

***In vitro* induction of fetal hemoglobin in erythroid cells derived from CD133⁺ cells by transforming growth factor- β and stem cell factor**

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Abstract

Increased fetal hemoglobin (HbF) in β -globin gene disorders ameliorates the clinical symptoms of the underlying disease. 5-azacytidine, butyrate and hydroxyurea, have been shown to activate γ -globin gene expression. It has also been found that hematopoietic growth factors can influence expression of γ -globin in erythroid cultures and in animal models. This study was designed to evaluate the *in vitro* effects of the stem cell factor (SCF) and transforming growth factor- β (TGF- β) on γ -globin gene reactivation of erythroid precursors derived from CD133⁺ cells *in vitro*. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analysis showed increased expression of the γ -globin transcript in cell culture groups containing either TGF- β or SCF or both as compared to control (2.2-, 2.7- and 5.5-fold, respectively) ($p < 0.01$). Production of HbF in a differentiated population was demonstrated using flow cytometry. The results of this study suggest that SCF and TGF- β warrant further evaluation as potential therapeutic drugs for the treatment of β -globin gene disorders.

Keywords: CD133⁺ cells; γ -globin; Fetal hemoglobin; SCF; TGF- β .

INTRODUCTION

Alleviation of the severity of symptoms of sickle cell disease (SCD) in patients with a hereditary persistence

of fetal hemoglobin (HPFH) phenotype has suggested that therapeutic efforts designed to elevate the level of fetal hemoglobin (HbF) would have clinical implications (Manca and Masala, 2008; Coleman and Inusa, 2007; Fathallah and Atweh, 2006).

Pharmacological induction of HbF has favorably influenced the course of disease in patients with SCD and β -thalassemia (Atweh and Schechter, 2001). Hence, efforts aimed at the clinical augmentation of HbF during adult erythropoiesis continue to be an active focus of research (Mentzer and Kan, 2001). Drug mediated HbF induction has been demonstrated for hydroxyurea (HU) (Lanzkron *et al.*, 2008), 5-deoxyazacytidine (DeSimone *et al.*, 2002), the histone deacetylase (HDAC) inhibitors sodium butyrate (NaB), trichostatin A (TSA) and adicypin (Gambari and Fibach, 2007; Witt *et al.*, 2002). Elevated HbF levels can be achieved in patients with SCD using hypomethylating agents (DeSimone *et al.*, 2002) or HDAC inhibitors (Cao and Stamatoyannopoulos, 2006; Cao H *et al.*, 2005). However, potential problems of bone marrow toxicity and teratogenicity especially in pediatric patients, complicate the use of some of these agents (Patrinos and Grosveld, 2008; Khayat *et al.*, 2006). Also, there often is a lack of correlation between *in vitro* γ -globin gene activation and HbF levels achieved *in vivo*. Therefore, efforts continue to identify additional compounds for human therapy. While even minimal increases in HbF levels are helpful in SCD, β -thalassemia requires much higher levels which are not

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reliably achieved by any of the currently used agents (Perrine, 2008).

It has long been suggested that the conditions of acute erythroid stress are associated with elevated HbF levels (Steinberg and Brugnara, 2003). Several investigators have attempted to reproduce this stress response *in vitro* using growth-related cytokines. Strong correlation between cytokine supplementation and HbF production has been reported (Gabbianelli *et al.*, 2008). Several reports revealed that the stem cell factor (SCF) (Wojda *et al.*, 2003; Gabbianelli *et al.*, 2008 and 2003) and transforming growth factor- β (TGF- β) (Bohmer, 2003; Bohmer *et al.*, 2000) or both (Bhanu *et al.*, 2005) can influence the growth and differentiation of hematopoietic cells as well as their globin switching. Since the CD133⁺ cells are more primitive and clonogenic than CD34⁺ cells (Jaatinen *et al.*, 2006). Here, the synergistic effects of SCF and TGF- β to reactivate γ -globin gene expression in cultures of erythroid precursors derived from bone marrow CD133⁺ cells were investigated.

