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ORIGINAL ARTICLE

# Human adipose tissue contains erythroid progenitors expressing fetal hemoglobin

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## Abstract

**AIM:** To investigate the origin of hematopoietic progenitors contained in the stromal vascular fraction (SVF) of human adipose tissue.

**METHODS:** Tissue samples obtained from lipectomies were subjected to enzymatic digestion with collagenase to obtain a single-cell suspension. The centrifuged cell pellet, termed SVF, was separated immunomagnetically into $CD45^+$ and $CD45^-$ cells and cultured in serum-free medium containing hematopoietic cytokines. The freshly isolated and cultured cells were evaluated to determine their ability to form hematopoietic colony-forming units in clonogenic assays and for the expression of certain hematopoietic transcription factors by reverse transcription-polymerase chain reaction; the gene expression level was compared to that in $CD34^+$ hematopoietic progenitor cells from cord blood (CB) and adult peripheral blood (PB). To characterize erythroid progenitors, burst-forming units-erythroid (BFU-E) were developed in a semisolid medium under different culture conditions, and the hemoglobin composition and globin gene expression in the erythroid colonies were determined.

**RESULTS:** The transcription factors *SCL/TAL1*, *RUNX1*, *RUNX2* and *GATA2* were expressed in both the $CD45^+$ and $CD45^-$ SVF populations; however, in contrast to our observations in the $CD34^+$ cells from CB and adult PB, *GATA1* was not detected. Nevertheless, *GATA1* could be detected in the SVF cells after seven days in culture, whereas its expression was upregulated in the CB $CD34^+$ cells. The analysis of BFU-E-derived colonies revealed that virtually all erythroid cells produced by SVF cells expressed fetal hemoglobin, and the γ-globin mRNA levels ranged between those obtained in the adult- and neonatal-derived erythroid cells. Moreover, the SVF-derived erythroid cells synthesized similar levels of α- and β-globin mRNA, whereas the α-globin transcript levels were consistently higher those of β-globin in the cells derived from CB or PB $CD34^+$ cells. Furthermore, although the cellular distribution of hemoglobin in the erythroid cells derived from the $CD34^+$ cells obtained from hematopoietic tissues was dependent on the presence or absence of serum in the culture medium, this did not affect the SVF-derived erythroid cells.

**CONCLUSION:** Our results demonstrate that hematopoietic progenitors in SVF have molecular and functional features that differ from those exhibited by circulating progenitors, suggesting the possibility of a different origin.



**Key words:** Hemoglobin; Adipose tissue; Stromal vascu-

lar fraction; Erythroid cells; Hematopoietic progenitors

**Core tip:** Stromal vascular fraction (SVF) from human adipose tissue contains mesodermal precursors with the ability to form mixed hematoendothelial colonies and hematopoietic colony-forming units, though this occurs at an extremely low frequency. It is well known that hematopoietic progenitors residing in the bone marrow are released into the circulation and enter peripheral tissues; therefore, the most plausible explanation for this hematopoietic activity is that these cells are actually circulating hematopoietic progenitors. However, it is also possible that they may originate from the adipose tissue itself. To address this hypothesis, we compared the expression levels of the most relevant hematopoietic transcription factors in cells isolated from SVF with their expression levels in $CD34^+$ cells isolated from adult peripheral blood and cord blood. Moreover, because the composition of hemoglobin in erythroid cells varies depending on the origin of the hematopoietic progenitors and their ontogenic stage, burst-forming units-erythroid were developed in culture, and the hemoglobin composition and globin gene expression in erythroid colonies were determined. Our results provide evidence that erythroid progenitors contained in SVF exhibit features that differ from those of circulating progenitors. These findings should encourage further research on stem cells and the microenvironment of human adipose tissue.

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## INTRODUCTION

Adipogenesis and angiogenesis are two closely related processes during prenatal and postnatal life[1,2], and it has been proposed that $CD34^+CD31^-$ cells are a common precursor for both adipocytes and endothelial cells[3]. In addition, angiogenesis is rapidly induced in growing adipose tissue[4-6]. Therefore, antiangiogenic factors could be potential targets for regulating fat cell development[7]. Because bone marrow-derived endothelial progenitor cells do not contribute significantly to neovascularization[8], endothelial cells must be present in the supportive stroma to enable the vascularization of the tissue. In support of this view, it has been reported that stromal-derived factor-1 produced by $CD34^+CD31^+$ cells induces the chemotaxis of $CD34^+CD31^-$ cells expressing CXCR4 and leads to their differentiation into endothelial cells[9]. Moreover, a primitive population of $CD34^+CD90^+$ cells in human adipose tissue with a high proliferative capacity has been shown to be capable of differentiating into endothelial cells, even in the absence of angiogenic factors[10]. We also previously described, for the first time, the existence of a rare $CD45^-KDR^+$ cell population that exhibits hemangioblastic properties, as they give rise to hematoendothelial colonies[11]. These findings demonstrate the existence of mesodermal progenitors that can provide adipose tissue with endothelial progenitor cells when required. Additionally, $CD45^-$ cells produce hematopoietic colony-forming units (CFUs) when seeded in a methylcellulose-based medium[11]. Because adipose tissue is not hematopoietic in origin, it is possible that these CFUs are generated by circulating hematopoietic progenitors that target adipose tissue[12]. However, given that hemangioblasts are bipotent cells that give rise to endothelial and hematopoietic progenitor cells, it is tempting to speculate that these cells could be responsible for the production of these hematopoietic progenitors. To address this hypothesis, we examined the expression of genes directly involved in the process of hematopoiesis and analyzed the hemoglobin levels and globin gene expression in burst-forming units-erythroid (BFU-E)-derived colonies.

## MATERIALS AND METHODS

### *Purification of human adipose tissue and cells*

Human adipose tissue was obtained, after informed consent, from female patients between 30 and 40 years of age who were undergoing lipectomy at the University General Hospital of Valencia. The stromal vascular fraction (SVF) was obtained as previously described[11]. The SVF cells were labeled with anti-CD45 microbeads and separated into $CD45^+$ and $CD45^-$ cells using a magnetic-activated cell sorting separation system (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany). In some experiments, the $CD45^-$ cells were separated on the basis of CD34 expression into $CD45^-CD34^+$ and $CD45^-CD34^-$ subpopulations using anti-CD34 microbeads. To achieve a high degree of purity, the positive and negative cells were passed through a second column. After informed consent, cord blood (CB) samples were obtained from normal full-term deliveries, and peripheral blood (PB) samples were obtained from healthy adults between 20 and 50 years of age. The $CD34^+$ cells were purified by positive selection using a CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec). The purity of the isolated cells was determined by flow cytometry.

