Fli-1 overexpression in erythroleukemic cells promotes erythroid de-differentiation while Spi-1/PU.1 exerts the opposite effect

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Abstract. The ETS transcription factors play a critical role during hematopoiesis. In F-MuLV-induced erythroleukemia, Fli-1 insertional activation producing high expression of this transcription factor required to promote proliferation. How deregulated Fli-1 expression alters the balance between erythroid differentiation and proliferation is unknown. To address this issue, we exogenously overexpressed Fli-1 in an erythroleukemic cell harboring activation of *spi-1/PU.1*, another ETS gene involved in erythroleukemogenesis. While the proliferation in culture remains unaffected, Fli-1 overexpression imparts morphological and immunohistochemical characteristics of immature erythroid progenitors. Fli-1 overexpression in erythroleukemic cells increased the numbers of erythroid colonies on methylcellulose and reduced tumorigenicity as evidenced by increase latency of erythroleukemogenesis in mice inoculated with these cells. Although all transplanted mice developed enlargement of the spleen and liver due to leukemic infiltration, Fli-1 overexpression altered the hematopoietic phenotype, significantly increasing the expression of regulatory hematopoietic genes cKIT, SCA-1, CD41 and CD71. In contrast, expression of Spi-1/PU.1 in a Fli-1 producing erythroleukemia cell line in which *fli-1* is activated, resulted in increased proliferation through activation of growth promoting proteins MAPK, AKT, cMYC and JAK2. Importantly, these progenitors express high levels of markers such as CD71 and TER119 associated with more mature erythroid cells. Thus, Fli-1 overexpression induces a de-differentiation program by reverting CFU-E to BFU-E erythroid progenitor activity, while Spi-1/PU.1 promoting maturation from BFU-E to CFU-E.

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Introduction

Hematopoietic differentiation occurs as a result of a distinct gene expression program, whereby the self-renewal of pluripotent hematopoietic stem cells and sequential commitment of intermediate progenitors, with a decreased capacity to proliferate, is governed by specific combinations of lineage-specific transcription factors (1). Transcription factors (TFs), such as those in the ETS gene family, play an integral role in hematopoiesis by coordinating the balance between proliferation and differentiation and influencing properties of self-renewal (2). Therefore, unsurprisingly the dysregulation of normal ETS transcriptional machinery plays a causal role in several human and murine hematological malignancies associated with chromosomal translocations or viral insertions.

Multi-stage erythroleukemia induced by Friend virus has served as an excellent mouse model to study the effects of specific ETS transcription factors associated with hematological pathogenesis. Friend virus-induced erythroleukemia is characterized by a marked expansion of erythroid progenitors. The two strains of the Friend virus complex, polycythemia- and anemia-inducing isolates, consist of a replication defective spleen-focus forming virus (SFFV) and a replication-competent Friend murine leukemia virus (F-MuLV) (3-5). The emergence of clonal tumorigenic erythroblasts is dependent upon retroviral insertional activation of the ETS transcription factors, *spi-1/PU.1* in SFFV-induced erythroleukemia (6), and *fli-1*, in F-MuLV-induced erythroleukemia (7,8). In both cases, tumorigenic erythroid progenitor cells are blocked in differentiation at the proerythroblast stage, with self-renewal capacities. SFFV induces enhancement of proerythroblasts exhibiting properties of erythroid colony-forming (CFU-E)

cells, whereas F-MuLV-induced erythroleukemic cells exhibit properties of erythroid burst forming (BFU-E) cells (9). Leukemic cells grown in methylcellulose have given rise to several established erythropoietin (Epo)-independent cell lines. Recent evidence suggests that the maintenance of the malignant phenotype in these cell lines is dependent upon the aberrant regulation of *fli-1* (10-12).

Fli-1, in addition to involvement in erythroleukemia, is also overexpressed in almost all hematological malignancies and activated as a result of translocation in Ewing's sarcoma resulting in generation of fusion protein EWS-Fli-1 with strong oncogenic activity (13). In human, Fli-1 deficiency was associated with both erythroid and megakaryocytic development (14,15). Studies of Friend virus-induced erythroleukemia have implied that activation of Fli-1 inhibits the commitment of erythroid progenitors to differentiate through disruption of critical erythroid signaling pathways, such as that of Epo and stem cell factor (SCF). Indeed, Fli-1 has been shown to alter the expression of erythroid lineage-associated genes, such as *Rb* (15), *bcl-2* (16) GATA1 (17) and *SHIP-1* (18).

To directly assess the role of ETS genes in erythroid transformation, an SFFV-induced erythroleukemia cell line was generated to ectopically express Fli-1 along with green fluorescent protein (GFP) reporter. Using this erythroleukemic cell line, we show that Fli-1 overexpression de-differentiates these cells to earlier progenitor status. However, contrary to Fli-1, when Spi-1/PU.1 is overexpressed in an F-MuLV-induced erythroleukemia cell line, these cells differentiate to a more mature erythroid progenitor. These data suggest that Fli-1 and Spi-1/PU.1 function differently and target distinct erythroid progenitors during erythroleukemogenesis.

Materials and methods

Cell culture and treatments. Erythroleukemia cell lines DP-17-17 and CB3 were maintained in alpha-minimum essential medium (α-MEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco). HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). To induce erythroid differentiation, FACS sorted DP17-17 cells were treated for two days with 2% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Oakville, ON, Canada). Differentiation assays were performed in triplicate by seeding ($1x10^5$) cells/well in 3 ml of a 6-well plate. After 48 h of induction with DMSO, adherent cells were removed from the culture dish using a cell scraper for cytospin preparation and histological analysis.

Enforced expression of Fli-1 and Spi-1. The MigR1-Fli-1, or empty vector control plasmid, MigR1, was triple-transfected with Lipofectamine 2000 (Invitrogen, Burlington, Canada) into HEK293T cells, following the manufacturer's protocol. In this transfection we included the vesicular stomatitis virus G glycoprotein (VSVG)-expressing vector, as well as the *gag* and *pol* virus packaging signals were provided by Dr D. Barber, University of Toronto. Viral supernatant was collected 48 h post-transfection. DP17-17 ($2.5x10^6$) were infected with virus, and incubated 16 h with polybrene (8 μg/ml final concentration), as previously described (10). Two days post-infection, cells were sorted by flow cytometry based on the intensity of green fluorescence. Sorted cell populations expanded in culture, and were sorted a second time based on high intensities of green fluorescence.

The MSCV-Spi-1/PU.1 or empty vector MSCV DNA was transfected into CB3 cells and after 3 days selected with neomycin (800 mg/ml) for two weeks. The G418 resistant cells were pooled and used in this study.

qRT-PCR analysis. RNA levels were determined by quantitative real-time PCR in a StepOne Plus thermal cycler (Applied Biosystems, Forest City, CA, USA) using specific primers and SYBR® select Master Mix (Life Technologies, Carlsbad, CA, USA), as described (19). The β-actin gene was used as control, the list of primers used for RT-PCR and qRT-PCR is as follows: all experiments were performed in triplicates and repeated at least two times.

