Figure 1

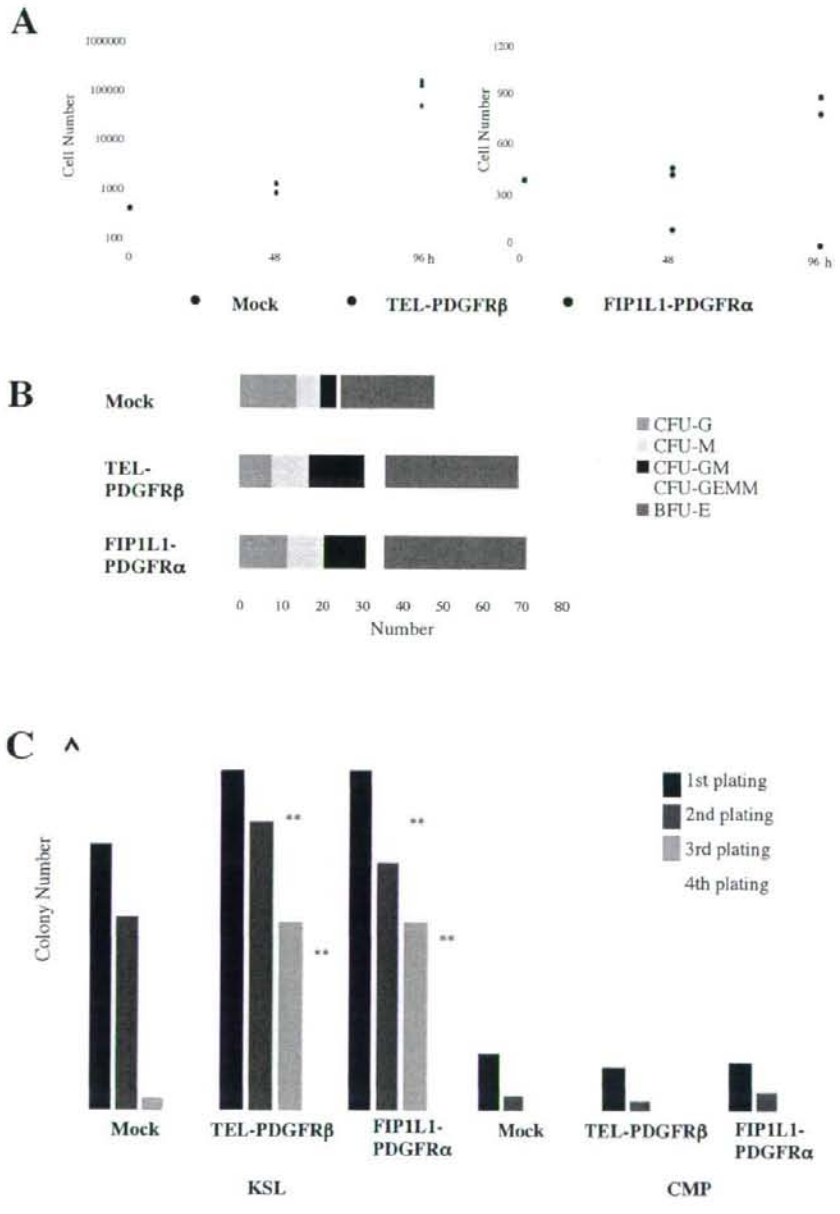


Figure 2

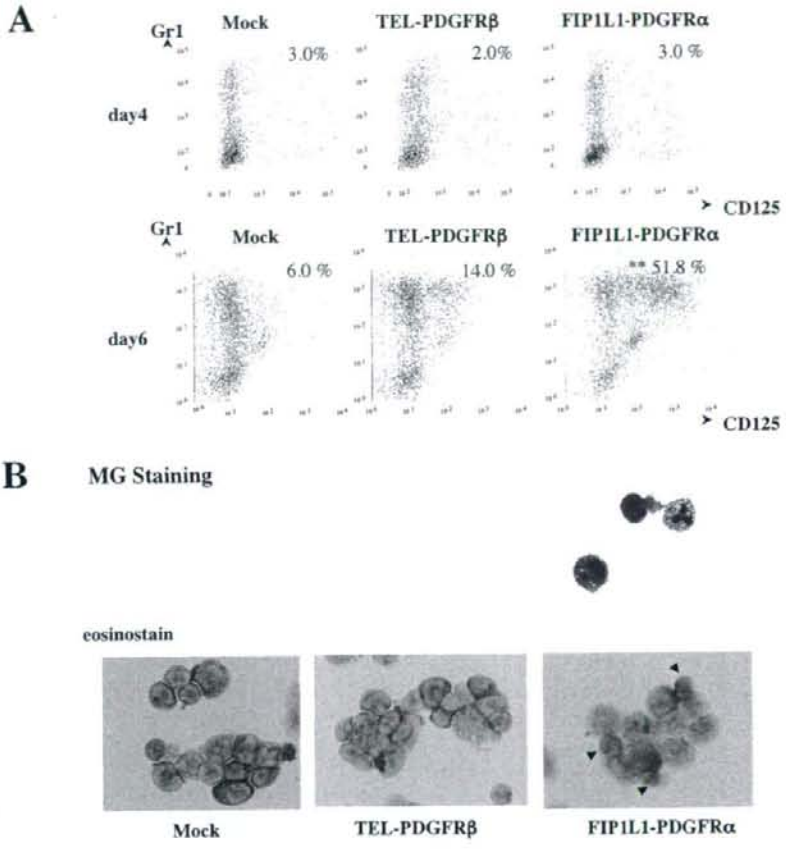
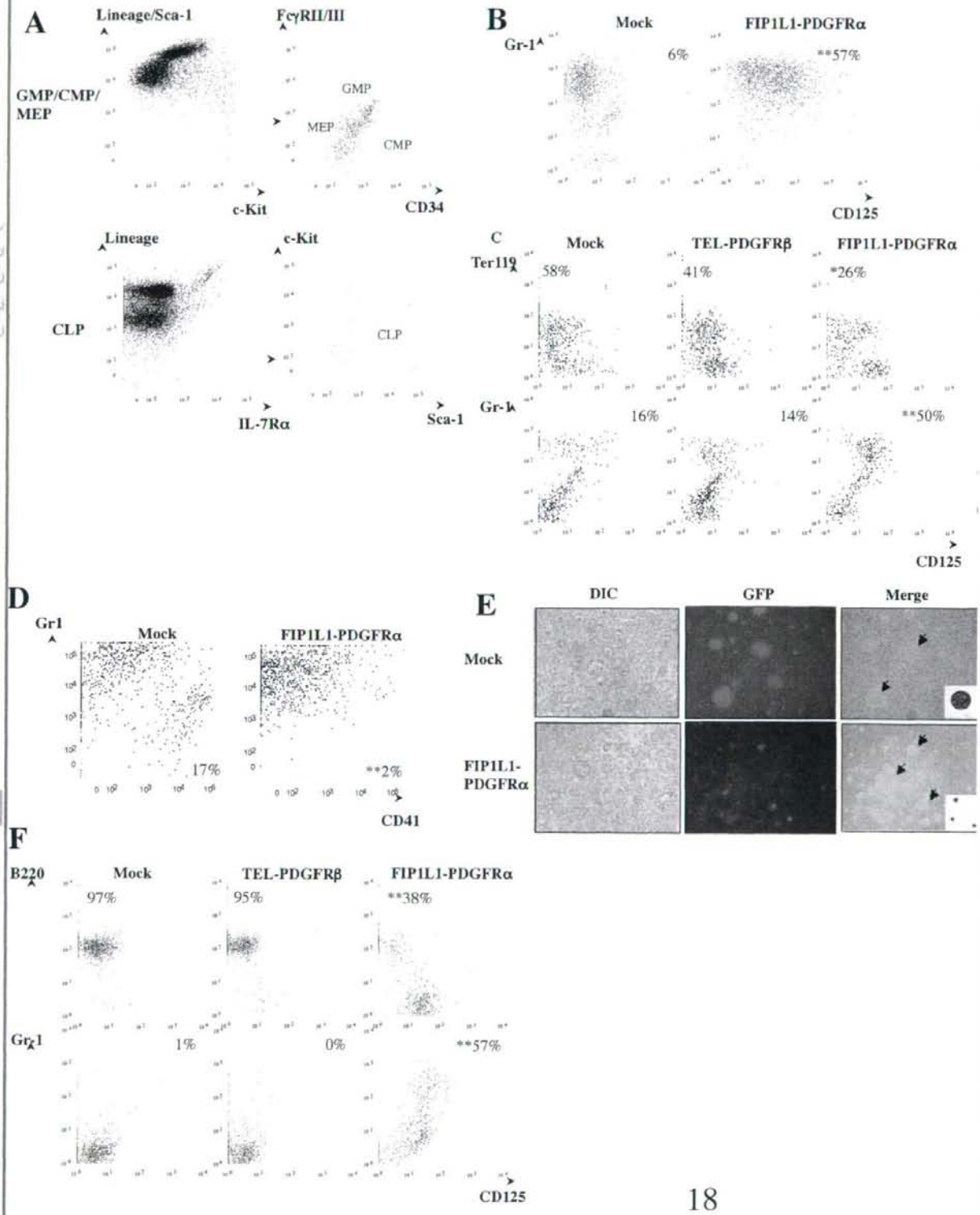

A
Gr1
Mock
TEL-PDGFRβ
FIP1L1-PDGFRα
3.0%
2.0%
3.0 %
day4
CD125
Gr1
Mock
TEL-PDGFRβ
FIP1L1-PDGFRα
6.0 %
14.0 %
** 51.8 %
day6
CD125
B
MG Staining
eosinostain
Mock
TEL-PDGFRβ
FIP1L1-PDGFRα
C
Mock
TEL-PDGFRβ
FIP1L1-PDGFRα
IL-5Rα
C/EBPε
GATA-1
MBP
HPRT

Figure 3


A
Lineage/Sca-1
FcγRII/III
GMP/CMP/
MEP
GMP
MEP
CMP
c-Kit
CD34
Lineage
c-Kit
CLP
CLP
IL-7Rα
Sca-1
B
Mock
FIP1L1-PDGFRα
Gr-1
6%
**57%
CD125
C
Mock
TEL-PDGFRβ
FIP1L1-PDGFRα
Ter119
58%
41%
*26%
Gr-1
16%
14%
**50%
CD125
D
Gr1
Mock
FIP1L1-PDGFRα
17%
**2%
CD41
E
DIC
GFP
Merge
Mock
FIP1L1-
PDGFRα
F
B220
Mock
TEL-PDGFRβ
FIP1L1-PDGFRα
97%
95%
**38%
Gr1
1%
0%
**57%
CD125

