Special Issue: Recent Advances in Stem Cell Biology in Regeneration and Disease

Mini Review

Identification of long-term repopulating hematopoietic stem cells by Evi1

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Tissue stem cells, such as hematopoietic stem cells (HSCs), have great promise for regenerative medicine. Establishment and maintenance of the hematopoietic system relies on self-renewing HSCs. Genetic studies have identified a number of transcription factors and signaling molecules that control HSC self-renewal, and have delineated the underlying molecular mechanisms. One molecule that has been a particularly useful marker of HSC is Ecotropic viral integration site 1 (Evi1). Evi1 is a transcription factor of the SET/PR domain protein family, and is essential for the maintenance of HSC. Evi1 is notorious for its involvement in leukemia, as Evi1 activation confers the worst prognosis in patients with acute myeloid leukemia. A recent study using Evi1-green fluorescent protein reporter mice demonstrated that *in vivo* repopulating HSCs are exclusively enriched within the Evi1-expressing fraction in both fetal and adult hematopoietic system. Consistent with predominant expression of Evi1 in HSCs, heterozygous knockout of the Evi1 gene leads to a marked reduction of long-term HSCs with defect of their self-renewal capacity. Here we will summarize the current knowledge regarding the role of Evi1 in hematopoiesis and focus on the specific relationship between Evi1 expression and HSC self-renewal activity.

Rec.6/6/2012, Acc.7/28/2013, pp175-180

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Key words ecotropic viral integration site 1, green fluorescent protein, hematopoietic stem cells, reporter-mouse, self-renewal

Introduction

The hematopoietic system serves as a paradigm for understanding tissue stem cells, their biology, and involvement in diseases, oncogenesis, and aging[1]. Hematopoietic stem cells (HSCs) are defined by their ability to generate multiple blood cell lineages through differentiation, while retaining the capacity to perpetuate themselves through self-renewal[2, 3]. The prospective isolation of HSCs is the most important step to dissect their function. The strategy that is most commonly used for HSC isolation is purifica-

tion based on the expression of a combination of cell surface markers. For instance, expression of the markers c-kit and Sca-1 but not any of lineage-specific markers defines a multipotential cell population, including long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs) and multipotent progenitors (MPPs)[2, 3]. LT-HSCs can be more precisely purified within this population by excluding cells that express CD34, Flt3 and CD48, or including only those cells that express CD150[4-6]. Another approach to the identification and purification of adult HSCs depends on their ability to efflux particular dyes such as Hoechst 33342 or Rhodamine 123[3]. However, some of these markers differ between mouse strains, change dramatically during development, and are expressed on other hematopoietic cells[7-9].

Ecotropic viral integration site 1 (Evi1)

Ecotropic viral integration site 1 (Evi1) is a nuclear transcription factor that belongs to the SET/PR domain protein family[10]. Evi1 activation can have distinct oncogenic potential in a variety of myeloid neoplasms, including acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and myelodysplastic syndrome[10]. Evi1 was first identified as a common locus of retroviral integration site in murine leukemia model[11, 12]. In humans, *EVI1* is located on chromosome 3q26, and its rearrangements often activate EVI1 expression in myeloid malignancies. EVI1 overexpression also occurs in approximately 5-10% of leukemia patients without 3q26 abnormalities, and elevated Evi1 expression predicts adverse outcome in patients with AML and chronic-phase CML, irrespective of the presence of 3q26 rearrangements[13-15]. In patients treated by gene-modified autologous HSCs, retroviral insertion at the Evi1 locus can be associated with long-term *in vivo* clonal dominance, occasionally leading to leukemic transformation[16]. In a murine bone marrow transplant model, forced expression of Evi1 in hematopoietic cells leads to myelodysplasia or leukemia[17, 18]. In addition to the pivotal role of EVI1 in leukemogenesis, accumulating evidences suggested that Evi1 accomplishes an essential regulatory function in normal hematopoiesis. Evi1 expression is predominantly expressed in hematopoietic stem/progenitor fraction in the fetal and adult hematopoietic systems. The number and function of HSCs are markedly decreased in Evi1-deficient embryos[19]. Moreover, conditional deletion of Evi1 in adult mice demonstrated that Evi1 is indispensable for the maintenance of HSC self-renewal, but is not needed for lineage differentiation[20].

