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Review

Hematopoietic stem cell enhancer: a powerful tool in stem cell biology

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Summary. There has been considerable interest in identifying a *cis*-regulatory element that targets gene expression to stem cells. Such an element, termed stem cell enhancer, holds the promise of providing important insights into the transcriptional programs responsible for inherent stem cell-specific properties such as self-renewal capacity. The element also serves as a molecular handle for stem cell-specific marking, transgenesis and gene targeting, thereby becoming invaluable to stem cell research. A series of candidate enhancers have been identified for hematopoietic stem cells (HSCs). This review summarizes currently known HSC enhancers with emphasis on an intronic enhancer in the *Runx1* gene which is critical for the generation and maintenance of HSCs. The element, named eR1 (+24m), is active specifically in HSCs, but not in progenitors, and is hence the most definitive HSC enhancer.

Key words: RUNX1, AML1, Hematopoietic stem cell, *Cis*-regulatory element

Introduction

Hematopoietic stem cells (HSCs) are among the most characterized adult tissue stem cells and serve as a poster child in the stem cell field owing to the great success of their use in bone marrow transplantation (BMT), or hematopoietic stem cell transplantation, for the treatment of hematopoietic disorders. Despite such a widespread clinical application, pivotal scientific questions about HSCs remain to be clarified.

HSCs are commonly identified on the basis of the combination of functional assay and immunophenotypic properties by employing multiple antibodies against HSC-specific antigens, namely HSC surface markers. Alternatively, HSCs can be identified by genetic marking where HSCs are visualized by the expression of reporters, such as enhanced green fluorescent protein (EGFP), under the control of HSC-specific *cis*-regulatory element.

There has been considerable interest in identifying a *cis*-regulatory element that targets gene expression to HSC, termed HSC enhancer. An HSC enhancer, in combination with reporters such as EGFP or LacZ, can be used for the identification of HSCs (marking). The use of fluorescence reporters, like EGFP, also enables *in vitro* and *in vivo* live imaging of HSCs. An HSC enhancer can drive an expression of any gene of interest specifically in the HSC compartment (transgenesis). In addition, when used to drive the expression of Cre recombinase gene, an HSC enhancer enables the knock-out (KO) of genes of interest specifically in the HSC compartment (gene targeting). Besides being a powerful tool for experimental manipulation of HSCs, characterization of HSC enhancer also holds the promise of generating important insights into the transcriptional programs responsible for "stemness". A series of candidate HSC enhancers have been identified as listed in Table 1. The specificity of individual candidates will

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be discussed below.

Promoters for pan-hematopoietic genes

The most frequently used genetic elements to target HSCs are the promoter (p) of pan-hematopoietic genes, *Mx1* or *Vav*. These genetic elements are active not only in the HSC compartment, but also in progenitors and differentiated cells in all hematopoietic lineages. However, they have been used for gene targeting in HSCs.

Mx1(p)-driven Cre transgenic [*Mx1*(p)-Cre Tg] mouse is an inducible mouse model used for gene targeting in the hematopoietic system. *Mx1* is activated by interferon α/β after a viral infection, but is otherwise silenced in normal cells. The injection of synthetic double-stranded RNA, polyinosine-polycytidylic acid (pIpC), into the mice is used to induce *Mx1*(p)-driven Cre recombinase expression by mimicking a virus-infected state (Kuhn et al., 1995). *Mx1*(p)-Cre Tg mouse confers dominant Cre activity in the hematopoietic system. However, Cre activity is also observed in other tissues such as heart, uterus, kidney, muscle and brain (Kuhn et al., 1995). Moreover, apart from hematopoietic cells (HPCs), *Mx1*(p)-Cre is also active in a subset of mesenchymal cells in the bone marrow (BM) (Joseph et al., 2013). Notably, *Mx1*(p)-mediated targeting does not enable complete excision of the gene of interest in lymphoid tissues, such as the thymus, as compared to the BM, where nearly complete excision is usually achieved.

Table 1. Comparison of promoters/enhancers related to HSC and hemogenic EC.

