ERG Is a Megakaryocytic Oncogene

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Abstract

Ets-related gene (*ERG*) is a member of the *ETS* transcription factor gene family located on Hsa21. *ERG* is known to have a crucial role in establishing definitive hematopoiesis and is required for normal megakaryopoiesis. Truncated forms of *ERG* are associated with multiple cancers such as Ewing's sarcoma, prostate cancer, and leukemia as part of oncogenic fusion translocations. Increased expression of *ERG* is highly indicative of poor prognosis in acute myeloid leukemia and *ERG* is expressed in acute megakaryoblastic leukemia (AMKL); however, it is unclear if expression of *ERG* per se has a leukemogenic activity. We show that ectopic expression of *ERG* in fetal hematopoietic progenitors promotes megakaryopoiesis and that *ERG* alone acts as a potent oncogene *in vivo* leading to rapid onset of leukemia in mice. We observe that the endogenous *ERG* is required for the proliferation and maintenance of AMKL cell lines. *ERG* also strongly cooperates with the GATA1s mutated protein, found in Down syndrome AMKL, to immortalize megakaryocyte progenitors, suggesting that the additional copy of *ERG* in trisomy 21 may have a role in Down syndrome AMKL. These data suggest that *ERG* is a hematopoietic oncogene that may play a direct role in myeloid leukemia pathogenesis. [Cancer Res 2009;69(11):4665–73]

Introduction

Ets-related gene (*ERG*) is a member of the *ETS* transcription factor gene family and is closely related to another *ETS* family member, *FLI-1*. There are at least five splice isoforms of *ERG* mRNA (1) and our previous study identified *ERG-3* as the main hematopoietic *ERG* isoform (2). *FLI-1* is required for normal megakaryopoiesis (3) and *ERG* has recently also been shown to have an essential role in establishing normal megakaryopoiesis (4). The mice harboring a missense mutation in *ERG* have also revealed the requirement for *ERG* to establish definitive hematopoiesis and for hematopoietic stem cell maintenance. ERG has been associated with multiple cancers. It is rearranged in 5% to 10% of patients with Ewing's sarcoma, where it is fused to EWS to create chimeric oncogenic protein (5) and is fused with TMPRSS2 in a large proportion of prostate cancers (6). An *ERG* fusion gene, TLS-ERG, has been found in some cases of acute megakaryoblastic leukemia (AMKL; refs. 7, 8). In both EWS-ERG and TLS-ERG, the fusion protein contains a truncated form of ERG that retains the ETS domain. A truncated form of *ERG* has also been found in some cases of childhood acute lymphoblastic leukemia (9), suggesting a possible role in leukemogenesis. Increased expression of *ERG* characterizes a subset of myeloid leukemia with complex karyotype (10), and a high expression level of *ERG* in cytogenetically normal acute myeloid leukemia is associated with poor prognosis (11). Although these data imply that *ERG* might be a hematopoietic oncogene, no explicit evidence for this has thus far been shown.

ERG is located on Hsa21 and children ages <4 years with germline trisomy 21 (Down syndrome) have a 500-fold increased risk for AMKL (12). Somatic mutations acquired during fetal hematopoiesis in the *GATA1* transcription factor are detected in megakaryoblasts from almost all the Down syndrome patients with AMKL (13–15), leading to the replacement of full-length GATA1 by a shorter isoform, GATA1s (13–15). The *GATA1* mutation alone is insufficient for leukemogenesis because *GATA1s* causes transient proliferation of immature fetal megakaryocytic progenitors but no postnatal hematopoietic abnormalities (16) and individuals with germ-line *GATA1s* show no reported malignancies (17), strongly suggesting that trisomy 21 is essential for leukemic development. Therefore, it has been proposed that overexpression of one or more genes on chromosome 21 (Hsa21) may have a crucial role in the leukemogenic transformation of megakaryoblasts (2, 18, 19). *ERG* is a potential candidate gene, as it has been shown to be expressed in both Down syndrome and non-Down syndrome AMKL patient samples (2) and to be required for megakaryopoiesis (2, 4).

Therefore, we wished to establish if *ERG* could act as an oncogene in myeloid leukemia and if the elevated expression of *ERG* in AMKL has functional significance. Combining ectopic expression experiments in fetal hematopoietic progenitors and RNA interference in AMKL cell lines, we show, for the first time, that full-length *ERG* is a myeloid oncogene. We also show that *ERG* strongly cooperates with *GATA1s* to immortalize megakaryocyte progenitors in colony-forming assays, suggesting a potential role in Down syndrome AMKL pathogenesis.