The murine β-actin primers: 5' forward (5'-GTGACGTTG ACATCCGTAAAGA-3') and 3' reverse (5'-GCCGGACTCATC GTACTCC-3'). The murine GATA-1: 5' forward (5'-CACCCT GAACTCGTCATACC-3') and 3' reverse (5'-ACCAGGGCAG AATCCACAAA-3'). The murine TAL-1: 5' forward (5'-CGGC AGCAGAATGTGAATGG-3') and 3' reverse (5'-CTCCTGG TCATTGAGTAACTTGG-3'). The murine RUNX1: 5' forward (5'-TGGTGGAGGTACTAGCTGACC-3') and 3' reverse (5'-CGAGTAGTTTTCATCGTTGCCT-3').

Immunoblotting. Cells were lysed with lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 50 mM NaF, plus 1 mM Na_3VO_4, 10 g/ml aprotinin, 100 g/ml leupeptin and 10 mM phenylmethylsulfonyl fluoride). Lysates (40 μg) were fractionated by SDS/PAGE and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Billerica, MA, USA). The following antibodies were used: SHIP-1, Fli-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); β-actin (Sigma-Aldrich); goat-anti-mouse, and goat anti-rabbit HRP-conjugated (Promega, Madison, WI, USA), ERK, phospho-AKT, AKT, MYC and JAK2 antibodies from Cell Signaling Technology (CST, Danvers, MA, USA).

Cellular proliferation assay. Transduced DP17-17 and CB3 cells, $1x10^4$, were plated in triplicates, removed at 24-h intervals, and cellular proliferation was measured by performing trypan-blue exclusion assay and counting using a hemocytometer.

Cytospin preparation and histochemical staining. Cells ($2x10^4$ per slide) were cytospun onto glass slides for 15 min at 1,000 rpm (Cytospin; Thermo Shandon, USA). Cells were fixed at room temperature in methanol for 5 min and air-dried. Fixed cells were stained with May-Grunwald stain followed by Giemsa stain per the manufacturer's protocol (Sigma). Light microscopy images were obtained using a Leica DM LB2 microscope, Leica DFC 300FX camera, and Leica Application Suite 3.1.0 software (Leica Microsystems, Switzerland). Blinded erythroid differential counts were performed by a hematopathologist, at Sunnybrook Health Sciences Centre, University of Toronto. A total of 6 cytospin slides with May-Grunwald Giemsa stains were prepared during two separate experiments for each non-transduced,

double-sorted MigR1, and MigR1-Fli-1 DP17-vector cell groups. Approximately 100-200 cells were counted on each slide and categorized into one of the three defined stages; R1, proerythroblast; R2, early basophilic erythroblast; R3, late basophilic erythroblast. Data are presented as the percentage of total cells analyzed.

Immunostaining and flow cytometric analysis. Freshly isolated cells were washed twice in PBS (Gibco) and immunostained for 15 min with the appropriate antibody; phycoerythrin (PE)-conjugated anti-TER119 (erythroid marker), PE-conjugated Anti-cKIT (SCF receptor), PE-conjugated anti-CD41 (glycoprotein IIb), PE-conjugated anti-CD61 (glycoprotein IIIa), PE-conjugated anti-Gr-1 (granulocytic marker), PE-conjugated anti-MAC-1 (monocytic marker), APC-conjugated anti-CD71 (transferrin receptor), APC-conjugated anti-SCA-1 (primitive hematopoietic cell marker) (1:200) (eBioscience, San Diego, CA, USA). Following antibody incubation, cells were washed once in PBS and resuspended in 500 μl PBS. Cell sorting and analysis of stained cells were performed using the Becton-Dickinson FACSCalibur (BD Biosciences, San Jose, CA, USA), and the FlowJo flow cytometry analysis software (FlowJo TreeStar Inc., Ashland, OR, USA). Relative mean fluorescence intensity (MFI) values were based on the unstained population controls and calculated using the Geometric Mean statistic (average of log fluorescence). Statistical analyses were performed using the two-tailed Student's t-test, where $P<0.05$ was considered to indicate a statistically significant difference.

Colony-forming cell assay. DP17-17 cells, transduced with the MigR1 empty vector control or MigR1 Fli-1 expressing vector and double sorted, were suspended in Iscove's modified Dulbecco's medium (IMDM) (Gibco) supplemented with 2% FBS (Gibco) and added to methylcellulose Medium (M334, or M3434, Stem Cell Technologies, Vancouver, BC, Canada) to assay for the presence of erythroid colony-forming units (CFU-E) and mature erythroid burst-forming units (BFU-E) (M3334 formulation consists of 15% FBS, 1% BSA, 10 μg/ml insulin, 200 μg/ml transferrin, 3 U/ml rh EPO, and M3434 formulation consists of 15% FBS, 1% BSA, 10 μg/ml insulin, 200 μg/ml transferrin, 50 ng/ml rm SCF, 10 ng/ml rm IL-3, 10 ng/ml IL-6, 3 U/ml rh EPO), according to the manufacturer's protocol. Colonies were counted after 12 days of culture. CFU-E and BFU-E colonies were detected by staining with benzidine solution, 0.4% benzidine (Sigma-Aldrich) in 12% acetic acid, with the addition of 0.3% hydrogen peroxide (Sigma). Individual colonies were isolated from methylcellulose cultures, cells were resuspended, and prepared for cytospins and histochemical staining, as described above.

Transplantation assay. DP17-17 cells, transduced with the MigR1 empty vector control or MigR1-Fli-1 expressing vector, were sorted by flow cytometry based on high intensities of green fluorescence, as described above. Double-sorted DP17-17 were suspended in phosphate-buffered saline (PBS) (Gibco), in a total volume of 200 μl, and administered intravenously into the tail veins of groups of female recipient eight-week-old DBA/2J mice, at concentrations of $1.0x10^6$, $1.0x10^5$ or $1.0x10^4$ cells. Injected mice were sacrificed if they presented with symptoms of disease progression, such as paleness, hunched posture, enlarged abdomen, and paralysis or difficulty breathing. Spleen and liver samples were isolated from recipient mice, cultured for two days, and subjected to flow cytometric analyses, as described above. The contribution and presence of the transduced DP17-17 cells injected was evaluated based on the detection of green fluorescence.

Survival and statistical analysis. The mouse survival rates were computed and plotted according to the non-parametric Kaplan-Meier analysis. Statistical analysis was performed using the two-tailed Student's t-test with significance considered at $P<0.05$, and by analysis of variance using Origin 3.5 software (Microcal Software, Northampton, MA, USA).

Animal care. Animal care was in accordance with the guidelines of the institution, animal care committee and University of Toronto.

