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MOLECULAR CHARACTERIZATION OF A LINEAGE NEGATIVE STEM-PROGENITOR CELL POPULATION FROM HUMAN PERIPHERAL BLOOD

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ABSTRACT

Current haematopoietic stem cell transplantation protocols rely heavily upon $CD34^+$ cells to estimate haematopoietic stem and progenitor cell yield. However, several studies nowadays emerged with evidence that $CD133^+$ cells represent a more primitive cell population than their $CD34^+$ counterparts. The objective of the present study was to isolate $CD133^+$ stem cells by a rapid negative isolation method which depletes human peripheral blood mononuclear cells (MNCs) from cells expressing haematopoietic lineage markers CD2, CD3, CD11b, CD14,CD15, CD16, CD19, CD56, CD123, and CD235a and isolates a discrete lineage negative (*Lin*$^-$) cell population (11±2% of MNC, n=6). Human peripheral blood MNCs were fractionated by density gradient centrifugation. After labelling with antibody against haematopoietic lineage markers, MNCs were subjected to magnetic assisted cell sorting (MACS). The expression of stem cells markers was examined by reverse transcriptase PCR (RT-PCR). We showed that *Lin*$^-$ stem-progenitor cell population encompassed the common markers used to characterize adult stem cells including SLAMF1, CD90 and CD133 and was also enriched for CD117, a surface receptor for stem cell factor. This cell population is more homogenous and primitive as compared to suspension MNCs which comprised of SLAMF1 and CD117 but the cells with haematopoietic lineage marker, CD14 and CD2 were also present. In conclusion, our approach was able to isolate a homogenous *Lin*$^-$ stem-progenitor cell population from human peripheral blood hence offers a promising alternative to current haematopoietic stem and progenitor selection methods.

Key words: Lineage negative, mononuclear cells, peripheral blood, stem cells

INTRODUCTION

Adult stem cells are now becoming an attractive alternative to the use of embryonic stem cells in regenerative medicine and congenital disease because of their availability and the manipulation of this type of stem cell are not bounded by ethical concern (Si *et al.*, 2011). Two examples of adult stem cells which are actively studied are hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) (Preston *et al.*, 2003).

Despite of increasing demands in adult stem cell research and their relevance in clinical field, it is agreed that a standard protocol for isolating homogeneous population is not yet established. The conventional isolation method of stem cells from various sources relies on the fractionation of mononuclear cells by gradient centrifugation and selection of fibroblast-like cells adhering to the culture plate surface by removing non-adherent floating cells. In order to obtain more homogenous stem cell populations, a cocktail antibody that depletes specific cell populations (Valenti *et al.*, 2008), fluorescence-activated cell sorting (FACS) isolation (Zohar *et al.*, 1997), and specific cell surface antibody selection (Battula *et al.*, 2009) have been employed, improving the purity of the final stem cell population isolated. However, although significant improvements have been made in purification techniques, these isolation methods have not yet been sufficient to isolate completely homogenous populations of stem cells. Therefore, this research is designed to isolate adult stem cell from human peripheral blood by adapting culture selection of peripheral blood mononuclear cells and enrichment of lineage negative stem-progenitor cells of human peripheral blood mononuclear cells.

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MATERIALS AND METHODS

Blood collection and mononuclear cells isolation

Blood of six healthy human between 18-25 years old with no history of blood disorders, infectious and systemic diseases was drawn into EDTA tubes (BD, USA) following informed consent. Subjects with conditions in which phlebotomy is contra-indicated or subject is a known carrier of a blood transmitted infectious disease were excluded from this study. The ethics approval reference number was UKM1.27/244/9.

Blood samples were diluted with Hanks Balanced Salt Solution (HBSS) pH 7.4 (Sigma, USA) and layered on top of Ficoll-Paque PLUS (G.E Healthcare, USA). After centrifugation at 400 g for 25 min at room temperature, the high density mononuclear cells in the interface were collected, washed with phosphate buffered saline (PBS) (Sigma, USA) pH 7.4 and resuspended in α-minimal essential medium (AMEM) supplemented with 10% (v/v) newborn calf serum (NBCS) and 2% (v/v) penicillin/streptomycin (Biowest, France) which herein referred as proliferation medium.

