TGF-β as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation

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Hematopoietic stem cells (HSCs) reside in a bone marrow niche in a nondividing state from which they occasionally are aroused to undergo cell division. Yet, the mechanism underlying this unique feature remains largely unknown. We have recently shown that freshly isolated CD34+ KSL hematopoietic stem cells (HSCs) in a hibernation state exhibit inhibited lipid raft clustering. Lipid raft clustering induced by cytokines is essential for HSCs to augment cytokine signals to the level enough to re-enter the cell cycle. Here we screened candidate niche signals that inhibit lipid raft clustering, and identified that transforming growth factor-β (TGF-β) efficiently inhibits cytokine-mediated lipid raft clustering and induces HSC hibernation ex vivo. Smad2 and Smad3, the signaling molecules directly downstream from and activated by TGF-β receptors were specifically activated in CD34+ KSL HSCs in a hibernation state, but not in cycling CD34+ KSL progenitors. These data uncover a critical role for TGF-β as a candidate niche signal in the control of HSC hibernation and provide TGF-β as a novel tool for ex vivo modeling of the HSC niche. (Blood. 2009;113:1250-1256)
for a single FoxO gene, FoxO3a, much milder but similar defects were observed. These results suggest that FoxOs play essential roles in the establishment of resistance to physiologic oxidative stress, a resistance necessary to ensure the quiescence, survival, and function of HSCs. These findings demonstrate a tight correlation between lipid raft status and Akt-FoxO signaling in the context of HSC hibernation and survival and indicate that LRC plays a key role in HSC emergence from hibernation and that LRC-inhibitory signals from the BM niche are critical in the induction and maintenance of HSC hibernation.

One of the niche signaling molecules, TGF-β, acts as a negative regulator of hematopoietic stem and progenitor cell proliferation in vitro. Upon association with TGF-β, TGF-β type II receptor (TβRII) forms a complex with TGF-β type I receptor (TβRI). Subsequently, the activated TGF-β receptor complex phosphorylates receptor-activated Smads (R-Smad2) and R-Smad3. R-Smads eventually heterodimerize with the common mediator Smad4, and the resulting complex translocates to the nucleus and recruits transcriptional cofactors to control expression of genes, including those involved in the cell cycle. It has been reported that TGF-β1-null mice and inducible TβRII knockout models develop a transplantable lethal inflammatory disorder affecting multiple organs. However, mice deficient in the TβRI, activin receptor-like kinase 5 (ALK-5), show no defects in HSC quiescence or in maintenance of the HSC pool. Mice deficient for the TGF-β type II receptor have not been well characterized with respect to HSC hibernation. TGF-β signaling deficiency so far has not revealed any effect on HSC proliferation and differentiation in vivo. Therefore, the outcome of TGF-β signaling is believed to be context dependent in hematopoiesis and the regulation of hematopoietic stem and progenitor cells is more complicated in the BM microenvironment in vivo than is seen in liquid cultures ex vivo.

In this study, we screened candidate niche signals that inhibit lipid raft clustering and identified that TGF-β efficiently inhibits cytokine-mediated LRC. We further characterized its role in HSC hibernation.

**Methods**

**Mice**

C57BL/6 (B6-Ly5.2) mice were purchased from Japan SLC (Shizuoka, Japan). C57BL/6 mice congenic for the Ly5 locus (B6-Ly5.1) were purchased from Sankyo-Lab Service (Tsukuba, Japan). C57BL/6 Ly5.1 × Ly5.2 F1 mice were bred and maintained in the Animal Research Facility of the Institute of Medical Science, University of Tokyo. Animal care in our laboratory was in accord with the guidance of Tokyo University for animal and recombinant DNA experiments.