Results

Exogenous expression of Fli-1 alters the state of erythroid differentiation. The spleen focus forming virus (SFFV) and the Friend murine leukemia virus (F-MuLV) induces erythroleukemia associated with insertional activation of *spi-1 (PU.1)* and *fli-1*, respectively (3). As shown in Fig. 1A, Spi-1/PU.1 is overexpressed in SFFV-induced erythroleukemia cell lines HB60-5, DP16-1, and DP17-17, however, its expression is absent in the F-MuLV-induced erythroleukemia cells CB7, CB3 and HB22.2, overexpressing Fli-1. The SFFV-induced erythroleukemic cell lines express Fli-1, albeit at significantly lower levels, compared to F-MuLV-induced erythroleukemia cell lines (Fig. 1A).

To decipher the role of Fli-1 in erythroid proliferation and differentiation, exogenous expression of Fli-1 was introduced into the SFFV-induced erythroleukemia cell line DP17-17. DP17-17 cells, transduced with either the MigR1 empty vector control, or MigR1-Fli-1 expressing retrovirus, were sorted by flow cytometry based on the intensity of green fluorescence two days post-infection. Sorted cell populations were isolated, and subjected to western blot analysis to confirm enforced expression of Fli-1 (Fig. 1B). Trypan blue exclusion assay was performed to reveal the effects of Fli-1 overexpression on the proliferation of the SFFV-induced erythroleukemic cells. The non-transduced (N/T), MigR1 and MigR1-Fli-1 transduced cell populations retained a comparable proliferation rate (Fig. 1C), indicating that Fli-1 overexpression has no additional effect on proliferation in these cells in culture. However, upon microscopic examination of transduced DP17-17 cultures, a clear distinction was observed in the gross morphology of the transduced populations. Typically, DP17-17 cells growing in culture have both an adherent and suspension population, and upon cell differentiation, using a chemical inducer, these cells become mainly adherent. Under normal culture conditions, exogenous Fli-1 expression in DP17-17 cells resulted in a remarkable shift in the proportion of these populations, increasing the numbers of suspension cells, compared to the MigR1 empty vector control (Fig. 1D). This observation suggested that Fli-1 overexpression might influence the differentiation of this erythroleukemia cell line.

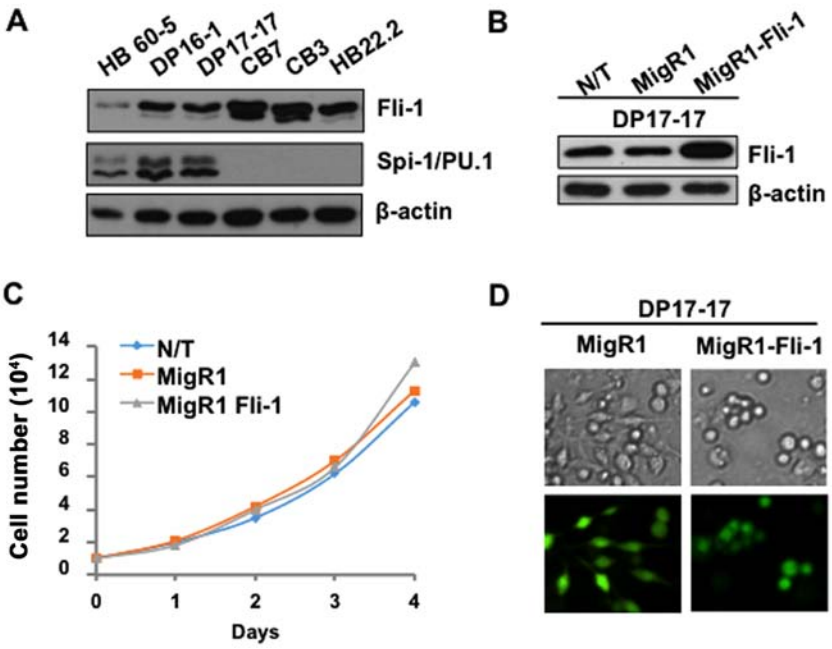


Figure 1. Exogenous expression of Fli-1 in SFFV-induced erythroleukemic cell line DP17-17. (A) Fli-1 and Spi-1/PU.1 expression levels in HB60-5, DP16-1, DP17-17, CB7, CB3 and HB22.2 cell lines. (B) Exogenous expression of Fli-1 in DP17-17 cells as detected by western blot analysis. β-actin was used as a loading control. (C) Unchanged proliferation rate of transduced DP17-17 cells as determined by trypan-blue dye exclusion. (D) Gross morphological examination revealed that Fli-1 overexpression increases the proportion of the non-adherent cell population under normal culture conditions, compared to the MigR1 empty vector transduced cells.

To confirm the above hypothesis, histochemical staining of transduced DP17-17 cells was performed to distinguish between different stages of erythroid development. Histology can detect the morphologically defined stages of erythropoiesis including, proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts, orthochromatophilic erythroblasts, reticulocytes and mature erythrocytes. Successive erythroid differentiation is characterized by a decrease in cell size, increase in condensation of nuclear chromatin, more abundant cytoplasm and increase in hemoglobinization as indicated by paler or less saturated dye. In two separate experiments, May-Grunwald Giemsa stains revealed that Fli-1 overexpressing DP17-17 cells exhibit darker staining of the nuclei, denser appearance of the nuclei chromatin (indicating immature chromatin), and deeply basophilic cytoplasm, compared to control cells (Fig. 2A). Overall, the morphological and expression characteristics of Fli-1 overexpressing DP17-17 cells was indicative of a more immature cell type compared to the control cells. To confirm our findings, a clinical hematopathologist performed blinded erythroid differential counts of transduced DP17-17 cell cytospin preparations stained with May-Grunwald/Giemsa and the result is depicted in Fig. 2B.

Exogenous Fli-1 expression does not affect the ability of Friend erythroleukemic cells to undergo erythroid differentiation in culture. The above-mentioned data suggest the ability of Fli-1 expression to induce features of a less-differentiated erythroid phenotype. To determine if exogenous Fli-1 expression also affects the potential or capacity of Friend erythroleukemic cells to differentiate along the erythroid lineage, DP17-17-MigR1 or MigR1-Fli-1 cells were grown in the presence of DMSO. Double sorted cell populations were seeded in triplicate and grown in culture medium supplemented with 2% DMSO. Following 48 h of induction, most of the cells became adherent to the culture dish (data not shown). To examine changes in the morphology of the DMSO-treated cells, suggestive of erythroid differentiation, DP17-17 cells were removed from the culture dish for cytospin preparation and histochemical staining. DMSO-induced cultures of non-transduced and MigR1 empty control DP17-17 cells consisted predominantly of cells resembling the orthochromatophilic stage, with small, condensed nuclei, and paler blue, more abundant cytoplasm (Fig. 2C), resembling late stage erythroid differentiation. DMSO-induced cultures of DP17-17 Fli-1 population consisted predominantly of cells resembling the polychromatophilic stage, with slightly larger nuclei and more deeply stained, less abundant cytoplasm compared to the control DP17-17 cells treated with DMSO (Fig. 2C). Flow cytometry analysis revealed that erythroid maturation by DMSO stimulation in DP17-17 Fli-1 and control cells coincides with a decrease in the expression of the transferrin receptor (CD71) and a slight increase in the erythroid lineage cell surface marker TER119 (Fig. 2D). This result is consistent with lower CD71 and higher TER119 expression in differentiated erythroid cells beyond CFU-E stage (20). Since Fli-1 is known to be downregulated by DMSO or during differentiation of erythroleukemia cells (15,21,22), we examined the level of this TF before and after chemical inducer treatment. Indeed, Fli-1 protein expression is significantly downregulated in DP17-17-vector and to a lesser extent in DP17-17 Fli-1 cells (Fig. 3A). Therefore, while exogenous expression of Fli-1 induces a less differentiated phenotype, it does not affect the potential of these leukemic cells to undergo chemically-induced erythroid differentiation.

