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Analysis of hematopoietic stem cells using a composite approach

Rohit P. Nagare[a], S. Sneha[a], S. Ramesh[c], Trivadi S. Ganesan[a,b,*]

[a] Laboratory for Cancer Biology, Cancer Institute (W.I.A), 38, Sardar Patel Road, Chennai, 600 036, Tamilnadu, India
[b] Department of Medical Oncology and Clinical Research, Cancer Institute (W.I.A), 38, Sardar Patel Road, Chennai, 600 036, Tamilnadu, India
[c] Department of Cytogenetics, Cancer Institute (W.I.A), 38, Sardar Patel Road, Chennai, 600 036, Tamilnadu, India

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ABSTRACT

Stem cells or Cancer stem cells (CSCs) have now been identified in different type of tissues by using surface markers. Functional assays such as ALDEFLUOR and side population which are marker independent have been additional approaches. However, whether all these approaches identify the same population of cells remain uncertain. To address this issue we have used hematopoietic stem cells as a model. Peripheral blood stem cells enumerated by CD34 are used routinely in bone marrow transplantation which supports the recovery of bone marrow after ablative chemotherapy or radiation. Hematopoietic stem cells (HSCs) were obtained from normal donor bone marrow (n = 5) and G-CSF stimulated peripheral blood stem cells (PBSCs) (n = 5) from patients undergoing leukapheresis prior to bone marrow transplantation. The stem cells were identified by combining CD34 expression with functional assays (ALDEFLUOR and side population). The cell cycle profile was further determined by simultaneous labeling of these cells with Hoechst and Pyronin Y. The simultaneous analysis showed that both CD34+ and CD34- cells co-exist with ALDH1A1+ cells but side population did not segregate with CD34+ cells. Though stem cell populations identified by functional assays were different, the cell cycle analysis showed that both ALDH1A1+ and CD34+ cells were in the G1 phase of cell cycle rather than in the quiescent (G0) phase.

1. Introduction

It is increasingly recognized that cancer stem cells exist and are necessary in the initiation and progression of cancer. However, the ability to identify CSCs with certainty by using cell surface markers from individual tumours has proved variable between laboratories (Nagare et al., 2017). To obviate this functional assays such as ALDEFLUOR and side population have been employed. However, it is uncertain whether these two functional assays identify the same stem cell population (Frandberg et al., 2015; Pearce and Bonnet, 2007; Pierre-Louis et al., 2009; Yasuda et al., 2013). CSCs resemble normal stem cells in self-renewal, expression of transcription factors, differentiation to multiple lineages and reproducible phenotype. Therefore stem cell research has become increasingly relevant to cancer research and CSCs identified in several types of cancers have been shown to be important in tumour initiation and progression (Lathia and Liu, 2017; Pattabiraman and Weinberg, 2014; Visvader and Lindeman, 2012). In view of these observations we chose peripheral blood stem cells as our model as it is easier to evaluate these questions.

Hematopoietic stem cells (HSCs), a rare population, have the potential to self- renew (for maintaining stem cell pool) and differentiate into other progenitor cells. Isolation of HSCs using CD34+/CD38-/Lin- phenotype have been shown to repopulate irradiated NOD/SCID mice which suggests self-renewal potential of these cells (Bhatia et al., 1998). However, cells with phenotype CD34-/Lin- have also been shown to enable long-term reconstitution of bone marrow in NOD/SCID mice (Bhatia et al., 1997; Gallacher et al., 2000; Hess et al., 2006). To define the properties of HSCs precisely, use of functional assays such as H33342 exclusion and ALDEFLUOR assay may be productive.

HSCs identified using the H33342 exclusion assay have been shown to reconstitute murine bone marrow at low doses. These cells have contributed to replenish the bone marrow with myeloid and lymphoid lineage cells (Goodell et al., 1996). The side population (H33342 -ve cells) has also been shown to have similar potential in rhesus and swine models (Goodell et al., 1997). ALDH1A1, a cytosolic enzyme, expressed at higher levels in cyclophosphamide resistant cell line has an

Abbreviations: HSCs, hematopoietic stem cells; PBSCs, peripheral blood stem cells; G-CSF, granulocyte stimulating factor; SP, side population (H33342 negative cells); DEAB, diethylaminobenzaldehyde (selective inhibitor for ALDH1A1 enzyme)

* Corresponding author at: Laboratory for Cancer Biology, Department of Medical Oncology and Clinical Research, Cancer Institute (W.I.A), 38, Sardar Patel Road, Chennai, 600 036, Tamilnadu, India.
E-mail address: ts.ganesan@cancerinstitutewia.org (T.S. Ganesan).

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Table 1
Summary for identification of HSCs.

Sr. No.	Source of HSCs	Stem cell Phenotype	Comment	Reference
1	HSCs from cord blood	Lin-/SP/CD34- CD133 + /CD38-	Overlap between SP and ALDH + /CD34 + /CD38- No overlap between SP and ALDH/CD34-/CD38-	(Pearce and Bonnet, 2007)
2	Bone marrow	Lin-/CD34 + /CD38-/ALDH + /SP PyYlow/CD34 + /CD38-/ALDH + /SP	Overlap between SP and ALDH + /CD34 + Quiescent cells (G0) are PyYlow/ALDH + /CD34 +	(Pierre-Louis et al., 2009)
3	HSCs from cord blood	CD34 + /CD117 + /ALDH +	CD34 and CD117 absent in SP No ALDH and SP overlap	(Frandberg et al., 2015)

