

The Effects of the Fanconi Anemia Zinc Finger (FAZF) on Cell Cycle, Apoptosis, and Proliferation Are Differentiation Stage-specific*

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FAZF, a member of the BTB/POZ family of transcriptional repressor proteins, has been shown to bind to FANCC, the protein defective in patients with the bone marrow failure syndrome Fanconi anemia complementation group C. Because bone marrow failure in Fanconi anemia has been attributed to a failure of the hematopoietic stem cell population to produce sufficient progeny, we documented the expression of FAZF in human CD34⁺ hematopoietic progenitor cells. FAZF was expressed at high levels in early stages of differentiation but declined during subsequent differentiation into erythroid and myeloid lineages. Consistent with its presumed role as a transcriptional repressor, FAZF was found in the nuclear compartment, where it resides in distinct nuclear speckles at or near sites of DNA replication. Using a FAZF-inducible myeloid cell line, we found that enforced expression of FAZF was accompanied by accumulation in the G₁ phase of the cell cycle followed later by apoptosis. These results suggest an essential role for FAZF during the proliferative stages of primitive hematopoietic progenitors, possibly acting in concert with (a subset of) the Fanconi anemia proteins.

FAZF¹ (also known as TZFP (1) and murine homologs ROG (2) and Tzfp (3)), a new member of the BTB/POZ (pox virus and zinc finger) protein family, was identified based on its ability to interact with the Fanconi anemia (FA) complementation group C protein, FANCC (4). FA is a genomic instability disorder characterized by progressive pancytopenia, diverse congenital

anomalies, and predisposition to cancer, particularly acute myeloid leukemia and squamous cell carcinoma (for review, see Ref. 5). Congenital malformations in FA are a striking feature of the disease. The most common are defects in the thumbs (*e.g.* absent, bifid, or hypoplastic thumb) (6), although abnormalities are variable and may involve any major organ system. The range and type of developmental defects observed suggest that FA genes are involved in morphogenesis. Cells from FA patients are hypersensitive to DNA cross-linking agents such as mitomycin C. In addition, FA cells have an abnormal cell cycle profile, and this abnormality is exacerbated by treatment with mitomycin C (7). CD34⁺ cells isolated from FA-C patients fail to thrive *in vitro* (8, 9), and treating normal CD34⁺ cells with antisense oligonucleotides directed against the FANCC gene recapitulates the defective phenotype (10). These results suggest that FA genes are involved in ensuring the proper growth and differentiation of primitive hematopoietic cells. Despite efforts to discover the function of the FANCC protein and the function of the proteins encoded by the other more recently cloned FA genes (FANCA, FANCD2, FANCE, FANCF, and FANCG), the basic defect is still unknown (11). There is strong evidence that several FA proteins functionally interact within a large protein complex, and recent experiments demonstrated that the Fanconi anemia protein FANCD2 is coupled to a DNA damage response pathway involving BRCA1 (12).

We reported previously that FAZF is a transcriptional repressor belonging to the BTB/POZ family of proteins and is similar to the PLZF protein (4). PLZF is involved in reciprocal chromosomal translocations with the retinoic acid receptor α leading to a form of acute promyelocytic leukemia (for review, see Melnick and Licht (13)). PLZF is a sequence-specific transcriptional repressor that functions by interacting with co-repressor complexes including HDAC1. Enforced expression of PLZF in the murine hematopoietic cell line 32Dcl3 blocks cells in G₁/S phase, inhibits cell growth and differentiation, and leads to apoptosis (14). Experiments with PLZF nullizygous mice showed that it is essential for axial skeleton patterning and normal limb development (15).

There are many unanswered questions regarding the function of FAZF in hematopoiesis and its relationship to the pathogenesis of FA, including its expression profile and its effects on growth and differentiation in hematopoietic cells. In the present study, we show that FAZF mRNA and protein are expressed in primary hematopoietic CD34⁺ progenitor cells, increase during early proliferation, and are then down-regulated

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¹ The abbreviations used are: FAZF, FA zinc finger; FA, Fanconi anemia; PLZF, promyelocytic zinc finger; SLF, stem cell factor; CSF, colony-stimulating factor; IL-3, interleukin-3; CFU, colony-forming unit; BrdUrd, bromodeoxyuridine; Epo, erythropoietin; DAPI, 4,6-diamidino-2-phenylindole.

during terminal differentiation in both erythroid and myeloid lineages. The pattern is similar but not identical with that of PLZF. Further analysis using a FAZF-inducible hematopoietic cell line demonstrated that enforced expression of FAZF triggers a G₁ phase cell cycle arrest followed by increased apoptosis. Thus, our data suggest that FAZF influences hematopoietic cell fate, and its effects are conditional, depending on the proliferation/differentiation status of the cell.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, Cytokines, Viruses, and Chemicals—The pFLAG-FAZF expression plasmid and 293-EBNA/FLAG-FAZF cell line have been described (4). FAZF-specific antibodies were prepared using a FAZF-glutathione *S*-transferase fusion protein as an antigen, essentially as described previously (16). The glutathione *S*-transferase-FAZF expression plasmid was constructed by inserting an internal *Xho*I-*Bam*HI fragment from FAZF cDNA, encoding amino acid residues 114–295 of the FAZF protein, into an *Xho*I-*Bam*HI-cut pGEX-5x-2 vector (Amersham Biosciences). U937T/FAZF cells, a line of U937 cells stably expressing FAZF and the autoregulatory tet-VP16 under the control of the tet-operator promoter (17), were routinely maintained in RPMI 1640 with 10% fetal bovine serum, 0.5 μ g/ml puromycin, and 0.1 μ g/ml tetracycline in 5% CO₂. Recombinant human Epo was purchased from Amgen Corp. (Thousand Oaks, CA) and used at 1 unit/ml. Recombinant human SLF and granulocyte-CSF were purchased from R & D Systems Inc. (Minneapolis, MN) and used at 50 and 10 ng/ml, respectively. Recombinant human GM-CSF and IL-3 were gifts from Immunex Corp. (Seattle, WA) and used at 200 units/ml each. Monoclonal mouse anti-PLZF antibody was purchased from Oncogene Research Products (Cambridge, MA). All other antibodies and chemicals were obtained from Sigma unless otherwise indicated.