Materials and Methods

Mice. All mice were maintained in the animal facilities of the University College London Institute of Child Health, and experiments were done according to United Kingdom Home Office regulations and local ethical guidelines.

Cell lines. The human megakaryoblastic cell line Meg01 and the Down syndrome AMKL cell line CMY (American Type Culture Collection) was

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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maintained in RPMI containing 10% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 g/mL streptomycin (Life Technologies).

Retroviral constructs. The pMSCV-*Gata1* and pMSCV-*Gata1s* retroviral vectors were generated by subcloning the FLAG-tagged murine *Gata1* and *Gata1s* cDNA fragments into a modified version of pMSCV-neo (BD Clontech) upstream of the phosphoglycerate kinase promoter and neo^R gene. The pMSCV-*ERG* retroviral construct was generated by cloning the human *ERG-3* (hematopoietic isoform of *ERG*) cDNA into *Eco* RI sites of the pMSCV-IRES-hCD2 vector constructed by modifying pMSCV-neo. The phosphoglycerate kinase promoter and neomycin resistance cassette was replaced by an internal ribosome entry site and truncated human CD2 cassette amplified by PCR from the pMI-IRES-hCD2 vector (gift from Dr. M.J. Bevan, Department of Immunology, University of Washington; Supplementary Fig. S1*A*). The expression of each protein from this vector was confirmed by immunoblotting of LinXE-transfected cells (Supplementary Fig. S1*B*).

Real-time quantitative PCR. RNA was isolated from immortalized cell line or from the spleen of two independent ERG or ERG + GATA1s leukemic mice using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was generated using MMLV reverse transcriptase, amplification grade DNase I, random primers, RNaseOUT, and dinucleotide triphosphates (all from Invitrogen) according to the manufacturer's instructions. Real-time quantitative PCR was done using TaqMan probe-based chemistry and an ABI Prism 7900HT Fast Sequence Detection System (Applied Biosystems). Expression levels of CD41 (Mm00439741), CD42 (Mm00497671_g1), and platelet factor 4 (PF4; Mm00451315_g1) were normalized to glyceraldehyde-3-phosphate dehydrogenase (Mm99999915_g1) expression. The 2-ΔΔCT relative quantitation method was used to determine the relative expression level. All primer/probe sets were from Applied Biosystems.

RNA interference and transduction of cell lines. Virus-mediated RNA interference was accomplished using the pRetroSuper construct. To generate pRetroSuper targeting human *ERG*, pRetroSuper was digested with *Bgl* II and *Hind III* and the annealed oligonucleotides were ligated into the vector. The 19-bp *ERG* target sequence is ATGCGCATCTCTTTCTTTG, and as control, a nontargeting sequence TCCAACACGACACTCACTA was used. The nontargeting sequence is not similar to any sequence revealed by BLAST search. 293T cells were transiently transfected with pRetroSuper-Control or pRetroSuper-ERG retroviral plasmids in combination with expression vectors coding for the Gag, Pol, and Env proteins. The culture medium was filtered through a 0.45 μm filter 48 h post-transfection, and the viral supernatant was used for infection of either Meg01 or CMY cells in the presence of 8 μg/mL polybrene (Sigma-Aldrich). The cells were doubly infected with a 24 h interval and allowed to recover for a further 48 h before selection. Transduced cells were selected for 3 weeks with 10 μg/mL puromycin (ant-pr-1; InvivoGen) for Meg01 cells and 1.5 μg/mL for CMY cells. Reverse transcription-PCR analysis of megakaryocytic markers was done as described previously (2).

Transduction of hematopoietic progenitor cells. Hematopoietic progenitor cells (HPC) were harvested from embryonic day 12.5 fetal liver of C57BL/6 mice. Single-cell suspensions were prepared. Monoclonal antibodies specific to c-Kit (2B8; BD Pharmingen) and Ter119 (BD Biosciences) were used to purify progenitor cells. The purification was carried out using a magnetic activated cell sorter (Miltenyi Biotech) according to the manufacturer's instructions. Isolated HPCs were cultured overnight in DMEM containing 10% FCS, 100 ng/mL stem cell factor (SCF), 10 ng/mL interleukin (IL)-3, 10 ng/mL IL-6 (PeproTech), and 50 mmol/L 2-mercaptoethanol. Retroviral supernatant was produced by transfecting the LinXE ecotropic retrovirus packaging cell line with retroviral vectors. Supernatant was harvested after 48 h and concentrated 8-fold by centrifugation for 1 h at 16,000 × *g*. The viral supernatant was used to transduce the purified c-$Kit^+Ter119^-$ HPCs by spinoculation (centrifugation at 700 × *g*, 25 C, 45 min) in the presence of 100 ng/mL SCF, 10 ng/mL IL-3, 10 ng/mL IL-6 (PeproTech), and 5 mg/mL polybrene (Sigma-Aldrich). Transduced HPCs were cultured with the same cytokine conditions for 24 h and subsequently used for colony-forming assays.

