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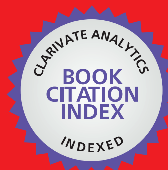

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Chapter 4

Canonical HSC Markers and Recent Achievements

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/54474

1. Introduction

A specific feature of hematopoietic stem cells (HSC) is the potency to supply all types of blood cells throughout life by self-renewal and differentiation. Bone marrow (BM) is actively producing differentiated blood cells with enormous cellular turnover. Under homeostatic state, primitive HSC in adult BM divide only rarely and are located in specialized regulatory environment to avoid exhaustion and DNA damages that are supposed to cumulatively develop hematopoietic disorders such as myelodysplastic syndrome or leukemia. However, those quiescent HSC can be proliferative on demand, particularly on systemic infection or myelo-suppressive treatment. Therefore, elaborate mechanisms regulating the self-renewal and differentiation of BM HSC is indispensable to maintain normal hematopoiesis throughout life. The fluctuating feature of HSC is thought to be associated with their regulatory environment, generally called “HSC niche”.

Technical improvement for purifying authentic HSC from heterogeneous cellular populations is necessary to understand the features of those extremely rare and precious cells and promote their therapeutic application. Many studies have attempted to identify their specific markers, and now flow cytometry- based strategies have made it possible to sort HSC with high purity in mice. However, the source and the stage of HSC change along ontogeny, which consequently influence not only their functional abilities but also their surface immunophenotypes. In the light of fluctuating nature of HSC, it should be very important to understand their phenotype specific to reconstitution activity of the immune system after myelo-suppressive events.

Hematopoietic cells and endothelial cells are both generated from mesodermal precursor cells in ontogeny [1]. Thereafter, HSC pool is formed in several anatomical sites such as aor-

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ta-gonad-mesonephros (AGM) region, placenta, fetal liver, and BM. At the early stage of ontogeny, HSC frequently undergo symmetrical and/or asymmetrical division to form entire hematopoietic system compared to adult HSC. Those early HSC and endothelial cells bear some common surface antigens, of which expression levels on HSC decline along aging. Interestingly, some of the endothelial-related surface molecules revive on HSC after BM injury, when the cells actively divide to regenerate BM cells.

We recently reported endothelial cell-selective adhesion molecule (ESAM) as a new marker for HSC [2]. Interestingly, ESAM levels on HSC clearly mirror the shift of HSC between quiescence and activation, and the up-regulation amplitude is prominent in comparison to other HSC-related antigens [3]. Furthermore, we found that ESAM is functionally indispensable for HSC to re-establish homeostatic hematopoiesis [3]. In this chapter, we review a wealth of information about traditional HSC markers, and introduce our recent findings.

2. Development of the strategy for purifying HSC from murine BM

HSC are defined by their capacity for both self-renewal and differentiation into all the blood cell types. In 1988, Spangrude *et al.* reported lineage (Lin; Mac-1, Gr-1, B220, CD4, and CD8)$^{-}$ Thy-1^{Lo} Sca-1^{+} cells in mouse BM as a multipotent HSC population. When these cells were transplanted into lethally irradiated mice, only thirty cells were sufficient to save 50% of the recipient mice and reconstitute B, T, and myeloid cells [4]. In 1991, Ogawa *et al.* reported that half of the c-kit^{+} BM cells do not express Lin (Mac-1, Gr-1, Ter119, and B220) markers, and c-kit^{-} population do not include hematopoietic progenitor cells [5]. From then on, Lin^{-} Sca-1^{+} c-kit^{+} (LSK) cells has been used as the population in which HSC are highly concentrated [6,7]. HSC can be functionally classified as either long-term (LT-HSC) or short-term (ST-HSC) according to their capacity to give rise to life-long or transient hematopoiesis. Osawa *et al.* showed that CD34^{+} LSK cells are capable of only short-term multilineage differentiation. In contrast, CD34$^{-/Lo}$ LSK cells have long-term multilineage reconstitution capacity. They also showed that CD34$^{-/Lo}$ LSK cells can differentiate into CD34^{+} LSK cells [7]. LSK fraction also can be divided into two populations by expression level of Flk-2. While LT-HSC are enriched in the Flk-2^{-} LSK fraction, the Flk-2^{+} LSK cells are mainly ST-HSC [8].