Construction of Inducible Cell Lines—Tetracycline-inducible FLAG-tagged FAZF cell lines were produced using the Tet-off system (18). Briefly, an *Eco*RI/*Bgl*II fragment containing FAZF cDNA was obtained from pSG5-FAZF (4) and cloned into pUHD10–3 cut with *Eco*RI and *Bam*HI (18). For each transfection 2×10^7 U937T cells were washed once and resuspended in 400 μ l of additive-free RPMI 1640. Ten micrograms of pUHD:FAZF and 1 μ g of pIND (Invitrogen), as a neomycin selection marker, were linearized and co-transfected by electroporation at 960 microfarads and 0.17 kV using a Bio-Rad electroporator. After 24 h cells were plated into methylcellulose containing 10% fetal bovine serum, 0.1 μ g/ml tetracycline, and 0.5 μ g/ml puromycin with 1 mg/ml G418. Resistant clones were isolated and analyzed for FAZF expression. Growth of triplicate cultures of U937 cells was measured by the metabolic conversion of tetrazolium to formazan (CellTiter 96 Proliferation Assay, Promega).

Separation and Culture of CD34⁺ Cells—Mononuclear cells were obtained using Ficoll-Hypaque centrifugation from normal human umbilical cord blood. CD34⁺ cells were isolated with magnetic-activated cell separation beads (Miltenyi Biotech Inc., Auburn, CA) according to the instructions of the manufacturer. The purification method routinely gave CD34⁺ cells that were more than 80–95% pure, as previously described (19, 20). CD34⁺ cells were cultured either in suspension culture or semi-solid culture for differentiation as previously described (19–22). Briefly, 200 cells/ml were seeded in semisolid cultures contained Iscove's modified Dulbecco's medium (Invitrogen), 1% methylcellulose, 30% fetal calf serum, 0.1 mmol/liter hemin (Eastman Kodak Co.), 2 mmol/liter L-glutamine (Invitrogen), 0.1 mmol/liter β -mercaptoethanol, 200 units/ml granulocyte-macrophage-CSF, 200 units/ml IL-3, 50 ng/ml SLF, 1 units/ml Epo, 10 μ g/ml vitamin B₁₂, 15 μ g/ml folic acid, 10 μ g/ml insulin. For erythroid differentiation in suspension culture, CD34⁺ cells were seeded at 2×10^4 cells/ml in Iscove's modified Dulbecco's medium containing 15% fetal calf serum in the presence of IL-3, granulocyte-macrophage-CSF, SLF, and Epo. For myeloid differentiation, CD34⁺ cells were cultured either in suspension or methylcellulose culture for CFU-granulocyte macrophage as for erythroid cultures except with the addition of 10 ng/ml granulocyte-CSF in lieu of Epo.

Reverse Transcriptase-PCR Analysis—Total RNA was isolated from freshly separated CD34⁺ cells (d0) or differentiated cells in both erythroid and myeloid lineages at different time courses (d3–d14) by Qiagen RNeasy mini kits (Qiagen, Valencia, CA) as suggested by the manufacturer. Total RNA was treated with DNase I for 15 min at room temperature followed by heating at 95 °C for 10 min before reverse transcription to eliminate any contamination of remaining genomic DNA. PCR was performed by a thermal cycler (PerkinElmer Life Sciences). The sequences of primers are as follows: FAZF, 5'-GAG ATG TTG CAC

AAG CAC TCG C-3', 5'-CAA CTG GTT CTG GCT CCA GAG C-3'; PLZF, 5'-GAA GCA TTC CAG CGA GGA GA-3', 5'-GGA GTA GAT GGC CAG ATG CT-3'. The primers for β -actin was described previously (20). The PCR profile used was denaturation at 94 °C for 45 s, annealing at 52–55 °C for 45 s, and polymerization at 72 °C for 2 min for 35 cycles. Ten μ l of amplification reaction was electrophoresed, transferred to membrane, and hybridized with [³²P]dCTP (Amersham Biosciences)-labeled FAZF, PLZF, or β -actin gene fragment as a probe. Hybridization was performed overnight at 42 °C, filters were washed with 0.1 \times SSC (1 \times SSC = 0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS at 55 °C for 60 min, dried, and exposed to x-ray at –70 °C.

Immunoblotting—Whole cell lysates were prepared by suspending cells at a concentration of 2×10^7 cells/ml in cell lysis buffer (50 mmol/liter Tris, pH 7.4, 0.25 mmol/liter NaCl, 0.5% Nonidet P-40, 0.1% SDS, 1 mmol/liter phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 100 mmol/liter NaF). Protein in the clear lysate was quantitated using the Bio-Rad protein assay kit and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were electroblotted onto Immobilon-P membranes (Millipore Corp., Bedford, MA) and incubated with mouse or rabbit antibodies specific for PLZF, FAZF, FLAG M2, or actin, as noted in text, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies. Proteins were detected with the ECL-Western blotting system (Amersham Biosciences).

Cell Cycle Analysis—Cells were seeded at a density of 5×10^4 cells/ml in RPMI 1640 plus 1% fetal calf serum and incubated for 1–7 days in 5% CO₂ at 37 °C. Cells were washed in phosphate-buffered saline (PBS) and fixed in 70% cold ethanol at –20 °C, washed twice in PBS, and incubated in 50 μ g/ml RNase A and 20 μ g/ml propidium iodide for 30 min at 37 °C. The cell cycle status was detected by fluorescence-activated cell sorter flow cytometry and analyzed using CellFit software (BD PharMingen).

