Altered Differentiation in Acute Myeloid Leukemias; Role of ERG and FUS-ERG Fusion Protein

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By

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In dedication to my Nana Wright and Grandpa Doarn
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Abbreviations

- AML: Acute Myeloid Leukemia
- APP: (β-Amyloid Precursor Protein)
- C/EBPα: (CCAAT/enhancer binding protein)
- CML: Chronic Myelogenous Leukemia
- CLP: Common Lymphoid Progenitor
- CMP: Common Myeloid Progenitor
- DNA: Deoxyribose Nucleic Acid
- ERG: ETS Related Gene
- ERK: Extracellular Regulated Kinase
- FBS: Fetal Bovine Serum
- G-CSF: Granulocyte Colony-Stimulating Factor
- GFP: Green Fluorescent Protein
- GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor
- HGF: Hematopoietic Growth Factor
- HSC: Hematopoietic Stem Cell
- IL: Interleukin
- LF: Lactoferrin
- MCL: Mantle Cell Lymphoma
- M-CSF: Macrophage Colony-Stimulating Factor
- MPO: Myeloperoxidase
- mRNA: Messenger RNA
- N-myc 1: Neuroblastoma myc-related oncogene 1
- PAGE: PolyAcrylamide Gel Electrophoresis
- RNA: Ribonucleic Acid
- SDS: Sodium Dodecyl Sulfate
- TBS: TRIS Buffer Saline
- TLS: Translocated in Liposarcoma
SUMMARY/ABSTRACT

The FUS-ERG chimeric oncogene has been associated with fatal acute myeloid leukemias (AML) carrying the non-random t(16;21) (p11;q22) chromosomal aberration. In these leukemias, the presence of the t(16;21) translocation product FUS-ERG is associated with a) an increase in blasts with a round or irregular nucleus, basophilic cytoplasm, vacuoles and, occasionally, with phagocytosis features; b) the presence of micromegakaryocytes; and c) impaired myeloid differentiation with dysplastic neutrophils. Reportedly, ectopic expression of FUS-ERG in murine myeloid precursor (L-G) and in human cord blood cells induces changes which, in part, might account for the phenotype observed in t(16;21) AML patients. However, the FUS-ERG-dependent mechanisms underlying multilineage hematopoietic differentiation defects are still unclear.

To understand how FUS-ERG affects granulocytic differentiation, we independently generated 32Dcl3-derived myeloid progenitor cell lines expressing HA-tagged ERG, FUS, and FUS-ERG oncoprotein. In the presence of the growth factor, IL-3, all cell lines showed similar proliferation rate without acquiring cytokine-independent growth. However, FUS and FUS-ERG, but not ERG, expression decreased the susceptibility to apoptosis induced by cytokine-deprivation in a dose-dependent manner. Interestingly, viable FUS-ERG-expressing cells (30%) were observed after five days in IL-3-deprived culture, whereas no parental cells were viable after 24 hours. Conversely, FUS-ERG-expressing cells showed enhanced proliferation and delayed differentiation when cultured in the presence of G-CSF, a growth factor important in granulocytic
differentiation. By contrast, marked apoptosis was observed in G-CSF-treated FUS- and ERG-expressing cells.

To dissect the FUS-ERG-dependent molecular mechanisms underlying altered differentiation, we assessed protein levels of G-CSF receptor (G-CSFR) and of the two major regulators of myelopoiesis, PU.1 and C/EBPα. Surprisingly, G-CSFR and C/EBPα, but not PU.1, levels were markedly increased in FUS-ERG-expressing cells but not in FUS- or ERG-transduced lines. Moreover, by microarray analysis we found that FUS-ERG expression specifically induced a) downregulation of genes either required for granulocytic differentiation or inhibiting G-CSF-induced proliferation; b) downregulation of pro-apoptotic and growth-suppressor genes and upregulation of positive regulators of survival and proliferation; and c) upregulation of genes that either promote megakaryocytic differentiation or are markers of differentiation into other hematopoietic lineages. Thus, it appears that FUS-ERG, rather than FUS or ERG overexpression, enhances survival and induces proliferation of myeloid progenitors by negatively affecting their ability to respond to differentiation stimuli.
INTRODUCTION

Stem cells are the totipotent cells of the body that are capable of extensive proliferation and differentiation (Gilbert, 2003). Stem cells are induced by the body to create all cells of an organism and constantly replenish various organic tissues such as blood, skin, and cells of the brain and liver. In between the stem cell and its terminally differentiated progeny cells there is an intermediate population of committed progenitor cells that have limited capacity to proliferate and regulated differentiation potentials (Watt and Hogan, 2000). The hematopoietic stem cell (HSC) has the ability of self-renewal and gives rise to the different types of blood cells (Figure 1). The adult pluripotent hematopoietic stem cells are found in the bone marrow and are responsible for generation of blood and lymph cells of the body. Active bone marrow is found in the central cavity of all bones at birth. However, by young adulthood the vertebrae, hips, shoulders, ribs, breastbone, and skull are the only bones of the body that continue to actively make blood cells (Beutler and Williams, 2001). Hematopoiesis is the process by which the HSCs proliferate and differentiate to give rise to committed progenitors of the blood; lymphoid and myeloid (white cells), and erythroid (red cells) (Figure 2).

Fig. 1: Effects of transcription factors (green arrows) on proliferation and commitment of the hematopoietic stem cell
These lineage-committed cells terminally differentiate into essential blood cells of the mammalian body: CTLs, plasma, helper T-cells, natural killer (NK) cells, platelets, basophils, eosinophils, neutrophils, macrophages, and erythrocytes (Figure 2). Red cells (erythrocytes) and platelets are confined to the blood system whereas the white blood cells can exit the blood system and enter tissues where they are able to battle infection.

