Human adipose tissue contains erythroid progenitors expressing fetal hemoglobin

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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.

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Key words: Hemoglobin; Adipose tissue; Stromal vascu-
lary fraction; Erythroid cells; Hematopoietic progenitors

Core tip: Stromal vascular fraction (SVF) from human adipose tissue contains mesodermal precursors with the ability to form mixed hematopoietical 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[3,4]. Therefore, angiogenic factors could be potential targets for regulating fat cell development[5]. Because bone marrow-derived endothelial progenitor cells do not contribute significantly to neovascularization[6], 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[6]. 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[7]. 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 hematopoietical colonies[8]. 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[9]. Because adipose tissue is not hematopoietical in origin, it is possible that these CFUs are generated by circulating hematopoietic progenitors that target adipose tissue[10]. 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 liposuction 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 x 10⁶ cells/mL, and the CB cells were plated at 5 x 10⁵ cells/mL. The cultures were incu-
the semisolid cultures, pooled and washed. The cells were scored according to their morphology using an inverted plated at 150 cells per dish. The cultures were incubated at 5 × 10⁶ gies). Isolated or liquid-cultured SVF cells were plated H4435 or Methocult SF H4436; STEMCELL Technology. Representative dot plots of selected cells are shown. The numbers sets 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 correspond - ing antigen. SVF: Stromal vascular fraction.

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 × 10⁵ cells per dish, and purified CD34⁻ cells were plated at 150 cells per dish. The cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and either 20% O₂ or 5% O₂. After seven days, the cells were harvested, counted and used for gene expression assays and clonogenic potential determination.

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, glycoporphin A (CD235A) conjugated to allophtycocyanin, 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 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. 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⁻ⁿ. 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 progeni - tor cells in human adipose tissue, SVF cells were separat - ed 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).
CD34+ 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 10^7 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 (CD45+ cells); cultured cells (CD45+ cells vs their corresponding isolated cells).

### Hematopoietic transcription factors are expressed in SVF 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% O2), the number of hematopoietic progenitors contained in the CD45− cell subset increased 3-fold over that under the normoxic condition (20% O2).

#### Table 1 Number of CFUs per 10^7 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 | Without serum |
| CD45+ cells   |          |          |          |          |
| Total CFUs    | 1.53 ± 0.26 | 1.55 ± 0.33 | 1.79 ± 0.42 | 10.13 ± 3.19 |
| CD45− cells   |          |          |          |          |
| Total CFUs    | 0.25 ± 0.05 | 0.30 ± 0.07 | 0.25 ± 0.21 | 0.67 ± 0.16 |

The CD45− cells from hematopoietic tissues and found that SCL/TAL1 and RUNX1 mRNAs were expressed at higher levels in hematopoietic CD34+ cells than in 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 2](https://www.wjgnet.com)
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+) 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 unchanged in both the CB cells and SVF CD45+ cells, its expression was slightly increased in the SVF CD45+ cells (Figure 2B).

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.
on the basis of α-globin transcript levels (designated as θ levels in the erythroid cells derived from SVF or from malized against

ingly, when the Ct values of the globin genes were nor -

between the levels obtained for the neonatal- and adult-

for the erythroid cells derived from SVF progenitors was

4.5-fold higher than that obtained for adult PB-derived

Table 2, the HbF/HbF + HbA ratio for erythroid cells

expression, the globin transcript

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 progeni-
tors 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 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 ex-

expression of the globin gene was also examined. Interest-

ingly, when the Ct values of the globin genes were nor-

malized against G-APDH 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. Addition-

ally, 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 ana-

lyzed, 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-

| CB CD34+ cells | HbF | HbA | HbF/HbF+HbA |
|----------------|-----|-----|-------------|
| A | 124082 ± 10917 | 129077 ± 9245 | 0.96 ± 0.14 |
| B | 25171 ± 3211 | 24544 ± 2598 | 1.02 ± 0.14 |