Apoptosis Assays—Apoptosis was determined using annexin V staining. Briefly, cells were incubated with annexin V-fluorescein isothiocyanate in binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) for 15 min at room temperature, then suspended in binding buffer containing 50 μ g/ml propidium iodide followed by flow cytometry analysis.

Immunofluorescence Microscopy—For FAZF expression in 293 EBNA cells (Invitrogen), cells were seeded on chamber slides and transfected using LipofectAMINE as directed by the manufacturer (Invitrogen). Plasmids encoding epitope-tagged FAZF or the parental vectors as negative controls for antibody specificity were used as indicated in text. Cells were processed as described previously (4) using monoclonal anti-HA 12CA5 (Roche Molecular Biochemicals) and/or rabbit polyclonal anti-FLAG (Zymed Laboratories Inc., South San Francisco, CA) and Oregon Green-conjugated goat anti-mouse and/or Texas Red-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR) as secondary antibodies. For analysis of CD34⁺ cells, cells were placed on chamber slides pretreated with poly-lysine. Sites of DNA synthesis were determined by a 2-bromo-2'-deoxy-uridine (BrdUrd)-labeling kit (Roche Molecular Biochemicals). Briefly, cells were incubated in the presence of 10 μ M BrdUrd for 4.5 h, permeabilized, and fixed with 70% ethanol in a 50 mM glycine buffer, pH 2.0. Cells were incubated with rabbit FAZF-specific antisera followed by Texas Red-conjugated goat anti-rabbit (Molecular Probes). BrdUrd incorporation was visualized with an anti-BrdUrd mouse monoclonal antibody followed by a fluorescein-labeled secondary antibody. Image collection and manipulation for overlapping signals were performed as described previously (4).

RESULTS

FAZF Localizes in Foci in Human Hematopoietic Progenitor Cells—To determine the subcellular localization of FAZF we generated FAZF-specific antibodies using a glutathione *S*-transferase-FAZF fusion protein as an antigen. An internal domain of FAZF with only 14% identity to PLZF was selected to reduce potential cross-reactivity with PLZF and other BTB/POZ proteins. To demonstrate that the antibody recognizes FAZF, we examined lysates from 293 EBNA cells transiently transfected with a plasmid encoding FLAG-tagged FAZF (293/F1-FAZF) or the parental plasmid (control) by immunoblot. As shown in Fig 1A, the antibody recognizes FAZF, and no signal was observed in negative control lysates. Reprobing the blot with anti-FLAG detected a band overlapping with that recognized by anti-FAZF (Fig 1B). In immunofluorescence assays

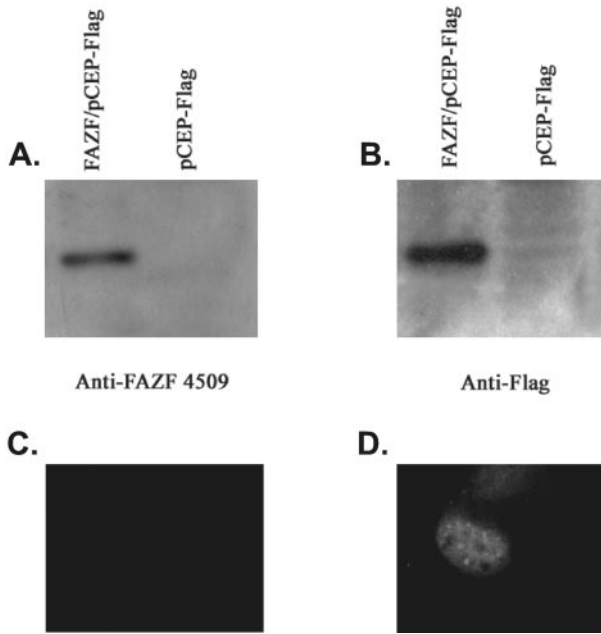


FIG. 1. FAZF-specific antibody. The lysates from 293 cells expressing FLAG-tagged FAZF (FAZF/pCEP-FLAG)- and pCEP-FLAG-transfected cells as mock controls were subjected to SDS-PAGE electrophoresis and probed with anti-FAZF 4509 antibody (A) or anti-FLAG (B). An equatorial confocal image of 293 EBNA cells expressing FLAG-FAZF was obtained by incubating the cells with anti-FAZF (D) followed by detection with an anti-rabbit antibody conjugated to Oregon Green compared with incubation with normal rabbit serum (C) as a control. The staining in panel D was identical to that using anti-FLAG (4).

using 293/FI-FAZF cells, preimmune serum lacked specific staining (Fig 1C), whereas FAZF-specific antiserum demonstrated that FAZF is localized in the nucleus of 293/FI-FAZF cells in speckles in a range of sizes from micropunctate to large foci (Fig 1D). The staining is indistinguishable from that using an anti-FLAG antibody that recognizes the epitope-tagged FAZF (4). We conclude that our antibody recognizes FAZF in both immunoblot and immunofluorescence assays.

To determine the expression pattern of endogenously expressed FAZF, CD34⁺ cells were isolated from human cord blood and grown in the presence of growth factors as described under "Experimental Procedures." The cells were fixed and stained with anti-FAZF and examined by immunofluorescence. As shown in Fig 2, FAZF is expressed in the nucleus in a speckled pattern, consistent with the pattern observed in 293 cells overexpressing the FLAG epitope-tagged FAZF (4). The speckled pattern is similar to that observed for other BTB/POZ-containing proteins including the related proteins, PLZF (23) and BCL6 (also known as LAZ3) (24, 25).