Culture selection of suspension MNCs and adherent MNCs

The mononuclear cells (MNCs) were cultured for 14 days at $2x10^6$ cells/mL in proliferation medium and maintained at 5% CO_2, 37°C. Within 14 days of culture, two cell populations emerged. The first cell population adhered to the culture dish and appeared as fibroblast-like cells. This cell population is termed as adherent MNCs. Another cell population is non-adherent MNCs or suspension MNCs. The suspension MNCs and adherent MNCs were collected and analysed for cell morphology and expression of molecular surface marker.

Enrichment of lineage negative stem-progenitor cells

Lineage negative (*Lin*$^-$) stem-progenitor cells were enriched from freshly isolated peripheral blood mononuclear cells. The enrichment protocol was performed according to manufacturer's recommendation (Miltenyi Biotec, Germany). Peripheral blood MNCs were incubated for 10 min at 4°C with biotinylated mouse monoclonal anti-human CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123 and CD235a. After washes in PBS-EDTA buffer (300 *g* for 10 min at 4°C), cells were subsequently labelled with anti-biotin secondary antibody conjugated to micro-beads for 15 min at 4°C. Cells were again washed and resuspended in PBS-EDTA. Antibody-labelled cells were passed through the mini-MACS selection column. *Lin*$^-$ fraction from the filtrate was collected prior to cell enumeration and viability assay by trypan blue exclusion assay. *Lin*$^+$ MNCs were reserved for morphological staining.

Determination of cell morphology and size

A total of $2 x10^5$ cells/mL of *Lin*$^-$ stem-progenitor cells and *Lin*$^+$ MNCs were washed with PBS before being attached to the glass slide by cytospin at 1000 g for 5 min at room temperature. The attached cells were air-dried for 15-20 min before subjected to methanol fixation. Following cell fixation, the cells were stained with May-Grunwald (v/v) (R&M chemical, USA) solution for 15 min and counter stained with Giemsa for 10 min. The cells were subsequently washed with deionised distilled water and air-dried. The morphology of *Lin*$^-$ stem-progenitor cells and *Lin*$^+$ MNCs stained by May-Grunwald Giemsa and the morphology of suspension MNCs, adherent MNCs, *Lin*$^-$ stem-progenitor cells and *Lin*$^+$ MNCs in culture medium were observed and photographed using inverted microscope equipped with digital camera (Olympus, Model: CKX75). Images were captured, and the diameter of *Lin*$^+$ MNCs and *Lin*$^-$ stem-progenitor cells was measured and analysed using Cell B software.

Reverse transcriptase-polymerase chain reaction analysis

Total RNA was extracted from *Lin*$^-$ stem-progenitor cells, suspension MNCs and adherent MNCs using Trizol (Invitrogen, New York, USA). One microgram of the total RNA was reverse transcribed to cDNA using Revert Aid First Strand cDNA synthesis Kit (ThermoScientific, Illinois, USA). PCR was performed with the initial cycle consisting of 94°C for 3 min and subsequently followed by 34 cycles of the following conditions: denaturation at 94°C for 1 min, primer annealing for 30 sec, extension at 72°C for 1 min. Final extension was at 72°C for 5 min. The PCR products were electrophoresed in a 1% (w/v) agarose gel and stained with ethidium bromide (EtBr) which was then photographed using the Alpha imaging system (Álpha Innotech, USA) under u.v light. The base pair size of the PCR generated DNA fragments were estimated relative to DNA ladder standards. Densitometry values were measured using Image J software. RT-PCR values are presented as a ratio of the specified gene's intensity divided by the GAPDH signal. The primer sequences and optimal PCR annealing temperatures are listed in Table 1. Each RT-PCR product was then sequenced and analysed. Sequences comparison using BLAST was performed to confirm that a correct product was obtained.