### *Short-term liquid culture*

$CD45^+$ and $CD45^-$ cells from the SVF and CB $CD34^+$ cells were cultured in a serum-free medium (StemSpan; STEMCELL Technologies, SARL, Grenoble, France) in the presence of stem cell factor and Flt3-L (100 ng/mL each) in addition to thrombopoietin and interleukin-6 (20 ng/mL each) (R and D Systems, Abingdon, United Kingdom). The medium was supplemented with 40 μg/mL lipoproteins (MP Biomedicals LLC, Solon, OH, United States) and 1% bovine serum albumin (BSA). The SVF cells were plated at $5 \times 10^5$ cells/mL, and the CB cells were plated at $5 \times 10^4$ cells/mL. The cultures were incu-

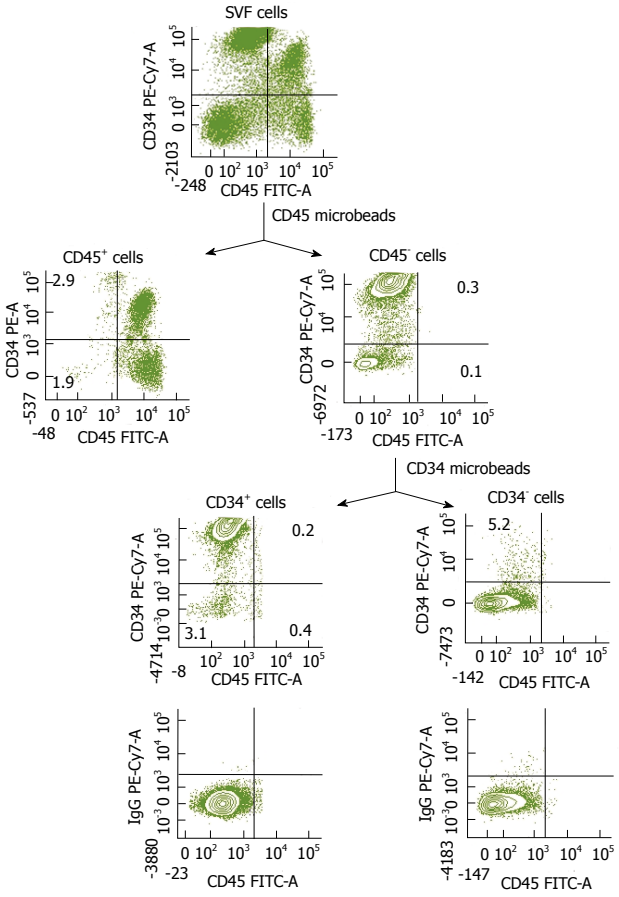


**Figure 1 Purity of stromal vascular fraction populations**. Selected cell subsets from stromal vascular fraction were separated using specific monoclonal antibodies coupled to magnetic particles following magnetic cell separation technology. Representative dot plots of selected cells are shown. The numbers in the quadrants indicate the percentages of cells expressing the corresponding antigen. SVF: Stromal vascular fraction.

bated at 37 ℃ in a humidified atmosphere containing 5% $CO_2$ and either 20% $O_2$ or 5% $O_2$. After seven days, the cells were harvested, counted and used for gene expression assays and clonogenic potential determination.

### *Clonogenic progenitor cell assay*

The SVF $CD45^-$ and $CD45^+$ cells (either freshly isolated or after seven days in liquid culture) and the CB or PB $CD34^+$ cells were seeded in methylcellulose-based media with or without fetal bovine serum (Methocult GF H4435 or Methocult SF H4436; STEMCELL Technologies). Isolated or liquid-cultured SVF cells were plated at $5 \times 10^5$ cells per dish, and purified $CD34^+$ cells were plated at 150 cells per dish. The cultures were incubated at 37 ℃ in a humidified atmosphere containing 5% $CO_2$ and either 20% $O_2$ or 5% $O_2$. After 14-21 d, CFUs were scored according to their morphology using an inverted microscope.

### *Hemoglobin analysis*

Individual BFU-E-derived colonies were selected from the semisolid cultures, pooled and washed. The cells were divided into two aliquots: one for FACS analysis to determine hemoglobin expression and the other globin gene expression analysis by real-time reverse transcription-polymerase chain reaction (RT-PCR). For the hemoglobin analysis, erythroid cells were fixed and permeabilized prior to staining with CD45-peridinin-chlorophyll protein complex, glycophorin A (CD235A) conjugated to allophycocyanin, fetal hemoglobin conjugated to FITC (all from BD Biosciences, Erembodegem, Belgium) and β-hemoglobin-PE (Santa Cruz Biotechnologies, Santa Cruz, CA, United States). The cells were analyzed using a FACSCanto Ⅱ (BD Biosciences) with FACSDiva software (BD Biosciences).

### *Gene expression analysis*

Total RNA was extracted using TRIzol Reagent and was subsequently treated with DNase(Deoxyribonuclease). cDNA was prepared using TaqMan Reverse Transcription Reagents. The real-time PCR experiments were performed using Mastermix and primers from Taqman Gene Expression Assays. All reagents and probes were purchased from Life Technologies (Foster City, CA, United States). The amplification protocol consisted of 95 ℃ for 10 min, followed by 40 cycles of 15 s at 95 ℃ and 1 min at 60 ℃. Each reaction was performed in duplicate. For each sample, the cycle threshold (Ct) readings were determined using the Sequence Detection Software. All expression data were calculated relative to *GAPDH* controls as $2^{-\Delta Ct}$. The following primers were used: SCL/TAL1 (Hs001097987_m1), RUNX1 (Hs01021971_m1), RUNX2 (Hs01047978_m1), GATA1 (Hs01085823_m1), GATA2 (Hs00231119_m1), α-globin (HS00361191_g1), β-globin (HS00747223_g1) and γ-globin (HS00361131_g1).

### *Statistical analysis*

Significant differences among the samples were tested using the Student *t* test or Mann-Whitney test where applicable. A *P* value less than 0.05 was considered statistically significant. The data were analyzed using GraphPad Prism Software 5.0 (GraphPad Software Inc., La Jolla, CA, United States).

## RESULTS

### *SVF cells have hematopoietic activity in vitro*

To demonstrate the presence of hematopoietic progenitor cells in human adipose tissue, SVF cells were separated into $CD45^+$ and $CD45^-$ populations, and $CD45^-$ cells were further separated into $CD45^-CD34^+$ and $CD45^-CD34^-$ populations (Figure 1). Clonogenic assays showed that the colony-forming ability of $CD45^-$ cells was restricted to CD34-expressing cells. As shown in Table 1, the $CD45^+$ cells, which accounted for approximately 10%-20% of the SVF cells, generated four times more CFUs than their complementary $CD45^-$ cells; however, no differences in CFU distribution were found. Notably, this colony-forming ability was not affected by either serum deprivation or a low oxygen concentration (Table 1).

**Table 1 Number of CFUs per $10^5$ CD45$^+$ or CD45$^-$ cells isolated from human adipose tissue stromal vascular fraction**

| | Freshly isolated cells | | | Liquid-cultured cells | |
|---|---|---|---|---|---|
| | Normoxia | | Hypoxia | Normoxia | Hypoxia |
| | With serum | Without serum | With serum | With serum | |
| CD45$^+$ cells | | | | | |
| Total CFUs | 1.53 ± 0.26$^b$ | 1.55 ± 0.35$^b$ | 1.79 ± 0.42$^b$ | 10.13 ± 3.19$^{a, d}$ | 13.73 ± 3.93$^d$ |
| Erythroid colonies | 0.59 ± 0.15$^b$ | 0.80 ± 0.05$^b$ | 0.72 ± 0.17$^b$ | 5.93 ± 1.58$^{a, d}$ | 7.01 ± 1.39$^d$ |
| Myeloid colonies | 0.94 ± 0.16$^b$ | 0.75 ± 0.35$^a$ | 1.01 ± 0.25$^a$ | 4.20 ± 1.67$^{a, d}$ | 6.72 ± 3.17$^d$ |
| CD45$^-$ cells | | | | | |
| Total CFUs | 0.37 ± 0.08 | 0.42 ± 0.11 | 0.34 ± 0.19 | 2.68 ± 0.86$^d$ | 7.91 ± 2.77$^d$ |
| Erythroid colonies | 0.12 ± 0.03 | 0.12 ± 0.05 | 0.09 ± 0.06 | 2.01 ± 0.99$^d$ | 4.70 ± 1.85$^d$ |
| Myeloid colonies | 0.25 ± 0.05 | 0.30 ± 0.07 | 0.25 ± 0.21 | 0.67 ± 0.16$^d$ | 3.21 ± 1.16$^d$ |

