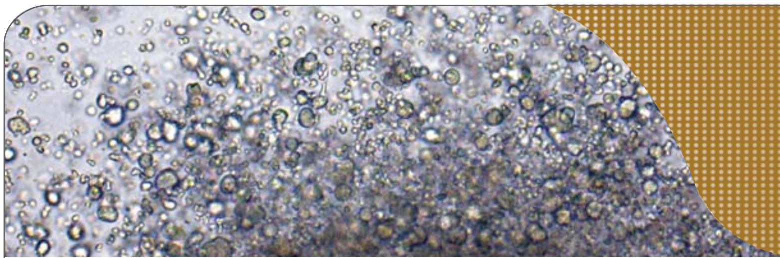


TECHNICAL BULLETIN

Endothelial Protein C Receptor (EPCR)

For Identification & Positive Selection of Mouse Hematopoietic Stem Cells

Background

Endothelial Protein C Receptor (EPCR, also known as CD201) is a recently discovered mouse hematopoietic stem cell (HSC) marker. EPCR was originally identified on the surface of endothelial cells and is involved in the regulation of coagulation and inflammation through binding to protein C and activated protein C.[1,2] EPCR was initially identified as an HSC marker by gene expression profiling and RT-PCR analysis of highly purified primitive hematopoietic cells that had been isolated using existing HSC markers, such as the c-KIT and SCA1 antigens.[3-5]

Subsequent studies demonstrated that purified $EPCR^+$ mouse bone marrow (BM) cells were highly enriched for in vivo repopulating stem cells.[6] Over 90% of primitive BM hematopoietic cells (identified on the basis of their Side Population (SP) phenotype after staining with the DNA-binding dye Hoechst 33342) were found to express EPCR, compared to only 1% of cells in the Main Population (MP).[6] EPCR expression was highest in the subset of SP cells with the lowest Hoechst 33342 fluorescence intensity, which is the fraction most enriched for HSCs.[6] $EPCR^+$ cells were also highly enriched for cells with the classical $Lin^-SCA1^+c\text{-}KIT^+$ (LSK) $CD34^-$ phenotype of hematopoietic stem cells and primitive progenitor cells.

Compared to unfractionated BM, purified EPCR-bright cells were approximately 1000-fold enriched for in vivo repopulating stem cells, as identified in competitive transplantation experiments.[6] Assuming an HSC frequency of ~1 in 10,000 in unfractionated BM,[7] this would translate to an HSC frequency of ~1 in 10 EPCR-bright cells. This stem cell frequency is similar to that of SP cells and at least 10-fold higher than that of LSK cells. EPCR expression appeared to be more predictive than Hoechst 33342 exclusion for stem cell activity, since $EPCR^+$ MP cells had significant in vivo repopulating activity, while EPCR-negative SP cells did not.[6]

EPCR has been used in combination with other markers to isolate mouse HSCs to high purity from adult mouse BM and fetal liver (FL). In FL, only the $EPCR^+$ subset of LSK cells (~1/3 of LSK cells) was able to reconstitute door type hematopoiesis in recipient mice for at least 4 months, whereas $EPCR^-$ LSK cells had little reconstituting ability.[8]

In BM and FL approximately 50% of $EPCR^+CD45^+CD48^-CD150^+$ cells (E-SLAM phenotype) had long-term multilineage repopulating ability in transplantation experiments in which single purified cells were transplanted into individual mice.[9] EPCR expression remained stable on HSCs that had been cultured for 5 – 7 days, suggesting that EPCR may be a useful marker to quantitate HSCs in expansion cultures. However, the association of $EPCR^+$ cells with stromal elements may be essential to maintain the quiescence and stem cell abilities of HSCs in vivo, as in one study, the reconstituting ability of $EPCR^+$ LSK cells was retained in co-cultures with FL stromal cells but lost after short-term culture in the absence of stroma.[8]