Table 1. A panel of markers used for characterization of adherent MNCs, suspension MNCs and *Lin*- stem-progenitor cells

Gene classification		Annealing T (^{0}C)	Product size(bp)	Sequence
Housekeeping gene	*GAPDH*	55	195	Fwd: 5'CCATGGAGAAGGCTGG 3 Rev: 5'CAAAGTTGTCAGGATGACC3'
MSC markers	*CD90*	54	143	Fwd: 5' TGGACCAGAGCCTTCG 3' Rev: 5' TCGGGAGCGGTATGTG 3'
	CD105	61	290	Fwd: 5'GCTCCCTCTGGCTGTTG3' Rev: 5'TTACACTGAGGACCAGAAGC3'
HSC markers	*CD133*	57	264	Fwd: 5' CCAAGGACAAGGCGTTCA 3' Rev: 5' GCACCAAGCACAGAGGG 3'
	SLAMF1	53	316	Fwd: 5'TCCTTGACCTTCGTGCTGT3' Rev: 5'CCTTCCTGCTTTCCCGTAT3'
Stemness	*CD117*	57	403	Fwd:5'CTCTGCGTTCTGCTCCTAC3' Rev: 5'TTGGTCACTTCTGGGTCTG3'
Hematopoietic lineage marker	*CD2*	60	345	Fwd: 5'CAACCCTGACCTGTGA 3' Rev: 5'CTACTCTGTGGGCTCTT3'
	CD14	59	448	Fwd:5'GGTGCCGCTGTGTAGGAAAGA3 Rev: 5'GGTCCTGGAGCGTCAGTTCCT3'

RESULTS

Morphology of suspension MNCs, adherent MNCs, *Lin*+ cells and *Lin*- stem-progenitor cells

Incubation of freshly isolated heterogeneous MNCs in complete medium for 14 days produced two distinguishable types of cells. Suspension MNCs were round shape and translucent under the light microscope (Figure 1A) whereas adherent MNCs (Figure 1B) showed a combination of spindle shaped, fibroblast-like cells and macrophage-like cells morphology which were strongly attached to the culture dish. *Lin*- stem-progenitor cells population enriched from freshly isolated peripheral blood MNCs was small, round in shape and suspended in the culture medium (Figure 1C). It was noted that *Lin*+ cells were also non-adherent, mononucleated with bigger in size as compared to *Lin*- stem-progenitor cells population (Figure 1D). Out of the original total MNCs, *Lin*- stem-progenitor cells was represented by 11±2% (n=6) (Figure 2A). Morphological staining of *Lin*+ MNCs and *Lin*- stem-progenitor cells showed that *Lin*+ MNCs consisted of lymphocytes. As a typical lymphocyte, *Lin*+ MNC has a large, dark-staining nucleus with little cytoplasm. In contrast, *Lin*- stem-progenitor cells were small, mononucleated with basophilic nucleus (Figure 2B). Based on cell diameter, *Lin*+ MNCs can be divided into small (7-11μm) and large (12-16μm) lymphocytes. The diameter of *Lin*- stem-progenitor cells were statistically smaller ($p<0.05$) than small lymphocyte and large lymphocyte with 6.3± 0.23μm in diameter (Figure 2C and Figure 2D).

Characterization of adherent MNCs by expression of surface markers

Adherent MNCs were found to express MSCs markers (CD90, CD117 and CD105) (Figure 3A) and macrophage marker (CD14) (Figure 3B). A few typical leukocyte-associated surface antigens, such as signalling lymphocytic activation molecule family member 1 (SLAMF1) and CD2 were also expressed in adherent MNCs (Figure 3B).

Characterization of suspension MNCs and *Lin*- stem-progenitor cells

In order to compare the efficiency of culture selection and lineage enrichment approach in