Endogenous FAZF Associates with Replication Foci in Primary Cells—The significance of the nuclear speckled pattern observed for certain BTB/POZ-containing proteins is unknown. However, recent experiments with BCL6 by Albagli *et al.* (25) demonstrate a close association of BCL6 and replication foci, suggesting that BCL6 may have a positioning or assembly role in replication. To test the possibility that FAZF associates with sites of DNA synthesis, we examined CD34⁺ cells that were grown in the presence of erythroid factors for 5 days by pulse-labeling the sites of DNA synthesis with BrdUrd. Stained cells were examined by confocal microscopy. Fig 3 shows that FAZF and BrdUrd labeling occur in foci and that these foci are often adjacent or overlapping, suggesting that FAZF is localized near sites of DNA replication.

FAZF Is Expressed during Erythroid and Myeloid Differentiation of Human Hematopoietic Progenitor Cells—To begin to

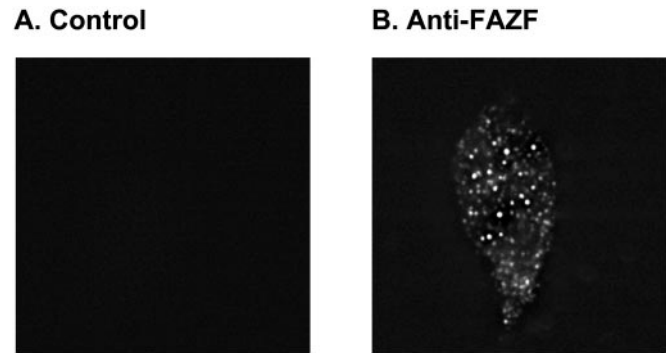


FIG. 2. FAZF localizes in foci in human hematopoietic progenitor cells. CD34⁺ cells were fixed, permeabilized, and then stained with a rabbit polyclonal antibody specific for FAZF or preimmune normal rabbit serum followed by staining with a fluorescent-conjugated secondary antibody (Texas Red, Molecular Probes). Immunofluorescence was examined by laser confocal microscopy (Deltavision, Applied Precision Inc.) using a 100 \times oil lens. Panel A, control cells were negative. Panel B, representative cell stained with anti-FAZF exhibit a micro-punctate nuclear pattern, similar to that of PLZF.

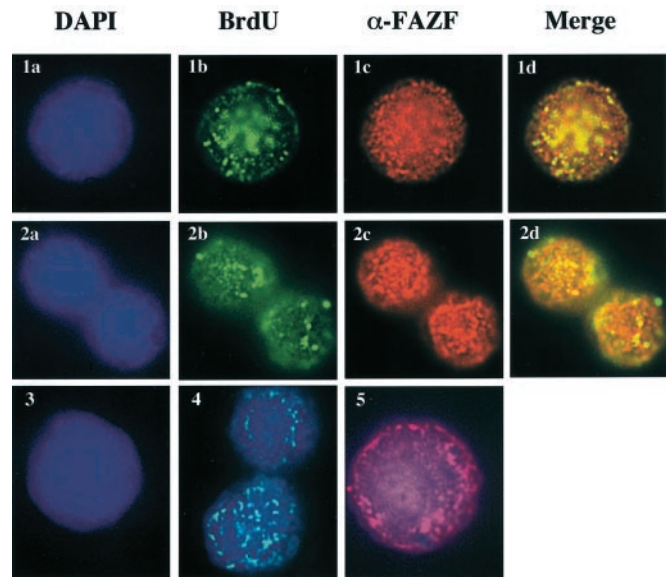
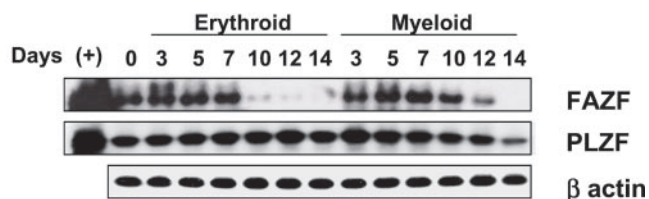


FIG. 3. FAZF associates with sites of DNA synthesis in human hematopoietic progenitors. The first two rows show representative CD34⁺ cells (1 and 2) grown for 3 days in the presence of erythroid growth factors and stained with a nuclear marker (DAPI), anti-BrdUrd (BrdU), and anti-FAZF. 1a, DAPI; 1b, anti-BrdUrd; 1c, anti-FAZF; 1d, merged image of anti-BrdUrd and anti-FAZF; 2a, DAPI; 2b, anti-BrdUrd; 2c, anti-FAZF; 2d, overlap of anti-BrdUrd and anti-FAZF; 3, cells stained with DAPI and preimmune rabbit serum; 4, cells stained with anti-BrdUrd and DAPI; 5, cells stained with anti-FAZF and DAPI.

define the role of FAZF in hematopoiesis, we determined the expression of FAZF during early proliferation and terminal differentiation stages of CD34⁺ progenitor cells directed to either erythroid or myeloid lineages by the presence of SLF, IL-3, granulocyte, macrophage-CSF, plus Epo (for erythroid) or granulocyte-CSF (for myeloid). The day-7–14 BFU-E (burst-forming unit-erythrocyte)-derived cells were described (19), and CFU-granulocyte macrophage-derived cells expressed myeloid lineage markers such as CD11b/CD14 by flow cytometry analysis (data not shown). The day-3 and -5 suspension culture-derived cells for both erythroid and myeloid lineages can form secondary CFU-erythrocyte and CFU-granulocyte macrophage colonies (data not shown), indicating that the cells differentiate to specific erythroid or myeloid lineages under these culture conditions. The expression of FAZF was compared with that of PLZF, the closest hematopoietic counterpart to FAZF.