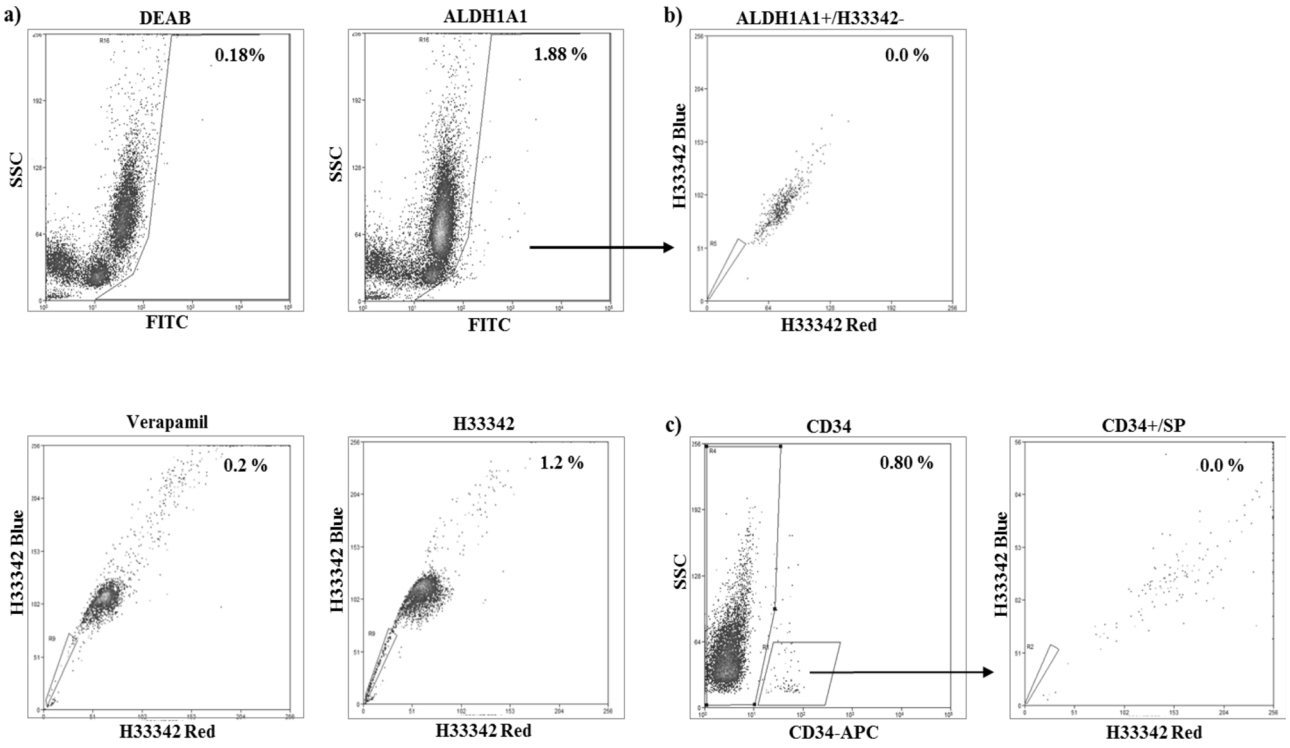

Fig. 1. ALDEFLUOR, H33342 exclusion assay and CD34 expression in bone marrow.
Cells from normal bone marrow were simultaneously labeled with Aldefluor reagent, H33342 and CD34 and analyzed as given below. Arrows indicate sequential gating of selected population of Cells (Table 2a; Sample#1).
(a) Cells from normal bone marrow were analyzed for expression of ALDH1A1 (top panel) and side population (bottom panel) along with DEAB and Verapamil as inhibitor control, respectively.
(b) Analysis of normal bone marrow cells using ALDEFLUOR and side population assay (top panel).
(c) Analysis of cells labeled with CD34 and H33342Co-expression of CD34 in side population. Cells from normal bone marrow were sequentially labeled with H33342 and CD34. These cells were analyzed using Verapamil and isotype specific antibody controls. Arrows indicate sequential gating of selected population of cells (Table 2a; Sample#1).

important role in ethanol oxidation and metabolism. Among multiple aldehyde dehydrogenase isoforms, ALDH1A1 isoform is highly expressed in embryonal tissue and adult stem cells. Increased expression of ALDH1A1 in cancer cells using ALDEFLUOR assay has become the technique of choice for identifying stem cells (Jones et al., 1995; Moreb, 2008). The simultaneous analysis of human cord blood and bone marrow using CD34, ALDEFLUOR and H33342 exclusion assay identified a small population of HSCs. The expression of ALDH1A1 + was restricted to CD34 + cells with the phenotype SP + /ALDH1A1 + / CD34 + (Pearce and Bonnet, 2007). In a similar study, bone marrow cells analyzed simultaneously for ALDH1A1, SP and CD34 expression revealed that majority of the CD34 + /CD38-/Lin- cells were identified as overlapping with ALDH1A1^{Br}/SP + subset (Table 1). Cell cycle analysis using H33342 and Pyronin Y showed presence of SP in quiescent phase (G0) while ALDH1A1^{Br} cells were in G1 phase of cell cycle. The simultaneous analysis with ALDEFLUOR, H33342 assays and surface markers enriches the stem cell compartment (Pierre-Louis et al., 2009). Thus, there is some uncertainty of the exact phenotype and exact phase of cell cycle of HSCs in the human bone marrow or cord blood. There is no data on HSCs from G-CSF stimulated peripheral blood stem cells.

In this study, we also wished to understand the cell cycle profile of putative stem cells identified using well established stem cell markers (CD34) and assays (ALDEFLUOR and H33342 exclusion). To discriminate between the cells from G0 (quiescent) and G1 phases of cell cycle phase of putative HSCs, we have performed simultaneous analysis using H33342 and Pyronin Y. As a composite approach is superior in identifying stem cells, we analyzed CD34 expression with that of ALDH1A1 and Hoechst exclusion abilities of HSCs.

