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The AML1/ETO target gene LAT2 interferes with differentiation of normal hematopoietic precursor cells

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Abstract

The adaptor protein linker activator of T-cells 2 (LAT2) is a known AML1/ETO target gene whose function during normal hematopoiesis is unknown. We addressed the role of LAT2 during erythroid and myeloid differentiation of normal human CD34+ hematopoietic cells. LAT2 is expressed at low levels in CD34+ cells and upregulated during cytokine-induced myeloid and erythroid differentiation. Forced LAT2 expression leads to a delay of erythroid and myeloid differentiation keeping CD34+ cells in a more immature state, whereas LAT2 knockdown accelerates differentiation. It is tempting to speculate that by affecting the differentiation capacity of normal hematopoietic progenitors, LAT2 may contribute to the pathogenesis of AML.

Keywords

Adaptor molecule; Acute myeloid leukemia; Myeloid differentiation; Erythroid differentiation

1. Introduction

During the last years, increasing evidence showed that adaptor molecules play an important role in oncogenesis. For example, the contribution of GRB2 to the pathophysiology of chronic myeloid leukemia (CML) [1] as well as the role of GAB family proteins in breast cancer [2] and of CRKL in lung cancer [3] have been widely described. Likewise, acquired mutations of C-CBL are found in juvenile myelomonocytic leukemia (JMML) [4] and acute myeloid leukemia (AML) [5] whereas germline missense mutations of C-CBL predispose to JMML [6]. C-CBLs dual role as a tumor suppressor and an oncogene was elegantly demonstrated in hematopoietic progenitors [7].

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Conflict of interest

The authors declare no conflict of interest.

Contributions. A.E., J.D., H.L.P. and M.L. contributed to the design and analysis of this study. A.E., J.D. and S.S. performed laboratory-based experiments. A.E., J.D., H.L.P. and M.L. wrote this manuscript, and all authors contributed to and agreed on the final version.

The adaptor protein linker activator of T-cells 2 (LAT2), also called non-T-cell activation linker (NTAL), linker activator for B-cells (LAB) or Williams Beuren Syndrome Critical Region 5 (WBSCR5) is expressed in normal B cells, plasma cells, NK and monocytes [8]. Interestingly, C-CBL and phosphorylated LAT2 interact in myeloid cells [9] (reviewed by [10]).

The role of the adaptor protein LAT2 in hematological malignancies was first revealed when it was identified as a AML1/ETO (RUNX1/RUNX1T1) target gene [11]. LAT2 expression is epigenetically modulated both in the presence of AML1/ETO and by histone deacetylase (HDAC) inhibitors [12]. Recently, genome-wide screening by Chromatin immunoprecipitation (ChIP)-sequencing further confirmed AML1/ETO binding to the *LAT2* locus [13].

LAT2 is expressed in mononuclear bone marrow cells from patients with AML. LAT2 levels vary depending on the FAB subtypes, suggesting a dichotomy in the function of LAT2 in the granulocytic and monocytic lineage. Furthermore, the forced expression of LAT2 blocks all-trans retinoid acid (ATRA)- and phorbol ester (PMA)-induced myeloid differentiation [14].

Although the role of LAT2 is very well characterized in normal B- [9,15], T- [16] and mast cell function and development [17], little is known about the function of LAT2 in normal erythro- and myelopoiesis.

In this study we addressed the role of LAT2 during erythroid and myeloid differentiation of normal human CD34+ cells. We hypothesized that changes in LAT2 expression may affect the differentiation capacity of normal hematopoietic progenitors, thereby contributing to the pathogenesis of AML.

2. Materials and methods

2.1. Purification of peripheral CD34+ cells

CD34+ cells were obtained from Buffy coats of healthy donors from the University Hospital Freiburg. CD34+ cells were purified by dextran sedimentation followed by Ficoll-Paque (Pharmacia, Freiburg, Germany) separation and antibody-based magnetic bead separation (MACS; Miltenyi, Bergisch Gladbach, Germany). In order to protect the privacy of each blood donor a unique patient number (UPN) was used.

