Improved \textit{ex vivo} expansion of adult hematopoietic stem cells by overcoming CUL4-mediated degradation of HOXB4

Jennifer Lee\(^1\), Jae-Hung Shieh\(^2\), Jianxuan Zhang\(^1\), Liren Liu\(^1\), Yue Zhang\(^1\), Jae Yong Eom\(^1\), Giovanni Morrone\(^3\), Malcolm A. S. Moore\(^2\), and Pengbo Zhou\(^1\)

\(^1\)Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, NY 10065.
\(^2\)Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065.
\(^3\)Department of Experimental and Clinical Medicine, University of Catanzaro "Magna Graecia", Catanzaro, Italy.

*Running title: HOXB4 ubiquitination in \textit{ex vivo} HSC expansion

To whom correspondence should be addressed:
Pengbo Zhou
Department of Pathology and Laboratory Medicine
Weill Cornell Medical College
1300 York Avenue
New York, NY, USA.
Tel.: (212)746-6415
Fax: (212)746-4483
E-mail: pez2001@med.cornell.edu

\textbf{Keywords:} HOXB4, hematopoiesis, CUL4A, ubiquitination, degron, HSC, \textit{ex vivo} expansion
Key Points

- The CUL4 ubiquitin ligases target HOXB4 for ubiquitination and degradation.
- A degradation-resistant HOXB4 variant markedly enhanced ex vivo expansion of adult hematopoietic stem cells.

Abstract

Direct transduction of the homeobox protein HOXB4 promotes the proliferation of hematopoietic stem cells (HSCs) without induction of leukemogenesis, but requires frequent administration to overcome its short protein half-life (~1 hour). We demonstrate here that HOXB4 protein levels are post-translationally regulated by the CUL4 ubiquitin ligase, and define the degradation signal sequence (degron) of HOXB4 required for CUL4-mediated destruction. Additional HOX paralogs share the conserved degron in the homeodomain and are also subjected to CUL4-mediated degradation, indicating that CUL4 likely controls the stability of all HOX proteins. Moreover, we engineered a degradation-resistant HOXB4 that conferred a growth advantage over wild-type HOXB4 in myeloid progenitor cells. Direct transduction of recombinant degradation-resistant HOXB4 protein to human adult HSCs significantly enhanced their maintenance in a more primitive state both in vitro and in transplanted NOD/SCID/IL2R-γnull (NSG) mice compared to transduction with wild-type HOXB4 protein. Our studies demonstrate the feasibility of engineering a stable HOXB4 variant to overcome a major technical hurdle in the ex vivo expansion of adult HSCs and early progenitors for human therapeutic use.
Introduction

Hematopoietic stem cells (HSCs) are pluripotent, asymmetrically self-renewing cells that give rise to all mature blood cells through successive rounds of differentiation. The signals that govern the self-renewal process have been intensively pursued as it may be possible to promote HSC expansion by transiently enforcing proliferation pathways or blocking differentiation cues, which would be highly desirable as a means to augment the number of HSCs for transplantation. Currently, HSC transplantation is used to treat hematological diseases, such as leukemia and bone marrow failure. HSCs are derived from three sources: umbilical cord blood (UCB), adult bone marrow, and peripheral blood following treatment with recombinant human granulocyte colony-stimulating factor (G-CSF), a cytokine that mobilizes HSCs from the bone marrow and allows for their harvest through peripheral blood apheresis. Ex vivo expansion of HSCs from these sources would improve transplantation outcomes and help meet the demand for stem cell transplants by permitting the use of samples of limited quantity (e.g., cord blood) or with low total numbers of HSCs (e.g., poor HSC mobilizers).

Ex vivo expansion of HSCs and progenitor cells has been the subject of considerable investigation with the use of combinations of hematopoietic growth factors\(^1\), synthetic small molecular weight compounds identified by high-throughput screening\(^2\), and the use of supportive bone marrow stroma\(^3,4\). In human studies, adult HSCs can undergo repeated rounds of asymmetric self-renewal with maintenance of the stem cell pool but with little or no expansion. In contrast, fetal and neonatal stem cells can be maintained in culture for 2-3 months with absolute increases in the number of HSCs. Ex vivo expansions of 7-9 fold in SCID-repopulating HSCs have been reported over 12-14 days in a number of studies\(^1,3,5\).
Homeobox (HOX) genes encode transcription factors that regulate patterning during embryonic development, and hematopoiesis both pre- and post-natally. In early development, HOX gene expression is both temporally and spatially regulated, as reflected by the sequential order of transcription with respect to their 3' to 5' chromosomal position. However, the spatio-temporal regulation of HOX gene expression is not observed in hematopoiesis, but instead assumes a complex, overlapping expression pattern that is not lineage-specific. Northern blotting analysis revealed that most HOX genes, excluding HOXD, are highly expressed in HSCs and progenitor cells, and are generally downregulated as cells terminally differentiate into mature, lineage-specific blood cells. To date, the mechanisms underlying transcriptional regulation of HOX genes during hematopoiesis remain largely unknown.