CD45$^+$ and CD45$^-$ cells from the stromal vascular fraction that were freshly isolated or cultured in a serum-free liquid medium for 7 d were seeded in a methylcellulose-based medium with or without serum, and colony-forming units (CFUs) were scored under a microscope. The numbers of CFUs per $10^5$ cells are provided. The total CFU values correspond to the sum of erythroid and myeloid colonies. The myeloid colonies include CFU-GM, CFU-M and CFU-GEMM. The data are provided as the mean ± standard error of the mean. Isolated cells: normoxia with serum ($n$ = 30-32), normoxia without serum (CD45$^+$, $n$ = 5; CD45$^-$, $n$ = 16), hypoxia ($n$ = 5); cultured cells ($n$ = 5). $^aP < 0.05$, $^bP < 0.01$ *vs* CD45$^-$ cells; $^dP < 0.01$ CD45$^-$ or CD45$^+$ cultured cells *vs* their corresponding isolated cells.

To evaluate the potential of hematopoietic progenitors to expand *in vitro*, SVF cells were cultured in a serum-free liquid medium containing early-acting cytokines for seven days and then tested for their colony-forming capacity. Under these culture conditions, the number of CFUs increased by approximately 7-fold in both the CD45$^+$ and CD45$^-$ cell populations. Moreover, when the cells were cultured under hypoxic conditions (5% $O_2$), the number of hematopoietic progenitors contained in the CD45$^-$ cell subset increased 3-fold over that under the normoxic condition (20% $O_2$).

### *Hematopoietic transcription factors are expressed in SVF cells*

To characterize the expression of hematopoietic progenitors in SVF, transcription factors involved in hematopoietic differentiation were analyzed by RT-PCR, and the gene expression levels were compared to those in the CD34$^+$ cells from CB and from adult PB. As shown in Figure 2A, *SCL/TAL1*, *RUNX1, RUNX2* and *GATA2* were expressed at significantly higher levels in the SVF CD45$^+$ cells than CD45$^-$ cells; however, *GATA1* was not detected in either cell subset. When CD34$^+$ hematopoietic cells were analyzed, the results showed that *SCL/TAL1* and *GATA2* were expressed at similar levels in the cells of neonatal and adult origin. However, the *GATA1*, *RUNX1* and *RUNX2* mRNA levels were significantly higher in the CD34$^+$ cells from adult PB compared to CB (Figure 2A). We also compared the gene expression profiles of the SVF cells with those of

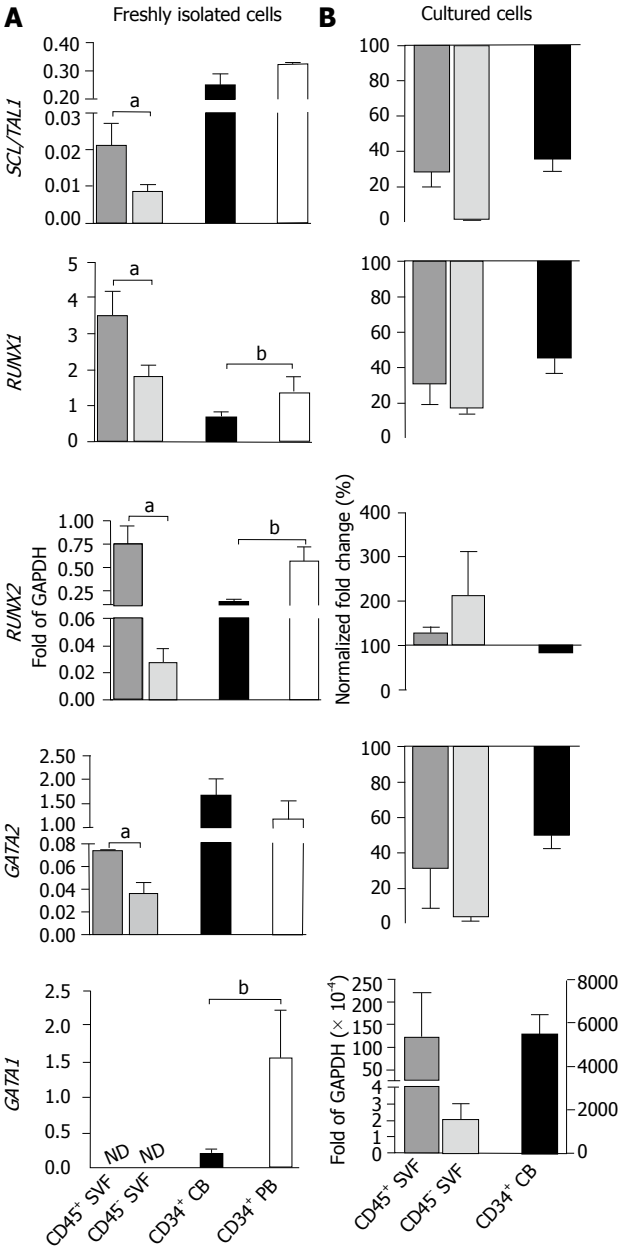


**Figure 2 Hematopoietic transcription factors genes are expressed in isolated stromal vascular fraction cell populations.** A: Freshly isolated CD45$^+$ and CD45$^-$ cells from stromal vascular fraction (SVF) and CD34$^+$ cells from cord blood (CB) or adult peripheral blood (PB) were isolated via immunomagnetic methods and used for a gene expression analysis by reverse transcription-polymerase chain reaction (RT-PCR). The transcripts were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); B: CB CD34$^+$ cells and CD45$^+$ and CD45$^-$ cells from the SVF were cultured in liquid serum-free medium containing early-acting cytokines for 7 d and then analyzed by RT-PCR to determine changes in the expression of selected genes. The transcripts were normalized to GAPDH, and the ratio between the gene quantity in cultured cells and the gene quantity in isolated cells was determined to yield a normalized fold change. CB CD34$^+$ cells, $n$ = 6-8; PB CD34$^+$ cells, $n$ = 3-4; SVF populations - CD45$^+$ cells, $n$ = 6; CD45$^-$ cells, $n$ = 6. Levels of statistical significance: $^aP < 0.02$, $^bP < 0.001$. ND: Not detected. All samples were assayed in duplicate. SVF: Stromal vascular fraction; CB: Cord blood; PB: Peripheral blood; GAPD: Glyceraldehyde-3-phosphate dehydrogenase.

the CD34$^+$ cells from hematopoietic tissues and found that *SCL/TAL1* and *GATA2* were expressed at significantly higher levels in hematopoietic CD34$^+$ cells than in

