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Near-maximal expansions of hematopoietic stem cells in culture using NUP98-HOX fusions

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Abstract

Objective—Strategies to expand hematopoietic stem cells (HSCs) ex vivo are of key interest. The objective of this study was to resolve if ability of HOXB4, previously documented to induce a significant expansion of HSCs in culture, may extend to other HOX genes and also to further analyze the HOX sequence requirements to achieve this effect.

Methods—To investigate the ability of Nucleoporin98-Homeobox fusion genes to stimulate HSC self-renewal, we evaluated their presence in 10- to 20-day cultures of transduced mouse bone marrow cells. Stem cell recovery was measured by limiting-dilution assay for long-term competitive repopulating cells (CRU Assay).

Results—These experiments revealed remarkable expansions of Nucleoporin98-Homeobox–transduced HSCs (1000-fold to 10,000-fold over input) in contrast to the expected decline of HSCs in control cultures. Nevertheless, the Nucleoporin98-Homeobox-expanded HSCs displayed no proliferative senescence and retained normal lympho-myeloid differentiation activity and a controlled pool size in vivo. Analysis of proviral integration patterns showed the cells regenerated in vivo were highly polyclonal, indicating they had derived from a large proportion of the initially targeted HSCs. Importantly, these effects were preserved when all HOX sequences flanking the homeodomain were removed, thus defining the homeodomain as a key and independent element in the fusion.

Conclusion—These findings create new possibilities for investigating HSCs biochemically and genetically and for achieving clinically significant expansion of human HSCs.

The self-renewal function of hematopoietic stem cells (HSCs) is essential to their ability to sustain lifelong hematopoiesis and to regenerate the hematopoietic system after myeloablative treatments. This property is the basis of an increasing range of applications of HSC transplants to treat various malignant and genetic disorders [1,2]. Further improvements in the safety and therapeutic utility of HSC transplants can be readily envisaged if robust methods for large-scale ex vivo expansion of HSCs were available. For example, the low absolute numbers of HSCs in most cord blood samples [3] or in harvests of mobilized autologous HSCs from some

patients [4] restrict the clinical utility of these products. A method for significantly expanding HSCs could not only address these insufficiencies but also broaden the exploitation of reduced conditioning regimens and associated therapeutic benefits.

One approach that has allowed some HSC expansion in vitro to be achieved has focused on the identification of optimized combinations and concentrations of externally acting growth factors and related molecules [5–12]. A complementary approach has been to identify intrinsic regulators such as transcription factors [13] and key mediators of signaling pathways [14,15] that can be manipulated to activate or promote HSC self-renewal divisions. A striking example of the latter strategy is the use of retrovirally engineered overexpression of the homeobox transcription factor HOXB4 to stimulate expansions of HSC numbers in vitro of up to 80-fold [16–20] with evidence of similarly enhanced HSC expansion in vivo [21–24]. Significant enhancement of HSC self-renewal has also been elicited by repeated delivery of HOXB4 protein to the cell as an exogenous supplied TAT-HOX fusion protein [25].

The studies described here were designed to determine whether even greater HSC expansions might be achieved by forced overexpression of modified HOX-containing fusion proteins. Assuming a HSC cell cycle time of 12 to 14 hours [26,27], the continuous execution of symmetric self-renewal divisions, and no cell death, the maximum expansion of HSCs that would be predicted to occur over a period of 5 to 8 days is 1000-fold, i.e., greater than 10 times that found to be achieved by overexpressing HOXB4. In a related paper, we show that HSC expansions of this magnitude can be realized by combining the overexpression of HOXB4 with the suppressed expression of the Hox cofactor, Pbx1. Here we investigated the effect on HSC expansion of a HOX fusion protein (either NUP98-HOXA10 or NUP98-HOXB4). We speculated that such fusion proteins might have a potent ability to promote HSC expansion based on our previous results showing that both stimulated very high expansions of cells with spleen colony-forming ability in short-term cultures [28]. In addition, we had observed that for at least 4 weeks in suspension culture, NUP98-HOXA10-transduced cells produced cells with at least transient myeloid repopulating activity [29]. Our present findings reveal a remarkable ability of the NUP98-HOXA10 fusion gene to stimulate near-maximal expansions of HSCs in short-term suspension culture. Moreover, the effect of NUP98-HOXA10 on HSC expansion was preserved when sequences flanking the homeodomain (HD) were removed, thus identifying the homeodomain as the key HOX gene sequence required in concert with the N-terminal region of NUP98.

Materials and methods

Retroviral vectors

The generation of MSCV-IRES-GFP, MSCV-HOXB4-IRES-GFP, MSCV-NUP98-HOXB4-IRES-GFP (NB4), MSCV-NUP98-HOXA10-IRES-GFP (NA10), and MSCV-NUP98-HOXA10HD (NA10HD) viral vectors (Fig. 1A) has been described [23,28]. The NB4, NA10, and NA10HD constructs were validated by sequencing and correct expression and transmission were confirmed by Western blot and Southern blot analysis [28].