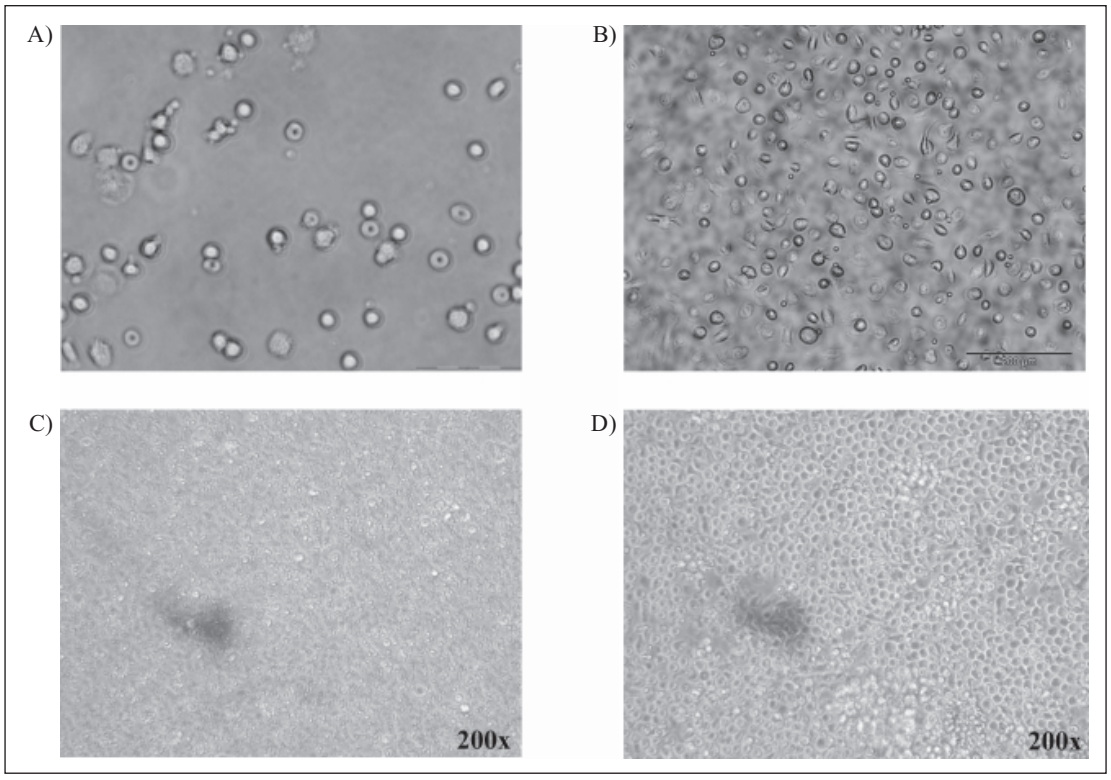

Fig. 1. Appearance of suspension MNCs, adherent MNCs, *Lin*$^+$ MNCs and *Lin*$^-$ stem-progenitor cells culture under the light microscope (A-D).