18

Figure 4

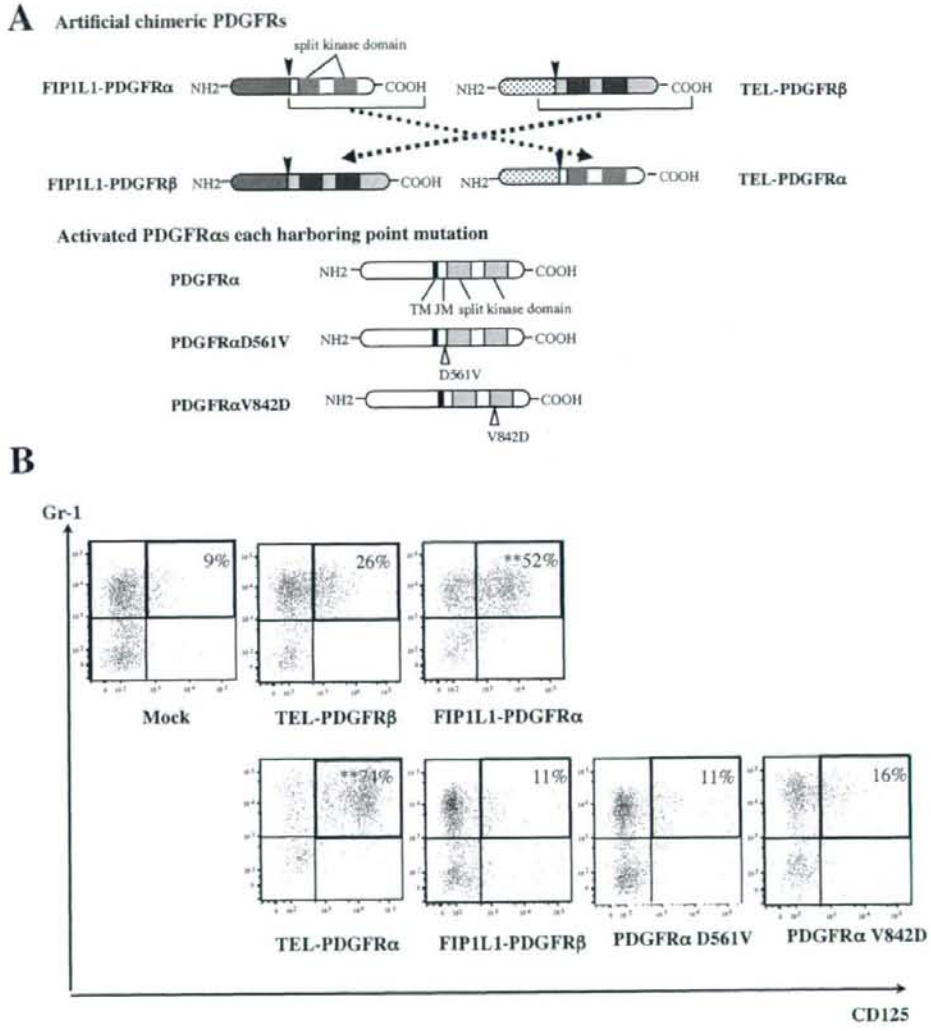

A
Artificial chimeric PDGFRs
split kinase domain
FIP1L1-PDGFRα
NH2
COOH
TEL-PDGFRβ
FIP1L1-PDGFRβ
TEL-PDGFRα
Activated PDGFRαs each harboring point mutation
PDGFRα
TM JM split kinase domain
PDGFRαD561V
D561V
PDGFRαV842D
V842D
B
Gr-1
9%
26%
**52%
Mock
TEL-PDGFRβ
FIP1L1-PDGFRα
**74%
11%
11%
16%
TEL-PDGFRα
FIP1L1-PDGFRβ
PDGFRα D561V
PDGFRα V842D
CD125