A. RT-PCR



B. Western Blot

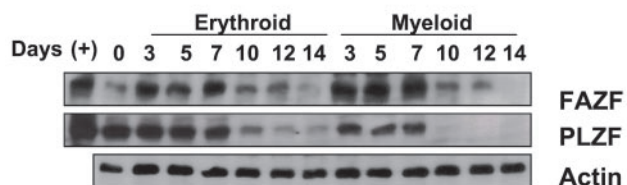


FIG. 4. FAZF is expressed during erythroid and myeloid differentiation of human CD34⁺ cells. Protein or RNA was prepared from freshly separated CD34⁺ cells (day 0), from suspension cultures of CD34⁺ cells grown for 3 and 5 days, and from colonies grown in 1% methyl cellulose cultures for 7, 10, 12, and 14 days in the presence of SLF, IL-3, granulocyte, macrophage-CSF, and with Epo (for erythroid differentiation) or granulocyte-CSF (for myeloid differentiation). *Panel A*, reverse transcriptase (RT)-PCR amplification was performed on total RNA prepared from the time points as indicated. *Panel B*, cell lysates were subjected to SDS/PAGE, and immunoblots were performed by using anti-FAZF or anti-PLZF antibodies. An actin immunoblot was used as a protein loading control.

Cell samples collected at days 0, 3, 5, 7, 10, and 14 were assayed for FAZF or PLZF protein by immunoblot using anti-actin as a control for protein loading. Transcripts from corresponding samples were also analyzed by reverse transcriptase-PCR. As shown in Fig. 4A, PLZF mRNA was expressed in CD34⁺ progenitor cells at a high level, consistent with previous reports (14, 23). PLZF was expressed during both early proliferation (before day 7) and terminal differentiation (after day 7) stages in both erythroid and myeloid lineages. No differences in expression were observed between lineages. In contrast, FAZF was expressed in CD34⁺ progenitor cells at a lower level relative to PLZF, which remained at high levels during early proliferation but was down-regulated during terminal differentiation in both lineages. As shown in Fig. 4B, the expression of both FAZF and PLZF proteins is high before day 7 and decreases during terminal differentiation stages, suggesting that PLZF but not FAZF is regulated at the post-transcriptional level during hematopoiesis *in vitro*. These results suggest that down-regulation of both PLZF and FAZF is required for the proper differentiation of both erythroid and myeloid lineages.

Generation of FAZF-inducible Cell Lines—We anticipated difficulty in directly assessing the biological function of FAZF by expressing the protein in cells because FAZF is similar to other proteins that negatively regulate growth. Thus, to complement the results obtained in primary cells and to examine the possible growth inhibitory and pro-apoptotic effects of FAZF in hematopoietic cells, we used a tetracycline-regulated expression system. A parental U937T cell line constructed to constitutively express the tetracycline repressor (17) was transfected with a FAZF construct under the control of the TET operator, and individual clones were isolated. Expression of FAZF upon tetracycline withdrawal was assessed in each independent clone by immunoblot with an anti-FAZF polyclonal antibody followed by a peroxidase-conjugated secondary antibody. We selected two clones with tight regulation and high

expression levels termed U937T/FAZF-6 and -30, respectively (Fig 5A). A cell line that was neomycin-resistant but did not produce FAZF as detected by immunoblot (U937T/NEO) was used as a negative control in all experiments. The growth rate, viability, and cell cycle profile before and after tetracycline withdrawal of the control U937T/NEO cell line was indistinguishable from the parental line U937T. In contrast, cultures of U937T/FAZF cell clones were significantly growth-suppressed upon withdrawal of tetracycline as shown in Fig 5B.

Enforced FAZF Expression Induces Apoptosis—BCL6 and PLZF, related BTB/POZ-containing proteins, can mediate apoptosis (14, 24). To determine whether FAZF triggers apoptosis, we tested the U937T/FAZF-inducible cell lines before and after FAZF expression. Apoptosis was demonstrated by an annexin V staining assay, which is a sensitive method for detection of cells in pre-apoptosis. We measured annexin V staining and propidium iodide staining over time in control cells and in cells induced to express FAZF. Annexin V staining labels cells that are undergoing apoptosis, whereas propidium iodide staining labels cells that have undergone programmed cell death that they become permeable to this nucleic acid stain. Thus, apoptotic cells bind annexin V and later become stained with propidium iodide. In cells where FAZF is not expressed, the population of annexin V-positive cells ranged from 3.7 to 6.1% over a 5-day period, and propidium iodide staining cells increased over this time period from 1.7 to 3.3% of the population (Fig 6). In contrast, cells with induced FAZF expression exhibit a significant increase in annexin V-positive cells during this time, and at day 7 post-induction, 32.8% of the population was stained in this representative experiment. A concomitant increase in cells stained by propidium iodide was observed, reaching 13% at day 7. We conclude that enforced FAZF expression in U937T/FAZF cells induces apoptosis.

Enforced FAZF Expression Results in G₁ Arrest—The combination of growth suppression and apoptosis led us to examine the cell cycle profiles in FAZF-expressing cells. U937/FAZF clones 6 and 30 were followed for 7 days after induction of FAZF expression. In the parental control cells and in FAZF clones in the presence of tetracycline (no FAZF expression), the cell cycle profile included ~60% of cells in G₁ and ~20% in each of the S and G₂/M phases. In contrast, in FAZF-expressing clones 3 days post-induction, 90% of cells accumulated in G₁, and cells in S and G₂/M phase decreased (Fig 7). We conclude that enforced FAZF expression in these cells causes accumulation in the G₁ phase of the cell cycle.

As shown in Fig 4, both PLZF and FAZF are highly expressed in CD34⁺ cells and in proliferating derivatives up to 7 days of culture *in vitro*, and then much lower levels of FAZF and PLZF proteins are detected thereafter. At this time cells in the population are predominantly in S phase and G₁ (70%, 29%, respectively) (19). Thus, physiologic expression of FAZF or PLZF is compatible with proliferation and differentiation in this context, at least up to about day 7. We infer from these data that appropriate expression of FAZF is required in the early stages of hematopoietic differentiation, and FAZF-dependent accumulation of cells in G₁ is conditional, depending on cell type and differentiation status.