Promoter (p)/ Enhancer (e)/ Knockin (KI)	Hematopoiesis specific?	Activities in non-hematopoietic tissues		HSC specific?	Activities within hematopoietic compartments			Hemogenic ECs specific?	Activities within embryonic ECs		Reference
		ECs	others		HSCs	Progenitors	Differentiated cells		Hemo-genic	non-Hemo-genic	
Mx1 (p)	dominant	++	++	no	++	++	++	no	++	++	Kemp et al., 2004; Kuhn et al., 1995; Joseph et al., 2013
Vav (p)	dominant	- ~ +	- ~ +	no	++	++	+ ~ ++ (BT)	no	-	-	Ogilvy et al., 1998; Georgiades et al., 2002; de Boer et al., 2003; Croker et al., 2004; Stadtfeld and Graf, 2005
Tie1 (p)	no	+++	+	no	+	+	-	no	+	++	Gustafsson et al., 2001
Tie2 (pe)	no	+++	+	no	-	-	-	no	+++	+++	Koni et al., 2001
VE-cadherin(p)	no	+++	- ?	no	+	-	-	no	+++	+++	Alva et al., 2006; Monvoisin et al., 2006; Chen et al., 2009
c-Kit (KI)	no	++	++	no	+++	+++	NA	NA	NA	NA	Tallini et al., 2009; Kimura et al., 2011
Sca-1 (p)	no	++	+	no	++	+	- ~ +++ (T)	yes ?	+	- ?	Ma et al., 2002
hCD34 (p)	no	++	++	no	+++	++	-	NA	NA	NA	Okuno et al., 2002
Tmtsp (KI)	no	++	NA	no	+++	- ~ ++	-	no	++	++	Takayanagi et al., 2006
Notch reporter	no	NA	+	no	+++	++	+ ~ ++	NA	NA	NA	Duncan et al., 2005
CD41 (p)	dominant	-	NA	no	-	++	- ~ ++ (Mk)	no	-	-	Emambokus and Frampton, 2003
Pf4 (p)	NA	NA	NA	no	++	++	++ ~ +++ (Mk)	NA	NA	NA	Calaminus et al., 2012
vWF (BAC Tg)	no	++	NA	no	++	- ~ ++	- ~ +++ (Mk)	NA	NA	NA	Sanjuan-Pla et al., 2013
Bmi1 (KI)	no	++	- ~ +++	no	+++	++	+	NA	NA	NA	Hosen et al., 2007
Gfi1 (BAC Tg)	no	-	- ~ ++	no	+	+	-	no	-	-	Wilson et al., 2010
Evi1 (KI)	NA	NA	NA	yes	+++	-	-	NA	NA	NA	Kataoka et al., 2011
Hoxb4 (KI)	no	+	- ~ +++	yes	++	-	-	yes ?	+ ?	- ?	Hills et al., 2011
C/EBPα (KI)	NA	NA	NA	no	+	+ ~ ++	- ~ +++ (M)	NA	NA	NA	Wolfler et al., 2010
Gata-1 (BAC Tg)	no	++	++	no	- ~ ++**	- ~ ++	- ~ +++ (ME)	NA	NA	NA	Mao et al., 2001; Takai et al., 2013
Gata-2 (e)	no	++	++	no	++	+	-	no	++	++	Khandekar et al., 2007
Gata-3 (KI)	no	NA	- ~ +	no	+	NA	- ~ +++ (T)	NA	NA	NA	Grote et al., 2006; Hosoya-Ohmura et al., 2011; Frelin et al., 2013
Lmo2 (e)	no	++	+	no	NA	+	++ (E)	no	+	+	Landry et al., 2009; Calero-Nieto et al., 2013
Scl (e)	no	++	+	no	+++	++	+	no	++	+ ?	Gottgens et al., 2002; Gothert et al., 2005
Fli1 (e)	dominant	+	++	no	++	+	-	yes ?	+ ?	-	Donaldson et al., 2005; Pimanda et al., 2007b
Prh (e)	dominant	+	+	no	+	+	-	no	+ ?	+ ?	Rodriguez et al., 2001; Donaldson et al., 2005
Smad6 (e)	dominant	+	++	no	++	+	-	no	++	++	Pimanda et al., 2007a
Erg (e)	NA	NA	NA	no	+++	- ~ ++	- ~ ++ (T)	NA	NA	NA	Thoms et al., 2011
Eng (e)	no	++	++	no	++	+	-	no	+	+	Pimanda et al., 2008
Flk-1 (pe)	no	++	+	no	-	-	-	no	-	++	Hirai et al., 2003, 2005
Runx1 (e)	dominant	-*	-	yes	+++	-	-	yes	++*	-	Ng et al., 2010

**Runx1* enhancer is active in hemogenic ECs at embryo stage but not active in adult ECs, **active when the interspacing sequence is removed. Abbreviations: Mk, megakaryocytes; M, myeloid cells; B, B cells; T, T cells; E, erythroids; ECs, endothelial cells; NA, not available; +, weak expression.

A disadvantage in using *Mx1*(p)-Cre system is its significant background Cre activity due to endogenous interferon production (Kemp et al., 2004).

Vav is a guanine nucleotide exchange factor that is important for hematopoiesis, especially for the development and activation of T and B cells. The constitutively active *Vav*(p)-Cre Tg mouse has been widely used to carry out gene deletions in the hematopoietic system. By virtue of its Cre activity in pan-hematopoietic tissues, *Vav*(p)-Cre Tg mice have been used to target HSCs. Three distinct *Vav*(p)-Cre Tg mice have been generated. Two of these Tg lines showed complete excision of targeted genes in pan-hematopoietic cells (Georgiades et al., 2002; de Boer et al., 2003; Croker et al., 2004; Stadtfeld and Graf, 2005). The Cre activity of *Vav*(p)-Cre Tg mouse is initiated at embryonic day E10.5 and becomes fully active in almost all HSCs at E13.5 (Chen et al., 2009; Gan et al., 2010). *Vav*(p)-Cre-mediated targeting in HPCs is maintained throughout the organism's lifespan. At the adult stage, *Vav*(p) activity is also detected in the heart [endothelial cells (ECs)], kidney, muscle and testis (Georgiades et al., 2002; Croker et al., 2004). Due to its activity in testis or ovaries, some extent of germline deletion is observed in *Vav*(p)-Cre Tg mouse strains (Georgiades et al., 2002; Croker et al., 2004).

As *Vav*(p)- and *Mx1*(p)-Cre activities are both prominent in hematopoietic tissues, the major phenotypes observed, including those in the HSC populations, are largely similar when utilizing either genetic element for targeting a specific gene. However, distinct phenotypes have also been observed when the same gene was targeted by these two systems. Deletion of Eed, a core component of the polycomb repressive complex 2 (PRC2), using the *Vav*(p)-Cre Tg system resulted in a differentiation block of HPCs, while targeted deletion by the *Mx1*(p)-Cre Tg system caused both a differentiation block and HSCs exhaustion (Xie et al., 2014). Another example is a defect in B lymphopoiesis in *Mll1* conditional KO mice, which was observed only in *Mll1* conditional KO mice using *Vav*(p)-Cre-mediated targeting, but not in *Mx1*(p)-Cre Tg mouse (Gan et al., 2010). Several factors might account for these phenotype differences: (i) Distinct requirement of a gene in developmental versus adult hematopoiesis. *Vav*(p) is activated at E10.5 onwards during the critical period of rapid HSC expansion, while *Mx1*(p) is induced at the adult stage when HSCs are largely in quiescence. (ii) Differential excision efficiencies in distinct cell types within the hematopoietic system. The above-mentioned B cell phenotypes in *Mll1* conditional KO may be explained by this mechanism. (iii) Significant targeting of non-hematopoietic cells in the BM and other hematopoietic tissues by *Mx1*(p)-Cre, but not by *Vav*(P)-Cre method. Niche constituting cells which serve as a micro-environment for HSCs are likely to be affected by *Mx1*(p)-Cre-mediated targeting (Joseph et al., 2013). (iv) *Mx1*(p)-Cre system is one-time-only induction whereas *Vav*(p)-Cre allows for continued targeting. Distinct phenotypes observed might be due to the significant number of residual non-targeted cells in *Mx1*(p)-Cre targeted mice. (v) Interferon-mediated bone marrow suppression in *Mx1*(p)-Cre might cause some differences shortly after pIpC injection.