| PB CD34+ cells | HbF | HbA | HbF/HbF+HbA |
|----------------|-----|-----|-------------|
| A | 90164 ± 23126 | 93478 ± 2049 | 0.97 ± 0.14 |
| B | 23495 ± 1326 | 24235 ± 1429 | 0.96 ± 0.14 |

| SVF CD45+ cells | HbF | HbA | HbF/HbF+HbA |
|----------------|-----|-----|-------------|
| A | 124082 ± 10917 | 129077 ± 9245 | 0.96 ± 0.14 |
| B | 25171 ± 3211 | 24544 ± 2598 | 1.02 ± 0.14 |

| SVF CD45− cells | HbF | HbA | HbF/HbF+HbA |
|----------------|-----|-----|-------------|
| A | 90164 ± 23126 | 93478 ± 2049 | 0.97 ± 0.14 |
| B | 23495 ± 1326 | 24235 ± 1429 | 0.96 ± 0.14 |

The mean fluorescence intensity values are expressed as the mean ± SE and correspond to the samples analyzed in Figure 2. Statistical significance: ‘P < 0.05, ‘P < 0.01 vs PB CD34+ cells; ‘P < 0.05, ‘P < 0.01 vs CB CD34+ cells; ‘P < 0.05 CD45+ cells vs CD45− cells. SVF: Stromal vascular fraction; CB: Cord blood; PB: Peripheral blood; HbF: Fetal hemoglobin; HbA: Adult hemoglobin.
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 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.
Recently been identified hematopoietic cells with hematopoietic activity. Progenitor cells reside in the lineage (Lin) fraction and are enriched in CD34+ cells that also express CD45 at moderate levels. However, Lin CD34 CD45+ primitive hematopoietic cells with hematopoietic activity in vivo (with or without clonogenicity in in vitro assays) have recently been identified. 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. 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, 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 O2 conditions compared to 20% O2, hypoxia has been shown to be essential for the proliferation of embryonic hematopoietic progenitors, and it remains controversial whether hematopoietic progenitors are better preserved or can be expanded under hypoxic conditions. 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. 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 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.
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% O2) on hemoglobin expression. Control cultures were grown under conditions of normoxia (20% O2)) 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. *P<0.02, *P<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 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. Although GATA1 and GATA2 are essential in embryonic and adult hematopoiesis and the expression of GATA2 overlaps that of GATA1 in hematopoietic lineages, 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, whereas GATA1 is required for erythroid differentiation. It has also been reported that the hematopoietic GATA factors are not functionally equivalent during adult hematopoiesis. 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. HbF/HbA cells were not only the most abundant cells in CB-derived erythroid colonies, but HbA was expressed at significantly lower levels in most abundant cells in CB-derived erythroid colonies, and GATA1 activities.

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. 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, the erythroid progenitors from both adult and neonatal origin gave rise to a significantly higher proportion of HbF/HbA2 cells and a lower proportion of HbF/HbA3 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. 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. However, it has also been proposed that fetal and neonatal cells produce HbF irrespective of oxygen concentration. Herein, we show that, although the neonatal-derived erythroid cells generated displayed the same hemoglobin profile under 20% O2 or 5% O2, 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.
In the manuscript, the authors demonstrated that stromal vascular fraction of adipocytic lineage cells can differentiate into hematopoietic and endothelial cells. Burst-forming units-erythroid is the earliest erythroid progenitor responsible for erythrocyte generation.

**Peel 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.

**REFERENCES**

1. Crandall DL, Hausman GJ, Kral JG. A review of the microcirculation of adipose tissue: anatomic, metabolic, and angiogenic perspectives. Microcirculation 1997; 4: 211-232 [PMID: 9219215]

2. Hausman GJ, Richardson RL. Adipose tissue angiogenesis. J Anim Sci 2004; 82: 925-934 [PMID: 15032451]

3. Planat-Beard V, Silvestre JS, Cousin B, André M, Nibbelink M, Tamarat R, Clergue M, Manneville C, Saillan-Barreau C, Duriez M, Tedgui A, Levy B, Pénicaud L, Castella L. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation 2004; 109: 656-663 [PMID: 14734516]

