Endomucin, a CD34-like sialomucin, marks hematopoietic stem cells throughout development

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To detect as yet unidentified cell-surface molecules specific to hematopoietic stem cells (HSCs), a modified signal sequence trap was successfully applied to mouse bone marrow (BM) CD34−c-Kit+Sca−1−Lin− (CD34−KSL) HSCs. One of the identified molecules, Endomucin, is an endothelial sialomucin closely related to CD34. High-level expression of Endomucin was confined to the BM KSL HSCs and progenitor cells, and, importantly, long-term repopulating (LTR)–HSCs were exclusively present in the Endomucin+CD34−KSL population. Notably, in the yolk sac, Endomucin expression separated multipotential hematopoietic cells from committed erythroid progenitors in the cell fraction positive for CD41, an early embryonic hematopoietic marker. Furthermore, developing HSCs in the intraembryonic aorta-gonad-mesonephros (AGM) region were highly enriched in the CD45+KSL HSCs and progenitor cells, and, importantly, long-term repopulating (LTR)–HSCs were exclusively present in the Endomucin+CD45+KSL population. Our findings establish Endomucin as a novel cell-surface marker for LTR-HSCs throughout development and provide a powerful tool in understanding HSC ontogeny.

Hematopoietic stem cells (HSCs) are defined as cells that retain the capacities for both self-renewal and multilineage differentiation. We have previously reported that in adult mouse BM, CD34<sup>+</sup>/−c-Kit<sup>+</sup>Sca−1<sup>+</sup>Lin<sup>−</sup> (CD34<sup>+</sup>KSL) cells, which constitute ~0.004% of BM cells, represent HSCs with long-term repopulating (LTR) ability, whereas CD34<sup>+</sup>KSL cells are progenitors with short-term repopulating capacity (1). In adult mice, HSCs reside in the so-called “stem-cell niche,” which forms the microenvironment for HSCs in the BM. HSC behaviors are regulated by signals from their niche through cell-surface and secreted molecules. Understanding the molecular mechanisms underlying these cell–cell interactions holds the key for HSC biology and is of biological and clinical interest. Moreover, identification of cell-surface molecules on HSCs is also important to obtain a truly specific marker for HSCs. However, experiments with HSCs have been hampered by the very low rate at which HSCs are found in BM, leaving their molecular nature unknown. Recent technological innovation is overcoming this hurdle, and extensive gene expression profiling is providing a list of genes potentially involved in HSC function (2–4). Yet the list of cell-surface molecules whose presence is currently used to mark HSCs is short; in addition, many of these molecules also are expressed by certain cells that bear lineage-differentiation markers (5, 6). For this reason, most approaches to HSC purification still include selection by the absence of certain molecules.
such as lineage markers, CD34, and Flk2/Flt3 (1, 7, 8). Even selection for dye efflux activity (9) is a selection by negative criterion.

Two waves of hematopoiesis occur in the mouse embryo. The first transient wave of primitive hematopoiesis is characterized by the presence of nucleated red cells expressing embryonic globin βH, and is detected in the yolk sac as early as day 7.5 (E7.5) of gestation (10). Definitive hematopoiesis that supplies adult-type red blood cells arises as the second wave in the E10.5 intraembryonic aorta-gonad-mesonephros (AGM) (11, 12). CD41 is known to mark the initiation of primitive and definitive hematopoiesis in the embryo, although its expression is down-regulated in hematopoietic progenitors by the fetal liver stage (13–15). In the AGM region, all adult-engrafting cells derived from the E10.5 to E12.5 AGM region reportedly express transcription factor Runx1 and, interestingly, adult-engrafting cells change their profile from CD45− to CD45+ between E10.5 and E11.5 (16). Two markers for adult HSCs, c-Kit and CD34, are also expressed on adult-engrafting cells in the AGM region and fetal liver (17). Despite extensive experiments that have defined the temporal and anatomical differences between primitive and definitive hematopoiesis, little is known about the development of HSCs in the embryo, and markers for developing HSCs are limited.

To identify novel cell-surface molecules on HSCs, we combined long-distance PCR amplification of full-length cDNA from purified HSCs with a signal sequence trap by retrovirus-mediated expression screening (SST-REX) (18). With this method, we identified several genes encoding cell-surface or secreted proteins preferentially expressed by mouse BM CD34+/KSL HSCs. One of these genes, Endomucin, has been shown to encode an endothelial sialomucin closely related to CD34 (19, 20). In this study, we show that Endomucin is preferentially expressed on both LTR–HSCs in adult BM and developing HSCs in the embryo. Of importance is that Endomucin expression in the embryo was specific to adult-engrafting HSCs and multipotential progenitors and was not detected on committed erythroid progenitor cells of both primitive and definitive types. Using Endomucin as a novel positive HSC marker, we present our success in tracing HSCs throughout development and define Endomucin as a new tool useful in understanding the ontogeny of HSCs.

