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Murine Hematopoietic Stem Cells and Multipotent Progenitors Express Truncated Intracellular Form of c-kit Receptor

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Abstract

The c-kit receptor plays a vital role in self-renewal and differentiation of hematopoietic stem cells (HSCs) and multipotent progenitors (MPPs). We have discovered that besides c-kit, the murine multipotent HSC/MPP-like cell line EML expresses the transcript and protein for a truncated intracellular form of c-kit receptor, called tr-kit. Notably, the tr-kit transcript and protein levels were down-regulated during cytokine-induced differentiation of the HSC/MPP-like cell line EML into myeloerythroid lineages. These findings prompted us to analyze tr-kit expression in purified murine fetal liver and bone marrow cell populations containing long-term repopulating (LTR) HSCs, short-term repopulating (STR) HSCs, MPPs, lineage-committed progenitors, and immature blood cells. Remarkably, these studies have revealed that in contrast to more widespread expression of c-kit, tr-kit is transcribed solely in cell populations enriched for LTR-HSCs, STR-HSCs, and MPPs. On the other hand, cell populations in which HSCs and MPPs are either present at a much lower frequency or are absent altogether, cells representing more advanced stages of differentiation into lymphoid and myeloid lineages do not express tr-kit. The observation that tr-kit is co-expressed with c-kit only in more primitive HSC- and MPP-enriched cell populations raises an exciting possibility that tr-kit functions either as a new component of the stem cell factor (SCF)/c-kit pathway or is involved in a novel signaling pathway, present exclusively in HSC and MPPs. Taken together, these findings necessitate functional characterization of tr-kit and analysis of its potential role in the self-renewal, proliferation, and/or differentiation of HSC and multipotent progenitors.

INTRODUCTION

The c-kit receptor and its ligand stem cell factor (SCF) play an important role in the maintenance and differentiation of hematopoietic stem cells (HSCs) and multipotent progenitors (MPPs) [1,2]. c-kit expression is also the phenotypic hallmark of HSCs and early hematopoietic progenitors [3-5].

Through studying the maintenance and differentiation of a SCF-dependent, murine HSC/MPP-like cell line EML [6], we discovered that in addition to the full-length c-kit receptor, EML cells express the truncated form of c-kit, called tr-kit. This intracellular truncated form of c-kit was first detected in mouse spermatids [7]. tr-kit is encoded by a 3.2-kb alternative transcript (GenBank Accession Number X65997), that originates from a cryptic promoter in

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intron 16 of the mouse *c-kit* gene (Fig. 1A). The tr-kit transcript contains a unique 415-bp-long 5' untranslated region (5'UTR) and a unique 36-bp-long start of the coding sequence, which differ from *c-kit* cDNA. The remaining coding and 3'UTR sequence are identical with the full-length, 5.5-kb-long *c-kit* transcript. Besides the extracellular, transmembrane, and juxtamembrane region, the 202-amino-acid (aa)-long tr-kit protein (MW 30 kD) also lacks the adenosine triphosphate (ATP) binding part of the kinase domain, as well as the hydrophilic kinase insert (Fig. 1A,B). Instead, tr-kit contains a unique 12-aa-long hydrophobic region (derived from translation of intronic sequences), which is in frame with the 190-aa-long carboxy-terminal part of the *c-kit* protein that encompasses the phosphotransferase domain and carboxy-terminal tail relevant for *c-kit* interaction with phospholipase C γ (PLC γ) (Fig. 1A,B) [8].

When injected into mouse oocytes, the tr-kit protein induces their activation by stimulating phosphorylation of PLC γ [9,10]. Although it lacks intrinsic kinase activity, in mouse oocytes and Hek293 cells tr-kit becomes phosphorylated on the carboxy-terminal tyrosine Y936 through interaction with the Src kinase Fyn and PL C γ . A model was proposed whereby tr-kit promotes the formation of a complex composed of Fyn, PL C γ , and Sam68 protein. Phosphorylation of tr-kit is necessary for these interactions to occur (Fig. 1A) [11].

In the present work we report that the multipotent hematopoietic cell line EML co-expresses *c-kit* and tr-kit, and that the tr-kit transcript and protein are down-regulated during its differentiation into myeloerythroid lineages. We have examined the expression of tr-kit during murine hematopoiesis and show that in contrast to *c-kit*, tr-kit expression is restricted to fetal liver and bone marrow cell populations enriched for HSCs and MPPs.

MATERIALS AND METHODS

Cells, flow cytometry, and reagents

The SCF-dependent EML cell line was maintained in Iscove's modified Dulbecco medium (IMDM; Gibco) with 20% equine serum (HyClone) and 10% SCF-conditioned medium from the BHK/MKL cell line [6]. The mouse pro-B cell line BaF3 was grown in RPMI medium supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT) and 10% WEHI-3B conditioned medium. The mouse pre-B cell line 70Z3 was grown in RPMI-1640 medium supplemented with 10% FCS and 0.05 mM 2-mercaptoethanol. The mouse monocyte cell lines RAW 309 and J774A.1 were grown in DMEM (Gibco) with 10% FCS.