While the techniques of purifying HSC by use of surface markers had been promoted, Goodell *et al.* reported the method for purifying HSC without use of surface markers. Hoechst33342 is a fluorescent dye which binds to DNA of live cells. When Hoechst fluorescence on whole BM was examined simultaneously at two emission wavelength (red and blue), one population of cells with increased ability to efflux Hoechst dye was observed. Goodell *et al.* named it “side population (SP)”, and showed that a majority of HSC were enriched in the SP by competitive repopulating experiments [9]. Subsequently, Matsuzaki *et al.* described a method of further purifying HSC by combining staining with antibodies to surface molecules with the Hoechst dye efflux. They showed the fraction of cells with the strongest dye efflux activity (termed as “Tip”-SP) has the highest marrow-repopulating activity. While 20% of “Tip”-SP cells are primitive hematopoietic cells, more than 90% of “Tip”-SP

$CD34^-$ KSL cells, which are extremely rare, representing only 0.001-0.01% of BM mononuclear cells, are almost pure primitive hematopoietic cells that have long-term multilineage repopulating potency [10]. More recently, the endothelial protein C receptor CD201 was found as a new endothelial-related HSC marker which marks approximately 70% of the SP cells. The marker seems to be useful to purify LT-HSC among the SP cells because only the $CD201^+$ subpopulation exhibited repopulating ability [11].

In recent years, Kiel *et al.* demonstrated that a simple combination of SLAM family markers (CD150, CD244 and CD48) could enrich primitive murine HSC. That is, one out of every 4.8 (21%) of $CD150^+$ $CD48^-$ cells from young adult murine BM gave long-term multilineage reconstitution [12]. Furthermore, they observed that one out of every 2.1 (47%) of $CD150^+$ $CD48^-$ LSK cells had long-term multilineage reconstituting potential. Approximately 15~20% of $CD150^+$ $CD48^-$ LSK or $CD34^-$ $CD150^+$ $CD48^-$ LSK cells, which divide only 5-6 times during the mouse life span, have more long-term repopulating potential than other cells [13,14]. We can now purify dormant LT-HSC from murine BM using the SLAM family markers in combination with LSK gating. The information regarding murine HSC markers is summarized in Table 1.

Markers	References
Lin^- $Thy\text{-}1^{Lo}$ $Sca\text{-}1^+$	Spangrude *et al. Science* (1988) [4]
$CD34^{-/Lo}$ LSK	Osawa *et al. Science* (1996) [7]
Side population (SP)	Goodell *et al. J Exp Med* (1996) [9]
Lin^- Rho^- SP	Uchida *et al. Exp Hematol* (2003) [15]
Tip-SP LSK	Matsuzaki *et al. Immunity* (2004) [10]
$CD48^-$ $CD150^+$ $CD41^-$ LSK	Kiel *et al. Cell* (2005) [12]
BrdU and histone 2B-retaining $CD48^-$ $CD150^+$ $CD34^-$ LSK	Wilson *et al. Cell* (2008) [14]
Histone 2B-retaining $CD48^-$ $CD150^+$ LSK	Foudi *et al. Nat Biotechnol* (2009) [13]

Lin, lineage; Rho, Rhodamine-123; LSK, Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$

Table 1. Markers for adult murine hematopoietic stem cells.

3. HSC markers during developmental stages

HSC markers during developmental stages are not identical to those of adult HSC. In the embryo, functional HSC that can reconstitute hematopoiesis in adult recipients are firstly found in the aorta-gonad-mesonephros (AGM) region at approximately embryonic day 10 (E10) [16-18]. Many reports have demonstrated that those earliest authentic HSC bud from endothelial-related cells, which involve the concept of “hemangioblast” or “hemogenic endothelium” [19-23]. In fact, emerging HSC and endothelial cells share various surface mark-

ers such as CD34 and VE-cadherin that do not mark adult murine LT-HSC [24-26]. On the contrary, the emerging HSC do not express either Sca-1 or CD45, a pan-hematopoietic marker [19,27]. Interestingly, those developing HSC express CD41/Integrin-α_v, a marker for megakaryocytes [28].