Mice

Mice were bred and maintained in our animal facility according to Canadian Council on Animal Care guidelines. Transplant donors were either C57Bl/6Ly-Pep3b (Pep3b) mice that express Ly5.1 or C57BL/6 J (B6) mice that express Ly5.2. Recipients of Pep3b cells were either B6 mice or C57Bl/6-W⁴¹/W⁴¹ (W⁴¹) mice that express Ly5.2 and recipients of B6 cells were Pep3b mice that express Ly5.1.

Purification of Sca1⁺lin⁻ (SL) or c-kit⁺Sca1⁺lin⁻ (KSL) cells

5-FU-pretreated bone marrow (BM) cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-Sca1, allophycocyanin (APC)-labeled anti-c-kit, and phycoerythrin (PE)-labeled anti-Gr-1, anti-B220, anti-CD4, anti-CD8, and Ter119. Sorting was performed on a FACS Aria system (Becton-Dickinson, San Jose, CA, USA) using gates set to exclude 99.9% of controls labeled with isotype control antibodies linked to the same fluorochromes. Sorted cells were counted by hemocytometer and plated into 96-well plates. For the phenotypic analysis of the donor-derived (GFP⁺) c-kit⁺Sca1⁺lin⁻ compartment and the nontransduced (GFP⁻) counterpart in a BM of repopulated recipients, a combination of markers including PE-conjugated Sca-1 (Ly6A/E), APC-conjugated c-kit (ACK2), and biotinylated lineage cocktail of antibodies (Gr-1: RB6-8C5, Mac-1: M1-70, B220: RA3-6B2, CD3: 145-2C11, TER119) was used. Biotinylated antibodies were visualized with ECD-conjugated streptavidin. These studies were conducted using a FACS-VantageSE with DiVa upgrade (BD Immunocytometry Systems, San Jose, CA, USA) equipped for three-laser excitation. All antibodies were purchased from BD-Biosciences Pharmingen (San Diego, CA, USA).

Infection of murine bone marrow cells

BM cells were obtained from mice injected 4 days previously with 150 mg/kg 5-fluorouracil (5-FU) (Faulding, Underdaler, Australia) and transduced as previously described [16]. Briefly, BM cells were cultured for 2 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 10 ng/mL human interleukin-6 (IL-6), 6 ng/mL murine interleukin-3 (IL-3), and 100 ng/mL murine stem cell factor (SF). Media and serum were purchased from StemCell Technologies (Vancouver, BC, Canada). Cells were then co-cultivated for 2 days with irradiated (40 Gy) GP+E-86 virus producer cells in the same medium supplemented with 5 µg/mL protamine sulfate (Sigma, Oakville, ON, Canada). At the end of this time, loosely adherent and nonadherent cells were recovered and cultured a further 6 days in the same medium without protamine sulfate. For bulk cultures, 3×10^6 cells were seeded in a 10-cm dish at day 0, and the equivalent of 3×10^5 starting cells (day 0) were replated into the same size dish on day 6 or 7. For cultures initiated with 5000 unseparated BM cells or 500 Sca1⁺lin⁻ cells, cells were cultured in a 96-well plate at day 0 and replated into a 24-well plate on day 7.

Competitive repopulating unit assay

HSCs were detected and quantified using the limiting-dilution competitive repopulating unit (CRU) assay, as described [30,31]. Briefly, lethally irradiated B6 or Pep3b mice (810 cGy of 250kVp x-rays followed by injection of a life-sparing dose of 10^5 normal B6 or Pep3b bone marrow cells) or sublethally irradiated W⁴¹ mice (450 cGy of ¹³⁷Cs gamma radiation alone) were injected with variable numbers of test cells and the proportion of mice in each group that showed multi-lineage repopulation with GFP⁺ cells was determined from FACS analyses of their peripheral blood leukocytes at least 16 weeks later. For these analyses, 100 µL of blood was taken from the tail vein, erythrocytes lysed with ammonium chloride (StemCell Technologies), and the leukocytes suspended in Hanks' Balanced Salt Solution with 2% FBS (HF, StemCell Technologies) and then incubated sequentially on ice with a combination of biotinylated anti-Ly5.1 (anti-Ly5.2) and either PE-labeled B220 or a combination of PE-labeled Gr-1 and Mac-1, or a combination of PE-labeled CD4 and CD8, and then APC-labeled streptavidin. All samples were washed with HF and 1 µg/mL propidium iodide (PI) prior to analysis on a FACSCalibur (Becton-Dickinson). For multiple staining flow studies peripheral blood was collected from each mouse in a single sample and after red blood cell lysis, a combination of monoclonal antibodies targeting leukocyte surface antigens toward CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), Ter119, B220 (RA3-6B2), Gr-1 (Ly6G), and Mac-1 (M1/70) was added together. All antibodies were purchased from BD-Pharmingen.