4. Lijnen HR. Angiogenesis and obesity. Cardiovasc Res 2008; 78: 286-293 [PMID: 18006485]

5. Cao Y. Adipose tissue angiogenesis as a therapeutic target for obesity and metabolic diseases. Nat Rev Drug Discov 2010; 9: 107-115 [PMID: 20118961 DOI: 10.1038/nrd3055]

6. Neels JG, Thimens T, Loskutoff DJ. Angiogenesis in an in vivo model of adipose tissue development. FASEB J 2004; 18: 983-985 [PMID: 15084517]

7. Sengénes C, Miraville A, Maumus M, de Barros S, Busse R, Bouloumié A. Chemotaxis and differentiation of human adipocytic lineage cells into committed progenitors: role of stromal derived factor-1 released by adipose tissue capillary endothelial cells. Stem Cells 2007; 25: 2269-2276 [PMID: 17525324]

8. De Francesco F, Tirino V, Desiderio V, Ferraro G, D’Andrea M, Giuliano M, Libondi G, Pirozzi G, De Rosa A, Papaccio G. Human CD34+/CD90 ASCs are capable of growing as sphere clusters, producing high levels of VEGF and forming capillaries. PLoS One 2009; 4: e6537 [PMID: 19657392 DOI: 10.1371/journal.pone.0006537]

9. Miñana MD, Carbonell-Ulbers F, Mirabet V, Marín S, Encabo A. IFATS collection: Identification of hematogloplants in the adult human adipose tissue. Stem Cells 2008; 26: 2696-2704 [PMID: 18450825 DOI: 10.1634/stem-cells.2007-0988]

10. Han J, Koh YJ, Moon HR, Ryoo HG, Cho CH, Kim I, Koh GY. Adipose tissue is an extramedullary reservoir for functional hematopoietic stem and progenitor cells. Blood 2010; 115: 957-964 [PMID: 19897586 DOI: 10.1182/blood-2009-05-219292]

11. Bhatia M, Wang JC, Kapp U, Bonnet D, Dick JE. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci USA 1997; 94: 5320-5325 [PMID: 9142435]

12. Bhatia M, Bonnet D, Murdoch B, Gan OI, Dick JE. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nat Med 1998; 4: 1038-1045 [PMID: 9743397]

13. Zanjani ED, Almeida-Porada G, Livingston AG, Flack AW, Ogawa M. Human bone marrow CD34+ cells engraft in vivo and undergo multilineage expression that includes giving rise to CD34+ cells. Exp Hematol 1998; 26: 353-360 [PMID: 9546319]

14. Gallacher L, Murdoch B, Wu DM, Karanu FN, Keeney M, Bhatia M. Isolation and characterization of human CD34(-)Lin(-) and CD34(+)/Lin(-) hematopoietic stem cells using cell surface markers CD133 and CD7. Blood 2005; 99: 2813-2820 [PMID: 15779426]

15. Jay KE, Rouleau A, Underhill TM, Bhatia M. Identification of a novel population of human cord blood cells with hematopoietic and chondrocytic potential. Cell Res 2004; 14: 268-282 [PMID: 15353124]

16. Ciraci E, Della Bella S, Salvucci O, Rofani C, Segarra M, Bason C, Molinari A, Maric D, Tosato G, Berardi AC. Adult human circulating CD34+ cells can differentiate into hematopoietic and endothelial cells. Blood 2011; 118: 2105-2115 [PMID: 21715308 DOI: 10.1182/blood-2010-10-316596]

17. Sun K, Kusminski CM, Scherer PE. Adipose tissue remodeling and obesity. J Clin Invest 2011; 121: 2094-2101 [PMID: 21633177 DOI: 10.1172/JCI45887]

18. Wood IS, de Heredia FP, Wang B, Trayhurn P. Cellular hypoxia and adipose tissue dysfunction in obesity. Proc Nutr Soc 2009; 68: 370-377 [PMID: 19698203 DOI: 10.1017/S0029665109990206]

