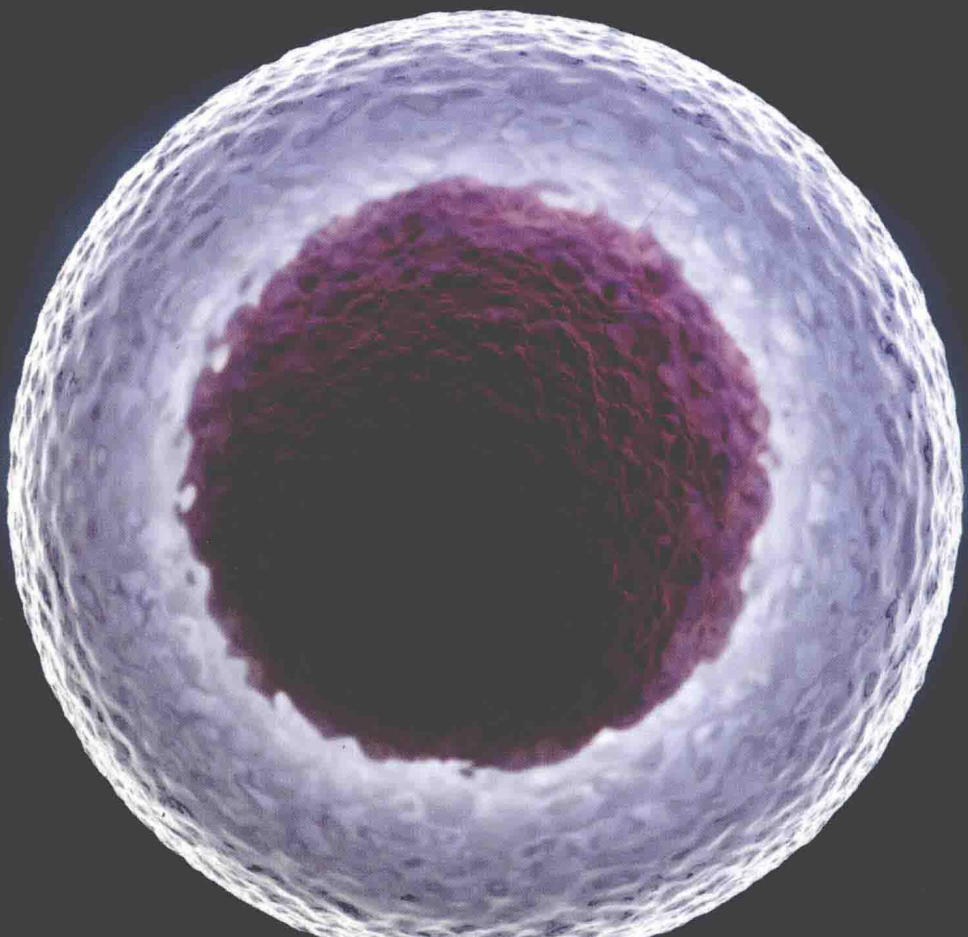
# Hematopoietic Stem Cell Research

Rex Turner



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# Preface

This book was inspired by the evolution of our times; to answer the curiosity of inquisitive minds. Many developments have occurred across the globe in the recent past which has transformed the progress in the field.

Researches are being conducted around the globe for a better understanding of hematopoietic stem cells. This book provides a complete analysis of the biology and healing possibilities of hematopoietic stem cells, and is meant for those involved in stem cell study. Beginning from primary principles in hematopoiesis, this book assembles a wealth of information related to central devices that may control separation and growth of hematopoietic stem cells in usual conditions and throughout disease. It discusses the properties and regulation factors of hematopoietic stem cells. The book compiles researches from renowned experts involved in this field.

This book was developed from a mere concept to drafts to chapters and finally compiled together as a complete text to benefit the readers across all nations. To ensure the quality of the content we instilled two significant steps in our procedure. The first was to appoint an editorial team that would verify the data and statistics provided in the book and also select the most appropriate and valuable contributions from the plentiful contributions we received from authors worldwide. The next step was to appoint an expert of the topic as the Editor-in-Chief, who would head the project and finally make the necessary amendments and modifications to make the text reader-friendly. I was then commissioned to examine all the material to present the topics in the most comprehensible and productive format.