Bone marrow cells and thymocytes were isolated from 6- to 8-week-old C57BL/6J mice. The following cell populations were isolated by sorting on an Aria cell sorter (Becton Dickinson) as described previously [4,5,12-]: (1) bone marrow Lin⁻ c-kit⁺ Sca-1⁺ (LKS) Flk2⁻ and LKS Flk2⁺ cells, Lin⁻ c-kit⁺CD34⁻ and Lin⁻ c-kit⁺ CD34⁺ cells, Lin⁻ c-kit⁺ Sca-1⁺ and Lin⁻ c-kit⁺ Sca-1⁻ cells, Lin⁻ Sca-1⁺ and Lin⁻ Sca-1⁻ cells, and Lin⁺ cells; (2) double-negative DN1 (CD44⁺CD25⁻) and DN2 (CD44⁺CD25⁺) thymocytes, and CD4⁺ and CD8⁺ T cells; (3) bone marrow pro/pre-B cells (B220⁺CD43⁻ and B220⁺CD43⁺ cells); and (4) bone marrow Ter119⁺ erythroid cells. The cells were analyzed on LSR flow cytometer (Becton Dickinson).

Cell-specific cDNAs from fetal liver HSC-enriched AA4.1⁺ LKS cells, fetal liver progenitors (AA4.1⁻ Lin⁻), and adult HSC-enriched Rho-123^{lo}Ho^{lo}LKS bone marrow cells were a kind gift from Dr. Ihor Lemischka (Princeton University).

Goat α -c-kit M-14, α -c-kit H300, and rabbit α -c-kit antibodies were purchased from Santa Cruz and Cell Signaling. Rabbit α -c-kit [pY⁷³⁰] and α -c-kit [pY⁹³⁶] phosphospecific antibodies were purchased from BioSource. fluorescein isothiocyanate (FITC)-conjugated α -

B220, Mac-1, Gr-1, Ter119, CD4, CD8, CD25, phycoerythrin (PE)-conjugated CD44 and CD43, and α -c-kit-APC, α -Sca-1-PE-Cy7, α -Flk-2-PE, and α -CD34-PE antibodies were purchased from BD Pharmingen or eBioscience. Recombinant human erythropoietin (EPO) and murine recombinant granulocyte/macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) were purchased from Ortho Biotech and PeproTech.

RNA extraction, reverse transcription and polymerase chain reaction

Total RNA from cell lines was isolated with Trizol (Life Technologies) and reverse transcribed using SuperScript II System (Invitrogen). The RNA from multiple batches of sorted cells (purity 98–99.5%) was isolated with RNeasy Micro Kit (Qiagen), and the RNA equivalents of 2×10^3 to 5×10^4 cells were reverse transcribed and amplified using SuperSCRIPT III System (Invitrogen). PCR reactions were performed in an Eppendorf Mastercycler for 35 cycles (95°C for 30 sec, 57–62°C for 45 sec, 72°C for 30 sec). The sequences of the primers for *c-kit*, *tr-kit* (primary and nested oligonucleotides), and other analyzed genes are provided in the Table 1. The primers for β -globin and lactoferrin were described before [15].

Quantitative real-time PCR reactions for *tr-kit* and hypoxanthine phosphoribosyl transferase (HPRT) were performed with LightCycler (Roche) under optimized conditions using SybrGreen, and analyzed with LightCycler software 3.5. A standard curve method was used for quantification of *tr-kit* and hypoxanthine phosphorybosil transferase (HPRT) expression according to LightCycler instructions. A water control and melting curve analysis were also performed to confirm the specificity of PCR. The RNA content of samples was normalized based on the amplification of HPRT as a control gene. Statistical analyses were performed using the Student *t*-test. $p < 0.05$ was considered significant.

Western blotting

The cells were lysed in 100 μ l of buffer consisting of 150 mM NaCl, 1% NP-40, 0.25 % deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS), and 50 mM Tris, pH 7.4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), supplemented with protease inhibitor (Roche). Up to 50 μ g of protein was run on 12% SDS-polyacrylamide gels (Gradipore), and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad). The membranes were incubated at 4°C overnight with primary antibodies at recommended dilutions, washed and incubated with goat α -rabbit immunoglobulin–horseradish peroxidase (Ig-HRP) or rabbit α -goat Ig-HRP secondary antibodies (Zymed) for 1 h at room temperature (1:5,000 to 1:10,000 dilutions). The proteins were detected with an electrochemiluminescence (ECL) detection system (Amersham) or SuperSignal West system for detection of HRP (Pierce). Where indicated, the membranes were stripped and reprobed with α -actin antibody (Sigma).