**Purification of mouse HSCs and CD34+ KSL cells**

Mouse CD34+ KSL HSCs and CD34+ KSL progenitor cells were purified from BM cells of 2-month-old mice. In brief, low-density cells were isolated on Lymphoprep (1.086 g/mL; Nycome, Oslo, Norway). The cells were stained with an antibody cocktail consisting of biotinylated anti–Gr-1, –Mac-1, –B220, –CD4, –CD8, and –Ter-119 monoclonal antibodies (Phar-Mingen, San Diego, CA). Lineage-positive cells were depleted with antibiotin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The remaining cells were further stained with fluorescein isothiocyanate (FITC)–conjugated anti-CD34, phycoerythrin (PE)–conjugated anti–Sca-1, and allophycocyanin (APC)–conjugated anti–c-Kit antibodies (PharMingen). Biotinylated antibodies were detected with streptavidin-APC Cy7 (Molecular Probes, Eugene, OR). Analysis and cell sorting were performed on a MoFlo using Summit software (Dako, Glostrup, Denmark) and results were analyzed with FlowJo software (TreeStar, Ashland, OR). Immunofluorescent staining and linearization analysis

The markers and antibodies used were the DNA marker 4,6-diamidino-2-phenylindole (DAPI), Alexa-488–conjugated cholera toxin B subunit (CTXB), Alexa-647–conjugated goat anti-rabbit IgG, goat anti–mouse IgG, and Alexa-488–conjugated goat anti–rabbit IgG (Molecular Probes, Carlsbad, CA), rabbit anti–phospho-Akt and rabbit anti-FOXO3a (Upstate Cell Signaling, Charlotteville, VA), rabbit anti-p57 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti–phospho-Smad2/3 (CHEMICON, Temecula, CA), and rabbit anti–phospho-Src (Y418; Biosource, Camarillo, CA). Individual CD34− KSL cells were sorted into a serum-free culture-medium drop supplemented with 50 ng/mL mouse SCF and/or 50 ng/mL human TPO on slide glasses. The sorted cells were incubated at 37°C for the indicated time periods. After fixation with 2% paraformaldehyde and blocking in 10% goat serum for 1 hour at room temperature, cells were incubated with a primary antibody for 12 hours at 4°C. The cells were then washed and were incubated with a secondary antibody for 30 minutes at room temperature. Immunofluorescence was observed with a Leica TCS SP2 AOS confocal microscope (Wetzlar, Germany) or with an Olympus Laser Scanning Cytometer 2 (LSC2; Tokyo, Japan).

**Single-cell culture**

CD34− KSL cells were clonally deposited into 96-well microtiter plates containing 200 μL S-Clone SF-03 (Sanko Junyaku, Tokyo, Japan) supplemented with 5 × 10−5 M 2-β-mercaptoethanol, 10% FCS, and the indicated cytokines (20 ng/mL mouse SCF, 50 ng/mL human TPO, 20 ng/mL mouse IL-3, and 2 U/mL human EPO) in the presence or absence of 5 ng/mL human TGF-β1, TGF-β2, TGF-β3, latent TGF-β1, Activin-A, and Nodal (R&D Systems, Minneapolis, MN). Survival and cell division of HSCs were monitored by microscopy. To allow colony formation, single HSCs were cultured in the presence of SCF, TPO, IL-3, EPO, and anti-TGF-β blocking antibody (R&D Systems) for 11 days. Colonies were recovered, cytospun onto glass slides, and subjected to May-Gru ¨nwald-Giemsa staining for morphologic examination.

**Competitive repopulation assays**

Competitive repopulation assays were performed using the Ly5 system. In brief, single cultured HSCs or pooled single cultured HSCs (B6-Ly5.1) were mixed with 2 × 105 BM competitor cells (B6-F1) and were transplanted into B6-Ly5.2 mice irradiated at a dose of 9.5 Gy. After transplantation, peripheral blood cells of the recipients were stained with biotinylated anti-Ly5.1 (A20) and FITC-conjugated anti-Ly5.2. The cells were simultaneously stained with PE-Cy7–conjugated anti-B220 antibody, a mixture of APC-conjugated anti–Mac-1 and –Gr-1 antibodies, or a mixture of PE-conjugated anti–CD4 and –CD8 antibodies (PharMingen). Biotinylated antibody was developed with streptavidin Alexa-594 (Molecular Probes, Carlsbad, CA). The cells were analyzed on a fluorescence-activated cell sorting (FACS) Vantage (BD. Franklin Lakes, NJ). Percentage chimerism was calculated as (percentage Ly5.1 cells) × 100/(percentage Ly5.1 cells + percentage F1 cells). When percentage chimerism of donor-derived cells was more than 1.0 (summed over myeloid, B-lymphoid, and T-lymphoid lineages), recipient mice were considered to be multilineage reconstituted (positive mice).

