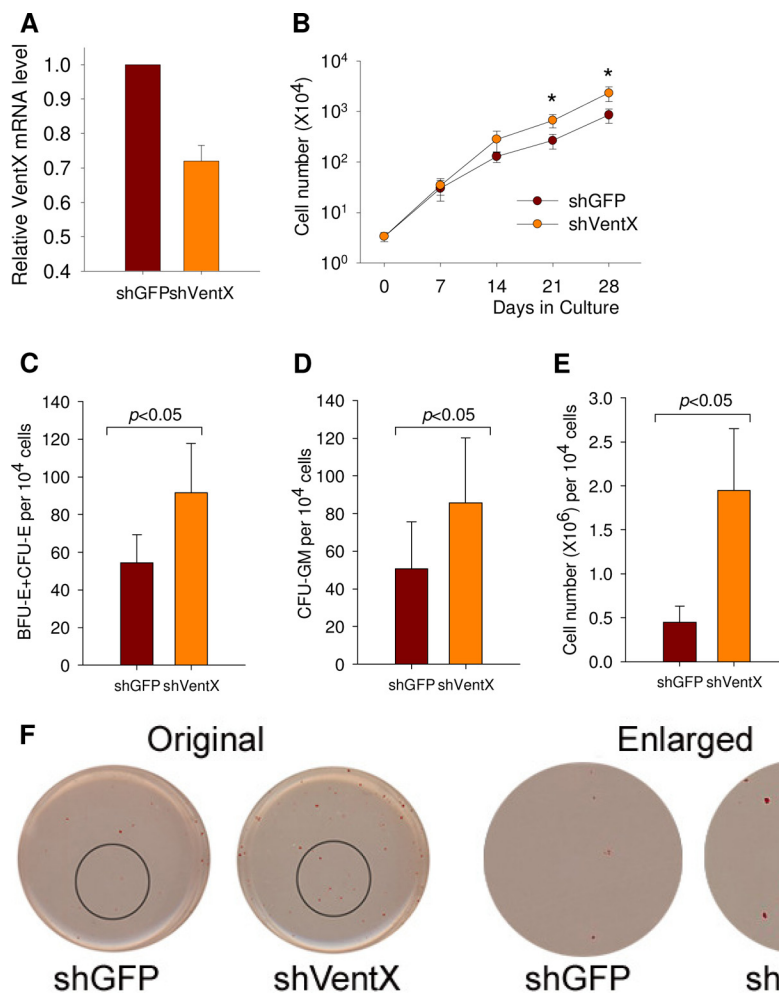
**VentX Regulates Expansion of Human HSCs *ex Vivo*—**Our previous studies have shown that VentX regulates cell proliferation by controlling key pathways involved in the cell cycle (15–17). The finding that VentX expression is up-regulated during hematopoiesis prompted us to explore the potential role of VentX in the regulation of HSC/MPP expansion. To achieve this goal, VentX expression in human bone marrow CD34<sup>+</sup> cells was knocked down with a lentivirus-based shRNA approach. The transduction rates of pHAGE-shGFP (which encodes a non-effective sequence targeting GFP) and pHAGE-

shVentX were ~20%. Using a quantitative PCR approach, the efficiency of VentX knockdown in pHAGE-shVentX-transduced CD34<sup>+</sup> cells was determined to be ~30% (Fig. 2A). Positively transduced cells were plated in cytokine-supplemented stroma-free liquid cultures, and the cell numbers were counted weekly up to 4 weeks. Plotting of weekly cell counts of semi-depopulated cultures showed that knockdown of VentX expression in CD34<sup>+</sup> cells led to a 3–4-fold increase in total cell numbers compared with control cells from three independent experiments (Fig. 2B). To determine the potential effects of VentX on the clonogenicity and lineage development of CD34<sup>+</sup> cells, we performed CFC assays. As shown in Fig. 2 (C–F), knockdown of VentX resulted in an ~2-fold increase in the formation of BFU-E/CFU-E and CFU-GM colonies. The total number of progenitor cells increased by ~3–4-fold in the CFC assays (Fig. 2E). Knockdown of VentX promoted multilineage expansion of hematopoietic cells, especially erythroid lineage colony formation (Fig. 2F). To determine the effects of VentX knockdown on the HSC pools, we examined the proportion of CD34<sup>+</sup> cells in the *ex vivo* liquid culture and found that knockdown of VentX led to a 30% increase in the percentage of CD34<sup>+</sup> cells (Fig. 3, A and B) and an ~2.5-fold increase in the absolute cell numbers (Fig. 3C). A time course FACS analysis revealed that knockdown of VentX helped preserve the CD34<sup>+</sup> cell population for at least 2 weeks (Fig. 3D). In addition, knockdown of VentX also increased the percentage (Fig. 3, E and F) and absolute number (Fig. 3G) of primitive CD34<sup>+</sup>CD38<sup>-</sup> cells. FACS staining showed that VentX knockdown did not change the lineage distribution of the differentiated progenitor cells (Fig. 3H). Furthermore, LTCIC frequency increased by ~3-fold in VentX shRNA-transduced CD34<sup>+</sup> cells (Fig. 3I). These results suggest that VentX knockdown may help maintain the CD34<sup>+</sup> stem cell pools, thereby promoting CD34<sup>+</sup> cell expansion.

