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Transcription factor SCL/TAL1 mediates the phosphorylation of MEK/ERK pathway in umbilical cord blood CD34$^+$ stem cells during hematopoietic differentiation

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ABSTRACT

Transcription factor stem cell leukemia (SCL), also known as the T-cell acute lymphocytic leukemia 1 (TAL1), plays a key role in the regulation of hematopoiesis, but the molecular mechanisms are not well understood. The aim of the present study is to elucidate the effects of the epidermal growth factor receptor (EGFR) signal pathways underlying the biologic activity of SCL/TAL1 on normal hematopoietic development. Lentiviral vectors with up or down-regulation of SCL/TAL1 were transfected into umbilical cord blood CD34 stem cells. EGFR signaling pathways (including MEK/ERK and Akt/mTOR) and surface hematopoietic markers were analyzed in the process of hematopoietic differentiation. The data revealed that up or down-regulation of SCL/TAL1 gene was accompanied positively by the expressions of p-MEK and p-ERK1/2 protein, but the changes of Akt/mTOR were unobvious. MEK/ERK inhibitor U0126 and SCL/TAL1 down-regulation showed similar inhibitory effects on erythroid, myeloid, and megakaryoid differentiation. However, Akt/mTOR pathway altered insignificantly. MEK/ERK inhibitor U0126 could not affect the expression of SCL/TAL1 mRNA or protein. Taken together, these findings fully illustrated that SCL/TAL1 is located in the up-stream of MEK/ERK pathway and partially regulates hematopoiesis by modulating the phosphorylation level of the key proteins in MEK/ERK pathway.

Introduction

Transcription factor stem cell leukemia (SCL), also known as the T-cell acute lymphocytic leukemia 1 (TAL1), is a member of the basic helix-loop-helix (bHLH) family of transcription factors that play key roles in the regulation of hematopoiesis [1,2]. It has been shown that SCL/TAL1 is essential for the development of all hematopoietic cell lineages and that the over-expression of SCL/TAL1 is the most common molecular abnormality found in human T cell leukemia. Although the role of SCL/TAL1 in adult hematopoiesis is well defined in human and mice, its precise molecular mechanism remains quite elusive [3,4]. Moreover, recent studies have suggested that the epidermal growth factor receptor (EGFR) families are required in hematopoietic regeneration and development [5,6]. Therefore, the aim of the present study was to elucidate the effect of SCL/TAL1 on normal hematopoietic development by modulating the EGFR signal pathway.

The CD34$^+$ stem cells are derived from umbilical cord blood (UCB) and belong to hematopoietic stem cells (HSCs) or hematopoietic progenitor cells. They are widely used as an in vitro model for hematopoietic differentiation researches because of the capacities of multi-lineage differentiation and colony-forming [7]. In this study, lentiviral vectors that allow high or low expression of SCL/TAL1 gene were transfected into CD34$^+$ cells sorted from human UCB cells. The roles of EGFR downstream signaling pathways, including MEK/ERK and Pten/Akt/mTOR, in the development of SCL/TAL1 mediated hematopoiesis were investigated in these cell lines with enhanced or decreased SCL/TAL1 expression. The preliminary results demonstrated that the phosphorylation level of ERK/MEK signaling pathway, but not Pten/Akt/mTOR, was positively modulated by transcription factor SCL/TAL1 and consequently played a key role in regulating the hematopoietic differentiation.

Methods

Plasmids and cell line

Plasmid pTRIPdU3-RNAiTALh-EF1a-GFP (shRNA to suppress TAL1 expression, SCL/TAL1^{low}), plasmid pTRIP-EF1a-TAL1 (cDNA to enhance TAL1 expression, SCL/TAL1^{high}), and plasmid pTRIP-dU3-RNAiluc-EF1-GFP (negative LUC control vector with enhanced GFP) were all provided by the Institute Cochin (Paris, France) [8,9]. The lentiviral packaging plasmid pCMVdeltaR8.91 containing Gag and Rev sequences were also

Abbreviations: UCB, umbilical cord blood; SCL, stem cell leukemia; TAL1, T-cell acute lymphocytic leukemia 1; bHLH, basic helix-loop-helix; EGFR, epidermal growth factor receptor; EPO, erythropoietin; HSCs, hematopoietic stem cells.