Polychromatic flow cytometry was performed on FACS Aria sorter equipped with three air-cooled lasers at 488-nm, 633-nm, and 407-nm wavelengths (Becton-Dickinson). Only mice whose blood contained greater than 1% donor-derived (GFP⁺) myeloid cells (Gr-1⁺ and/or Mac-1⁺), B cells (B220⁺), and T cells (CD4⁺ and/or CD8⁺) were considered to be positive. All other mice were scored as negative. CRU frequencies were calculated by applying Poisson statistics to the proportion of negative recipients in groups transplanted with different numbers of cells using the L-Calcul software (StemCell Technologies).

Proviral integration analysis

Genomic DNA was isolated with DNAzol reagent (Invitrogen, Carlsbad, CA, USA), as recommended by the manufacturer. Southern blot analysis was performed as previously described [32]. Unique proviral integrations were identified by digestion of DNA with EcoRI, which cleaves once within the provirus and at various distances within the genome. Digested DNA was then separated in 0.8% agarose gel by electrophoresis and transferred to zeta-probe membranes (Bio-Rad, Mississauga, ON, Canada). Membranes were probed with a [³²P]dCTP GFP sequence.

Results

NUP98-HOX fusion genes potentially stimulate HSC expansion in vitro

In a first series of experiments we compared the effects of forced expression of various NUP98-HOX fusion genes to HOXB4 on HSC expansion over a 10-day period in vitro. The constructs evaluated are shown in Figure 1A and the general design of the experiments is shown schematically in Figure 1B. Changes in transduced HSC numbers were inferred from a comparison of the number of CRUs measured in the starting population of 5-FU-treated BM cells (day 0) versus the number of GFP⁺ CRUs measured at the end of the culture period (day 10). On day 10, more than 75% of the total cells in all cultures were consistently GFP⁺.

The data used to calculate CRU numbers are shown in graphic form in Figure 2A for a representative experiment. In cultures initiated with cells transduced with the control green fluorescent protein vector (GFP), the total number of GFP⁺ CRUs present in the 10-day cultures had markedly declined (from ~1 per 5000 starting cells to less than 1 per 230,000 starting cell equivalents on day 10, i.e., an absolute decrease of ~50-fold). In contrast, the number of HOXB4-transduced CRUs significantly increased during the same period (to 1 per 218 starting cell equivalents, an expansion of ~25-fold, as expected [16]). Strikingly, the CRUs transduced with NUP98-HOXB4 or NUP98-HOXA10 underwent much more pronounced expansions (to 1 per 47 and 1 per 5 starting cell equivalents, respectively, indicative of expansions of ~100- (NUP98-HOXB4) and ~1000-fold (NUP98-HOXA10), respectively).

These findings were reproduced in two other experiments. The pooled data confirmed net expansions in CRU numbers of 290-fold for NUP98-HOXB4 and greater than 2000-fold for NUP98-HOXA10 as compared to 80-fold for HOXB4 (Fig. 2B). Total levels of GFP⁺ white blood cells present after 16 weeks in the blood of individual recipients of different sources of transduced cells are shown in Figure 3A and representative FACS profiles demonstrating B- and T-lymphoid and myeloid repopulation by the transduced cells are shown in Figure 3B. In all of these experiments, there was complete concordance between the presence of regenerated donor-derived cells (identified by CD45 allotype markers) and transduced (GFP⁺) cells in the reconstituted recipients (data not shown). It can be seen that mice transplanted with a cell dose as low as 2 starting cell equivalents of NUP98-HOXA10-transduced cells showed high levels (>10%) of donor cell-derived long-term lympho-myeloid reconstitution. To achieve comparable levels of reconstitution, 10-fold higher transplant doses of NUP98-HOXB4-transduced cells were required and ~100-fold and greater than 1000-fold higher transplant

doses of HOXB4- or GFP-transduced cells were needed. As shown in Figure 3B, in vitro-expanded HSC transduced with either NUP98-HOXB4 or NUP98-HOXA10 made substantial contributions to all major peripheral blood lineages, as well as matching high-level contributions to the red blood cell compartment, although the proportion of myeloid cells in some recipients was modestly increased and the proportions of B and T cells correspondingly decreased, a phenomenon previously observed for recipients of HOXB4-transduced cells [33–35]. However, no recipients followed over the 6- to 8-month time frame of the experiments showed any evidence of a myeloproliferative disorder or leukemia consistent with a previously observed lack of leukemogenic activity for NUP98-HOXB4 and long disease latency in recipients of large doses of NUP98-HOXA10-transduced cells [28].

To verify the multi-lineage differentiation activity of the ex vivo-expanded NUP98-HOXA10-transduced HSCs, BM, spleen, and thymus cells were isolated or Gr1⁺/Mac1⁺, B220⁺, and CD3⁺ cells were purified from corresponding tissues and examined for proviral integration patterns by Southern blot analysis of the DNA extracted from each population. As shown for representative mice (Figure 3C), identical inserts in these different tissues were seen in mice transplanted with relatively small numbers of cells.