The identification of HOX genes that are highly expressed in CD34+ HSCs and early progenitors led to several overexpression studies that revealed the effects of specific HOX genes on HSC proliferation. In particular, sustained HOXB4 expression by retroviral transduction promoted the selective expansion of murine HSCs in cell culture and following bone marrow transplantation. Moreover, the enhanced proliferation of HOXB4-transduced HSCs was not leukemogenic in transplanted mice, indicating that enforced HOXB4 expression did not alter HSC differentiation. In marked contrast, the ectopic expression of other HOX genes highly expressed in HSCs, such as HOXA9 and HOXB7, did result in myeloid leukemia. Comparison of the effects of HOXB4 overexpression in different species further revealed species-specific variations in the magnitude of HOXB4-induced HSC proliferation, with more modest effects observed in cells from humans and baboons than mice and dogs. Thus, the effect of ectopic HOXB4 expression in promoting human HSC proliferation is likely more equivocal than the robust outcome obtained in murine HSCs.
Follow-up studies revealed the development of myeloid leukemia in large mammals two years post-transplantation with HSCs expressing retrovirally-transduced HOXB4\(^\text{12}\), highlighting the risks of such genetic manipulation. While the molecular basis underlying the observed leukemogenesis appears to be HOXB4-dependent, integration of a transgene at chromosomal “hotspots” may also lead to the activation of adjacent oncogenes, and is believed to contribute to gene therapy-associated malignancy\(^\text{13-15}\). Therefore, a transient, non-viral delivery method to augment HOXB4 levels in HSCs is necessary for potential human therapeutic applications. To that end, direct protein transduction has been established as an alternative method of introducing HOXB4 into HSCs\(^\text{16,17}\). Comparable levels of \textit{ex vivo} murine HSC proliferation were achieved with the protein transduction of recombinant human HOXB4 as with retroviral integration\(^\text{17}\), but the short half-life of HOXB4 necessitated frequent replenishing of the recombinant protein, which is impractical for large-scale \textit{ex vivo} expansion of HSCs. Studies using human embryonic stem cells\(^\text{18}\) or bone marrow cells\(^\text{19,20}\) also demonstrate the effects of recombinant HOXB4 protein for therapeutic use. Stabilization of the HOXB4 protein through post-translational manipulation would represent a major advance in providing a renewable source of HSCs for human therapeutic use.

We previously studied the post-translational regulation of homeodomain-containing proteins by the CUL4A ubiquitin ligase, and determined that CUL4A targets HOXA9, a transcription factor that promotes HSC proliferation, for ubiquitination and proteosome-mediated degradation\(^\text{21}\). Additionally, the AML-associated Nup98-HOXA9 fusion protein is resistant to CUL4A-mediated ubiquitination, and the resulting protein stabilization may contribute to its potent leukemogenic activity\(^\text{22}\). In this study, we showed that representative members of multiple HOX paralogous groups, including HOXB4, are subjected to ubiquitination and subsequent
degradation by CUL4A. The degradation signal in HOXB4 was identified as the LEXE motif in helix I of the homeodomain, which is conserved in all 39 HOX HD proteins. Mutation of this motif resulted in the profound stabilization of HOXB4 protein levels. Retroviral transduction of 32D cells, a murine myeloid progenitor cell line, with degradation-resistant HOXB4 mutant dramatically enhanced their proliferation potential compared to wild-type HOXB4. Finally, the addition of recombinant degradation-resistant HOXB4 protein to adult HSCs resulted in higher numbers of colony-forming cells in vitro, and remarkable efficiency in the maintenance of primitive CD34+ cells in the engrafted bone marrow of NOD/SCID/IL2R-γnull mice.