2. Materials and Methods

The experiments described in this report have been approved by the Scientific Advisory Committee, Institute Ethics Committee and Institutional Stem cell committee.

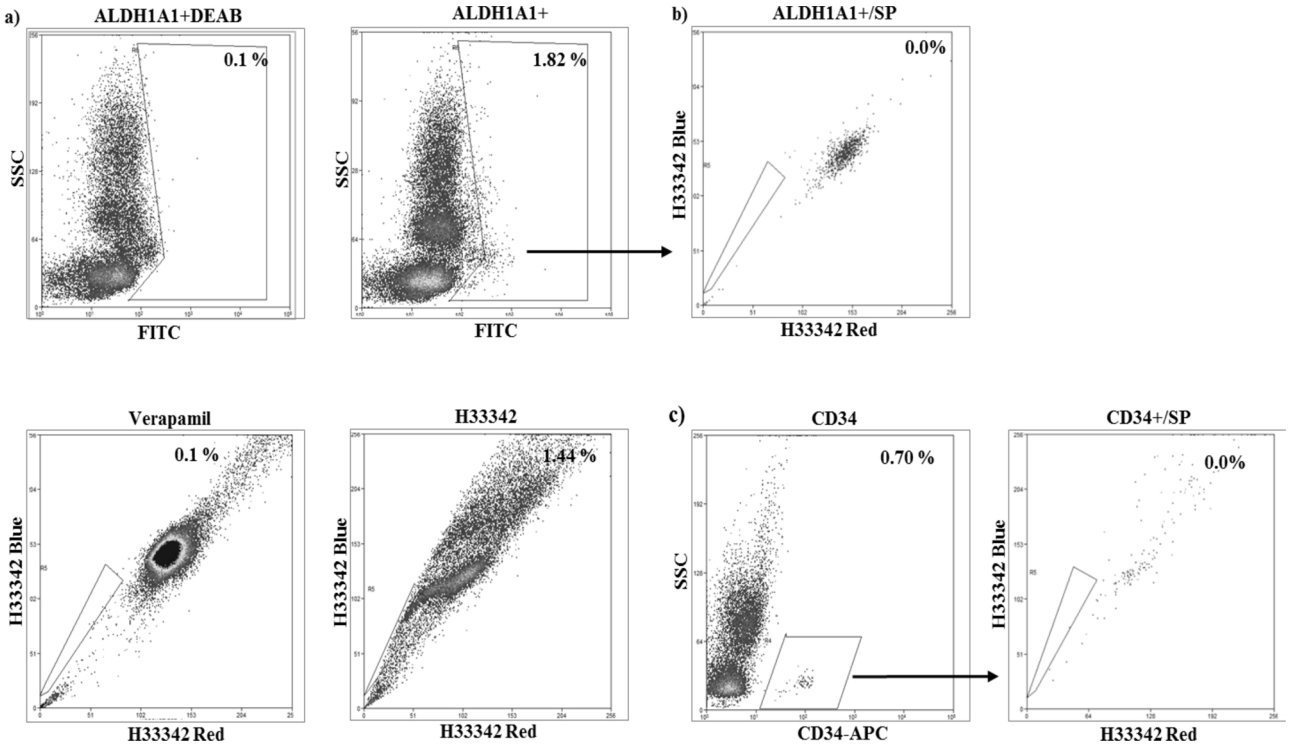

Fig. 2. ALDEFLUOR, H33342 exclusion assay and CD34 expression in PBSCs.
Cells from G-CSF stimulated blood simultaneously labeled with ALDEFLUOR reagent, H33342 and CD34 and analyzed as given below. Arrows indicate sequential gating of selected population of Cells (Table 2b; Sample#1).
(a) PBSCs were analyzed for expression of ALDH1A1 (top panel) and side population (bottom panel) along with DEAB and Verapamil as inhibitor control, respectively.
(b) Analysis of PBSCs using ALDEFLUOR and side population assay (top panel).
(c) Analysis of PBSCs labeled with CD34 and H33342. Cells from PBSCs were sequentially labeled with H33342 and CD34. These cells were analyzed using Verapamil and isotype specific antibody controls. Arrows indicate sequential gating of selected population of cells (Table 2b; Sample#1).

Table 2
ALDEFLUOR, Side population assay and CD34 expression.

a. Bone marrow

Sr.	Markers	1	2	3	4	5	Mean ± SE
1	ALDH1A1 +	1.88	2.5	2.2	2.8	2.50	2.38 ± 0.16
2	Side population (H33342-)	1.2	1.5	0.79	1.8	1.67	1.4 ± 0.80
3	ALDH1A1 + /SP	0.0	0.0	0.0	0.0	0.0	0.0
4	CD34 +	0.80	0.76	0.65	0.70	0.57	0.70 ± 0.05
5	SP/CD34 +	0.0	0.0	0.0	0.0	0.0	0.0

b. Peripheral blood

Sr.	Marker	1	2	3	4	5	Mean ± SE
1	ALDH1A1 +	1.82	1.46	1.60	1.50	1.3	1.54 ± 0.10
2	Side population (H33342-)	1.44	1.60	1.79	1.50	1.2	1.51 ± 0.09
3	ALDH1A1 + / SP	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0
4	CD34 +	0.70	0.60	0.75	0.62	0.55	0.65 ± 0.04
5	SP / CD34 +	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0

2.1. Processing of samples

Bone marrow from healthy donors (N = 5) and Granulocyte colony stimulating factor (GCSF) stimulated Leukapheresis blood (N = 5) from normal healthy donors that remained after transplantation were used for these experiments. The samples were collected in a tube containing Sodium Citrate as anti-coagulant. Nucleated cells, separated by Histopaque (Grigoryan et al., 2018), were collected in a separate tube and re-suspended in DMEM with 10% FBS for immediate experiments. Cell density was adjusted to 10^6 cells per ml of medium. Typically for peripheral blood stem cells, patients were administered G-CSF for 5 days prior to collection of PBSCs. The cells were usually transfused into the patient the following day. The remainder was used for our experiments for analysis. Experiments were done on the same day.