Figure 5

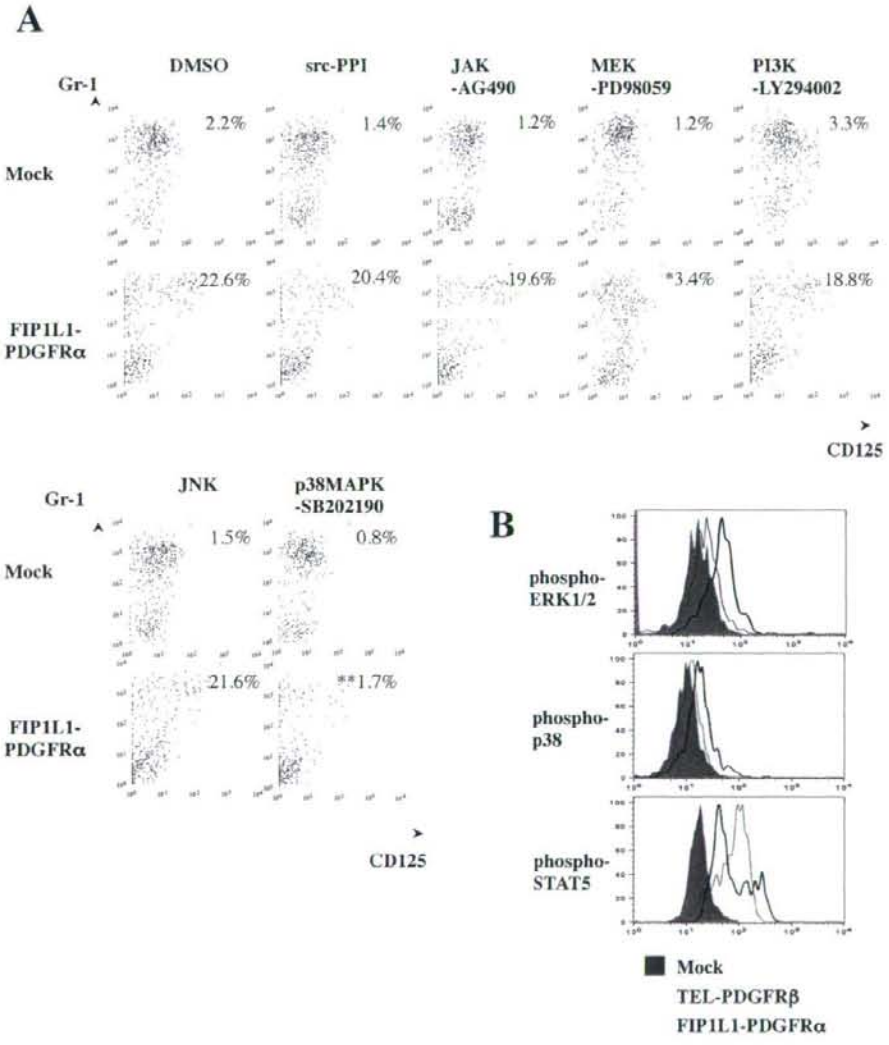

A
DMSO
src-PP1
JAK
-AG490
MEK
-PD98059
PI3K
-LY294002
Gr-1
2.2%
1.4%
1.2%
1.2%
3.3%
Mock
22.6%
20.4%
19.6%
*3.4%
18.8%
FIP1L1-
PDGFRα
CD125
JNK
p38MAPK
-SB202190
Gr-1
1.5%
0.8%
Mock
21.6%
**1.7%
FIP1L1-
PDGFRα
CD125
B
phospho-
ERK1/2
phospho-
p38
phospho-
STAT5
Mock
TEL-PDGFRβ
FIP1L1-PDGFRα

Figure 6

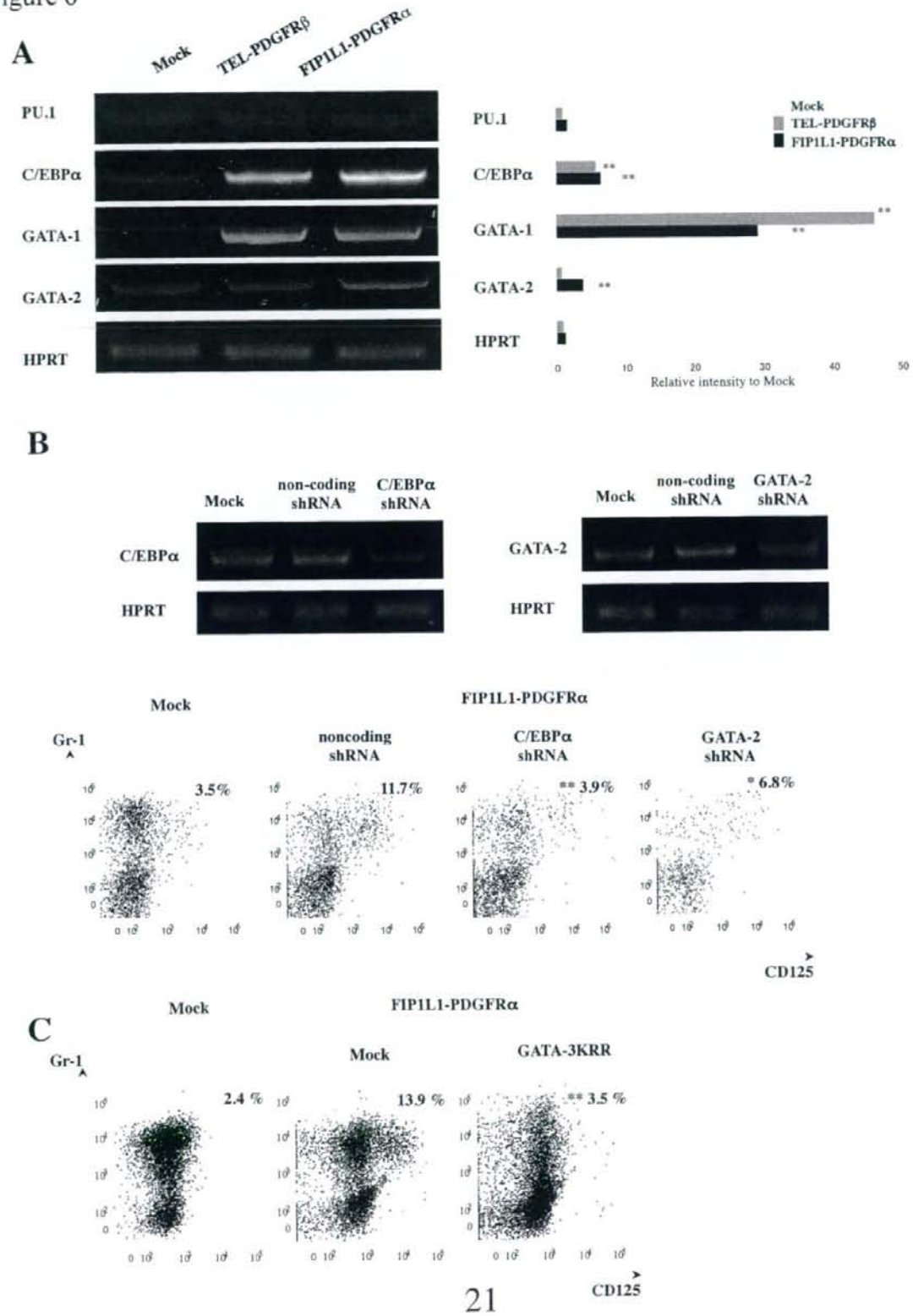

A
Mock
TEL-PDGFRβ
FIP1L1-PDGFRα
PU.1
C/EBPα
GATA-1
GATA-2
HPRT
Mock
TEL-PDGFRβ
FIP1L1-PDGFRα
0
10
20
30
40
50
Relative intensity to Mock
B
Mock
non-coding shRNA
C/EBPα shRNA
GATA-2 shRNA
Mock
FIP1L1-PDGFRα
Gr-1
noncoding shRNA
C/EBPα shRNA
GATA-2 shRNA
3.5%
11.7%
** 3.9%
* 6.8%
CD125
C
Mock
FIP1L1-PDGFRα
Gr-1
Mock
GATA-3KRR
2.4 %
13.9 %
** 3.5 %
CD125