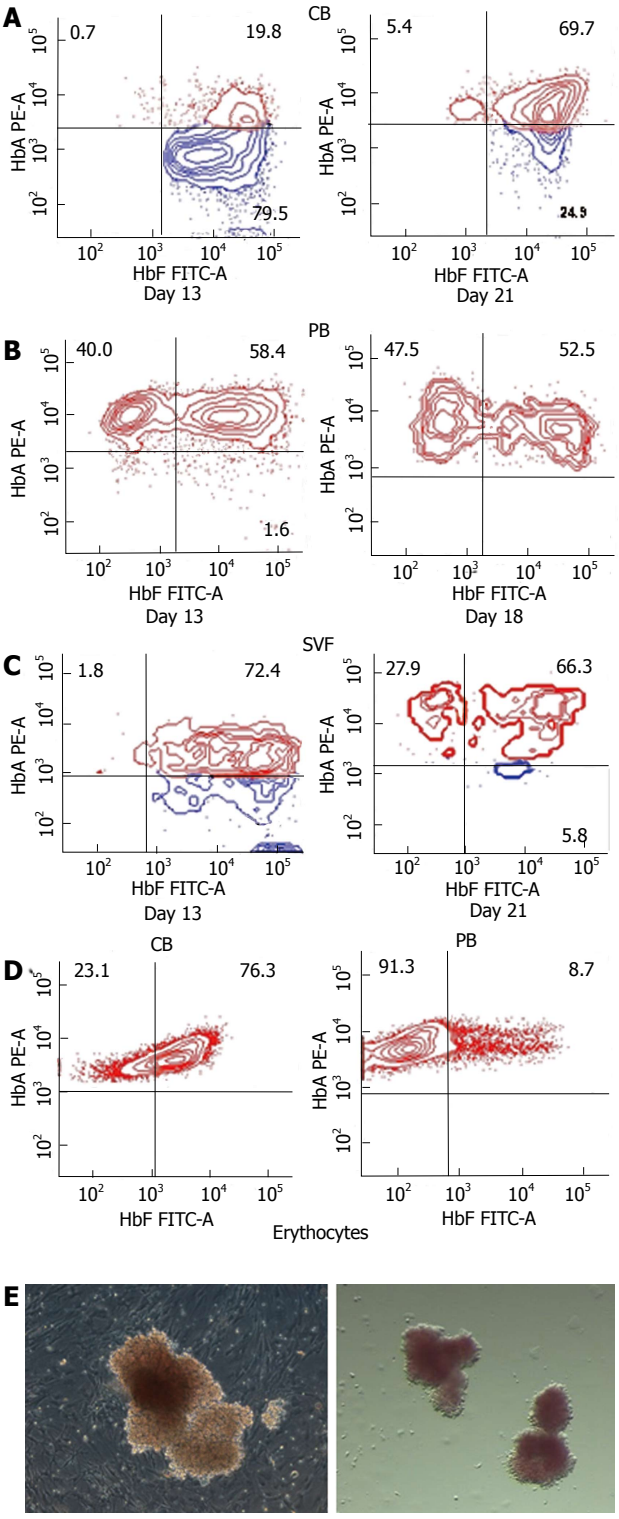


**Figure 3 Representative flow cytometric dot plots of hemoglobin expression in erythroid cells.** Burst-forming units-erythroid (BFU-E)-derived colonies were analyzed for HbF and HbA expression by flow cytometry after different lengths of time in culture. Erythroid cells generated from cord blood (CB) CD34$^+$ cells (A), from peripheral blood (PB) CD34$^+$ cells (B), and from stromal vascular fraction (SVF) cells (C). Representative fluorescence-activated cell sorting plots showing hemoglobin expression in circulating erythrocytes from CB and PB (D). Representative phase-contrast photomicrographs showing the morphological aspect of the BFU-Es generated from CD45$^-$ (left) and CD45$^+$(right) cells isolated from SVF (E). HbF: Fetal hemoglobin; HbA: Adult hemoglobin; CB: Cord blood; PB: Peripheral blood.

SVF cells. However, the adult PB CD34$^+$ cells expressed *RUNX1* and *RUNX2* at levels similar to the CD45$^-$ and CD45$^+$ cells from SVF, respectively. Lastly, the SVF cells and CB CD34$^+$ cells were cultured in liquid for seven days, and changes in their gene expression patterns were compared. The most important finding was that *GATA1* could only be detected in the SVF cells after seven days of culture, whereas *GATA1* expression was upregulated in the CB CD34$^+$ cells. The *SCL/TAL1, RUNX1* and *GATA2* mRNA levels were decreased in all the cultured cells. Additionally, although *RUNX2* gene expression was unchanged in both the CB cells and SVF CD45$^+$ cells, its expression was slightly increased in the SVF CD45$^-$ cells (Figure 2B).

### *Erythroid cells derived from SVF express fetal hemoglobin*

BFU-E-derived erythroid cells generated in semisolid culture from selected SVF cells or CB cells or PB CD34$^+$ cells were analyzed for hemoglobin expression. As expected, virtually all the neonatal erythroid cells expressed fetal hemoglobin (HbF), whereas adult hemoglobin (HbA) was expressed in all the erythroid cells derived from adult PB CD34$^+$ cells (Figure 3A and B). As shown in Figure 3E, the BFU-E-derived colonies generated from the SVF CD45$^-$ cells developed on a monolayer of stromal cells, whereas adherent cells were not found in the SVF CD45$^+$ cell cultures. It is important to note that when the SVF-derived erythroid cells were analyzed, a high proportion of cells containing HbF together with HbA (HbF$^+$HbA$^+$) were observed; unexpectedly, some cells expressing only HbF (HbF$^+$HbA$^-$) and a very small proportion of cells expressing only HbA (HbF$^-$HbA$^+$) were also detected (Figure 3C).

The mean fluorescence intensity (MFI) of intracellular Hb-associated immunofluorescence was used to estimate the amount of intracellular hemoglobin per cell. However, because the majority of CB- and SVF-derived erythroid cells were growing in clusters containing a wide range of HbF and HbA levels, the samples were separated into two groups based on the MFI of HbF in the HbF$^+$HbA$^+$ cells. First, a comparison between the neonatal- and adult-derived erythroid cells was established. As shown in Table 2, the neonatal erythroid cells in group A exhibited the highest MFIs for HbF and HbA. The samples in group B exhibited an MFI for HbF similar to that observed in the adult-derived cells, but HbA was significantly lower in both, HbF$^+$HbA$^+$ and HbF$^-$HbA$^+$ cells compared to their corresponding adult-derived cells. Next, the MFIs of HbF and HbA in the SVF-derived cells were compared to those observed in the erythroid cells derived from the CD34$^+$ hematopoietic cells from CB and adult PB. An analysis of the HbF$^+$HbA$^-$ cells showed that HbF was expressed at similar and higher levels in the cells derived from the SVF CD45$^-$ population than in the neonatal cells corresponding to group A and B, respectively. In contrast, the erythroid cells derived from the SVF CD45$^+$ population expressed HbF at a lower

**Table 2 Mean fluorescence intensity of fetal hemoglobin and adult hemoglobin in hemoglobin-expressing erythroid cells**

| | | HbF | | HbA | | HbF/HbF + HbA |
|---|---|---|---|---|---|---|
| | | $HbF^+HbA^-$ cells | $HbF^+HbA^+$ cells | $HbF^-HbA^+$ cells | $HbF^+HbA^+$ cells | |
| CB $CD34^+$ cells | A | 124082 ± 10917 | 204968 ± 17070[b] | 22139 ± 4457[a] | 25171 ± 3211[a] | 8.49 ± 1.40 |
| | B | 16989 ± 1382 | 21627 ± 1027 | 6139 ± 182[a] | 5248 ± 398[a] | 5.58 ± 0.38 |
| PB $CD34^+$ cells | | | 23495 ± 1326 | 10746 ± 893 | 9496 ± 548 | 1.26 ± 0.14 |
| SVF $CD45^-$ cells | A | 90164 ± 23126 | 78566 ± 2697[b,c,f] | 28229 ± 5204[a] | 21022 ± 3720[a] | 3.86 ± 0.71 |
| | B | 34612 ± 2701[c] | 29051 ± 1514 | 9637 ± 1094 | 5433 ± 325[a] | 5.92 ± 1.10 |
| SVF $CD45^+$ cells | A | 61429 ± 4085[c] | 47907 ± 2011[b,d] | 16388 ± 986 | 16668 ± 587[a] | 3.53 ± 0.18 |
| | B | 20971 ± 4696 | 15731 ± 1875 | 8006 ± 1653 | 9219 ± 1533 | 3.46 ± 1.03 |