In vitro HSC expansion by a NUP98-Homeobox fusion gene

We next asked whether the effects obtained with a NUP98-HOXA10 fusion gene could be attributed to particular domains in the molecule. The second exon of NUP98-HOXA10 encodes the HD plus another 16 N-terminal amino acids that provide a PBX-binding motif [36] and another 15 C-terminal amino acids of unknown function. Recent studies of the leukemogenic properties of various NUP98-HOX fusion genes, including NUP98-HOXA10, indicated that the homeodomain is essential for blocking hematopoietic differentiation [28]. Other studies had shown that PBX1 knockdown enhances the in vivo competitiveness of HOXB4-overexpressing HSCs more than 20-fold [18]. Therefore it was of interest to investigate whether or not the HD contributes all of the HSC expanding function of NUP98-HOX fusion proteins.

Accordingly, we constructed a NUP98-HOX fusion gene retaining only the cDNA sequence corresponding to the HD of HOXA10, placed it in a murine stem cell virus (MSCV) vector (NUP98-HOXA10HD, Fig. 1A), and then tested the ability of this cDNA to stimulate HSC expansion using the same short-term culture protocol described in Figure 1B. These experiments showed that the starting equivalent transplant doses of NUP98-HOXA10HD-transduced cells required to match the day-10 culture yields of nontransduced control CRUs were at least 200-fold lower (Fig. 4A), corresponding to over 1000-fold net expansion of the NUP98-HOXA10HD-transduced CRUs (Fig. 4B). Moreover, in one series of mice tested following expansion culture for a further 10 days (total of 21 days), significant long-term lympho-myeloid reconstitution was observed down to a transplant dose equivalent of 0.25 starting cells or representing at least a 10,000-fold net expansion of CRUs (Fig. 4A, Experiment VII). Thus NUP98-HOXA10HD retains its extremely potent capacity to stimulate HSC expansion in vitro for at least 17 days.

Detailed immunophenotypic multicolor flow cytometric analysis was performed on peripheral blood samples obtained from mice a minimum of 24 weeks after they had been transplanted with NUP98-HOXA10HD-transduced cells. The concurrent presence of myeloid, B lymphoid, and T lymphoid cells within the donor-derived (GFP⁺) compartment of circulating white blood cells was confirmed in a representative from each group and served to demonstrate a similar lineage distribution in the recipients of test and control cells (Fig. 4C). In addition, Southern blot analyses carried out on BM, spleen, and thymus cells obtained from recipients of expanded NUP98-HOXA10HD-transduced cells confirmed common integration patterns consistent with expansion and repopulation of long-term lympho-myeloid HSCs (Fig. 4C). Intriguingly, HSCs expanded with NUP98-HOXA10HD did not show any evidence of skewed differentiation,

suggesting that removal of sequences flanking the homeodomain may further reduce or eliminate the modest skewing seen in HSCs transduced with NUP98-HOXB4 or NUP98-HOXA10. Consistent with this normal differentiation pattern, mice followed for periods extending to over 1 year posttransplant had normal white blood cell counts and on sacrifice did not have any evidence of splenomegaly or myelodysplasia.

These findings indicate that the HOX DNA-binding homeodomain alone, within a NUP98-HOX fusion gene, is sufficient to promote marked levels of HSC expansion in culture without altering normal differentiation and repopulating activity.

NUP98-HOX-expanded HSCs retain significant proliferative potential

The proliferative potential of the expanded NUP98-HOX-transduced HSCs, and hence their ability to regenerate the HSC compartment *in vivo*, was evaluated further by determining the HSC content of the BM of highly reconstituted mice (>75%) at least 6 months posttransplant using the limiting-dilution CRU assay (performed in secondary recipients) to quantify the number of transduced HSCs regenerated. As shown in Figure 5A, these measurements showed that the HSC pool regenerated in mice that had been initially transplanted with *ex vivo*-expanded NUP98-HOXA10 or NUP98-HOXA10HD-transduced HSCs had reached normal levels by 4 months, consistent with their ability to reconstitute the HSC compartment as well as the mature blood cell compartment.

In vivo self-renewal of NUP98-HOXA10HD-transduced CRUs was additionally confirmed by phenotypic analysis of the donor-derived (GFP⁺) c-kit⁺Sca1⁺lin⁻ compartment in the BM of reconstituted recipients (Fig. 5B). The proportion of such cells was equivalent to that of the nontransduced counterparts. Moreover, in these experiments, we confirmed that reconstitution of the mature compartments by transduced cells was similar to that obtained by nontransduced (GFP⁻) cells in the same mice and as seen in normal, unmanipulated mice (data not shown).