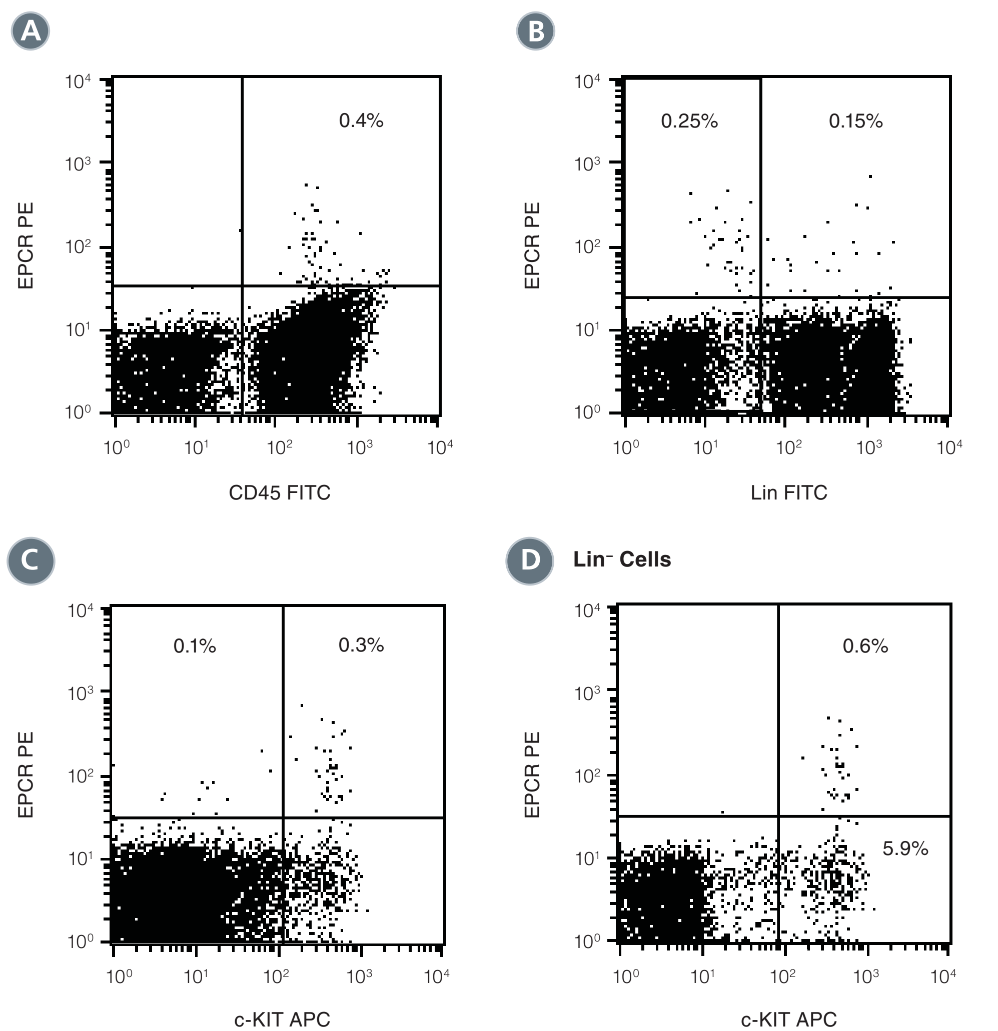
In order to assess the correlation between EPCR expression and the frequency of lineage-committed progenitor cells, standard colony assays were performed in semi-solid methylcellulose-based culture medium.[6] While EPCR-bright cells (~0.1% of BM) had little colony-forming ability, EPCR-intermediate cells (~1% of BM cells) contained 60% of the total BM colony-forming cells. This indicates that HSCs may gradually lose EPCR expression as they differentiate into lineage-committed progenitor cells that are detectable by their colony-forming ability.

The majority of mature blood cells do not appear to express EPCR at detectable levels. Indeed, typically less than 1% of unfractionated mouse BM cells show detectable staining with anti-EPCR antibody, and most of these cells do not express mature blood cell (lineage) antigens (for example, see Figure 1B). It should be noted however that small subsets of mature monocytes, neutrophils and eosinophils can be positively stained with anti-EPCR antibodies, either due to endogenous EPCR expression on these cells or binding of soluble EPCR from the plasma.[10-13] These rare $EPCR^+$ mature cells are much less frequent than mature blood cells that express other commonly used stem cell markers, such as c-KIT and SCA1.