2.2. LAT2 overexpression in CD34+ cells

LAT2 cDNA was cloned into the pMYSIG-GFP retroviral vector [18], a kind gift of Dr. C. Stocking, Heinrich-Pette-Institut, Hamburg, Germany. The virus was produced in 293T cells and titrated in TE671 cells. In order to have enough CD34+ cells two or three blood donates from different volunteers were pooled and cultured for 24-h in expansion medium consisting of 20% BIT in IMDM supplemented with FLT-3 (10 ng/ml), IL-6 (20 ng/ml), SCF (100 ng/ml) and TPO (100 ng/ml). All cytokines were purchased form PeproTech. After this time period three cycles of retroviral infections were performed over a 48-h period with transduction efficiencies ranged from 25% to 60%. GFP-positive cells were than sorted by

fluorescent-activated cell sorting (FACS) and cultured either in erythroid or myeloid differentiation media or used for colony assay.

2.3. LAT2 shRNA knock down in CD34+ cells

For shRNA-mediated knockdown of the *LAT2* gene, oligonucleotides (MWG, Germany) encoding the knockdown sequence [19] as well as the loop and overhang sequences were cloned into the lentiviral LeGO-G vector (http://www.lentigo-vectors.de/vectors.htm).

2.4. LAT2 shRNA: 5′-AACCCCGTACCAGAACTTCAGCAAATTCAAGAGATTTGCTGAAGTTCTGGTACTTTTTTC-3′

Virus production was done in 293T cells followed by titration. Pooled CD34+ from two or three blood donates were used and cultured in expansion medium as described above. Then over a period of 48-h two cycles of lentivial infections were performed with transduction efficiencies ranged from 30% to 70%. GFP-positive cells were sorted by fluorescent-activated cell sorting (FACS) and cultured either in erythroid or myeloid differentiation media.

2.5. Erythroid and myeloid differentiation culture

For erythroid differentiation CD34+ cells were cultured in StemSpan® Serum-Free Expansion Medium (SFEM) supplemented with SCF (50 ng/ml; PeproTech), IL-3 (10 ng/ml; PeproTech), LDL (40 ng/ml; Harbor Bio-Products) and EPO (1 IU/ml; Erypo®). For myeloid differentiation cells were cultured in 20% BIT in IMDM supplemented with SCF (100 ng/ml; PeproTech), FLT-3 (10 ng/ml; PeproTech), IL-3 (20 ng/ml; PeproTech), IL-6 (20 ng/ml; PeproTech), GM-CSF (20 ng/ml; PeproTech) and G-CSF (20 ng/ml; Amgen). Cells were maintained at a density of 5×10^5 per ml, verified by daily counting and addition of medium.

2.6. Colony assay

2200 Cells were seeded in methylcellulose media (1000 cells/ml) containing SCF, IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF) and EPO (H4434; Stem Cell Technologies) incubated for 14 days at 37 °C, 5% CO_2. Followed incubation colonies were counted.

2.7. Western blot

LAT2 protein was detected with a mouse anti-LAT2 (ExBio, Prague, Czech Republic) and a horseradish peroxidase (HRP)-coupled anti-mouse secondary antibody (Amersham, München, Germany). To control equal loading mouse anti-β-actin (Sigma) was used. The amount of total protein, which was loaded, was 30 μg.

2.8. Flow cytometry

Cells were stained with a phycoerythrin (PE)-conjugated antiCD235a/GpA antibody (555570) as well as an allophycocyanin (APC)-conjugated anti-CD36 antibody (550956), or with a PE-conjugated anti-CD11b antibody (555388), PE-mouse IgG (555743) and APC-mouse IgG isotype controls (555576) were used, all from BD Biosciences, Heidelberg,

Germany. Analysis were done on a FACS Calibur (BD Bioscience) using both cellquest (BD Bioscience) and flowjo software (FlowJo, Ashland, OR, USA).

2.9. Statistics

Student's *t*-test was used to determine the significant difference ($P < 0.05$) between two groups.