Methods

Cell Cultures, Plasmids and Protein Extracts

Steady-state levels of epitope-tagged HOX proteins in response to increasing levels of MYC-CUL4A were determined following transfection of 293T cells with 1 µg of the indicated HOX plasmids (lanes 1-4) and 1, 3, or 9 µg MYC-CUL4A (lane 2-4). All DNA amounts were normalized with vector DNA, and protein levels were detected using standard Western blotting techniques with anti-MYC (Roche), anti-HA (Covance) and anti-β-actin (Santa Cruz) antibodies.

To measure the effect of CUL4A silencing on HOXB4 half-life, HeLa cells were transiently transfected with 6 µg MYC-HOXB4 and 6 µg shCONTROL or 6 µg shCUL4A. Pulse-chase of transfected HeLa cells was performed as previously described23. The half-lives of 35S-labeled-MYC-HOXB4 and HOXB4 degron mutants were determined by immunoprecipitation and immunoblotting with anti-MYC antibody, and quantitated via phosphorimaging.
To determine the effect of CUL4A inhibition on the ubiquitination of MYC-HOXB4, 293T cells were transiently transfected with 3 μg MYC-HOXB4 (lanes 1-5), 3 μg HIS-ubiquitin (lanes 2-5), and 1, 3, or 9 μg V5-tagged dominant negative CUL4A (DN-CUL4A, lane 3-5)\textsuperscript{23}. Transfected cells were treated with 25 μM MG132 for 4 hrs prior to harvesting. Cell lysates were precipitated with Ni\textsuperscript{2+}-NTA agarose beads (Qiagen), and immunoblotted with antibodies against MYC, V5 (Invitrogen), and α-tubulin (Sigma).

To determine the effect of the CUL4 ubiquitin ligases on endogenous HOXB4 protein levels in hematopoietic progenitor cells, bone marrow cells from wild-type and null Cul4a and Cul4b mice were isolated and progenitor cells were enriched using the EasySep\textsuperscript{TM} Mouse Hematopoietic Progenitor Cell Enrichment Kit (Stemcell Technologies). Genotyping was performed as previously described\textsuperscript{24}. HOXB4 protein levels were analyzed by Western blot analysis using standard techniques with an anti-HOXB4 antibody (Cell Signaling).

Mutations of the HOXB4 LEXE motif were generated using the QuikChange\textsuperscript{®} Multi Site-Directed Mutagenesis kit (Stratagene). The eGFP-HOXB4 homeodomain (wild-type and mutant) fusion constructs were generated by PCR amplification of wild-type and mutant HOXB4 homeodomain (HD) and ligation to pEGFP-C2 (BD Bioscience). Steady-state levels of eGFP-HOXB4 HD in response to increasing levels of MYC-CUL4A expression were determined following transfection of 293T cells with 1 μg eGFP-HOXB4 WT HD (lanes 1-4) or 1 μg eGFP-HOXB4 M3 HD (lanes 5-8), together with 1 μg (lanes 2 and 6), 3 μg (lanes 3 and 7), or 9 μg (lanes 4 and 8) MYC-CUL4A. All DNA amounts were normalized with vector DNA, and protein levels were detected using standard Western blotting techniques.

Wild-type and mutant HA-HOXB4 were retrovirally transduced into 32D cells as previously described\textsuperscript{21} and sorted by GFP expression. The MIGRI retroviral vector alone
(lacking a transgene) was also transduced into 32D cells as a negative control. Ectopic expression of wild-type and mutant HA-HOXB4 was confirmed by standard Western blotting techniques. The stable lines were cultured in IMDM (Mediatech) supplemented with 20% Serum Replacement (Invitrogen), 1% penicillin/streptomycin (Mediatech) and 2 ng/mL murine IL-3 (Miltenyi Biotech). Cells were passaged every three days, and counted daily. HOXB4 half-life was measured following addition of 50 μM cycloheximide (final concentration). Samples were harvested at the indicated times, and HA-HOXB4 levels were determined by Western blotting.