NUP98-HOX fusions stimulate all transduced HSCs to expand *ex vivo*

If the large HSC expansions stimulated by the NUP98-HOXA10 or NUP98-HOXA10HD fusion genes were due to the responses of all transduced HSCs rather than a minor subset of these cells, then one would expect that the mature progeny produced in mice transplanted with nonlimiting numbers of CRUs, from individual cultures initiated with large number of bulk bone marrow cells, would be polyclonal. To investigate this possibility, proviral integration patterns in the DNA of BM from recipients of varying doses of cells from single cultures were examined by Southern blot analysis. As shown in Figure 6, different recipients of cells from the same cultures of NUP98-HOXA10- and NUP98-HOXA10HD-transduced cells showed distinct proviral integration patterns over a wide range of transplant cell doses and the number of clones present in each mouse was clearly related to the dose of transduced cells that had been transplanted. Recipients of higher transplant doses (i.e., 2500 or 250 and 200 or 20 starting cell equivalents, respectively) showed highly complex and distinct proviral integration patterns. In contrast, for recipients of the lowest dose of NUP98-HOXA10- or NUP98-HOXA10HD-transduced cells used (i.e., 2 starting cell equivalents/mouse), variations in the autoradiographic intensities of bands that represent unique integrations suggest that each of the mice in these latter groups was reconstituted by one or two GFP⁺ clones (i.e., one or two differently marked GFP⁺ HSCs) with up to 3 proviral integrations per clone (per marked HSC). Therefore the 10-day cultures from which the transplants of transduced HSCs had been derived had not become dominated by a small number of clonally expanded HSCs but, in fact, contained the HSC progeny of many originally transduced cells. In sharp contrast, the GFP⁺ BM cells regenerated in all of the recipients of HSCs transduced with the control GFP vector showed the same proviral integration pattern, regardless of the dose of cells transplanted (250,000

starting cell equivalents; data not shown), confirming the limited ex vivo expansion activity of most HSCs.

The polyclonal nature of the expansion of HSCs in cultures of NUP98-HOXA10- or NUP98-HOXA10HD-transduced BM cells suggested a general susceptibility of HSCs to the effects of these fusion genes. To test this more directly, a series of mini-cultures were initiated and manipulated as in Figure 7A but the number of input cells was reduced to contain an estimated 1 to 2 CRUs (either from unseparated BM from 5-FU-pretreated donors or after isolation of the Sca1⁺lin⁻ or c-kit⁺Sca1⁺lin⁻ cells). The number of GFP⁺ CRUs present in each culture was then separately determined after 6 or 10 days of expansion period. The results show that all wells in which the cells were exposed to the NUP98-HOXA10 (6 of 6) or NUP98-HOXA10HD (2 of 2) fusion genes contained highly expanded numbers of GFP⁺ CRUs. In contrast, none of the cultures of cells exposed to the control vector contained detectable GFP⁺ CRUs after 6 or 10 days of expansion in vitro (Fig. 7B). The fact that recipients of as little as 1/2000 of a culture initiated with whole BM cells or 1/25,000 of a culture initiated with Sca1⁺lin⁻ cells or 1/4000 of a culture initiated with c-kit⁺Sca1⁺lin⁻ cells were highly reconstituted in all lineages provides further strong evidence that HSC expansions of greater than 1000-fold were typical and obtained from most HSCs.

Proviral integration analysis of BM DNA from representative recipients of cultured NUP98-HOXA10- or NUP98-HOXA10HD-transduced cells from these experiments showed that most recipients of cells from the same culture had been repopulated by the progeny of the same initially transduced cell, irrespective of the number of CRUs transplanted (Fig. 7C). Moreover, similar results were obtained for one of the 10-day cultures initiated with Sca1⁺lin⁻ cells, where we transplanted only the amplified Sca1⁺lin⁻ subset of cells present in the harvested cells (Fig. 7C–Resorted SL), indicating that the amplified CRUs had retained a Sca1⁺lin⁻ phenotype.

Discussion

Recent studies have suggested the ability of HOXB4 to induce a significant expansion of HSCs in culture may extend to other HOX genes. These include results of experiments testing the effect of forced overexpression of HOXA9 [37] and preliminary data using engineered NUP98-HOX fusion genes [28], identified because of their implied role in leukemogenesis [38]. All NUP98-HOX fusions reported to date include the N-terminus of NUP98, which contains a region of multiple phenylalanine-glycine repeats that may act as a transcriptional co-activator through binding to CBP/p300 [39] and the C-terminus of the HOX gene product, including the intact homeodomain and a variable portion of the flanking amino acids [38]. The engineered NUP98 fusions we had previously studied included both HOXB4, a member of the Ant-class group, as well as HOXA10, which belongs to a different Abd-B class of HOX genes; and both of these were found to stimulate a marked expansion in vitro of multipotent spleen colony-forming cells. Moreover, overexpression of the NUP98-HOXA10 fusion gene appeared to block terminal differentiation, leading to a sustained output of cells with a “primitive” phenotype [28]. This stands in marked contrast to HOXB4, which has little effect on terminal differentiation events, or on cells with transient myeloid repopulating activity [29]. Accordingly we predicted that overexpression of the NUP98-HOXA10 fusion gene might have a more potent ability to stimulate HSC expansion in short-term culture than HOXB4.