MATERIALS AND METHODS

Hematopoietic growth factors: Recombinant human stem cell factor (SCF, or c-kit ligand; Sigma-Aldrich, St. Louis, MO, USA), recombinant human (rh)erythropoietin (EPO; R&D systems, Minneapolis, MN, USA), interleukin-3 (IL-3; Stem cell Technology, Vancouver, BC, Canada) and transforming growth factor- β (TGF- β ; PeproTech Inc., Rocky Hill, NJ, USA) were used in this study.

Isolation of mononuclear cells from bone marrow: Human bone marrow (BM) was extracted by aspiration from the posterior iliac crest of healthy adult volunteers after obtaining informed consent (Taleghani hospital, Tehran, IRAN) (age range, 22-35 years). Heparin (100 U/ml, Becton Dickinson, France) was added to prevent coagulation. The marrow was mixed with the same volume of Hank's balanced salt solution (HBSS), and layered onto Ficoll-Paque (Amersham Pharmacia, Piscataway, NJ) density gradient to deplete red blood cells (RBCs) and polymorphonuclear cells. The mononuclear cell (MNC) interface was collected, diluted into three volumes of HBSS, and pelleted at 250 rpm for 10 minutes. The cell pellet was washed twice after centrifugation.

Positive Selection of CD133⁺ cells: CD133⁺ cells were enriched using a magnetic activated cell sorting (MACS) CD133 isolation kit (Miltenyi Biotech, Germany), according to manufacturer's instruction. Briefly, 10⁷ MNCs were incubated with 400 μ l of phosphate-buffered saline (PBS, pH 7.2) supplemented with 3% (v/v) human serum as a blocking reagent for 30 min at 4°C. After washing, 100 μ l of anti-human CD133 monoclonal antibody conjugated with magnetic beads was subsequently added to the mixture and incubated for 30 min at 4°C. After incubation, the cells were washed in PBS supplemented with 2 mM EDTA and 0.5% (w/v) bovine serum albumin (BSA; Sigma-Aldrich). The cells were passed through a MiniMACS column retained in a magnetic field, and the column was washed three times with degassed PBS supplemented with 2 mM EDTA and 0.5% (w/v) BSA to remove unbound cells. Cell harvest was performed by firmly flushing out of retained fraction in using the plunger supplied with the column. Remaining erythrocytes were lysed using ammonium chloride solution 8% (w/v) (Stem cell Technologies, Canada). Then, the purified CD133⁺ cells were centrifuged for 5 min at 1200 rpm and resuspended in Iscove's Modified Dulbecco's Medium (IMDM) containing 2% (v/v) fetal calf serum (FCS). Cell count and viability were measured with a hemocytometer using trypan blue. The cells were analyzed by flow cytometry using the monoclonal anti-CD133 antibody (clone, AC141; Miltenyi Biotech, Germany) to assess the purity of separated cells. Finally, 1-3 \times 10⁴ CD133⁺ cells with 92-95% purity were obtained from 10⁷ MNCs.

Cell culture and induction of HbF: CD133⁺ cells were cultured in a medium consisting of IMDM, 30% (v/v) fetal bovine serum (FBS) (Cambrex, Belgium), 1% (w/v) BSA, 3 μ g/ml of insulin, 70 μ g/ml of iron-saturated transferrin, 2 mM L-glutamine, β -mercaptoethanol (10⁻⁵ M) penicillin (100 U/ml) and streptomycin (100 μ g/ml). The medium was further supplemented with 4 U/ml of recombinant human EPO. For attaining maximum HbF production, the cultures were treated with 1.25 ng/ml of rhTGF- β within the first four days of culture or 50 ng/ml of rhSCF after seventh days or both were used, as described by Bohmer (2003) and Wojda *et al.* (2003). The refeeding of medium was performed twice a week. Four groups with different cytokine treatments [(EPO only (E); EPO+SCF (ES); EPO+TGF- β (ET) and EPO+SCF+TGF- β

(EST)] were considered to evaluate HbF inducing activity. After fourteen days of incubation at 37°C and 5% CO₂, the cells were analyzed. CD133⁺ cells from 6 donors were cultured separately for this study.