3. Results

3.1. LAT2 is transiently upregulated during normal hematopoiesis

First, to explore the possible role of LAT2 in normal human hematopoiesis, we determined the protein expression level of LAT2 during induced granulocytic, monocytic and erythroid differentiation of normal CD34+ cells. CD34+ cells were cultured either in granulocytic differentiation medium containing G-CSF, SCF, FLT-3, IL-3 and IL-6, monocytic differentiation medium containing GM-CSF, IL-4, SCF, FLT-3, IL-3 and IL-6 or erythroid differentiation medium containing EPO, SCF, IL-3 and IL-6. Protein was harvested at different time points of culture. A transient up-regulation of LAT2 was shown, with highest protein levels on day 7 and day 9, respectively, of differentiation culture (Fig. 1), indicating that LAT2 is regulated during granulocytic, monocytic and erythroid differentiation of normal CD34+ cells. Due to low cell numbers, cells could not be harvested at early time points of erythroid differentiation.

3.2. Forced LAT2 expression delays erythroid and myeloid differentiation

To investigate the function of LAT2 on myeloid and erythroid differentiation of normal hematopoietic progenitors, we used a cell culture system of retrovirally transduced normal peripheral blood CD34+ cells. CD34+ cells were transduced with pMYSIG-LAT2-GFP (LAT2) or with an empty pMYSIG-GFP (control), GFP-positive cells, which were successfully transduced, were sorted and were cultured either in erythroid differentiation medium containing EPO, IL-3 and SCF [20] or in myeloid differentiation medium containing GM-CSF, G-CSF, FLT-3, IL-3, IL-6 and SCF [21]. Erythroid maturation was assessed by CD36 and Glycophorin A (GpA) staining and flow cytometry analysis was performed daily from day 4 to day 13 [20]. Two different cell populations were characterized, $CD36^{-}/GpA^{-}$ double negative cells and $CD36^{+}/GpA^{+}$ double positive cells. As differentiation precedes the number of double negative cells decreased, while the number of double positive cells increased (Fig. 2A). For the CD34+ cells transduced with the control-vector the appearance of mature erythroid precursor cells ($CD36^{+}/GpA^{+}$) was consistently higher and more efficient compared with LAT2-overexpressing cells (Fig. 2B). At the same time increased proportions of $CD36^{-}/GpA^{-}$ double negative cells remained in LAT2-overexpressing culture (Fig. 2C) suggesting a role of LAT2 in inhibiting erythroid differentiation.

To evaluate the effect of the forced LAT2 expression during myeloid differentiation, normal CD34+ cells transduced with LAT2-expressing and control-vector were induced to differentiate into the myeloid lineage by a cytokine cocktail. LAT2 overexpression was confirmed by Western blot analysis at day 13 of the erythroid and at day 15 of the myeloid

culture (Fig. 2D and F). From day 9 to day 15 their CD11b expression was analyzed by flow cytometry. A delay of maturation was observed in the LAT2-overexpressing cells in comparison to the control cells (Fig. 2E), confirming our previous results on the induced myeloid differentiation of leukemia cell lines [14].

3.3. LAT2 overexpression reduces colony formation

To further test the hypothesis that LAT2 overexpression delays erythroid and myeloid maturation, we next analyzed colony-forming capacity of LAT2- and control-transduced normal CD34+ cells. CD34+ cells were transduced with pMYSIG-LAT2-GFP (LAT2) or with an empty pMYSIG-GFP (control), sorted for GFP expression and are seeded in methylcellulose for 14 days to determine colony-forming potential. A significant reduction in the total colony number (CFU-GM, BFU-E, CFU-E), both myeloid colonies (CFU-GMs) and erythroid colonies (BFU-E and CFU-E) was observed (Fig. 3A). The colonies also showed changes in morphology. In the experiment with LAT2-transduced cells the colonies were notably smaller and more compact than those found in the experiment initiated form control cells (Fig. 3C). After colony counting, cells were harvested and persistent LAT2 expression was again confirmed by Western blot (Fig. 3B). These results support our findings of impaired erythroid and myeloid differentiation of LAT2 overexpressing CD34+ cells.