Here we present the results of a systematic study that bears out this prediction. We quantified HSCs defined by rigorous functional endpoints and confirmed their pluripotency by examination of their clonal contributions to all lineages of regenerated lymphoid and myeloid cells using FACS and, proviral integration analysis. The average magnitude of the HSC expansions achieved by overexpression of the NUP98-HOXB4 fusion gene was ~300-fold, i.e., ~4 times the effect of HOXB4 alone using the same vector. The greater than 2000-fold

expansions of HSCs obtained using NUP98-HOXA10 fusion genes are unprecedented and come close to the theoretical limit in a maximum period of 7 to 8 days of gene expression, assuming no significant shortening of the reported 12- to 14-hour cell cycle time for these cells [26,27]. Even further levels of HSC expansion could be anticipated in more prolonged cultures (~10-fold expansion every 2 additional days). Indeed, extension of the culture period to 17 days in a preliminary experiment showed that the transduced HSC numbers continued to increase up to a total of greater than 10,000-fold (Fig. 4A and B). Moreover, the proliferation rate of control and NUP98-HOXA10-transduced Sca1⁺Lin⁻ cells in culture was comparable (data not shown), suggesting that any increase in HSC numbers is the result of altered self-renewal rather than an effect on cell cycle. Nevertheless, when these cells were transplanted into lethally irradiated recipients, they were able to repopulate the BM and respond to local regulatory mechanisms that directed their production of a balanced supply of phenotypically normal lymphoid and myeloid cells despite the continued presence of the integrated pro-virus and continued transgene expression. In addition, measurements of the number of progeny CRUs regenerated in the primary mice suggest that these reach normal CRU levels in the BM but do not become excessive (Fig. 5A). This dramatic differential behavior of Hox and NUP98-Hox transcription factors on HSC function in vitro versus in vivo points to a major interplay between intrinsic and extrinsic regulators in control of self-renewal. Thus it should prove of great interest to now dissect out the possible key role of growth factors (e.g., identity, combination, concentrations) and other microenvironmental factors such as stromal-niche that enable effectively unrestrained self-renewal behavior in vitro in contrast to normal regenerative and differentiation behavior in vivo.

The mechanism underlying the greater potency of NUP98-HOXA10 and even NUP98-HOXB4 over HOXB4 in stimulating HSC expansion in vitro is currently unclear. It may be due simply to an increased stability of the HOX component within the fusion protein. Alternatively, it may reflect the dominant transcriptional activation properties of NUP98 rather than intrinsic properties of the intact HOX protein. The ability of NUP98-HOXA10 to stimulate a greater HSC expansion than either NUP98-HOXB4 or HOXB4 may similarly reflect qualitative differences in the transcriptional activities of the encoded proteins and target genes affected. Given that the major function of HOX genes appears to lie in their ability to modulate gene expression, further pursuit of this possibility could provide a novel approach to elucidating the molecular mechanisms involved in HSC self-renewal control. In this regard, the greatly expanded populations of NUP98-HOXB4- and NUP98-HOXA10-transduced HSCs now readily obtained after a few days in culture should greatly facilitate further investigation of these questions.

An additional important finding was the ability of the HOXA10 homeodomain denuded of all adjacent sequences to mimic the effects of the complete exon 2 of HOXA10 when incorporated into a fusion protein with the same NUP98 sequences. Consistent with previous findings that the capacity of HOXB4 to induce HSC expansions does not require direct HOX/PBX interaction [40] and given the ability of antisense pbx1 to greatly enhance the competitive expansion of HOXB4-transduced HSCs in vivo [18] and in vitro (Cellot et al., accompanying paper), it is of particular interest that the PBX binding motif was not required for achieving high levels of HSC expansion by NUP98-HOX fusion genes. HOXA10 was reported to bind the p21 (Cip1/Waf1) promoter and activate p21 transcription in the presence of the cofactors PBX1 and MEIS1 [41]. Thus, consistent with the recent finding of an apparent synergistic effect of p21 deficiency and HOXB4-mediated expansion [20], it might be speculated that NUP98-HOX fusions somehow inhibit p21, facilitating the cell cycle entry of HSCs. However, such a mechanism is difficult to reconcile with the fact that the effects seen in vitro occurred under conditions optimized for HSC mitogenesis [7].

As anticipated and seen in our previous study [28], we confirmed the absence of any long-term hematological defects in recipients repopulated with NUP98-HOXB4-expanded HSCs. This was also true for recipients of NUP98-HOXA10-expanded HSCs that were followed for 6 to 8 months—consistent with the previously documented long latency of any myeloproliferative effect of NUP98-HOXA10. Strikingly, for NUP98-HOXA10HD, mice did not manifest disease even followed routinely for over 1 year posttransplant. We had previously documented the potent collaboration of NUP98-HOXA10HD with Meis1 in induction of leukemia [28, 29]; however, in those earlier studies (data not shown) and now confirmed here, NUP98-HOXA10HD (like HOXB4 or NUP98-HOXB4) has on its own no apparent leukemogenic activity. Of further interest, the lineage contributions from NUP98-HOXA10HD-transduced HSC were indistinguishable from normal. Together these findings suggest that restriction of the NUP98-fusion to the homeodomain sequence alone further blunted any differentiation abnormalities or intrinsic transforming potential. Whether this derives from removal of the PBX interacting domain remains to be determined. Together the shared ability of HOXB4, NUP98-HOXB4, and NUP98-HOXA10HD to stimulate HSC expansion, albeit with different potencies, should provide powerful new tools to identify key molecular pathways, e.g., target genes underlying the self-renewal program.