**Ectopic Expression of VentX Blocks CD34<sup>+</sup> Cell Proliferation and Expansion—**To further determine the effects of VentX on the expansion of human bone marrow HSC/MPPs, we tested whether ectopic expression of VentX would prevent CD34<sup>+</sup> cell expansion. As shown in Fig. 4 (A–D), the colony formation of CD34<sup>+</sup> cells was largely abrogated by the ectopic expression of VentX, suggesting that VentX is a critical regulator of CD34<sup>+</sup> cell proliferation and expansion. A recent study demonstrated that an aryl hydrocarbon receptor antagonist, StemRegenin 1 (SR1), robustly promoted *ex vivo* expansion of human CD34<sup>+</sup> cells (19). To determine whether VentX plays an essential regulatory role in the expansion of human HSC/MPPs, we explored the effects of SR1 treatment on the expression levels of VentX and the effects of VentX on SR1-induced CD34<sup>+</sup> expansion. SR1 treatment accelerated the proliferation of CD34<sup>+</sup> cells (Fig. 4F, first two bars) and decreased the expression levels of VentX (Fig. 4E). Consistent with the notion that VentX plays an essential regulatory role in CD34<sup>+</sup> expansion, we found that ectopic expression of VentX prevented SR1-induced expansion of CD34<sup>+</sup> cells (Fig. 4F). Our data indicate that down-regulation of VentX is a prerequisite for proliferation and expansion of CD34<sup>+</sup> cells.

**VentX Regulates HSC/MPP Expansion *in Vivo*—**We next sought to determine whether VentX knockdown promotes expansion of HSC/MPPs *in vivo* using the NOD/SCID/