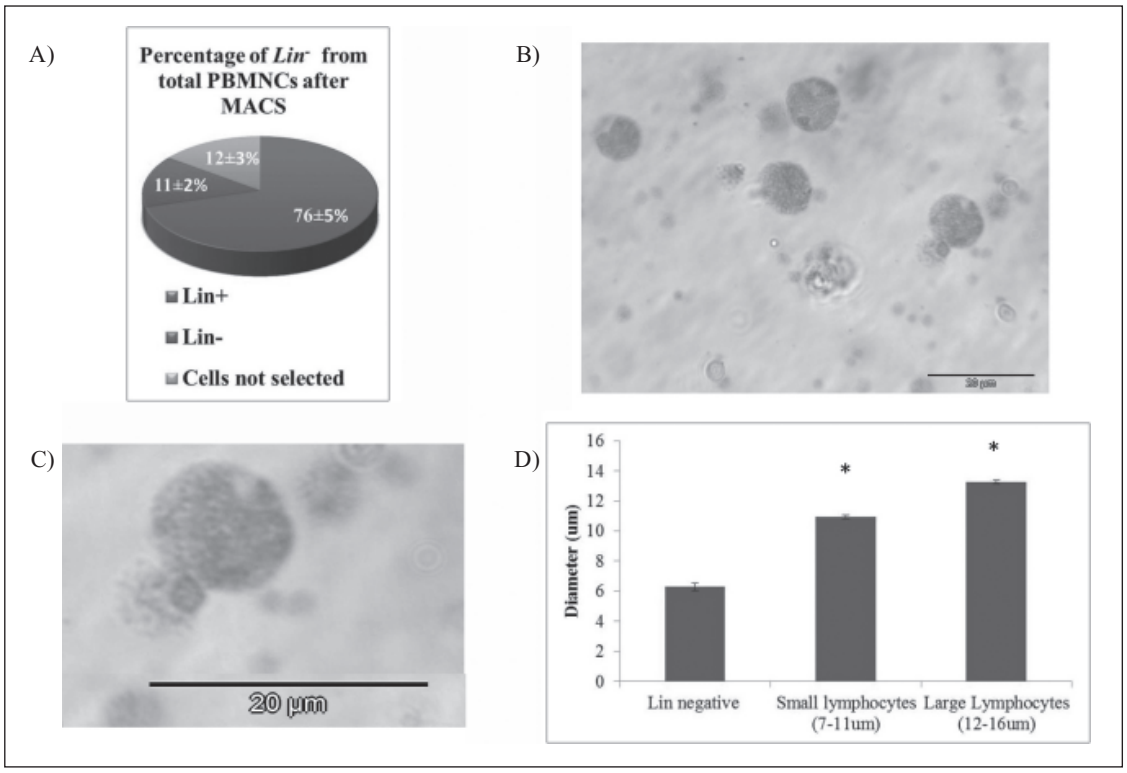

Fig. 2. Percentage of *Lin*$^-$ stem-progenitor cells from total peripheral blood MNCs after MACS enrichment (A). Morphological comparison between *Lin*$^-$ cells and Lin$^+$ cells stained by May-Grunwald Giemsa (B). Comparisons of cell size between *Lin*$^-$ stem-progenitor cells and *Lin*$^+$ which comprised of small lymphocytes and large lymphocytes (C). *Lin*$^-$ stem-progenitor cells were significantly smaller compared to small and large lymphocytes (D).

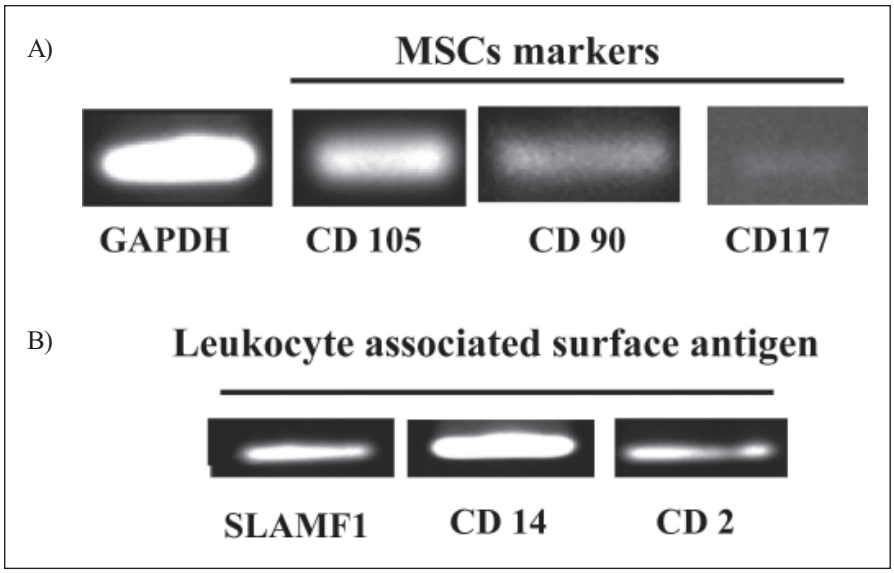

Fig. 3. Characterization of adherent MNCs by RT-PCR. Adherent MNCs expressed MSCs markers (A). Adherent MNCs was also positive for SLAMF1, CD14 and CD2 (B).

producing homogenous stem cells population, the analysis of cell phenotype at the RNA level was evaluated. When the suspension MNCs were characterized following 14 days of culture selection, a heterogeneous cell population was obtained. Although the cells expressed CD117, SLAMF1 and CD133 which are general markers used to identify HSC, suspension MNCs were also positive for CD14 and CD2 (Figure 4A). In contrast, enrichment of *Lin*- stem-progenitor cells from freshly isolated MNCs generated more homogenous hematopoietic stem-progenitor cells population characterized by highly positive CD133, SLAMF1 and CD117 expression (Figure 4B). Relative expression of CD133 to GAPDH showed that *Lin*- stem-progenitor cells isolation yield a higher expression of CD133 as compared to suspension MNCs (Figure 4A and Figure 4B). The correct expression of the markers used in the study was confirmed by gene sequences comparison using BLAST.