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provided by the Institute Cochin. The Stem Cell Research Lab of West China Hospital (Chengdu, China) provided Escherichia coli TOP10 as well as envelope plasmid pMD.G expressing VSV-G gene, which encodes VSVG protein to replace outer membrane protein of HIV. The batches of the three concentrated vector particles were prepared together to ensure similar transduction efficiencies. The 293FT cell line, which was derived from human embryonal kidney cells transformed with SV40 large T antigen, was brought from Invitrogen (California, USA).

Purification of hematopoietic CD34$^+$ cells

UCB samples (80–130 mL each sample) were collected from 10 healthy parturient women who had signed the informed consent. The erythrocytes were removed from the samples with 1% methylcellulose, and mononuclear cells were isolated by density gradient centrifugation with human lymphocyte separating medium Ficoll. CD34$^+$ cells were purified from mononuclear cells using immunomagnetic cell-sorting program Mini-MACS and CD34 isolation kit (Miltenyi Biotec, Paris, France) in accordance with manufacturer's instruction. The purity of CD34-selected cells was analyzed by FACScan using a FITC-conjugated monoclonal antibody (BD Company, NJ, USA). The purified CD34$^+$ cells (>95% purity) were ready for the next experiments.

Plasmids transduction and cell culture

The transduction procedure of CD34$^+$ stem cells was performed as the previous report by Ye et al. [10]. Briefly, CD34$^+$ stem cells were transduced with plasmid pTRIP-dU3-RNAiluc-EF1-GFP (LUC), pTRIPdU3-RNAiTALh-EF1a-GFP (SCL/TAL1^{low}) or pTRIP-EF1a-TAL1 (SCL/TAL1^{high}), respectively, according to our optimized procedure. Briefly, freshly purified UCB CD34$^+$ cells (10^5 cells/mL) were divided into three groups (LUC, SCL/TAL1^{low} and SCL/TAL1^{high}) and incubated separately with the three lentiviral pellets (10^7 TU) in a flat-bottomed 12-well plate with 1 ml opti-MEM (Gibco, CA, USA) containing 2 μg/mL Polybrene, 20 ng/ml thrombopoietin, 100 ng/ml stem cell factor (SCF) and 100 ng/ml FLT3 ligand (TSF). After one hour of centrifugation with 200×*g* force, the CD34$^+$ cells and the lentiviral pellets were co-cultured for 8 h in the incubator under 37 °C, 5% CO_2. The supernatant was then discarded and new lentiviral pellets (10^7 TU) were replenished for the next 48-hour co-culture. At the end of the co-culture, the transfected cells were planted in 12-well plates at a density of 500 cells per well cultured in methylcellulose semisolid medium (H4435; Stem Cell Technologies, Vancouver, Canada) containing 50 ng/mL SCF, 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), 20 ng/mL interleukin-3 (IL-3), 20 ng/mL IL-6, 20 ng/mL granulocyte colony-stimulating factor (G-CSF), and 3 units/mL erythropoietin with the ratio of 1:1 in 37 °C, 5% CO_2. The cytokines mentioned above were all purchased from Peprotech (NJ, USA). For the CD34$^+$ cells we have already achieved a transfection efficiency of over 80%.

Colony counting

The CD34$^+$ stem cells transfected with SCL/TAL1^{low}, SCL/TAL1^{high} and LUC, as well as the non-transfected CD34$^+$ stem cells (Control), were cultured as described above for 14 days. Colonies were counted and colony types, including CFU-GM, CFU-G, CFU-M, CFU-E, BFU-E, and CFU-GEMM (Mix) were identified by light microscope ($n = 10$). Atypical colonies were identified by smear and Wright's stain.