Detection of EPCR Expression

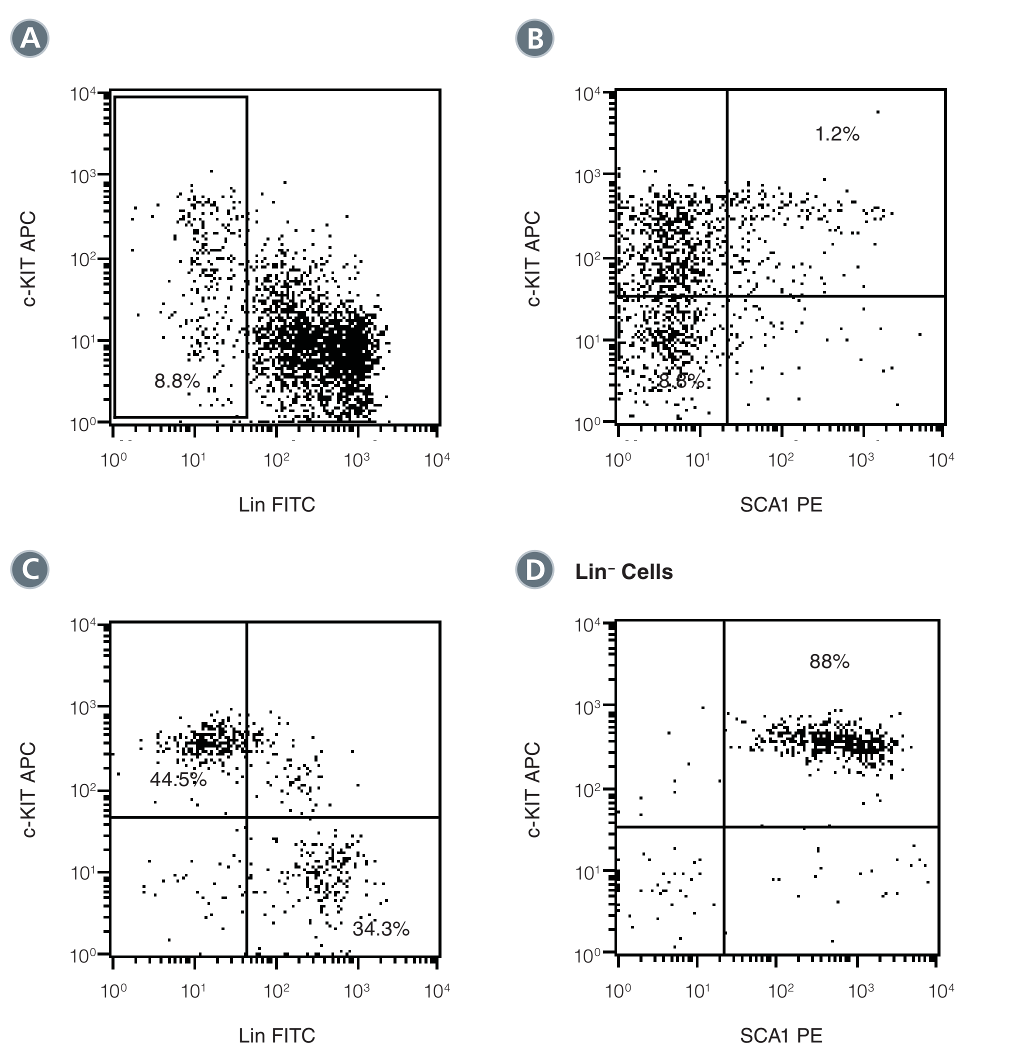
EPCR⁺ cells can be detected by flow cytometry using conjugated antibodies directed against the cell surface EPCR protein. In the example given in Figure 1, unfractionated BM cells from C57BL/6 mice were stained with anti-EPCR biotin (Catalog #10156) and streptavidin-PE. BM EPCR⁺ cells represent an extremely rare cell population (0.4% in this example). Almost all EPCR⁺ cells express CD45 (Figure 1A), confirming their hematopoietic origin. More than half of the EPCR⁺ cells are Lin⁻ or c-KIT⁺ (Figures 1B and 1C). Lin⁻EPCR⁺ cells were detected almost exclusively in the c-KIT⁺ BM subset, and comprised only ~10% of c-KIT⁺ cells (Figure 1D). Further analysis indicated that the Lin⁻EPCR⁺ cells also expressed SCA1 (Figure 2).