RESULTS

Multipotent hematopoietic cell line EML co-expresses the full-length and truncated c-kit receptor

The multipotent, SCF-dependent HSC/MPP-like cell line EML [6] shares phenotypic and functional features with murine $\text{Lin}^- \text{c-kit}^+ \text{Sca-1}^+$ (LKS) and LKS Flk2^- bone marrow cells, enriched for HSCs and MPPs [13,16–18]. Similar to HSCs and MPPs, almost all EML cells are $\text{Sca-1}^+ \text{c-kit}^+ \text{Flk2}^-$ (Fig. 2A) and transcribe all functionally relevant genes (e.g., *Hoxb4*, *Meis1*, *Rae28*, *SCL*, *GATA-2*, *AML1*, *AML2*, *Ikaros*, *PU.1*, *Notch1*, *Bmi-1*, etc.) (Figs. 2B and 4B, below).

In the presence of SCF, EML cells undergo proliferative self-renewal and remain undifferentiated, whereas in the presence of cytokines and/or stroma EML cells differentiate into erythroid, myeloid and lymphoid lineages (Fig. 2C) [6,19,20]. For example, in colony-forming assays, EML cells generate colony-forming units granulocyte/macrophage (CFU-GM), burst-forming unit erythroid (BFU-E), and colony-forming units megakaryocytic (CFU-Meg) colonies in the presence of interleukin-3 (IL-3) or GM-CSF, EPO, and IL-3 and thyropoietin (TPO), respectively (Fig. 2C). In co-culture with stromal cell line W20 and IL-7, EML cells give rise to the pro-B cells that express RAG-1 and undergo D-J rearrangements [6]. Furthermore, we have recently found that EML cells can undergo T cell differentiation in co-culture with OP9 stromal cells expressing Notch ligand Delta-like 1 [21] (Fig. 2C, unpublished data).

By studying the role of the SCF/c-kit pathway in their maintenance and differentiation, we discovered that undifferentiated EML cells express tr-kit protein as well as c-kit. The tr-kit protein was detected by western blotting using goat α -c-kit (M-14, Santa Cruz) and rabbit α -c-kit (Cell Signaling) antibodies that recognize the carboxyl terminus of c-kit, and the phosphotransferase part of the c-kit kinase domain (Fig. 1). Both antibodies have detected the full-length c-kit protein (160/145 kD) and tr-kit protein (30 kD) in EML cells, with the expression level of tr-kit being consistently lower than the level of c-kit protein (Fig. 3A,B). However, c-kit and tr-kit proteins were not detected in the pre-B cell line 70Z3, pro-B cell line BaF3, and 293T and NIH-3T3 cell lines, which are known not to express c-kit (Fig. 3B) [22,23]. In contrast, the H-300 α -c-kit antibody (Santa Cruz), which recognizes the amino terminus of c-kit, did not detect tr-kit protein in EML cells (Figs. 1 and 3A).

Transcription of tr-kit in EML cells was confirmed by RT-PCR, using a primer set (tr-kit', Table 1) that specifically amplifies an 800-bp fragment of tr-kit cDNA and spans several introns in the *c-kit* gene. The 5' oligonucleotide is specific for the unique 5' UTR of tr-kit, whereas the 3' oligonucleotide is specific for the beginning of the 3'UTR, common for tr-kit and *c-kit* (Fig. 3C). The identity of amplified tr-kit PCR product was confirmed by sequencing and secondary PCR using a set of nested tr-kit (tr-kit'') primers (Fig. 3C and Table 1).

Previous studies have reported that the exogenously expressed tr-kit protein in oocytes and Hek293 cells becomes phosphorylated on the carboxy-terminal tyrosine Y936 [11]. Thus, we sought to determine whether the tr-kit protein is phosphorylated in EML cells. Notably, western analysis with α -c-kit [pY⁹³⁶] phosphospecific Ab (BioSource) has shown that both c-kit and tr-kit protein in EML cells are phosphorylated at the carboxy-terminal tyrosine Y936 (Fig. 3D). On the other hand, western analysis with α -c-kit [pY⁷³⁰] antibody (BioSource) has demonstrated that only the c-kit protein is phosphorylated on tyrosine Y730, which is not present in the tr-kit protein (Figs. 1A and 3D).

Expression of tr-kit is restricted to cell populations enriched for HSCs and MPPs

The presence of tr-kit transcript and protein in EML cells indicated that besides germ-line cells the cells of the hematopoietic origin express tr-kit as well. Thus, we examined the pattern of tr-kit expression during murine blood cell development.