Although HSC do not emerge in the fetal liver de novo, the organ is the main site of HSC expansion before birth. Circulating HSC seed in the fetal liver, where they robustly expand and differentiate. Indeed, numbers of HSC increase ~40-fold in the fetal liver between E12 and E16 [29]. Unlike the emerging HSC in the AGM region, HSC in fetal liver express CD45 and Sca-1. Morrison *et al.* showed that HSC are highly enriched in Thy1^{Lo} Sca-1^{+} Lin^{-} Mac1^{+} fraction of fetal liver cells [30]. His group later demonstrated that the SLAM family markers (CD150^{+} CD48^{-}) are also useful to enrich for HSC in E14.5 fetal liver just as in adult BM by the fact that 37% of CD150^{+} CD48^{-} Sca-1^{+} Lin^{-} Mac1^{+} fetal liver cells had long-term reconstituting capacity [31]. Although the expression levels of AA4.1 and VE-cadherin are very high at the early stage of fetal hematopoiesis, they become gradually down-regulated after E12-E13 [32,33]. Interestingly, the phenotype of HSC in fetal liver rapidly changes after E16, when their number is reaching to a plateau level [30]. Recently, our group reported ESAM as a novel HSC marker in fetal liver (see below). HSC markers during mouse ontogeny are summarized in Table 2.

Fetal age	Location	Markers	References
E8.5-E10.5	AGM region	CD41^{+} CD34^{+} CD45^{-} VE-cadherin^{+} Sca-1^{-} AA4.1^{+} ESAM^{+}	Petrenko *et al. Immunity* (1999) [33], Hsu *et al. Blood* (2000) [34], Baumann *et al. Blood* (2004) [24], Fraser *et al. Exp Hematol* (2002) [25], Ogawa *Exp Hematol* (2002) [26], de Brujin *et al. Immunity* (2002) [19], Mikkola *et al. Blood* (2003) [28], Matsubara *et al. J Exp Med* (2005) [27], Kim *et al. Blood* (2005) [32], Kim *et al. Blood* (2006) [31], Mansson *et al. Immunity* (2007) [35], Yokota *et al.* Blood (2009) [2]
E11.5-E16.5	Fetal liver	CD41^{-} CD34^{+} CD45^{+} CD31^{+} Sca-1^{+} Mac1^{+} Tie-2^{+} Flt3^{-} c-kit^{+} AA4.1^{+} VE-cadherin$^{+/-}$ CD150^{+} CD48^{-} ESAM^{+}	

Table 2. Markers for hematopoietic stem cells during mouse ontogeny.

Adult and fetal HSC are not the same with regard to not only surface phenotypes but also cell-cycle status. Recent studies have shown that the long-term reconstituting activity of adult BM is sustained mostly in very quiescent HSC [13,14]. However, cycling HSC from the fetal liver give rise to higher levels of reconstitution than HSC obtained from adult BM [30,36]. The microenvironments, known as "HSC niches", are believed to influence cell-cycle status of HSC, and adult HSC niches in BM seem to be different from HSC niches in the fetal liver [12,37-40]. More precise analyses of hematopoietic environment in the embryo should give us valuable information regarding what are the imperative conditions for HSC expansion and how the alteration of surface molecules on HSC is functionally involved in that process. Furthermore, such cell surface antigens that mirror the HSC state are invaluable for understanding the relationship between HSC and their niches.

4. Niche signals regulating HSC pool

We think it seems meaningful to deal with the "HSC niche" briefly here, although another chapter in this book provides more detailed information about its function. Molecular cross-talk between HSC and their niches has been considered to be important to provide signals for self-renewing division that maintain HSC pool. Although precise mechanisms regulating HSC status still remain unknown, there are accumulating evidences to involve several specific cells, or cytokines and chemokines secreted from stromal cells in this process.