Hematopoietic colony assay: Methylcellulose medium (MethoCult H4230, Stem Cell Technologies Inc., Vancouver, BC, Canada) was prepared in 5.0 ml tubes, each containing 10 ng/ml of interleukin-3 (Stem cell Technologies) and 3 U/ml of erythropoietin (R&D systems) in addition to TGF-β, SCF or both in the experimental groups. Also, appropriate dilutions of stock reagents were added to each tube of MethoCult. Purified CD133⁺ cells were resuspended in IMDM containing 2% (v/v) FCS. 1.5×10⁴ CD133⁺ cells were added to each tube. After deep mixing, the 1.1 ml of the mixture was plated in triplicate onto each of the 35-mm pre-tested Petri dishes (Stem Cell Technologies Inc.) at 1.1 ml. Cultures were grown in an incubator with a fully humidified atmosphere of 5% CO₂, at 37°C. Total (erythroid, myeloid and mix) and erythroid colonies were scored on day 14 using a phase-contrast inversion microscope and a checkered scoring dish (Stem Cell Technologies Inc).

Flow cytometry: For HbF detection, purified antibody against γ-chain of HbF (IQ-Products, Groningen, The Netherlands; IQP-172P) and FITC conjugated anti-mouse antibody (IQ-Products, The Netherlands; IQP-190F) (IQP-190F) were used according to the manufacturer's instructions. FITC-conjugated mouse IgG1 antibody (IQ-Products, the Netherlands; IQP-191F) was used as an isotype matched control. After labeling, the cells were washed and suspended in 1% (w/v) paraformaldehyde. The cells were processed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). WinMDI 2.8 software (Joseph Trotter, Scripps, San Diego, CA) was used to create the histograms. Dead cells were gated out with a forward versus side scatter window and then with propidium iodide staining.

Semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR): Semi-quantitative RT-PCR was performed to assess the expression of γ- and β-globin genes. To selectively amplify cDNA, the primers were designed such that at least one primer from a pair spans an exon junction. After fourteen days, total cells were harvested,

gently washed in PBS and immediately prepared for RNA extraction. RNA extractions were carried out using TRI REAGENT® BD (Sigma-Aldrich), according to the manufacturer's instructions. A DNase I (EN0521; Fermentas, Lithuania) treatment was applied to remove traces of contaminating DNA. Then, pure RNA was reverse transcribed in the presence of oligo (dT) 18 as primer, using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (K1622; Fermentas), according to the manufacturer's instructions. Reaction mixtures for PCR, included 2.5 μl of cDNA, 1X PCR buffer (AMS) (Cinnagen, Iran), 400 μM dNTPs, 0.4 μM of each primer pair and 1.25 U of *Taq* DNA polymerase (EP0403; Fermentas). The following primer sequences were used for PCR: β-globin sense primer CCT GAG GAG AAG TCT GCC GTT AC, β-globin antisense primer CCA GCA CAC AGA CCA GCA CG, γ-globin sense primer GGA GGA CAA GGC TAC TAT CA, γ-globin antisense primer GAA TTC TTT GCC GAA ATG GA, β-actin sense primer GTC CTC TCC CAA GTC CAC AC and β-actin antisense primer GGGAGACCAAAGCCTTCA. Polymerase chain reactions were accomplished on a Mastercycler gradient machine (Eppendorf, Germany). Amplification conditions were as follows: initial denaturation at 94°C for 5 min followed by 30 cycles (for β-actin 20 cycles) of denaturation at 94°C for 45 s, annealing temperatures at 56°C (γ-globin), 64°C (β-globin) and 60°C (β-actin) for 45 s, extension at 72°C for 30 s, and a final polymerization at 72°C for 10 min. Each PCR was performed under linear conditions and β-actin was used as an internal standard. Products were electrophoresed on a 3% (w/v) agarose gel. The gels were stained with ethidium bromide (10 μg/ml) and photographed on a UV transilluminator (Uvidoc, UK). The gel images were analyzed using the UVI bandmap program (Uvitec, Cambridge, UK). Band intensity was expressed as relative absorbance units. The ratio between the target RNA to be determined and β-actin was calculated to normalize variations in reaction efficiency. Semi-quantitative RT-PCR values were presented as a ratio of the signal of the specified gene divided by the β-actin signal. RT-PCR signals were averaged from three separate experiments. Statistical significance for all experiments was determined by the Student paired t-test analyses. A probability of less than 0.05 ($p < 0.05$) was considered significant.