FIGURE 1. Flow cytometry profiles of EPCR expression on unfractionated C57BL/6 BM cells



(A) Cells were stained with anti-EPCR biotin and streptavidin-PE and counterstained with anti-CD45 FITC. (B) Cell staining with a cocktail of FITC-conjugated antibodies directed against myeloid and lymphoid lineage antigens (CD4, CD8, CD11b, CD11c, CD19, CD45R, Ly-6C/G, NK1.1). (C, D) Cell staining with anti-EPCR biotin/streptavidin-PE and anti-c-KIT APC antibodies. (A), (B) and (C) represent all BM cells after gating out debris and dead (PI⁺) cells; (D) represents further cell gating for lineage-negative (Lin⁻) cells

FIGURE 2. Flow cytometry profiles of C57BL/6 BM cells before and after EPCR+ cell selection using anti-EPCR biotin and the EasySep™ Biotin Selection Kit for Mouse Cells



(A, B) Unfractionated C57BL/6 BM cells and (C, D) EPCR+-selected cells were stained with anti-lineage FITC antibodies in combination with either anti-c-KIT APC or anti-SCA1 PE antibodies. (A) and (C) represent cells after gating out debris and dead (PI+) cells; (B) and (D) represent further gating for lineage-negative cells.

Isolation of EPCR⁺ Cells

EPCR⁺ cells can be rapidly enriched using EasySep™ immunomagnetic cell separation technology. Cells are labeled with biotin- or PE-conjugated anti-EPCR antibody and are bound by bispecific tetrameric antibody complexes (TACs) that recognize the conjugated primary antibody and EasySep™ magnetic particles (Figure 3). Magnetically labeled EPCR⁺ cells are then retained in the hand-held EasySep™ magnet, while unlabeled cells are simply poured away (Figure 4). The EasySep™ magnetic particles are compatible with flow cytometry and other downstream applications, and do not need to be removed following EPCR⁺ cell selection.

The optimal method of EPCR⁺ cell selection is determined by the proposed downstream usage of the selected cells. It is sometimes desirable for the selected cells to remain unstained with fluorescently labeled antibodies (e.g. to enable three-color flow cytometric analysis with FITC-, PE- and APC-conjugated antibodies). EPCR⁺ cell selection using anti-EPCR biotin is recommended in such cases. Accurate measurement of EPCR expression on anti-EPCR-biotin-selected cells is not possible due to blocking of both the EPCR antigen and the biotin molecule with primary antibody and TAC (see Figure 3 for illustration).

If accurate measurement of EPCR expression on the enriched cells is desired, a PE-conjugated anti-EPCR antibody should be used for cell selection. The PE-labeled EPCR⁺ cells will be available for flow cytometric analysis immediately after cell separation. The expression of other cell surface proteins can be further analyzed by using appropriately conjugated antibodies (e.g. anti-Lin FITC, anti-SCA1 APC and anti-c-KIT PE-Cy5 for LSK cells).

Tools for Detection and Enrichment of EPCR⁺ Cells

STEMCELL Technologies Inc. has developed several reagents for the detection of EPCR⁺ cells by flow cytometry, as well as for the enrichment of EPCR⁺ cells using the EasySep™ immunomagnetic cell separation system (Table 1). Biotin- and Phycoerythrin (PE)-conjugated anti-EPCR antibodies are the most appropriate products for these purposes; FITC-conjugated anti-EPCR is not recommended for the detection and isolation of EPCR⁺ hematopoietic cells by flow cytometry due to lower fluorescence intensity.

FIGURE 3. Tetrameric Antibody Complex (TAC) crosslinking a cell to a dextran-coated magnetic particle