There are various factors affecting each step of the lineage pathways called hematopoietic growth factors (HGFs). These factors are also known as cytokines and include granulocyte colony-stimulating factor (G-CSF), interleukin-3 (IL-3), granulocyte-macrophage colony stimulating factor (GM-CSF), CSF-1, macrophage colony stimulating factor (M-CSF), stem cell factor (SCF), and tumor necrosis factor (TNF) (Gilbert, 2003). Expression of these regulators is controlled at the transcriptional level (Figure 1) by specific molecules (transcription factors) which upon binding DNA, activate or repress gene expression. Transcription is the process where mRNA is synthesized from a DNA template. This process in eukaryotic cells is controlled by the RNA polymerase II (Roeder, 2005). This polymerase binds to specific sequences present...
at the 5’ end of each gene called promoters and thus initiates RNA synthesis. Following this step is elongation of the polynucleotide at its 3’ end. Finally, the polymerase reaches the termination sequence and ceases transcription. Following transcription, the newly formed messenger RNA (mRNA) leaves the cell nucleus and is translated into protein by ribosomes in the cell’s cytoplasm (Rodnina et al., 2005). The proteins created serve in many areas and functions of a cell and many are transcription factors themselves. These factors assist RNA polymerases in transcribing DNA into RNA. Some of the transcription factors are involved in hematopoiesis and include PU.1 and C/EBPα (Gilbert, 2003). The two major HGFs involved in this study are G-CSF and IL-3 and the two major transcription factors are PU.1 and C/EBPα (Figures 2 and 3).

**Fig. 3: Role of C/EBPα and PU.1 in hematopoietic cell differentiation.** (HSC) Hematopoietic stem cell; (CMP) common myeloid progenitors; (CLP) common lymphoid progenitors; Ikaros, PU.1, GATA-1, NFE2 and C/EBPα are transcription factors.
G-CSF is produced by fibroblasts, monocytes, and endothelial cells, and the protein primarily stimulates neutrophil differentiation (Figure 2). G-CSF promotes maturation and proliferation of acute myelogenous blast cells in addition to stimulating proliferation and maturation of normal common myeloid progenitors (CMP) (Beutler and Williams, 2001). IL-3 is a multi-colony stimulating factor; it stimulates the growth and maturation of immature myeloid progenitors into granulocytes, monocytes, erythrocytes, megakaryocytes, mast cells, and T-cells (Beutler and Williams, 2001) (Figure 2).

PU.1 is expressed in the HSCs in addition to the multipotential progenitors, CMP and common lymphoid progenitors (CLP), and has been shown to be a transcription factor essential for development of myeloid lineages in hematopoiesis (Scott et al., 1994). This factor regulates promoter activity of a multitude of other myeloid genes encoding growth factors (i.e. SCL, G-CSF, M-CSF, and GM-CSF). PU.1 is a member of the ETS transcription family and is important in lineage development. Alterations in this gene have been shown to create a loss of development in myeloid and B-Cells (Scott et al., 1994). In some cases of acute myeloid leukemia (AML), activity of this factor has been shown to be suppressed, which implicates that its inhibition is a crucial step in the development of leukemia (Mueller et al., 2002). Various data collected on PU.1 suggest that its degradation increases the self-renewal capacity of erythroid precursors and inhibits their differentiation (Tenen et al., 1997). C/EBPα (CCAAT/enhancer binding protein) is a member of a family of leucine zipper transcription factors (Tenen et al., 1997). This factor is essential for the induction of genes that regulate granulocytic differentiation and expression of cytokine receptors, and is important for normal myelopoiesis. These cytokine receptors include M-CSF, GM-CSF, G-CSF, and IL-3, and
genes required for granulocytic differentiation are MPO, LF, Myeloblastin, and others. Various studies on C/EBPα have suggested that it is a general inhibitor of cell proliferation and, therefore, resembles a tumor suppressor (Watkins et al., 1996). C/EBPα is genetically or functionally inactivated in AML and also inhibits the transactivation activity of PU.1 (Dahl and Simon, 2003). Alterations in any step of the hematopoietic process (from stem-cell to mature blood cells) can disrupt the normal development of these cells and lead to clonal outgrowth of one or more cell lineages which can lead to deadly disease such as lymphomas and leukemias (Pereira et al., 1998).

Lymphomas originate mostly in the lymph node system and are malignancies of the B and T cells (Hellman et al., 2001). Leukemia is a cancer of the blood cells and affects the lymphoid or myeloid compartments. Leukemia is categorized as being either chronic or acute. Chronic leukemias develop in primitive myeloid or lymphoid progenitors which retain their ability to function as normal HSCs and continue to differentiate normally (Beutler and Williams, 2001). However, these cells also have the tendency to undergo further transformation which leads to inhibited differentiation and loss of normal hematopoiesis. There are four different types of leukemia; chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), and acute myeloid leukemia (AML) (Figure 4). This study focuses on the last type, AML, one of the most common types of leukemia in the Western Hemisphere. AML is a clonal, malignant disease of the hematopoietic system that is characterized by increased proliferation and survival of myeloid immature cells, primarily in the bone marrow, and by impaired normal blood cell production (Beutler and Williams, 2001). As the mutant cells gain growth and/or survival advantages over the
normal stem cells, its progeny proliferate to form billions of cells. These processes usually result in bone marrow failure, with inhibition of normal hematopoiesis, arrested differentiation, neutropenia, anemia, and thrombocytopenia (Beutler and Williams, 2001). These place patients at risk for life-threatening infections, end-organ dysfunctions, and bleeding (Beutler and Williams, 2001).

Management of AML is complex with only approximately 40% of treated patients achieving a long-term complete remission. Treatment in general consists of induction with cytarabine/anthracycline-based chemotherapy regimen (“7+3”) followed by consolidation treatments for those patients who achieve complete remission. The latter include high-dose cytarabine-based regimen or myeloblative doses of chemotherapy with autologous stem cell support. For those patients at high risk of relapse; cases with
secondary AML developed from prior hematologic disorders or after receiving chemotherapy for unrelated cancers, and for those with cytogenetic or molecular aberration predicting early disease recurrence, therapeutic strategies with allogenic stem cell transplantation are usually pursued (Beutler and Williams, 2001).

Acute myeloid leukemia is the primary form of leukemia during neonatal years; however, it represents only a small number of childhood and adolescent cases. AML incidence increases with age; it accounts for 15-20% of acute leukemias in children and around 80% of those in adults (Beutler and Williams, 2001). AML is only slightly more common in males over females and there is minimal difference between incidences in African or European descent at all ages. Per year, there are around 2.3 cases per 100,000 people at all ages. Approximately 60% of AML cases are in people at ages of greater than 60 years, with a median age of 65-70 (Godwin and Smith, 2003).