2.2. Co-labelling using ALDEFLUOR and H33342 dye

To identify overlap between cells with high ALDH expression and side population, cells were simultaneously labeled with ALDEFLUOR reagent (Stem cell Technologies, USA) and H33342 dye (Sigma). Cells

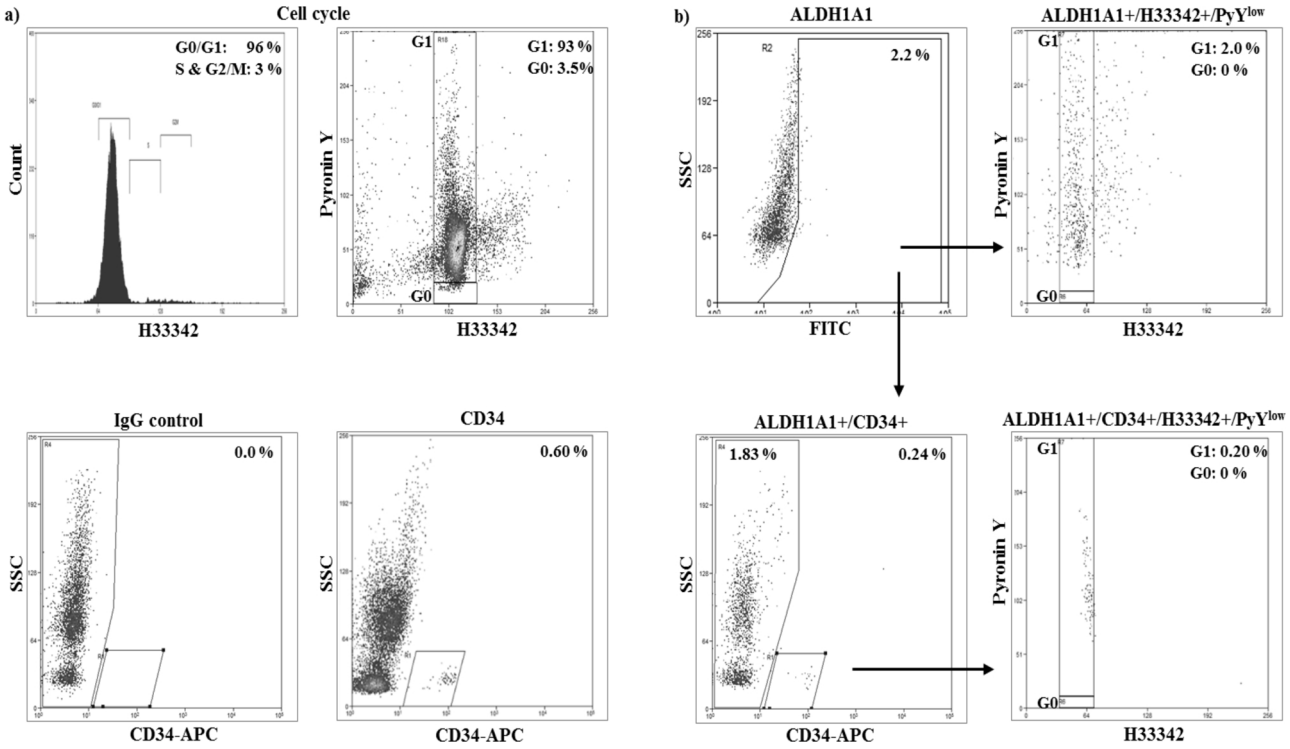

Fig. 3. Cell cycle analyses of ALDH1A1 + and CD34 + cells in normal bone marrow.
Cells were labeled with ALDEFLUOR reagent, H33342, Pyronin Y and CD34 for analysis. Arrows indicate sequential gating of selected population of cells (; Sample#3)
(a) Live cell cycle analysis of cells from bone marrow using H33342 dye (top left), separation of G0 and G1 phases of cell cycle by simultaneous labeling of cells using H33342 and Pyronin Y (top right), and Expression of CD34 surface marker in normal bone marrow cells (bottom right) with respect to IgG isotype control (bottom left)
(b) Expression of ALDH1A1 in cells from bone marrow (top left), simultaneous analysis of ALDH1A1 + cells with H33342 and Pyronin Y (top right), with CD34 (bottom left) and together with CD34, H33342 and Pyronin Y (bottom right). Cells are shown as a percentage of the original gated population.

Table 3a
Identification of HSCs by simultaneous analysis in bone marrow.