In 1994, human osteoblasts were shown to maintained hematopoiesis by constitutively producing G-CSF in vitro [41]. In the first decade of the 21st century, a notion that connects osteoblasts with the HSC niche rapidly developed. Parathyroid hormone (PTH), which is a main regulator of calcium homeostasis, was reported to increase in the number of both osteoblasts and HSC, suggesting osteoblasts as the candidate for HSC niche [42]. In addition, it was also reported that BrdU label retaining cells (LRC) were attached to spindle-shaped N-cadherin$^+$ osteoblasts (SNO) cells, and that bone morphogenetic protein (BMP) signalling controlled the number of HSC by regulating SNO cells [37].

On the other hand, Kiel *et al.* reported that many CD150$^+$ CD48$^-$ CD41$^-$ Lin$^-$ LT-HSC were in contact with sinusoidal endothelial cells in spleen or BM, suggesting that endothelial cells are also essential components of the HSC niche. With regard to cytokine-chemokine sinalings, the CXC chemokine ligand 12 (CXCL12) -CXC chemokine receptor 4 (CXCR4) pathway was found to be important. In vitro, HSC expressing CXCR4 migrate in response to CXCL12 which is the ligand for CXCR4 [43]. Nagasawa's laboratory reported that a majority of CD150$^+$ CD48$^-$ CD41$^-$ HSC were in contact with CXCL12-abundant reticular (CAR) cells, and that the numbers of HSC in CAR cell-depleted mice were reduced in comparison with control mice. These data are supportive of the idea that CXCL12-CXCR4 pathway is essential for HSC pool [39,44]. Recently, Yamazaki *et al.* reported TGF- β as a candidate niche signal in the control of HSC hibernation [45]. The same group advocated that glial cells, regulating activation of TGF-β signal, might be a component of the HSC niche in adult BM and maintain HSC hibernation [46].

5. Differences between murine and human HSC markers

A critical issue that has been an obstacle in applying the information of murine HSC to human is the lack of common HSC markers between the two species. Researchers described above have made great efforts to purify authentic HSC from murine hematopoietic organs. Owing to those achievements, we can now sort LT-HSC with very high purity from the murine BM. However, human HSC cannot be purified with the same markers. Human HSC do not express Sca-1 or CD150 that are the established HSC markers in mice. In addition, the long-term HSC of human BM are enriched in CD34$^+$ CD38$^-$ population, while murine BM HSC are CD34$^-$ CD38$^+$ [26,47,48].

Early studies in the 1980s proved by using monoclonal antibody technique that $CD34^+$ population of human BM includes immature hematopoietic progenitors [49-51]. Berenson *et al.* showed that autologous $CD34^+$ cells enriched from baboon BM were able to reconstitute normal hematopoiesis after lethal irradiation. Animals transplanted with $CD34^-$ cells, however, did not recover sufficient hematopoiesis [52]. Afterwards, over the past two decades, CD34-positive has been used as a reliable marker for human HSC or hematopoietic progenitor cells (HPC). Indeed, transplantation of $CD34^+$ cells obtained from donor BM, peripheral blood, or cord blood (CB) can provide long-term and multilineage hematopoietic reconstitution in recipients.

As CD34 marks human HSC as well as more differentiated progenitor cells, researchers have sought additional markers to further enrich $CD34^+$ population for LT-HSC. Baum *et al.* reported that CD90/Thy-1^+ population in Lin^- $CD34^+$ cells contained pluripotent hematopoietic progenitors [53]. Recently a series of studies of John Dick's laboratory have successfully improved the techniques to more purify human HSC. His group reported that human HSC activity was restricted to $CD49f^+$ fraction, and that single Lin^- $CD34^+$ $CD38^-$ $CD45RA^-$ Thy-1^+ Rhodamin123^{Lo} $CD49f^+$ cells in CB cells accomplished multilineage engraftment in immune-deficient mice [54].

While LT-HSC can be enriched mainly in the $CD34^+$ population, the possibility that $CD34^-$ cells also contain LT-HSC has been reported. Bhatia *et al.* showed human $CD34^-$ population in Lin^- cells of BM and CB also contained LT-HSC [55]. It should be important to compare the features of primate $CD34^-$ HSC with those of murine $CD34^-$ LSK cells. In addition, a new positive marker for human HSC could resolve the relationship between the $CD34^+$ and $CD34^-$ HSC. Markers for human HSC are summarized in Table 3.