I would like to take this opportunity to thank all the contributing authors who were supportive enough to contribute their time and knowledge to this project. I also wish to convey my regards to my family who have been extremely supportive during the entire project.

**Editor**

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# Part 1

## Hematopoietic Stem Cell Properties

1

# Markers for Hematopoietic Stem Cells: Histories and Recent Achievements

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## 1. Introduction

Hematopoietic stem cells (HSC) are characterized with the capacity for self-renewal as well as multi-lineage differentiation, maintaining the immune system and blood cell formation throughout life. Although studies for the HSC biology have been in the forefront of the stem cell research field, many questions still remain with regard to the origin, development, and aging of HSC. Furthermore, needless to say, HSC are very useful for clinical medicine, particularly in the transplantation and/or regeneration therapy for hematological malignancies. Success of those therapies depends on how effectively HSC are purified and transplanted to the patients. In order to address those important issues in both basic and clinical science, information of cell surface molecules that selectively mark HSC is essential.

Since the frequency of HSC in bone marrow or peripheral blood is extremely low, many studies have attempted to identify unique markers associated with those rare cells. As a result, it is now possible to purify long-term reconstituting HSC from mouse bone marrow with very high efficiency. However, many of these parameters change dramatically during ontogeny or inflammation, and what is worse still, they differ between mouse and man. Efficient HSC-based therapies and the emerging field of tissue-regenerative medicine will benefit from more precise information about what defines HSC.

In this chapter, we summarize a large body of information with respect to the HSC-related markers and introduce Endothelial cell-selective adhesion molecule (ESAM) as a novel marker for HSC (Yokota et al., 2009). Indeed, ESAM is expressed throughout the ontogeny in mouse and can be used as a gating parameter for sorting long-term repopulating HSC. In addition, the marker appears to be useful for the purification of human HSC.

## 2. Development of methodology for HSC purification from mouse bone marrow

In 1988, Spangrude et al tried to find a set of cell surface proteins that were associated with multi-lineage reconstitution ability, and succeeded to enrich such multipotential progenitors in the Lineage marker (Lin; generally including TER119, Mac1, Gr1, CD45R/B220, CD3, CD4, CD8)$^-$ Thy-1$^{Low}$ Sca-1$^+$ fraction of mouse bone marrow (Spangrude et al, 1988). Indeed, they showed that only 30 Lin$^-$ Thy-1$^{Low}$ Sca-1$^+$ cells injected via a tail vein could rescue 50% of lethally irradiated mice. Three years later, in 1991, Ogawa et al reported that hematopoietic progenitor activity of mouse bone marrow was excusive to the cells expressing c-kit, which is a receptor for stem cell factor (Ogawa et al., 1991). Since then, Lin$^-$ Sca-1$^+$ c-kit$^+$ (LSK) has been generally used as a canonical marker set for HSC enrichment.

It has been gradually recognized that the LSK fraction is heterogeneous, including long-term self-renewing HSC, short-term non-self-renewing HSC and lineage-committed progenitors. In 1996, Osawa et al reported that long-term HSC in adult bone marrow exist in the CD34 low to negative fraction among LSK cells (Osawa et al., 1996). Injection of a single CD34$^{-/Low}$ LSK cell resulted in multi-lineage long-term reconstitution in 21% of lethally irradiated mice whereas CD34$^+$ LSK cells revealed early but only short-term reconstitution. Transplantation of graded numbers of CD34$^{-/Low}$ LSK cells showed that the CD34$^{-/Low}$ LSK fraction contains long-term HSC at the frequency of 1 out of 5 cells. In 2001, Christensen and Weissman also showed that the LSK fraction is heterogeneous and long-term HSC are highly enriched in the Flk2/Flt3 receptor tyrosine kinase negative cells (Christensen & Weissman, 2001).

In addition to the cell surface markers, another approach has been developed to enrich long-term HSC activity by focusing on their high efflux activity. Using the fluorescent DNA-binding dye Hoechst33342, in 1996, Goodell et al found that cells in a small Hoechst$^{low}$-stained population (termed "Side population") can protect recipients from lethal irradiation at low cell doses (Goodell et al., 1996). A following study by Matsuzaki et al showed that, in combination with the CD34$^{-/Low}$ LSK phenotype, the strongest Hoechst33342 efflux activity (Tip-side population) can purify long-term multi-lineage HSC with almost absolute efficiency (Matsuzaki et al., 2004).