RESULTS
Identification of Endomucin as an HSC-specific gene
To identify cell-surface molecules specific to HSCs, we took advantage of an SST–REX cloning method. This method detects signal sequences in cDNA libraries based on the sequences’ ability to redirect a constitutively active mutant of c-Mpl to the cell surface, thereby permitting IL-3–independent growth of Ba/F3 cells (18). We successfully applied SST-REX to a limited number of CD34+ KSL HSCs (Fig. 1 A). From 231 IL-3–independent clones, we isolated 128 cDNAs and identified 46 genes. Of these, 36 were known murine genes, 4 were putative murine homologues of human genes, and 6 were unknown murine genes (Table S1, available at http://www.jem.org/cgi/content/full/jem.20051325/DC1). RT–PCR analysis of selected genes revealed that several genes were preferentially expressed in hematopoietic stem and progenitor fractions (Fig. 1 B). Among these genes, mRNA expression of Endomucin was restricted to CD34+ KSL cells. The extracellular domain of Endomucin is highly glycosylated with O-linked glycans (Fig. 1 C) (19). Its structure indicates membership in a sialomucin family that includes CD34, a well-characterized molecule specific to hematopoietic and endothelial systems. Endomucin was originally identified as an endothelium-associated marker (19, 21), but it had not yet been investigated in hematopoietic lineage cells. We therefore examined the expression of Endomucin on HSCs.

Expression of Endomucin on adult BM HSCs
Using anti-Endomucin mAbs, clone V.7C7 (19) and clone 2D4 (newly generated for this study), expression of Endomucin was extensively analyzed in adult hematopoietic cells of BM, spleen, and thymus. Endomucin was not expressed

Figure 1. SST screening of CD34+ KSL cells. (A) Overview of SST cloning. Full-length cDNA amplified from 5,000 CD34+ KSL cells was digested into pieces, ligated with BST-ⅩⅠ adaptor, and subcloned into an SST-REX vector. TM, transmembrane domain. (B) Expression of representative clones detected by RT-PCR. Genes depicted are Endomucin, CLEC (c-type lectin-like receptor), PEDF (pigment epithelium-derived factor), and Serpine (sequences are available from GenBank/EMBL/DDBJ under accession nos. AF060883, BC052840, NM_011340, and AF166382, respectively). Cells analyzed include BM CD34− KSL HSCs, progenitors, Lin+ cells, Gr1− neutrophils, Mac-1+ monocytes/macrophages, TER119+ erythroblasts, B220+ B cells, spleen Thy-1.2+ T cells, NK1.1+ NK cells, B220+ B cells, thymic CD4+ CD8− T cells (DN), CD4+ CD8+ T cells (DP), CD4− CD8− T cells (CD4SP), and CD4− CD8+ (CD8SP). (C) Schematic representation of Endomucin protein. N- and O-linked glycosylation in the extracellular domain and the potential protein kinase C phosphorylation sites (serine and threonine residues) in the cytoplasmic domain are indicated.
on a vast majority of cells bearing lineage markers, including Gr-1−, Mac-1−, B220−, CD4−, CD8−, and TER119-positive cells. Only small populations of BM B220+ (0.66%) and spleen B220+ cells (1.8%), which appeared to be B220+ CD43−IgM+ mature B cells, expressed Endomucin (unpublished data). A fraction of Mac-1low cells, representing 0.39% of total BM cells, also expressed Endomucin (unpublished data). Mac-1 is a surface antigen that is highly expressed on myeloid cells but also moderately expressed on some short-term repopulating hematopoietic progenitor cells (22). These findings showed good correlation with our RT-PCR data.

By using magnetic cell sorting to deplete Lin+ cells, Lin− BM mononuclear cells were enriched to 85.7 ± 4.8% of the preparations. Lineage-depleted BM cells were segregated into subpopulations defined by levels of Endomucin expression. Each subpopulation was tested for expression of c-Kit and Sca-1. The subpopulation with the highest level of Endomucin expression was enriched for KSL immature hematopoietic cells (Fig. 2 A). Conversely, 83.4% of KSL cells expressed Endomucin (Fig. 2 B). These data indicated that Endomucin is abundantly expressed on immature hematopoietic cells.

To evaluate Endomucin expression on HSCs directly, we next analyzed Endomucin expression on CD34− KSL cells.