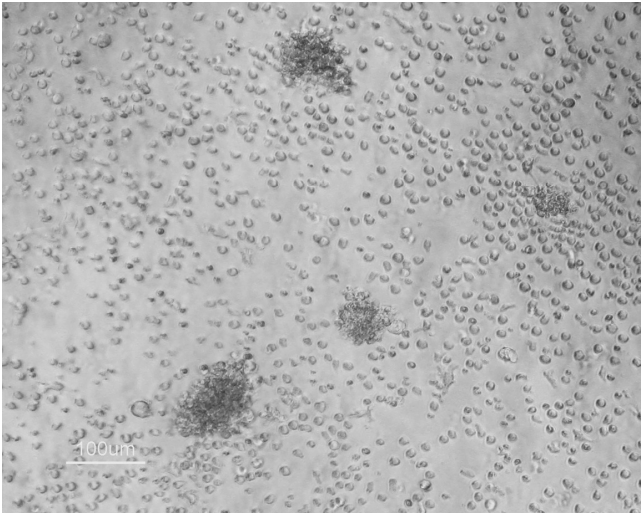


Figure 1. CD133⁺ cells in culture. CD133⁺ cells were isolated from human bone marrow using the Mini-MACS separation system. The cells were then cultured in a medium containing EPO, TGF- β , SCF or all of them (100 x).

RESULTS

Opposite effects of SCF versus TGF- β on cell proliferation and colony formation: CD133⁺ cells were isolated from human bone marrow and cultured in a medium containing EPO, TGF- β , SCF or all of them (Fig. 1). The opposite effects of SCF versus TGF- β on hematopoietic cell proliferation and colony formation were shown. As shown in Figure 2, SCF significantly increased cell proliferation (average cell count in EPO

= 4.4×10^4 cells/ml; EPO+SCF= 1.3×10^5 cells/ml; $p < 0.008$) (Fig. 2a). Adding TGF- β to the cultures had an opposite effect on growth. TGF- β significantly inhibited the proliferation of the cells compared with EPO alone (EPO= 4.4×10^4 cells/ml; EPO+TGF- β = 1.8×10^4 cells/ml; $p < 0.02$). However, with combination of three cytokines (EPO+SCF+TGF- β = 4.9×10^4 cells/ml), a slight increase in cell proliferation was observed, but the difference was not significant ($p < 0.2$). Therefore, it seems that the opposite effect of SCF and TGF- β on cell proliferation has been balanced.

Also, the effect of mentioned cytokines on total and hematopoietic colony formation was evaluated (Fig. 2b). Total colony formation was higher in the presence of SCF ($p < 0.05$) and lower in presence of TGF- β . Benzidine staining of total colonies showed that TGF- β has hematopoietic differentiating activity. Erythroid colony formation potential of TGF- β and SCF in comparison with the control (E) was significantly higher ($p < 0.001$). Again, E and EST groups had nearly identical potentials for colony formation ($p < 0.1$) (Fig. 2b). The results demonstrated the opposite effects of SCF and TGF- β on proliferation and colony formation.