Genetic elements for pan-hematopoietic factors appear useful for gene targeting in HSCs; however, differentiated hematopoietic cells are also targeted. Thus, HSC-specificity cannot be achieved by using genetic elements active in pan-hematopoietic tissues.

Promoters for pan-endothelial genes

Adult BM HSCs, or definitive HSCs, are derived from a subset of endothelial cells (ECs), termed hemogenic ECs, at a specific embryonic stage. In E10.5 murine embryos, definitive HSCs first emerge within major arteries, including the dorsal aorta (DA) in the aorta-gonad-mesonephros (AGM) region. HSCs reside within clusters of HPCs budding from their immediate precursor cells, hemogenic ECs, in the luminal wall of these arteries. Hence, HPCs are all progenies of hemogenic ECs, and genetic elements of pan-endothelial genes are expected to mark all HPCs, including HSCs.

Tie1 and Tie2 are cell surface receptors expressed on ECs and both are activated by their ligands, angiopoietin-1 (Ang-1) and -2, in blood vessel formation. *Tie2* is essential for definitive hematopoiesis during embryogenesis (Takakura et al., 1998). Cre expression driven by *Tie2* promoter and enhancer (pe) was seen in all ECs including hemogenic ECs in the embryo (Schlaeger et al., 1997). Hence, a complete deletion of the targeted gene was observed in adult BM cells using *Tie2*(pe)-Cre Tg mice (Koni et al., 2001). In contrast to *Tie2*(pe)-Cre Tg mouse, relatively weak Cre activity was observed in hemogenic ECs of *Tie1*(p)-Cre Tg embryos although Cre activity was seen in most ECs at E10. Only 13% $LacZ^+$ HPCs were observed in E10 embryos of *Tie1*(p)-Cre; Rosa-LSL-LacZ mice (Gustafsson et al., 2001) and approximately 10-20% $LacZ^+$ cells were found in differentiated blood cells of adult BM. Therefore, *Tie2*(pe)-Cre Tg mouse, but not *Tie1*(p)-Cre Tg mouse, is preferentially used to target pan-HPCs, including HSCs.

Vascular endothelium-specific cadherin (*VE-Cadherin*), which is responsible for vascularization, exhibited robust activities in ECs (Alva et al., 2006; Chen et al., 2009). Two distinct *VE-Cadherin*(p)-Cre Tg lines have been generated but the gene targeting efficiencies in adult BM differ from each other: one demonstrated 50-60% gene targeting efficiency, while the other exhibited nearly 100% efficiency (Alva et al., 2006; Chen et al., 2009). Two tamoxifen inducible *VE-Cadherin*(p)-driven $CreER^{T2}$ Tg lines were also generated (Monvoisin et al., 2006; Wang et al., 2010). Tamoxifen injection into *VE-Cadherin*(p)-$CreER^{T2}$; Rosa-LSL-LacZ mother mice on day 9.5 post coitum produced 2-24% of $LacZ^+$ HPCs in adult BM. In contrast, tamoxifen injection into adult mouse generated

0.3% LacZ$^+$ HPCs in the BM (Monvoisin et al., 2006).

In summary, genetic elements for pan-ECs enable highly efficient marking of HSCs at embryonic stage as all HPCs are derived from hemogenic ECs. However, most of these elements do not serve as a useful HSC-specific gene targeting tool at the adult stage due to their null activity in HPCs.

Genetic elements for HSC surface markers

Several cell surface markers abundantly expressed on HSCs have been identified and utilized to study HSCs behavior. For example, *c-kit* and *Sca-1* expression is used to isolate HSCs in the Lineage$^-$ (Lin) population.

c-Kit-EGFP BAC Tg and *c-Kit*-IRES-EGFP knock-in (KI) mice were generated to recapitulate endogenous c-Kit expression which is seen in various tissues such as hematopoietic, germ, mast, stellate, epithelial, endothelial and smooth muscle cells (Tallini et al., 2009; Kimura et al., 2011). *c-Kit*-IRES-EGFP KI mice demonstrated EGFP expression in almost all c-Kit$^+$ hematopoietic cells, including the progenitor and differentiated cells, in adult BM (Kimura et al., 2011). This broad expression of EGFP seems to limit the use of *c-Kit*-IRES-EGFP KI mouse for HSC marking.

Sca-1(p)-EGFP Tg mouse allows for the marking of both hemogenic ECs and HPCs within the AGM region at E11.5 (de Bruijn et al., 2002). In adults, *Sca-1*(p)-driven EGFP expression was observed in around 15% of BM cells. Of these EGFP$^+$ cells, 72% were Mac1$^+$ myeloid cells. In addition, 80% lymph node cells expressed EGFP (Ma et al., 2002), suggesting that *Sca-1*(p) activity is very strong in mature cells and is hence not useful for HSC marking.

To further enrich for HSCs, CD34 is employed in combination with other HSC surface markers. Human HSCs are shown to reside in human CD34$^+$ (hCD34$^+$) population whereas mouse HSCs are enriched in CD34$^-$ (mCD34$^-$) c-Kit$^+$Sca-1$^+$Lin$^-$ (KSL) population (Osawa et al., 1996). CD34 expression patterns between mouse and human HSCs were carefully examined by generating *hCD34*(p)-EGFP Tg mouse. mCD34$^-$ hCD34$^+$ KSL population, but not mCD34$^+$ hCD34$^+$ KSL fraction, was capable of repopulating the whole hematopoietic system in lethally irradiated recipient mice (Okuno et al., 2002). As such, HSCs appear to reside in the *hCD34*(p)-EGFP$^+$ population. However, a significant frequency of *hCD34*(p)-EGFP$^+$ cells was also observed in the hematopoietic progenitor cell fraction. Hence, HSC-specificity is not fully conferred by using *hCD34*(p)-EGFP Tg system.

Transmembrane molecule with thrombospondin module (Tmtsp) was recently identified as a cell surface marker highly expressed in mouse immature HPCs and ECs. In embryos, *Tmtsp*-Venus KI mouse demonstrated *Tmtsp*-Venus$^+$ cells at initial stages of definitive hematopoiesis in the AGM region. *Tmtsp*-Venus$^+$ cells were also found in ECs within the AGM region (Takayanagi et al., 2006). In adult BM, Venus was highly expressed in almost all CD34$^-$ KSL HSCs and CD34$^+$ KSL multipotent progenitor cells. Transplantation of Lineage$^-$ Tmtsp-Venus^{high} cells into wild-type mice demonstrated long-term hematopoietic reconstitution. High frequency of *Tmtsp*-Venus$^+$ cells was also detected in myeloid and lymphoid progenitors, but no *Tmtsp*-Venus$^+$ cells were observed in the differentiated HPC population (Takayanagi et al., 2006).