Figure 2. Endomucin expression on BM hematopoietic cells. (A) Level of Endomucin expression correlates with immature cell phenotype. Lin− BM cells were divided into five subgroups (i–v) defined by Endomucin expression levels (top left). Each group was further analyzed with respect to expression of c-Kit and Sca-1. Panel numbers correspond to each subgroup. The percentage of c-Kit+Sca-1+ cells is indicated in panel v. (B) Endomucin expression in KSL cells. Expression of Endomucin in the KSL cell fraction (left, inset) is presented (right). KSL cells were stained with an isotype control IgG (continuous line) and the anti-Endomucin antibody (dashed line). The percentage of Endomucin− cells is indicated.

Lineage-depleted BM cells were further analyzed for the expression of CD34, c-Kit, Sca-1, and Endomucin. Most CD34−KSL cells (71%) strongly expressed Endomucin (Fig. 3 A). Although the expression level of Endomucin on CD34+ KSL cells was relatively low compared with that on CD34− KSL cells, Endomucin protein persisted on most CD34+ KSL cells (78%). The KSL fraction was then segregated by expression of Endomucin and of CD34, and single cells from each fraction were sorted into 96 microwell plates to allow colony formation. Endomucin−CD34+ KSL cells showed the highest efficiency of colony formation. Moreover, this fraction was most enriched for CFU-neutrophil/macrophage/erythroblast/megakaryocyte that retained multilineage differentiation capacity (Fig. 3 A). To determine whether Endomucin expression identifies cells with LTR capacity, we transplanted cells from each fraction into lethally irradiated mice (Fig. 3 C). 6 mo after transplantation, long-term repopulation was detected only with Endomucin−CD34+ KSL cells but not with Endomucin+CD34+ KSL cells. These data clearly show that all LTR-HSCs express Endomucin.

The engraftment rate of Endomucin−CD34+ KSL cells (8/18 recipient mice) was lower than that which we have previously reported for CD34 KSL cells (1, 23). We ascribe this to incomplete depletion of Lin+ cells. To include an anti-Endomucin antibody in four-color FACS analysis and cell sorting, Lin+ cells were depleted only by using magnetic beads, and the remaining Lin+ cells were not gated out on FACS cell sorting. This appears to have lowered the purity of HSCs in this population. To exclude the possibility that anti-Endomucin antibody inhibits HSC homing in vivo, we transplanted HSCs preincubated with anti-Endomucin antibody. However, it did not affect the engraftment rate (unpublished data).

Knowing that Endomucin expression marks all HSCs and correlates well with c-Kit and Sca-1 expression, we next investigated whether Endomucin, as a marker, could replace c-Kit and Sca-1. In competitive repopulation assays, again only Endomucin+CD34−Lin− cells contributed to long-term repopulation. As few as 100 cells were enough to obtain a higher order of repopulation (Fig. 3 B and C), indicating that the single marker Endomucin could substitute for c-Kit and Sca-1 to a large extent.

Erythroid progenitors are devoid of Endomucin expression in the yolk sac

We next asked whether Endomucin is expressed on hematopoietic cells of the developing embryo. In mouse embryos, blood cells first appear in the extraembryonic yolk sac (10). Consistent with other experiments, colony-forming cells (CFCs) in methycellulose medium were present only in the yolk sac at E8.5. Most CFCs were confirmed as primitive erythroid progenitor (Ery) cells by identification of unique morphologic features and of expression of ßH₄ embryonic hemoglobin specific to primitive hematopoiesis (unpublished data). Endomucin expression was then analyzed in
correlation with CD41, one of the most reliable markers for embryonic hematopoietic progenitor cells (13, 14), and with CD45, a panhematopoietic marker. In the yolk sac at E8.5, methylcellulose colony assays demonstrated that CFCs, including EryE -CFCs, are present only in the Endomucin fraction irrespective of cellular expression of CD41 or CD45 (Fig. 4, A and B). In the yolk sac at E10.5, again, erythroid progenitor cells of a definitive type, including CFU-E and BFU-E, were observed only in the Endomucin fraction in methylcellulose culture medium. In contrast, Endomucin+ cells gave rise exclusively to mixed colonies, which consisted mostly of macrophages and erythroid cells, although their origin (i.e., in primitive or definitive hematopoiesis) remains obscure (Fig. 4, C and D). These data indicate that Endomucin is not expressed on erythroid progenitor cells of either primitive or definitive type but is expressed on multipotent progenitor cells that develop in the yolk sac.