Sr.	Markers	1	2	3	4	5	Mean ± SE
1	ALDH1A1 +	1.24	1.50	2.20	1.32	1.60	1.57 ± 0.17
2	CD34 +	0.50	0.88	0.60	0.66	0.75	0.68 ± 0.64
3	ALDH1A1 + / CD34 +	0.12	0.18	0.24	0.17	0.30	0.20 ± 0.03
4	ALDH1A1 + / CD34-	1.08	1.28	1.83	1.12	1.22	1.31 ± 0.13
5	H33342 + / PyY^{Low} (G0)	4.00	5.0	3.50	3.20	4.10	4.18 ± 0.30
6	H33342- / PyY^{Low}	1.40	1.30	1.00	1.30	1.22	1.25 ± 0.06
7	ALDH1A1 + /H33342 + / PyY^{Low} (G0)	0.0	0.0	0.0	0.0	0.0	0.0
8	ALDH1A1 + / H33342 + / PyY + (G1)	1.20	1.42	2.0	1.22	1.48	1.45 ± 0.14
9	ALDH1A1 + /Cd34 + /H33342 + /PyY^{Low} (G0)	0.0	0.0	0.0	0.0	0.0	0.0
10	ALDH1A1 + /CD34 + /H33342 + /PyY + (G1)	0.12	0.15	0.20	0.15	0.22	0.17 ± 0.09

(10^6 cells / mL) were mixed thoroughly in a tube with 5ul of ALDEFLUOR reagent to enhance diffusion. Half of these cells (500 µl) were transferred to another tube containing 5 µl of DEAB as the inhibitor and mixed thoroughly. To the cells containing ALDEFLUOR alone, 5 µl of H33342 dye (1.2 mg/ml; Sigma) was added and mixed gently. In addition, 5 µl of H33342 dye was also added separately another tube containing Verapamil (4.91 mg/ml; Sigma) and 1 × 10^6 cells as an inhibitor control for H33342 dye. Cells were incubated at 37 °C for 45 min, centrifuged and re-suspended in 100 µl ice-cold ALDEFLUOR assay buffer. To these cells, 5 µl of APC conjugated CD34 antibody was added and mixed gently. Cells were incubated for 1 h on ice protected from light. Cells were centrifuged, washed and re-suspended in 1 ml of ice-cold ALDEFLUOR buffer and kept on ice till analysis.

2.3. Cell cycle analysis

Cells in G0 phase or quiescent cells show low mRNA content which can be identified by simultaneous analysis using H33342 and Pyronin Y (Darzynkiewicz et al., 2004). To identify quiescent cells accurately, we have optimized the method using normal bone marrow (N = 5). To separate the G0/G1 phase of cell cycle, first cells (10^6 cells/ml) were labelled with 1.5 ml of H33342 dye (12 µg / ml) and incubated for 45 min at 37 °C followed by labeling with 7.5 µl of Pyronin Y (200 µg/ml; Sigma) for 45 min at 37 °C. Cells were centrifuged and pellet was re-suspended in ice cold wash buffer (Phosphate buffered saline + 10% FBS + 0.1% Glucose) (Darzynkiewicz et al., 2004). Cells (1 × 10^6/ml) were individually labeled with the same concentrations of H33342 and Pyronin Y as controls and were included in the analysis. Verapamil was added as an inhibitory control for H33342 dye for identification of the side population. H33342 binds to DNA alone while Pyronin Y binds to