Taken together, while some genetic elements for HSC surface markers may be useful for HSC marking to some extent, most HSC surface markers identified so far are also expressed in progenitors and differentiated cells. Thus, HSC-specific marking is not achieved by utilizing genetic elements for HSC surface markers.

Reporter mouse for signaling molecule active in HSCs

Extracellular signaling molecules, such as Notch, Wnt and Hedgehog have been found to play important roles in maintaining HSC properties (Maillard et al., 2003; Reya and Clevers, 2005). In particular, the Notch signaling pathway is essential for the generation and maintenance of definitive HSCs, particularly through the interaction between HSCs and their niche. A Notch reporter mouse strain, containing a transgene composed of a Notch response element and a minimal SV40 promoter followed by EGFP sequence, was generated to capture the behavior of Notch signaling receiving cells. Notch reporter mice revealed high activity of Notch signaling in HSCs and T-cells, and this activity gradually declined as HSCs differentiated (Duncan et al., 2005). Due to such widespread importance of Notch pathway involvement, HSC specificity is not achieved in Notch reporter mice.

Megakaryocyte-related genes and HSCs marking

The HSC compartment is known to be a heterogeneous population within which differentially lineage biased HSCs, namely lymphoid-, myeloid- and platelet-biased HSCs, can be found (Muller-Sieburg et al., 2002; Dykstra et al., 2007; Copley et al., 2012; Sanjuan-Pla et al., 2013; Yamamoto et al., 2013).

A megakaryocyte marker, CD41 (integrin α IIb) glycoprotein, has long been used as one of the embryonic HSC markers in zebrafish and mouse. In adult mouse BM, CD41 is classically used as a megakaryocyte- and platelet-specific marker. Endogenous CD41 expression is observed in a subset of murine fetal liver (FL) HSCs (Morrison et al., 1995). In *CD41*(p)-Cre Tg mouse, gene targeting activity in a few ECs budding from the aorta wall and HPCs within the AGM region was observed in E10.5 embryos. As a result, 20-50% of HPCs of E18.5 embryos were LacZ$^+$ in *CD41*(p)-Cre; Rosa-LSL-LacZ Tg mouse. However, HPCs targeted by *CD41*(p)-mediated Cre activity were significantly reduced to around 2-3% in adult BM (Emambokus and Frampton, 2003). These results

suggest that *CD41*(p) preferentially targets embryonic stage-specific HPCs, but not adult HSCs.

Platelet factor 4 (Pf4), also known as Cxcl4, is a chemokine secreted by activated platelets during platelet aggregation. Endogenous *Pf4* expression was observed in $CD150^+CD48^-$ KSL and $CD150^+CD48^+$ KSL cells with the highest expression found in $CD150^+CD48^+$ KSL cells (Calaminus et al., 2012). The FL of *Pf4*(p)-Cre Tg E14.5 embryo showed low percentage of Cre active cells (around 10%) in $CD150^+CD48^-$ KSL fraction whereas a higher percentage of Cre active cells (40-60%) was observed in the corresponding HSC fraction of adult BM, indicating that there is a certain level of *Pf4*(p) activity in BM long-term (LT)-HSCs. As *Pf4* is highly expressed in megakaryocytes, the Cre activity was also clearly detected in these cells.

Recently, a megakaryocyte-biased HSC subset has been identified through the use of von *Willebrand factor* (*vWF*)-EGFP BAC Tg mouse system (Sanjuan-Pla et al., 2013). vWF is a blood glycoprotein responsible for platelet aggregation. High endogenous *vWF* expression was reported in mouse HSC-enriched BM cells (Kent et al., 2009). At embryonic stage, almost all $CD150^+CD48^-$ KSL FL cells were *vWF*-$EGFP^+$. In contrast, *vWF*-$EGFP^+$ cells were reduced to around 60% in adult $CD150^+CD48^-CD34^-$ KSL BM cells. Transplantation of *vWF*-$EGFP^+$ and *vWF*-$EGFP^-$ cells from the $CD150^+CD48^-CD34^-$ KSL fraction of adult BM achieved long-term hematopoietic reconstitution in recipient mice. However, recipient mice of $CD150^+CD48^-CD34^-$ KSL *vWF*-$EGFP^+$ cells predominantly demonstrated platelet/myeloid biased reconstitution. Remarkably, a high percentage of *vWF*-$EGFP^+$ cells was observed in megakaryocyte progenitors of *vWF*-EGFP BAC Tg mouse.

Altogether, megakaryocyte-related genetic elements appear to mark a subset of megakaryocyte-biased HSCs. However, their continuous expression from HSCs to megakaryocyte lineage cells prevents HSC-specific marking.

Knock-in or BAC Tg mice of HSC-associated transcription factors

HSCs specification, maintenance and subsequent differentiation are tightly regulated by key transcription factors, such as *Bmi1, Gfi1, Evi1, Hoxb4, C/EBPα,* Gata factors, *Lmo2* and *Runx1*.

Bmi1 is a component of the polycomb repressive complex 1 (PRC1) responsible for gene repression. *Bmi1* plays an important role in the maintenance of "stemness" in multiple tissue stem cells, in particular neural stem cells and HSCs. In adult BM, *Bmi1*-EGFP KI mice displayed the strongest EGFP signals in $CD34^-Flk2^-$ KSL and $CD34^-CD150^+$ KSL population but significant EGFP signals remain in progenitors and further differentiated cells (Hosen et al., 2007). Lethally irradiated mice which received *Bmi1*-$EGFP^{high}$ KSL cells were capable of reconstituting their hematopoietic system but not those who received *Bmi1*-$EGFP^{low}$ KSL cells. The multilineage analysis showed highly myeloid-biased reconstitution. Through limiting dilution assay, the frequency of HSCs in *Bmi1*-$EGFP^{high}$ KSL fraction was estimated at 1 in 16 cells.