There are numerous predisposing conditions that can lead to the development of AML. These include environmental factors such as exposure to radiation, benzene, and alkylating agents. Some acquired diseases that lead to AML are primary thrombocythemia, CML, paroxysmal nocturnal hemoglobinuria, and myeloma (Beutler and Williams, 2001). There are also a number of inherited conditions that increase the chance of developing the disease: Down syndrome, identical sibling with AML, Werner syndrome, familial AML, and neurofibromatosis 1 are just a few (Beutler and Williams, 2001). Cytogenetic alterations are very common and frequent in the development of AML as well (Figure 5). Some of these mutations include trisomy of chromosomes 8, 4, and 13, and deletions of all or part of chromosomes 7 and 5. In addition to these mutations a number of chromosom al translocations have been noted in various leukemias.
that often cause a rearrangement of a critical region of a protoncogene resulting from fusion between two genes. The fusion genes are often transcription factors that alter regulatory sequences that control growth, differentiation, and survival (Hellman et al., 2001).

One well-known translocation is that of the Philadelphia chromosome in CML discovered in 1960. This is caused by the translocation between the c-ABL gene on chromosome 9 and the BCR gene on chromosome 22 which forms the well-known BCR-ABL fusion gene (reviewed in Calabretta and Perrotti, 2004). This translocation is also seen in approximately 30% of adults suffering from acute lymphoblastic leukemia (Ph1 ALL).

In approximately forty to fifty percent of those affected with AML, the somatic mutation results from a chromosomal translocation (Beutler and Williams, 2001). These include the translocation (t) between chromosomes 8 and 21 AML1/ETO, t(15;17) PML-RARα, t(9;11) AF9-MLL, t(7;11) Nup98-HOXA9, and t(16;21)(p11;q22) FUS-ERG (reviewed in Marcucci et al., 2005 and in Bloomfield, 2002) (Figure 5).
This last translocation between the FUS gene on chromosome 16 and the ERG gene on chromosome 21 leads to the production of the FUS-ERG fusion protein (Figure 6). The FUS-ERG translocation is very rare and, to date has only been diagnosed in approximately 30 patients with AML. This non-random translocation was also observed in one patient with blast crisis of CML and in a few patients with myelodysplastic syndrome (MDS) that progressed to AML (Yamamoto et al., 1997). In the FUS-ERG translocation, the RNA-binding domain of FUS is substituted with the DNA-binding domain of ERG and the chimeric transcript has been shown to function as a regulator of transcription (Yamamoto et al., 1997).

Fig. 6: (left) Generation of the chimeric FUS-ERG oncogene and possible mechanisms of leukemogenesis; (right) Clinical features of the t(16;21) AML.

Because AML shows diverse cytomorphological features, it is classified into various subtypes (M0, M1, M2, M3, M4, M5, M6 and M7) of the French-American-British (FAB) classification (Shimizu et al., 1993). Occurrence in each subtype is dependent on cell type and degree of differentiation. AML with the t(16;21) translocation shows arrested differentiation at different stages (M0, M1, M2, M4, M5, and M7) (Figure 6). The M0 stage is characterized by cells that are myeloblastic with minimal differentiation.

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M1 is characterized by myeloblastic cells without maturation. M2 cells are myeloblastic with maturation. M3 are promyelocytic, M4 are myelomonocytic, M5 are monocytic, M6 are Erythroleukemia, and M7 are megakaryocytic (Krause, 2000). One group has identified 4 different types of mRNA transcripts for FUS-ERG, and although the biological implication of the heterogeneity of these transcripts has not been determined, it is predicted that these varying transcripts enable the translocation to be implicated in these various subtypes of leukemia (Panagopoulos et al., 1995). The prognosis of patients diagnosed with this genetic aberration in AML is very poor and no patients achieve complete remission.

The FUS/TLS (translocated in liposarcoma) gene encodes an RNA/DNA-binding protein and was first discovered as a translocated gene in myxoid liposarcoma (Crozat et al., 1993). In this disease, FUS is fused to the CHOP (c/EBP homologous protein) gene (Yang et al., 2000). The FUS protein serves in transcription regulation and RNA metabolism. It should also be noted that the nuclear RNA-binding protein encoded by FUS is homologous in amino-acid sequence to the EWS protein. It has been shown in a subset of Ewing’s Sarcomas that ERG is fused with the EWS gene. FUS is also closely related to TAF\textsubscript{II}68 (TATA-binding protein-associated factor). TAF\textsubscript{II}68, TLS (FUS), and EWS interact with RNA polymerase II which implicates that TLS is a transcriptional activator (Yang et al., 2000). In addition, experiments have shown that FUS binds to RNA polymerase II through the N-terminal domain and associates with various splicing factors via the C-terminal domain (Chansky et al., 2001). The FUS gene contains a highly conserved C-terminus region composed of 80 amino acids called the ribonucleoprotein consensus sequence (RNP-CS) (Crozat et al., 1993). Flanking this is
an RNA recognition motif (RRM) and further down from this are Arg-Gly-Gly (RGG) repeats and a C2-C2 zinc finger domain (Hackl and Luhrmann, 1996). It has been demonstrated that suppression of FUS expression in myeloid precursor 32Dcl3 cells is correlated with upregulation of granulocyte-colony stimulating factor receptor (G-CSFR) and downregulation of interleukin-3 receptor (IL-3R) (Perrotti et al., 1998). In addition, G-CSF-stimulated differentiation was seen to be accelerated in FUS-knock-down myeloid cells (Perrotti et al., 2000). The FUS gene has also been found to be upregulated in CML-BC (blast crisis) (Perrotti et al., 1998).