In the present studies we have also investigated the spectrum of target cells capable of producing expanded HSC populations and their individual ability to be amplified. This is a key question given the extreme heterogeneity of self-renewal activity displayed by individual normal HSCs in vivo [42]. By designing experiments that allowed the expansion of individual HSCs transduced with either NUP98-HOXA10 or the NUP98-HOXA10HD fusion gene to be followed, we could show that most if not all transduced HSCs could be greatly expanded within 8 to 12 days in culture, indicating that the effects seen were not due to a minor subset of HSCs. In addition, the use of partially purified populations of HSC both as transduction targets and as the harvested cells to be tested in vivo provided further evidence that the introduced NUP98-HOX fusion genes exert their effects by priming a pre-existing self-renewal mechanism already in place in HSCs. However, further confirmation of the precise target cell(s) responsive to the expanding properties of NUP98-HOX fusions would require systems for tracking highly purified, single HSCs and their progeny. The elimination of nontarget cells that along with HSC may undergo extensive expansion in culture would also allow studying the relationship between changes in HSC numbers and phenotype of NUP98-HOX-expanded cells. Consistent with this, in preliminary experiments initiated with single $\text{lin}^{-}\text{Rho}^{\text{dull}}\text{SP}$ cells [27] followed by NUP98-HOXA10HD transduction and in vitro expansion, we have observed the generation of almost entirely lineage-negative cultures that include cells with robust in vivo HSC activity.

In summary, we have developed a new strategy for consistently achieving very high expansion of HSCs ex vivo using a single agent. Together with recent publication revealing the potency of another NUP98-HOX fusion to increase the numbers of human long-term culture-initiating cells [43,44], our findings raise optimism for future extensions of this technology to enable even greater levels of HSC expansion to be obtained in vitro after longer times and/or different growth factor conditions. The approach described here also appears ideally suited to the type of protein delivery system afforded using TAT-fusion protein technology [25], which might allow HSC expansion without gene manipulation and be of considerable interest for both basic and clinical applications to human HSCs.

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Figure 1.

Experimental elements of the study. **(A)** Retroviruses used. A light gray rectangle indicates PBX-binding motif and the HD are illustrated as dark gray rectangles. LTR, long terminal repeat; IRES, internal ribosomal entry site; eGFP, enhanced green fluorescent protein. **(B)** General experimental design. BM cells were obtained from 5-FU-pretreated donor mice and then placed directly into medium containing DMEM, 15% FBS, IL-3, IL-6, and SCF, then incubated for 2 days (prestimulation) prior to virus exposure for another 2 days (infection) and then incubated for another 6 (or where indicated up to another 10 or 17 days) (expansion phase) in fresh medium of the same composition not including virus and without selection of GFP-expressing cells. Aliquots of cells used to initiate the cultures and harvested at the end of the culture period were then injected directly into irradiated Ly5 congenic recipients and the proportion of circulating B, T, and myeloid WBCs that were GFP⁺ and of the donor Ly5 allotype was then determined 4 to 6 months later. LDA, limiting-dilution analysis.

Figure 2.

Ex vivo expansion of HSCs in cultures of BM cells transduced with various NUP98-HOX fusions. **(A)** Limiting-dilution data from a representative experiment (see also Fig. 3A, Experiment III). In order to allow comparisons of CRU outputs from different experiments, all CRU “frequencies” are expressed “per starting equivalent cells”; i.e., per a constant fraction of the culture, regardless of the total nucleated cell output. **(B)** Summary of the HSC expansions obtained from 3 experiments in which the viruses shown were compared (see also Fig. 3A). Results are expressed as the mean $\pm$ SEM of the CRU numbers per culture after 6-day expansion phase. Horizontal bar represents an estimated range (based on the mean $\pm$ SEM) of CRU numbers measured in the starting population of 5-FU-treated BM cells.

Figure 3.

Robust and multi-lineage reconstitution in recipients of HSCs expanded in cultures after transduction of NUP98-HOX fusions. **(A)** Percent reconstitution (proportion of white blood cells that were GFP⁺) in each individual recipient of cells in 3 experiments investigating the vectors indicated. Solid symbols represent mice in which the criteria (described in the Methods) for multi-lineage positivity were met. Open symbols represent mice where this was not the case. † symbolizes a dead mouse; Tx (transplantation) dose is expressed in starting cell equivalents. **(B)** Representative peripheral blood FACS profiles of recipients transplanted ≥ 16 weeks previously with NUP98-HOXB4- or NUP98-HOXA10-transduced cells. Recipients received relatively high (starting equivalent of 200 NUP98-HOXB4- or 200 NUP98-HOXA10-transduced cells, mouse a and b, respectively) or low (starting equivalent of 20 NUP98-HOXB4- or 2 NUP98-HOXA10-transduced cells, mouse c and d, respectively) transplant dose. WBCs, white blood cells; RBCs, red blood cells. **(C)** Southern blot of DNA from myeloid, T lymphoid, and B lymphoid populations enriched or purified from BM, spleen (Sp), and thymus (Thy) of two representative recipients transplanted 24 weeks previously with starting cell equivalents of 500 (left panel) or 2 (right panel) NUP98-HOXA10-transduced cells.