**Endomucin expression marks HSCs in the embryo**

It is reported that the E10.5 AGM autonomously produces HSCs that repopulate hematopoiesis in adults (11, 12). In a previous immunohistochemical analysis, specific Endomucin expression was observed at E8.5 to E11.5 on the endothelium of the dorsal aorta and in cell clusters associated with the luminal surface of the endothelium (24). These findings indicate tight correlation of Endomucin expression with HSC development and prompted us to analyze AGM hematopoiesis at E10.5 to E11.5.

In contrast with the findings in the E10.5 yolk sac (Fig. 4 D), the vast majority of hematopoietic activity in the E10.5 AGM was detected within CD45+ cells (Fig. 5, A and D). FACS analysis demonstrated that ~0.16% of CD45+ cells were CD41+ Endomucin+ and that Endomucin expression was restricted to a population of CD41+E (Fig. 5 A). Among CD45+ cells, those which were CD41+ Endomucin+ composed the main population that gave rise to colonies in methylcellulose medium (Fig. 5 D). Co-culture of early murine AGM cells with stromal cells is reported to facilitate hematopoiesis from hemangioblasts (25). We used this co-culture system as well as methylcellulose culture. Surprisingly, when co-cultured with OP-9 stromal cells, CD45 CD41+ Endomucin+ cells showed a plating efficiency 38-fold higher than that in methylcellulose medium and a plating efficiency 4.6-fold higher than that of CD45 CD41+ Endomucin+ cells (Fig. 5 D). This CD45 CD41+ Endomucin+ population proliferated extensively (Fig. 5 E) and differentiated into multiple lineages (unpublished data). In contrast, CD45−CD41−Endomucin− cells formed VE-cadherin+ endothelial cell colonies on OP-9 cells in the presence of vascular endothelial growth factor (unpublished data) but formed no hematopoietic colonies (Fig. 5 D), suggesting that this population includes pure endothelial cells. Embryonic stem (ES) cell differentiation in vitro recapitulates the embryonic development. We additionally used an ES cell differentiation model to confirm the correlation of Endomucin expression with developing HSCs. ES cells were...
allowed to differentiate by forming embryoid bodies (EB; i.e., aggregates containing three germ layers), and their mesodermal differentiation was monitored by flow cytometry up to day 10 (EB10). Endomucin cells appeared around EB5 (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20051325/DC1), which is later than the onset of formation of hemangioblasts (EB3) that give rise to primitive hematopoiesis. With respect to hematopoiesis, EB4 to EB6 corresponds to the developmental stage around E8.5 to E10.5 (13). As observed with AGM cells, CD45 cells at EB4 to EB6 showed robust expansion on OP-9 stromal cells (Fig. S1, B and C), whereas CD45 cells did not proliferate at all (not depicted). These data correlate well with and strongly support the findings in the AGM (Fig. 5, D and E).

We next analyzed cells from the E11.5 AGM, which reportedly exhibit higher hematopoietic activity (17). Interestingly, hematopoietic activity largely shifted to CD45 cells in the E11.5 AGM (Fig. 5, D and E). Although only 5% of AGM cells were CD45, Endomucin expression on CD41 cells identified cells with the highest hematopoietic potential. These CD45 Endomucin cells notably exhibited high plating efficiency both in methylcellulose medium and on OP-9 stromal cells (Fig. 5, D and E).

To define HSC properties of Endomucin AGM cells, we directly evaluated the capacity of Endomucin cells to repopulate adult BM hematopoiesis in vivo. Fractionated AGM cells at E10.5 were transplanted directly into the BM of lethally irradiated adult mice. Although the degree of chimerism was low, as few as 750 CD45 CD41 Endomucin cells were enough to establish long-term repopulation (Table I), whereas other fractions did not contribute to long-term repopulation at all. Additionally, and in keeping with the in vitro data, LTR-HSCs with enhanced engrafting capacity were detected only in the CD45 Endomucin fraction.

Figure 4. Endomucin is not expressed on mouse Ery cells during development. FACS profiles of E8.5 (A) and E10.5 (C) yolk sac cells. Sorting gates for colony assays are depicted and the percentage of cells in each gate is shown. Numbers of CFUs in methylcellulose culture contained in indicated E8.5 (B) and E10.5 (D) yolk sac fractions are shown. CFU-M, CFU-macrophage; CFU-E, CFU-erythroid; CFU-Mix, mixed CFU; BFU-E, burst-forming unit–erythroid. The results are shown as means ± S.D. of triplicate cultures.