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Figure 7

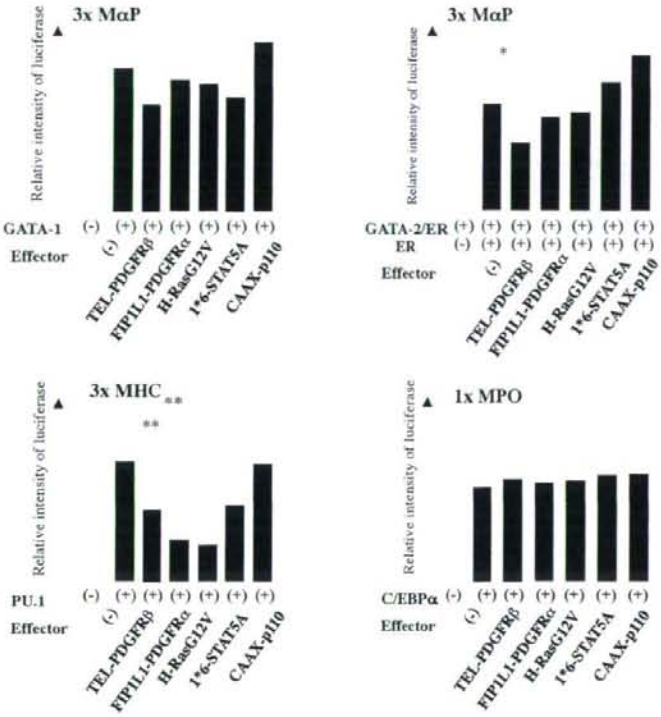

Table1
Peripheral blood examinations on 16 weeks after transplantation

mouse	WBCx10^9/L	eosinophil (%)	
Mock-1	88.3	1.2	
Mock-2	96.2	2.4	
Mock-3	83.5	2.6	
Mock-4	102.2	0.8	
Mock-5	88.2	2	
FIP1L1-PDGFRα-1	563.2	3.6	
FIP1L1-PDGFRα-2	121.1	1.2	
FIP1L1-PDGFRα-3	492.3	4.8	
FIP1L1-PDGFRα-4	140.1	3.6	
FIP1L1-PDGFRα-5	662.3	0.2	CD3, CD8positive cell 96%

blood

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The endothelial antigen ESAM marks primitive hematopoietic progenitors throughout life in mice

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STEM CELLS IN HEMATOLOGY

Title

The Endothelial Antigen ESAM Marks Primitive Hematopoietic Progenitors Throughout Life in Mice

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Abstract

Although recent advances have enabled hematopoietic stem cells (HSC) to be enriched to near purity, more information about their characteristics will improve our understanding of their development and stage-related functions. Here, using microarray technology we identified endothelial cell-selective adhesion molecule (ESAM) as a novel marker for murine HSC in fetal liver. ESAM was expressed at high levels within a Rag1$^-$ c-kit^{Hi} Sca1$^+$ HSC-enriched fraction, but sharply down-regulated with activation of the Rag1 locus; a valid marker for the most primitive lymphoid progenitors in E14.5 liver. The HSC-enriched fraction could be subdivided into two on the basis of ESAM levels. Among endothelial antigens on hematopoietic progenitors, ESAM expression showed intimate correlation with HSC activity. The ESAM^{Hi} population was highly enriched for multipotent myeloid-erythroid progenitors and primitive progenitors with lymphopoietic activity, and exclusively reconstituted long-term lympho-hematopoiesis in lethally irradiated recipients. Tie2$^+$ c-kit$^+$ lympho-hematopoietic cells in the E9.5-10.5 aorta-gonad-mesonephros region also expressed high levels of ESAM. Furthermore, ESAM was detected on primitive hematopoietic progenitors in adult bone marrow. Interestingly, ESAM expression in the HSC-enriched fraction was up-regulated in aged mice. We conclude that ESAM marks HSC in murine fetal liver and will facilitate studies of hematopoiesis throughout life.

Introduction

Hematopoietic stem cells (HSC) are defined as cells with the capacity for self-renewal as well as differentiation into multi-lineage blood cells, maintaining the immune system throughout life. A large body of information exists about molecular mechanisms involved in maintaining their integrity, and many studies have attempted to identify unique markers associated with these extremely rare cells. In bone marrow of adult mice, the Lin^- c-kit^{Hi} $Sca1^+$ $CD34^{-/Lo}$ $Thy1.1^{Lo}$ subset is known to include HSC with long-term repopulating capacity.[1] However, several of these parameters differ between strains of mice, change dramatically during developmental age or inflammation, and are expressed on many non-HSC.[2-4] The recent identification of CD150/SLAM as stable markers made it possible to increase the purity of HSC even in aged mice or cyclophosphamide/G-CSF-treated mice with mobilized progenitors.[5] However, even the most highly purified HSC are heterogeneous and it may eventually be possible to associate discrete functions or activity states with sub-populations. Additional authentic HSC markers could have utility in attempts to rescue hematopoietic disorders by using hematopoietic progenitors obtained from reprogrammed adult tissues.[6,7]