**RT-PCR**

Semi-quantitative RT-PCR was carried out using normalized cDNA and quantitative PCR with TaqMan rodent GAPDH control reagent (Perkin-Elmer Applied Biosystems, Foster City, CA) as previously described. Cycling parameters were as follows: denaturation at 95°C for 15 seconds, annealing at 58°C for 15 seconds, and extension at 72°C for 30 seconds. Amplification proceeded for 38 cycles. PCR products were separated on 1.4% agarose gels and visualized by ethidium bromide staining.
We then asked whether TGF-β induces quiescence in HSCs. Single CD34+ KSL HSCs were cultured in the presence of SCF, TPO, and TGF-β1. In the presence of SCF and TPO, more than 85% of HSCs proliferated robustly, but in the absence of SCF or TPO, none survived more than 24 hours (data not shown). In contrast, addition of TGF-β1 in culture strongly suppressed colony formation of single HSCs in a dose-dependent manner (Figure S2). Detailed observation revealed that addition of 5 ng/mL TGF-β1 in culture strongly suppressed division of single HSCs that, however, remained alive. During 5-day culture, 57% of single HSCs stayed dormant, that is, persisted as living single cells, and 22% of single HSCs divided only once (Figure 2A). Similar results were obtained when HSCs were cultured under another HSC-supporting cytokine condition, SCF plus IL-11 (Figure S3). After culture medium was changed to an optimal medium supplemented with SCF, TPO, IL-3, and EPO, 72.2% of single HSCs, which had stayed dormant for 5 days, gave rise to colonies; of these, 47% were neutrophil/macrophage/erythroblast/megakaryocyte (nmEM) colonies, derived from colony-forming units–nmEM (CFU-nmEMs) with multipotency, that is, a full range of differentiation capacity along myeloid lineages (Figure 2B). Thus, 33.9% of surviving single HSCs could retrospectively be inferred to have been CFU-nmEMs. Even after 7 days of culture, most single HSCs retained multipotency (Figure 2B). These data demonstrate that TGF-β can keep HSCs in hibernation without loss of higher-order biologic potential ex vivo. Furthermore, when we compared the activities of TGF-β1, TGF-β2, and TGF-β3 with respect to induction of the hibernating state, some HSCs survived as single cells for more than 15 days in culture in the presence of TGF-β3 (Figure S4).

To obtain direct evidence of HSC activity, we performed competitive hematopoiesis repopulation assays in vivo. We again selected single HSCs that had not divided during 5-day clonal single-cell culture in the presence of SCF, TPO, and TGF-β. Single HSCs or pools comprising 20 individual HSCs were transplanted into lethally irradiated recipient mice. As a control, freshly isolated single HSCs or pools comprising 20 individual HSCs were similarly transplanted. Comparable proportions of freshly isolated and cultured hibernating (in the presence of TGF-β) single HSCs exhibited LTR activity (26% and 20%, respectively); establishment of chimerism also was comparable (13.5% and 9.5%, respectively) (Figure 2C). In contrast, when single HSCs were cultured in the presence of SCF and TPO without TGF-β, they robustly proliferated but lost LTR activity (data not shown). All recipient mice infused with pools of 20 freshly isolated HSCs showed donor cell repopulation. So did those infused with pools of 20 cultured single
HSCs, although established chimerism declined compared with that established by freshly isolated HSCs. All these data strongly support the proposition that TGF-β induces hibernation in HSCs ex vivo without affecting HSC capacity to self-renew and to differentiate into a full range of hematopoietic cell lineages.