The expression of tr-kit and c-kit was analyzed first by RT-PCR in fluorescence-activated cell sorting (FACS)-purified (1) Lin⁻ c-kit⁺ Sca-1⁺ Flk2⁻ (LKS Flk2⁻) bone marrow cells, highly enriched for long-term repopulating (LTR) HSCs, (2) Lin⁻ c-kit⁺ Sca-1⁺ Flk2⁺ (LKS Flk2⁺) bone marrow cells, highly enriched for short-term re-populating (STR) HSCs and MPPs, (3) more heterogeneous populations of Lin⁻ c-kit⁺ CD34⁻, Lin⁻ c-kit⁺ Sca-1⁺ and Lin⁻ Sca-1⁺ bone marrow cells, containing HSC and progenitors, (4) Lin⁻ c-kit⁺ CD34⁺, Lin⁻ c-kit⁺ Sca-1⁻ and Lin⁻ Sca-1⁻ bone marrow cells, enriched for progenitors but lacking

LTR-HSCs, and (4) Lin⁺ bone marrow cells, containing immature and mature blood cells (Fig. 4A) [3-5,12-14].

Interestingly, tr-kit transcript was reproducibly detected in cDNA prepared from LKS Flk2⁻, LKS Flk2⁺, Lin⁻ c-kit⁺ CD34⁻, Lin⁻ c-kit⁺ CD34⁺, and Lin⁻ c-kit⁺ Sca-1⁺ bone marrow cells (equivalent to 5–12 × 10³ cells), which also express c-kit (Fig. 4B). Tr-kit expression was detectable in Lin⁻ c-kit⁺ Sca-1⁻ cells also, but only when the cDNA equivalent of 3 × 10⁴ cells or more was used as a template for amplification (Fig. 4B). However, the tr-kit transcript was not found in the Lin⁻ Sca-1⁺, Lin⁻ Sca-1⁻ and Lin⁺ bone marrow cells, even after using increasing numbers of sorted cells (ranging from 5 × 10³ to 10⁵ cells) to isolate and transcribe RNA (Fig. 4B and data not shown).

Transcription of tr-kit and c-kit was also examined by PCR in cDNA libraries prepared from (1) fetal liver (FL) AA4.1⁺ Lin⁻ Sca-1⁺ c-kit⁺ (AA4.1⁺ LKS) cells highly enriched for fetal HSC, (2) FL AA4.1⁻ Lin⁻ cells lacking HSCs but enriched for progenitors, and (3) Rho-123^{lo}Ho^{lo}Lin⁻ Sca-1⁺ c-kit⁺ (Rho^{lo}Ho^{lo} LKS) bone marrow cells, highly enriched for HSCs and MPPs [24-26]. Although the c-kit Transcript was detected in all three cell populations, only the HSC-enriched AA4.1⁺ LKS and Rho^{lo}Ho^{lo} LKS cells transcribe tr-kit (Fig. 4C). Moreover, tr-kit exhibits an identical expression pattern with GATA-2 and HoxA9 in these cell populations (Fig. 4C) [17,18].

Next, we examined tr-kit expression in primary hematopoietic cells and cell lines representing more advanced stages of differentiation into lymphoid and myeloerythroid lineages. The c-kit transcript was detected by RT-PCR in the thymus, spleen, and double-negative DN1 (CD44⁺ CD25⁻) and DN2 (CD44⁺ CD25⁺) thymocytes, but none of these tissues and cells expressed tr-kit (Fig. 4D). Although CD4⁺ and CD8⁺ T cells express GATA-3 as a control, they do not express c-kit or tr-kit (Fig. 4D and data not shown). The bone marrow pro/pre-B cells (B220⁺ CD43⁻ and B220⁺ CD43⁺ cells), and the J774A.1 monocyte cell line expresses c-kit, whereas the BaF3 pro-B cell line, RAW 309 monocyte cell line, and erythroid Ter119⁺ bone marrow cells do not [22]. However, tr-kit was not expressed in any of these cell types (Fig. 4D). These results have demonstrated that during murine blood cell development tr-kit expression is restricted to cell populations enriched for HSC and MPPs.

tr-kit expression is down-regulated during differentiation of EML cell line into myeloerythroid lineages

When cultured in the presence of cytokines such as EPO, IL-3, GM-CSF, and G-CSF, EML cells differentiate into myeloerythroid progenitors, which in turn give rise to mature erythrocytes, granulocytes, and macrophages [6,20]. To analyze tr-kit expression during the differentiation of EML cells into the erythroid lineage, EML cells were cultured in triplicate with recombinant EPO (8 U/ml) for 72 and 96 h. tr-kit transcription in undifferentiated and differentiated EML cells was analyzed by RTPCR and quantitative real-time PCR, whereas the tr-kit protein expression was analyzed by western using rabbit α -c-kit Ab.