Gfi1 belongs to a small family of transcription factors which function as a repressor of gene expression. *Gfi1* expression was reported in HSCs and T cell lineages. The E11.5 embryo of *Gfi1*-LacZ BAC Tg mouse showed LacZ expression in hematopoietic tissues and other organs, such as somites, stomach, snout and eyes (Wilson et al., 2010). A *Gfi1* hematopoietic enhancer, -35 enhancer (e), was identified within an intron of the neighboring gene *Evi5* and drove high expression of LacZ in hematopoietic organs of E11.5 embryos (Wilson et al., 2010). The E11.5 embryos of both *Gfi1* -35(e)-LacZ and *Gfi1*-LacZ BAC Tg lines showed $LacZ^+$ cells in HPCs within AGM. Notably, no LacZ signals were observed in ECs of both *Gfi1*-35(e)-LacZ and *Gfi1*-LacZ BAC Tg mouse embryos.

Evi1 was first identified as a gene located at a common retroviral integration site in AKXD murine myeloid tumors. Evi1 is known to be one of the key factors in maintaining integrity of HSCs. *Evi1*-IRES-EGFP KI mice showed EGFP signals specifically in the KSL compartment. Within the KSL compartment, a relatively high percentage of *Evi1*-IRES-$EGFP^+$ cells was observed in $CD150^+CD48^-$ KSL and $CD34^-Flk2^-$ KSL fractions. Remarkably, EGFP signals were not observed in hematopoietic progenitors and differentiated cells. In addition, $EGFP^+$ cells, but not $EGFP^-$ cells, isolated from $CD150^+CD48^-$ KSL and $CD34^-Flk2^-$ KSL fractions of *Evi1*-IRES-EGFP KI mice showed long-term multilineage hematopoietic reconstitution in adult BM (Kataoka et al., 2011). Therefore, *Evi1*-IRES-EGFP KI mice appear to be successful in HSC-specific marking.

A homeobox gene, *Hoxb4*, is known to be important for HSC behavior. Its importance is highlighted by the fact that overexpression of *Hoxb4* in embryonic stem cells allows for the generation of definitive HSCs in *in vitro* culture (Kyba et al., 2002). *Hoxb4*-EYFP KI mouse was generated to trace *Hoxb4* expression in HSC development. The strongest *Hoxb4*-EYFP signals were observed in the neural tube posterior to the rhombomere 6/7 boundary at embryonic stage. In the hematopoietic system, *Hoxb4*-$EYFP^+$ cells were found in roughly 50% of both ECs and HPCs within AGM of E11.5 embryo (Hills et al., 2011). In adult BM, *Hoxb4*-$EYFP^+$ cells were observed in KSL fraction, though the EYFP signal intensity was very weak, and the slightly brighter EYFP cells were found in $CD150^+$ KSL population. Notably, weak or no *Hoxb4*-$EYFP^+$ cells were found in hematopoietic progenitors and differentiated cells. Long-term hematopoietic reconstitution was observed in wild-type mice transplanted with adult BM $Lineage^-Sca\text{-}1^+EYFP^+$ cells, but not in those with $Lineage^-Sca\text{-}1^+EYFP^-$ cells. Therefore, the *Hoxb4*-EYFP signal seems useful for HSC marking, but the weak signal intensity

may limit its usefulness.

C/EBPα is widely believed to be a myeloid lineage specific transcription factor, but *C/EBPα* is also shown to be expressed and functioning in the HSC compartment. *C/EBPα*-Cre KI mouse showed the highest Cre activity in granulocyte/macrophage progenitors (GMP) and descendent myeloid cells with comparatively low activity in HSCs (Wolfler et al., 2010).

Gata factors belong to the C_4 zinc-finger transcription factor family and are important in the development of many organs. In particular, *Gata-1, Gata-2* and *Gata-3* are critical for the development of various hematopoietic lineage cells (Pevny et al., 1991; Tsai et al., 1994; Pandolfi et al., 1995). *Gata-1* plays a role in erythroid and megakaryocyte differentiation but not in HSCs. However, recent analyses of *cis*-regulatory elements in the *Gata-1* locus identified 3.2-kb interspacing sequences in its upstream region which inactivates *Gata-1* expression in HSCs (Takai et al., 2013). Removal of this interspacing sequence in *Gata-1*-GFP BAC Tg mouse led to a significant increase in frequency of GFP^+ cells in $CD150^+CD48^-$ KSL fraction. This transgenic mouse could be used to mark HSCs.

Gata-2 has an essential role in definitive HSC development. *Gata-2* null mice die at mid-gestation due to failure of hematopoietic stem/progenitor pool expansion (Tsai et al., 1994). *Gata-2*-GFP KI mice showed GFP expression in pan-endothelial cells (Khandekar et al., 2007). The hematopoietic enhancer for *Gata-2* has been identified and will be discussed later.

Gata-3-EGFP KI mouse was originally generated to study kidney development (Grote et al., 2006). In the hematopoietic system, *Gata-3* is well known to function in T cells and is expressed in HSCs (Zhong et al., 2005; Kent et al., 2009). Sorted *Gata-3*-$EGFP^-$ cells from BM displayed intermediate-term reconstituting activity (8-16 weeks) while *Gata-3*-$EGFP^+$ cells from BM of *Gata-3*-EGFP KI mouse showed long-term reconstituting activity in a transplantation experiment (Frelin et al., 2013). However, a strong EGFP signal in the T lymphocyte lineage (Hosoya-Ohmura et al., 2011) may limit the potential of *Gata-3*-EGFP KI mice for assessing HSC behaviors.

Lmo2 belongs to the LIM domain family which is a bridging molecule in multiprotein transcription complexes. Lmo2 interacts with HSC-associated transcription factors, such as Scl/Tal1 (Wadman et al., 1997). *Lmo2* is essential in hematopoiesis and vascular development during ontogeny. *Lmo2* null mice die at E10.5 due to the failure of yolk sac erythropoiesis (Warren et al., 1994). *Lmo2*-LacZ KI mouse showed LacZ activity in FL and ECs of the major blood vessel walls and capillaries of embryos (Yamada et al., 2000). Three promoters and eight enhancer elements that regulate *Lmo2* expression have been identified (Landry et al., 2009). *Lmo2* +1(e)-LacZ showed expression in ECs, developing limbs and tail (Calero-Nieto et al., 2013). No significant LacZ expression was observed in the blood of *Lmo2* +1(e)-LacZ Tg mouse embryo, although *Lmo2* +1(e) was functionally active in leukemic cell lines. Lmo2 -75(e)-LacZ Tg mouse showed weak LacZ expression within circulating blood cells (Landry et al., 2009). A combination of *Lmo2* +1 and -75 enhancers showed specific and significantly high erythrocyte marking at embryonic stage. The transcriptional regulation of *Lmo2* in HSCs warrants further investigation.