**In vitro HSC assays**

To express recombinant protein, wild-type and mutant HOXB4 were subcloned into the pTAT-HA expression vector (generous gift from Dr. Steven Dowdy). Recombinant protein was purified as previously described and desalted using PD-10 columns (GE Healthcare Life Sciences). G-CSF-mobilized CD34+ cells were grown in serum-free QBSF medium (Quality Biological) supplemented with 100ng/mL thrombopoietin (Miltenyi Biotech), c-Kit ligand (Amgen) and Flt3 ligand (Amgen), and 1μg/mL recombinant protein every other day. Colony-forming cell (CFC) assays were performed with G-CSF-mobilized CD34+ cells cultured in triplicate (500 cells/plate). Each plate contained 1mL of semisolid medium with 1.2% methylcellulose (Corning Chemicals), 20% Serum Replacement, 80 μM 2-mercaptoethanol, 2 mM L-glutamine, 1% penicillin/streptomycin, 0.125mM hemin (Sigma), 20 ng/mL G-CSF (Amgen), c-Kit ligand and IL-3 (Amgen), and 6 U/mL erythropoietin (Amgen) and 1 μg/mL recombinant proteins. Colonies were scored after two weeks of incubation at 37°C.

Limiting dilution cobblestone area-forming colony (CAFC) assays were performed in 96-well format with the indicated numbers of G-CSF-mobilized CD34+ cells added to MS-5 stromal
cells concurrently with transplantation into NSG mice. Cultures were changed weekly with the replacement of one-half of the culture volume with fresh media (MEM Alpha (MSKCC) supplemented with 12.5% horse serum (HyClone), 12.5% fetal calf serum, 80 μM 2-mercaptoethanol, 2 mM L-glutamine, 1% penicillin/streptomycin and 1 μM hydrocortisone (Sigma)). After 5 weeks, CAFCs were scored using an inverted-phase microscope as phase-dark hematopoietic areas of at least five cells beneath the MS-5 stromal layer.

**Transplantation experiments**

NOD/SCID/IL2R-γnull (NSG) mice bred in the MSKCC animal facility were irradiated at 250 cGy (cesium source). Each irradiated mouse (five mice/group) was transplanted via tail vein injection with 3x10^5 CD34+ G-CSF-mobilized adult HSCs (AllCells, LLC, Emeryville, CA) treated with two rounds of purified recombinant wild-type or degradation-resistant HOXB4 protein (1 μg/mL) added immediately following and 24 hours after overnight cytokine stimulation (100 ng/mL thrombopoietin, c-Kit ligand and Flt3 ligand). Mice were sacrificed at twelve weeks post-transplantation, and bone marrow was harvested for FACS analysis using monoclonal antibodies against human CD19, CD33, CD34 and CD45 (Miltenyi Biotech). All protocols were IRB- and IACUC-approved.

**Results**

**CUL4A mediates ubiquitination of HOXB4**

We previously determined that HOXA9 is specifically targeted for ubiquitination and degradation by CUL4A\(^{21}\), and observed that members of the HOX4 family also interacted with CUL4A by yeast two-hybrid assay (Zhang and Zhou, unpublished). Due to its well-established ability to promote HSC self-renewal, we first evaluated whether HOXB4 is subject to CUL4A-
mediated degradation. The steady-state levels of MYC-tagged HOXB4 decreased corresponding to increasing levels of MYC-CUL4A (Fig. 1A). Conversely, knockdown of CUL4A by shRNA prolonged the half-life of HOXB4 (Fig. 1B, S1). Following the addition of MG132, we observed that HOXB4 is polyubiquitinated, and enforced expression of dominant-negative CUL4A (previously described in\textsuperscript{23}) dramatically reduced ubiquitination of HOXB4 in a dose-dependent manner (Fig. 1C).

CUL4A and CUL4B are 82\% identical, and target similar substrates for ubiquitination and degradation\textsuperscript{24,25}. To determine the role of CUL4A and CUL4B on HOXB4 stability in hematopoietic progenitor cells, we compared the endogenous HOXB4 protein levels in hematopoietic stem and progenitor cells derived from\textit{Cul4a-} and\textit{Cul4b-null} mice versus their wild-type littermates, and observed that the deletion of either\textit{Cul4a} or\textit{Cul4b} resulted in the increased accumulation of HOXB4 protein levels (Fig. 1D). Taken together, these results indicate a role for both CUL4A and CUL4B as regulators of HOXB4 stability. Our studies hereafter utilized CUL4A as the representative CUL4 E3 ligase to investigate the biochemical mechanisms and functional implications of this E3 family in orchestrating the ubiquitination and degradation of HOXB4.