3.4. LAT2 knockdown accelerates erythroid and myeloid differentiation

Using the same cellular system and analysis methods, we employed the converse approach to investigate the function of LAT2 during the erythroid and myeloid differentiation by decreasing LAT2 expression in normal CD34+ progenitor cells with a shRNA. CD34+ cells were transduced with a lentiviral LeGO-G vector containing the shRNA against *LAT2* (shLAT2) or with a LeGO-G vector with a shRNA against a scrambled GFP sequence (control), sorted for GFP expression and cultured either (A–D) in erythroid differentiation medium or (E and F) in myeloid differentiation medium. Down-regulation of LAT2 expression by shRNA-knockdown was confirmed by Western blot during the later erythroid (Fig. 4D) and myeloid (Fig. 4F) differentiation.

During the earlier phase of erythroid differentiation the appearance of mature erythroid precursor cells ($CD36^+/GpA^+$) was consistently elevated in LAT2-knockdown transduced cells (Fig. 4A). Consequently the proportion of early erythroid precursor cells ($CD36^+/GpA^-$) was reduced in the same group (Fig. 4B). Only a slight decrease in the $CD36^-/GpA^-$ fraction was seen in the LAT2 knockdown culture in comparison to the control culture (Fig. 4C). These data confirm the inhibitory role of LAT2 in erythroid differentiation shown above (Fig. 2B). Concordantly, there was an increased number of CD11b positive cells in the LAT2-knockdown culture during myeloid differentiation (Fig. 4E). These data suggests that the reduction of LAT2 expression in normal CD34+ cells may accelerate erythroid and myeloid differentiation. By counting the cells every day of culture, no effect on cell survival could be detected (data not shown).

4. Discussion

The leukemia-specific chimeric transcription factor AML1/ETO (RUNX1/RUX1T1) results from one of the most common chromosomal translocation *t*(8;21) in AML [22]. We previously identified the *LAT2* gene as a novel in vivo AML1/ETO target gene. Its expression is strongly down-regulated in *t*(8;21) AML. We here confirm these results by showing low to absent levels of LAT2 mRNA and protein in primary AML blasts with the *t*(8;21) [11,14].

Furthermore, we addressed the molecular mechanisms of AML1/ETO-mediated *LAT2* repression. We could show that, in the presence of AML1/ETO, LAT2 is repressed by epigenetic mechanisms and treatment with a class-I specific HDAC inhibitor reverted epigenetic silencing marks [12]. It is increasingly recognized that epigenetic gene silencing events play an important role in leukemogenesis [23]. To study the functional implications of LAT2 in an AML1/ETO background, we overexpressed LAT2 in Kasumi-1 cells. Forced LAT2 expression blocked ATRA induced myeloid differentiation *in vitro* suggesting a contributing role of LAT2 in the aberrant differentiation of leukemia cells [14].

We therefore hypothesized that LAT2 is regulated during differentiation of normal hematopoietic cells and may play a functional role in the pathophysiology of AML.

In the current study we analyzed the effect of LAT2-overexpression and -knockdown on cytokine-induced erythroid and myeloid differentiation of primary human CD34+ cells. LAT2 knockdown enhances both myeloid and erythroid differentiation of normal CD34+ cells (Fig. 4) whereas LAT2 overexpression delays maturation (Figs. 2 and 3). In immature, slightly differentiated normal CD34+ cells, only low levels of LAT2 expression can be detected (Fig. 1). Low levels of LAT2 might lead to a stimulation of physiological differentiation. At the time when differentiation proceeds, higher LAT2 levels can be detected which might result in a restriction of rash and uncontrolled maturation. Therefore, LAT2 might play an important role in controlling physiological differentiation. Unbalance of this control mechanism might lead to pathological differentiation of hematopoietic progenitors.

It has been suggested that LAT2 may play a role as a tumor suppressor gene in lymphoid malignancies, affecting signaling pathways of lymphoid malignant cells [24]. In a recent study it was hypothesized that LAT2 down-regulation leads to an impairment of the AKT activation, which might induce an anti-leukemic effect and reduce cell proliferation [25].