In summary, KI or BAC Tg mice of the HSC-associated transcription factors actively drive reporter genes in the HSC compartment; however, HSC-specificity cannot be achieved as most of these transcription factors also function in differentiated cells. Interestingly, for some genes, expression in HSCs and differentiated cells can be regulated by distinct cis-regulatory elements. Hence, an HSC-specific regulatory element could be identified in the above-mentioned HSC-associated transcription factor genes.

Known HSC enhancers

In several genes, such as *Scl/Tal1* (henceforth *Scl*) (Porcher et al., 1996; Robb et al., 1996), *Gata-2* (Tsai et al., 1994) and *Runx1* (Okuda et al., 1996; Wang et al., 1996), potential HSC-specific enhancers, which are clearly dissected out from differentiated cell-specific *cis*-regulatory elements, have been identified.

Scl, encoding a basic helix-loop-helix transcription factor, is important for hematopoiesis and development of the endothelium (Sanchez et al., 1999). Among HSC-related genes, *cis*-regulatory elements of *Scl* have been most extensively studied. Tg mouse studies have shown that *Scl* +19(e) targets expression of a reporter gene to ECs and HPCs at E11.5 (Gottgens et al., 2002). Tamoxifen-inducible $CreER^T$ recombinase under the control of *Scl* +19(e) showed 17% marking within the KSL compartment of FL at E14.5. In adult BM, *Scl* +19(e)-driven $CreER^T$ activity was observed in 21% of KSL cells and 93% of $Flk2^-$ KSL fraction (Gothert et al., 2005). Lethally irradiated mice, which received $EYFP^+$ cells from *Scl* +19(e)-$CreER^T$; Rosa-LSL-EYFP Tg mouse after tamoxifen induction, showed long-term hematopoietic reconstitution.

Gata-2 is indispensible for HSCs development. *Gata-2* -3(e)-GFP Tg mouse showed GFP signals in ECs and HPCs at E9.5 (Kobayashi-Osaki et al., 2005). *Gata-2* -3(e)-GFP^+ cells, but not *Gata-2* -3(e)-GFP^- cells, were capable of differentiating into multiple hematopoietic lineages after co-culture with OP9 stromal cells.

Among the well characterized HSC enhancers, such as *Scl* +19(e) and *Gata-2* -3(e), the orientation and spacing constraints of binding sites for HSC-associated transcription factors share a remarkable feature, named Ets/Ets/Gata (E/E/G) signature. Genome wide computational screen based on the Ets/Ets/Gata

signature identified several regulatory elements in other genes, such as *Fli1* +12(e), *Prh +1*(e) and *Smad6* -57(e).

Fli1 is a transcription factor, which was first identified in erythroleukemias induced by friend murine leukemia virus. *Fli1* +12(e)-LacZ Tg mouse embryos showed dominant activity in HPCs in AGM with weak activity in ECs (Donaldson et al., 2005; Pimanda et al., 2007b). Sorted LacZ$^+$ FL cells from *Fli* +12(e)-Tg mouse embryos showed long-term multilineage reconstitution in recipient mice.

Proline-Rich Homeodomain protein (Prh, also known as Hex) is an essential transcription factor which regulates various gene expressions during development. Embryonic lethality of *Prh* null mouse was observed at E14.5 due to abnormal cardiac development and defective vasculogenesis (Hallaq et al., 2004). A 3-kb regulatory element isolated from the *Prh* gene locus, encompassing *Prh* +1(e), drove reporter gene expression in the blood island of yolk sac at E7.5 (Rodriguez et al., 2001). *Prh* +1(e)-LacZ Tg mouse embryos showed LacZ expression in a subset of FL HPCs and occasionally in ECs (Donaldson et al., 2005).

Smad6 is an inhibitor for the BMP signaling pathway. *Smad6* -57(e)-LacZ$^+$ cells were observed in the HPC population within the AGM of E11.5 embryos (Pimanda et al., 2007a). A similar frequency of LacZ$^+$ cells (around 8%) was found in whole BM of *Smad6* -57(e)-LacZ and *Scl* +19(e)-LacZ Tg mice. Remarkably, cells targeted by both *Scl* +19(e) and *Smad6* -57(e) are enriched in the CD150$^+$CD48$^-$CD41$^-$ KSL population.

Ets-related gene (Erg) is a transcription factor responsible for platelet adhesion to the subendothelium and hematopoiesis. *Erg* is highly expressed in HSCs and myeloid cells (Murakami et al., 1993; Thoms et al., 2011). The E11.5 embryo of *Erg* +85(e)-Venus Tg mouse showed *Erg* +85(e)-Venus^{high} signal in HPCs (Thoms et al., 2011). In adult BM, *Erg* +85(e)-Venus^{high} cells were observed in T cell lineages, KSL and myeloid progenitor fractions. *Erg* +85(e)-Venus signals gradually reduced as HPCs differentiate.

Endoglin (Eng) is a cell surface receptor important for TGF-β signaling and modulates hematopoiesis and angiogenesis during embryo development. *Eng* -8(e), +7(e), and +9(e) are functionally active chromatin regions in the HPC7 hematopoietic progenitor cell line. *Eng* +7(e) is a hematopoietic enhancer albeit its activity was weak in HPCs of *Eng* +7(e)-LacZ Tg mouse embryos. In combination with *Eng* -8(e) and +9(e), which are endothelial enhancers, *Eng* -8(e), +7(e), +9(e)-LacZ Tg mouse embryos showed significantly high LacZ expression in both ECs and HPCs (Pimanda et al., 2008). An *in vitro* colony assay showed that *Eng* -8(e), +7(e), +9(e)-LacZ$^+$ cells have higher multilineage colony forming ability compared to LacZ$^-$ cells.