With the evidence that CUL4A targets both HOXA9 and HOXB4 for ubiquitination, we next sought to determine whether other HOX proteins in the Class I homeodomain family are subject to CUL4A-mediated degradation. Indeed, the steady-state levels of representative HOX paralogous group proteins, including HOXA1, A2, B7, B8, A11 and A13, also decreased corresponding to increasing levels of MYC-CUL4A (Fig. 2), while the HOX co-factor Meis1 was not subjected to CUL4A-mediated degradation (Fig. S2). Thus, the post-translational
regulation of HOX protein stability by the CUL4A ubiquitin ligase may be mediated through a conserved mechanism, such as recognition of a common HOX protein domain.

**CUL4A-specific HOXB4 degron**

Our previous studies on HOXA9 indicated that the homeodomain, which is required for DNA-binding activity of HOX proteins, is also necessary for CUL4A-mediated HOXA9 turnover. A sequence alignment of the highly conserved homeodomain among all HOX proteins revealed a conserved LEXE motif in helix I that is not involved in DNA binding (Fig. 3A). Given our observation that several HOX paralogous groups are post-translationally regulated by CUL4A, we examined whether the conserved LEXE motif may comprise the CUL4A-dependent degron, or the substrate sequence that directs its recognition by a specific ubiquitin ligase for degradation. While mutation of the first two residues of the LEXE motif prolonged the HOXB4 protein half-life compared to wild-type protein, the triple mutant, HOXB4(M3), dramatically extended HOXB4 protein stability (Fig. 3B, C). Accordingly, we designated the triple mutant as degradation-resistant HOXB4 or HOXB4(m).

To determine if the LEXE motif in the context of its native tertiary structure is required to target proteins for CUL4A-mediated degradation, wild-type or mutant HOXB4 homeodomain was fused to eGFP, a long-lived protein that is not a native CUL4A substrate. The fusion of eGFP to the wild-type HOXB4 homeodomain conferred sensitivity to CUL4A-mediated degradation, while the eGFP-HOXB4 mutant homeodomain protein remained resistant to increasing levels of CUL4A (Fig. 3D). We conclude from these studies that the LEXE motif within helix I of the HOXB4 homeodomain constitutes a transferable signal that is both necessary and sufficient to mediate CUL4A-dependent degradation.
**HOXB4(m) enhances 32D proliferation**

To determine whether HOXB4 degradation by CUL4A impacts the proliferation potential of hematopoietic cells, we retrovirally transduced HOXB4 or HOXB4(m) into the 32D murine myeloid progenitor cell line and observed that steady-state levels of HOXB4(m) were higher than wild-type HOXB4, while the mRNA levels were comparable (Fig. 4A, S3). Next, we compared their growth in serum-free medium and found that ectopic expression of either wild-type or degradation-resistant HOXB4 resulted in a distinct growth advantage over control cells. Remarkably, 32D cells expressing HOXB4(m) showed a further 10-fold increase in cell number compared to wild-type HOXB4-transduced cells after 15 days in culture, representing a 10,000-fold increase compared to 32D cells transduced with vector alone (Fig. 4B). Correspondingly, the half-life of HOXB4(m) was substantially longer than wild-type HOXB4 (Fig. 4C), indicating that the enhanced protein stability of HOXB4(m) contributes to the growth advantage of 32D myeloid progenitors.

**HOXB4(m) maintains HSC potential**

HSCs derived from peripheral blood are more readily available and abundant than those derived from umbilical cord blood (UCB) for stem cell transplantation. To determine the effect of HOXB4 on postnatal HSC expansion, we first compared the expression of HOXB4 in CD34+ cells from cord blood versus G-CSF-mobilized adult peripheral blood. As determined by quantitative RT-qPCR in Fig. 5A, HOXB4 mRNA levels are 12-fold lower in G-CSF-mobilized adult CD34+ cells than UCB CD34+ cells, suggesting that HOXB4 may be a limiting factor in the *ex vivo* expansion of G-CSF-mobilized adult HSCs, and that ectopic introduction of HOXB4
protein into G-CSF-mobilized adult CD34⁺ cells may promote more dramatic HSC expansion than UCB CD34⁺ cells.