Evi1 expression as a sensor for HSC activity in the adult hematopoietic system

Recently, as Evi1 may be a distinctive marker of HSC, an Evi1- internal ribosome entry site (IRES) - green fluorescent protein (GFP) knock-in mouse line has been developed[21]. In this Evi1-IRES-GFP knock-in mouse line, GFP is expressed under the endogenous transcriptional regulatory elements of Evi1 gene, which enable to detect Evi1 expression on an individual cell basis. Flow cytometric analysis of bone marrow revealed a small, but distinct population of GFP$^+$ cells (0.15 ± 0.6%; n = 8), and these GFP$^+$ cells were highly restricted to the Lin$^-$ Sca-1$^+$ c-kit$^+$ (LSK) fraction (Fig.1). When the percentages of GFP$^+$ cells were

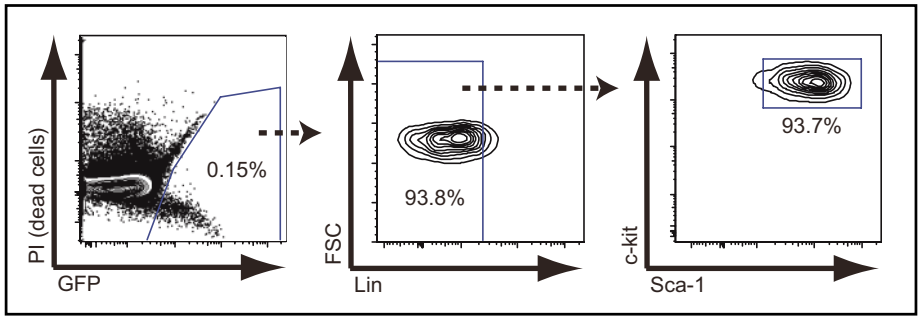

Fig.1 Evi1 expression is highly restricted to the hematopoietic stem/progenitor fraction in adult bone marrow

Flow cytometric analysis of expression of lineage markers (Lin), c-kit, and Sca-1 on GFP$^+$ cells in adult bone from Evi1-IRES-GFP knock-in mice. PI, propidium iodide; FSC, forward scatter. (©Kataoka et al., 2011. Originally published in The Journal of Experimental Medicine. doi:10.1084/jem.20110447.)

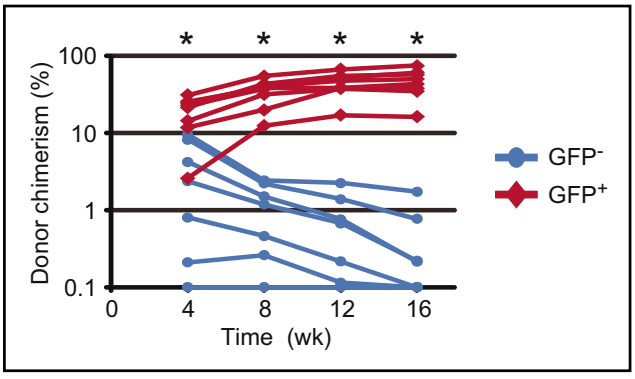

Fig. 2 Evi1 expression marks *in vivo* long-term multilineage repopulating HSCs in adult bone marrow

To determine whether Evi1 expression marks *in vivo* long-term repopulating HSCs, we performed competitive repopulation assays, in which 100 CD48$^-$ CD150$^+$ LSK GFP$^-$ or CD48$^-$ CD150$^+$ LSK GFP$^+$ cells sorted from Evi1-IRES-GFP knock-in mice (Ly5.1) were transplanted into lethally irradiated recipients (Ly5.2) together with 2x10^5 competitor bone marrow cells (Ly5.2). Percentages of donor-derived cells (Ly5.1) in the peripheral blood after transplantation are shown ($*p < 0.005$; n = 7-8). (©Kataoka et al., 2011. Originally published in The Journal of Experimental Medicine. doi:10.1084/jem.20110447.)