The mean fluorescence intensity values are expressed as the mean ± SE and correspond to the samples analyzed in Figure 2. Statistical significance: [a]$P < 0.05$, [b]$P < 0.01$ *vs* PB $CD34^+$ cells; [c]$P < 0.05$, [d]$P < 0.01$ *vs* CB $CD34^+$ cells; [f]$P < 0.05$ $CD45^+$ cells *vs* $CD45^-$ cells. SVF: Stromal vascular fraction; CB: Cord blood; PB: Peripheral blood; HbF: Fetal hemoglobin; HbA: Adult hemoglobin.

and similar intensity than the neonatal cells in group A and B, respectively. The expression level of HbF in the SVF $HbF^+HbA^+$ cells from group A was significantly lower than that observed in the cells of neonatal origin from the same group. However, the HbF expression level in the SVF-derived erythroid cells in group B was similar to that in both the neonatal and adult-derived erythroid cells of the same group.

Although the proportion of $HbF^-HbA^+$ cells in the SVF-derived erythroid colonies was very small, the MFI values showed that the cells from group B expressed HbA at levels similar to the adult-derived cells. However, no significant differences were found between the SVF-derived cells from group B and neonatal-derived cells in the same group. Lastly, an analysis of HbA in the SVF-derived $HbF^+HbA^+$ cells in group B revealed that HbA was expressed at similar levels (*i.e.*, a similar MFI intensity) in the erythroid cells derived from $CD45^-$ progenitors and neonatal-derived cells in the same group. However, the MFI of HbA in the cells derived from $CD45^+$ progenitors was similar to that of the adult-derived cells, suggesting that the erythroid progenitors contained within the $CD45^+$ population must be at a later stage of maturation or differentiation.

MFI values were also used to estimate the contribution of HbF to the total hemoglobin levels. As shown in Table 2, the HbF/HbF + HbA ratio for erythroid cells derived from CB $CD34^+$ cells was approximately 6.5- and 4.5-fold higher than that obtained for adult PB-derived cells, providing evidence for the neonatal origin of CB hematopoietic progenitors. The HbF/HbF + HbA ratio for the erythroid cells derived from SVF progenitors was between the levels obtained for the neonatal- and adult-derived cells.

In addition to our analysis at the cellular level, the expression of the globin gene was also examined. Interestingly, when the Ct values of the globin genes were normalized against *GAPDH* expression, the globin transcript levels in the erythroid cells derived from SVF or from CB varied widely. Therefore, three groups were defined on the basis of α-globin transcript levels (designated as Ⅰ, Ⅱ and Ⅲ in Figure 4). We observed that the level of α-globin mRNA was always higher than that of β-globin in the erythroid cells generated from $CD34^+$ cells from hematopoietic tissues (either CB or PB) (Figure 4A and D), whereas similar levels of α- and β-globin mRNA were observed in the SVF-derived erythroid cells (Figure 4B and C). As expected, the highest and lowest levels of γ-globin transcripts corresponded to neonatal- and adult-derived erythroid cells, respectively, whereas the erythroid cells generated from SVFs expressed γ-globin mRNA at levels ranging between those observed in the CB- and PB-derived cells (Figure 4A-D).

The globin chain mRNA ratios were also calculated. As shown in Figure 5A, the α/(β + γ) globin ratio was 2.55 for the PB-derived cells and 0.55, 1.15 and 2.50 for CB-derived groups Ⅰ, Ⅱ and Ⅲ, respectively. Additionally, the γ/(β + γ) globin ratios were 0.74, 0.57 and 0.56 for groups Ⅰ, Ⅱ and Ⅲ, respectively, though this ratio decreased to 0.04 in the PB-derived cells (Figure 5B).

When erythroid cells derived from the SVF were analyzed, the α/(β + γ) globin gene mRNA ratios were very similar to those obtained in the CB-derived cells from groups Ⅰ and Ⅱ, ranging from 0.54 to 1.11 and from 0.56 to 0.87 in the cells derived from $CD45^-$ cells or $CD45^+$ cells, respectively (Figure 5A). However, the γ/(β + γ) globin gene ratios were between those obtained for the CB- and PB-derived cells (Figure 5B).

### *Cellular distribution of hemoglobin in erythroid cells derived from SVF is not affected by serum deprivation*

An analysis of the hemoglobin expression profile over the culture period showed that the proportion of $HbF^+HbA^-$ cells gradually decreased and that $HbF^+HbA^+$ and $HbF^-HbA^+$ cells increased in the erythroid colonies generated from CB $CD34^+$ progenitor cells (Figure 6A). Indeed, at the end of the culture period, the cellular distribution of hemoglobin was very similar to that exhibited by circulating erythrocytes in CB/placenta (Figure 3D). Therefore, this culture system recapitulates the neonatal pattern of hemoglobin expression *in vivo*. However, the cellular distribution of hemoglobin in the adult PB-derived erythroid colonies changed only slightly during the culture period. In contrast, in the cells derived from SVF, the most notable effect was a gradual decrease in the proportion of $HbF^+HbA^-$ cells, which was accompa-