DISCUSSION

The pre-requisite for studying the biological function and molecular characterization of HSCs is to obtain a purified suspension of HSCs. However, the extremely low frequency of HSCs in any tissue and the absence of a specific HSC phenotype have made their purification and characterization a highly challenging goal. The present study was comparing between culture selection protocol and lineage negative enrichment approach for acquiring more homogenous subpopulation of HSCs. The rationale behind culturing peripheral blood MNCs for 14 days was to eliminate mature blood cells which have a finite life-span *in-vitro* (Mackinney *et al.*, 1962; Perillo *et al.*, 1989) as compared to primitive stem cells. Within 14 days of culture, the adherent MNCs would attach to the culture flask, the mature cells died, leaving non-adherent or suspension MNCs floating in the culture medium. Meanwhile, the lineage cell depletion approach used in present study is an indirect magnetic labelling system for the depletion of mature hematopoietic cells such as T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes, erythroid cells, and their committed precursors from mononuclear cells of bone marrow, cord blood, or peripheral blood. The depletion of *Lin*+ cells results in the enrichment of untouched stem and progenitor cells. Negative selection protocols offer a real advantage to positive immunomagnetic selection as this technique does not cause activation of HSC adhesion molecules. Direct antibody ligation as used in positive selection can potentially mediate cell-adhesion molecule activity, including the CD34 molecule (McGuckin *et al.*, 2003).

HSCs and primitive hematopoietic cells can be recognized from mature blood cells by the absence of lineage-specific markers and presence of certain other cell-surface antigens, such as CD133, SLAMF1 and CD117 (Wognum *et al.*, 2003). By targeting various combinations of markers, whose expression is gained (or lost) at different rates as primitive hematopoietic cells differentiate thus could subdivide functionally heterogeneous mixture of total MNCs into more homogenous subpopulations. CD117, also known as stem cell factor receptor was reported to play important role