DISCUSSION

FAZF was identified as an interacting partner of the Fanconi anemia protein FANCC by a yeast two-hybrid assay, subcellular colocalization, and coimmunoprecipitation (4). FAZF also interacts with another FA protein, FANCG, in yeast two-hybrid and in coimmunoprecipitation experiments, reinforcing the notion that FAZF might be part of the FA protein complex (26).

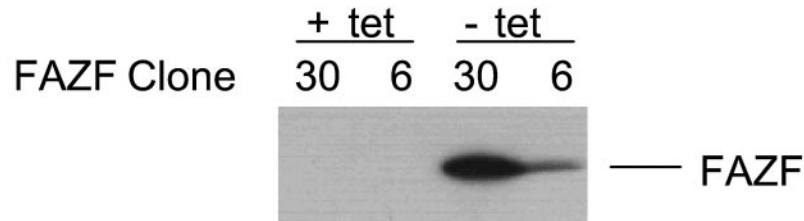
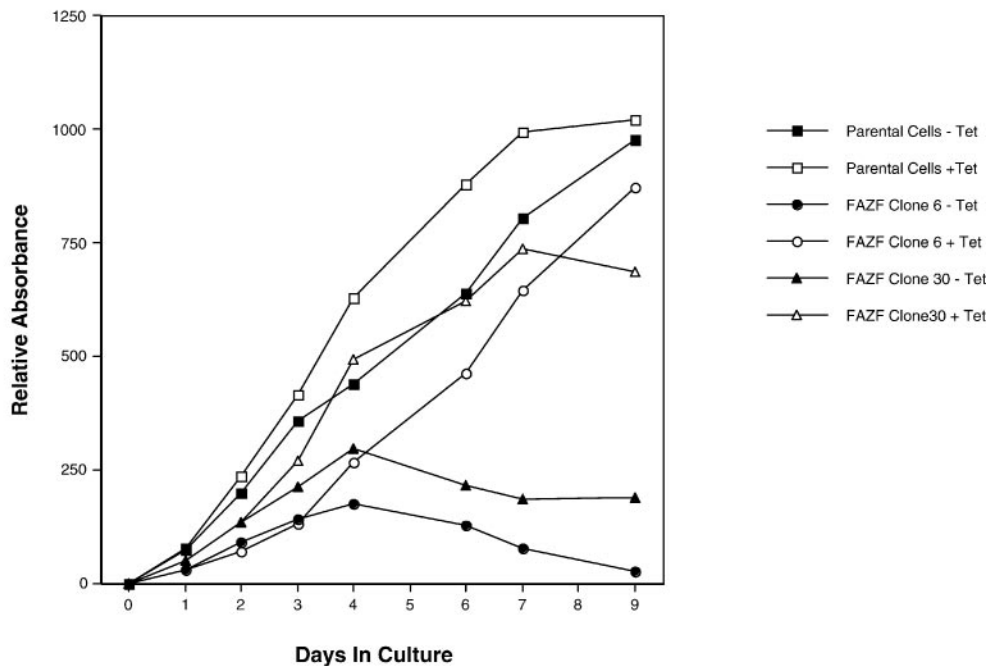
A.**1 Day after induction****4 Days after induction****B.**

FIG. 5. Characteristics of FAZF-inducible cell lines. A, U937T/FAZF cell clones were grown in the presence or absence of tetracycline for 1 and 4 days. Cell lysates were analyzed by SDS-PAGE followed by immunoblot with anti-FAZF. B, expression of FAZF has a growth-suppressive effect. Control U937T parental cells and FAZF-expressing clones 6 and 30 were seeded in the presence and absence of tetracycline. Proliferation assays on cultured cells were performed daily.

Because so little is known about the function(s) of FAZF or of the FA proteins, the current work is focused on the basic biology of FAZF, emphasizing early hematopoiesis. We chose to examine hematopoietic progenitor cells because this compartment is a likely intersection between FAZF and FANCC func-

tion, based on the structural similarity of FAZF to PLZF and on the pancytopenia observed in FA. To analyze role of FAZF in early hematopoiesis, we determined expression at the mRNA and protein levels, determined subcellular localization, and measured the effects of its enforced expression. A unique aspect

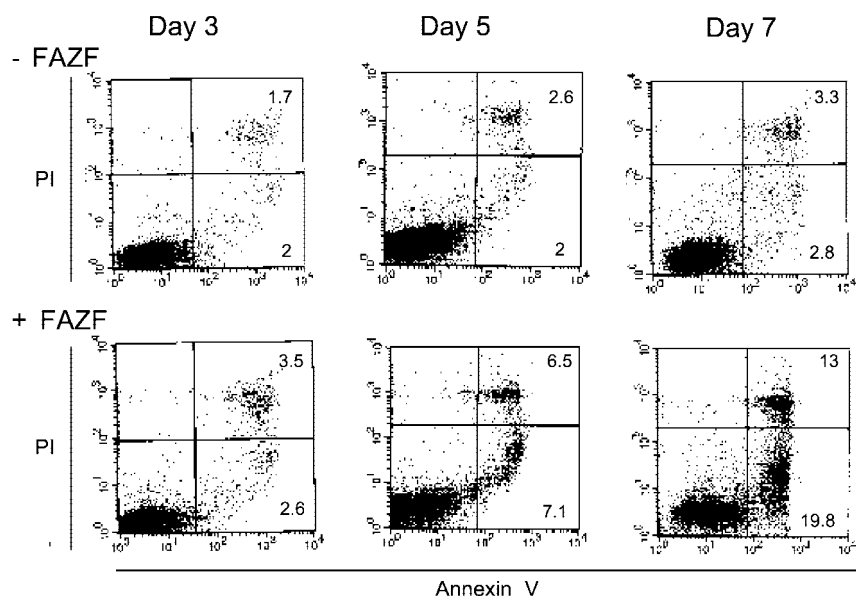
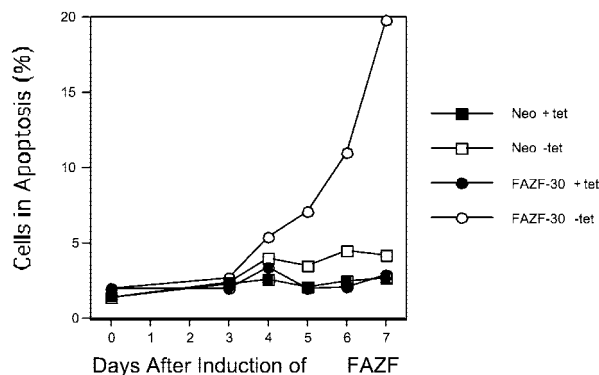