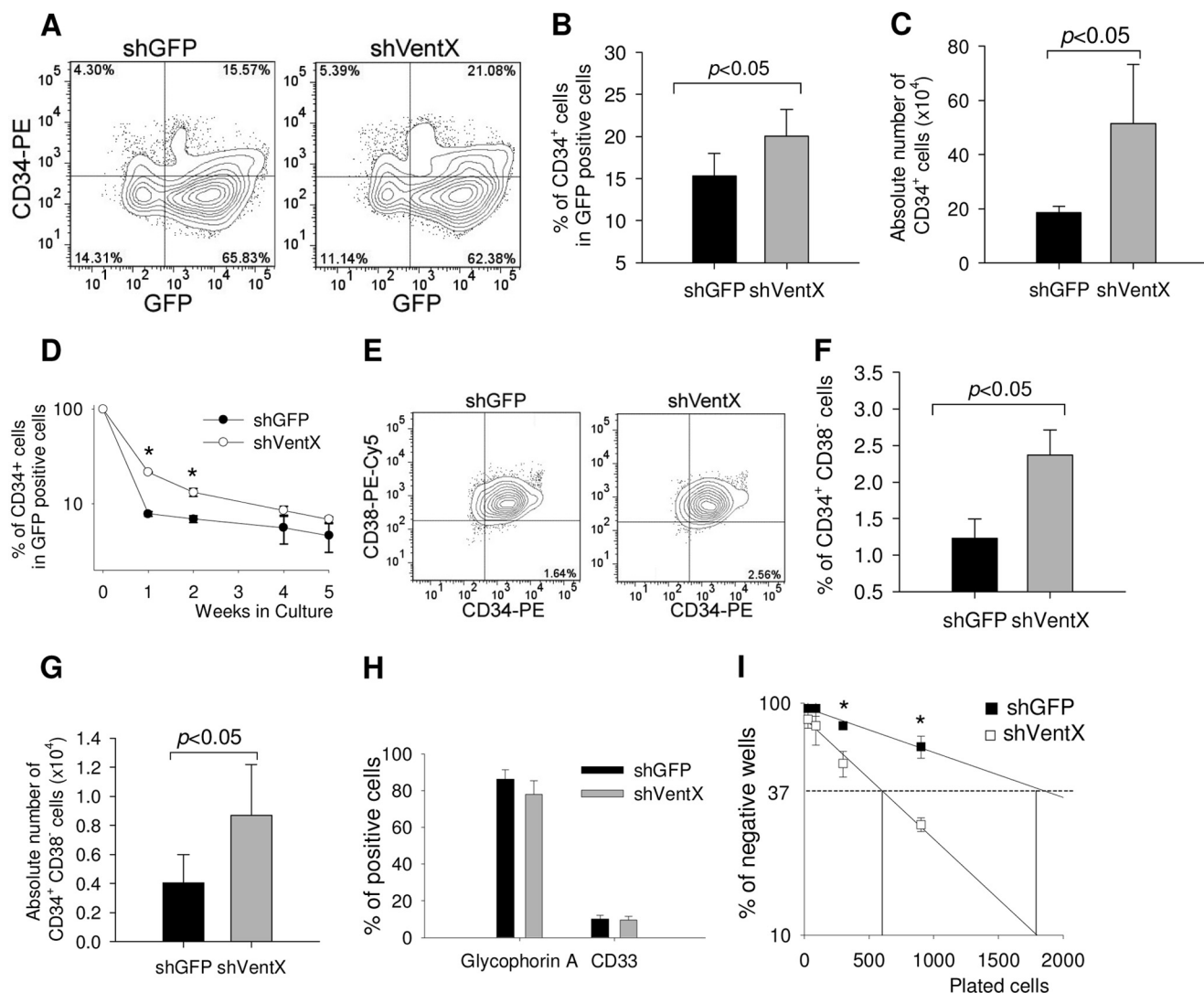
## Regulation of HSC Expansion by VentX



**FIGURE 2. Knockdown of VentX promotes expansion of human CD34<sup>+</sup> cells ex vivo.** *A*, real-time PCR showing the knockdown efficiency of VentX shRNA (*shVentX*) lentivirus in CD34<sup>+</sup> cells. *shGFP*, GFP shRNA. *B*, VentX knockdown promotes human bone marrow CD34<sup>+</sup> cell growth in liquid culture. Human CD34<sup>+</sup> cells were transduced with either non-effective GFP shRNA or VentX shRNA lentivirus. Positively transduced cells ( $2 \times 10^4$ ) were sorted by GFP and grown in stroma-free liquid cultures using a mixture of cytokines as described under "Experimental Procedures." The cells were counted weekly for 4 weeks, and the mean  $\pm$  S.D. of three independent experiments is shown. The number of cells at Day 0 represents the number of total transduced cells that were plated. The statistically significant difference ( $p < 0.05$ ) is marked with asterisks. *C–F*, knockdown of VentX in CD34<sup>+</sup> cells increases the colony formation in semisolid culture. Positively transduced CD34<sup>+</sup> cells as described above were plated in triplicate in methylcellulose-based medium and allowed to form colonies for 2 weeks. *C* and *D* show the colony numbers of BFU-E/CFU-E and CFU-GM, respectively. *E* shows total progenitor cells from  $2 \times 10^4$  CD34<sup>+</sup> cells after culturing for 2 weeks. The significant difference between the two groups (three independent experiments for each group) was determined by Student's *t* test ( $p < 0.05$ ). *F*, representative images of CFC assays showing the erythroid colonies in GFP shRNA- or VentX shRNA-transduced CD34<sup>+</sup> cells.

IL2R $\gamma$ <sup>2null</sup> (NSG) mouse model. Human bone marrow CD34<sup>+</sup> cells were transduced with lentiviruses expressing either GFP or VentX shRNA. The unsorted transduced CD34<sup>+</sup> cells were then transplanted into three pairs of NSG mice. At 8 weeks post-transplantation, engraftment efficiency was determined by FACS analysis of human CD45<sup>+</sup> cells from bone marrow of recipient mice. We found that the human cell engraftment rate increased by ~1.5–2.5-fold in VentX shRNA-transduced CD34<sup>+</sup> cells compared with GFP shRNA-transduced CD34<sup>+</sup> cells (Fig. 5, *A* and *B*). Notably, knockdown of VentX allowed multilineage development of hematopoietic cells (Table 1). Given that the lentiviral transduction rate of the CD34<sup>+</sup> cells was only ~20%, to further explore the effects of VentX knockdown on the expansion of HSC/MPPs, we sorted out the positively transduced CD34<sup>+</sup> cells and then transplanted the sorted CD34<sup>+</sup> cells into NSG mice. At 13 weeks post-transplantation, the percentage of human CD45<sup>+</sup> cells in the bone marrow of recipient mice was determined by FACS analysis. Strikingly, the

percentage of human CD45<sup>+</sup> cells was consistently increased by 20-fold in VentX shRNA-transduced CD34<sup>+</sup> cells compared with GFP shRNA-transduced CD34<sup>+</sup> cells (Fig. 5, *C* and *D*). Supporting a potential role of VentX knockdown in the expansion of HSC/MPPs, we found that the number of human CD34<sup>+</sup> cells increased significantly in bone marrow of mice transplanted with VentX shRNA-transduced CD34<sup>+</sup> cells compared with control mice transplanted with GFP shRNA-transduced CD34<sup>+</sup> cells ( $45 \pm 7$  and  $720 \pm 13$  per  $10^5$  total mononuclear cells for GFP shRNA- and VentX shRNA-transduced cells, respectively). To examine the effects of VentX on long-term engraftment of human CD34<sup>+</sup> cells, we performed secondary transplantation experiments. Whereas no detectable secondary engraftment was observed in mice transplanted with the control GFP shRNA-transduced CD34<sup>+</sup> cells, both of the two mice transplanted with the VentX shRNA-transduced CD34<sup>+</sup> cells showed positive human cell engraftment (0.25 and 0.28%, respectively) (Fig. 5*E*).



**FIGURE 3. Knockdown of VentX in CD34<sup>+</sup> cells helps preserve stem cell pools of HSC/MPPs.** Human CD34<sup>+</sup> cells transduced with either GFP shRNA (*shGFP*) or VentX shRNA (*shVentX*) were grown in liquid culture as described in the legend to Fig. 2B. The cells were harvested at the indicated time intervals and stained with PE-conjugated anti-CD34 and PE/Cy5-conjugated anti-CD38 antibodies for FACS analysis. *A*, representative FACS profile showing the percentage of CD34<sup>+</sup> cells from the GFP-positive populations at 2 weeks after plating. *B*, three independent experiments from *A* are shown. The significant difference between the two groups was determined by Student's *t* test ( $p < 0.05$ ). *C*, absolute numbers of the CD34<sup>+</sup> cells in the two groups at 2 weeks after plating. *D*, GFP-positive CD34<sup>+</sup> cells were harvested at the indicated time points, and the percentage of the remaining CD34<sup>+</sup> cells was shown. The statistically significant difference ( $p < 0.05$ ) is marked with asterisks. *E*, representative FACS profile showing the percentage of CD34<sup>+</sup>CD38<sup>-</sup> cells from GFP-positive populations at 2 weeks after plating. *F*, three independent experiments from *E* are shown. The significant difference between the two groups was determined by Student's *t* test ( $p < 0.05$ ). *G*, absolute numbers of CD34<sup>+</sup>CD38<sup>-</sup> cells in the two groups at 2 weeks after plating. *H*, FACS analysis of the erythroid (glycophorin A) and myeloid (CD33) marker staining in the two groups at 2 weeks after plating. *I*, LTCIC assay of the GFP shRNA- and VentX shRNA-transduced CD34<sup>+</sup> cells. The statistically significant difference ( $p < 0.05$ ) is marked with asterisks.