19. Ivanovic Z, Hermitte F, Brunet de la Grange P, Dazezy B, Beloc F, Lacombe F, Vezon G, Praloran V. Simultaneous maintenance of human cord blood SCID-repopulating cells and expansion of committed progenitors at low O2 concentration (3%). Stem Cells 2004; 22: 716-724 [PMID: 15342936]

20. Danet GH, Pan Y, Luongo JL, Bonnet DA, Simon MC. Expansion of human SCID-repopulating cells under hypoxic conditions. J Clin Invest 2003; 112: 126-135 [PMID: 12840067]

21. Ivanovic Z, Dello Scarpa P, Trimoreau F, Faucher JL, Praloran V. Primitive human HPCs are better maintained and expanded in vitro at 1 percent oxygen than at 20 percent. Transfusion 2000; 40: 1482-1488 [PMID: 11134568]

22. Adelman DM, Maltepe E, Simon MC. Multilineage embryonic hematopoiesis requires hypoxic ARNT activity. Genes Dev 1999; 13: 2478-2486 [PMID: 10521392]

23. Cipollechi MG, D’Ippolito G, Bernabei PA, Caporale R, Nannini R, Mariani M, Fabbiani M, Rossi-Ferrini P, Olivotto M, Dello Scarpa P. Severe hypoxia enhances the formation of erythroid bursts from human cord blood cells and the maintenance of BFU-E in vitro. Exp Hematol 1997; 25: 1187-1194 [PMID: 9328456]

24. Okumura AJ, Peterson LF, Lo MC, Zhang DE. Expression of AML/Runx and ETO/MTG family members during hematopoietic differentiation of embryonic stem cells. Exp Hematol 2007; 35: 978-988 [PMID: 17533052]

25. Ng YY, van Kessel B, Lukhorst HM, Baert MR, van den Burg CM, Bloem AC, Staal FJ. Gene-expression profiling of CD34+ cells from various hematopoietic stem-cell sources reveals functional differences in stem-cell activity. J Leukoc Biol 2004;
Navarro A et al. Fetal hemoglobin and human adipose tissue