FIG. 6. Enforced FAZF expression induces apoptosis. The U937/FAZF-inducible cell line was assayed for annexin V and propidium iodide staining with and without induction of FAZF expression at days 3, 5, and 7 post-induction. The lower panel shows annexin V staining (apoptosis) over the course of induction. *tet*, tetracycline.



of our work is that these experiments were performed in primary cells as well as with a FAZF-inducible cell line. As a result, we had the opportunity to observe that the effects of FAZF protein expression depended on the differentiation stage of the cells. Our results support the hypothesis that FAZF and PLZF have similar but non-overlapping characteristics and that FAZF plays a role in cell fate decisions during normal hematopoiesis.

We found that expression of FAZF protein in quiescent CD34⁺ cells was reproducibly lower than that in proliferating derivatives, whereas PLZF was highly expressed in both quiescent and proliferating stages of CD34⁺ cells. Both FAZF and PLZF are expressed at high levels between days 3 and 7 and then decline. As protein expression begins to decrease for PLZF in both erythroid and myeloid lineages, PLZF mRNA is still abundant. Thus, our data suggest that unlike FAZF, PLZF is post-transcriptionally regulated in both myeloid and erythroid lineages as CD34⁺ cells proliferate and express their commitment to differentiate. The overlapping expression pattern during early proliferation and late differentiation stages of primary CD34⁺ cells between FAZF and PLZF suggests that FAZF expression must decrease to allow for the normal differentiation program. These conclusions are in agreement with those made previously for PLZF using a PLZF-inducible cell line (14, 27).

Like PLZF, FAZF is localized in discrete nuclear foci of various sizes in an asynchronous population of hematopoietic progenitors. BCL6, another BTB/POZ protein, which is involved in translocations frequently found in non-Hodgkin lym-

phomas (28, 29), is also located in distinct nuclear foci in osteosarcoma cells (24). BCL6 and PLZF proteins function by interacting with co-repressor complexes including histone deacetylase. Recent evidence suggests that BCL6 foci coincide or are nearby DNA replication foci, suggesting that this structurally related transcriptional repressor is involved in some processes related to DNA synthesis (25). The functional significance of the foci is unknown, although BCL6 derivatives lacking the BTB/POZ domain fail to form foci, do not associate with ongoing DNA synthesis, and also fail to function as transcriptional repressors (24). Because FAZF can associate with sites of DNA synthesis, FAZF may have a role in initiation or progression of synthesis. Alternatively, with regard to its interaction with FANCC, FAZF could have a role in DNA repair processes that occur during replication. In this regard, the recent work by Garcia-Higuera *et al.* (12) demonstrating the DNA damage-induced co-localization of FANCD2 and BRCA1 in nuclear foci is especially intriguing. BRCA1, a protein with multiple functions involved in the maintenance of genomic stability, has been shown to associate with components of the histone deacetylase complex (30). Further underscoring the possible connection between FA proteins and chromatin remodeling, recent work by Otsuki *et al.* (31) demonstrate that FANCA interacts with the BRG1 gene product, a subunit of the SWI/SNF chromatin remodeling complex.

The strong homology between FAZF and PLZF and our initial work demonstrating an overlap in transcriptional repression activity suggested that the function of FAZF and PLZF were related. Based on previous experiments with PLZF, we