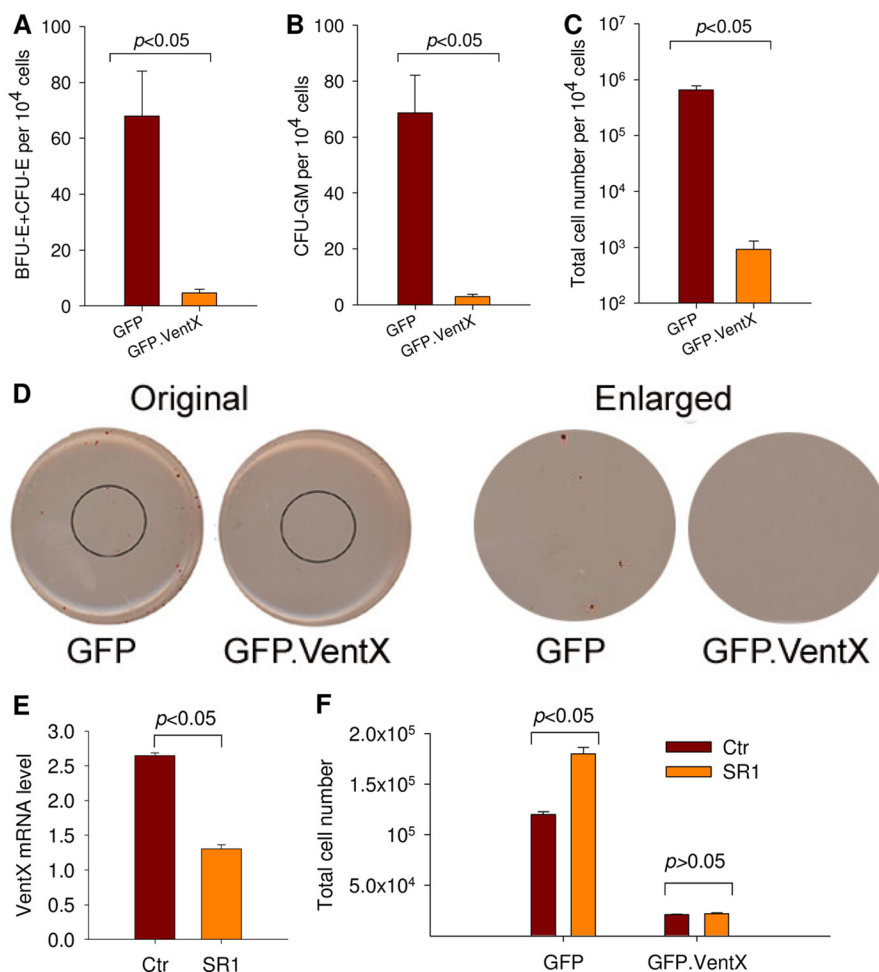
**VentX Targets Cell Cycle Regulators in CD34<sup>+</sup> Cells**—Our recent studies have shown that VentX is a regulator of the Wnt signaling pathway and several components of the cell cycle machinery, such as cyclin D<sub>1</sub>, p21, and p16<sup>ink4a</sup> (15, 16). To explore the mechanisms underlying VentX-regulated HSC/MPP expansion, we performed a targeted screen to determine the potential effects of VentX on these critical cell cycle regulators in CD34<sup>+</sup> cells. As shown in Fig. 6A, quantitative PCR demonstrated that overexpression of VentX increased the expression of p21 and p16<sup>ink4a</sup> but down-regulated the expression of cyclin D<sub>1</sub> and *c-myc* in primary CD34<sup>+</sup> cells. The results were further confirmed by a loss-of-function approach in which knockdown of VentX by siRNA decreased the expression of p21 but increased the expression of cyclin D<sub>1</sub> and *c-myc* (Fig. 6B).

Cyclin D<sub>1</sub> has been implicated in CD34<sup>+</sup> cell proliferation (20–22). The finding that the cyclin D<sub>1</sub> mRNA level was pronouncedly regulated by VentX prompted us to further examine whether the protein levels of cyclin D<sub>1</sub> are also subject to VentX regulation. As shown by FACS analysis of the intracellular staining of cyclin D<sub>1</sub>, VentX regulated the expression of cyclin D<sub>1</sub> at the protein level (Fig. 6, C and D).

## DISCUSSION

Molecular mechanisms controlling HSC/MPP proliferation and differentiation are targets of extensive investigations for their role in the management of diseases involving HSCs, such as primary and secondary bone marrow failure syndrome, refractory anemia, and myelodysplastic syndromes. Difficulties