Markers	References
$CD34^+$	Berenson *et al. J Clin Invest* (1988) [52]
$CD34^+$ $CD38^-$	Terstappen *et al. Blood* (1991) [48]
$CD34^+$ Lin^- Thy-1^+	Baum *et al. Proc Natl Acad Sci USA* (1992) [53]
Lin^- $CD34^-$ $CD38^-$	Bhatia *et al. Nat Med* (1998) [55]
$CD34^+$ $CD38^-$ Lin^- Rho^{Lo}	McKenzie *et al. Blood* (2007) [47]
Lin^- $CD34^+$ $CD38^-$ $CD45RA^-$ Thy-1^+ Rho^{Lo} $CD49f^+$	Notta *et al. Science* (2011) [54]

Table 3. Markers for human hematopoietic stem cells.

6. Differences between quiescent and activated HSC markers

After mice are treated with cytotoxic agents or irradiation, most of cell-cycling hematopoietic cells are killed and dormant primitive HSC start to proliferate. The patterns of surface molecules expressed on activated HSC change from those under steady-state con-

dition. While activated HSC increase the expression level of Sca-1, CD150, Tie2, Endoglin, Mac-1, and CD34, they clearly decrease that of c-kit and N-cadherin [26,56,57]. Some endothelial-related antigens, which mark actively dividing fetal HSC but do not mark quiescent adult HSC, are up-regulated again on the activated HSC after BM injury. The characteristics of those activated HSC are reminiscent of fetal HSC. Since no obvious phenotypes have been documented regarding CD34 or CD150-deficient mice, how the up-regulation of those molecules contributes to the functions and/or characteristics of activated HSC remains unknown [12,58]. Tie2 and Endoglin, which are the receptors for angiopoietin and TGF, respectively, might transduce important signals to regulate dividing speed of HSC. If we could accurately monitor the fluctuation of HSC status with a set of surface markers, that should yield significant insight regarding HSC biology and HSC applications for clinical purposes. As a very recent achievement, our group has demonstrated that ESAM is a useful marker for activated HSC.

7. An endothelial-related antigen ESAM as a new novel HSC marker

We previously reported sorting strategy of HSC and early lymphoid progenitors (ELP) from Rag1/GFP knockin mice [59,60]. We searched for genes whose expression levels are significantly different between Rag1^{-} c-kit^{Hi} Sca-1^{+} HSC and Rag1^{Lo} c-kit^{Hi} Sca-1^{+} ELP by analyzing micro-array data. Among the HSC related genes ESAM drew our attention because its transcripts were conspicuous in the HSC fraction whereas the expression was drastically down-regulated in the ELP fraction. ESAM molecule is an immunoglobulin superfamily protein that is exposed on cell surface and originally identified as an endothelial cell-specific protein [61,62]. We found the ESAM^{Hi} population of Rag1^{-} c-kit^{Hi} Sca-1^{+} fraction of E14.5 fetal liver was highly enriched for LT-HSC compared with ESAM$^{-/Lo}$ subset. Among Rag1/GFP^{-} Tie2^{Hi} E10.5 AGM cells, only ESAM^{+} cells could effectively produce both CD19^{+} lymphoid cells and Mac1^{+} myeloid cells [2].

ESAM is also expressed on adult murine HSC-enriched fraction in BM. Ooi *et al.* reported that ESAM^{+} Sca-1^{+} Lin^{-} BM cells could more effectively enrich for LT-HSC than the conventional HSC-enriched LSK cells, and that ESAM expression on HSC was conserved among various mouse strains [63]. ESAM levels on HSC are variable according to developing stages or advancing age. Interestingly, the intensity of ESAM expression on HSC gradually increased with age after reaching adulthood [2]. Based on these observations, ESAM can be a novel murine HSC marker throughout life including developmental stages.