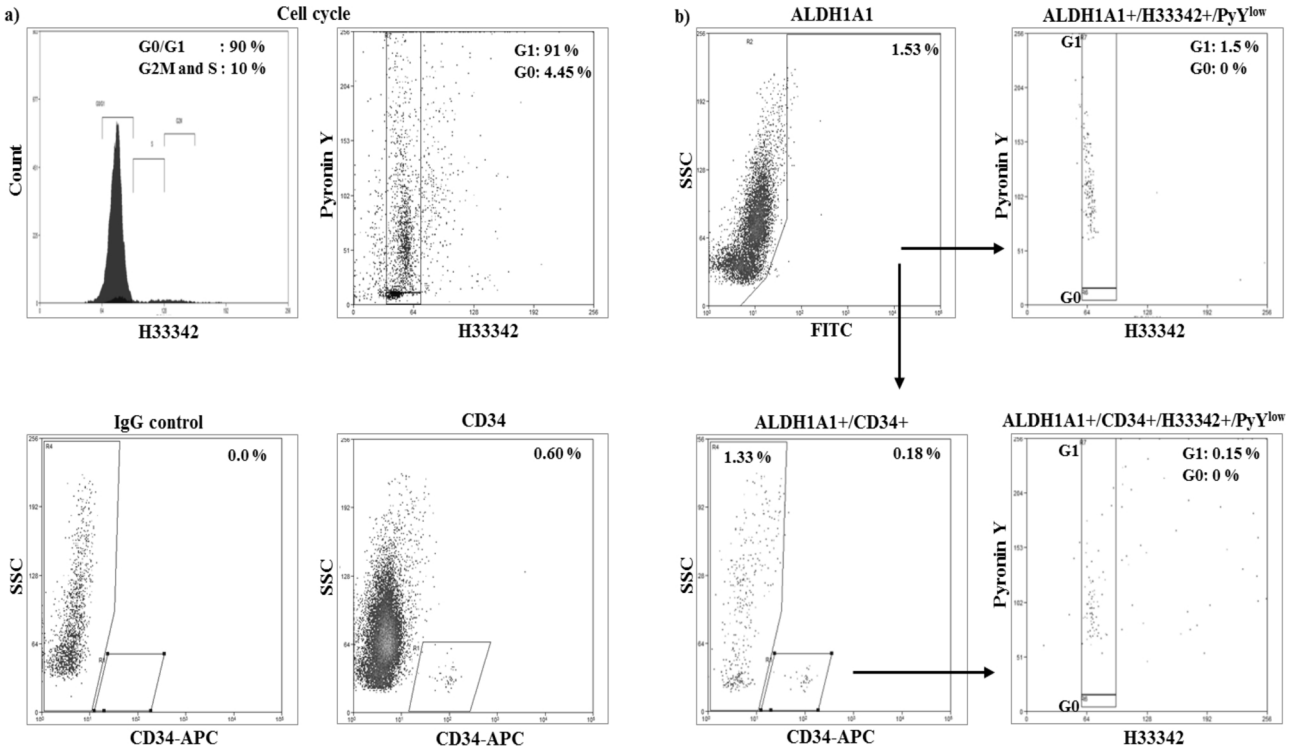

Fig. 4. Cell cycle analyses of ALDH1A1 + and CD34 + cells in PBSCs.
Cells were labeled with ALDEFLUOR reagent, H33342, Pyronin Y and CD34 for analysis. Arrows indicate sequential gating of selected population of cells (Table 3b Sample#1)
(a) Live cell cycle analysis of PBSCs using H33342 dye (top left), separation of G0 and G1 phases of cell cycle by simultaneous labeling of cells using H33342 and Pyronin Y (top right), and Expression of CD34 surface marker in normal bone marrow cells (bottom right) with respect to IgG isotype control (bottom left).
(b) Expression of ALDH1A1 in PBSCs from bone marrow (top left), simultaneous analysis of ALDH1A1 + cells with H33342 and Pyronin Y. (top right), with CD34 (bottom left) and together with CD34, H33342 and Pyronin Y (bottom right). Cells are shown as a percentage of the original gated population (Table 3b; Sample#1).

Table 3b
ALDEFLUOR, Side population assay and CD34 expression in PBSCs.

Sr.	Markers	1	2	3	4	5	Mean ± SE
1	ALDH1A1 +	1.53	1.50	2.70	1.25	1.40	1.70 ± 0.26
2	CD34 +	0.60	0.90	0.80	0.62	0.50	0.69 ± 0.07
3	ALDH1A1 + / CD34 +	0.18	0.23	0.35	0.15	0.20	0.23 ± 0.04
4	ALDH1A1 + / CD34-	1.33	1.26	2.30	1.08	1.18	1.43 ± 0.22
5	H33342 + / PyY^{Low} (G0)	4.45	4.00	3.50	6.00	3.20	4.24 ± 0.49
6	H33342- / PyY^{Low}	1.44	0.63	1.72	1.5	1.01	1.26 ± 0.20
7	ALDH1A1 + /H33342 + / PyY^{Low} (G0)	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0
8	ALDH1A1 + / H33342 + / PyY + (G1)	1.5	1.44	2.3	1.0	1.38	1.5 ± 0.21
9	ALDH1A1 + /CD34 + /H33342 + /PyY^{Low} (G0)	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0
10	ALDH1A1 + /CD34 + /H33342 + /PyY + (G1)	0.16	0.21	0.30	0.15	0.19	0.20 ± 0.02

RNA only in the presence of H33342 reflecting cellular state. Cells in G0 phase or quiescent cells show low mRNA content which is identified by simultaneous analysis using H33342 and Pyronin Y.

2.4. Characterization of HSCs

To analyze phases of cell cycle, cells were initially suspended in DMEM with 10% FBS and labeled with H33342 dye (1.5 ml; 12 µg / ml) followed by incubation for 45 min at 37 °C. Cells were centrifuged, washed and were re-suspended in DMEM + 10%FBS. Further, these cells were labeled with ALDEFLUOR reagent (5 µl) and Pyronin Y (7.5 µl; 200 µg/ ml), simultaneously. Cells were incubated for 45 min at 37 °C.

Cells were centrifuged and re-suspended in 100 µl ALDEFLUOR assay buffer. These cells were further labelled with anti-human primary labelled CD34-APC (5 µl; BD Pharmingen) with respective IgG controls and incubated on ice for 1 h. Excess antibody was removed from cell suspension by centrifugation and subsequent wash with ice-cold PBS. Cells were re-suspended in 1 ml ice-cold ALDEFLUOR assay buffer. Cells were analyzed immediately after staining was performed or were kept on ice until further analysis.

The Mo-Flo XDP flow cytometer from Beckman Coulter was used for these experiments. The instrument consists of Blue (488 nm), Red (645 nm), Violet (405 nm) and UV (355 nm) laser. Each sample (1×10^6 cells) was analyzed at 10,000 events per seconds using 100 µm filters (total events analyzed 0.5 to 1×10^6). Cells were analyzed for ALDFLUOR and Pyronin Y (488 nm), Hoechst dye (355 nm) and primary labeled CD34 antibody conjugated with APC (645 nm) using respective lasers. The labeled cells were analyzed individually or simultaneously according to experimental requirement.

2.5. Flow cytometry analysis

For identification of overlap between ALDH and SP, initially labeled

cells were analyzed using side scatter to remove cell debris. Further, this gated population (P1) was analyzed in respective fluorochromes channels (ALDEFLUOR: FITC, Side population: Hoechst Red Vs Hoechst Blue, CD34-APC: Red). For simultaneous analysis of ALDH and side population, P1 was analyzed in FITC channel to identify ALDH1A1 + cells (P2). This population of ALDH1A1 + cells (P2) was further analyzed to identify side population in H33342 Red Vs H33342 Blue channel using UV laser (355 nm).

The PBSCs were stained with ALDEFLUOR reagent, H33342, Pyronin Y and CD34 antibody conjugated with APC. The stained cells from P1 were individually analyzed for ALDEFLUOR, CD34-APC, H33342 and Pyronin Y to ensure the staining and respective controls. For identification of quiescent (G0) cells (stained with ALDEFLUOR reagent, H33342, Pyronin Y and CD34) P1 population was sequentially analyzed for ALDH1A1 (P2) and co-expression with CD34 (P3). These cells (P3) with phenotype (ALDH1A1 + /CD34 +) were further analyzed for identification of cell cycle phase using H33342 and Pyronin Y staining. Cells were gated as H33342 + /Pyronin Y^{Low} depending upon the fluorescence intensity of these cells.