ERG (ets-related gene) is a member of the ETS gene superfamily which consists of a number of genes that encode for transcriptional regulators in a variety of developmental mechanisms. These transcription factors play important roles in cell development, proliferation, differentiation, apoptosis, tissue remodeling, and deregulation of ETS genes leads to malignant transformation of cells (Oikawa, 2004). ETS proteins bind to ETS-binding sequences of DNA through a uniquely conserved winged helix-turn-helix motif (Donaldson et al., 1994). Two of these genes are ETS-1 and ETS-2, both of which have been implicated in oncogenesis and cellular transformation when overexpressed (Myers et al., 2005). The ERG gene encodes for five proteins; Erg-1, Erg-2, Ergp55, Ergp49, and Ergp38 (Carrere et al., 1998). ERG overexpression decreases apoptosis of serum-deprived NIH3T3 cells (Yi et al., 1997). Furthermore, it has been shown that ERG overexpression in mouse fibroblast NIH3T3 cell lines alters morphological features, allows cells to grow in low and serum-free media, and gives rise to colonies in soft agar (Hart et al., 1995), thus, ERG may contribute to oncogenesis. In addition, mice injected with ERG-expressing cells ectopically developed tumors, which
suggests that ERG overexpression may contribute to tumor transformation (Ichikawa et al., 1999). In addition to ERG, the APP (β-amyloid precursor protein) gene is located on chromosome 21 in close proximity to ERG and ETS-2 transcription factor and has been shown to be overexpressed in AML (Baldus et al., 2004). This protein is also associated with Down Syndrome and has previously been implicated in Alzheimer’s disease (Wolvetang et al., 2003).

FUS-ERG has been shown to arrest erythroid and alter myeloid differentiation and to increase proliferation and self-renewal capacity of human myeloid progenitors (Pereira et al., 1998). The translocation increases the number of blast cells which exhibit the following characteristics: round or irregular nucleus, basophilic cytoplasm, vacuoles, and phagocytic features. The presence of micromegakaryocytes is also observed. In addition, the chimeric protein has been demonstrated to induce anchorage-independent growth in 3T3 fibroblasts and to block neutrophilic differentiation of myeloid L-G cells (Ichikawa et al., 1999). It seems that FUS is required for FUS-ERG-dependent suppression of differentiation. This fusion protein has also been suggested to cause cellular abnormalities by interfering with FUS-mediated RNA splicing by serine-arginine proteins (Yang et al., 2000). While FUS-ERG is known to be associated with AML, the mechanism by which the fusion protein acts is still unclear. Thus, the goal of this study is to investigate whether the effects of the translocated fusion gene, FUS-ERG, in acute myeloid leukemia cells depend on interference with FUS RNA regulatory activity or with ERG transcriptional activating function.
RESULTS

1. Effect of ERG and FUS-ERG on proliferation and survival of myeloid progenitors.

To assess the effect of FUS-ERG expression on proliferation and survival of myeloid progenitor cells, the IL-3-dependent 32Dcl3 mouse myeloid precursors were infected with neomycin- or green fluorescent protein (GFP)-containing bicistronic retroviruses carrying the hemagglutinin (HA)-tagged FUS-ERG, ERG, and FUS cDNAs (Figure 7). After antibiotic (G418)-mediated selection or FACS-mediated sorting of GFP positive cells, the newly established cell lines were used in proliferation and survival assays.

To assess the effects of ERG and FUS-ERG on proliferation and survival of myeloid progenitors, parental 32Dcl3, FUS-, ERG-, and FUS-ERG-expressing cells were grown in the presence of IL-3 (Figure 8) and absence of IL-3 for 0 to 36 hours (Figure 9). As shown, proliferation of the IL-3-growing cell lines was identical to that of parental cells (Figure 8). While parental 32Dcl3 and 32D-ERG cells died by 24 hours of culture in the
absence of IL-3 (Figures 9), 32D-FUS-ERG cells were less prone to undergo apoptosis induced by IL-3 deprivation (Figure 9).

In fact, 30% of FUS-ERG cells were still alive after five days in culture with absence of IL-3 (not shown). Interestingly, the 32D-FUS cells also behaved as the FUS-ERG cells and exhibited delayed apoptosis in absence of IL-3 (Figures 9). These results suggest that the fusion protein gains an oncogenic function by circumventing cytokine-generated survival signals. However, FUS-ERG cells failed to become completely growth factor-independent and were all apoptotic within a week of culture (not shown).

![Fig. 8. Effects of ERG, FUS, and FUS-ERG on IL-3-dependent proliferation of myeloid 32Dc13 cells.](image)

Growth of parental, FUS-ERG-, ERG- and FUS-expressing 32Dc13 cells in IL-3-supplemented cultures. Viable cells were counted over a 72 hour time period by trypan-blue exclusion test. Lines in the graph represent Mean±SD. Representative of three different experiments with similar results.

![Fig. 9. Effects of IL-3 deprivation on survival of parental, ERG-, FUS-, and FUS-ERG-expressing myeloid 32Dc13 cells.](image)

Graph shows survival of parental 32Dc13 and derivative cell lines upon IL-3 starvation. Viable cells were assessed by trypan-blue exclusion test. Representative of three independent experiments with similar results.
2. Effect of ERG and FUS-ERG expression on G-CSF-driven granulocytic differentiation of myeloid progenitors.

Because dysplastic myeloid maturation is a characteristic feature of patient-derived t(16;21) AML cells (Hiyoshi et al., 1995), and ectopic FUS expression impairs G-CSF-driven granulocytic differentiation and induces apoptosis of G-CSF-cultured myeloid precursors (Perrotti et al., 1998; Perrotti et al., 2000), we investigated the effects of FUS-ERG and ERG overexpression to determine whether altered differentiation in t(16;21) AML depends on FUS-ERG expression, and whether ERG overexpression might account for those effects. Thus, growth and morphology were monitored in parental, low and high ERG-, and FUS-ERG-expressing 32Dcl3 cells exposed to G-CSF for 7 to 9 days, (10% of U87MG conditioned medium as source of crude G-CSF). Parental 32Dcl3 cells ceased to proliferate after 4 days of culture in the presence of G-CSF (Figure 10) and underwent complete granulocytic differentiation by day 7 (Figure 11).