HSC are thought to arise initially from hemogenic endothelium which can produce hematopoietic cells as well as endothelial cells. Therefore, it is not surprising that HSC share some endothelial properties at early developmental stages.[8,9] For example, the CD34 sialomucin and Tie2, an angiopoietin receptor, are expressed on HSC in E10-11 embryos.[10,11] Endoglin and VE-cadherin are additional endothelial markers found on fetal HSC.[8,12] However, the expression of many of these antigens declines on HSC at later stages of development.[3,4,13] It is interesting that the expression of CD34 is restored when adult HSC are driven into cycle by 5-FU or G-CSF administration.[14,15] CD11b/Mac-1 is an adhesion molecule that is similarly dependent on developmental age and activation status.[16] In contrast to these patterns, endomucin is a CD34-like sialomucin that marks HSC from E10 and throughout subsequent development.[17] Each of these advances offered the promise of learning more about how HSC arise de novo and function throughout life.

We previously determined that the most primitive cells with lymphopoietic potential first develop in the paraaortic splanchnopleura (PSp) / aorta-gonad-mesonephros (AGM) region of embryos, and we tracked expression of the Rag1 lymphoid gene.[18,19] To extend those findings, we searched for genes that might be differentially expressed at the very earliest stages of lymphopoiesis. We here show that endothelial cell-selective adhesion molecule (ESAM) is a durable and effective marker of HSC. Indeed, ESAM was expressed throughout life and could be used as a gating parameter for sorting long-term repopulating HSC.

Materials and Methods

Animals.

Rag1/GFP knock-in mice (CD45.2 alloantigen) were described.[20,21] Mice of the corresponding wild-type (WT) C57BL/6 were obtained from Japan Clea (Shizuoka, Japan). Mating homozygous male Rag1/GFP knock-in mice with WT C57BL/6 female mice generated heterozygous Rag1/GFP knock-in fetuses. The day of vaginal plug observation was considered as day 0.5 postcoitum (E0.5). In some experiments, we purchased pregnant C57BL/6 mice from Japan Clea and used their fetuses. The congenic C57BL/6 strain (C57BL/6SJL; CD45.1 alloantigen) was purchased from The Jackson Labs (Bar Harbor, ME) and used in transplantation experiments. The experimental designs of this study were approved by the committee of Osaka University for animal studies.

Antibodies.

Phycoerythrin (PE)-conjugated anti-Sca1 (Ly6A/E; D7), CD48 (HM48-1), CD11b/Mac-1 (M1/70), Gr-1 (RB6-8C5), CD19 (1D3), CD4 (L3T4), and CD8a (53-6.7) monoclonal antibodies (mAbs), biotinylated anti-CD45.2 (104) mAb, allophycocyanin (APC)-conjugated anti-CD11b/Mac-1 (M1/70) and c-kit (2B8) mAbs, and PE-Texas red tandem-conjugated (PE-TR) streptavidin were purchased from BD Pharmingen (San Diego, CA). PE-conjugated anti-CD34 (RAM34), CD31/PECAM-1 (390), CD105/Endoglin (MJ7/18), and Tie2 (TEK4) mAbs, PE-Cy7-conjugated anti-Sca1 (Ly6A/E; D7) mAb, and APC-conjugated anti CD45.1 (A20) mAb were purchased from eBioscience (San Diego, CA). A rat anti-mouse ESAM mAb (1G8), a rabbit anti-mouse ESAM polyclonal Ab (VE19), and a rabbit preimmune IgG were prepared in our hand.[22] A fluorescein isothiocyanate (FITC)-conjugated goat-anti rat IgG (H+L) Ab purchased from Southern Biotechnology (Birmingham, AL), a PE-conjugated goat anti-rat Ig Ab purchased from BD Pharmingen, or AlexaFluor® 488goat anti-rabbit IgG (H+L) Ab purchased from Invitrogen (Eugene, OR) was used as a second Ab for the anti-ESAM Abs. A PE-conjugated hamster IgG1 was purchased from BD Pharmingen and used as a control for a PE-conjugated anti-CD48. FITC-conjugated anti- CD11b/Mac-1 (M1/70), Gr-1 (RB6-8C5),

TER-119, CD45R/B220 (RA3-6B2) and CD3e (145-2C11) were purchased from BD Pharmingen and used as lineage markers in adult studies.

Cell Sorting.

Fetal liver or cells obtained from adult femurs and tibias of heterozygous Rag1/GFP knock-in mice were harvested and subjected to cell sorting as previously described.[18] In the first step, $Rag1^{-}$ or $Rag1^{Lo}$ cells were sorted according to levels of GFP expression. Background auto-fluorescence was discriminated from authentic GFP by collecting data in two fluorescence channels without compensation. Under these conditions, even extremely low levels of fluorescence specific to GFP knock-in mice were obvious on two-color diagonal plots. The purity of the sorted cells in the first step was more than 95%. The sorted cells were incubated with anti-FcR (2.4G2) before staining with PE-anti-Sca1 and APC-anti-c-kit antibodies, suspended in 7AAD-containing buffer, and subjected to a second round of sorting. Dead cells stained with 7AAD and a few contaminating cells with inappropriate GFP levels were gated out, and the cells were then fractionated according to Sca1 and c-kit to obtain $Rag1^{-}$ $c\text{-}kit^{Hi}$ $Sca1^{+}$ HSC and $Rag1^{Lo}$ $ckit^{Hi}$ $Sca1^{+}$, early lymphoid progenitors (ELP). The cell sorting was performed with FACSaria using the FACSDiva program (Becton Dickinson, San Jose, CA). The sorting gates used to isolate HSC and ELP from E14.5 fetal liver for gene array experiments (see below) are shown in Supplemental Figure 1.