Further investigations are needed to elucidate the underlying signal pathway in myeloid malignancies. We suggest that C-CBL, which is mutated in AML [5] and interacts with phosphorylated LAT2 [9], might be relevant in the LAT2-mediated leukemogenesis. Mutated C-CBL is thought to loose its E3-ubiquitin ligase activity, which might prevent LAT2 degradation resulting in higher protein expression levels [10]. As shown, higher LAT2 expression levels lead to a delay in differentiation keeping CD34+ cells in an immature state. Surely, other unknown mechanisms are also likely especially due to the fact that not all subtypes of AML show the same LAT2 expression levels.

In conclusion, we describe a novel role for LAT2 in the differentiation of normal CD34+ cells.

Acknowledgments

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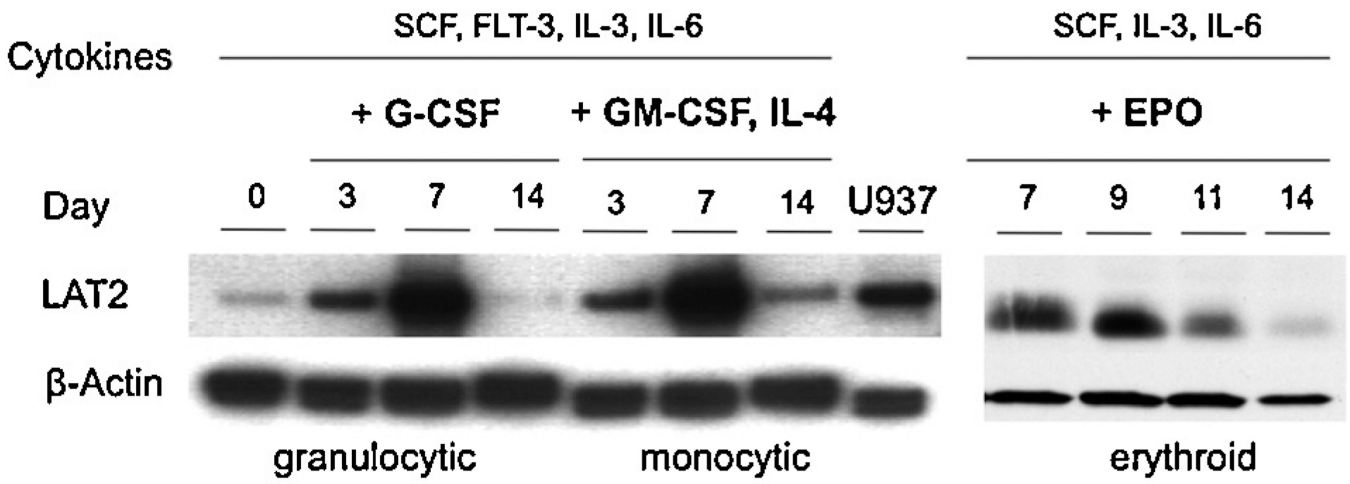

Fig. 1.

Western Blot analysis of LAT2 expression in normal CD34+ cells during granulocytic, monocytic and erythroid differentiation. Equal loading was assured by re-probing with an antibody against β-actin.