By contrast, G-CSF-treated FUS-ERG-expressing cells showed enhanced proliferation (Figure 10) and delayed differentiation, as mitotic cells, vacuolated myeloblasts, cells at intermediate stages of maturation (myelocytes and metamyelocytes) and cells with

Fig. 10. Effect of ERG and FUS-ERG on G-CSF-driven proliferation of myeloid 32Dcl3 cells. Graph shows growth of parental 32Dcl3 and derivative cell lines upon exposure to G-CSF. Viable cells were assessed by trypan-blue exclusion test. Representative of three independent experiments with similar results.
segmented nuclei (polymorphonuclear cells; neutrophils) were evident after 9 days of culture in G-CSF-containing medium (Figure 11). Thus, altered myeloid differentiation of t(16;21) leukemia cells might be a direct consequence of FUS-ERG expression.

Unexpectedly, 32D-ERG-expressing cells, like FUS-expressing 32Dcl3 myeloid progenitor cells (Perrotti et al., 1998), did not show enhanced proliferation or differentiation in response to G-CSF but underwent apoptosis by day 5-6 (Figures 10 and 11). Interestingly, in parental 32Dcl3 cells, levels of endogenous ERG progressively decrease during G-CSF-induced neutrophilic maturation (Figure 12), suggesting that downregulation of ERG expression is required for differentiation.
Thus, these results suggest that overexpression of ERG or FUS alone does not account for the effect of FUS-ERG on differentiation of myeloid cells, which is most likely the result of either interference with ERG transcriptional activity or FUS-dependent regulation of mRNA transcription and splicing. Alternatively, the effects of FUS-ERG on myeloid differentiation might be a specific effect of the fusion protein itself.

3. Effect of FUS, ERG, and FUS-ERG expression on levels of transcription factors important for myeloid differentiation.

To dissect the FUS-ERG-dependent molecular mechanisms underlying altered differentiation, we assessed the effects of FUS, ERG, and FUS-ERG expression on the protein levels of the transcription factors PU.1, C/EBPα, and C/EBPε that, as extensively reported (reviewed in Tenen, 2003), are essential for differentiation of CMP cells toward the more committed granulocytic precursors, initial maturation of myeloblasts, and terminal maturation of myelocytes into neutrophilic cells, respectively.

In 32Dcl3 cells, overexpression of FUS suppresses C/EBPα expression (Figure 13, lane 2). Consistent with this finding, expression of a FUS mutant with dominant negative activity (S256A FUS), but not of the dominant active S256D FUS mutant (Perrotti et al., 2000), rescued C/EBPα expression back to normal levels (Figure 13, lanes
1, 3, and 4), suggesting that altered differentiation of FUS-ERG cells might depend on the effect of the FUS portion of FUS-ERG on C/EBPα expression.

In agreement with this hypothesis, C/EBPα levels were clearly higher in three single clones of FUS-ERG-expressing cells than in parental 32Dcl3 cells (Figure 14, left panel). Furthermore, upregulation of C/EBPα by FUS-ERG appears to be dependent on the levels of the fusion protein itself, as C/EBPα protein levels are higher in 32Dcl3 cells highly expressing FUS-ERG than in a 32Dcl3 cell line expressing lower levels of FUS-ERG (Figure 14, right panel, lanes 2 and 3).
Consistent with this finding, levels of the C/EBPα-regulated G-CSFR were also increased in FUS-ERG-expressing 32Dcl3 myeloid progenitors (Figure 14, left panel). By contrast, levels of C/EBPε and PU.1 were not affected by FUS-ERG expression (Figure 14), suggesting that PU.1 and C/EBPε transcription factors are not targets of FUS-ERG pro-leukemogenic activity.

In 32Dcl3 myeloid precursors ectopically expressing low (32D-M12) or high (32D-M4) levels of ERG protein, C/EBPα expression was similar to that of parental 32Dcl3 cells (Figure 15, lanes 3 and 4).

![Western blots showing expression of active STAT3, APP, C/EBPα, active MAPK ERK1/2, and Myb in 32Dcl3 cells ectopically expressing FUS-ERG (32D-FE3) (lane 2), High ERG (lane 3) and low ERG (lane 4) levels. Level of ectopic HA-tagged FUS-ERG and ERG are shown in the 6th panel. Levels of GRB2 were monitored as control for equal loading.](image)

However, expression and activity of other molecules involved in the regulation of myeloid differentiation were also aberrantly regulated by ERG and/or FUS-ERG overexpression. Interestingly, expression of some of these factors appears to be regulated at the transcriptional level because they have also been detected by microarray performed on FUS, ERG, and FUS-ERG-expressing cells (see section 5 of results). For example, levels of Myb, a transcription factor essential for proliferation and initial maturation of immature myeloid cells (Gonda, 1998), was markedly inhibited by FUS-ERG expression.
Moreover, both FUS-ERG and ERG expression enhanced activity of the signal transducer and activator of transcription 3 (STAT3), a G-CSF-induced transcription factor frequently activated in AML that augments proliferation and C/EBPα differentiation-inducing activity (Benekli et al., 2003; Coffer et al., 2000; Numata et al., 2005; McLemore et al., 2001; Lai et al., 2003). Similarly, activity of the mitogen activated protein kinase ERK1/2 was induced by FUS/ERG overexpression (Figure 15). Of note, it has been shown that ERK1/2 activity inhibits myeloid differentiation upon phosphorylation of C/EBPα on serine 21 (Ross et al., 2004).

4. Effect of ERG and FUS-ERG expression on APP levels and cellular localization in myeloid progenitor 32Dcl3 cells.

Changes in amyloid precursor protein (APP) expression were investigated because it has been recently shown that its levels are increased in AML and correlated with that of ERG (Baldus et al., 2004). As shown, APP expression is strongly enhanced in ERG and FUS-ERG expressing cells (Figure 15). However, APP expression dramatically decreases upon G-CSF treatment of parental and FUS-ERG-expressing 32Dcl3 cells (Figure 16), suggesting that either high APP levels might be non-compatible with myeloid differentiation or APP expression is associated with growth, and levels decline when cells cease to proliferate.
The effect of ERG and FUS-ERG is not only evident on APP expression; in fact, by confocal microscopy performed on anti-APP antibody-stained cytopspinned cells, we found APP primarily localized in the cytoplasm of parental 32Dcl3 cells whereas it was relocated in the nucleus in 32D-FUS-ERG and 32D-ERG cells (Figure 17). These data suggest that overexpression of ERG and, consequently, increased nuclear APP levels may play an important role in the development of AML, including the t(16;21) leukemias.
5. **Global effect of ERG and FUS-ERG on the transcription profile of myeloid progenitor 32Dcl3 cells.**

To determine the effect of ERG and FUS-ERG on gene expression, oligonucleotide array analysis was performed using total RNA obtained from the IL-3-cultured parental, FUS-ERG- (FE3 and FEP), and ERG- (M4 and M12), and FUS-expressing 32Dcl3 cells. Interrogation of Affymetrix Microarray revealed a discrete number of genes regulated by ERG, and/or FUS-ERG (Table 1). For example, cell lines expressing FUS-ERG had upregulated genes required for survival and proliferation, megakaryocytic differentiation and lymphocytic markers, whereas genes required for G-CSF induced differentiation, and growth suppression were downregulated.