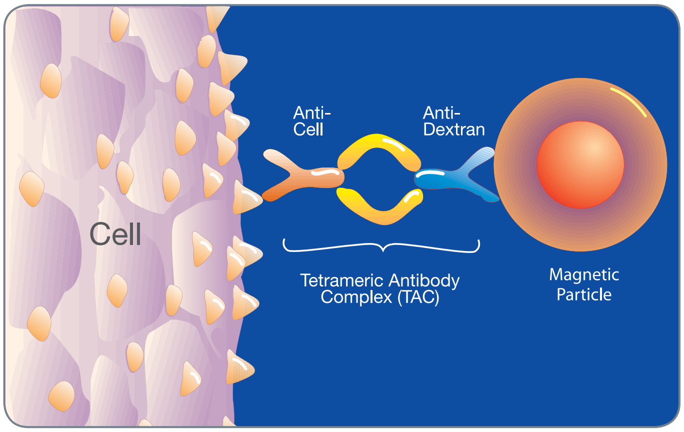
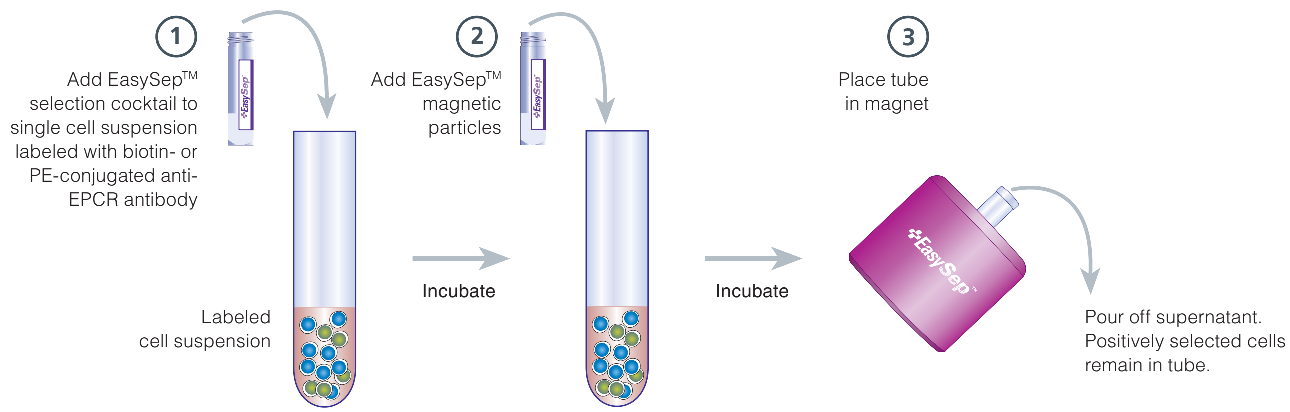


FIGURE 4. Schematic drawing showing EasySep™ cell separation protocol



EPCR⁺ cells are targeted with anti-EPCR antibodies and are then crosslinked to magnetic particles. The sample is placed in the EasySep™ Magnet, and the magnetically labeled EPCR⁺ cells remain in the tube when the supernatant is removed. The EPCR⁺ cells are easily collected by removing the tube from the magnet.

TABLE 1. STEMCELL Technologies provides various solutions for detecting and selecting EPCR⁺ cells

PRODUCT	CATALOG #	RECOMMENDED USE
Anti-Mouse EPCR (purified antibody) Clone: RMEPCR 1560	01511	Detection of EPCR⁺ cells, in combination with a suitable secondary reagent.
Biotinylated Anti-Mouse EPCR (anti-EPCR biotin) Clone: RMEPCR 1560	10156	Detection of EPCR⁺ cells from mouse BM or other tissues by flow cytometry, in combination with a fluorescent streptavidin-conjugate (Figure 1). Positive selection of EPCR⁺ cells using EasySep™ Biotin Selection Kit for Mouse Cells (Figure 2).
PE-conjugated Anti-Mouse EPCR (anti-EPCR PE) Clone: RMEPCR 1560	10856	Detection of EPCR⁺ cells from mouse BM or other tissues by flow cytometry. Positive selection of EPCR⁺ cells using EasySep™ PE Selection Kit for Mouse Cells (Figure 5).
RELATED REAGENTS	**CATALOG #**	**RECOMMENDED USE**
EasySep™ Biotin Selection Kit for Mouse Cells	18556	Immunomagnetic positive selection of EPCR⁺ cells labeled with biotin anti-EPCR.
EasySep™ PE Selection Kit for Mouse Cells	18554	Immunomagnetic positive selection of EPCR⁺ cells labeled with PE anti-EPCR.

Example 1: EPCR^{+} Cell Selection Using a Biotinylated Anti-EPCR Antibody

Unfractionated BM cells (Figures 2A, B) and EasySep™-selected EPCR^{+} cells (Figures 2C, D) were analyzed for lineage antigen, SCA1 and c-KIT expression. EPCR-selected cells were highly enriched for Lin^{-}SCA1^{+}c-KIT^{+} cells, whereas Lin^{+} and Lin^{-}SCA1^{-} c-KIT^{+} cells were generally not recovered (Figures 2C, D). Enrichment of EPCR^{+} cells increased the frequency of Lin^{-}SCA1^{+} c-KIT^{+} cells 15-fold to 16.9 ± 3.9% from a start frequency of 1.2 ± 0.5% (n=4). The frequency of colony-forming cells (CFCs), as identified in colony assays using MethoCult™ GF M3434 medium (Catalog #03434/03444), increased 11-fold compared to unfractionated BM, from 244 ± 61 to 2600 ± 350 CFC/10^5 plated cells (Table 2, n=3).