Our goal of engineering the degradation-resistant HOXB4(m) protein is to overcome the labile nature of HOXB4 (t½ ~1 hour) used in protein-based ex vivo expansion of HSC and progenitor cells¹⁷. To this end, HOXB4 and HOXB4(m) were tagged with the cell-penetrating HIV TAT protein transduction domain that allows efficient translocation across cellular membranes (reviewed in²⁷ and references therein). It is also noteworthy that the homeodomain of HOXB4 possesses membrane-penetrating activity¹⁶. TAT-HA-HOXB4 and TAT-HA-HOXB4(m) were expressed as hexahistidine-tagged fusions in E. coli, affinity-purified, and applied to G-CSF-mobilized adult CD34⁺ cells for evaluation in clonogenic assays. Cell entry and peri-nuclear localization of recombinant HOXB4 and HOXB4(m) protein were observed in G-CSF-mobilized CD34⁺ cells (Fig. S4), consistent with the typical punctate distribution of transduced TAT-fusion proteins²⁸. Diffused nuclear localization was also observed, especially for TAT-HA-HOXB4(m) (Fig. S4). The transcriptional activity of putative HOXB4 target genes was similar following the addition of wild-type or degradation-resistant HOXB4 protein (Fig. S5). As shown in Fig. 5B-C, the total number of both myeloid and erythroid colony-forming cells increased >10-fold with the addition of HOXB4(m) compared to wild-type HOXB4 protein. Moreover, myeloid (CFU-GM) and erythroid burst-forming cells (BFU-E) grown in the presence of HOXB4(m) were significantly larger and more frequent (Fig. 5B-C). In contrast, UCB CD34⁺ cells responded similarly following the addition of recombinant wild-type or degradation-resistant HOXB4 protein (Fig. S6) further highlighting the effect of HOXB4(m) in G-CSF-mobilized adult HSCs. We also performed limiting dilution cobblestone area-forming colony (CAFC) assays to quantify the number of HSCs in G-CSF-mobilized adult CD34⁺ cells that were
treated with either wild-type or degradation-resistant HOXB4 recombinant protein. Untreated cells served as the baseline control. Addition of degradation-resistant HOXB4(m) protein doubled the number of HSCs measured by limiting dilution compared to both control and G-CSF-mobilized CD34⁺ cells treated with wild-type HOXB4 protein (Fig. 5D), indicating that the prolonged half-life of HOXB4 recombinant protein increased the efficiency in maintaining and expanding G-CSF-mobilized HSCs \textit{ex vivo}.

To determine the effect of wild-type versus degradation-resistant HOXB4 protein on the engraftment of G-CSF-mobilized adult HSCs and progenitors, CD34⁺ cells were treated with recombinant protein, transplanted into sublethally irradiated NOD/SCID/IL2R-γnull (NSG) mice, and analyzed 10 weeks post-transplantation. HOXB4(m) protein significantly enhanced the number of human CD34⁺ cells in the bone marrow of primary transplanted mice compared to both control and wild-type HOXB4 protein-treated mice, and multi-lineage engraftment was also observed (Fig. 5E). Strikingly, a 5-fold increase in the percentage of primitive CD34⁺ HSCs and progenitors was detected in the engrafted bone marrow of HOXB4(m)-transduced NSG mice. Thus, the degradation-resistant HOXB4(m) protein enhances proliferation of G-CSF-mobilized HSCs \textit{in vitro} and maintains the primitive state of HSCs more effectively than wild-type HOXB4 \textit{in vivo}.

\textbf{Discussion}

Previous studies have demonstrated the ability of HOXB4 to expand HSCs, but a practical method of delivering HOXB4 had yet to be devised that ensures safety and pharmaceutical compatibility. Although the ectopic expression of HOXB4 following retroviral transduction was not reported to induce leukemia in mice, follow-up studies have since identified
a causal relationship between the development of leukemia in large mammals and their transplantation with HSCs constitutively expressing HOXB4\textsuperscript{12}. Direct transduction of wild-type HOXB4 protein recapitulated the expansion of HSCs similar to enforced expression of the \textit{HOXB4} gene, but required frequent replenishing of the recombinant protein due to the short protein half-life\textsuperscript{17}. Subsequent studies have indicated a longer protein half-life is possible with the truncation of the HOXB4 N-terminus, but the exact mechanism that accounts for this effect is unknown\textsuperscript{18}. In this study, we have determined the biochemical mechanism for the post-translational regulation of HOXB4 that can be exploited to advance the field of \textit{ex vivo} HSC expansion. We previously demonstrated that HOXA9 was targeted for CUL4A-mediated degradation, and found that the degradation signal resided in the homeodomain\textsuperscript{21}. Here, we determined that CUL4A is responsible for the proteasome-mediated degradation of multiple HOX proteins, and identified a conserved homeodomain motif as a significant determinant for CUL4A-directed activity. Fusion of the HOXB4 homeodomain to a non-native CUL4A substrate was sufficient to target the protein for CUL4A-mediated degradation, indicating that the HOXB4 homeodomain contains a bona fide CUL4A degron. Future studies may exploit this finding to direct the degradation of proteins in a CUL4A-dependent manner.