<p>| Table 1. Genes regulated by ERG, FUS-ERG, and FUS in relation to parental 32Dcl3 cells. |
|---------------------------------|---------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>ERG</th>
<th>Fold *</th>
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<tr>
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<td>N-myc 1</td>
<td>6.08</td>
<td>13.98</td>
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<td>transglutaminase 2, C polypeptide</td>
<td>Tgm2</td>
<td>13.93</td>
<td>10.18</td>
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<td>interleukin 2 receptor, alpha chain</td>
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<td>9.30</td>
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<tr>
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<td>Hoxc8</td>
<td>1.89</td>
<td></td>
</tr>
<tr>
<td>v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian) Mafb</td>
<td>-1.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAD homolog 4 interacting transcription coactivator 1</td>
<td>Mitc1</td>
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<td>frizzled-related protein</td>
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<tr>
<td>gosdermin</td>
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<td></td>
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<tr>
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<td>Csf3r(G-CSFR)</td>
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<td></td>
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<td>Nfkβ1</td>
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<td>5.27</td>
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<td>Von Willebrand factor homolog</td>
<td>Vwf</td>
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<td></td>
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<tr>
<td>amyloid beta precursor protein (cytoplasmic tail) binding protein 2 Appbp2</td>
<td>-1.50</td>
<td></td>
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</table>

a. Listed are genes with at least a 1.5 fold change in regulation.
Interestingly, a gene upregulated by FUS-ERG is the G-CSFR which, as we previously showed, is overexpressed in FUS-ERG cells in a dose-dependent manner (Figure 14). Similarly, other transcription factors like NF-Kb were upregulated by FUS-ERG. Indeed, anti-p65 NFkB western blot shows that NFkB is a true transcriptional target of FUS-ERG (Figure 18).

Genes encoding markers of differentiation of different hematopoietic lineages (Mpl, vWf, and IL-2Rα) were also upregulated in FUS-ERG cells, consistent with the observation that leukemic cells from t(16;21) AML patients present lymphoid and megakaryocytic markers of differentiation (Hiyoshi et al., 1995).

Some downregulated genes in FUS-ERG-expressing cells included myeloblastin, myeloperoxidase (MPO), and lactoferrin (LF), three factors whose activity is required for differentiation into mature granulocytes (Berliner et al., 1995; Lutz et al., 2001). The levels of the mRNAs encoding the proto-oncogene Myb, the inhibitor of differentiation Id2 and the suppressor of cytokine signaling 3 (SOCS3), all of which are inhibitors of G-CSF induced differentiation (Yanagisawa et al., 1991; Cooper and Newburger, 1998; Croker et al., 2004), were downregulated in FUS-ERG-expressing cells as well. Consistent with this data we have already shown that Myb protein is downregulated by FUS-ERG expression (Figure 15). Other genes transcriptionally downregulated by FUS-

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Fig. 18. Effect of FUS-ERG on NFkB expression.
Western blot show expression of NFkB in parental, high (lane 2) and low (lane 3) FUS-ERG-expressing cell lines. Levels of GRB2 were monitored as control for equal loading.
ERG were the pro-apoptotic and growth suppressors Wig-1 and BNIP-3 (Hellborg et al., 2001; reviewed in Zhang et al., 2003).

The proto-oncogene N-myc 1 was found among those genes upregulated in microarrays of ERG and FUS-ERG overexpressing 32Dcl3 cells. Anti-N-Myc immunoblotting confirmed the microarray data; in fact, levels of N-Myc protein were markedly increased in 32Dcl3 cells ectopically expressing ERG and FUS-ERG (Figure 19).

![Fig. 19. Effect of FUS-ERG, ERG, and FUS overexpression on N-Myc expression.](image)

Another gene upregulated in ERG-expressing cells was HOXC8, a transcription factor belonging to the homeobox family whose enhanced expression correlates with loss of differentiation (Waltregny et al., 2002). Other genes downregulated in ERG-expressing cells include: MafB (v-maf) which is a leucine zipper transcription factor; the MAD homolog 4 interacting transcriptional coactivator Mtc1; gasdermin (GSDM), a protein whose expression is suppressed in Gastro-Intestinal cancer (Saeki et al., 2000); and Frzb, an inhibitor of the wnt signaling pathway whose loss is apparently associated with tumor development (Bafico et al., 1999; reviewed in Polakis, 2000).
DISCUSSION

In the rare and fatal AML with the recurrent t(16;21) translocation, leukemic cells show enhanced proliferation and dysplastic myeloid differentiation (Hiyoshi et al., 1995); in fact, the t(16;21) translocation was found not associated to a specific FAB phenotype (Kong et al., 1997). Although few studies indicate that FUS(TLS)-ERG, the product of the t(16;21) translocation (Panagopoulos et al., 1994), plays a pivotal role in promoting leukemogenesis (Pereira et al., 1998; Ichikawa et al., 1999), there is no formal evidence showing that it can transform hematopoietic stem cells or committed myeloid progenitors and induce leukemia in mice.