Recently, Morrison and colleagues reported an alternative method for HSC purification based on the expression pattern of the signaling lymphocytic activation molecule (SLAM) family proteins, i.e. CD150, CD244, and CD48 (Kiel et al., 2005). They showed that CD150$^+$ CD48$^-$ cells were uniformly CD244$^-$ and a simple gating for CD150$^+$ CD48$^-$ could enrich long-term HSC at approximately 1 in 5 cells. Moreover, combined with the canonical HSC marker LSK, the SLAM code could purify the HSC at 1 in 2 cells (Kiel et al., 2005).

Representative achievements during these 2 decades are summarized in Table 1. With surface markers, we can now purify the long-term multi-lineage HSC from adult mouse bone marrow with extremely high efficiency as Lin$^-$ Sca-1$^+$ c-kit$^+$ Thy1$^{Low}$ CD34$^{-/low}$ CD150$^+$ CD48$^-$ cells. In fact, recent studies have demonstrated that the Lin$^-$ Sca-1$^+$ c-kit$^+$ CD34$^-$ CD150$^+$ CD48$^-$ fraction in adult mouse bone marrow contains truly dormant HSC, which divide only 5-6 times during the life span (Wilson et al., 2008; Foudi et al., 2009).

| Markers | references |
|---|---|
| $Lin^- Thy\text{-}1^{Low} Sca\text{-}1^+$ | Spangrude et al., 1988 |
| $CD34^{-/Low} Lin^- Sca\text{-}1^+ c\text{-}kit^+$ | Osawa et al., 1996 |
| Side Population (high Hoechst-efflux ability) | Goodell et al., 1996 |
| *Tip-SP $Lin^- Sca\text{-}1^+ c\text{-}kit^+$ | Matsuzaki et al., 2004 |
| $CD150^+ CD244 CD48^-$ | Kiel et al., 2005 |
| BrdU or Histone 2B-GFP-retaining, $CD150^+ CD48^- CD34^- Lin^- Sca\text{-}1^+ c\text{-}kit^+$ | Wilson et al., 2008<br>Foudi et al., 2009 |

*Tip-SP: The highest Hoechst-efflux fraction in the Side Population

Table 1. Markers for hematopoietic stem cells in adult mouse bone marrow

## 3. Fickleness of HSC surface markers

It is important to stress here that none of the surface markers shown above is entirely specific to the long-term HSC. In addition, many of these parameters differ between strains of mice and change dramatically during developmental age. For example, Sca1, which has been a center in the canonical HSC marker "LSK", is not detectable on the emerging HSC in the aorta-gonad-mesonephros (AGM) area and only appears after day 11.5 of gestation on HSC in fetal liver (Matsubara et al., 2005; Our unpublished observation). Furthermore, the expression level of Sca1 on HSC differs between strains and is not very effective to enrich HSC from Balb/c mice (Spangrude & Brooks, 1993). Likewise, the SLAM family CD150 is not useful for the emerging and developing HSC in embryos (McKinney-Freeman et al., 2009). On the contrary, CD41, CD11b/Mac1, vascular endothelial (VE)-cadherin, and CD34 are known to mark the emerging and developing HSC during the fetal period, but gradually disappear along the ontogeny (Mikkola & Orkin, 2006).

It is also a well-recognized fact that cell surface markers on HSC in adult bone marrow do fluctuate according to the cell-cycle status and the differentiating behavior, which change depending on the physiological requirement. Bone marrow suppression by irradiation and/or chemotherapy revives several disappeared markers including CD11b/Mac1 and CD34 whereas it significantly down-regulates the expression level of c-kit on long-term HSC (Randall & Weissman, 1997; Ogawa 2002). Molecular crosstalk between HSC and bone marrow microenvironment is thought to control the status of HSC and influence their surface phenotypes, but precise mechanisms remain largely unknown. Therefore, researchers in the HSC field need to carefully choose an appropriate marker set and a sorting gate depending on the HSC characteristics, otherwise they would miss important target cells even in the lineage depletion step.