Example 2: EPCR^{+} Cell Selection Using a PE-Conjugated Anti-EPCR Antibody

As selected cells are already labeled with anti-EPCR PE, this approach enables direct analysis of EPCR^{+} cell purity and recovery after enrichment. The EPCR^{+} selected cells were stained with anti-Lin FITC and anti-c-KIT APC. Magnetic separation increased the frequency of EPCR^{+} cells over 20-fold compared to unfractionated BM, from approximately 1% to 25.0 ± 7.2% (Figures 5A and 5B; n=6). There was a 12-fold increase in CFC frequency from 244 ± 61 to 3000 ± 955 CFC/10^5 plated cells (Table 2, n=4). Gating on EPCR^{+} cells revealed that ~26% of EPCR^{+} cells were Lin^{-} and c-KIT^{+} (Figure 5D). As demonstrated above (Figure 2), the majority of the Lin^{-}EPCR^{+}c-KIT^{+} cells are SCA1^{+} and probably represent very immature progenitors, including HSCs. These Lin^{-}EPCR^{+} c-KIT^{+}SCA1^{+} cells represent only a small subset of Lin^{-}c-KIT^{+} cells; most of the Lin^{-}c-KIT^{+} cells are EPCR^{-}SCA1^{-} and represent more differentiated cells. Approximately 50% of the EPCR^{+} cells were Lin^{+} and c-KIT (Figure 5D), which is consistent with the expression of EPCR on small numbers of mature cells.[10-13] Although these Lin^{+}EPCR^{+} mature cells are relatively abundant compared to the primitive Lin^{-}EPCR^{+}c-KIT^{+} cells, they represent less than 1% of lineage^{+} cells in unfractionated BM.

TABLE 2. Enrichment of hematopoietic cells by EasySep™ positive selection of EPCR^{+} cells using either biotinylated or PE-conjugated anti-EPCR antibodies

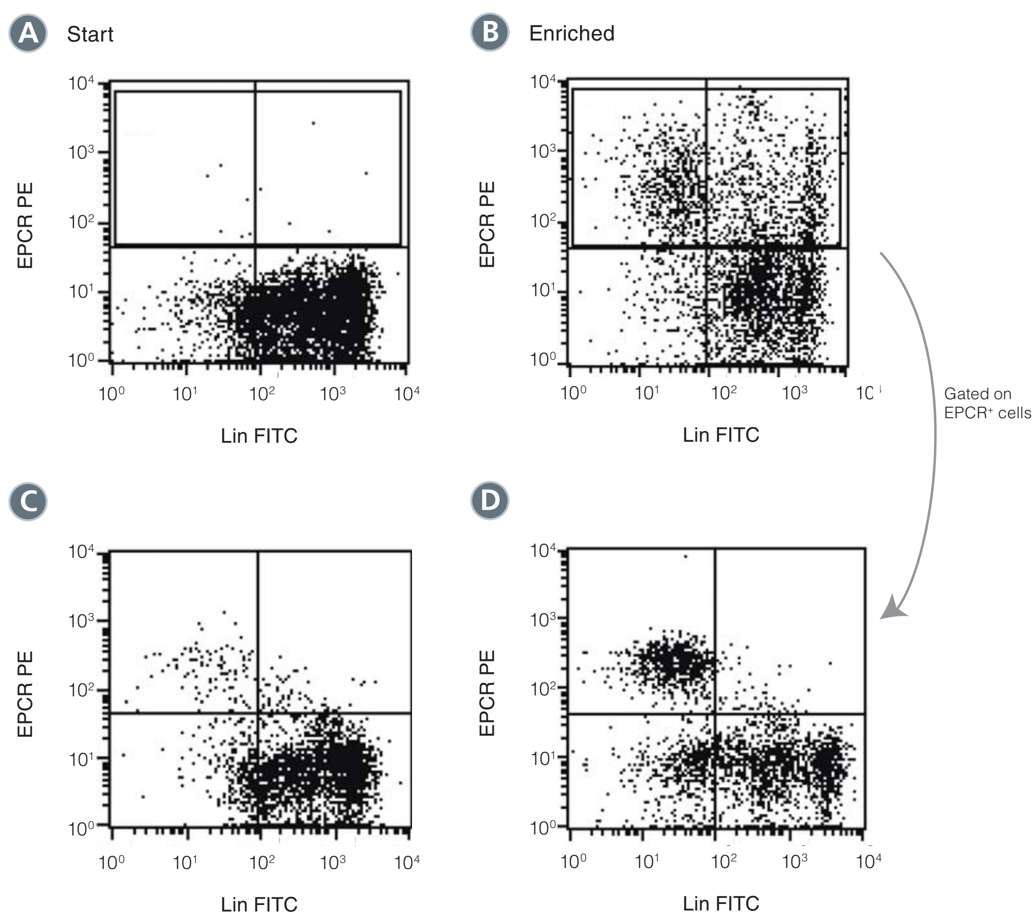
	UNFRACTIONATED BM	ANTI-EPCR BIOTIN SELECTED CELLS[1]	ANTI-EPCR PE SELECTED CELLS[2]
% EPCR^{+}	~1.3 (n=6)	ND[3]	25.0 ± 7.2 (n=6)
% Lin^{-}SCA1^{+}c-KIT^{+}	1.2 ± 0.5 (n=4)	16.9 ± 3.9 (n=4)	ND[3]
CFCs/10^5 cells	244 ± 61 (n=4)	2600 ± 350 (n=3)	3000 ± 955 (n=4)