## Regulation of HSC Expansion by VentX



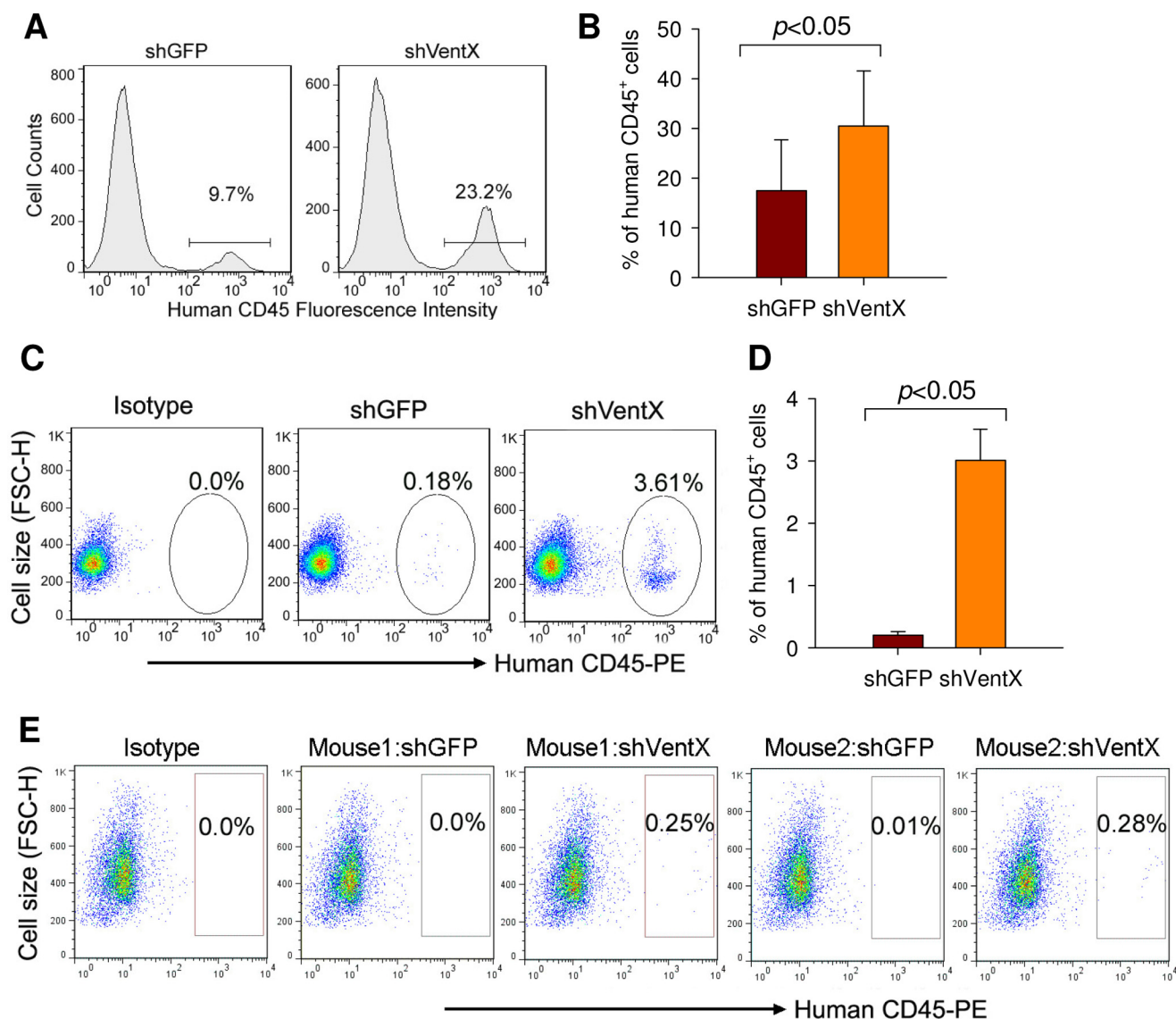
**FIGURE 4. Ectopic expression of VentX in CD34<sup>+</sup> cells inhibits colony formation.** A–C, human bone marrow CD34<sup>+</sup> cells were transfected with either the GFP VentX or GFP VentX plasmid as described under “Experimental Procedures.” Positively transfected cells ( $2 \times 10^4$ ) were sorted and cultured in triplicates in methylcellulose-based medium for CFC enumeration. The number of BFU-E/CFU-E colonies is shown in A, and the number of CFU-GM colonies in B. C, total progenitor cells from  $2 \times 10^4$  CD34<sup>+</sup> cells after culturing for 2 weeks. The significant difference was determined by Student’s *t* test ( $p < 0.05$ ). D, representative images of the CFC assay showing erythroid colonies in GFP- or GFP-VentX-transfected CD34<sup>+</sup> cells. E, human CD34<sup>+</sup> cells were treated with 1  $\mu$ M SR1 for 1 week or mock-treated (control (Ctr)). The treated cells were then harvested for analysis of VentX mRNA expression levels. F, GFP- or GFP-VentX-transfected CD34<sup>+</sup> cells ( $2 \times 10^4$ ) were sorted and treated with 1  $\mu$ M SR1 for 1 week or mock-treated. The total cell numbers were then counted and plotted.

in obtaining sufficient numbers of HSC/MPPs also present a major hurdle for potential clinical applications of HSC/MPP transplantation. Multiple cell intrinsic factors and signaling pathways have been implicated in HSC/MPP expansion; nevertheless, the functions and mechanisms of these pathways in HSC/MPP expansion remain as topics of active debate (23–25). For example, although Wnt signaling was shown to be able to expand mouse HSCs, it seems that the levels of Wnt signaling play an important role in HSC/MPP expansion (26). In addition to the complex signaling events and their interpretations, there is growing appreciation of the species-specific property of HSC/MPPs (27). Evidently, although it is relatively easy to expand mouse HSCs, it remained a fundamental challenge to expand human HSC/MPPs by targeting cell intrinsic factors.

In this study, we have shown that VentX, a recently appreciated hematopoietic homeobox transcription factor, is a novel regulator of HSC/MPP expansion. We showed that suppression of VentX expression led to significant expansion of CD34<sup>+</sup> cells *ex vivo* and a >20-fold increase in engraftment potential in the NOD/SCID/IL2R $\gamma$ <sup>2null</sup> mouse model (Fig. 5). We showed

that knockdown of VentX helped preserve the stem cell pools (Fig. 3) of HSC/MPPs, promoted clonogenicity, and allowed multilineage development of hematopoietic cells. The essential role of VentX in controlling expansion of CD34<sup>+</sup> cells was further illustrated by the finding that VentX expression was down-regulated upon application of SR1, a recently identified small molecule that promotes expansion of CD34<sup>+</sup> cells, and by the finding that ectopic expression of VentX blocked SR1-induced CD34<sup>+</sup> cell expansion.