## 4. Difference between mouse and man

Another very critical issue on the topic of the HSC markers is their diversity between species. Although essential difference has not been observed between mouse and man regarding either the organs producing HSC or the transcription factors regulating their differentiation, completely different markers have been used to sort HSC in the two species. Human HSC do not express Sca1 or the SLAM family CD150 (Larochelle et al., 2011). While the $CD34^+$ $CD38^-$ phenotype has been regarded as the canonical marker set for human HSC, it has been repeatedly reported that murine adult HSC locate in the $CD34^-$ $CD38^+$ fraction (Randall et al., 1996; Matsuoka et al., 2001; Tajima et al., 2001). There is no reasonable explanation so far for the change along evolution, and such phenotypic differences between murine and human HSC have been an obstacle to apply achievement in mouse studies to human.

Early studies by Berenson et al demonstrated that autologous $CD34^+$ cells enriched from bone marrow effectively radioprotected baboons and promoted hematopoietic recovery in human patients after marrow ablative therapy (Berenson et al, 1988, 1991). Over the past 2 decades, the use of CD34 as a marker for hematopoietic stem/progenitor cells has been a strong tool in the field of clinical hematology. Since the $CD34^+$ fraction of human bone marrow contains lineage-committed progenitors as well as long-term multi-lineage HSC, many laboratories have sought additional markers to further enrich the $CD34^+$ population for long-term HSC. CD90/Thy1, Tie, CD117/c-kit, and CD133/AC133 have been found as positive markers to enrich long-term-HSC whereas several negative markers including CD38 have been reported (Baum et al., 1992; Hasiyama et al., 1996; Gunji et al., 1993; Yin et al., 1997; Terstappen et al., 1991).

Recent advances of xenotransplantation models and techniques have enabled the assessment of pluripotency as well as self-renewal of human hematopoietic progenitors in vivo (Shultz et al., 2007). A series of studies by John Dick's laboratory have successfully enriched human long-term HSC within the $Lin^-$ $CD34^+$ $CD38^-$ population (McKenzie et al., 2007; Doulatov et al., 2010). In a very recent report, they have purified human HSC from cord blood with a maker set of $Lin^-$ $CD34^+$ $CD38^-$ $CD45RA^-$ $CD90/Thy1^+$ $Rhodamin123^{Low}$ $CD49f^+$. Indeed, those cells were capable of long-term multilineage engraftment in NOD/SCID/IL2 receptor common-γ chain null mice at a single-cell level (Notta et al., 2011). The information regarding human HSC markers is summarized in Table 2.

While CD34 has been playing an important role as a reliable marker for human hematopoietic stem/progenitor cells in the practical medicine, several studies have demonstrated that long-term reconstituting activity is also detectable in the $CD34^-$ $Lin^-$ population (Bhatia et al., 1998; Gallacher et al., 2000; Wang et al., 2003). A prior study using Hoechst 33342 by Goodell et al also identified $CD34^-$ cells in the side-population of human and rhesus bone marrow, and actually rhesus $CD34^-$ side-population cells acquired the ability to form hematopoietic colonies after long-term cultivation on bone marrow stromal cells (Goodell et al., 1997). It should be interesting to examine molecular signatures associated with those $CD34^-$ HSC in primates, and compare their features with murine $CD34^-$ LSK cells.

| Markers | references |
|---|---|
| $CD34^+$ | Berenson et al., 1988, 1991 |
| $CD34^+$ $CD38^-$ | Terstappen et al., 1991 |
| $CD34^+$ $Lin^-$ $Thy1^+$ | Baum et al., 1992 |
| $CD34^+$ c-$kit^+$ | Gunji et al., 1993 |
| $CD34^+$ $Tie^+$ | Hashiyama et al., 1996 |
| $CD34^+$ CD133/$AC133^+$ | Yin et al., 1997 |
| $CD34^-$ $Lin^-$ CD133/$AC133^+$ $CD7^-$ | Gallacher et al., 2000 |
| $CD34^+$ $CD38^-$ $Lin^-$ $Rhodamine123^{Low}$ | McKenzie et al., 2007 |
| $CD34^+$ $CD38^-$ $Lin^-$ $CD45RA^-$ $Rhodamine123^{Low}$ $CD49f^+$ | Notta et al., 2011 |