Relative increase in γ -globin mRNA expression: In order to evaluate the effects of SCF and TGF- β on the expression of globin genes, the γ and β -globin transcripts were quantified using semi-quantitative RT-PCR (Fig. 3). On day fourteen, RNA was collected

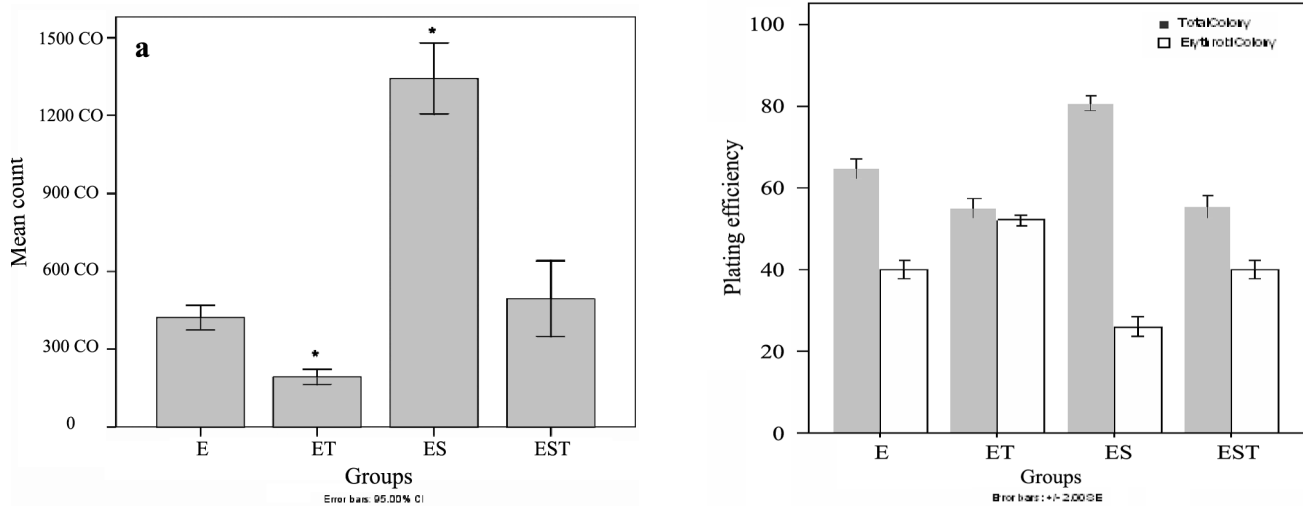


Figure 2. The effects of SCF and TGF- β on hematopoietic cell proliferation and colony formation. Total cell count (a), plating efficiency (b) in different culture groups (E=EPO; ET=EPO+TGF- β ; ES=EPO+SCF; EST=EPO+SCF+ TGF- β) after 14 days. *indicates that the obtained data in comparison with the control group (E) are significantly different ($p < 0.05$).