In the sorting experiments with ESAM expression, $Rag1/GFP^{-}$ cells were firstly sorted from fetal tissues or adult bone marrow. Then the cells were stained with rat anti-mouse ESAM mAb (1G8) followed by FITC-goat anti-rat IgG or PE-goat anti-rat Ig, respectively. After the staining for ESAM, E14.5 fetal liver $Rag1/GFP^{-}$ cells were stained with PE-anti-Sca1 and APC-anti-c-kit antibodies. For PSp/AGM or yolk sac (YS) cells of E9.5-10.5 embryos, a PE-anti-Tie2 Ab was used instead of a PE-anti-Sca1 Ab. The adult marrow $Rag1/GFP^{-}$ cells were stained with FITC-anti-Lin (Mac-1, Gr-1, TER119, CD45R/B220, CD3e), PE-Cy7-anti-Sca1 and APC-anti-c-kit Abs. Then the cells were suspended in 7AAD-containing buffer, and subjected to a second round of sorting.

Gene arrays.

Total RNAs were isolated from the Rag1^{-} ckit^{Hi} Sca1^{+}, HSC-enriched fraction and the Rag1^{Lo} ckit^{Hi} Sca1^{+}, early lymphoid progenitors (ELP)-enriched fraction, both of which were isolated from mouse E14.5 fetal liver. From the aliquot of each RNA (24 ng), biotin-labeled cRNA was prepared using GeneChip Two-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA) according to the manual. The cRNA was fragmented and hybridized to Mouse Genome 430 2.0 Array (Affymetrix) using a GeneChip Hybridization, Wash, and Stain Kit (Affymetrix). Fluorescent signals were scanned by GeneChip Scanner 3000, and the data were analyzed by a GeneSpring GX software (Agilent Technologies, Palo Alto, CA). Bacillus subtilis genes on the arrays were used as negative controls for background subtraction. All values of the genes in each array were divided by the value of glyceraldehyde-3-phosphate dehydrogenase (Gapdh) gene. The Cross-Gene Error Model was used to estimate measurement precision by combining variability of gene expression data. The experiments were duplicated to confirm reproducibility of the data. All microarray data has been deposited with CIBEX, National Institute of Genetics DDBJ (DNA Data Bank of Japan), under the accession number CBX73.

Flow cytometry.

Rag1/GFP^{-} cells of E14.5 fetal liver were incubated with rat anti-mouse ESAM mAb (1G8) followed by FITC-goat anti-rat IgG. Then the cells were incubated with a rat anti-mouse FcRII/III (2.4G2), and subsequently stained with PE-Cy7-anti-Sca1 and APC-anti-c-kit in combination with PE-anti-CD34, CD31/PECAM1, CD105/Endoglin, or Tie2 mAbs. The cells were suspended in 7AAD-containing buffer, and subjected to flow cytometry analyses, performed with FACSaria using the FACSDiva program (Becton Dickinson).

In the other experiments, cultured cells or recovered cells from transplanted mice were incubated with anti-FcR and then stained with PE-, APC-conjugated mAbs and/or a biotinylated Ab followed by PE-TR-streptavidin

as indicated in each figure. The flow cytometry analyses were performed with FACScalibur using the Cellquest program (Becton Dickinson). The data analyses were done with FlowJo software (Tree Star, San Carlos, CA).

Methylcellulose culture.

Five hundred or one hundred cells of each sorted fraction (Rag1/GFP$^-$ ckit^{Hi} Sca1$^+$ ESAM1$^{-/Lo}$ or Rag1/GFP$^-$ ckit^{Hi} Sca1$^+$ ESAM1^{Hi} of fetal liver or adult marrow, respectively) were cultured in Iscove's MDM-based methylcellulose medium supplemented with 50 ng/mL of recombinant mouse (rm) SCF, 10 ng/mL of rm IL-3, 10 ng/mL of recombinant human IL-6, and 3 units /mL of recombinant human erythropoietin (Methocult GF 3434; StemCell Technologies, Vancouver, Canada). After 8-10 days, colonies were enumerated and classified as CFU-GM/M, BFU-E or CFU-Mix (GEM(M)) according to shape and color under an inverted microscope. In some experiments, colony types were morphologically certified by examination of cytospin preparations after May-Grünwald/Giemsa staining.

Stromal coculture and limiting dilution assays.

The murine bone marrow stromal cell line, MS-5 was generously provided by Dr. J. Mori (Niigata University, Japan). Non-irradiated MS-5 stromal cells were prepared at a concentration of 5 x 10^4 cells/well in 24-well tissue plates 1 day prior to the seeding of sorted cells at a concentration of 50 cells / mL. Cells were cultured in α-MEM (Invitrogen, Grand Island, NY) supplemented with 10% FCS, rm SCF (10 ng/mL), rm Flt3-ligand (20 ng/mL), and rm IL7 (1ng/mL). The cultures were fed every 4 days by removing half of the medium and replacing it with fresh medium, and maintained for 6 or 10 days. Cytokines were freshly added with each feeding.