Figure 5. Endomucin traces the ontogeny of definitive HSCs in AGM. (A) FACS profile and sorting gates of E10.5 AGM cells. (B) RT-PCR mRNA expression profiles of HSC-affiliated genes in indicated fractions of E10.5 AGM cells. (C) FACS profile and sorting gates of CD45 (left) and CD45 (right) E11.5 AGM cells. (D) E10.5 and E11.5 AGM fractions were subjected to methylcellulose culture (MC) and OP-9 co-culture (OP-9) assays. In both assays, cells were incubated in the presence of SCF, TPO, IL-3, and EPO for 6–8 d. Plating efficiency (percentages of colony numbers against plated cell numbers) is presented as mean ± S.D. of triplicate cultures. (E) Growth of the E10.5 and E11.5 AGM fractions in OP-9 co-culture. Cell numbers at day 6 are presented per 104 starting cells as means ± S.D. of triplicate cultures.
E11.5 AGM cells (Table I). Of interest was that E10.5 AGM cells preferentially contributed to B cell repopulation, whereas E11.5 AGM cells established multilineage repopulation (Table I).

To understand the genetic background of E10.5 AGM cells, fractionated cells were analyzed on the mRNA expression of HSC-related genes (Fig. 5 B). CD45<sup>−</sup>CD41<sup>+</sup> Endomucin<sup>+</sup> cells coexpressed CD34 and c-kit, the cell-surface marker genes for AGM HSCs (17). In contrast, CD45<sup>−</sup>CD41<sup>+</sup>Endomucin<sup>−</sup> cells expressed c-kit at a considerably lower level. Relatively high levels of transcription factors SCL, Runx1, and GATA-2, which are indispensable for HSC development (16, 26, 27), were detected in CD45<sup>−</sup>CD41<sup>+</sup>Endomucin<sup>−</sup> cells, whereas GATA-1, which is highly expressed in erythroid-committed cells (28), was preferentially expressed in CD45<sup>−</sup>CD41<sup>+</sup>Endomucin<sup>−</sup> cells. These expression profiles strongly support our findings that CD45<sup>−</sup>CD41<sup>+</sup>Endomucin<sup>−</sup> cells are enriched for developing HSCs, whereas CD45<sup>−</sup>CD41<sup>+</sup>Endomucin<sup>+</sup> cells are enriched for erythroid-committed progenitor cells.

At the developmental stage of E11 to E11.5, HSCs and progenitor cells could already be detected in the liver, into which they are presumed to have migrated via the circulation. Fetal liver serves as a major hematopoietic site from E11 until hematopoiesis is taken over by the BM. To characterize Endomucin<sup>+</sup> cells in the fetal liver, we next used c-Kit as a hematopoietic marker because CD41 expression on primitive hematopoietic cells gradually declines after E11 (14). In the fetal liver, a small population of Lin<sup>−</sup>c-Kit<sup>high</sup> cells appeared to express Endomucin (Fig. S2 A, available at http://www.jem.org/cgi/content/full/jem.20051325/DC1). Among these Lin<sup>−</sup>c-Kit<sup>high</sup>Endomucin<sup>+</sup> cells, Sca-1 expression was up-regulated between E11.5 and E12.5, and all CD34<sup>+</sup>KSL HSCs in E14.5 fetal liver were identified as Endomucin<sup>+</sup> (Fig. S2 B).

**DISCUSSION**

Among several genes identified by SST-REX cloning, Endomucin is noteworthy, as it encodes a protein that belongs to the sialomucin family. CD34 is the prototypic member of this family and has been widely used as a marker of HSCs and/or progenitor cells and of endothelial cells. The highly conserved amino acid sequence, protein structure, and genomic organization of CD34, endoglycan, and podocalyxin suggest that they are closely related to each other (29). These molecules reportedly are expressed on HSCs and/or progenitor cells and on endothelial cells and are supposed to have redundant functions (29, 30). Although Endomucin exhibits a protein structure highly related to that of these three molecules, its genomic structure is distant, indicating a different phylogeny (19).

Endomucin was originally identified as an endothelium-specific protein (19), but we and others have reported possible involvement of Endomucin in HSC development in the embryo (24, 31). Identification of Endomucin on SST-REX screening of adult BM HSCs thus encouraged us to perform

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### Table I. Endomucin marks LTR-HSCs in AGM

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Results of competitive adult BM repopulation assays using E10.5 and E11.5 AGM cells. The indicated number of cells from each subpopulation (B6-Ly5.1) was mixed with 1 × 10<sup>5</sup> B6-Ly5.1 competitor cells. The mixture was injected directly into the BM (intra-BM transplantation) of lethally irradiated B6-Ly5.1 recipient mice. An additional 2 × 10<sup>5</sup> B6-Ly5.1 competitor cells were injected intravenously. E, Endomucin.
thorough studies of the role of Endomucin in the hematopoietic system.