Despite it being shown that the FUS-ERG fusion protein has potent transcriptional transactivation activity (Prasad et al., 1994), the mechanisms whereby it alters the phenotype and, perhaps, induces leukemic transformation of myeloid progenitors is still largely unknown. Here, we reported that FUS-ERG expression decrease susceptibility to apoptosis induced by cytokine deprivation and enhances G-CSF-driven proliferation of mouse myeloid progenitor 32Dc13 cells. Furthermore, we also provided evidence showing that increased survival was a characteristic shared between FUS-ERG and FUS but not ERG overexpressing cells, whereas the G-CSF-induced proliferative advantage was a specific feature of FUS-ERG cells. Aberrant G-CSF-dependent proliferation was also accompanied by delayed granulocytic differentiation of FUS-ERG cells. Consistent with this observation, it has been reported that expression of FUS-ERG in primary CD34+ cord blood cells markedly enhances self renewal and inhibits erythroid development (Pereira et al., 1998). Seemingly, the patient-derived myeloblastic YNH1 cells, which carry the t(16;21) translocation and express the
FUS-ERG fusion protein, do not undergo granulocytic differentiation but continuously proliferate if exposed to G-CSF (Yamamoto et al., 1997). Although the FUS-ERG-induced phenotypic changes might resemble those of leukemic cells, FUS-ERG expression did not confer growth factor independence to 32Dcl3 myeloid progenitors. Similarly, proliferation of L-G myeloid precursors transduced with a FUS-ERG-expressing construct remained dependent on the exogenous support of cytokines (Ichikawa et al., 1999).

Although FUS-ERG expression may be not sufficient to fully transform cells, altered differentiation of FUS-ERG-expressing myeloid progenitor 32Dcl3 cells seems to be a direct consequence of FUS-ERG expression. In fact, the presence of vacuolated blast cells with irregular nuclei, frequent mitosis, cells arrested at different stages of maturation, and expression of lymphoid and megakaryocytic markers in myeloid 32D-FUS-ERG cells exposed for 9 days to G-CSF has also been observed in specimens from t(16;21) AML patients (Hiyoshi et al., 1995).

Because differentiation arrest is a common feature of acute leukemias, and genetic or functional inactivation of C/EBPα, the major regulator of granulocytic differentiation (Zhang et al., 1997), has been found associated to myeloid leukemias with normal cytogenetic or carrying specific chromosomal abnormalities (Perrotti et al., 2004), we asked whether the aberrant differentiation of FUS-ERG-expressing cells results from loss of C/EBPα function. Unexpectedly, C/EBPα expression was upregulated in FUS-ERG-expressing cells, downregulated in FUS-expressing cells, and unaffected in ERG-expressing cells. Moreover, increased C/EBPα expression appears to correlate with increased C/EBPα transcriptional activity, as levels of the C/EBPα target G-CSFR
(Zhang et al., 1998) were also upregulated. Increased expression of the G-CSF receptor (G-CSFR) may explain the enhanced survival of FUS-ERG cells cultured in the presence of G-CSF; however, the presence of high levels of C/EBPα argue against this simple interpretation. In fact, C/EBPα is not only an inducer of differentiation but also a strong growth suppressor (Friedman, 2002). Since G-CSF can amplify its mitogenic signals by inducing the expression of its own receptor (Steinman and Tweardy, 1994) and induce granulocytic differentiation in a C/EBPα-independent manner (Collins et al., 2001), it is possible that increased C/EBPα expression reflects a mechanism that tries to compensate the growth advantage induced by the effects of G-CSF through STAT3 signaling. Indeed, we have shown that STAT3 activity is enhanced in FUS-ERG-expressing myeloid cells. In this scenario, we must expect that FUS-ERG activates pathways leading to post-translational inactivation of C/EBPα that, in turn, becomes unable to induce G1/S arrest and differentiation. In line with this hypothesis, we found that FUS-ERG augments MAPK ERK1/2 activity that, as previously reported, inhibits C/EBPα transcriptional and growth suppressive functions upon phosphorylation of C/EBPα serine 21 (Ross et al., 2004).

Although altered differentiation of FUS-ERG-expressing cells might, in part, depend on functional inactivation of C/EBPα, the expression profile of 32D-FUS-ERG cells indicate that upregulation of growth-promoting genes together with downregulation of genes with pro-apoptotic, growth suppressive, and differentiation inhibitory function might account for enhanced proliferation and dysplastic differentiation of committed myeloid progenitor cells.
Interestingly, we also found the amyloid precursor protein (APP) to be upregulated by FUS-ERG and ERG expression. Although we still do not understand the function of APP in myelopoiesis and in leukemia, it appears clear that it might have an important role in these processes because of the ability of FUS-ERG and ERG to induce APP expression and relocation into the nuclear compartment. Note that it has been recently reported that increased APP and ERG expression correlate with poor prognosis of AML patients with chromosome 21 abnormalities (Baldus et al., 2004). Interestingly, APP was one of the few genes whose expression was regulated in a similar manner by both ERG and FUS-ERG. Moreover, we did not find factors (with the exception of C/EBPα) which were similarly or antagonistically modulated by FUS and FUS-ERG expression, suggesting that FUS-ERG neither represents a more powerful transcription factor enhancing expression of ERG-target genes, nor a protein that strongly interferes with FUS activities.

In summary, we favor the hypothesis that FUS-ERG predisposes myeloid cells to leukemic transformation which, eventually, will be induced by a subsequent hit (Figure 20).

![Fig. 20. Role of FUS-ERG in leukemogenesis. FUS-ERG may alter differentiation of myeloid progenitors and, eventually, predispose them to become leukemic upon oncogenic activation and/or loss of genes with tumor suppressor activity.](image-url)
Indeed, the t(16;21) translocation was also found in patients with myelodysplastic syndrome (MDS) that subsequently developed AML (Hiyoshi et al., 1995). However, we cannot exclude the possibility that FUS-ERG might be capable of transforming primary hematopoietic stem cells and induce a series of phenotypic changes characteristic of the t(16;21) AML. Additional studies aimed at investigating the molecular mechanism whereby FUS-ERG regulates gene expression will provide further insight into the role of FUS-ERG in leukemogenesis. Specifically, experiments aimed at assessing the effects of FUS-ERG and ERG on proliferation, survival, and differentiation of primary CD34+ human bone marrow cells and at determining whether FUS-ERG expression in normal marrow cells induces leukemia in immunocompromised NOD/SCID mice might unveil new insights into the molecular mechanisms responsible for differentiation arrest of acute myeloid leukemias cells. A better understanding of the complexity of these mechanisms might help the identification of novel therapeutic drugs aimed at restoring the normal development of the leukemic myeloid progenitors.
MATERIALS AND METHODS