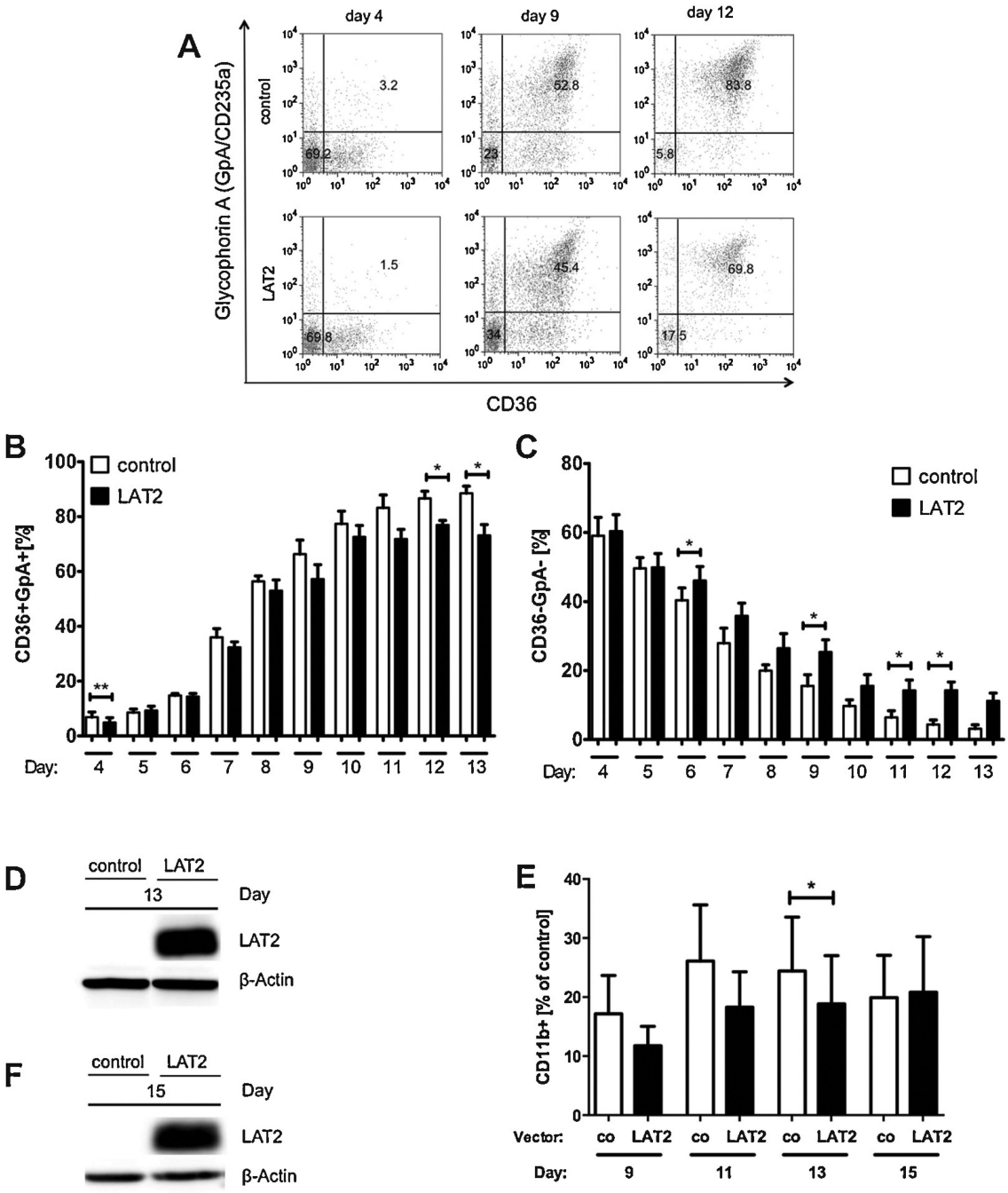

Fig. 2.

Forced expression of LAT2 affects erythroid and myeloid differentiation of normal CD34+ cells. (A) To assess erythroid differentiation cells were analyzed by FACS for CD36− and GpA-expression at various time points. A representative FACS-analysis is shown. In order to characterize cells at different maturation stages two phenotypic populations were analyzed: (B) CD36 and GpA double positive and (C) CD36 and GpA double negative cells. (D) LAT2 protein expression was detected by Western Blot on day 13 of erythroid differentiation and assessed for equal protein loading using an antibody against β-actin. A

representative experiment is depicted. (E) To assess myeloid differentiation cells were analyzed by FACS for CD11b expression at various time points. (F) Protein extracts were analyzed on day 15 of myeloid differentiation for LAT2 expression by Western Blot and assessed for equal protein loading using an antibody against β-actin. All results in this figure represent means ± S.D. *$P < 0.05$; **$P < 0.01$; $n = 3$.