analyzed in various hematopoietic cell populations, the LSK fraction showed a heterogeneous expression of GFP, whereas almost no GFP expression was found in hematopoietic progenitors, mature hematopoietic lineages, or non-hematopoietic cells. Further enrichment for LT-HSCs within the LSK fraction using CD34 and Flk-2 expression[4, 5], or CD48 and CD150[6], demonstrated the Flk-2$^-$ CD34$^-$ LSK or CD48$^-$ CD150$^+$ LSK fraction, in which LT-HSCs are highly enriched, had the highest expression of GFP, and its expression diminished with differentiation to hematopoietic progenitors. These findings give rise to the hypothesis that Evi1 expression would have the potential to effectively mark long-term multilineage repopulating HSCs. Indeed, competitive repopulation assays using LSK or CD48$^-$ CD150$^+$ LSK cells in the bone marrow showed that GFP$^+$ cells had remarkable long-term multilineage reconstitution capacity, while significantly impaired engraftment was observed in recipients of GFP$^-$ cells (Fig.2), suggesting *in vivo* long-term multilineage repopulating HSCs are exclusively enriched in the Evi1-expressing fraction in the adult bone marrow.

Specific relationship between Evi1 expression and HSC self-renewal capacity throughout hematopoietic ontogeny

In mammals, the sequential sites of hematopoiesis include the yolk sac, the aorta-gonad-mesonephros (AGM) region, the placenta, the fetal liver, and finally the bone marrow. There are a variety of phenotypic and functional differences between fetal and adult HSCs in surface marker expression, self-renewal capacity, cell-cycle status, gene expression profile, and regulatory mechanism[1, 7]. During embryonic development, HSCs divide rapidly and undergo massive expansion, whereas HSCs in the postnatal bone marrow are mostly quiescent starting from 3-4 weeks of age[22]. Moreover, fetal HSCs differ from adult HSCs in the expression of specific markers such as Mac-1, CD34, and AA4.1[7]. Despite their distinct features from adult HSCs, Evi1 expression was highly enriched in the hematopoietic stem/progenitor fractions in the various tissues of Evi1-IRES-GFP knock-in embryos[21]; CD45$^+$ CD34$^+$ c-kit$^+$ cells in the embryonic day 10.5 (E10.5) AGM[23], CD34$^+$ c-kit$^+$ CD48$^-$ cells in the E12.5 placenta[24], and Mac-1$^+$ Sca-1$^+$ Lin$^-$ CD48$^-$ cells in the E14.5 fetal liver[25]. Competitive repopulation assays using CD34$^+$ c-kit$^+$ CD48$^-$ cells from E12.5 placenta exhibited that GFP$^+$ cells are the exclusive reservoir of HSC activity, with no reconstitution ability being observed in GFP$^-$ cells. Furthermore, within Mac-1$^+$ Sca-1$^+$ Lin$^-$ cells from the E14.5 fetal liver, GFP$^+$ cells contributed to the long-term reconstitution of irradiated recipients, whereas donor chimerism was almost undetectable in mice transplanted with GFP$^-$ cells. Therefore, irrespective of the functional differences between fetal and adult HSCs, Evi1 expression can mark long-term multilineage repopulating HSCs throughout hematopoietic ontogeny, suggesting a unique association between HSC self-renewal capacity and Evi1 expression.

Dominant role of Evi1 in LT-HSC regulation

As mentioned above, it is well known that Evi1 is essential for the maintenance of HSC self-renewal. Recently, using Evi1 heterozygous knockout mice, it was shown that Evi1 has a predominant effect on LT-HSCs by specifically regulating their self-renewal capacity[21]. Evi1 heterozygosity led to an almost complete loss of phenotypic LT-HSCs (Fig.3), while the differentiation potential to all mature lineages and committed progenitors was maintained. Evi1-