[1] This approach is recommended if the selected cells are to remain unstained with fluorescently labeled antibodies, e.g. to enable three-color flow cytometry analysis with FITC-, PE- and APC-conjugated antibodies. Accurate measurement of EPCR expression on anti-EPCR biotin selected cells is not possible due to blocking of both the EPCR antigen and the biotin molecule with primary antibody and TAC.

[2] This approach is recommended if accurate measurement of EPCR expression on the enriched cells is desired. The expression of other cell surface proteins can be further analyzed by using appropriately conjugated antibodies (e.g. anti-Lin FITC, anti-SCA1 APC and anti-c-KIT PE-Cy5 for LSK cells).

[3] ND: Not Determined

FIGURE 5. Flow cytometry profiles of C57BL/6 BM cells before and after EPCR+ cell selection using anti-EPCR PE and the EasySep™ PE Selection Kit for Mouse Cells



(A, C) Unfractionated C57BL/6 BM cells and (B, D) EPCR+-selected cells were stained with anti-lineage FITC antibodies in combination with either anti-EPCR PE or anti-cKIT APC antibodies. (A), (B) and (C) represent cells after gating out debris and dead (PI+) cells; (D) represents further gating for EPCR+ cells.

Concluding Remarks

EPCR expression may be used as an alternative to, or in combination with, other markers, such as the LSK, SP or SLAM receptor phenotypes, to identify and isolate mouse HSCs and primitive progenitors.[6] Unlike SCA1, EPCR expression can be used to detect and isolate HSCs from BALB/c mice as well as C57BL/6 mice. However, EPCR expression on hematopoietic cells from other mouse strains remains to be demonstrated. Isolation of mouse HSCs on the basis of SP phenotype requires staining with Hoechst 33342 dye and exposure to UV laser light, which is toxic to cells and technically challenging to use. Staining with anti-EPCR antibodies is much more straightforward than Hoechst 33342 staining, does not require a flow cytometer or cell sorter equipped with a UV laser, and does not appear to be harmful to the cells. In addition, it allows rapid pre-enrichment of HSCs and primitive progenitors by immunomagnetic separation. Due to the very low frequency of $EPCR^+$ cells in unfractionated BM, the use of pre-enrichment strategies prior to flow cytometric sorting is highly recommended. This approach will reduce sorting time significantly and may result in better cell purities and recoveries. The recommended pre-enrichment strategies include EasySep™ positive selection of $EPCR^+$ cells (Table 1), $SCA1^+$ cells (Catalog #18756) or $c\text{-}KIT^+$ cells (Catalog #18757), or depletion of lineage-positive cells (Catalog #19756).

Finally, although gene expression analysis of purified human $CD34^+CD38^-Lin^-$ cells suggest that EPCR is expressed on primitive human hematopoietic cells,[14] there is currently no evidence that EPCR is a useful marker for identification and isolation of human HSCs and progenitors.

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