Cell Lines

The murine IL-3-dependent 32Dcl3 myeloid precursor cells and derivatives were maintained in culture in Iscove’s modified Dulbecco medium (IMDM) supplemented with 10% heat-inactivated FBS (Gibco, BR, Gaithersburg, MD), 2mM L-Glutamine (Gibco, BR, Gaithersburg, MD), 1% penicillin/streptomycin (Gibco, BR, Gaithersburg, MD), and 10% WEHI-conditioned medium as a source of IL-3. The 32Dcl3 HA-FUS-, HA-ERG-, and HA-FUS-ERG-expressing cells were generated by retroviral infection as previously described (Perrotti et al., 1998). Low-expressing lines of 32Dcl3-FUS-ERG cells were created (32D-FEP, -FEA, and -FEAB) in addition to high expressing FUS-ERG (32D-FE1, -FE2, and -FE3). Two ERG-expressing cell lines, high ERG (32D-M4) and low ERG (32D-M12) were created. All cDNA were tagged with HA. Granulocytic differentiation of 32Dcl3 cells and derivates was induced by G-CSF.

The amphotropic-packaging cell line Phoenix (Dr. G. P. Noloan, Stanford University of Medicine) was maintained in culture in 10% Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, BR, Gaithersburg, MD), FBS medium, 2mM L-glutamine, and 1% penicillin/streptomycin, and grown for 24hr to 80% confluence prior to transfection by calcium phosphate-DNA precipitation (ProFection system, Promega, Madison, WI).

Plasmids

The MIGR1-HA-ERG plasmid was generated as follows: ERG cDNA was amplified by RT-PCR using two sets of primers spanning the BamHI site present in the ERG cDNA, and containing at the 3’ end the hemagglutinin (HA) sequence followed by
a BglII restriction site. The RT-PCR products were then phosphorylated, digested with BglII and BamHI, and subcloned into previously BglII/HpaI digested retroviral vector MIGR1. The LXSN plasmids containing the HA-tagged wild-type and S256A or S256D FUS cDNAs were created as previously described (Perrotti et al., 2000). Briefly, pSK-HA S256A FUS (inactive mutant) and pSK-HA S256D FUS (dominant active mutant) were XbaI-HindIII digested, blunted, and subcloned into the blunted EcoRI site of the LXSP retroviral vector as previously described. FUS-ERG plasmid was obtained from Dr. Ichikawa H. (NCCRI, Tokyo Japan). The HA-tagged FUS-ERG cDNA was cloned into a HindIII site of pLNCX retroviral expression vector as previously described (Ichikawa et al., 1999).

### Western Blot Analysis

Cells were harvested and lysed: approximately $10^6$ cells/100 μl of isotonic lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Hepes at pH 7). Lysates were clarified by centrifugation (12,000g, 15 min, 4°C), denatured (10 min, 100°C), after being incubated on ice for 30 minutes and subjected to one cycle of freezing-thawing. Protein lysates were denatured in boiling water bath for 10 minutes and subjected to Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE). Proteins in gel were electro-transferred to nitrocellulose filter and used for Western blot. Briefly, the filter was subjected to washing with TRIS Buffer Saline (TBS) solution (100 mM TRIS, 1 mM NaCl at pH 7.6) and blocking with 5% Nestle Carnation Dry Milk. Primary antibody was added followed by secondary antibody. Primary antibodies used were: monoclonal anti-GRB2, monoclonal anti-HA (Covance, Princeton NJ), polyclonal antit-C/EBPα (Santa Cruz Biotechnology), polyclonal anti-G-CSFR (Santa Cruz Biotechnology), polyclonal
anti-PU.1 (Santa Cruz Biotechnology), monoclonal anti-N-myc 1 (Upstate Biotechnology), and polyclonal anti-APP (Cell Signaling Technology). Secondary antibodies used were anti-mouse and anti-rabbit (Amersham Biosciences). Protein levels were detected by chemiluminescence (ECL Western Blotting Detection Agents).

**Cytospins**

Morphologic differentiation was monitored by May-Grunwald/Giemsa staining of cytospin preparations. At each day of culture in G-CSF-supplemented medium, approximately 50-75x10³ cells were obtained through centrifugation and re-suspension in PBS. Cells were transferred to Fisher Brand microscope slides via Thermo Shandon Cytospin 4 machine. Stained cells were observed under a Zeiss Axioskop 2 Plus microscope for analysis.

**Proliferation, Survival, and Differentiation Assays**

In order to observe the effect of ERG and FUS-ERG on proliferation and survival, infected murine cells were grown in either Interleukin-3 (IL-3)-containing or deprived medium for the indicated time. Percentage of dead and alive cells was monitored by Trypan-Blue exclusion test. The effect of ERG and FUS-ERG on G-CSF-driven myeloid proliferation and neutrophilic differentiation was also assessed at the indicated time by Trypan-Blue exclusion test.

**Immunofluorescence Microscopy**

5-7.5x10⁴ parental, HA-tagged ERG- and FUS-ERG-expressing 32Dcl3 cells were cytopspinned on glass slides and fixed for 10 minutes in PBS-containing 3.7%
formaldehyde. Thereafter, cells were washed three times with PBS, permeabilized by incubation (10 minutes) in PBS-0.05% Triton X-100 (Sigma), rinsed again with PBS and then blocked for 10 minutes in PBS-4% goat serum. Incubation with the anti-APP antibody (1:200 dilution) and with Rhodamine Red-x goat anti-mouse IgG (H+L) (1:300 dilution). Molecular probes were carried out at room temperature for 30 minutes. Slides were rinsed three times with PBS, treated with SlowFade Antifade reagent (Molecular Probes), and analyzed.

**Microarray Analysis**

Microarray analysis was performed to further understand which genes are up or downregulated by FUS-ERG, ERG, and FUS. Total RNA, isolated by acid phenol guanidium extraction and isopropanol precipitation, was used to interrogate mouse oligonucleotide arrays (The Ohio State University Comprehensive Cancer Center microarray facility).
LITERATURE CITED