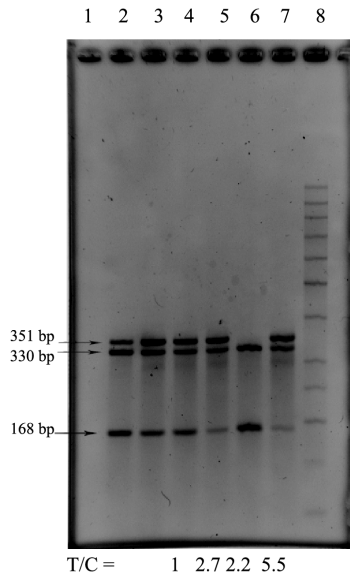


Figure 3. Semi quantitative RT-PCR. RT-PCR was performed for detection of γ (351 bp), β -globin (330 bp) and β -actin (168 bp) mRNA in hematopoietic differentiated cells [E (lane 2), ES (lane 3), ET (lane 4), and EST (lane 5)], bone marrow (lane 6) and cord blood (lane 7) as positive controls for β and γ -globin expression, respectively. Lane 1 is negative control (without reverse transcriptase (RT) and lane 8 is DNA ladder (Fermentas, Lithuania). Samples from hematopoietic differentiated groups were collected on days 14 and immediately lysed using TRI Reagent. T/C indicates the relative level of the γ -globin amplification product (T) over the β -actin amplification product (C) as calculated by UVI bandmap program.

from ET, ES and EST groups and compared with the control grown in culture containing EPO alone. Samples from bone marrow and cord blood were used as positive control for the β and γ -globin transcripts, respectively (Fig. 3, lane 6, 7). RT-PCR product without reverse transcriptase was used as a negative control (Fig. 3, lane 1).

The results of this study indicated that there was a significant change in the pattern of γ -globin gene transcription in the presence of SCF, TGF- β or both. β -actin was the housekeeping gene used for semi-quantification. Based on the relative absorbance units obtained from the UVI bandmap program and after normalization, values derived from gene expression analyses of different groups were compared. The average values from ET and ES groups showed elevation of γ -globin expression compared with EPO alone (2.2- and 2.7-fold increase, respectively) ($p < 0.01$ and $p < 0.02$). The difference between values of the E and EST groups was much higher than other groups (5.5-fold increase) ($p < 0.005$). These results were confirmed and quantified by flow cytometry for HbF. The statistical data for β -globin gene expression showed no significant differences.

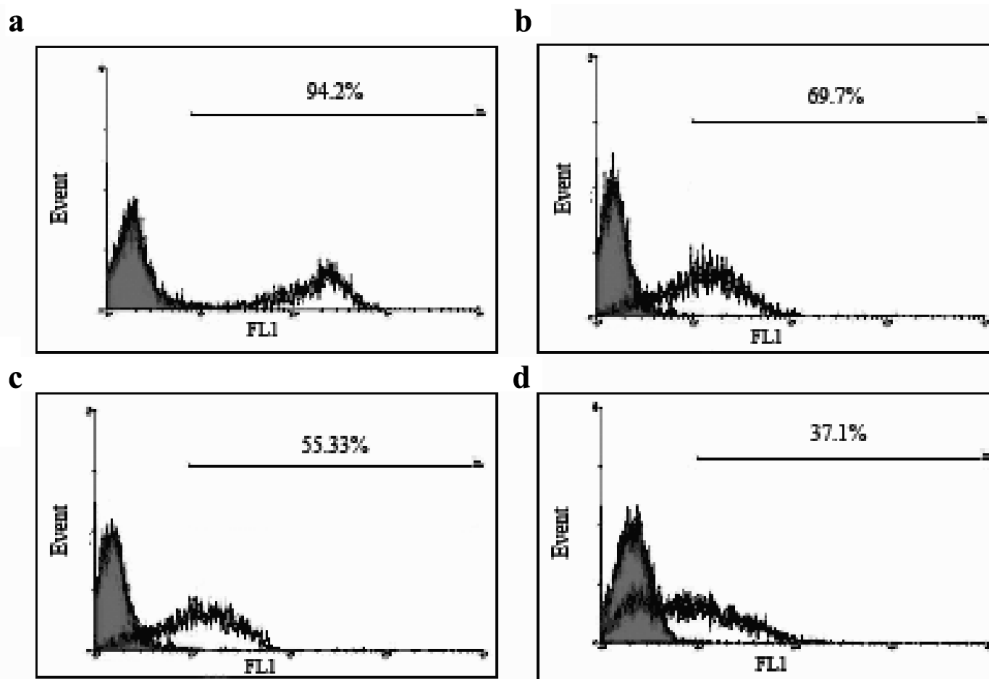


Figure 4. Cellular distribution of HbF. Flow cytometric analysis of cells cultured in EPO (d), EPO+TGF- β (c), EPO+SCF (b) or all three of them (a) for 14 days. Isotype control has been shown as a grey histogram. At least 5000 cells were analyzed in each panel. The percentage of HbF-positive cells have been shown on the top of the bar in each panel. FL1 is fluorescence channel 1 that green fluorescent (FITC) is detected in this channel.