The regulatory elements from the above mentioned genes showed specificity in HSCs to some extent. However, none of them have met the stringent requirements of HSC-specificity as a HSC enhancer.

A Runx1 intronic enhancer, eR1, marks long term-HSCs

Runx1 is a transcription factor that is essential for HSC generation and maintenance (Okuda et al., 1996; Motoda et al., 2007; Jacob et al., 2010). Despite such a well-established role of Runx1 in hematopoiesis and its frequent association with human diseases, the transcriptional regulation of the Runx1 gene is not well characterized. We recently succeeded in identifying an intronic enhancer in the *Runx1* gene. The element, named eR1 (+24m) or *Runx1* +24mCNE, was discovered through a combinatorial *in silico* approach and subsequent *in vivo* verification using zebrafish and mouse transgenic models. Interestingly, eR1 also contains the above-mentioned E/E/G signature which is commonly seen in HSC-related regulatory elements. A chromatin immunoprecipitation assay showed that several hematopoietic-related transcription factors, such as Scl, Gata-2, Fli1, Lmo2 and Lbd1, bind to eR1(+24m) (Nottingham et al., 2007). Accumulation of all these proteins at *Runx1* enhancer is believed to activate *Runx1* expression in HSCs.

In eR1-EGFP Tg mouse, only a low percentage of Lin$^+$ differentiated cells were marked by very weak EGFP fluorescence (Fig. 1A). Consistent with endogenous *Runx1* expression, eR1 was largely inactive in erythroid cells (Lorsbach et al., 2004; North et al., 2004). Surprisingly, however, myeloid and lymphoid cells which express *Runx1* were not marked by eR1 activity, suggesting that eR1 does not recapitulate the wider expression of endogenous *Runx1* within the adult hematopoietic compartment. Other *cis*-regulatory element(s) are likely to be involved in regulating the Runx1 gene expression in these differentiated HPCs.

In line with high expression of *Runx1* in HSCs (Lorsbach et al., 2004; North et al., 2004), 50% of the cells within the BM KSL compartment were marked by strong EGFP fluorescence (Fig. 1, fraction ii). In contrast, 20% of the immature progenitor cells exhibited significantly reduced EGFP fluorescence (Fig. 1A, fraction i). In addition, further enrichment for HSCs within KSL (Fig. 1A, fraction ii) using CD34 or SLAM makers (CD150, CD48) showed that EGFP^{high} cells (red arrow in Fig. 1B) were found in greatest abundance within LT-HSC, whereas EGFP^{high} cells are significantly reduced in ST-HSC. These observations suggest that the eR1 is strongly and specifically active in LT-HSC and its enhancer activity is likely to be rapidly "shut down" upon commitment to progenitor cells. This LT-HSC specific activity of eR1 was further confirmed by rigorously conducted long-term multilineage engraftment assay (Ng et al., 2010), lending credence to the role of the eR1 as a HSC enhancer.

eR1 in hemogenic ECs

HSCs are derived from a subset of ECs, namely

hemogenic ECs, from the artery wall of dorsa aorta. Currently, no enhancer has been shown to specifically target expression of a reporter gene to hemogenic ECs as the majority of the reported enhancers show the activities in the entire EC compartment.

eR1 activity was found in hemogenic ECs at E10.5. We analyzed eR1 activity in the AGM region of E10.5 Tg mouse embryos by flow cytometry analysis using antibodies against Pecam-1 / CD31 (an EC marker), CD45 (a pan-leukocyte marker) and Ter119 (an erythrocyte marker). Consistent with results obtained from wholemount immunostainings showing that emerging HPCs are Pecam-1$^+$, a significant percentage of CD45$^+$ HPCs are also Pecam-1$^+$. Remarkably, almost all CD45$^+$ HPCs were EGFP$^+$ while Ter119$^+$ EryP cells were EGFP$^-$. These observations suggest that HPCs which emerge from the AGM region are all marked by eR1-driven EGFP expression. On the other hand, not all Pecam-1$^+$ ECs were EGFP$^+$, as expected from observations made in wholemount immunostainings.

It was previously shown that ECs from wild-type, but not from *Runx1*$^{-/-}$ mouse embryos, gave rise to HPCs when cultured *in vitro* on an OP9 stromal layer in the presence of cytokines (Hills et al., 2011). Using the same system, we investigated the hemogenic capability of EGFP$^-$ or EGFP$^+$ fractions within Pecam-1$^+$CD45$^-$ CD41$^-$Ter119$^-$ ECs from eR1-EGFP Tg mouse embryos. Strikingly, only the eR1-GFP$^+$ but not eR1-GFP$^-$ ECs gave rise to HPCs (Ng et al., 2010; Swiers et al., 2013). Therefore, the eR1 activity is specifically restricted to hemogenic ECs, establishing the enhancer activity of this element as a surrogate marker for hemogenic ECs.

Flk-1(pe) was also identified as a genetic element associated with hemogenic ECs. However, *Flk-1*(pe) displayed a completely opposite expression pattern. Only *Flk-1*(pe)-GFP$^-$ ECs cells can produce HPCs on OP9 stromal cell layer (Hirai et al., 2003). Hence eR1 acts as a positive marker for hemogenic ECs, whilst *Flk-*