In contrast to undifferentiated cells, the EML cells cultured with EPO express β -globin as an indicator of their differentiation into erythroid lineage [15] (Fig. 5A). More importantly, in comparison to EML cells maintained with SCF, the cells cultured for 72 h with EPO have significantly down-regulated expression of tr-kit, whereas in the cells cultured with EPO for 96 h tr-kit transcript is barely detectable (Fig. 5A). The β -globin was amplified in the bone marrow Lin⁺ cells as a positive control.

The quantitative real-time PCR analysis has revealed that the levels of tr-kit transcript (normalized against HPRT) have decreased more than five-fold in EML cells cultured with

EPO for 72 h, and more than eight-fold in cells cultured with EPO for 96 h in comparison to undifferentiated EML cells (Fig. 5B). Remarkably, western analysis has demonstrated a significant decrease of tr-kit protein level in EML cells cultured for 72 and 96 h with EPO (Fig. 5C). Similarly, the expression of c-kit protein was also significantly down-regulated during erythroid differentiation of EML cells (Fig. 5C).

Next, EML cells were cultured in triplicate with the combination of recombinant G-CSF (0.1 ng/ml) and GM-CSF (2 ng/ml) for 72 and 96 h, and tr-kit expression was analyzed during their differentiation into granulocyte and macrophage lineages. After 72 h of culture in the presence of G-CSF and GM-CSF (G/GM-CSF), tr-kit transcription was only slightly down-regulated in EML cells (Fig. 6A). However, after 96 h tr-kit expression was significantly down-regulated in comparison to undifferentiated EML cells (Fig. 6A). In addition, EML cells cultured with G/GM-CSF transcribed lactoferrin as a marker of their differentiation into granulocyte-macrophage lineage [15] (Fig. 6A). Real-time PCR analysis has shown that the levels of tr-kit transcript (normalized against HPRT) in EML cells cultured with G/GM-CSF for 72 and 96 h have decreased about 2- and 2.5-fold when compared with undifferentiated EML cells (Fig. 6B). Similarly, the expression of tr-kit and c-kit protein was only slightly decreased in EML cells cultured with G/GM-CSF for 72 h, but was significantly down-regulated in EML cells treated with G/GM-CSF for 96 h (Fig. 6C). Nevertheless, the down-regulation of tr-kit expression in EML cells differentiated with G/GM-CSF was not as prominent as in EML cells differentiated with EPO.

Previous studies have shown that GM-CSF cannot induce differentiation of all EML cells into granulocyte-macrophage progenitors and mature granulocytes and macrophages [6,20]. Thus, the residual expression of trkit transcript and protein seen in EML cells cultured with G/GM-CSF is most likely due to the presence of remaining undifferentiated EML cells in these cultures.

Simultaneous treatment of EML cells with an increased concentration of GM-CSF and a high concentration of retinoic acid (RA) (10^{-5} M) has been reported to augment their differentiation into granulocyte-macrophage progenitors and granulocytes and macrophages dramatically [6]. Hence, in the next set of experiments, the expression of tr-kit was examined in EML cells cultured in the presence of G-CSF (0.1 ng/ml), GM-CSF (10 ng/ml), and RA (10^{-5} M) for 72 and 96 h, respectively.

The RT-PCR analysis has shown that in comparison to undifferentiated EML cells and cells differentiated with G/GM-CSF (Fig. 6A-C), EML cells cultured with G/GM-CSF and RA for 72 and 96 h do not express detectable levels of tr-kit transcript (Fig. 6D,E). More importantly, the expression of tr-kit protein in these cells is significantly down-regulated at 72 h and barely detectable at 96 h (Fig. 6D,E). Moreover, in comparison to EML cells differentiated with G/GM-CSF alone (Fig. 6A), the EML cells treated with G/GM-CSF and RA also show elevated expression of lactoferrin (Fig. 6D).

Overall, these results demonstrate that the expression of tr-kit is rapidly and significantly down-regulated in EML cells undergoing terminal differentiation into erythroid and myeloid lineages.

DISCUSSION

Discovery of tr-kit in the multipotent HSC/MPP-like cell line EML prompted us to determine its expression pattern during mouse hematopoiesis. Remarkably, tr-kit transcript was detected only in the fetal liver (FL) and bone marrow-derived cell populations that are highly enriched for LTR-HSCs, STR-HSCs, and MPPs, and are expressing c-kit as well. Notably, an online search of several public HSC databases generated through differential

gene expression profiling [26-30], has revealed that trkit was found in the cDNA library from HSC-enriched Thy-1.1^{lo} LKS bone marrow cells, which was subtracted with cDNA from Lin⁺ bone marrow cells (http://db.systemsbio.org/projects/stem_cell/) [29].