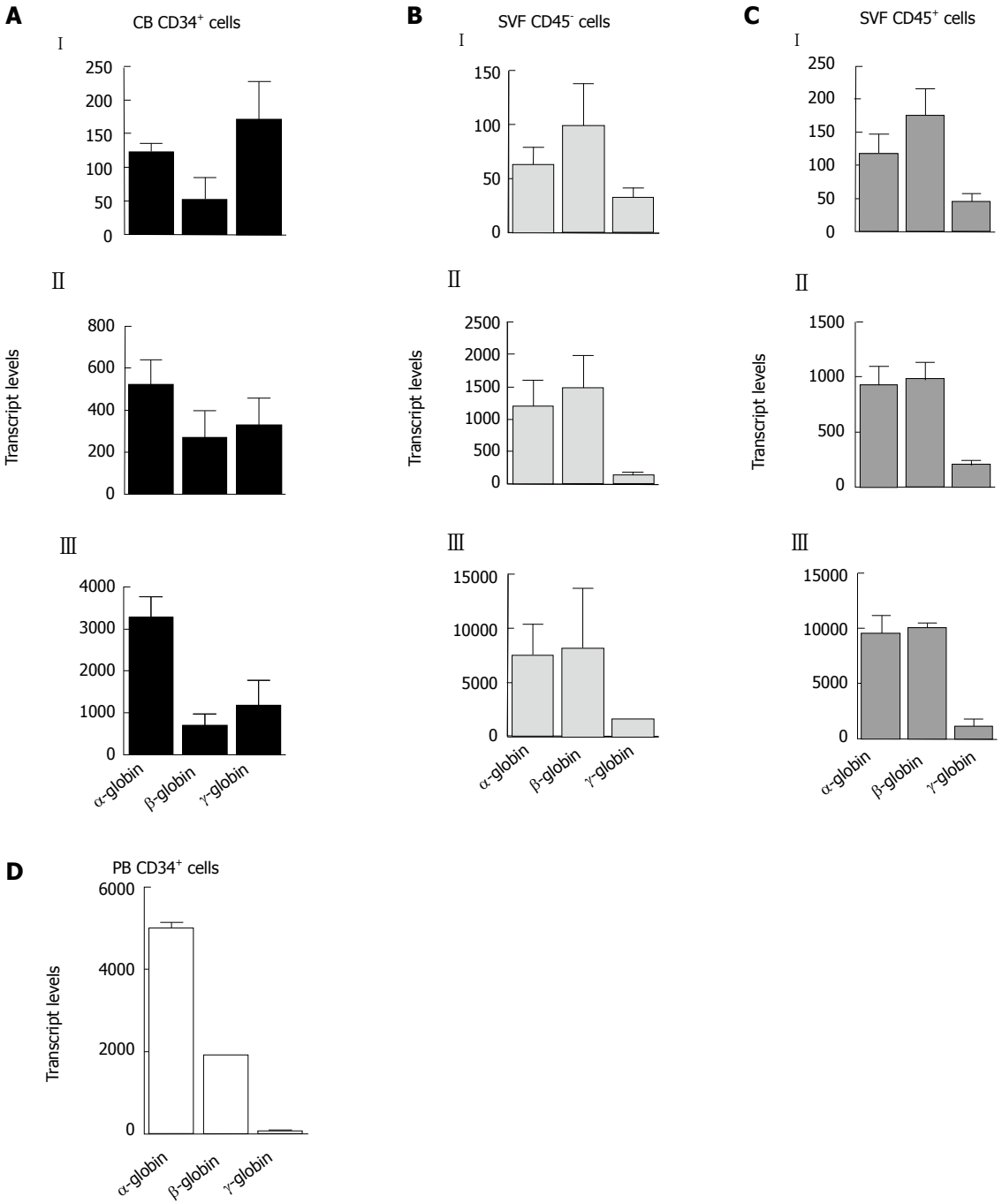


**Figure 4 Analysis of globin gene expression in erythroid cells.** $CD45^+$ and $CD45^-$ cells isolated from the stromal vascular fraction (SVF) and $CD34^+$ cells from cord blood (CB) or adult peripheral blood (PB) were cultured in a methylcellulose-based medium, and burst-forming units-erythroid -derived erythroid cells were isolated at day 15 of culture to determine globin gene expression by reverse transcription-polymerase chain reaction. The transcripts were normalized to glyceraldehyde-3-phosphate dehydrogenase. Based on the α-globin levels, the values obtained for SVF- and CB-derived cells were placed into three groups (Ⅰ, Ⅱ and Ⅲ). A: CB $CD34^+$ cells, $n$ = 10; B: SVF $CD45^-$ cells, $n$ = 17; C: SVF $CD45^+$ cells, $n$ = 17; D: PB $CD34^+$ cells, $n$ = 4. All samples were assayed in duplicate.

nied by an increase in the proportion of the $HbF^-HbA^+$ cells. Therefore, at the end of the culture period, a large proportion of $HbF^+HbA^+$ cells continued to be observed in the erythroid colonies derived from both adult PB and SVF hematopoietic progenitors (Figure 6A).

The effect of serum deprivation and hypoxic conditions on hemoglobin expression was also examined. As shown in Figure 6B, when erythroid colonies derived from CB or PB $CD34^+$ cells were developed in serum-free medium, a switch from HbF to HbA production was observed at the cellular level. Thus, the proportion of $HbF^+HbA^+$ cells was significantly decreased, whereas the proportion of $HbF^-HbA^+$ cells increased. However, no change in the proportion of $HbF^+HbA^-$ cells was observed in the CB-derived colonies. Under these conditions, the cellular distribution of hemoglobin in the adult-derived erythroid cells was nearly identical to that observed in the circulating adult erythrocytes (Figure

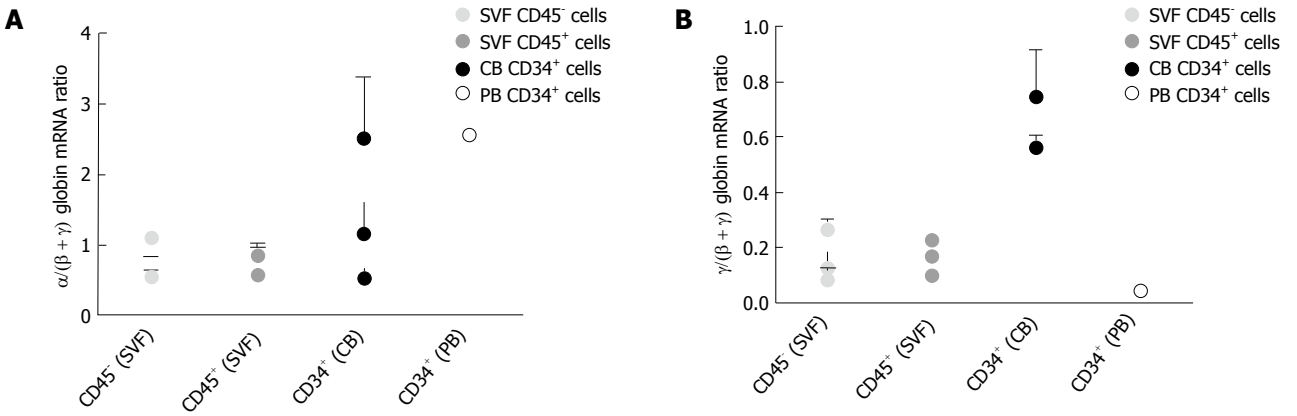


**Figure 5 Ratio of α to (β + γ) and of γ to (β + γ) globin mRNA in erythroid cells.** The values of transcript levels corresponding to the samples analyzed in Figure 4 were used to calculate the α/(β + γ) globin ratios (A) and γ/(β + γ ) globin ratios (B). The results are expressed as the mean ± SE. SVF: Stromal vascular fraction; CB: Cord blood; PB: Peripheral blood.

3D). In sharp contrast, serum deprivation did not induce significant changes in the cellular distribution of hemoglobin in the erythroid colonies generated from SVF progenitors (Figure 6B). Serum deprivation also induced a dramatic reduction in the mRNA levels of all globins: the α- and β-globin transcript levels were reduced by approximately 90%-95% in erythroid cells derived from all three tissue sources. However, although the γ-globin transcript level was reduced by 95% in the SVF-derived erythroid cells, its level was decreased by 75% and 85% in the CB- and PB-derived cells, respectively.

Lastly, the effect of a low concentration of oxygen (5% $O_2$) on hemoglobin synthesis was studied. As shown in Figure 6B, the erythroid colonies generated from CB $CD34^+$ cells exhibited the same hemoglobin profile in both normoxia (20% $O_2$) and hypoxia (5% $O_2$). However, hypoxia induced a significant decrease in the proportion of $HbF^+HbA^+$ cells in the SVF erythroid cells, though no significant changes in HbF-expressing cell populations were observed.