75: 314-323 [PMID: 14634063]
28 Ichikawa M, Goyama S, Asai T, Kawazu M, Nakagawa M, Takeshita M, Chiba S, Ogawa S, Kurokawa M. AML1/Runx1 negatively regulates quiescent hematopoietic stem cells in adult hematopoiesis. J Immunol 2008; 180: 4402-4408 [PMID: 18354160]
29 Weiss MJ, Orkin SH. Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. Proc Natl Acad Sci USA 1995; 92: 9623-9627 [PMID: 7568185]
30 Vodyanyik MA, Bork JA, Thomason JA, Slukvin II. Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. Blood 2005; 105: 617-626 [PMID: 15374881]
31 Anguita E, Hughes J, Heyworth C, Blobel GA, Wood WG, Higgs DR. Globin gene activation during haemopoiesis is driven by protein complexes nucleated by GATA-1 and GATA-2. EMBO J 2004; 23: 2841-2852 [PMID: 15215894]
32 Bresnick EH, Lee HY, Fujiiwara T, Johnson KD, Keles S. GATA switches as developmental drivers. J Biol Chem 2010; 285: 31087-31093 [PMID: 20670937 DOI: 10.1074/jbc.R110.159079]
33 Leonard M, Brice M, Engel JD, Papayannopoulou T. Dynamics of GATA transcription factor expression during erythroid differentiation. Blood 1993; 82: 1071-1079 [PMID: 8353273]
34 Venezia TA, Merchant AA, Ramos CA, Whitehouse NL, Young AS, Shaw CA, Goodell MA. Molecular signatures of proliferation and quiescence in hematopoietic stem cells. PLoS Biol 2004; 2: e301 [PMID: 15497755]
35 Tipping AJ, Pina C, Castor A, Hong D, Rodrigues NP, Lazzari L, May GE, Jacobsen SE, Enver T. High GATA-2 expression inhibits human hematopoietic stem and progenitor cell function by effects on cell cycle. Blood 2009; 113: 2661-2672 [PMID: 19168794 DOI: 10.1182/blood-2008-06-161117]
36 Tsai FY, Orkin SH. Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. Blood 1997; 89: 3636-3643 [PMID: 9160668]
37 Takahashi S, Shimizu R, Suwade N, Kuroha T, Yoh K, Ohta J, Nishimura S, Lim KC, Engel JD, Yamamoto M. GATA factor transgenes under GATA-1 locus control rescue germline GATA-1 mutant deficiencies. Blood 2000; 96: 910-916 [PMID: 10910904]
38 Chang KH, Nelson AM, Cao H, Wang L, Nakamoto B, Ware CB, Papayannopoulou T. Definitive-like erythroid cells derived from human embryonic stem cells coexpress high levels of embryonic and fetal globins with little or no adult globin. Blood 2006; 108: 1515-1523 [PMID: 16645170]
39 Qiu C, Olivier EN, Velho M, Bouhassira EE. Globin switches in yolk sac-like primitive and fetal-like definitive red blood cells produced from human embryonic stem cells. Blood 2008; 111: 2400-2408 [PMID: 18204790]
40 Zhang X, Ma YN, Zhang JW. Human erythroid progenitors from adult bone marrow and cord blood in optimized liquid culture systems respectively maintained adult and neonatal characteristics of globin gene expression. Biol Res 2007; 40: 41-53 [PMID: 17657354]
41 Lodish HF. Alpha and beta globin messenger ribonucleic acid. Different amounts and rates of initiation of translation. J Biol Chem 1971; 246: 7131-7138 [PMID: 5167019]
42 Papayannopoulou TH, Brice M, Stamatoyannopoulos G. Stimulation of fetal hemoglobin synthesis in bone marrow cultures from adult individuals. Proc Natl Acad Sci USA 1976; 73: 2033-2037 [PMID: 1064874]
43 Fujimori Y, Ogawa M, Clark SC, Dover GJ. Serum-free culture of enriched hematopoietic progenitors reflects physiologic levels of fetal hemoglobin biosynthesis. Blood 1990; 75: 1718-1722 [PMID: 1691666]
44 Bohner RM, Campbell TA, Bianchi DW. Selectively increased growth of fetal hemoglobin-expressing adult erythroid progenitors after brief treatment of early progenitors with transforming growth factor beta. Blood 2000; 95: 2967-2974 [PMID: 10779447]
45 Migliaccio AR, Migliaccio G, Brice M, Constantoulakis P, Stamatoyannopoulos G, Papayannopoulou T. Influence of recombinant hematopoietins and of fetal bovine serum on the globin synthetic pattern of human BFUe. Blood 1990; 76: 1150-1157 [PMID: 1698099]
46 Bohmer RM, Zhen D, Bianchi DW. Differential development of fetal and adult haemoglobin profiles in colony culture: isolation of fetal nucleated red cells by two-colour fluorescence labelling. Br J Haematol 1998; 103: 351-360 [PMID: 9827904]
47 Rissu A, Fabbro D, Damante G, Antonutto G. Expression of fetal hemoglobin in adult humans exposed to high altitude hypoxia. Blood Cells Mol Dis 2012; 48: 147-153 [PMID: 22260786 DOI: 10.1016/j.bcmd.2011.12.004]
48 Weinberg RS, Acosta R, Knobloch ME, Garber M, Alter BP. Low oxygen enhances sickle and normal erythropoiesis and fetal hemoglobin synthesis in vitro. Hemoglobin 1991; 15: 263-275 [PMID: 8537230]
49 Narayan AD, Ersek A, Campbell TA, Colon DM, Pidley JS, Zanjani ED. The effect of hypoxia and stem cell source on haemoglobin switching. Br J Haematol 2005; 128: 562-570 [PMID: 15686468]
50 Rogers HM, Yu X, Wen J, Smith R, Fibach E, Noguchi CT. Hypoxia alters progression of the erythroid program. Exp Hematol 2008; 36: 17-27 [PMID: 17936496]
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