Fli-1 overexpression increases the number of colony-forming cells and expression of stem cell genes. To further investigate

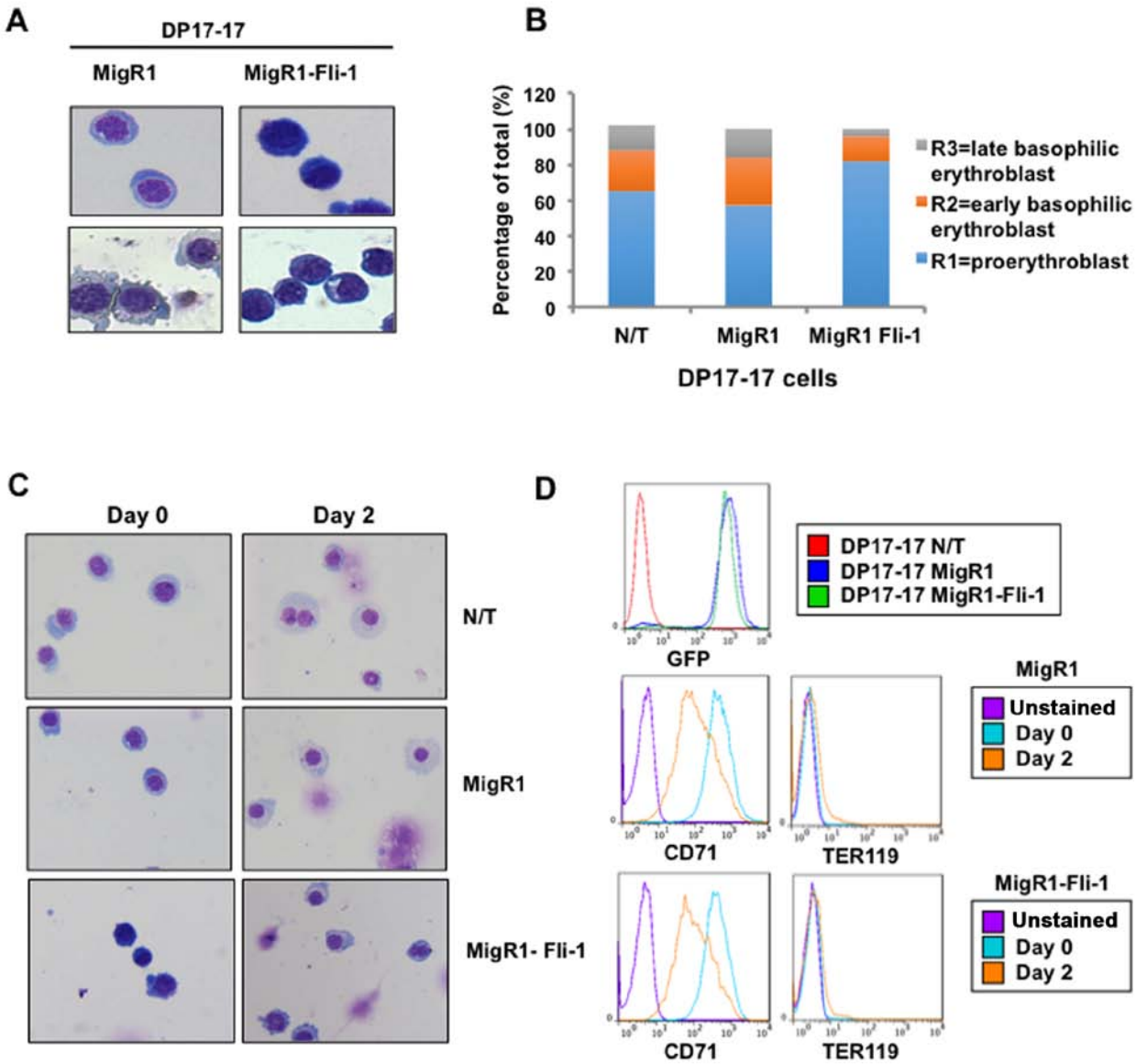


Figure 2. Exogenous expression of Fli-1 alters morphology of transduced DP17-17 cells. (A) May-Grunwald Giemsa stained cytospin preparations of double-sorted DP17-17 cells transduced with the MigR1 and MigR1-Fli-1 vectors. (B) Erythroid differential counts from stained cytospin preparations of transduced DP17-17 cells were performed in a blinded manner by a clinical hematopathologist. The bar graph represents percentages of total cells analyzed. R1 represents the proerythroblast; R2 represents the early basophilic erythroblast, and R3 the late basophilic erythroblast stage (42). (C and D) Stained cytospin preparations (C) and flow cytometric analysis (D) of untreated and DMSO-treated DP17-17, DP17-17 MigR1 and DP17-17 Fli-1.

the role of exogenous Fli-1 overexpression in erythroid differentiation, a colony-forming cell (CFC) assay was performed to quantify changes in the number of lineage-restricted progenitors of the erythroid or megakaryocytic lineage. Double-sorted DP17-17 cells transduced with the MigR1 or MigR1-Fli-1 were suspended in semi-solid methylcellulose medium supplemented with Epo, or Epo plus other additional cytokines. CFU-E and BFU-E colonies were enumerated by staining with benzidine solution. Individual colonies were quantified and the lineage composition was classified based on morphological recognition by light microscopy, and cytochemical staining of cellular cytospins with May-Grunwald/Giemsa staining. DP17-17 Fli-1 cells produced ~2.5x more erythroid colonies compared to the appropriate controls (Fig. 3B). Typical morphologies of benzidine-stained cells for MigR1 or MigR1 Fli-1 expressing colonies are presented in Fig. 3C. Benzidine positive staining revealed that the clear majority of colonies generated by both groups of DP17-17 cells are of erythroid origin. Gross morphological examination by light microscopy also revealed that most Fli-1 overexpressing colonies grew larger in size, compared to controls (data not shown), suggesting that Fli-1 may enhance the proliferation of erythroid progenitors. Isolation of individual colonies, and subsequent cytospin preparation for May-Grunwald Giemsa staining confirmed the erythroid composition, as stained cells displayed characteristic morphologies of mid to late stage erythroblasts (Fig. 3D). The data further suggest that Fli-1 promotes hematopoietic progenitor de-differentiation program along erythroid pathway, and was further supported by flow cytometry, in which a higher number of DP17-17 Fli-1 cells with higher intensity express the stem/progenitor cell markers cKIT and CD41, when compared to DP17-17 control cells (Fig. 4A). No significance difference in