In contrast, the expression of tr-kit was not detected in (1) FL and bone marrow cell populations in which HSCs and MPPs are either present at a much lower frequency or are absent altogether, and (2) in cells representing more advanced stages of differentiation into lymphoid and myeloid lineages. These findings were corroborated by the observation that the expression of tr-kit transcript and protein is rapidly and significantly down-regulated in EML cells as they differentiate into erythroid and myeloid lineage cells in response to EPO, G-CSF, GM-CSF, and RA.

Taken together, our studies have revealed that in contrast to c-kit, whose expression is more widespread during murine hematopoiesis [1], tr-kit expression is restricted to cell populations enriched for HSCs and MPPs. The absence of tr-kit in later stages of blood cell development and down-regulation of tr-kit expression during myelo-erythroid differentiation of EML cells suggest that tr-kit expression is regulated in a developmental stage-specific manner.

Despite documented importance for self-renewal and differentiation of HSC and MPPs, the mechanisms through which c-kit regulates these processes are still not well understood [1,31]. The novel finding that HSC- and MPP-enriched cell populations co-express c-kit and tr-kit raises an interesting possibility that tr-kit functions either as a new component of canonical SCF/c-kit pathway, or it participates in a novel signaling pathway, present exclusively in HSCs and MPPs.

Interestingly, similar to a previous report [11], in EML cells tr-kit is phosphorylated at the carboxy-terminal tyrosine Y936, indicating that tr-kit is activated in primitive hematopoietic cells through an as yet unidentified process. Thus, it will be very important to elucidate whether tr-kit interacts with c-kit in HSCs and MPPs and participates in c-kit signaling pathway. Interactions between the full-length and naturally occurring or engineered truncated forms of tyrosine kinase receptors (e.g., EGF-R, trkB, and c-kit) have been observed before, and can lead to ligand-independent receptor dimerization and activation [32-34].

Along with c-kit, tr-kit could also be involved in the regulation of self-renewal and differentiation of HSCs and MPPs. For example, the W-41 and W-42 mutations in the c-kit phosphotransferase domain that affect c-kit function could affect the function of tr-kit as well. Hence, the HSC functional defects found in mice heterozygous for W-41 and W-42 mutations [31,35], could be due to attenuated activity of both c-kit and tr-kit.

Thus, it will be important to determine how the gain and loss of tr-kit function impacts the maintenance and differentiation of EML cell line, and elucidate potential role of tr-kit in self-renewal and/or differentiation of bone marrow-derived HSCs and multipotent progenitors.

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FIG. 1. Schematic structure of c-kit and tr-kit protein. (A) The 202-aa-tr-kit protein (MW 30 kD) is encoded by a 3.2-kb alternative transcript. The tr-kit protein lacks the extracellular, transmembrane, and juxtamembrane regions, as well as the ATP-binding part of the kinase domain. Instead of the typical hydrophilic kinase insert, tr-kit contains a unique 12-aa-long hydrophobic region, which is in frame with the carboxyl terminus of c-kit, which contains the phosphotransferase domain and carboxy-terminal tail. Epitopes in the c-kit and tr-kit protein for three different α -c-kit antibodies used in this study. The tyrosines (Y) phosphorylated in the c-kit protein are also shown. (B) ClustalW alignment of amino acid sequence of c-kit and trkit proteins (GenBank Accession Number CAA46798). Twelve amino-terminal amino acids specific for tr-kit are underlined.

FIG. 2.

The phenotype, maintenance, and multilineage differentiation of the multipotent hematopoietic cell line EML. **(A)** Flow cytometry analysis of c-kit, Sca-1, and Flk-2 HSC marker expression on EML cells. **(B)** RT-PCR expression analysis of several important hematopoietic genes in EML cells from three separate cultures. HPRT was amplified as a positive control. **(C)** Maintenance and multilineage differentiation model of a multipotent hematopoietic cell line EML. Microphotographs on the right show granulocyte-macrophage (CFU-GM), megakaryocytic (CFU-Meg), and erythroid (BFU-E) colonies formed by EML cells in colony-forming assays in the presence of IL-3, TPO, and EPO.

FIG. 3.