When globin gene expression was analyzed, the results showed that the β-globin transcript level was significantly reduced (by approximately 55%, $P < 0.05$) in the CB-derived erythroid cells, whereas α- and γ-globin mRNA levels were only slightly decreased (by 20% and 25%, respectively). In contrast, α-globin expression was reduced (by approximately 45%, $P < 0.04$) in the erythroid cells derived from SVF, whereas the expression levels of β- and γ-globins were decreased and increased, respectively, by approximately 25%.

## DISCUSSION

It is widely accepted that human hematopoietic stem and progenitor cells reside in the lineage $(Lin)^-$ fraction and are enriched in $CD34^+$ cells that also express CD45 at moderate levels[13]. However, $Lin^-CD34^-CD45^-$ primitive hematopoietic cells with hematopoietic activity *in vivo* (with or without clonogenicity in *in vitro* assays) have recently been identified[14-18]. Although bone marrow is the primary site of hematopoiesis in adult humans, our group first described the presence of $CD45^-$ cells with hemangioblastic properties in human adipose tissue SVF[11]. Indeed, the finding that hematopoietic progenitors exist in adipose tissue is very exciting.

The present study shows that CD34-expressing cells are responsible for the ability of SVF-derived $CD45^-$ cells to produce CFUs and that the clonogenic efficiency of $CD45^-$ cells is significantly lower than that of their counterpart $CD45^+$ cells. It has been proposed that adipose tissue in mouse models of obesity and in obese human subjects is in a hypoxic state[19,20]; thus, unlike circulating hematopoietic progenitors, hematopoietic progenitors in SVF may be exposed to mild hypoxia. Although hematopoietic stem cells are better preserved in culture under low $O_2$ conditions compared to 20% $O_2$[21-23] and hypoxia has been shown to be essential for the proliferation of embryonic hematopoietic progenitors[24], it remains controversial whether hematopoietic progenitors are better preserved or can be expanded under hypoxic conditions[23,25]. Herein, we show that hematopoietic progenitors derived from the SVF $CD45^-$ cell fraction, but not those derived from the SVF $CD45^+$ cell fraction, were expanded under hypoxic conditions. These results could indicate that $CD45^-$ progenitors are at an earlier stage of differentiation than $CD45^+$ progenitors.

In support of this hypothesis, the $CD45^+$ cells expressed much higher levels of *SCL/TAL1*, *GATA2*, *RUNX1* and *RUNX2* compared to $CD45^-$ cells, which is consistent with their clonogenic potential. *RUNX1* and *RUNX2* mRNA levels were also significantly increased in the adult $CD34^+$ cells compared to neonatal $CD34^+$ cells. Because purified $CD34^+$ cells include both hematopoietic stem and progenitor cells, these differences in gene expression could be explained by the higher number of proliferating hematopoietic progenitor cells in PB, as previously suggested[26-28]. However, *GATA1* was unexpectedly not detected in either SVF population, in contrast to what was observed in the $CD34^+$ cells from adult PB or CB. This finding was very surprising because *GATA1*,

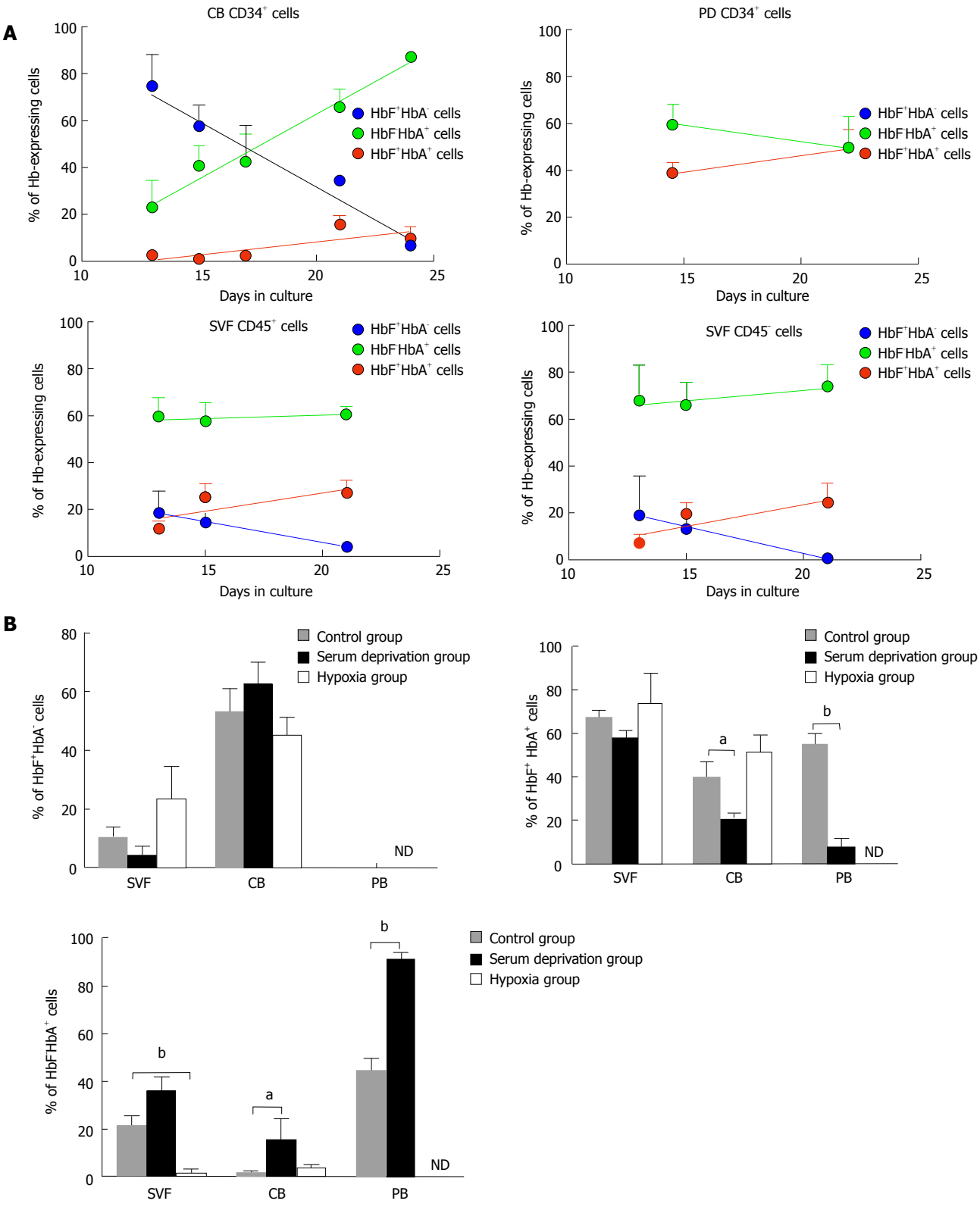


**Figure 6 Effects of time and cell culture conditions on the pattern of hemoglobin expression in erythroid cells.** Individual burst-forming units-erythroid (BFU-E)-derived colonies generated in a methylcellulose-based medium were selected, pooled and analyzed for hemoglobin expression by flow cytometry. A: The kinetics of $HbF^+HbA^-$ cells, $HbF^-HbA^+$ cells and $HbF^+HbA^+$ cells of erythroid cells in the different cultures over time are given. From top to bottom: erythroid cells derived from cord blood (CB) $CD34^+$ cells, from peripheral blood (PB) $CD34^+$ cells, from stromal vascular fraction (SVF) $CD45^+$ cells and from SVF $CD45^-$ cells. The results are expressed as the percentage of total Hb-expressing cells (CB $CD34^+$ cells, $n$ = 7; PB $CD34^+$ cells, $n$ = 4; SVF $CD45^+$ cells, $n$ = 10 and SVF $CD45^-$cells, $n$ = 10). The data are provided as the means ± SE; B: A parallel series of cultures were performed to examine the effect of serum deprivation and hypoxia (5% $O_2$) on hemoglobin expression. Control cultures were grown under conditions of normoxia (20% $O_2$)) using a serum-containing medium . SVF cells, $n$ = 10; CB $CD34^+$ cells, $n$ = 10; PB $CD34^+$ cells, $n$ = 4. BFU-E-derived erythroid cells were analyzed by flow cytometry for hemoglobin composition at day 15 of culture. The results are expressed as the percentage of total Hb-expressing cells. The data are provided as the mean ± SE. $^aP<0.02$, $^bP<0.0001$. ND: Not detected. CB: Cord blood; PB: Peripheral blood; SVF: Stromal vascular fraction.