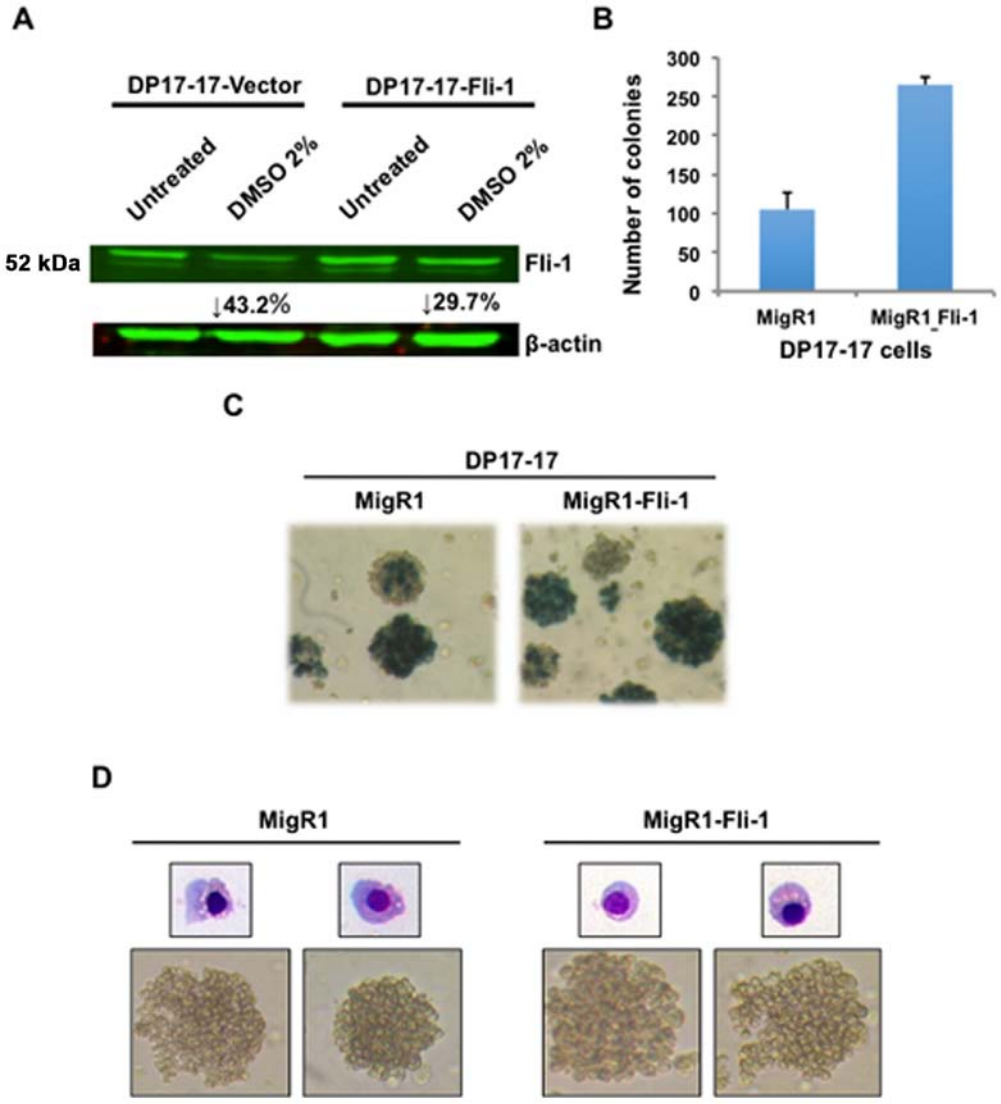


Figure 3. DP17-17 cells overexpressing Fli-1 display increased numbers of erythroid colonies. (A) Western blot analysis of Fli-1 expression after treatment of the indicated cells for 3 day with 2% DMSO. Densitometry was used to determine the level of Fli-1 downregulation by DMSO. (B) Transduced DP17-17 cells were plated in triplicate on methylcellulose media in the presence of cytokines. Erythroid colonies were quantified by staining with benzidine following 12 days of culture. Representative data indicating benzidine positive staining of methylcellulose cultures on day 12. (C) Analysis of individual methylcellulose colonies. (D) Cellular cytospins and May-Grunwald-Giemsa stains of sampled colonies from transduced DP17-17 cells display characteristic morphologies of mid to late stage erythroblasts.

expression of CD71 was observed between DP17-17 Fli-1 and control cells (Fig. 4A).

Fli-1 binds and forms heptad complexes with other members of master hematopoietic TFs GATA1/GATA2, RUNX1 and SCL/TAL1 (23). The composition of this heptad TFs is critical for differentiation of progenitors to erythroid and megakaryocytic lineage (22). We then determined the expression of these genes in DP17-17 cells. Indeed, a lower expression of GATA1 was detected in DP17-17 Fli-1 than DP17-17 control cells (Fig. 4B). This result is consistent with negative regulation of GATA-1 by Fli-1 (17). Fli-1 expression in DP17-17 Fli-1 cells increased the expression of RUNX1, but significantly reduced SCL/TAL1 transcription when compared to the control cells (Fig. 4C and D), as previously observed during erythroid differentiation (2).

Fli-1 overexpression in SFFV-induced erythroleukemia increases the latency of disease progression and alters the hematopoietic phenotype. To determine whether Fli-1 overexpression, and the concurrent alteration of differentiation status, affects the progression of FV-P-induced erythroleukemia, DP17-17-vector and DP17-17 Fli-1 cells were transplanted into syngeneic DBA/2J mice. Transduced and double-sorted DP17-17 cells ($1x10^6$, $1x10^5$, $1x10^4$) were intravenously injected into the tail veins of eight-week-old DBA/2J mice, and monitored for physiological signs of FV-P-induced erythroleukemia progression and survival. No appreciable difference in survival rate was observed when mice received $1x10^6$ cells (Fig. 5A). However, mice injected with $1x10^5$ and $1x10^4$ Fli-1 overexpressing cells displayed a statistically significant increase in survival, indicating growth suppression (Fig. 5A).