The frequencies of lympho-hematopoietic progenitors in the fetal liver-derived Rag1/GFP$^-$ ckit^{Hi} Sca1$^+$ ESAM$^{-/Lo}$ or Rag1/GFP$^-$ ckit^{Hi} Sca1$^+$ ESAM^{Hi} fraction were determined by plating the sorted cells in limiting dilution assays using 96-well flat bottom plates. Pre-established MS-5 stromal cell layers were plated with 1, 2, 4, 8, or 50 cells each using the Automated Cell Deposition Unit of the FACSaria (BD Bioscience). Cells were cultured in

α-MEM supplemented with 10% FCS, rm SCF (10 ng/mL), rm Flt3-ligand (20 ng/mL), and rm IL7 (1ng/mL) as described above. At 10 days of culture, wells were inspected for the presence of hematopoietic clones. Positive wells were harvested, stained, and analyzed by flow cytometry for the presence of $CD19^+$ $Gr\text{-}1^-$ B lineage cells. The frequencies of progenitors were calculated by linear regression analysis on the basis of Poisson distribution as the reciprocal of the concentration of test cells that gave 37% negative cultures.

Competitive repopulation assay.

The Ly5 system was adapted to a competitive repopulation assay. One thousand of Rag1/GFP^- c-kit^{Hi} $Sca1^+$ $ESAM^{+/Lo}$ or Rag1/GFP^- c-kit^{Hi} $Sca1^+$ $ESAM^{Hi}$ cells sorted from fetal liver of E14.5 Rag1/GFP heterozygous embryos (CD45.2) were mixed with 2×10^5 unfractionated adult bone marrow cells obtained from wild type C57BL/6-Ly5.1 (CD45.1) mice, and were transplanted into C57BL/6-Ly5.1 mice irradiated at a dose of 10 Gy. At 5w after transplantation, peripheral blood cells of the recipients were colleted by retroorbital bleeding and were stained with APC-conjugated anti-CD45.1 and biotinylated anti-CD45.2 Abs followed by PE-TR streptavidin. The cells were simultaneously stained with PE-conjugated anti-Mac-1and Gr-1 Abs, or PE-conjugated anti-CD19 Ab, or PE-conjugated anti-CD4 and CD8a Abs. Twenty-weeks after transplantation, all recipients were killed, and reconstitution of $CD45.2^+$ myeloid, B or T cells was confirmed by flow cytometry in the bone marrow, spleen and thymus, respectively. For the second transplantation, bone marrow cells of primary recipients with CD45.2 engraftment were transferred into 10 Gy-irradiated C57BL/6-Ly5.1 mice (1×10^6 whole bone marrow cells per recipient). After 20w contribution of CD45.2 cells to the hematopoietic reconstitution was evaluated in the second recipients.

Cell preparation from PSp/AGM and YS.

To examine the phenotype or function of hematopoietic progenitors in the PSp/AGM and the YS of early embryos, cells were prepared as previously described.[19] Briefly, tissues were dissociated by incubation with dispase II (Roche Diagnosis, Mannheim, Germany) for 20 min at 37°C and cell dissociation

buffer (Invitrogen) for 20 min at 37°C followed by vigorous pipetting. The cells were then suspended in PBS containing 3% FCS and stained with the Abs indicated in each experiment.

Statistical methods

Statistical analysis was carried out by standard student t-tests. Error bars used throughout indicate SD of the mean.

Results

Identification of ESAM as a marker of primitive hematopoietic cells.

Microarrays were performed as part of an effort to characterize the initial transition of fetal HSC to primitive lymphopoietic cells. The comparisons involved mRNA from Rag1^{Lo} ckit^{Hi} Sca1^{+}, early lymphoid progenitors (ELP) and the HSC-enriched Rag1^{-} ckit^{Hi} Sca1^{+} fraction isolated from E14.5 fetal liver (Supplemental Figure 1).

Consistent with previous analyses [12,23,24], Evi1, CD41, and Endoglin were highly expressed by fetal HSC (Figure 1). ESAM transcripts were conspicuous in the HSC fraction and attracted attention because of a sharp down-regulation on differentiation to ELP (Figure 1). Real-time PCR using ESAM specific primers verified the results of these microarrays (data not shown).

ESAM can be used to sub-divide hematopoietic progenitors in fetal Liver.

The availability of a rat anti-mouse ESAM mAb (clone 1G8) facilitated characterization of mononuclear cells obtained from E14.5 fetal liver. A majority of ESAM^{+} cells were found in the c-kit^{Hi} fraction (Figure 2A, left). ESAM expression also correlated with Sca1 (Figure 2A, middle). These observations suggested that ESAM might substitute for at least one of the two most widely used HSC markers. Indeed, cells in the Sca1^{Hi} ESAM^{Hi} gate were almost homogeneous with respect to high c-kit expression (Figure 2A, right).

The c-kit^{Hi} Sca1^{+} fraction, known to include all conventional HSC, was divided into two categories according to ESAM staining (Figure 2B left and middle). The subpopulation with the highest density of ESAM was enriched for c-kit^{Hi} Sca1^{Hi} cells, while ones with negative or low levels of ESAM were found in the c-kit^{Hi} Sca1^{Lo} subset (Figure 2B right). The latter are thought to be enriched with respect to committed myelo-erythroid progenitors.[25] Similar results were obtained with a rabbit anti-mouse ESAM polyclonal Ab (VE19) (data not shown).

When E14.5 fetal liver cells of wild type C57B6 mice and traditional markers of fetal liver HSC were used instead of the Rag1/GFP knock-in mice, essentially identical results were obtained (Figure 2C). This analysis was performed with CD48 expression, one of the SLAM family members recently