Determination of frequencies of HbF-positive erythroblasts: Flow cytometry analysis was performed to confirm data obtained from γ -globin gene expression. Representative HbF-based histograms of the day four-teen analyses from several donors have been shown in Figure 4. SCF had a significant effect on HbF elevation. The percentage of HbF positive cells in this group was 69.7% (Fig. 4b) versus 55.3% in the ET group (Fig. 4c). It was shown that both SCF and TGF- β had a stimulating effect on HbF production. In EPO alone (as control) (Fig. 4d), 37.1% of the cells were HbF-positive. Flow cytometry results in EST showed 94.2% (Fig. 4a) HbF positive cells. Considering the final results, synergistic effects of TGF- β and SCF on HbF expression at the protein level were demonstrated. These results were also consistent with the semi-quantitative RT-PCR study.

DISCUSSION

In steady state hematopoiesis, the default program is based on low Hb F expression in erythroblast cells (Gambari and Fibach, 2007). An interesting event in erythroleukemia is HbF elevation. Also, SCF can induce HbF production. These events are related to increased proliferation associated with inhibited differentiation. In contrast, chemotherapy drugs are acting by decreased proliferation and increased differentiation. Based on our data and other researches, it can be proposed that HbF elevation in a hematopoietic stress situation results from increases in erythroid growth and differentiation as demonstrated in this study by the combination of SCF and TGF- β .

This investigation showed that the TGF- β and SCF treatments lead to dramatically increased levels of HbF-containing cells during hematopoiesis of CD133⁺ cells in culture. This effect is achieved by TGF- β and SCF treatment within the first four days and the last seven days of culture, respectively. Both TGF- β and SCF could demonstrate a reversed hemoglobin switching pattern. An increase in the proportions of F⁺ cells, initiated early in culture and measured after the first week, may be due to different mechanisms: proliferation of F⁺ cells or a selective inhibition of F-A⁺ cells. As shown in this study by semi-quantitative RT-PCR, the γ -globin transcripts increased in comparison to those of the control group. According to this research, HbF elevation during SCF or TGF- β treatments was

not solely due to Hb switching because β -globin gene expression was not significantly reduced. Bhanu *et al.* (2005) have shown an Hb switching mechanism by demonstrating the decrease of β -globin gene expression using real-time RT-PCR, their results, therefore, being more accurate and precise. Nevertheless, the data from the present study and other researches indicate a complex situation that may be explained by superposition of many effects. Wojda *et al.* (2003) have shown that an EPO supplemented culture leads to production of hemoglobinized populations of erythroblasts with low levels of HbF. Several experiments have shown that SCF can increase HbF in erythroid cells, *in vitro* and *in vivo*. Other researches have focused on the effect of TGF- β in elevation of HbF in culture. The synergistic effect of SCF and TGF- β on γ -globin induction in hematopoietic culture has been already been described (Bhanu *et al.*, 2005). In previous studies, CD34⁺ cells were used as a hematopoietic stem/progenitor cell population for *in vitro* hematopoiesis. From this study, it can be concluded that the pattern of CD133⁺ hematopoiesis and HbF accumulation in erythroid cells is similar to hematopoiesis from CD34⁺ cells. This investigation suggests that balanced increases in growth and differentiation beyond steady-state levels lead to persistent expression of HbF in adult erythroid precursor cells. Due to undesirable effects of mentioned cytokines on nonerythroid cells *in vivo*, the signaling pathways involved should be further investigated.

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