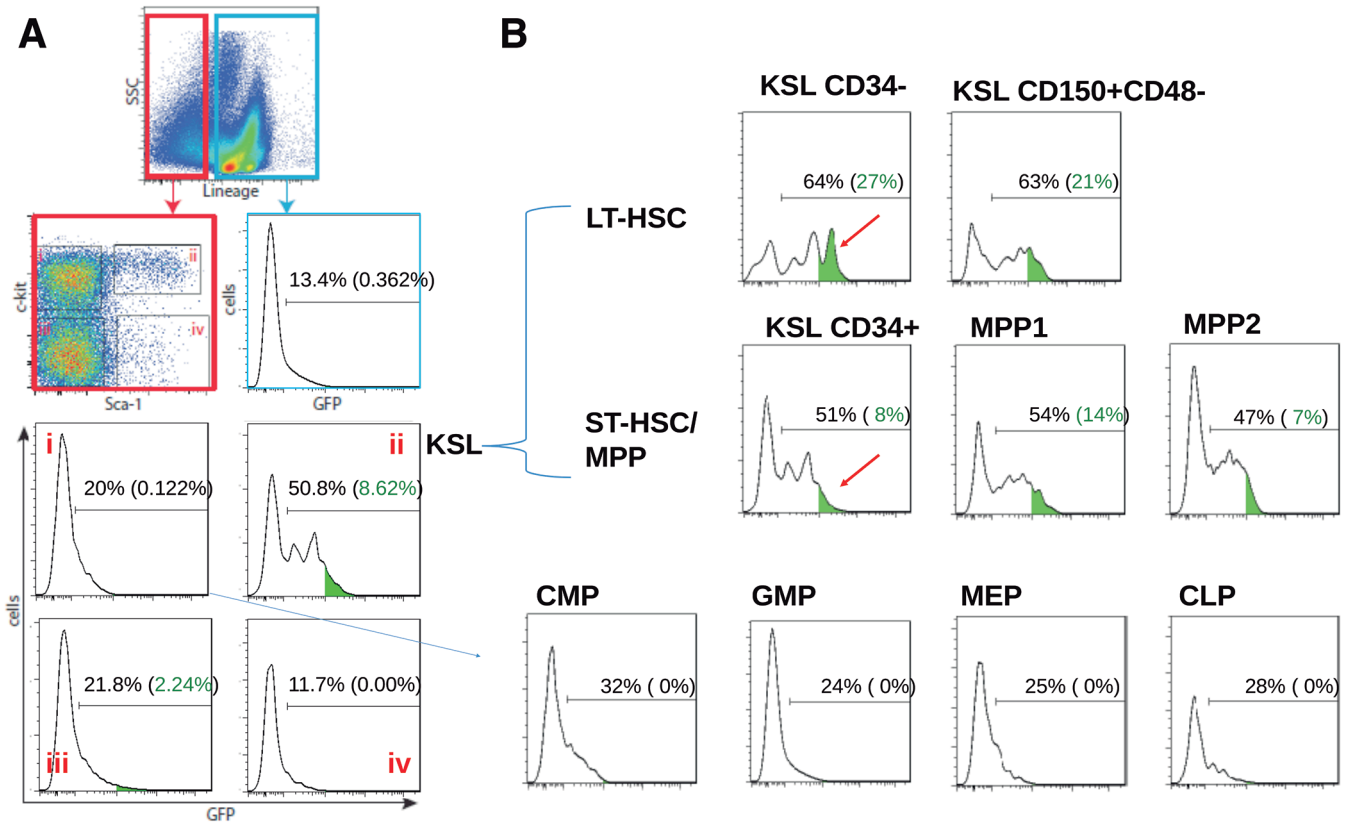

Fig. 1. eR1 activity marks LT-HSCs. **A.** Bone marrow cells from adult eR1-EGFP Tg mice were harvested and analyzed by flow cytometry. eR1-driven high expression EGFP (EGFP^{high}, shaded in green) is seen only in the hematopoietic stem/progenitor (HSPC) fraction defined as the c-kit$^+$Sca-1$^+$Lin$^-$ (KSL) (fraction ii), while a very low percentage of EGFP^{high} cells in c-kit$^+$Sca-1$^-$Lin$^-$ progenitor fraction (fraction i). Lineage$^+$ fraction (differentiated cells) is boxed out in blue with very few EGFP^{high} cells. **B.** HSCs within KSL (fraction ii) were further enriched using CD34 or SLAM markers. EGFP^{high} cells (red arrow) were found in greatest abundance within LT-HSCs, whereas EGFP^{high} cells are significantly reduced in ST-HSC/MPP fractions, suggesting that eR1 is specifically and strongly active in LT-HSCs. Abbreviations: LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; MPP, multipotent progenitors.

1(pe) can be used as a negative marker.

Future perspectives

A series of candidate enhancers which target gene expression to HSCs have been identified (summarized in Table 1). However, none of them except for *Runx1* enhancer (eR1) show activities restricted to HSC compartment, but much broader expressions including less immature progenitor fractions. *Evi1* KI mice exhibited HSC-specificity; however, its regulatory element(s) responsible for HSC-specific expression remains to be identified.

Single marker HSC visualization is desirable not only for *in vitro* HSC isolation, but also for *in situ* or *in vivo* HSC detection. In current immunohistochemical techniques, HSCs are identified through a combination of multiple markers. To visualize HSC localization, tissues are subjected to a multistep staining process. Bone staining is, in general, difficult because of high background signals mainly due to complex skeletal structure. Hence, the simpler staining using one single marker like HSC enhancer driven EGFP would drastically improve histological images. Furthermore, live cell imaging is also likely to become feasible. With the generation of HSC-specific enhancer driven reporter transgenic mouse, previously unappreciated *in vivo* HSC behavior would be unraveled.

The developmental process of HSC generation has drawn scientists' intense attention as such knowledge facilitates the generation of HSCs from embryonic stem (ES) cells or induced pluripotent stem (iPS) cells. eR1-EGFP Tg mouse is the first reporter mouse model that specifically distinguishes between hemogenic and non-hemogenic ECs. Hence, eR1-driven fluorescence signal appears to act as a surrogate marker for successful induction of hemogenic ECs, and HSCs, from ES or iPS cells.

HSCs and leukemia stem cells (LSCs) are anticipated to share some common features for maintaining their "stemness". The enhancer for normal HSCs is expected to be active in LSCs as well. For example, two distinct *Bmi1* expression populations were observed within a subset of progenitor population ($Flk2^+$ KSL) in mouse leukemia model. A high level of *Bmi1* expression was believed to confer a leukemia-prone status (Hosen et al., 2007). Hence the HSC-marking method would also work as a LSC marking system, thereby serving as a powerful tool to better understand the behaviors of LSC and consequently lead to drug development.

Deregulation of a HSC enhancer could be associated with human disease (Kleinjan and Coutinho, 2009). Alteration in the HSC enhancer, eR1, was recently reported in familial leukemia patients (Buijs et al., 2012). The separation of eR1 from the promoter of RUNX1 due to germline chromosomal translocation, t(16;21), is considered to result in decreased expression of RUNX1 and subsequent pathogenesis including leukemogenesis. With the increment of defined HSC enhancers, hitherto unknown disease etiology would be unveiled.

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