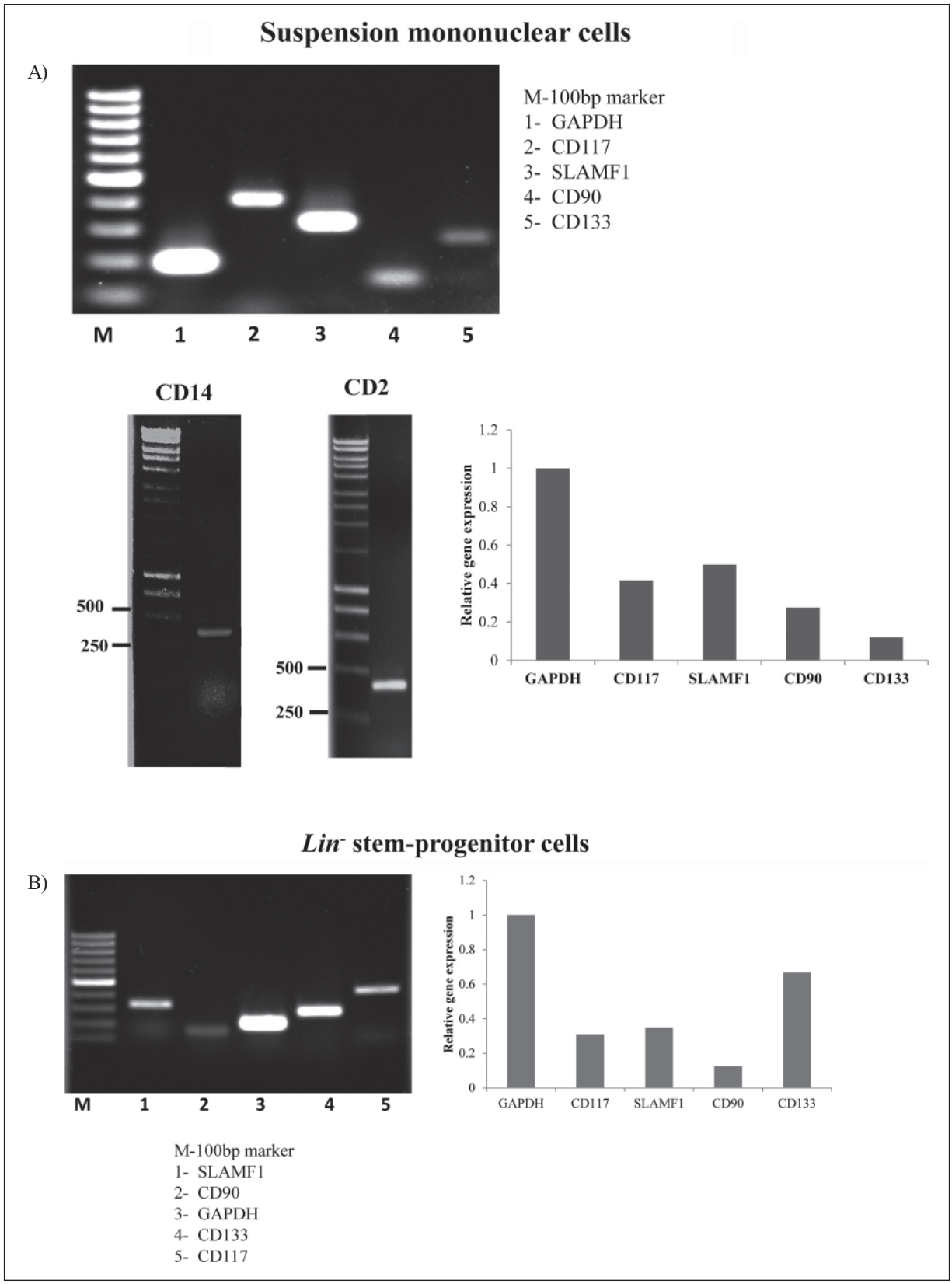

Fig. 4. Comparisons between culture selection approach and lineage negative enrichment by MACS for stem cells isolation. Suspension MNCs expressed markers for HSCs (SLAMF1, CD133 and CD117) and haematopoietic lineage markers (CD2, CD14). Gene transcript quantity was measured by relative RT-PCR using the internal standard GAPDH RT-PCR signal as the denominator as described in the materials and methods section. (A). Characterization of *Lin*- stem-progenitor cells. Expression intensity was measured by relative RT-PCR using the internal standard GAPDH RT-PCR signal as the denominator as described in the Materials and Methods section (B).

in gametogenesis, melanogenesis, and haematopoiesis (Chabot *et al.*, 1988). This receptor protein is present on human embryonic stem cells (Hoffman *et al.*, 2005), primordial germ cells and many somatic cells (De Coppi *et al.*, 2007) hence it has been used as a marker for stemness determination.

Molecular characterization of HSCs surface markers on suspension MNCs showed that these cells were positive for HSCs markers. However, the presence of hematopoietic lineage markers such as CD2 and CD14 indicates that this cell population still contain a few mature blood cells. Conversely, we have shown that enrichment of *Lin*$^-$ stem-progenitor cell from total MNCs of freshly isolated blood produced a homogenous cell population as compared to culture selection approach. This is supported by higher expression of CD133 in comparison to suspension MNCs. CD34 and CD133 are co-expressed on primitive progenitors and some leukaemia and therefore both can be considered as early antigens (Yin *et al.*, 1997). Morphological comparison between suspension mononuclear cells and *Lin*$^-$ stem-progenitor cells demonstrated that *Lin*$^-$ stem-progenitor cells were significantly smaller compared to normal mononuclear cells. The basophilic cytoplasmic appearance of *Lin*$^-$ stem-progenitor cells stained by May-Grunwald Giemsa suggests for the presence of ribosomal RNA which is reminiscent to primitive or blast cells. These morphological observations together with the expression of CD133, SLAMF1 and CD117 further support the notion that *Lin*$^-$ stem-progenitor cells are primitive and undifferentiated.

MSCs were originally isolated 40 years ago by Fredenstein and colleagues (1968) and termed as mesenchymal stem cells based on their capacity of differentiating into a variety of mesodermal tissues including bone, cartilage, and adipose (Si *et al.*, 2011). To date, bone marrow, adipose tissue, umbilical cord blood, and umbilical cord have been recognised as a major source for MSCs for tissue engineering and regenerative medicine. Although similar in basic features, MSCs derived from various tissues have substantial differences in the expansion potential as well as the difference in age-related functional properties (Kern *et al.*, 2006). Based on these, it is essential to decide the most suitable MSCs source for future applications.

The present study reported the possibility to obtain MSCs from human peripheral blood. By expansion in a basic medium consisting AMEM, 10% (v/v) NBCS and 2% (v/v) pen/strep without additional growth factors for 14 days, we obtained a cell population morphologically typical of MSCs. Gene expression profile shows the adherent mononuclear cells share the same markers of MSCs defined by International Society for Cellular Therapy (ISCT) which are CD90 and CD105 (Dominici *et al.*, 2006). In addition, the activation of CD117 provides the evidence of the stemness of this cell population.

CONCLUSION

Magnetic assisted cell sorting approach could be used to isolate a discrete *Lin*$^-$ cell population enriched with CD133$^+$ stem cells. This approach is superior to culture selection approach in producing homogenous stem cells.

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