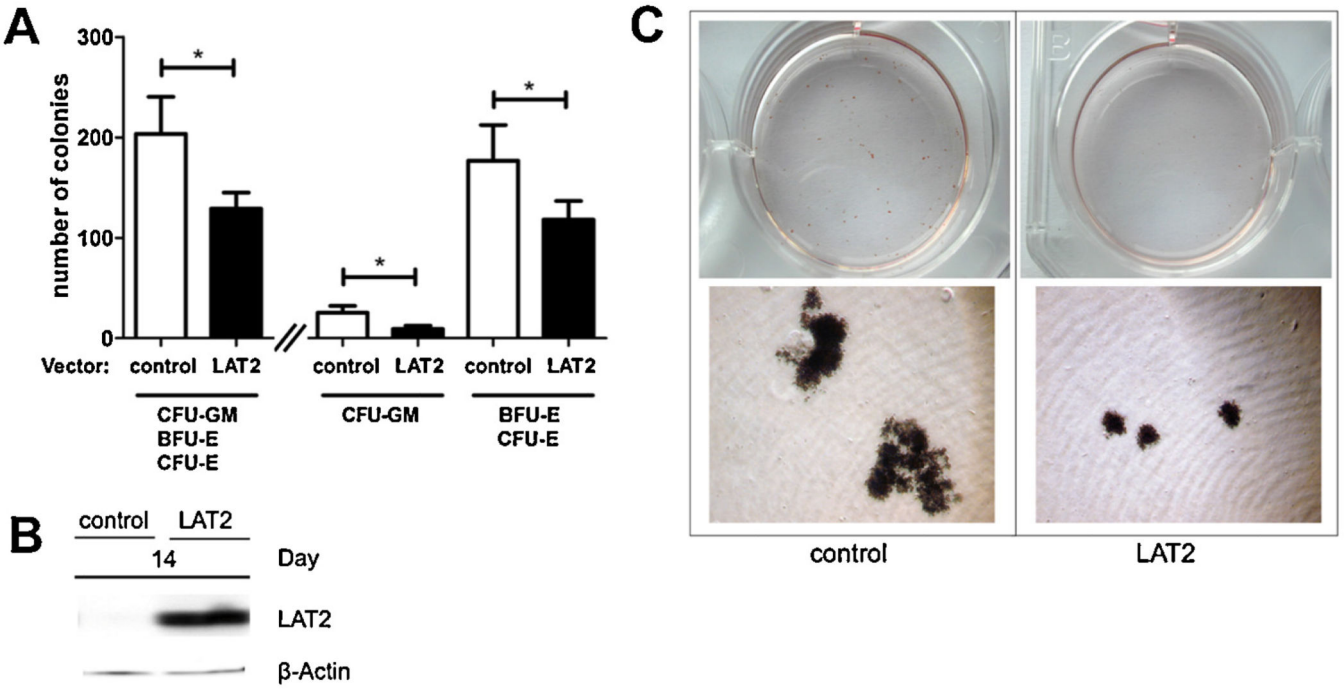

Fig. 3.

Colony-forming assay in methylcellulose enriched with cytokines. (A) Total number of colonies (CFU-GM, BFU-E, CFU-E), myeloid (CFU-GM) and erythroid colonies (BFU-E and CFU-E) of CD34+ cells transduced with LAT2- or a control-vector were compared after 14 days of culture. The data represent means ± S.D. $*P < 0.05$; $n = 3$. (B) On day 14 LAT2 expression was detected by Western Blot. Equal loading was assured by re-probing with an antibody against β-actin. (C) A representative picture of colonies of CD34+ cells transduced with the LAT2- and control-vector.