Figure 4.

NUP98-HOXA10 fusion gene containing just the HOX homeodomain effectively expands transduced HSCs. **(A)** Percent reconstitution (proportion of white blood cells that were GFP⁺) in each individual recipient of cells from experiments IV, V, VI, and VII investigating the vectors indicated. Solid symbols represent mice in which the criteria (described in the Methods) for multi-lineage positivity were met. Open symbols represent mice where this was not the case. † symbolizes a dead mouse; Tx (transplantation) dose is expressed in starting cell equivalents. **(B)** Summary of the NUP98-HOXA10HD-induced HSC expansions obtained from 3 experiments shown in (A). Results are expressed as the mean $\pm$ SEM of the CRU numbers per culture after 6-day expansion phase (10 days total culture time). Horizontal bar represents an estimated range (based on the mean $\pm$ SEM) of CRU numbers measured in the starting population of 5-FU-treated BM cells. **(C)** Representative peripheral blood FACS profiles of recipients transplanted 8 months previously with starting equivalents of 250 (mouse e, f) or 0.25 (mouse g) NUP98-HOXA10HD-transduced cells and Southern blot of DNA from BM, spleen (Sp), and thymus (Thy) of the recipient transplanted 6 months previously with starting equivalent of 200 NUP98-HOXA10HD-transduced cells.

Figure 5.

Regeneration of HSCs following ex vivo expansion. **(A)** CRU regeneration following transplantation of the progeny of 200 day-0 NUP98-HOXA10- or 2000 day-0 NUP98-HOXA10HD-transduced cells. HSC (CRU) content (± 1 SE) in the bone marrow of primary recipients was determined at 16 weeks posttransplant by limiting-dilution assay in secondary recipients. HSC number per primary mouse were calculated from CRU frequencies and estimates of a total of 2×10^8 BM cells per adult mouse. **(B)** Representative bone marrow FACS profiles of two recipients transplanted 8 months previously with starting equivalents of 250 NUP98-HOXA10HD-transduced cells after ex vivo expansion, showing the percentage of c-kit⁺Sca1⁺lin⁻ cells within donor-derived (GFP⁺) BM and the nontransduced (GFP⁻) counterpart.

Figure 6.

Polyclonal expansion of NUP98-HOX-transduced HSCs in bulk cultures. DNA from representative recipients of NUP98-HOXA10-transduced cells (Experiment VI, mouse #1–#9) and NUP98-HOXA10HD-transduced cells (Experiments IV [mouse #1, #2, #3] and VI [mouse #1–#9] as shown in Figure 4A) was analyzed by Southern blotting for proviral integrants as described in the in the Methods. Tx (transplantation) dose is expressed in starting cell equivalents.

Figure 7.

NUP98-HOX fusion genes stimulate most HSCs to undergo large clonal expansions. **(A)** Experimental protocol for examining the ex vivo expansion of NUP98-HOX-transduced BM cells in cultures initiated with 1 to 2 CRUs. A series of mini-cultures were initiated with reduced numbers of input 5-FU-pretreated mouse BM cells, presumably containing 1 or 2 CRUs. Cells were placed directly, or after isolation of the Sca⁺Lin⁻ (SL) or c-kit⁺Sca⁺Lin⁻ subset as indicated, into medium containing DMEM, 15% FBS, IL-3, IL-6, and SCF, then incubated for 2 days (prestimulation, PS) prior to virus exposure for another 2 days (infection) and then incubated for another 6 or 10 days (expansion phase) in fresh medium of the same composition not including virus and without selection of GFP-expressing cells. Various fractions of cells

used to initiate each individual mini-culture were harvested at the end of the culture period and then injected directly into multiple irradiated Ly5 congenic recipients and the proportion of circulating B, T, and myeloid white blood cells that were GFP⁺ and of the donor Ly5 allotype was then determined 6 months later. **(B)** Percent reconstitution (proportion of white blood cells that were GFP⁺) in each individual recipient of cells harvested from minicultures initiated with small numbers of unseparated cells (after 6 or 10 days of expansion) or Sca1⁺lin⁻ or c-kit⁺Sca1⁺lin⁻ cells (after 6 days of expansion) estimated to initially contain 1 to 2 CRUs. Solid symbols represent mice in which the criteria (described in the Methods) for multi-lineage positivity were met. Open symbols represent mice where this was not the case. Tx (transplantation) dose is expressed as the fraction of the total culture transplanted. **(C)** DNA from different recipients shown in (B) was analyzed by Southern blotting for proviral integrants as described in the Methods.