Constitutive \textit{Cul4a}- and \textit{Cul4b}-knockout mice exhibited no defects in hematopoiesis\textsuperscript{24,25}. Here, we showed that the deletion of either CUL4 family members led to increased accumulation of HOXB4, indicating a redundant role for CUL4A and CUL4B \textit{in vivo}. Future experiments are required to address the regulatory mechanisms that trigger CUL4-mediated degradation of HOX proteins, which may have significant effects on the proliferation and/or differentiation of hematopoietic cells. Quantitative RT-PCR revealed higher levels of HOXB4 mRNA in CD34\textsuperscript{+} umbilical cord blood cells than adult CD34\textsuperscript{+} cells, which may account for the modest effect of
exogenous HOXB4 protein on UCB CD34+ cells compared to G-CSF mobilized adult CD34+ progenitor cells in colony-forming cell assays. Moreover, degradation-resistant HOXB4 enhanced the proliferation of the murine myeloid progenitor 32D cell line and the generation of more primitive myeloid and erythroid colonies by G-CSF CD34+ progenitor cells compared to wild-type HOXB4, further indicating that the effect of degradation-resistant HOXB4 may be augmented in hematopoietic progenitor cells with limiting levels of HOXB4.

Over 50,000 hematopoietic stem cell transplants are performed each year worldwide, of which the vast majority employ adult HSCs mobilized into the peripheral blood following treatment with G-CSF. HSCs from umbilical cord blood or adult G-CSF-mobilized bone marrow/peripheral blood are not equivalent in their transplantation outcome. Cord blood is limited in the number of CD34+ stem/progenitor cells that can be obtained (1-2x10^6), an amount sufficient for the engraftment of children or small adults. For larger adults, the use of two cord blood units has proven effective but requires a double match and considerable increased cost. Transplantation with HSCs derived from a sibling or unrelated matched bone marrow or G-CSF-mobilized peripheral blood donors requires a minimum of 2x10^6 CD34+ cells/kg. In the case of poor mobilizers, combinations of AMD3100 and G-CSF can be used to increase CD34+ yield and/or multiple leukaphereses may be undertaken. A disadvantage is the time required to identify a suitable match and to schedule the harvest. In light of the unstable nature of recombinant wild type HOXB4 protein that impedes the development of protein-based ex vivo expansion of adult HSCs and progenitors, our studies represent an important advance in elucidating a post-translational regulatory mechanism of HOXB4 protein stability that may be exploited to maintain the proliferative and repopulative potential of HSCs and early progenitors. Up to one-third of autologous transplant patients require additional rounds of G-CSF treatment to mobilize
sufficient numbers of HSCs for transplant. We demonstrated that degradation-resistant HOXB4 protein showed a stronger effect in expanding G-CSF-mobilized HSCs, which may be used to eliminate the need for repeat HSC mobilization procedures. Future studies will seek to fully reconstitute the HOXB4 ubiquitination reaction in vitro, which may offer mechanistic insight into the specific inhibition of HOXB4 degradation to promote the expansion of hematopoietic stem and progenitor cells.

Acknowledgements

We thank Lorraine Gudas (Weill Cornell Medical College), Steve Dowdy and Mark Kamps (University of California, San Diego) for plasmids, Lisa Prevedel for technical assistance, and Jeffrey Hannah for critical reading of the manuscript. This work is supported by National Institutes of Health Grant CA118085 and CA098210, the Leukemia and Lymphoma Society Scholar Award, the Irma T. Hirschl Career Scientist Award to P.Z, the AIRC IG-9204 grant to G.M., and the Roberta C. Rudin Leukemia Research Fund, the Gar Reichman Fund of the Cancer Research Institute and National Institutes of Health Contract NIAID HHSN272200900059C to M.A.S.M.

Authorship Contributions

J.L. and J-H.S. designed and performed research, and analyzed and interpreted the data. J.Z., L.L., Y.Z. and J.Y.E. performed part of the assays. P.Z. and M.A.S.M. designed and directed the research and provided funding. G.M. contributed to data analysis and interpretation. J.L. and P.Z. wrote the manuscript. G.M. and M.A.S.M. edited the paper.