**The TGF-β signal is active in hibernating niche HSCs**

The activated TGF-β receptor complex phosphorylates Smad2 and Smad3. Smad2 and Smad3 use Smad4 as a partner to form a transcriptionally active complex. To obtain physiologic evidence that supports active TGF-β signaling in niche HSCs, we next examined TGF-β signals in freshly isolated HSCs. Smad2/3, the signaling molecules directly downstream from and activated by TGF-β receptors, were highly phosphorylated in freshly isolated CD34+ KSL HSCs, where they accumulated in the nucleus. In contrast, Smad2/3 were scarcely phosphorylated in CD34+ KSL progenitor cells (Figure 3A). Quantification of the levels of Smad2/3 phosphorylation by laser scanning microscopy showed a striking contrast between freshly isolated CD34+ KSL HSCs and CD34+ KSL progenitors (Figure 3A). These data strongly indicate that the TGF-β signaling pathway is active in HSCs in the BM niche, but not in progenitor cells. We observed, in keeping with this, that Smad2/3 in HSCs were rapidly dephosphorylated by cytokine stimulation. Pretreatment of HSCs with TGF-β, however, again counteracted cytokine stimulation and Smad2/3 remained phosphorylated (Figure 3A).
We previously reported that CD34−KSL HSCs express a high level of p57Kip2, whereas CD34+KSL progenitor cells do not. Of note was that p57 as well as cyclin D1, D2, and D3 localize in the cytoplasm in HSCs. That TGF-β up-regulates p57 expression in human primitive hematopoietic cells to induce cell-cycle arrest intrigued us. To verify this finding in mouse HSCs, we stimulated freshly isolated CD34−KSL HSCs with SCF and TPO for 12 hours, to down-regulate p57; we then treated the cells with TGF-β. Twelve hours after the addition of TGF-β, p57 was abundantly reinduced at both mRNA and protein levels, whereas expression of p21 and p27 did not change at all (Figure 3B). These data indicate that TGF-β regulates the expression of p57, which supposedly functions as a specific CDKI that binds to and suppresses the activity of the cyclin D/CDK complexes in HSCs.

TGF-β induces hibernation, but other TGF-β family members do not

Smad2 and Smad3 are activated not only by TGF-β, but also by Activin and Nodal. We evaluated the effects of these agents on HSC cell cycle. Single CD34−KSL HSCs were cultured in the presence of SCF, TPO, and Activin or Nodal. TGF-β strongly suppressed division of single HSCs; 65.7% of them stayed dormant during 2-day culture. Activin-A and Nodal were not efficient in suppressing division of single HSCs; they, respectively maintained dormancy in only 6.6% and 6.9% of HSCs during 2-day culture (Figure 4). These data establish that within its family TGF-β has a major role in maintenance of HSC hibernation.

Activation of latent TGF-β is required for TGF-β bioactivity

TGFβ reportedly is produced not only by niche cells, but also by HSCs themselves. As expected, HSCs expressed a significant level of TGF-β1 and a low level of TGF-β3, but not Activin A or Nodal, indicating the presence of both autocrine and paracrine regulatory loops of TGF-β signaling (Figure 5A). Importantly, however, TGF-β is produced as an inactive form, latent TGF-β. We asked whether HSCs themselves could activate latent TGF-β to establish an autocrine TGF-β signaling loop. We seeded single CD34−KSL HSCs in the presence of SCF and TPO along with either active-form TGF-β or latent TGF-β, and allowed the HSCs to form colonies. TGF-β strongly suppressed colony formation, whereas latent TGF-β did not affect colony formation at all. These data indicate that HSCs can produce latent TGF-β but cannot activate it by themselves. Since TGF-β is produced by a variety of cells as an inactive form, the capacity to activate latent TGF-β could be a key property of BM niche cells.

Discussion

The cell-cycle status of HSCs in the niche is supposed to be precisely regulated by a specific combination of niche signals. We have reported an unexpected role of lipid raft organization in the maintenance of HSC hibernation through regulating the PI3K-Akt-FoxO pathway that lies downstream of cytokine signaling. HSCs are exposed to a variety of secreted and membrane-bound growth factors in the niche. Nonetheless, our findings clearly demonstrate that lipid raft reorganization is strictly inhibited in HSCs in the niche. We inferred that nonclustered lipid raft microdomains finely tune cytokine signals and mediate them toward suitability for HSC survival in the hibernating state, and that some niche signals inhibit lipid raft reorganization to maintain HSC hibernation. These findings support a novel model in which HSC fate, that is, hibernation or cell-cycle re-entry, largely depends on lipid raft regulation.