VentX represents the first cell intrinsic factor whose down-regulation leads to significant expansion of human bone marrow CD34<sup>+</sup> cells. In comparison with other known cell intrinsic factors, such as HoxB4 and BMI1 (9–11), VentX knockdown allowed more significant expansion of CD34<sup>+</sup> cells. In our current experimental system, a 30% knockdown of VentX led to a 20-fold increase in engraftment potential in NOD/SCID/IL2R $\gamma$ <sup>2null</sup> mice (Fig. 5). Theoretically, the efficacy of HSC/MPP expansion by VentX down-regulation could still be improved through more efficient knockdown of VentX. It should be noted, however, that we focused on the effects of VentX knockdown on the expansion of CD34<sup>+</sup> cells from



**FIGURE 5. VentX knockdown promotes expansion of human CD34<sup>+</sup> cells *in vivo* in NSG mice.** *A* and *B*, human bone marrow CD34<sup>+</sup> cells were transduced with GFP shRNA (*shGFP*) or VentX shRNA (*shVentX*) lentivirus. The total unsorted transduced cells ( $1 \times 10^6$ , with transduction efficiency at  $\sim 20\%$ ) were injected into sublethally irradiated NSG mice. The human CD45<sup>+</sup> chimerism levels were determined from the bone marrow of recipient mice at 8 weeks post-transplantation. *A*, representative FACS profile showing the percentage of human CD45<sup>+</sup> cells. *B*, mean  $\pm$  S.D. of three independent experiments from *A*. The significant difference was determined by Student's *t* test ( $p < 0.05$ ). *C* and *D*, human bone marrow CD34<sup>+</sup> cells were transduced with either GFP shRNA or VentX shRNA lentivirus. Positively transduced cells ( $2 \times 10^4$ ) were sorted and then injected into sublethally irradiated NSG mice. The human CD45<sup>+</sup> chimerism levels were determined from the bone marrow of recipient mice at 13 weeks post-transplantation. *C*, representative FACS profile showing the percentage of human CD45<sup>+</sup> cells. *D*, mean  $\pm$  S.D. of four independent experiments from *C*. The significant difference was determined by Student's *t* test ( $p < 0.05$ ). *E*, bone marrow cells from the primary recipients were used to perform secondary bone marrow transplants, and the chimerism levels were analyzed after an additional 6 weeks. Results from two pairs of mice are shown. *FSC-H*, forward scatter height.

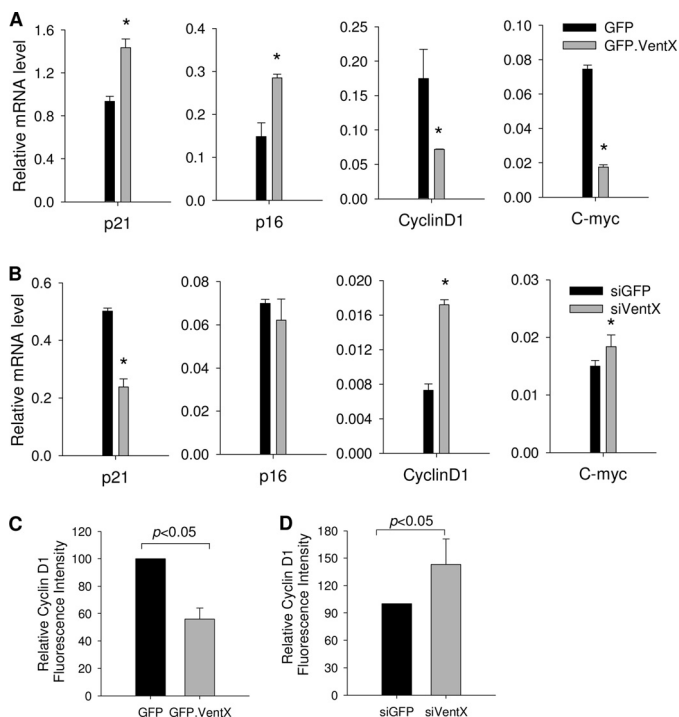
**TABLE 1**  
Knockdown of VentX allows multilineage differentiation of human CD34<sup>+</sup> cells *in vivo*

GPA, glycophorin A.

	Human CD45 cells					
	CD45	CD3	CD19	CD14	CD33	GPA
	%					
<b>Mouse1</b>						
GFP shRNA	100.0	20.2	60.6	37.5	32.7	21.2
VentX shRNA	100.0	18.5	59.2	30.3	35.5	19.9
<b>Mouse2</b>						
GFP shRNA	100.0	7.5	47.6	27.6	47.0	7.2
VentX shRNA	100.0	8.5	50.5	20.8	38.0	6.9
<b>Mouse3</b>						
GFP shRNA	100.0	6.1	63.6	35.4	23.2	12.1
VentX shRNA	100.0	5.0	75.7	24.3	14.0	8.6

human bone marrow. With the increasing application of cord blood in HSC transplantation and the notion that even modest HSC expansion would significantly increase the potential application of cord blood in adult HSC transplantation (28), the potential effects of VentX knockdown on the expansion of human cord blood CD34<sup>+</sup> cells would be of great interest for future exploration. The description of cell surface markers of *bona fide* human HSCs is constantly improving (29). In this study, we focused on CD34<sup>+</sup> HSC/MPPs. The clinical importance of these cells was reflected by the direct correlation with time to neutrophil recovery, an indicator of post-transplant mortality (27). The finding of VentX as the first cell intrinsic factor whose down-regulation promotes CD34<sup>+</sup> cell expansion