Friend virus-induced erythroleukemia is marked by the emergence and expansion of transformed erythroblasts in the spleen and liver (5). Indeed, the size of both spleens and liver increased in DP17-17 vector and DP17-17-Fli-1 injected mice, although no significance difference was observed (data

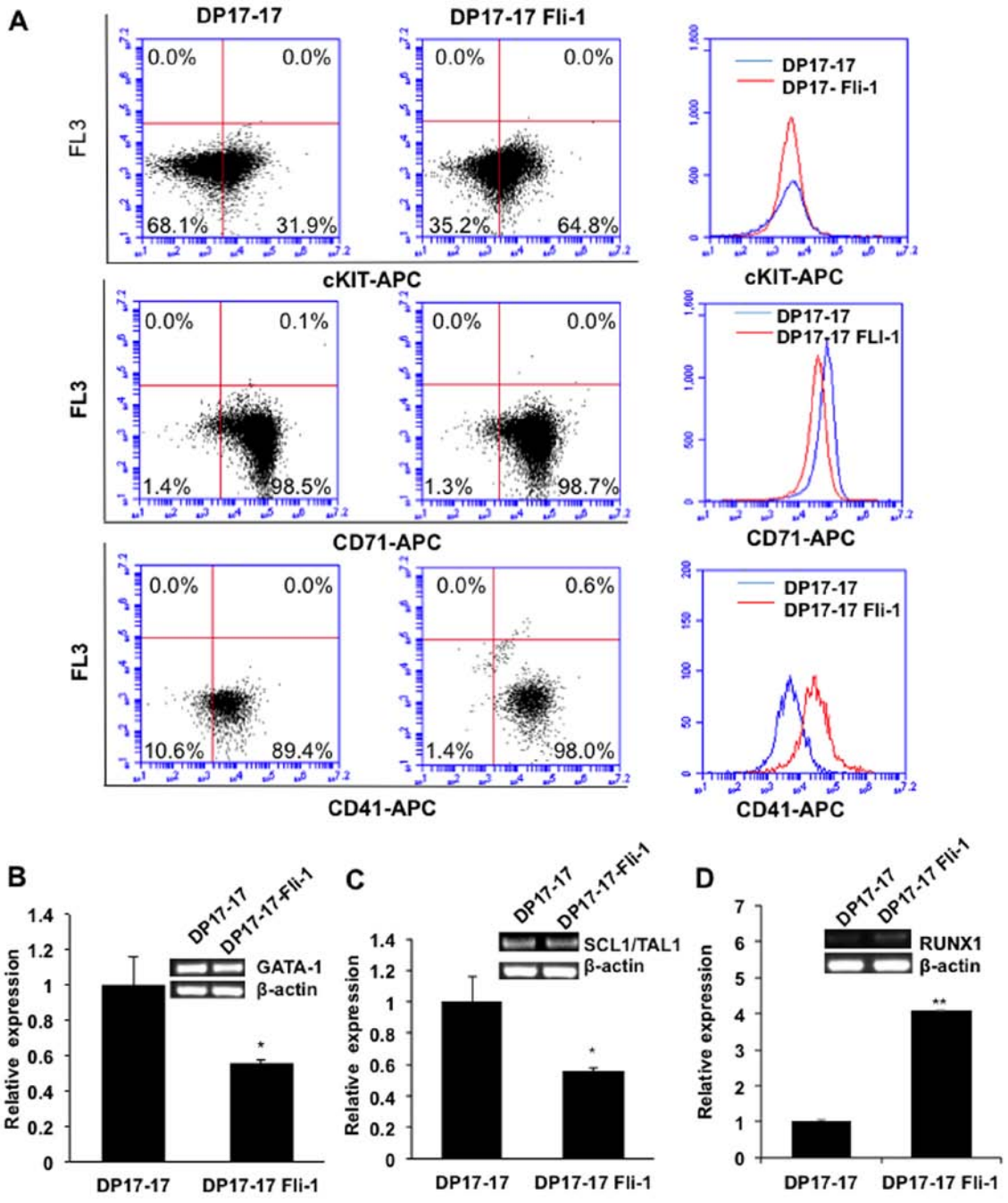
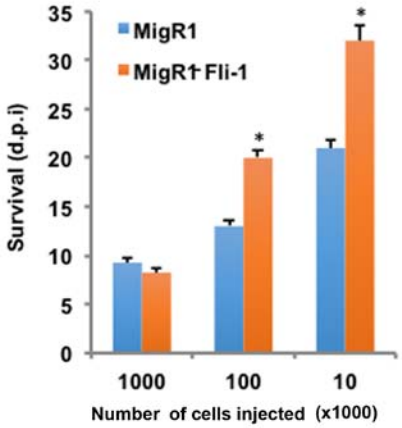


Figure 4. DP17-17 cells overexpressing Fli-1 express markers of megakaryocytic cells. (A) Flow cytometry analysis demonstrated a higher expression of CD41 and cKIT expression in DP17-17 Fli-1 than the control DP17-17 vector cells. (B) qRT-PCR analysis for expression of GATA1 (B), SCL/TAL1 (C) and RUNX1 (D) in DP17-17 Fli-1 and DP17-17-vector cells. β-actin waas used as loading control. *P<0.05; **P<0.005.

not shown). To further characterize the erythroid malignancy generated in the recipient mice, tumor spleen and liver cultures isolated from these tumorigenic mice were subjected to flow cytometric immunophenotyping. The contribution and presence of intravenously injected DP17-17 vector (n=9) or DP17-17 Fli-1 (n=7) cells ($1x10^4$) was detected through green fluorescence. Thus, the frequencies of several hematopoietic cell surface markers were determined as a percentage of the total GFP-positive cell population in diseased recipient mice. The enlarged spleens and livers of both vector and Fli-1 injected mice contained similar percentages of cells positive for CD71 and TER119 (Fig. 5B). Although the percentages of cells positive for CD41, CD61, cKIT, and MAC-1 in the spleen, as well as CD41 and cKIT in the liver, were significantly higher in Fli-1 cells injected mice compared to vector cells injected mice (Fig. 5B). Taken together, the data suggested that increased latency of FV disease progression caused by Fli-1 overexpression might result from an inherent change in the hematopoietic phenotype of erythroid progenitors.

Spi-1/PU.1 transduction in the negative producing erythroleukemia cell line CB3 promotes further erythroid differentiation. In F-MuLV-induced erythroleukemias cell line CB3, insertional activation of *fli-1* increases the

A



B

	Fli-1	MigR1	Fli-1	MigR1
Lineage markers	Spleen	Spleen	Liver	Liver
CD41	33.8 ± 5.9*	19 ± 3.8	28.5 ± 10.9*	15.2 ± 4.8
CD61	66 ± 6.7*	46.2 ± 4.3	55.2 ± 10.1	44.8 ± 17.3
CD71	98.5± 0.6	95.8 ± 2.8	98.8 ± 0.5	93.5 ± 1.9
TER119	2.1 ± 0.8	3.25 ± 1.9	1.1 ± 0.8	1.4 ± 0.8
cKIT	86 ± 4.8*	71 ± 3.6	79.8 ± 3.9*	61.9 ± 3.5
SCA-1	40.2 ± 32.6	12.6 ± 9.5	32.5 ± 42.4	12 ± 8.1
MAC-1	31.5 ± 9.3*	9.5 ± 2.1	39.5 ± 18.4	11.6 ± 2.1
GR-1	2.8 ± 4.8	0.4 ± 0.1	0.4 ± 0.2	0.2 ± 0.4