Table 2. Markers for human hematopoietic stem cells

## 5. Endothelial-related markers

Hematopoietic cells are thought to originate from the hemangioblast and/or the hemogenic endothelium, which can produce hematopoietic cells and endothelial cells. Therefore, it seems quite natural that HSC share some surface molecules with the endothelial lineage. CD34, PECAM-1/CD31, endoglin, Tie2 and VE-cadherin are well-known endothelial antigens that also mark HSC particularly at early developmental stages (Mikkola & Orkin 2006; Takakura et al., 1998; Yokota et al, 2006). In addition, recent studies have identified endomucin, endothelial protein-C receptor/CD201, and junctional adhesion molecule-A that are common to HSC and endothelial cells (Matsubara et al., 2005; Balazs et al., 2006; Sugano et al., 2008). Although, as discussed above, the expression level of some of these antigens declines or even diminishes at later stages of development (Mikkola & Orkin 2006), each of these advances offered the promise of learning more about how HSC arise de novo and function throughout life. It is crucial to define the means to identify the authentic HSC at all developmental stages so that we can ultimately understand the precise molecular mechanisms of the HSC development.

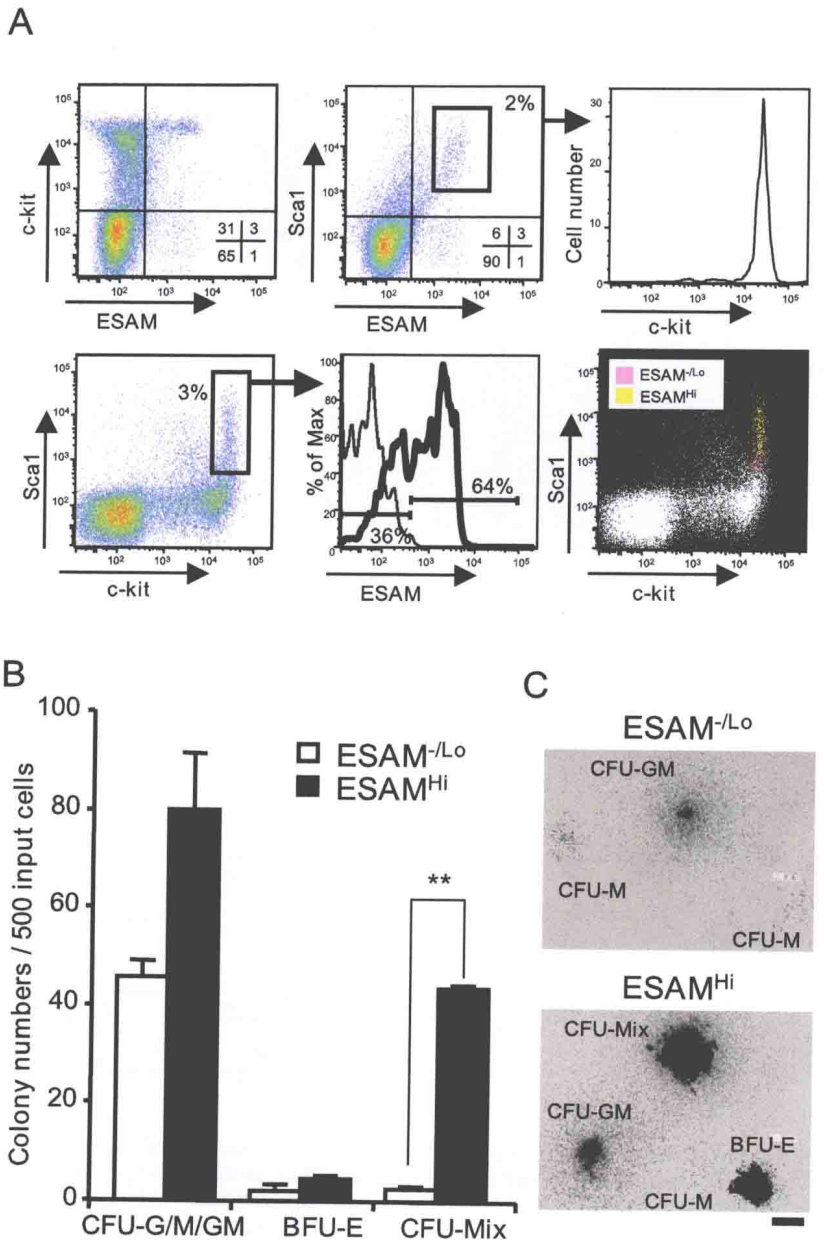
## 6. Identification of ESAM as a novel HSC marker