Multipotent hematopoietic cell line EML expresses truncated c-kit receptor. **(A)** Western analysis of tr-kit and c-kit protein expression in EML cells. The goat and rabbit α -c-kit antibodies have detected the full-length c-kit protein (160/145 kD) and tr-kit protein (30 kD) in EML cells. H-300 α -c-kit Ab, which recognizes the amino-terminus of c-kit, did not detect tr-kit protein in EML cells. The blots were stripped and reprobed with α -actin antibody. **(B)** Western analysis of c-kit and tr-kit protein expression in EML cells, 70Z3 pre-B cell line, the BaF3 pro-B cell line, and 293T and NIH-3T3 cell lines with rabbit α -c-kit Ab. **(C)** RT-PCR analysis of c-kit and tr-kit expression in EML cells, BaF3 pro-B cell line, and mouse testis as a positive control. The expression of tr-kit was analyzed with two different sets of primers (tr-kit' and tr-kit'', Table 1). An aliquot (2 μ l) of the primary RT-PCR reaction (tr-kit') was used as a template in the secondary reaction with nested primers (tr-kit''). **(D)** Western analysis with rabbit α -c-kit [pY⁹³⁶] phosphospecific Ab has shown that c-kit and tr-kit protein in EML cells are both phosphorylated at the carboxy-terminal tyrosine Y936. In contrast, the analysis with α -c-kit [pY⁷³⁰] phosphospecific Ab has demonstrated that only the c-kit protein is phosphorylated on tyrosine Y730, which is absent in tr-kit protein.

FIG. 4.

Expression of *tr-kit* is restricted to cell populations enriched for HSC and MPPs. **(A)** Multiparameter sorting of $\text{Lin}^- \text{c-kit}^+ \text{CD34}^-$ and $\text{Lin}^- \text{c-kit}^+ \text{CD34}^+$ cells, $\text{Lin}^- \text{c-kit}^+ \text{Sca-1}^-$ and $\text{Lin}^- \text{c-kit}^+ \text{Sca-1}^+$, and $\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^+$ (LKS) Flk2^- and LKS Flk2^+ cells from the bone marrow of C57BL/6/J mice. **(B)** RT-PCR analysis of *tr-kit* and *c-kit* expression in (1) LKS Flk2^- bone marrow cells, highly enriched for LTR-HSC, (2) LKS Flk2^+ bone marrow cells, highly enriched for STR-HSCs and MPPs, but lacking LTR-HSCs, (3) more heterogeneous populations of $\text{Lin}^- \text{c-kit}^+ \text{CD34}^-$, $\text{Lin}^- \text{c-kit}^+ \text{Sca-1}^+$ and $\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^+$ bone marrow cells, containing HSCs and progenitors, (4) $\text{Lin}^- \text{c-kit}^+ \text{CD34}^+$, $\text{Lin}^- \text{c-kit}^+ \text{Sca-1}^-$ and $\text{Lin}^- \text{Sca-1}^-$ bone marrow cells, enriched for progenitors but lacking LTR-HSCs, and (5) Lin^+ bone marrow cells, containing immature and mature blood cells. The expression of *tr-kit* was analyzed with two different sets of primers (*tr-kit'* and *tr-kit''*, Table 1). An aliquot (2 μl) of the primary RT-PCR reaction (*tr-kit'*) was used as a template in the secondary reaction with nested primers (*tr-kit''*). *Bmi-1*, required for maintenance of HSCs [36,37], and *HPRT* were amplified as controls. **(C)** The analysis of *tr-kit* and *c-kit* expression in cDNA libraries prepared from fetal liver (FL) AA4.1 $^+$ LKS cells highly enriched for fetal HSC, FL AA4.1 $^-$ Lin $^+$ cells lacking HSCs but enriched for progenitors, and bone marrow-derived $\text{Rho}^{\text{lo}}\text{Ho}^{\text{lo}}$ LKS cells, highly enriched for LTR-HSC. The expression of *c-kit* and *tr-kit* in these cell populations was compared with the expression of *GATA-1*, *GATA-2*, *HoxA9*, and *Meis1*. **(D)** The expression of *tr-kit* in primary cells and cell lines representing various stages of differentiated into lymphoid and myeloerythroid lineages. The expression of *tr-kit* and *c-kit* was analyzed by RT-PCR in the thymus, spleen, double-negative DN1 ($\text{CD44}^+ \text{CD25}^-$) and DN2 ($\text{CD44}^+ \text{CD25}^+$) thymocytes, CD4 and CD8 T cells from thymus, bone marrow-derived pro/pre-B cells ($\text{B220}^+ \text{CD43}^-$ and $\text{B220}^+ \text{CD43}^+$ cells), BaF3 pro-B cell line, 70Z3 pre-B cell line, J774A.1 and RAW 309 monocyte cell lines, and bone marrow-derived erythroid Ter119 $^+$ cells. *GATA-3* was amplified as a control for CD4 and CD8 cells, and β -globin as a control for Ter119 $^+$ cells.

FIG. 5.