Percentage of expressing cells

Figure 5. Increased disease latency in mice inoculated with Fli-1 overexpressing DP17-17 erythroleukemic cells. (A) The mean survival rate of eight-week-old DBA/2J mice i.v injected with $1x10^6$ (n=6), $1x10^5$ (n=6), or $1x10^4$ (n=6) DP17-17 cells transduced with MigR1 or MigR1-Fli-1 retroviruses. *P<0.05. (B) Frequency of hematopoietic cell surface marker expression, as a percentage (% ± standard deviation) of total GFP-positive cells isolated from the spleens and livers of CB3 (10^6) recipient DBA/2J mice, where Fli-1; n=7 and MigR1 control; n=9.

expression of this TF, while negligible level of Spi-1 was detected in these cells (8). We next examined if expression of Spi-1/PU.1 in CB3 cells can alter the phenotype of these cells through erythroid differentiation pathway. CB3-Spi-1 cells proliferate at a higher rate that CB3-vector cells in culture (Fig. 6A). Accordingly, these cells express a higher level of growth promoting genes, including phospho-MAPK/ERK, phospho-AKT, cMYC and JAK2 (Fig. 6B). The Spi-1 overexpressing CB3 cells exhibit lighter staining of the nuclei with less density of the nuclei chromatin (indicating mature chromatin), and weaker basophilic cytoplasm, compared to control CB3-vector cells (Fig. 6C). Moreover, while Spi-1/PU.1 expression in CB3 cells did not affect the level of SCA-1 on cells, it significantly increased CD71 and moderately decreased cKIT expression (Fig. 6D). TER119 is only slightly increased in Spi-1/PU.1 expressing CB3 cells (Fig. 6D). Higher CD71 expression is consistent with highest level of this cell surface protein detected in CFU-E progenitors (20). Thus, while Spi-1/PU.1 expression in erythroid progenitors transform erythroblasts at CFU-E stage of erythroid differentiation, Fli-1 overexpression target progenitors at BFU-E stage during erythroleukemogenesis (Fig. 6E).

Discussion

The ETS family member, *fli-1*, is aberrantly expressed in several cancers, including erythroleukemia and Ewing's sarcoma (7,8,13). Mice homozygous for a targeted deletion of *fli-1* revealed an indispensable function for Fli-1 during embryonic development and hematopoiesis, specifically proper megakaryocytic development (24). Moreover, fetal livers of homozygous Fli-1 mutants contain a significant reduction in the number of multilineage, erythroid, and myeloid progenitors compared to those of the wild-type and heterozygous embryos (25). Loss of function studies performed in the *Xenopus* and zebrafish embryos have provided conclusive evidence implicating Fli-1 at the top of the transcriptional network regulating blood and endothelial cell development within the mesoderm (26). To explore a more specific contribution of Fli-1 aberrant regulation in malignant transformation, we compared the phenotypes of an SFFV-induced erythroleukemia cell line, with and without exogenous Fli-1 expression. This study demonstrated that the enforced expression of Fli-1 in erythroleukemia cells induces de-differentiation of progenitors toward more immature state, an observation consistent with the