We previously reported that Rag1/GFP$^{-}$ Lin$^{-}$ c-kit$^{High}$ Sca1$^{+}$ cells derived from bone marrow or fetal liver of the Rag1/GFP reporter mice reconstituted lympho-hematopoiesis in lethally irradiated recipients, while Rag1/GFP$^{+}$ Lin$^{-}$ c-kit$^{High}$ Sca1$^{+}$ cells only transiently contributed to T and B lymphopoiesis (Igarashi et al., 2002; Yokota et al., 2003). Those data demonstrated that Rag1 expression is useful to distinguish early lymphoid progenitors (ELP) from the long-term HSC. To learn more about the first step of HSC differentiation to the lymphoid lineage, microarray analyses were conducted to search for genes that characterize the initial transition of HSC to ELP. The search brought us a large body of information about genes potentially related to early lymphopoiesis whereas it also identified genes whose expression seemed to correlate with HSC. Among the HSC-related genes, ESAM strongly drew our attention because of its conspicuous expression in the HSC fraction and sharp down-regulation on differentiation to ELP.

ESAM was originally identified as an endothelial cell-specific protein (Hirata et al., 2001; Nasdala et al., 2002). Flow cytometry analyses with anti-ESAM antibodies showed that the HSC-enriched Rag1$^{-}$ c-kit$^{High}$ Sca1$^{+}$ fraction of E14.5 fetal liver could be subdivided into two on the basis of ESAM level (Figure 1A). The subpopulation with the high density of ESAM was enriched for c-kit$^{High}$ Sca1$^{High}$ cells, while ones with negative or low levels of ESAM were found in the c-kit$^{High}$ Sca1$^{Low}$ subset. In addition, ESAM expression well correlated with hematopoietic stem/progenitor activity (Figure 1B). Cells in the ESAM$^{High}$ Rag1$^{-}$ c-kit$^{High}$ Sca1$^{+}$ fraction formed more and larger colonies than those in the ESAM$^{-/Low}$ Rag1$^{-}$ c-kit$^{High}$ Sca1$^{+}$ fraction. Particularly, majority of CFU-Mix, multi-potent primitive progenitors, were found in the ESAM$^{High}$ fraction (Figure 1B and 1C). In limiting dilution stromal cell co-cultures, we found that 1 in 2.1 ESAM$^{High}$ Rag1$^{-}$ c-kit$^{High}$ Sca1$^{+}$ cells and 1 in 3.5 ESAM$^{-/Low}$ Rag1$^{-}$ c-kit$^{High}$ Sca1$^{+}$ cells gave rise to blood cells. However, 1 in 8 ESAM$^{High}$ Rag1$^{-}$ c-kit$^{High}$ Sca1$^{+}$ cells produced CD19$^{+}$ B lineage cells whereas only 1 in 125 ESAM$^{-/Low}$ Rag1$^{-}$ c-kit$^{High}$ Sca1$^{+}$ cells were lymphopoietic under these conditions. Furthermore, in long-term reconstituting assays, ESAM$^{High}$ Rag1$^{-}$ c-kit$^{High}$ Sca1$^{+}$ cells contributed highly to the multi-lineage recovery of lympho-hematopoiesis in recipients, but no chimerism was detected in mice transplanted with ESAM$^{-/Low}$ Rag1$^{-}$ c-kit$^{High}$ Sca1$^{+}$ cells. These results suggested that the long-term multi-lineage HSC in E14.5 fetal liver are exclusively present in the ESAM$^{High}$ fraction.