In adult BM, Endomucin expression correlated with immature hematopoietic phenotype, and cells with the highest levels of Endomucin expression were enriched for KSL cells, a population enriched for HSCs (Fig. 2). Conversely, most KSL cells, both CD34−KSL HSCs and CD34+KSL progenitor cells, expressed Endomucin. Importantly, LTR-HSCs were identified only in the Endomucin+ subpopulation of CD34+KSL cells. As Endomucin was identified on ~71% (10% / [10% + 4%]) of CD34−KSL cells, to detect its expression did not largely promote further HSC enrichment of the CD34−KSL HSC fraction (Fig. 3 A). However, we demonstrated that, as a marker, Endomucin could substitute for both c-Kit and Sca-1 to a large extent (Fig. 3 C). In this regard, Endomucin might be a useful HSC marker for simplifying mouse HSC enrichment. We also have confirmed that expression of Endomucin is specific to the human HSC fraction at the mRNA level (unpublished data). This suggests that Endomucin would promote enrichment of human HSCs as well.

In the developing mouse, hematopoiesis is detected as early as E7. In this stage, hematopoietic elements consist of primitive erythroid cells (32). During HSC development in the embryo, the expression pattern of Endomucin was distinct from that of CD41 in that Endomucin was not expressed on Eryp cells in the yolk sac. This finding is supported by the lack of reaction in blood islands of the extraembryonic yolk sac at E8 when reacted with an anti-Endomucin antibody (24). On the other hand, Endomucin expression was demonstrated on all LTR-HSCs in the AGM (Table I). After a transient wave of primitive hematopoiesis in the yolk sac, LTR-HSCs capable of engrafting adult mice emerge in the AGM at E10.5 (11, 12). These LTR-HSCs lose medium but exhibit a strikingly high plating efficiency of ~23% and generated multilineage hematopoietic cells when co-cultured with stromal cells (Fig. 5, D and E). From these findings, we suggest that CD45−CD41+ Endomucin+ cells in E10.5 AGM are enriched for the earliest HSC and progenitor cells, which require further maturation both to attain an engrafting capacity equivalent to that of adult HSCs and to respond to conventional mitogenic cytokines in vitro. This unique property of Endomucin+ AGM cells in vitro was also observed in similar cells obtained from the yolk sac at E10.5 (Fig. 4 D and not depicted), suggesting that Endomucin marks developing HSCs and progenitor cells not only in the AGM but also in the yolk sac. Intriguingly, HSC activity in the AGM largely shifted to CD45+CD41+Endomucin+ cells by E11.5; this subpopulation also exhibited high proliferative capacity both in vitro and in vivo (Fig. 5, D and E, and Table I).

In summarizing our data and their implications, we propose that HSCs initially branch from putative hemogenic endothelium as CD45−CD41+Endomucin+ cells with limited repopulation capacity in vivo. These immature HSCs, or “developing HSCs,” undergo a maturation process and become CD45+ “definitive HSCs” with nearly complete engrafting capacity in adult recipient mice as well as multilineage repopulation capacity (Fig. 6). In AGM, Endomucin is highly expressed on the endothelium of the dorsal aorta, on budding cells from the endothelium, and on cell clusters associated with the luminal surface of the endothelium (24). In contrast, CD41 expression is restricted to budding cells and to hematopoietic clusters (15, 33, 35). These immunohistological findings further indicate that in the E10.5 AGM, CD45−CD41+Endomucin+ cells may represent budding HSCs arising from the hemogenic endothelium of the dorsal aorta; these then mature into CD45+CD41+Endomucin+—definitive HSCs in the E11.5 AGM.

Injection of HSCs into BM has considerably improved the engrafting capacity of human CD34− HSCs and human ES-derived HSCs in adult recipient mice. This technique supposedly overcomes both defects in human CD34− HSCs homing to recipient mouse BM and intravenous aggregation of human ES-derived HSCs (36, 37). Because AGM cells, particularly E10.5 AGM cells, reportedly are scarcely capable of engrafting adult recipients, we expected that intra-BM injection would improve engraftment. As expected, engraft-

![Figure 6. Endomucin and developing HSCs.](image-url)
ment by E10.5 AGM cells was observed using this method. Its efficacy in embryonic HSC transplantation, however, needs further evaluation, including direct comparison with intravenous injection and transplantation into busulfan-treated newborn mice or immunodeficient recipients.