The usefulness of ESAM as a HSC marker has been further enhanced by the findings that its expression in human HSC is also detected. Ooi *et al.* reported that robust levels of ESAM transcripts were detected in Lin^{-} CD34^{+} CD38^{-} CD90^{+} human HSC, while the levels of ESAM transcripts in unfractionated CB cells were very low [63]. We have confirmed that ESAM expression is clearly detectable on human CB CD34^{+} cells by using its specific antibody and flow cytometry [64]. In addition, our group has also observed that the marker is effective as well for adult human HSC in both BM and mobilized peripheral blood. (Ishibashi *et al.* manuscript in preparation).

8. ESAM monitors HSC status between quiescence and self-renewal

As mentioned above, the expression pattern of surface antigens on activated HSC after BM injury substantially differs from that on quiescent HSC. Administration of an anti-cancer drug 5-FU causes apoptosis of dividing hematopoietic progenitors, while the treatment retains quiescent LT-HSC and induces their proliferation afterward. We have observed that remarkable increase of ESAM expression levels transiently occurs on BM HSC after a 5-FU treatment. Furthermore, we have proved that the long-term hematopoietic reconstituting activity is almost exclusive to LSK cells bearing up-regulated ESAM expression [3].

Although expression levels of CD34, Tie2, and Endoglin on LSK show modest increases after 5-FU injection, up-regulation of ESAM is remarkable (Figure 1). Why does ESAM need to revive so vividly on HSC after BM injury? One possible reason is that HSC might directly receive necessary signals which regulate self-renewal or differentiation via interaction with ESAM. Another possibility is that high amounts of ESAM might change the polarity or mobility of HSC, which consequently facilitate them to settle in adequate supporting niches (Figure 2). The latter assumption is likely because Wegmann and colleagues reported that ESAM deficiency causes insufficient Rho signalling in endothelial cells, which regulates the stabilization of endothelial tight junctions [65]. Rho is also expressed in hematopoietic progenitors and involved in their polarity and mobility [66]. It is noteworthy that more than 80% of ESAM^{Hi} HSC were located around perivascular areas in 5-FU-treated BM[3].

In any case, ESAM is likely to play an indispensable role during the recovery from BM injury. Because, while ESAM deficient mice do not show significant hematopoietic defects in homeostatic stage, the mice fall into severe and prolonged pancytopenia after myelo-suppressive treatment. In particular, they suffer from severe anemia and frequently die before hematopoietic recovery. Our findings indicate that ESAM not only marks activated HSC but also functionally supports their proliferation and differentiation.

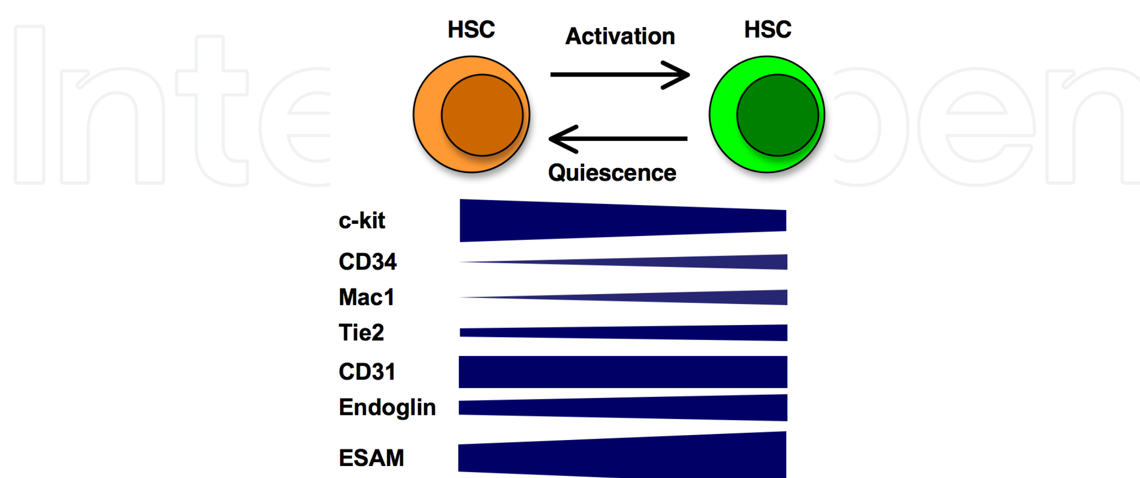

Figure 1. Overview of cell surface expression levels on quiescent steady-state HSC and activated HSC.