Tr-kit transcription and protein expression are down-regulated during differentiation of the EML cell line into erythroid lineage. (A) RT-PCR analysis of tr-kit expression during differentiation of EML cells into erythroid lineage. EML cells were cultured in triplicate with EPO (8 U/ml) for 72 and 96 h. The expression of β -globin demonstrates differentiation of EML cells into erythroid lineage. β -Globin was amplified in bone marrow Lin⁺ cells as a positive control. (B) Quantitative real-time PCR analysis of tr-kit expression in undifferentiated EML cells and EML cells that are undergoing differentiation into erythroid lineage in the presence of EPO. The levels of tr-kit transcript were normalized based on the amplification of HPRT as a control gene. The Student *t*-test was used for statistical analysis, where *p* 0.05 was considered significant. (C) Western analysis of tr-kit and c-kit protein expression during EML cell differentiation into erythroid lineage. The blots were stripped and reprobed with α -actin Ab.

FIG. 6.

Tr-kit transcription and protein expression are down-regulated during differentiation of EML cell line into myeloid lineages. **(A)** RT-PCR analysis of tr-kit expression during differentiation of EML cells into granulocyte-macrophage lineages. EML cells were cultured in triplicate with the combination of G-CSF (0.1 ng/ml) and GM-CSF (2 ng/ml) for 72 and 96 h. The expression of lactoferrin demonstrates differentiation of EML cells into granulocyte-macrophage lineages. Lactoferrin was amplified in the bone marrow Lin⁺ cells as a positive control. **(B)** Quantitative real-time PCR analysis of tr-kit expression in undifferentiated EML cells and EML cells that are undergoing differentiation into granulocyte-macrophage lineages in the presence of GM-CSF and G-CSF. The levels of tr-kit transcript were normalized based on the amplification of HPRT as a control gene. **(C)** Western analysis of tr-kit and c-kit protein expression during EML cell differentiation into granulocyte-macrophage lineages. The blots were reprobed with α -actin Ab. **(D)** RT-PCR analysis of tr-kit expression in EML cells differentiating into granulocyte-macrophage lineages in the presence of G-CSF (0.1 ng/ml), GM-CSF (2 ng/ml), and RA (10^{-5} M) for 72 and 96 h. The expression of lactoferrin demonstrates differentiation of EML cells into granulocyte-macrophage lineages. **(E)** Western analysis of tr-kit and c-kit protein expression in EML cells differentiating into granulocyte-macrophage lineages in the presence of G-CSF, GM-CSF, and RA for 72 and 96 h. The blots were reprobed with α -actin Ab.

Table 1

The Sequence of the Primers Used for Gene Expression Analysis

Gene	5' Oligonucleotide	3' Oligonucleotide
<i>c-kit</i>	TCCTCACTCACGGGCGGATC	TGGAGGTGGGGTGGGGAAC
<i>tr-kit</i>	TTAGAGCCCCGATCCTGTGA	AGCAGCAAAGCCTGTGGAC
<i>tr-kit</i> (nested primers)	AAATGAATGGCTGTTGCTGT	AACCATCACAGAAGCCAGAA
<i>Ikaros</i>	CACTACCTCTGGAGCAGCAGAA	CATAGGGCATGTCTGACAGGCACT
<i>Sca-1</i>	CAATGTAGCAGTTCCCAATG	CAGGGGCTATAAAGGCAAAA
<i>GATA-1</i>	GGAGCCCTCTCAGCTCAGC	GACTTCCTGGTCGACCACCG
<i>GATA-2</i>	GGCGTCAAGTACCAAGTGTCAC	CTCCCGGCCTTCTGAGCAGGAG
<i>GATA-3</i>	GCGGTCTCAACGGTCAGCAC	TCGGGCACATAGGGCGGATAG
<i>PU.1</i>	CGGATGACTTGGTTACTTACG	TTGGACGAGAACTGGAAGGTA
<i>Rae-28</i>	GTGCTACATGGTGACAGCTT	AGCTAGGAAAGCTGACCTCT
<i>Meis1</i>	ATGGGTTCCTCGGTCAATGAC	ATGGTCTCTATTCCAAGAGGGC
<i>HoxA9</i>	CCCACGCTTGACACTCACACTTTG	GAGTGGAGCGAGCATGTAG
<i>HoxB4</i>	TCCAGTGCAGCCAACAGCCG	GTGCCACCCGAGCGGATCTT
<i>AML1</i>	CCAGCAAGCTGAGGAGCGGCG	CGGATTGTAAAGACGGTGA
<i>AML2</i>	CACTCAGTCCATTTCCTCAA	TTCCGTCTTTCTTTCTGTCC
<i>SCL</i>	TATGAGATGGAGATTTCTGATG	GCTCCTCTGTGTAACGTGCC
<i>Notch1</i>	CAATCAGGGCACCTGTGAGCCCACAT	TAGAGCGCTTGATTGGGTGCTTGCGC
<i>Bmi-1</i>	AAGGAAGAGGTGAATGATAAAAGG	ACTACAAAGGAAGCAAAGTGGAC
<i>HPRT</i>	GTTGAGAGATCATCTCCACC	AGCGATGATGAACCAGGTTA