## Regulation of HSC Expansion by VentX



**FIGURE 6. VentX targets critical cell cycle regulators in CD34<sup>+</sup> cells.** A, human bone marrow CD34<sup>+</sup> cells were transfected with either GFP or GFP-VentX as described under "Experimental Procedures." GFP-positive cells were sorted at 2 days post-transfection and then used for quantitative analysis of mRNA levels of the indicated genes. \*,  $p < 0.05$ . B, CD34<sup>+</sup> cells were transfected with either GFP siRNA (siGFP) or VentX siRNA (siVentX), and cells were harvested 3 days post-transfection. Quantitative PCR analysis was carried out to determine the mRNA levels of the indicated genes. \*,  $p < 0.05$ . Results show the mean  $\pm$  S.D. of triplicates from one of two independent experiments. C, CD34<sup>+</sup> cells were transfected with either GFP or GFP-VentX, and the intracellular staining of cyclin D<sub>1</sub> and FACS analysis were performed at 2 days post-transfection. GFP-positive cells were gated for analysis of mean fluorescence intensity. D, CD34<sup>+</sup> cells were cotransfected with either GFP siRNA (non-effective) or VentX siRNA and with plasmid encoding GFP. The intracellular staining of cyclin D<sub>1</sub> and FACS analysis were performed at 3 days post-transfection. GFP-positive cells were gated for analysis. Results show the mean  $\pm$  S.D. of three independent experiments.

provides a novel therapeutic target for diseases involving HSC/MPPs and HSC/MPP-based clinical therapy.

We began to explore the mechanisms underlying VentX regulation of CD34<sup>+</sup> cell proliferation and differentiation. VentX expression is relatively low in early HSC/MPPs but increases dramatically during the proliferative differentiation of CD34<sup>+</sup> cells. Consistent with its role as a pro-differentiation and anti-proliferation hematopoietic transcription factor, we found that knockdown of VentX promoted proliferation of CD34<sup>+</sup> cells (Fig. 2) and deferred their differentiation into lineage-specific progenitor cells (Fig. 3). As the stemness of HSC/MPPs decreases gradually during the proliferative differentiation of HSCs, our data suggest that VentX knockdown promotes the expansion of human HSC/MPPs by deferring differentiation of human HSC/MPPs. Although VentX knockdown promoted proliferation of CD34<sup>+</sup> cells, no obvious alteration of the cell cycle profile was observed by knockdown of VentX (data not shown).

At the molecular level, we found that VentX regulates the expression of genes downstream of canonical Wnt signaling, such as cyclin D<sub>1</sub> and *c-myc*, and other critical cell cycle regu-

lators involved in HSC/MPP proliferation and differentiation, such as p21 (30–32). Interestingly, we found that although overexpression of VentX increases the expression of p16, knockdown of VentX does not alter p16 expression significantly, suggesting that other factors are involved in controlling p16 expression in HSC/MPPs (Fig. 6, A and B). As such, the exact position of VentX in the molecular hierarchy of controlling HSC/MPP proliferation and differentiation remains to be further defined.

VentX was identified as an inhibitor of the oncogenic Wnt and as an activator of the p53/p21 and p16/pRb tumor suppressor pathways. Therefore, caution needs to be exercised for potential application of VentX knockdown in the expansion of human HSC/MPPs.

*Acknowledgments*—We thank Drs. D. Cohen and R. Blumberg for critical reagents and use of their facilities. We thank Yinke Yang and Weixiong Ke for technical support.

## REFERENCES

- Sorrentino, B. P. (2004) Clinical strategies for expansion of hematopoietic stem cells. *Nat. Rev. Immunol.* **4**, 878–888
- Mimeault, M., Hauke, R., and Batra, S. K. (2007) Stem cells: a revolution in therapeutics—recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. *Clin. Pharmacol. Ther.* **82**, 252–264
- Bhardwaj, G., Murdoch, B., Wu, D., Baker, D. P., Williams, K. P., Chadwick, K., Ling, L. E., Karanu, F. N., and Bhatia, M. (2001) Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat. Immunol.* **2**, 172–180
- Antonchuk, J., Sauvageau, G., and Humphries, R. K. (2001) HOXB4 overexpression mediates very rapid stem cell regeneration and competitive hematopoietic repopulation. *Exp. Hematol.* **29**, 1125–1134
- Reya, T., Duncan, A. W., Ailles, L., Domen, J., Scherer, D. C., Willert, K., Hintz, L., Nusse, R., and Weissman, I. L. (2003) A role for Wnt signaling in self-renewal of hematopoietic stem cells. *Nature* **423**, 409–414
- Antonchuk, J., Sauvageau, G., and Humphries, R. K. (2002) HOXB4-induced expansion of adult hematopoietic stem cells *ex vivo*. *Cell* **109**, 39–45
- Bhatia, M., Bonnet, D., Wu, D., Murdoch, B., Wrana, J., Gallacher, L., and Dick, J. E. (1999) Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells. *J. Exp. Med.* **189**, 1139–1148
- Zhang, C. C., Kaba, M., Iizuka, S., Huynh, H., and Lodish, H. F. (2008) Angiopoietin-like 5 and IGFBP2 stimulate *ex vivo* expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. *Blood* **111**, 3415–3423
- Amsellem, S., Pflumio, F., Bardin, D., Izac, B., Charneau, P., Romeo, P. H., Dubart-Kupferschmitt, A., and Fichelson, S. (2003) *Ex vivo* expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. *Nat. Med.* **9**, 1423–1427
- Krosl, J., Austin, P., Beslu, N., Kroon, E., Humphries, R. K., and Sauvageau, G. (2003) *In vitro* expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. *Nat. Med.* **9**, 1428–1432
- Rizo, A., Dontje, B., Vellenga, E., de Haan, G., and Schuringa, J. J. (2008) Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMI1. *Blood* **111**, 2621–2630
- Gao, H., Wu, B., Giese, R., and Zhu, Z. (2007) Xom interacts with and stimulates transcriptional activity of LEF1/TCFs: implications for ventral cell fate determination during vertebrate embryogenesis. *Cell Res.* **17**, 345–356
- Yang, Y. (2007) Xom as a novel partner of LEF/TCFs during dorsal-ventral patterning of the *Xenopus* embryo. *Cell Res.* **17**, 307–308
- Moretti, P. A., Davidson, A. J., Baker, E., Lilley, B., Zon, L. I., and D'Andrea,