Real-time quantitative reverse transcription PCR (qRT-PCR)

The qRT-PCR was used to measure the effect of SCL/TAL1 on the mRNA expressions of PU.1, LMO1, LMO2, GATA1, GATA2, and RUNX1. The CD34$^+$ stem cells from SCL/TAL1^{low}, SCL/TAL1^{high}, LUC, and Control groups were cultured as mentioned above. On the day 0, 3, 7, and 14 of culture, the total RNA (1 μg) of each sample was reversely transcribed by 7500 Fast RT-PCR System (ABI, Vernon, CA, USA). The amplification was carried out in a total volume of 20 μl containing 0.4 μl of each primer, 0.4 μl ROX Reference Dye, 10 μl SYBR Premix Ex Taq (Takara, Liaoning, China), and 2 μl of 1:10 diluted cDNA. All the primers (Table 1) were designed and tested by Takara. PCR was prepared in duplicate and heated to 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 5 s, and extension at 72 °C for 20 s. Relative gene expression quantifications were calculated according to the comparative Ct method using β-actin as an endogenous control.

Flow cytometry

The CD34$^+$ stem cells of SCL/TAL1^{low}, SCL/TAL1^{high}, LUC, Control and U0126 (5 μmol/L) groups were cultured as mentioned above. On the day 7 of culture, 5×10^5 cells were collected and divided into 4 subgroups: A. blank control; B. erythrocyte surface marker (CD71-PE and CD235a-APC); C. granulocyte surface marked antibodies (CD33-PEcy5 and CD13-APC); D. megakaryocytes surface marked antibodies (CD41a-AP and CD42b-PEcy). The direct fluorescent antibodies (all purchased from BD Pharmingen, CA, USA) for erythroid, granulocyte, and megakaryocyte were added respectively. After 30 min of incubation, the cells were washed by PBS, and the expression of surface markers was tested by FACScan (BD Company).

Western blot analysis

The time course of the phosphorylation of MEK/ERK signaling and Akt/mTOR signaling was analyzed in CD34$^+$ cells on day 0, 7 and 14 by western blot ($n = 3$). Briefly, 1×10^7 CD34$^+$ cells from the SCL/TAL1^{low}, SCL/TAL1^{high}, LUC, and Control group were prepared in RIPA lysis buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, pH 8.0; 0.1% Triton-X100, 0.01 mg/mL aprotinin, 0.005 mg/mL leupeptin, 0.4 mM PMSF, 4 mM NaVO4). Quantitated proteins were loaded on 6% to 15% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, proteins were electro-transferred to nitrocellulose membrane. The primary antibodies are rabbit monoclonal antibodies to phospho-Akt (ser473), phospho-mTOR (ser2448), phospho-MEK1/2 (ser217/221), phospho-ERK1/2 (Thr202/Tyr204), ERK and SCL/TAL1 (all by Cell Signaling Technology, MA, USA). The membranes were incubated with the above primary antibody overnight at 4 °C. Consequently, it was washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature and finally detected by enhanced chemiluminescence (ECL) detection system and film (Bio-Rad Laboratories, CA, USA) according to manufacturer's instruction. GAPDH was used

Table 1
Primers of hematopoietic genes in qRT-PCR analysis.

Gene	Primer
SCL/TAL1	5′- AGGAGACCTTCCCCCTATGAG -3′
	3′- GGCTGTTGGTGAAGATACGC -5′
GATA1	5′- GGGCCCTGACTTTTCCAGTA -3′
	3′- AGTTGGTGCACTGAGTACCTG -5′
GATA2	5′- AGCTCCTACCCTGTAAGC -3′
	3′- GGTTCCATGTAGTTGTGCG -5′
LMO1	5′- TATCCTCGGCGCTAGTCC -3′
	3′- GCCAGTACTTGTCCAATGCC -5′
LMO2	5′- ACATTGGGGACCGCTACTTC -3′
	3′- TCATAGGCACGAATCCGCTT -5′
PU.1	5′- TTACAGGCGTGCAAAATGGA -3′
	3′- AGGGGTAATACTCGTGCGTT -5′
RUNX1	5′- CTCGAAGACATCGGCAGAAA -3′
	3′- AGGGATGGACAGAAGAACTGA -5′