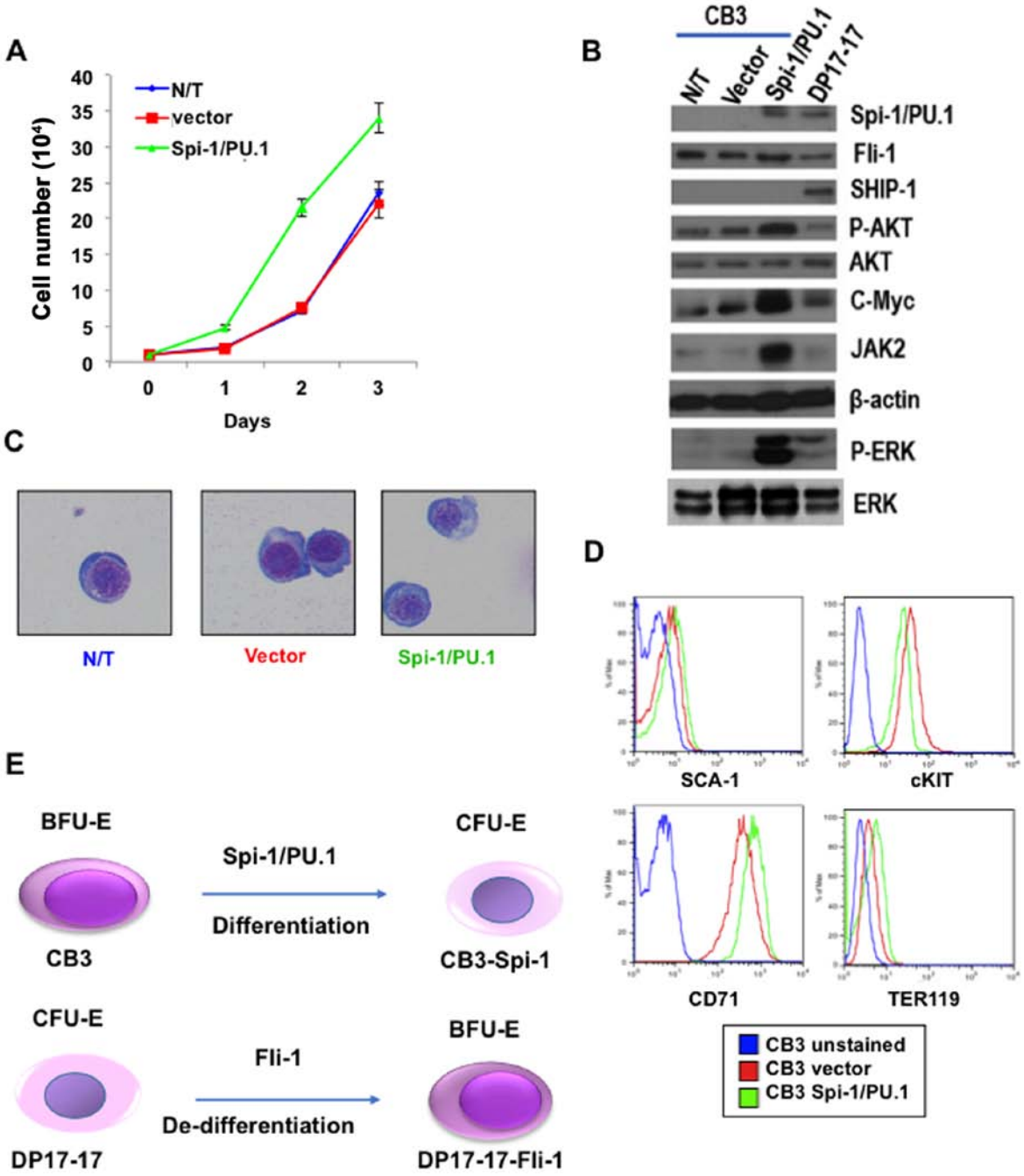


Figure 6. CB3 cells transduced with exogenous Spi1/PU.1 express markers of more mature erythroid progenitors. (A) Expression of Spi-1/PU.1 in CB3 cells accelerates the growth of these cells in culture when compared to CB3-vector cells. (B) Expression of the indicated protein in untreated CB3 (N/T), CB3-vector, CB3-Spi-1/PU.1 and DP17-17 cells. β-actin is used as loading control. (C) May-Grunwald Giemsa stained cytospin preparations of CB3-Spi-1 and CB3-vector cells transduced with the MSCV-Spi-1 and empty vector plasmids. (D) Flow cytometric analysis of CB3-Spi-1 and CB3-vector cells using the indicated antibodies. (E) A proposed model of erythroid de-differentiation and differentiation by Fli-1 and Spi-1/PU.1, respectively. In this model, expression of Fli-1 in DP17-17 cells (CFU-E like progenitors) induces a de-differentiation program resulting in generation of cells resembling BFU-E progenitors. In contrast, expression of Spi-1/PU.1 in CB3 cells (BFU-E like progenitors) promotes differentiation to cells resembling CFU-E like progenitors.

role of this TF in regulation of hematopoiesis. In contrast, when Spi-1/PU.1 was introduced into a Fli-1 overexpressing erythroleukemia cell line, these leukemic cells expressed markers of more mature erythroid progenitors. These results indicate that both Fli-1 and Spi-1 block erythroid differentiation in distinct progenitors leading to the development of erythroleukemias with distinct maturation phenotype.

TFs play a critical role in controlling hematopoietic precursor cell fate decision, function and behavior (1). Indeed, while exogenous expression of Fli-1 into DP17-17 cells did not affect the proliferation rate of these cells in culture, it resulted in morphological characteristics, indicative of more primitive erythroid progenitors (27). Fli-1 overexpression in these cells induces changes in the expression of key hematopoietic TFs such as SCL/TAL1, GATA-1, RUNX1 that may promote properties of self-renewal and favor the transition to a more immature erythroid phenotype. Among these, GATA1 is well known to be negatively regulated by Fli-1 (17). Although Fli-1

overexpression leads to de-differentiation, it does not inhibit the ability of this erythroleukemia cell line to differentiate along the erythroid lineage in response to DMSO. Both Fli-1 and Spi-1 is known to be downregulated during normal erythropoiesis and by DMSO (15,21,22), as observed for Fli-1 in this study. This result then further confirms the role of these ETS genes in hematopoiesis.

The cell surface antigens CD41 (integrin α_{IIb}) and CD61 (integrin β_3) are expressed in cells of the megakaryocytic lineage, and increased in DP17-17 Fli-1 cells. Megakaryocytes and erythroblasts originate from a common myeloid progenitor (28,29), and accordingly cells derived from megakaryocytic leukemia or erythroleukemia often display traits of both erythroid and megakaryocytic progenitors (30-32). The molecular mechanisms regulating differentiation of either lineage remain unclear, however, it is likely that activation of signaling pathways regulating self-renewal, survival, and proliferation of the erythroid/myeloid progenitor are governed through alterations of gene expression profiles. The expression of Fli-1, along with the RUNX-1 transcription factor, is associated with megakaryocytic differentiation (33,34). The differentiation of megakaryocytes is primarily driven by thrombopoietin (TPO), and several genes known to play an important role in megakaryopoiesis, including the TPO receptor (TPOR) or c-MPL (35), glycoprotein (GP) IX and IIb, and cyclin D1 (36), all of which contain ETS binding sites and are regulated by Fli-1 (24,37). Indeed, Fli-1 overexpressing DP17-17 cells express higher CD41. Moreover, leukemic cells isolated from the organs of mice injected with DP17-17 Fli-1 cells revealed a statistically significant increase in the percentage of cells positive for CD41 and CD61 expression. The data indicate that Fli-1 overexpression enhanced acquisition of megakaryocytic features while maintaining erythroid features, indicative of a megakaryocytic/erythroid progenitor phenotype, and are consistent with our recent observation that Fli-1 agonist compounds can induce the expression of megakaryocytic-specific markers and promote megakaryocytic differentiation in erythroleukemic cells (38).

The ectopic expression of Fli-1 also stimulated an increase in the percentage and intensity of cKIT expressing cells in culture and in transplanted mice. Interestingly, both MYB and ETS proteins are candidate regulators of cKIT expression since its promoter contains potential binding sites for these TFs (39). The positive relationship between cKIT and Fli-1 expression may partially explain the ability of this ETS transcriptional regulator to increase progenitor cell activity and induce changes that resemble an earlier stage of erythroid development. Future studies involving the regulation of cKIT by Fli-1 should uncover the role of this TF in malignant transformation, and its ability to alter the balance between hematopoietic differentiation and proliferation.

When Spi-1/PU.1 was expressed in F-MuLV-induced erythroleukemia cell line CB3, these cells exhibited characteristic opposite to Fli-1 overexpressing DP17-17 cells. Spi1/PU.1 expressing CB3 cells grow faster in culture associated with an increase in expression of growth promoting genes including JAK-2, cMYC and activation of PI3K and Ras pathway. Since JAK-2 activation regulates all these growth promoting genes and pathways, this kinase is likely a direct or indirect target of Spi-1/PU.1 (40), which should be investigated in an independent study. The Spi-1.PU.1 cells also exhibited morphology of more differentiated CFU-E progenitors associated with higher expression of CD71 and TER119, as previously described (20). In addition, a lower expression of cKIT and SCA-1, associated with more primitive erythroid progenitors, detected in Spi-1/PU.1 transduced cells. These results suggested that while Fli-1 and Spi-1/PU.1 play a critical role in the induction of different erythroleukemias, their oncogenic function can only be exerted in distinct erythroid progenitors. As summarized in Fig. 6E, we propose that Fli-1 overexpression in CFU-E progenitors can initiate a de-differentiation phenotype like BFU.E. This BFU-E phenotype can be induced to become CFU-E through Spi-1/PU.1 overexpression.

The overexpression of Fli-1 led to increased latency of disease progression, upon transplantation of transduced DP17-17 cells in DBA/2J syngeneic mice. This increase in disease latency of SFFV-induced erythroleukemia seems to support the notion that Fli-1 overexpression imparts the expansion of a more immature erythroid phenotype. This is consistent with lower proliferation ability of cancer stem cells versus more differentiated cells arisen from these cells within the tumors (41).

In conclusion, we have shown that Fli-1 overexpression in erythroid progenitors induces de-differentiation program, while Spi-1/PU.1 reverses this process. This phenomenon may be partially mediated through direct activation of distinct Fli-1 target genes, including cKIT, CD41, GATA1, CD71 and Spi-1/PU.1 target genes JAK-2. These results for the first time demonstrate the role for these ETS genes in progenitor proliferation/self-renewal and differentiation. Most importantly, the discovery of a role for Fli-1 in self-renewal may also shed light on the pathogenesis of diseases associated with Fli-1 aberrant regulation including erythroleukemia and Ewing's sarcoma.

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