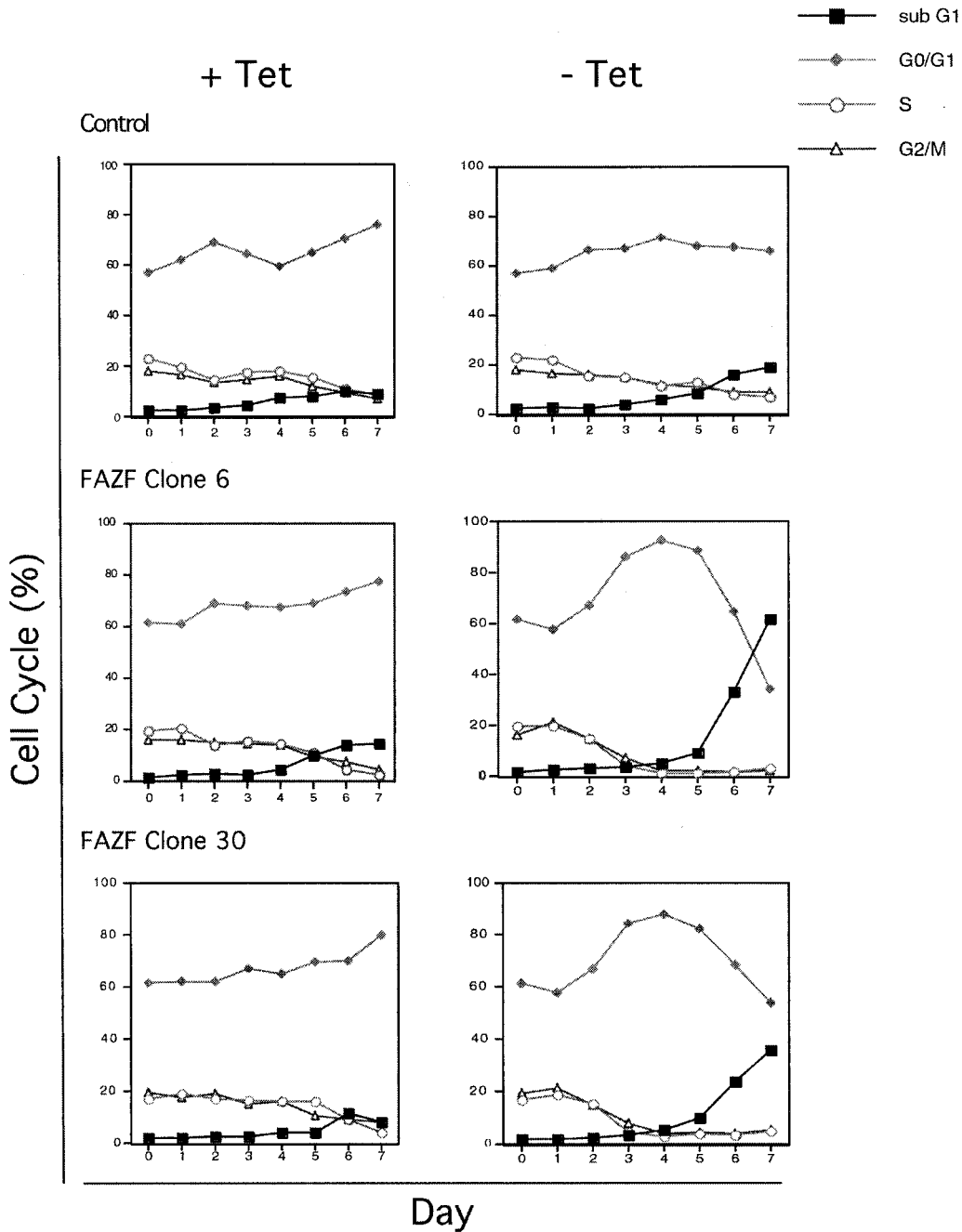


FIG. 7. **Enforced FAZF expression results in G₁ arrest in U937 cells.** Control U937T clone and two FAZF-expressing clones were grown in the presence or absence of tetracycline (*Tet*). Cell cycle analysis was performed daily over a 7-day period.

hypothesized that enforced expression of FAZF would have a profound effect on cell cycle progression, differentiation/proliferation, and apoptosis (14, 27). When expression was induced in the cell lines, FAZF acted as an effective negative regulator of cell growth. Cell cycle analysis of the U937T/FAZF cell lines 3 days after FAZF induction demonstrated that the cells accumulated in the G₁ phase of the cell cycle in significant numbers. Moreover, the accumulation in G₁ presaged a shift to apoptosis, with apoptotic cells beginning to rise at day 4 post-induction to 32.8% of the cell population at day 7 post-induction.

One major conclusion that can be drawn from our studies comparing FAZF-inducible cell lines and physiologic expression of FAZF in CD34⁺ cells is that the high physiologic expression of FAZF (and PLZF) in CD34⁺ (days 3–7) cells is compatible with rapid growth in these cells. In contrast, FAZF expression in U937T/FAZF cells has the effect of rapidly caus-

ing G₁ accumulation followed by apoptosis. One might have concluded from enforced (inducible) expression that PLZF and FAZF have negative growth effects, as reported earlier for PLZF and BCL-6 (14, 24). However, because our studies show that the expression of FAZF and PLZF is abundant in rapidly proliferating derivatives of CD34⁺ cells, this effect apparently depends on the cell type where FAZF and PLZF are expressed. A possible mechanism for these different effects is that repression of critical target genes required for growth suppression might not occur in the CD34⁺ cells because certain differentiation stage-specific co-repressors are absent. Alternatively, the action of certain specific corepressors could be consistent with survival and proliferation of CD34⁺ cells. Specific co-repressors for BTB/POZ-containing proteins are possible in addition to the HDAC1-containing corepressor complex that interacts with (among others) PLZF and BCL6. Indeed, a specific co-repressor

has been identified for BCL6 (32). It remains to be seen if FAZF has unique interaction partners that can act as co-repressors or for other functional roles. In this regard, it is interesting to speculate on the contribution of one of the FAZF specific interacting partners, FANCC. FANCC mRNA is expressed in CD34⁺ cells (33, 34) and is evidently necessary for survival (10). Experiments with CD34⁺ cells from FA patients as well as evidence from *Fancc*^{-/-} mouse models reinforces the idea that FANCC is critical for early hematopoietic cell development (7, 35–38). Thus, as an early step in defining the functions of FAZF and FANCC, it will be important to determine whether FAZF is a member of the FA protein core complex, particularly in early hematopoietic cells. Specifically, it will be of interest to determine whether FAZF, like PLZF, is a component of a high molecular weight complex with Cdc2 (39). Evidence from co-immunoprecipitation studies suggest that binding of FANCC and Cdc2 is required to relieve the cell cycle defect observed in FA cells (40), and other work has led to the hypothesis that FANCC is involved in a cross-link damage avoidance pathway that signals through Cdc2 (41). Recent work by Qiao *et al.* (42) demonstrates that Fanconi anemia proteins are associated with chromatin and the nuclear matrix in wild-type cells and in FA complementation group D cells but not in cells from FA patients belonging to other complementation groups. This is consistent with the idea that the FA protein complex may have a role in chromatin changes during replication and DNA damage repair. Further studies will focus on the role of FAZF in this process.

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