a late hematopoietic transcription factor, is directly involved in erythropoiesis[29] and the SVF cells were able to generate erythroid colonies. Importantly, after seven days in culture under conditions that facilitate the expansion of hematopoietic progenitor cells, *GATA1* was detected in the SVF-derived cells and was overexpressed in the $CD34^+$ neonatal cells. Moreover, *GATA1* expression was higher in the PB $CD34^+$ cells than in neonatal $CD34^+$ cells, which was expected because GATA1 is upregulated in the later stages of commitment and differentiation[30-32]. Although *GATA1* and *GATA2* are essential in embryonic and adult hematopoiesis and the expression of GATA2 overlaps that of GATA1 in hematopoietic lineages[33], there are substantial differences between the GATA2 and GATA1 activities. *GATA2* is highly expressed in quiescent hematopoietic stem cells and is necessary for their maintenance and expansion[34-36], whereas *GATA1* is required for erythroid differentiation[29,32,36]. It has also been reported that the hematopoietic GATA factors are not functionally equivalent during adult hematopoiesis[37]. Therefore, differences in the expression levels of these key hematopoietic transcription factors between $CD34^+$ cells from hematopoietic tissues and SVF cells lead us to suggest that these progenitors have a different origin.

To further characterize the hematopoietic function of the SVF-derived cells, we utilized the results from the study of BFU-E-derived colonies because the composition of hemoglobin in erythroid cells varies depending on the origin of the hematopoietic progenitors and their ontogenic stage[38-40]. $HbF^+HbA^-$ cells were not only the most abundant cells in CB-derived erythroid colonies, but HbA was expressed at significantly lower levels in these cells compared to the adult PB-derived cells, indicating their early ontogenic stage. However, unlike the observations in the PB-derived erythroid cells, HbF was expressed by the majority of the erythroid cells produced by SVF progenitors, and γ-globin mRNA was more highly expressed in these cells than in the PB-derived cells.

Several important findings support the hypothesis that SVF hematopoietic progenitors may not have originated in the bone marrow. The first of these findings indicated that the mRNA level of α-globin was always higher than that of β-globin in the erythroid cells derived from hematopoietic tissues, which may be interpreted as a signature of their hematopoietic origin[41]. In contrast, α- and β-globins were synthesized at similar levels in SVF-derived cells.

The second of these findings is based on the different patterns of hemoglobin "switching" in response to culture conditions. As expected based on previous studies showing the serum-induced reversal of the hemoglobin switch[42-46], the erythroid progenitors from both adult and neonatal origin gave rise to a significantly higher proportion of $HbF^+HbA^+$ cells and a lower proportion of $HbF^-HbA^+$ cells when cultured in serum-containing medium. However, the profile of the hemoglobin synthesized by erythroid progenitors from the SVF was nearly identical in the presence or absence of serum.

Additionally, it is largely known that low oxygen concentrations affect erythropoiesis and the synthesis of the HbF[25,47-49]. Furthermore, in erythroid cells derived from erythroid progenitors from bone marrow or PB, it has been reported that the increase in HbF associated with hypoxia is concomitant with the early induction of γ-globin mRNA, and, in some circumstances, with the reduction of the β-globin mRNA, most likely as a consequence of the presence or absence of serum in the culture medium[49,50]. However, it has also been proposed that fetal and neonatal cells produce HbF irrespective of oxygen concentration[49]. Herein, we show that, although the neonatal-derived erythroid cells generated displayed the same hemoglobin profile under 20% $O_2$ or 5% $O_2$, importantly, β-globin expression was reduced by 55% in hypoxia, whereas γ-globin expression was not affected. However, in sharp contrast to the reactivation of HbF observed in the erythroid cells derived from adults, hypoxia did not induce an increase in the proportion of cells expressing HbF among the erythroid cells derived from SVF; rather, hypoxia caused a decrease in the number of cells expressing only HbA. A significant reduction in the level of α-globin, but not β-globin, was also observed.

Taken together, these results indicate the presence of early erythroid progenitors within the SVF and show that there are both differences in the regulation of globin gene expression at the transcriptional level and differences in the mechanisms that control the hemoglobin switch in these cells when compared to erythroid cells derived from hematopoietic tissues. Although this finding suggests that the origin of the early erythroid progenitors and therefore the origin of hematopoietic progenitors within the SVF is the adipose tissue rather than the hematopoietic tissue, this result is not conclusive. We cannot rule out that the adipose tissue niche might influence epigenetic patterns and the tissue-specific regulation of globin gene expression and hemoglobin production.

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## COMMENTS

### Background

The authors' previous observation of the existence of a small subset of $CD45^-$ cells in the stromal vascular fraction (SVF) of human adipose tissue with hemangioblastic properties *in vitro* and with the ability to produce hematopoietic colony-forming units led to the determination of the molecular and functional features of the hematopoietic progenitors contained in SVF.

### Research frontiers

In this study, the authors demonstrated that the cellular distribution of hemoglobin and globin gene expression in the erythroid cells produced by the early erythroid progenitors in SVF are different from that observed in the erythroid cells derived from $CD34^+$ hematopoietic progenitors from cord blood and adult peripheral blood, indicating a different origin of these cells. However, it is also possible that the adipose tissue niche might influence epigenetic patterns. Further studies on the basic biology of adipose-derived primitive mesodermal cells

and the adipose tissue niche may be important for a better understanding of adult stem cells and for cell-based therapies.

### Innovations and breakthroughs

Adipose tissue, similar to bone marrow, is derived from the embryonic mesoderm and contains a highly heterogeneous stromal cell population; however, unlike bone marrow, adipose is not a hematopoietic tissue. To our knowledge, this is the first report to demonstrate the existence of hematopoietic progenitors in human adipose tissue and reveal their characteristics at the cellular and molecular levels.

### Applications

Evidence is provided that the erythroid progenitors contained in human adipose tissue are more primitive than those in the circulation and that the regulation of globin gene expression is tissue-specific. This study may provide new insights that will be helpful for elucidating the potential of adipose-derived primitive mesodermal cells and for understanding the characteristics and function of the adipose stem cell niche.

### Terminology

Hematopoietic stem cells are responsible for the maintenance of all blood cell types and are characterized by their self-renewal capacity. These cells give rise to multipotent, oligopotent and unipotent hematopoietic progenitor cells. Burst-forming units-erythroid is the earliest erythroid progenitor responsible for erythrocyte generation.

### Peer review

In the manuscript, the authors demonstrated that stromal vascular fraction of human adipose tissues expressed fetal hemoglobin. This is an interesting study. The methods of this study are reasonable and results are clear.

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