- R. J. (2001) Molecular cloning of a human Vent-like homeobox gene. *Genomics* **76**, 21–29
15. Gao, H., Le, Y., Wu, X., Silberstein, L. E., Giese, R. W., and Zhu, Z. (2010) VentX, a novel lymphoid-enhancing factor/T-cell factor-associated transcription repressor, is a putative tumor suppressor. *Cancer Res.* **70**, 202–211
  16. Wu, X., Gao, H., Ke, W., Giese, R. W., and Zhu, Z. (2011) The homeobox transcription factor VentX controls human macrophage terminal differentiation and proinflammatory activation. *J. Clin. Invest.* **121**, 2599–2613
  17. Wu, X., Gao, H., Ke, W., Hager, M., Xiao, S., Freeman, M. R., and Zhu, Z. (2011) VentX transactivates p53 and p16<sup>ink4a</sup> to regulate cellular senescence. *J. Biol. Chem.* **286**, 12693–12701
  18. Rawat, V. P., Arseni, N., Ahmed, F., Mulaw, M. A., Thoene, S., Heilmeier, B., Sadlon, T., D'Andrea, R. J., Hiddemann, W., Bohlander, S. K., Buske, C., and Feuring-Buske, M. (2010) The vent-like homeobox gene *VENTX* promotes human myeloid differentiation and is highly expressed in acute myeloid leukemia. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 16946–16951
  19. Boitano, A. E., Wang, J., Romeo, R., Bouchez, L. C., Parker, A. E., Sutton, S. E., Walker, J. R., Flaveny, C. A., Perdew, G. H., Denison, M. S., Schultz, P. G., and Cooke, M. P. (2010) Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science* **329**, 1345–1348
  20. Istvanffy, R., Kröger, M., Eckl, C., Gitzelmann, S., Vilne, B., Bock, F., Graf, S., Schiemann, M., Keller, U. B., Peschel, C., and Oostendorp, R. A. (2011) Stromal pleiotrophin regulates repopulation behavior of hematopoietic stem cells. *Blood* **118**, 2712–2722
  21. Ko, K. H., Holmes, T., Palladinetti, P., Song, E., Nordon, R., O'Brien, T. A., and Dolnikov, A. (2011) GSK-3 $\beta$  inhibition promotes engraftment of *ex vivo* expanded hematopoietic stem cells and modulates gene expression. *Stem Cells* **29**, 108–118
  22. Zou, P., Yoshihara, H., Hosokawa, K., Tai, I., Shinmyozu, K., Tsukahara, F., Maru, Y., Nakayama, K., Nakayama, K. I., and Suda, T. (2011) p57<sup>Kip2</sup> and p27<sup>Kip1</sup> cooperate to maintain hematopoietic stem cell quiescence through interactions with Hsc70. *Cell Stem Cell* **9**, 247–261
  23. Cobas, M., Wilson, A., Ernst, B., Mancini, S. J., MacDonald, H. R., Kemler, R., and Radtke, F. (2004)  $\beta$ -Catenin is dispensable for hematopoiesis and lymphopoiesis. *J. Exp. Med.* **199**, 221–229
  24. Kirstetter, P., Anderson, K., Porse, B. T., Jacobsen, S. E., and Nerlov, C. (2006) Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nat. Immunol.* **7**, 1048–1056
  25. Koch, U., Wilson, A., Cobas, M., Kemler, R., Macdonald, H. R., and Radtke, F. (2008) Simultaneous loss of  $\beta$ - and  $\gamma$ -catenin does not perturb hematopoiesis or lymphopoiesis. *Blood* **111**, 160–164
  26. Luis, N. M., Morey, L., Mejetta, S., Pascual, G., Janich, P., Kuebler, B., Cozutto, L., Roma, G., Nascimento, E., Frye, M., Di Croce, L., and Benitah, S. A. (2011) Regulation of human epidermal stem cell proliferation and senescence requires Polycomb-dependent and -independent functions of Cbx4. *Cell Stem Cell* **9**, 233–246
  27. Doulatov, S., Notta, F., Laurenti, E., and Dick, J. E. (2012) Hematopoiesis: a human perspective. *Cell Stem Cell* **10**, 120–136
  28. Brunstein, C. G., and Wagner, J. E. (2006) Umbilical cord blood transplantation and banking. *Annu. Rev. Med.* **57**, 403–417
  29. Notta, F., Doulatov, S., Laurenti, E., Poeppl, A., Jurisica, I., and Dick, J. E. (2011) Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* **333**, 218–221
  30. Liu, Y., Elf, S. E., Miyata, Y., Sashida, G., Liu, Y., Huang, G., Di Giandomenico, S., Lee, J. M., Deblasio, A., Menendez, S., Antipin, J., Reva, B., Koff, A., and Nimer, S. D. (2009) p53 regulates hematopoietic stem cell quiescence. *Cell Stem Cell* **4**, 37–48
  31. Fleming, H. E., Janzen, V., Lo Celso, C., Guo, J., Leahy, K. M., Kronenberg, H. M., and Scadden, D. T. (2008) Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal *in vivo*. *Cell Stem Cell* **2**, 274–283
  32. Baena, E., Ortiz, M., Martínez-A. C., and de Alborán, I. M. (2007) c-Myc is essential for hematopoietic stem cell differentiation and regulates Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>-</sup> cell generation through p21. *Exp. Hematol.* **35**, 1333–1343