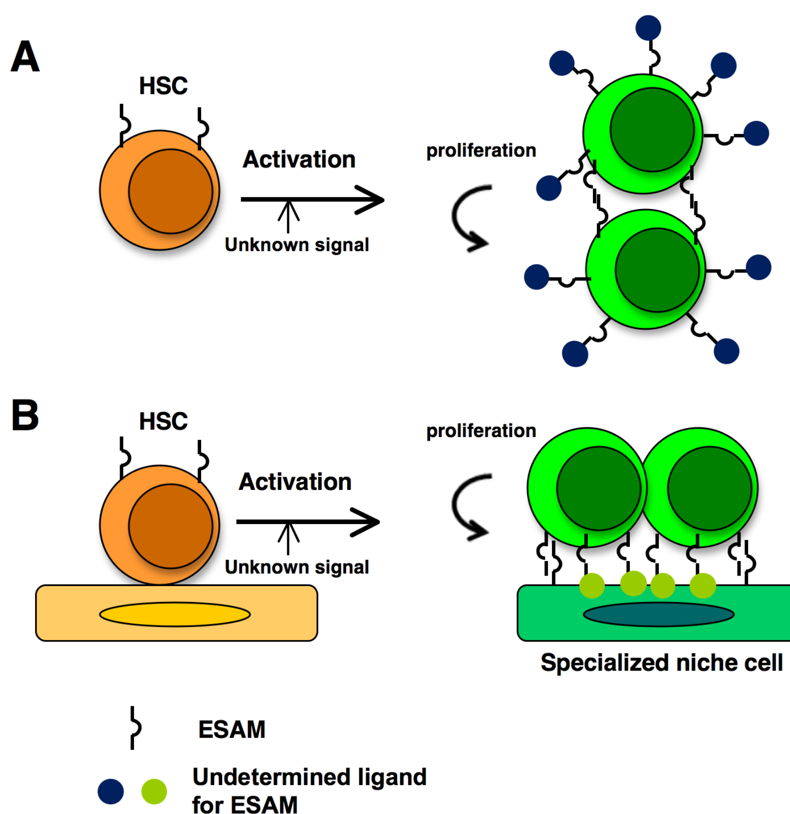

Figure 2. Tentative models of ESAM function. (A) In this model, activated HSC directly receive necessary signals which regulate self-renewal or differentiation via interaction with ESAM. (B) In this model, HSC change their polarity or mobility, and consequently, they can move to appropriate niches. ESAM may function as an adhesion factor between HSC and their niches.

9. Concluding remarks

In this chapter, we summarized achievements for identification of murine and human HSC, and introduced endothelial-related antigen ESAM as a useful HSC marker. While we can now purify murine LT-HSC with high efficiency, characterization of human HSC is less well understood because of insufficient information about surface antigens. Over two decades CD34-positive has been believed to be a reliable marker for human HSC/HPC. Although there are now accumulating evidences regarding surface markers to further enrich human LT-HSC in the CD34^{+} fraction, more information about human HSC-related antigens should be useful to improve strategies of HSC application to the clinical medicine. Although ESAM was originally identified with an endothelial specific molecule, we have demonstrated that it is a positive marker for both murine and human HSC. Because ESAM seems to play an essential role for hematopoietic recovery after BM injury, it would be significant to elucidate downstream signals of ESAM, and the possibility of ESAM as niche components. In addition, we now know that the up-regulation of ESAM is observed on cultured murine embryonic stem (ES) cells cultured in the OP9 system which recapitulate very primitive stages of

hematopoietic development [67] (Doi et al. manuscript in preparation). ESAM might have some roles in embryonic hematopoiesis at very early stages. As an on-going study, we are now investigating whether ESAM can be a useful biomarker for inducing hematopoietic cells from ES or induced pluripotent stem cells.

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