3. Results

3.1. Analysis of putative stem cells

The analysis of bone marrow cells (HSCs) individually by flow cytometry showed the presence of ALDH1A1 + cells (1.88%), side population (1.2%) and CD34 + cells (0.80%) (Fig. 1a). For simultaneous analysis cells were stained with ALDEFLUOR reagent, H33342 dye followed by APC conjugated CD34 antibody. This analysis showed that cells in side population (H33342-) did not express ALDH1A1 and CD34 (Fig. 1b and 1c).

Similar experiments with PBSCs showed ALDH1A1 (1.82%), side population (1.44%) and CD34 + cells (0.70%) (Fig. 2a) Simultaneous analysis of PBSCs using assays like ALDEFLUOR, H33342 exclusion and CD34 antibody revealed that ALDH1A1 and CD34 expression was absent in the side population (H33342-) cells (Fig. 2b and 2c).

The above results showed that H33342 exclusion and ALDEFLUOR assay identify different population of cells in bone marrow and PBSCs. ALDH1A1 and CD34 did not co-express with side population (H33342-) and appeared in the middle of the side population cytometry plot. The mean values of normal bone marrow (N = 5) and G-CSF stimulated blood samples (N = 5) analyzed are shown in Table 2a and b, respectively.

3.2. Simultaneous analysis for identification of phase of cell cycles

To identify if CD34 + cells segregated with ALDH1A1 + cells and to determine the phase of the cell cycle of either population, we labeled them simultaneously with Pyronin Y. The flow cytometry analysis of bone marrow revealed the presence of a quiescent population (G0) denoted by H33342 + /Pyronin Y^{Low} (3.5%). Bone marrow cells showed the expression of both ALDH1A1 (2.2%) and CD34 (0.60%) when analyzed separately (Fig. 3a). The simultaneous analysis of cells showed a small fraction of ALDH1A1 + cells co-expressed CD34 (0.24%) while the majority of ALDH1A1 + cells did not express CD34 (1.83%) (Fig. 3b) To define phase of the cell cycle, cells with the phenotype ALDH1A1 + (2.2%) and ALDH1A1 + /CD34 + (0.24%) were subjected to cell cycle analysis using H33342 and Pyronin Y. The cell cycle analysis revealed that all the cells were present in G1 phase with ALDH1A1 + /H33342 + /Pyronin Y + (2%) or ALDH1A1 + /CD34 + / H33342 + /Pyronin Y + (0.20%) (Fig. 3b) The mean values of normal bone marrow samples (N = 5) analyzed are shown in Table 3a.

We performed a similar analysis of G-CSF stimulated PBSCs. The flow cytometry analysis identified a small population of cells with the phenotype H33342 + /Pyronin Y^{Low} (4.45%) marked as quiescent (G0). PBSCs revealed the presence of ALDH1A1 + (1.53%) and CD34 (0.60%) when analyzed separately (Fig. 4a). Simultaneous analysis identified a small population of cells with the phenotype ALDH1A1 + /CD34 + (0.18%) while cells with phenotype ALDH1A1 + /CD34- (1.33%) constituted the majority of ALDH1A1 + cells (Fig. 4b). Further, the cells (ALDH1A1 + and ALDH1A1 + /CD34 +) were analyzed to define the cell cycle phase using H33342 and Pyronin Y. This analysis showed that G1 constituted with majority of ALDH1A1 + /H33342 + /Pyronin Y + (1.5%) and ALDH1A1 + /CD34 + /H33342 + /Pyronin Y + (0.15%) cells were in G1 phase of the cell cycle (Fig. 4b). The mean values of G-CSF stimulated blood samples (N = 5) analyzed are shown in Table 3b.

These results represent that the majority of putative stem cells identified by either functional assay or surface markers or together are present in G1 and not in quiescent (G0) phase of cell cycle denoted as ALDH1A1 + /H33342 + /Pyronin Y^{Low} and ALDH1A1 + /CD34 + / H33342 + /Pyronin Y^{Low}.

4. Discussion

This study was initiated for two reasons. Firstly, data from in vitro experiments are not entirely consistent regarding ALDH/SP positive cells and their relationship to expression of CD34, as well phase of the cell cycle (Table 1). Secondly, the expression of ALDH1A1 as measured by the ALEDFLUOR kit or the side population assay (Hoechst negative cells) has been used to identify a tumour initiating cells or cancer stem cells (CSCs) (Pearce et al., 2004).

It is in this context we wished to determine whether the two functional assays i.e, side-population and ALDEFLUOR identify same set of cells which also co-express CD34, a well-known HSCs specific surface marker. The easiest normal tissue to evaluate this is the hematopoietic system. There is an expansion of the stem cell pool following G-CSF administration and leukapheresis is performed for their isolation. Analysis of HSCs has not been performed in peripheral blood stem cells using these assays.

The previous 3 studies (Table 1) which attempted to address this question used either cord blood or human bone marrow. Our data using bone marrow as well as G-CSF stimulated peripheral blood stem cells shows that the types of cells identified by the ALDEFLUOR assay and side population are completely different (Figs. 1b and 2 b). As the expression of CD34 marks HSCs, we evaluated whether these cells segregated with either the side population or ALDH1A1 + cells. This showed that there were no cells identified with the phenotype SP/CD34 + in both normal bone marrow as well as PBSCs (Figs. 1c and 2 c) but a small fraction of cells co-expressed ALDH1A1 with CD34 in both normal bone marrow and PBSCs (0.24% and 0.18% respectively) which could represent the true stem cell compartment (Figs. 3b and 4 b).