Although controversy persists, cells originating from both the yolk sac and AGM are supposed to seed the fetal liver, where they acquire the capacity to engraft and to constitute the BM (38, 39). We and others have previously reported that HSCs repopulating adult recipient mice are first detectable in the fetal liver at E11.5 to E12 and markedly expand in number by E12.5 (34, 40). Focusing on Lin−c-KithighEndomucin+ cells in the fetal liver, we observed a drastic up-regulation of Sca-1 expression at E12.5 (Fig. S2 A). The phenotypic change in Sca-1 expression on Lin−c-KithighEndomucin+ cells may track the maturation process of embryonic HSCs as they acquire a higher capacity to engraft the adult BM niche.

Even though the function of Endomucin in HSCs is unknown, impaired BM engraftment of CD34-deficient BM cells recently has been demonstrated. This impairment was attributed in part to antiadhesive activity on the part of CD34 (41). It is intriguing that CD34 homologues and Endomucin also have antiadhesive activity (31, 41–43). In this regard, antiadhesive activity of Endomucin may also play a role in HSC behavior in vivo.

Finally, our findings establish Endomucin as a novel LTR-HSC marker from their developing stage onward, and will provide a powerful tool in understanding HSC ontogeny.

MATERIALS AND METHODS

Mice. C57BL/6-Ly5.2 mice were purchased from SLC. C57BL/6-Ly5.1 mice and Ly5.1 × Ly5.2 F1 mice were bred and maintained in the Animal Research Facility of the Institute of Medical Science at the University of Tokyo. All experiments using mice received approval from the University of Tokyo Administrative Panel for Animal Care.

Signal sequence trap cloning, SST-REX cloning (18) was performed with partial modification. 5,000 CD34+ KSL cells were obtained by cell sorting, and total RNA was extracted using ISOGEN reagent (Nippon Gene). cDNA was synthesized using a PCR cDNA synthesis kit (SMART; CLONTECH Laboratories, Inc.) and oligo–dT primer. cDNA was digested with Restriction enzyme and linked with a BstXI adaptor. cDNA fragments ranging from 0.5 to 2.0 kb were size selected by electrophoresis on a 1% agarose gel. cDNA was synthesized using a PCR cDNA synthesis kit (SMART; CLONTECH Laboratories, Inc.) as previously described (44). The cDNA and quantitative PCR, with rodent GAPDH control reagent (TaqMan; Perkin-Elmer Applied Biosystems) as previously described (45). The amplification proceeded for 36–38 cycles. PCR products were separated on an agarose gel and visualized by ethidium bromide staining.

FACS analysis and cell sorting. Mouse CD34+ KSL cells were purified from BM of 2- to 6-mo-old mice. In brief, low-density cells were isolated on 1.086 g/ml Ficoll-Paque PLUS (GE Healthcare). The cells were stained with an antibody cocktail consisting of biotinylated anti-Gr-1, –Mac-1, –B220, –CD4, –CD8, and –TER119 mAbs (eBioscience). Lin+ cells were depleted with streptavidin–conjugated magnetic beads (M-280; Dynal). The cells were further stained with FITC-conjugated anti-CD34, PE-conjugated anti-Sca-1, and allophtocyanin-conjugated anti-c-Kit antibodies (BD Biosciences). Biotinylated antibodies were detected with streptavidin–Texas red (Invitrogen). Four-color analysis and sorting were performed on a FACS Vantage (Becton Dickenson). To detect Endomucin expression, BM mononuclear cells were first reacted with a mixture of purified antibodies against lineage markers (Gr-1, Mac-1, CD4, CD8, B220, and TER119; eBioscience), followed by reaction with anti-rat IgG microbeads (Miltenyi Biotech). Magnetically labeled cells were then passed through an LD column (Miltenyi Biotech), and the flow-through cells were recovered as cells depleted in populations bearing lineage markers. Cells depleted in such populations were stained with biotinylated anti-Endomucin mAb (clone V.7.C7) (19), FITC-conjugated anti-CD34, allophtocyanin–conjugated anti-c-Kit, and PE-Cy5.5-conjugated anti-Sca-1 (Caltag). A mixture of antibodies against lineage markers was not included in this staining. Biotinylated anti-mouse Endomucin mAb was detected with streptavidin–PE (BD Biosciences). FACS analysis and cell sorting were performed on a FACS Vantage using CellQuest software (Becton Dickinson), a FACS Aria using FACS Diva software (Becton Dickinson), or a MoFlo using Summit software (DakoCytomation).