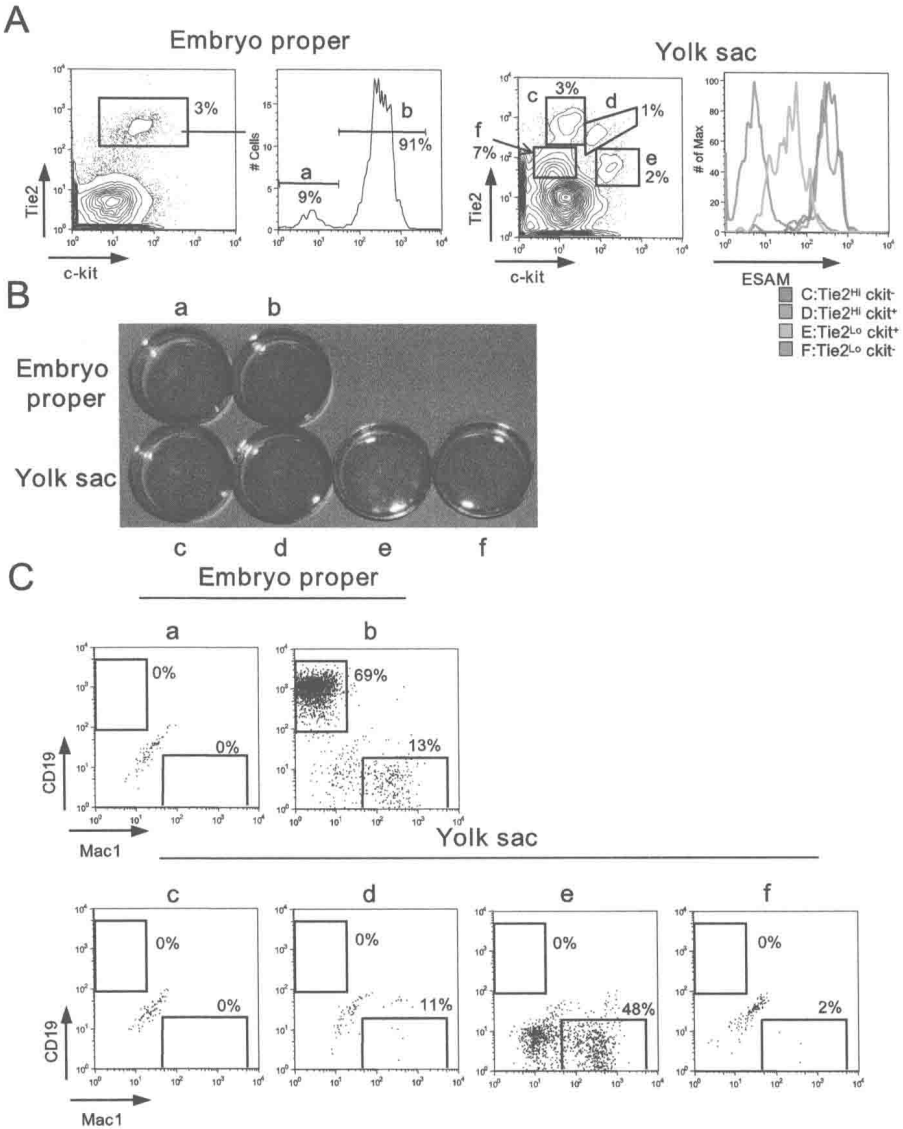
## 7. ESAM marks HSC in different developmental stages and in different species

Hematopoietic cells arise from mesoderm precursors at different sites and stages of development (de Bruijn et al., 2000; Oberlin et al., 2002). We previously determined that, while myelo-erythroid progenitors emerge from the yolk sac, hematopoietic progenitors with lymphopoietic potential first develop in the paraaortic splanchnopleura (pSp) / AGM region (Yokota et al., 2006). ESAM$^{+}$ cells in the AGM were found to co-express c-kit and endothelial antigens, Tie2, CD34 and CD31/PECAM-1 that are known as a marker set for emerging HSC. However, the earlier hematopoietic progenitors in the yolk sac that have limited life span and little lymphopoietic activity were harbored in the ESAM$^{Low}$ Tie2$^{Low}$ c-kit$^{High}$ fraction (Figure 2).




(A) Flow cytometry analysis was performed for mouse E14.5 fetal liver cells using anti-c-kit, anti- Sca1, and anti-ESAM Abs. ESAM$^{-/Lo}$ or ESAM$^{Hi}$ cells of the Rag1/GFP$^{-}$ ckit$^{Hi}$ Sca1$^{+}$ fraction were sorted and subjected to methylcellulose colony formation assay. Numbers of CFUs (B) and morphology of the colonies (C) are shown. (Modified from reference Yokota et al., 2009)