We have now identified that TGF-β inhibits cytokine-induced LRC (Figure 1A). TGF-β suppresses Akt activation and induces nuclear accumulation of FoxO3a in HSCs. It also inhibits translocation of cyclin D1 into the nucleus and maintains high cytoplasmic accumulations of p57 (Figure 1D and data not shown). Through these mechanisms, TGF-β strongly inhibits cell division and maintains HSCs in the hibernating state ex vivo. Together with our finding that Smad2 and Smad3, which are activated by the TGFβ receptor complex, are selectively and highly phosphorylated in CD34−KSL HSCs, but not in CD34−KSL progenitor cells, these findings strongly indicate a physiologic role for TGF-β in HSC hibernation in the
niches (Figure 3A). This notion is also supported by the study of *C elegans*, which indicated a critical role of Daf-7 as a positive regulator of Daf-16.22 Daf-7 is a TGF-β-like molecule. Via its receptor and downstream signaling molecules (Daf-4, Daf-1, Daf-8, and Daf-14), it up-regulates Daf-16 expression and exerts a dauer larval gene program.23

TGF-β is widely expressed in BM by elements that include osteoblasts and other stromal cells. Importantly, however, TGF-β is produced as a latent form. Latent TGF-β must be processed and activated. As shown in Figure 5B, HSCs are not able to activate latent TGF-β. That the BM niche is where TGF-β can be processed/activated and where TGF-β induces HSC hibernation is thus a tempting hypothesis. In contrast, Ang-1, another regulator of HSC hibernation, was much less effective than TGF-β in inhibiting cytokine-induced LRC and subsequent Akt activation (data not shown). Recently, the TPO signal was proposed as an essential component for HSC hibernation in the osteoblastic niche.24 TPO efficiently induces LRC and activates the PI3K-Akt pathway in vitro. However, its signal is supposedly attenuated by inhibited LRC in hibernating HSCs in the niche. We assume that the attenuated TPO signal by inhibitory niche signals including TGF-β acts as a survival signal but not proliferation signal on HSCs and holds the key in keeping HSCs in hibernation. These data highlight lipid raft assembly and its regulation by TGF-β as a novel regulatory component of HSC hibernation. Our findings thus indicate that HSC hibernation is regulated by at least 2 different routes, the Ang-1–Tie-2 and TGF-β–Smad signaling pathways, and establish a central role for TGF-β in regulating the lipid raft–PI3K–Akt–FOXO pathway.

Although TGF-β has been well characterized as a negative regulator of hematopoietic stem and progenitor cell proliferation in vitro,7 mice models deficient for TGF-β signaling molecules, including ALK-5 TβRI, show no defects in maintenance or quiescence of HSCs.17 These discrepancies may be at least partly explained by the considerably low mRNA expression of ALK-5 in HSCs compared with that in E12.5 total embryo (Figure 5A), which is indicative of alternative TβRIs in HSCs. Mice deficient for the TβRII have not been well characterized with respect to HSC hibernation because of the lethal inflammatory disorder affecting multiple organs.16 Furthermore, overlapping receptor and Smad usage by different TGF-β superfamily ligands (TGF-βs, BMPs, and Activins) accounts for their functional redundancies, making their signaling pathways in HSCs hibernating in the BM niche.25 Whether the latent TGF-β can transduce alternative signals via an as-yet-unrecognized pathway in HSCs is a tempting question to be addressed.

Our findings stress the critical role of lipid rafts in regulating the cell-cycle status of HSCs and demonstrate a novel interplay between the lipid raft–PI3K–Akt–FoxO and the TGF-β–Smad signaling pathways in HSCs hibernating in the BM niche. Smad proteins activated by TGF-β could form a complex with FoxO proteins FoxO1, FoxO3a, and FoxO4.32 TGF-β–mediated interaction between these 2 signaling pathways thus could hold a key role in the regulation of gene expression that controls HSC hibernation.

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Authorship

Contribution: S.Y., A.I., and H.E. designed the research and analyzed data; S.Y., A.I., and H.N. wrote the paper; K.E. contributed vital new data; S.Y., A.I., and H.N. wrote the paper. Correspondence: Hiromitsu Nakauchi, 4-6-1 Shirokanedai, Minato-ku, Tokyo, Japan; e-mail: nakauchi@ims.u-tokyo.ac.jp.

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