Previous studies reported more than 90% of CD34 + cells in the ALDH1A1 fraction in human cord blood which is very high as compared to this study where less than 1% of CD34 + cells were present in the ALDH1A1 fraction from peripheral blood stem cells (Pearce and Bonnet, 2007). The expression of CD34 in side population is still debatable due to variability in results which suggests either CD34-/SP/Lin- or CD34 + /CD38-/Lin^- /ALDH + /SP + as a putative stem cell phenotype (Table 1). Our results further strengthen the finding which indicates lack of overlap between ALDH1A1 + cells and side population in human cord blood cells (Frandberg et al., 2015). This also contradicts the previous reports suggesting the existence of stem cell population marked by both ALDEFLUOR and H33342 exclusion assay simultaneously (Pearce and Bonnet, 2007; Pierre-Louis et al., 2009).

These results may suggest that different populations of putative stem cells exist whose phenotype is unclear. However, there is a caveat, the identification of rare population of cells depend on the number of events. We have analyzed a million events which should yield any population that is at 0.1% level. More recently, it has been shown that expression of CD133 marks HSCs and CD34-/CD133 + are able to repopulate the bone marrow in irradiated mice (Takahashi et al., 2014). Although stem cells from human bone marrow have been analyzed,

direct comparison with G-CSF stimulated peripheral blood has not been performed previously. Our results show similarity between individual populations in the bone marrow and peripheral blood stem cells. This may be different numerically if the comparison is made between both sources following G-CSF stimulation.

To identify the exact phase of the cell cycle of these cells, an experiment with a combination of Pyronin Y, Hoechst dye, ALDEFLUOR reagent and anti-CD34 antibody was performed. Simultaneous Pyronin Y and H33342 dye staining is known for identifying quiescent (G0) cells depending upon the RNA content in cells (Darzynkiewicz et al., 2004; Shapiro, 1981). Normally stem cells should be predominantly quiescent and so in G0. Usually they appear together with cells in G1 phase of cell cycle (Figs. 3a and 4 a). By using H33342 and Pyronin Y dyes one can discriminate the quiescent (G0) cells from active (G1) phases of cell cycle with ($H33342+/PyY^{Low}$) phenotype. The previous studies in bone marrow showed that the phenotype of putative HSCs (PyYLow/CD34+/CD38-/ALDH+/SP+) differed from quiescent (G0) cells (PyYLow/CD34+/ALDH+) (Pierre-Louis et al., 2009).

In this regard, our results showed that in both normal bone marrow (Fig. 3b) and PBSCs (Fig. 4b) samples, quiescent stem cells ($H33342+/PyY^{Low}$) did not express ALDH1A1 and CD34. Further this suggests that these cells are present in metabolically active phase (G1) of cell cycle and not in the quiescent (G0) phase (Table 3a and 3b). Ultimately, sorting of cells defined by ALDH1A1/CD34 or SP/CD34 has to be performed and assessed whether these will reconstitute hemopoiesis in mice. There is now some evidence that $PyY^{Low}/H33342^{Low/-}$ cells may be truly quiescent (Yilmaz et al., 2006) for normal bone marrow and G-CSF stimulated blood samples (Table 3a and 3b).

Previously in the murine hematopoietic system, SP cells repopulated mice that had been lethally irradiated (Goodell et al., 1997). However, $ADH1A1^{-/-}$ mice did not have any defects in the stem cell pool (Bhatia et al., 1997; Levi et al., 2009). Inhibition of ALDH1A1 expression using drugs such as DEAB or siRNA showed that there is an expansion of CD34+ cells. ALDH1A1 regulates HSC differentiation via augmentation of retinoid signalling (Muramoto et al., 2010). It has also been confirmed by another group that ALDH1A1 is dispensable for murine hematopoietic stem cells (Levi et al., 2009). However, the lack of ALDH1A1 is compensated by increased expression of ALDH3A1 as shown elegantly using knockout mice. This is supported by $ALDH1A1^{-/-}$ and $ALDH3A1^{-/-}$ compound homozygotes that have reduced numbers of HSCs as well as aberrant cell cycle distribution, increased reactive oxygen species levels, p38 mitogen-activated protein kinase activity and sensitivity to DNA damage (Gasparetto et al., 2012). The above data suggest that at least ALDH1A1 is dispensable for stem cell function.

Some of the cell surface markers that have been commonly identified in tumours are CD34, CD44, CD133, CD117 and CD24 with variable frequency of expression. In general, ALDH1A1 expression has been in the range 1–5% in different tumour types. Side population reports have been less frequent and again range from 1 to 5% (Abbaszadegan et al., 2017; Nagare et al., 2017). Similarly, identification and characterization of HSCs is mainly based upon functional properties like self-renewal, H33342 exclusion and ALDH1A1 expression. Combining surface markers with functional properties has identified a small population of HSCs which can reconstitute the bone marrow with different lineages. These cells show typical phenotype with expression of CD34, c-kit and lack of lineage markers (Lin^-) (Ng and Alexander, 2017).

Our results suggest that CD34 alone may not be sufficient to identify more primitive quiescent stem cells. Although clinically CD34+ cells are used primarily to evaluate the adequacy of stem cell content, the repopulation of bone marrow may suggest the presence of as yet unidentified more primitive population of HSCs (Takahashi et al., 2014). Alternatively; CD34+ cells retain the capacity to repopulate the marrow, although they do not have the functionality of pluripotent stem cells. This indicates that putative HSCs cannot be discriminated on the basis of surface marker or intrinsic properties alone. More experiments are required to identify putative stem cells using conventional markers and evaluate their stemness properties. The mechanistic understanding of this must await further experiments.

Author's contribution

T.S.G. conceived the study, critically read and corrected the manuscript.

R.P.N. designed and performed the experiments, analyzed data and wrote manuscript.

S.S. analyzed data and R.S. collected and processed the Leukapheresis blood samples.

Conflict of interest

All authors have read and approved the manuscript for publication. The authors declare no competing interests associated with this manuscript.

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