Fig. 1. ESAM expression on the HSC-enriched population of mouse fetal liver

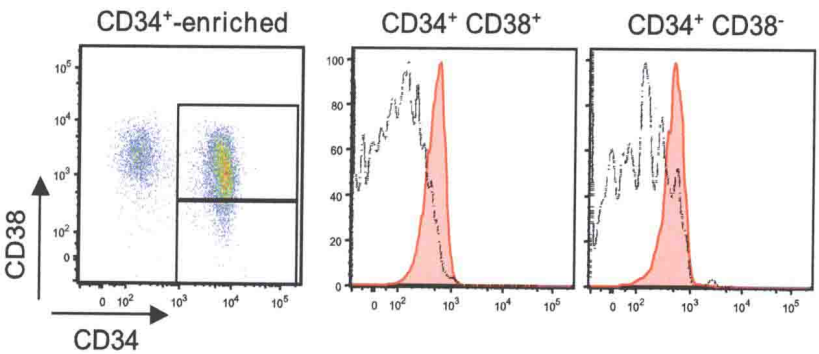



Yolk sac or the caudal half of embryo proper were obtained and pooled from E9.5 embryos of wild type C57B6 mice. The obtained cells were stained with the anti-ESAM Ab followed by goat anti-rat IgG-FITC, anti-c-kit-APC, anti-Tie2-PE, and 7AAD. (A) The profiles of Tie2 and c-kit expression are shown in the left panels. In the right panels, ESAM expression in each gate is shown in histograms. The sorted fractions were labeled with "a" to "f". The sorted cells were subjected to methylcellulose colony formation assay (B) and tested in the MS5 co-culture system (C). (Modified from reference Yokota et al., 2009)

Fig. 2. Yolk sac hematopoietic cells differ from those in the embryo proper with respect to ESAM expression and lymphopoietic activity.

ESAM expression was also detected on HSC within the Lin- c-kit$^{High}$ Sca1$^{+}$ fraction in adult bone marrow. Interestingly, while the expression level was slightly decreased in the adolescent period, it was up-regulated again in aged mice. In addition, Ooi et al showed that the ESAM$^{+}$ Lin$^{-}$ Sca1$^{+}$ gating could more effectively enrich adult bone marrow for the long-term reconstituting HSC than the conventional LSK gating, and that ESAM expression in HSC is conserved between different mouse strains (Ooi et al., 2009). Based on these observations, we conclude that ESAM serves as an effective and durable marker for HSC throughout life in mice.

The importance of ESAM as a HSC marker has been further enhanced by the findings that its expression in HSC is conserved between mouse and man. Ooi et al detected abundant ESAM transcripts in human cord blood CD34$^{+}$ CD38$^{-}$ Lin$^{-}$ Thy1/CD90$^{+}$ cells (Ooi et al., 2009). Furthermore, by using a rabbit anti-human polyclonal ESAM antibody and flow cytometry, we also detected ESAM expression on human cord blood CD34$^{+}$ cells (Figure 3). The intensity of ESAM expression, however, was similar between CD34$^{+}$ CD38$^{-}$ and CD34$^{+}$ CD38$^{+}$ cells, suggesting that the ESAM$^{+}$ gate covers committed as well as non-committed hematopoietic progenitors. ESAM expression might serve as an alternative marker to CD34 for the selection of hematopoietic stem/progenitor cells in human. It is noteworthy that, although majority of human cord blood CD34$^{-}$ CD38$^{+}$ fraction were negative for the ESAM staining, the fraction contains a small ESAM$^{+}$ population. Further study is necessary to characterize those ESAM$^{+}$ CD34$^{-}$ cells.




CD34$^{+}$ cells were firstly enriched from cord blood mononuclear cells by magnetic beads conjugated with an anti-human CD34 antibody, and then stained with anti-CD34, anti-CD38, and anti-ESAM antibodies. The left panel shows CD34 and CD38 expression profile of the CD34$^{+}$ enriched population. The middle and right panels indicate ESAM expression (red tinted) on CD34$^{+}$ CD38$^{+}$ or CD34$^{+}$ CD38$^{-}$ cells, respectively. Dot lines show background staining levels with control IgG for an anti-ESAM antibody.

Fig. 3. ESAM expression on human cord blood CD34$^{+}$ cells

## 8. Conclusion

In this chapter, we summarized 2 decades achievements for the identification of HSC and introduced our recent discovery of human ESAM as a new HSC marker. Although it is possible in mouse to purify the long-term multi-lineage HSC with high efficiency, characterization of human HSC has lagged behind partly due to insufficient information about their cell surface antigens. As a new tool, ESAM expression might contribute to improve the purification strategy of human HSC, not only from human hematopoietic