Colony assay. Single cells were directly sorted into 96-well roundbottom plates containing 150 µl S–clones (Sanko Junyaku) supplemented with 10% FCS (Sigma-Aldrich), 5 × 10−4 M 2-mercaptoethanol (Sigma-Aldrich), 10 ng/ml mouse IL-3, 10 ng/ml mouse stem cell factor (mSCF), 2 U/ml human erythropoietin (EPO), and 50 ng/ml mouse thrombopoietin (TPO; PeproTech). After 14 d of culture in a humidified 5% CO2 atmosphere, colonies were scored by light microscopical examination and were recovered, cytospun onto slide glasses, and subjected to May–Grunwald Giemsa staining for morphological examination.

Competitive repopulation assays for adult donor cells. The Ly5 system was adopted for competitive repopulation assay as previously described (1). C57BL/6-Ly5.1 donor-derived test cells were collected by cell sorting and injected intravenously together with 2 × 105 total BM competitor cells from B6-Ly5.1 × Ly5.2 F1 mice into 9.5-Gy irradiated B6-Ly5.2 mice. 6 mo after transplantation, peripheral blood cells of recipient mice were collected and stained with biotinylated anti-Ly5.1 (eBioscience), FITC-conjugated anti-Ly5.2, allophtocyanin-conjugated anti-Gr-1, allophtocyanin–conjugated anti-Mac-1, PE-conjugated anti-CD4, PE-conjugated anti-CD8 (BD Biosciences), PE-Cy7-conjugated anti-B220 (Caltag), and streptavidin–Texas red (Invitrogen). Four-color analysis and sorting were performed on a FACS Vantage (Becton Dickinson) using CellQuest software (Becton Dickinson) for lineage analysis. Percent chimerism was calculated as follows: total percent chimerism = donor-derived cells / (donor-derived cells + competitor-derived cells) × 100; and percent chimerism lineage = donor-derived lineage-positive cells / (donor-derived lineage-positive cells + competitor-derived lineage-positive cells) × 100. Mice that had >1% chimerism in each lineage were considered to be multilineage reconstituted. Median and standard deviation values were calculated among the reconstituted recipients.

Analyses of embryonic hematopoiesis. Fetuses were collected in ice-cold PBS from C57BL/6 pregnant mice. Yolk sacs and AGM regions from E10.5 fetuses and yolk sacs from E8.5 fetuses were collected separately. Sin-
Single-cell suspensions were prepared by treating fetal tissues with 0.05% collagenase for 20–40 min at 37°C. Cells were stained with biotinylated anti-Endomucin (V-7C7) and FITC-conjugated anti-CD41 or PE-conjugated anti-CD145 (BD Biosciences). Cell fractions were recovered by cell sorting and plated in triplicate on an OP-9 layer for co-culture assays. For colony assays, sorted cells were suspended in α-MEM-based 1% methylcellullose (Sigma-Aldrich), 30% FCS (Sigma-Aldrich), 1% BSA (Sigma-Aldrich), 10−4 M 2-mercaptoethanol (100 U/ml), 10 ng/ml mouse IL-3, 10 ng/ml mSCF, 2 U/ml EPO, and 50 ng/ml TPO. Numbers of CFU-Cs were determined at day 12 of culture. Numbers of recipient mice were collected and stained with biotinylated anti-Ly5.1, PE–Cy7-conjugated anti-B220, and streptavidin–Texas red. Phycocyanin-conjugated anti–Mac-1, PE-conjugated anti-CD4, PE-conjugated anti-CD8, PE–Cy7-conjugated anti-B220, and streptavidin–Texas red. Percent chimerism was calculated as in the previous section. Mice that had $>0.1\%$ chimerism in each lineage were considered positive.

**OP-9 co-culture.** OP-9 cells (density of 40,000 cells/ well) were plated on four-chamber culture slides (BD Biosciences) or on 24-well culture plates 1 day before the initiation of co-culture. Sorted cells were placed onto OP-9 layers in triplicate and were co-cultured in α-MEM supplemented with 10% FCS, 2 mM l-glutamine, 5 $×$ 10−4 M 2-mercaptoethanol (100 U/ml), 10 ng/ml mouse IL-3, 10 ng/ml mSCF, 2 U/ml EPO, and 50 ng/ml TPO. At day 2 of co-culture, the colonies growing were counted. At day 6, single-cell suspensions were prepared and the hematopoietic cells were counted, using a hemocytometer (Erma), while excluding OP-9 cells by their size and morphology.

**Online supplemental material.** Fig. S1 provides data for Endomucin expression during hematopoietic development from ES cells. Fig. S2 provides data for Endomucin expression during fetal liver hematopoiesis. Table S1 provides the list of SST–R鑫–selected clones. Supplemental Materials and methods provides the methods for induction of hematopoiesis from ES cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20051325/DC1.

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