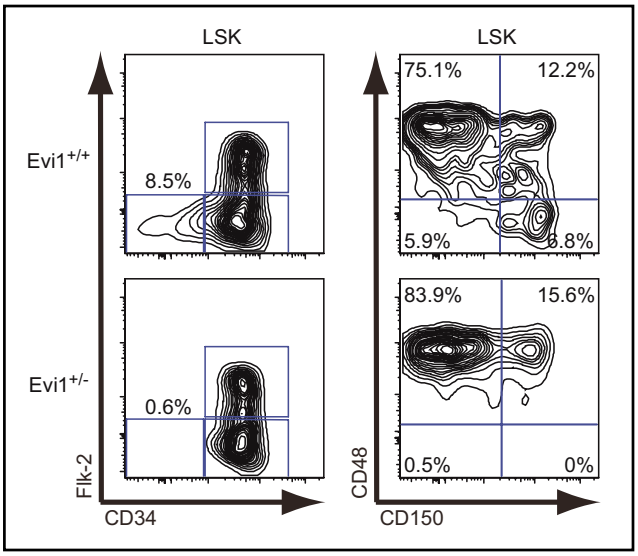

Fig.3 Evi1 heterozygosity leads to an almost complete loss of LT-HSCs

Flow cytometric analysis of expression of Flk-2 and CD34 (left) or C48 and CD150 (right) on LSK cells in adult bone marrow from Evi1 heterozygous knockout mice. (©Kataoka et al., 2011. Originally published in The Journal of Experimental Medicine. doi:10.1084/jem.20110447.)

heterozygous MPPs/ST-HSCs ($CD34^+$ LSK cells) had normal *in vitro* colony-forming capacity and *in vivo* colony-forming unit-spleen activity. In contrast, LT-HSCs ($Flk\text{-}2^-$ $CD34^-$ LSK cells) exhibited a specific defect of *in vivo* repopulating capacity in Evi1 heterozygous knockout mice. These data suggest that Evi1 is considered to play a more specific role in LT-HSCs than in other hematopoietic cells. However, in contrast with the physiological states, it has been reported that EVI1 overexpression can affect hematopoietic differentiation, such as blocking granulocytic and erythroid differentiation, or inducing megakaryocytic commitment[10, 26]. In addition, as there have been conflicting studies regarding this issue, no definitive conclusion about the role of EVI1 in hematopoietic differentiation has so far been drawn[26].

Evi1 as a central regulator in HSC self-renewal

The balance between self-renewal and differentiation is controlled by the competition between transcription factor complexes. Cross-antagonism of transcription factors and subsequent competition for target genes could induce a rapid shift leading to blocked differentiation and maintained self-renewal[2]. Along with the stem cell-specific expression profile of Evi1, the fact that Evi1 heterozygosity induces a near total loss of self-renewing HSCs indicates Evi1 as a potential key regulator in this competitive transcription-factor model for HSC self-renewal. Furthermore, a microarray analysis of gene expression demonstrated that DNA binding sites for Evi1 are enriched in the upstream region of a set of genes which are preferentially expressed in quiescent HSCs[27]. Indeed, a variety of molecules associated with HSC self-renewal, such as Gata2[19, 28], Pbx1[29], Runx1[30], TGF-β [31], PTEN[18] and polycomb group proteins[18], have been identified as downstream targets or interacting proteins of Evi1. Therefore, these findings suggest that the gene dosage of Evi1 is a critical determinant of HSC self-renewal capacity.

Conclusions and future directions

The current interest in stem cell-based therapies in regenerative medicine emphasized the significance of understanding how tissue-specific stem cells are regulated. Studies on the expression pattern and function of Evi1 have firmly established this transcription factor as a pivotal player in HSC self-renewal, and designating Evi1 expression as a robust and reliable HSC marker. Thus, the functional identification of self-renewing HSC by Evi1 expression provides a powerful approach for investigating HSC biology. Given the link between deregulation of Evi1 function and leukemia, such a framework will be helpful for exploring leukemia stem cells.

Source of funding

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and by Health and Labour Sciences Research grants from the Ministry of Health, Labour and Welfare.

Conflict of interests

The authors have no conflicting financial interests.

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