Disclosure of Conflicts of Interest

The authors declare no competing interests.
References


Figure Legends

Figure 1. CUL4A targets HOXB4 for ubiquitin-dependent degradation. A. Steady-state levels of MYC-HOXB4 were determined in response to increasing levels of co-transfected MYC-CUL4A in 293T cells by immunoblotting with the anti-MYC and β-actin (loading control) antibodies. B. Pulse-chase analysis of HOXB4 half-life was performed following shCUL4A knockdown. C. In vivo ubiquitination of HOXB4 in response to increasing levels of dominant-negative CUL4A was detected in 293T cells following tranfection with the indicated plasmids, treatment with MG132, precipitation with Ni²⁺-NTA agarose beads under denaturing conditions and immunoblotting with anti-MYC antibody. D. To determine the physiological effect of CUL4A and CUL4B on endogenous HOXB4 protein levels, bone marrow stem and progenitor cells from Cul4a and Cul4b knockout mice and their wild-type littermates were purified and analyzed by immunoblotting with anti-HOXB4. The upper panel showed the genotyping of Cul4a⁻/⁻ and Cul4b⁺/⁻ mice by PCR.

Figure 2. CUL4A targets multiple HOX proteins for degradation. Steady-state levels of indicated HOX proteins were reduced in response to increasing levels of transfected CUL4A, as determined by immunoblotting with the appropriate antibodies. β-actin levels were determined by immunoblotting as loading controls.

Figure 3. HOXB4 homeodomain contains the CUL4A-dependent degron.
A. A sequence alignment of the homeodomains of all HOX paralogous groups revealed a conserved LEXE motif. B, C. Half-lives of HOXB4 LEXE mutants. Pulse-chase analysis of
HOXB4 LEXE mutants (M1: L175D; M2: L175D, E176K; M3: L175D, E176K, E178K) was performed following transfection of the MYC-tagged proteins into HeLa cells, $^{35}$S-Met labeling, and immunoprecipitation with the anti-MYC antibody. The percentages of HOXB4 and the LEXE mutant proteins remaining at each time point were normalized relative to time 0 of each protein. The percentages of HOXB4 or LEXE mutant proteins remaining at each time point are indicated. D. Determination of the steady-state levels of GFP-HOXB4 homeodomain fusion protein in response to increasing MYC-CUL4A levels by immunoblotting with antibodies against GFP or MYC epitope tags or β-actin as loading control.

**Figure 4. Degradation-resistant HOXB4 confers proliferative advantage to 32D myeloid cell line.**

A. Expression levels of HA-HOXB4 (wild-type or mutant) in 32D stable lines, as determined by immunoblotting with the anti-HA antibody. B. Cumulative expansion of 32D stable lines in serum-free medium. C. Cycloheximide chase analysis for the half-lives of HA-HOXB4 (wild-type or mutant) in 32D stable lines. HA-HOXB4 or HA-HOXB4(m) levels were determined by immunoblotting at the indicated time points. β-actin levels were determined as loading control.

**Figure 5. Direct transduction of recombinant degradation-resistant HOXB4 protein maintains G-CSF-mobilized CD34⁺ cells in a more primitive state than wild-type HOXB4.**

A. Comparison of HOXB4 mRNA expression levels in CD34⁺ UCB cells vs. CD34⁺ G-CSF-mobilized cells. B, C. Comparison of granulocyte-monocyte (CFU-GM) and erythroid (BFU-E, CFU-E) colony-forming cells following addition of wild-type or degradation-resistant recombinant HOXB4 protein to CD34⁺ G-CSF-mobilized cells. D. Limiting dilution CAFC
assays of G-CSF-mobilized CD34+ cells following addition of wild-type or degradation-resistant HOXB4 protein. E. Multi-lineage engraftment of G-CSF-mobilized CD34+ cells treated with wild-type or degradation-resistant recombinant HOXB4 protein in NSG mice (n=8 per group) 12 weeks post-primary transplantation. The experiment was independently repeated three times.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Improved ex vivo expansion of adult hematopoietic stem cells by overcoming CUL4-mediated degradation of HOXB4

Jennifer Lee, Jae-Hung Shieh, Jianxuan Zhang, Liren Liu, Yue Zhang, Jae Yong Eom, Giovanni Morrone, Malcolm A. S. Moore and Pengbo Zhou