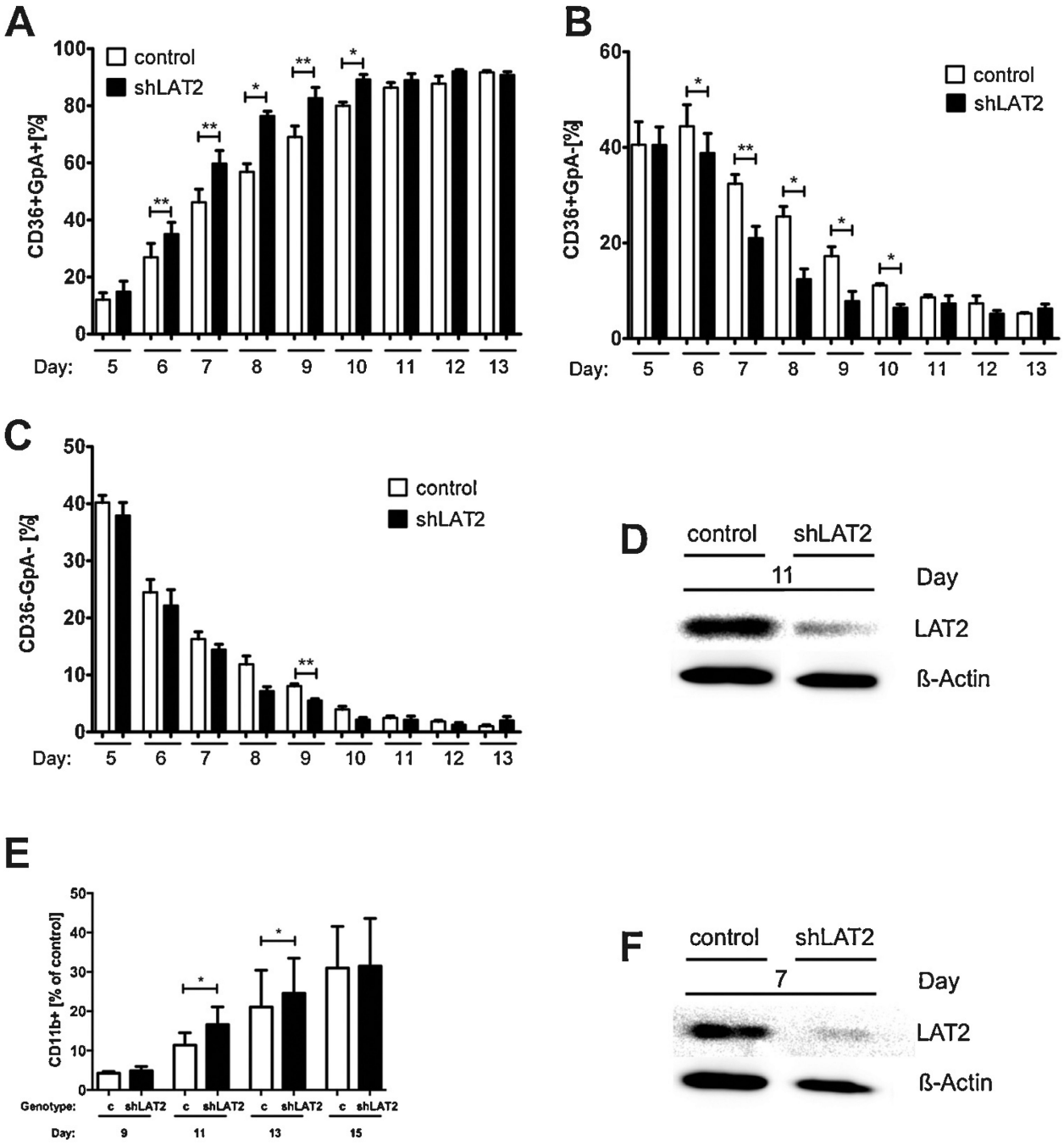

Fig. 4.

The effect of LAT2 depletion by shRNA during erythroid and myeloid differentiation of normal CD34+ cells. To assess erythroid differentiation cells were divided into different maturation stages described by three phenotypes: (A) CD36 and GpA double positive, (B) single positive for CD36 and (C) double negative. (D) LAT2 expression level was analyzed by Western Blot and assessed for equal protein loading using an antibody against β-actin after 11 days of erythroid differentiation. (E) CD11b expression during myeloid differentiation was analyzed by flow cytometry. (F) A representative Western Blot of LAT2

protein expression level on day 7 of myeloid differentiation culture is shown. Equal loading was assured by re-probing with an antibody against β-actin. All results in this figure represent means ± S.D. *$P < 0.05$; **$P < 0.01$; $n = 3$.