Colony-forming assays and generation of immortalized cell lines. Transduced HPCs were cultured in 1.1 mL duplicate methylcellulose cultures in 35 mm plates. For megakaryocytic colony assays, cells were plated in Methocult 3234 (Stem Cell Technologies) supplemented with 50 ng/mL thrombopoietin (Research and Diagnostics) and 100 ng/mL SCF or Methocult containing 50 ng/mL thrombopoietin, 100 ng/mL SCF, and 10 ng/mL IL-3 in the presence of 1 mg/mL G418 (Life Technologies). Cells were cultured at 37°C for 6 to 10 days after which colonies were scored and cells were harvested and analyzed by flow cytometry. Colonies were stained with 1 mg/mL *p*-iodonitrotetrazolium (Sigma-Aldrich) in PBS. The colonies were scanned on GS-800 calibrated densitometer machine (Bio-Rad) 48 h following staining. Single-cell suspensions were serially replated in methylcellulose medium supplemented with the same growth factors without G418. Cell lines were established by picking single colonies following the third round of plating and propagation in DMEM liquid medium containing 10% FCS, L-glutamine, and 50 mmol/L 2-mercaptoethanol in the presence of thrombopoietin, and SCF or thrombopoietin, SCF, and IL-3. The ERG immortalized cell lines were maintained in 100 ng/mL SCF, 50 ng/mL thrombopoietin, and 10 ng/mL IL-3. The ERG + GATA1s immortalized cell lines were maintained in 100 ng/mL SCF and 50 ng/mL thrombopoietin.

***In vivo* leukemogenesis assay.** HPCs (2×10^5-3×10^5) transduced with empty vector, *ERG* alone, *GATA1s* alone, or *ERG* + *GATA1s* retroviruses were intravenously injected into sublethally irradiated (6 Gy) C57BL/6 recipient mice 48 h after infection. Mice were sacrificed when they developed signs of disease.

Histopathology. Murine tissues were fixed in 10% neutral buffered formalin and paraffin-embedded. H&E staining was carried out on 4 μm sections.

Immunoblotting. Protein lysates were fractionated in 10% polyacrylamide gel and transferred on to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) using one transfer buffer (*N*-cyclohexyl-3-aminopanesulfonic acid; pH 11.0) for 5 h at 100 V. Following blocking for 5 h in 5% skim milk-PBS + 0.05% Tween 20, membranes were probed with an anti-FLAG antibody (M2; Sigma-Aldrich) or an anti-ERG antibody (C-17; Santa Cruz Biotechnology). For loading control, an anti-NPM (Zymed Labs), anti-actin (Santa Cruz Biotechnology), or anti-GAPDH (Chemicon) was used. The membrane was washed twice in PBS-Tween 20 and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for a further hour. The membrane was washed twice with PBS-Tween 20 and subjected to chemiluminescence detection (Amersham) according to the manufacturer's instructions.

Flow cytometry. Cells were stained with fluorochrome-conjugated monoclonal antibodies to c-Kit (2B8; BD Pharmingen), CD41 (MW/Reg30; Abcam), CD2 (LFA2; BD Pharmingen), CD61 (F0803; DAKOCytomation), CD9 (KMC8; BD Pharmingen), and isotype control antibodies. For the analysis of leukemic cells, red cells were lysed in ammonium chloride solution at room temperature for 10 min before primary antibody staining. The cells were washed in staining buffer (PBS with 0.05% sodium azide and 0.1% bovine serum albumin) and preincubated with unlabeled anti-Fc II/III receptor monoclonal antibody 2.4G2 for 15 min on ice. Cells were stained with monoclonal antibodies conjugated with FITC, phycoerythrin, or biotin. Biotin-conjugated monoclonal antibodies were detected using peridinin chlorophyll a protein-conjugated streptavidin.

Results

***ERG* promotes megakaryopoiesis and immortalizes HPCs in the presence of IL-3.** We first investigated the potential role of *ERG* in megakaryopoiesis by transducing HPCs derived from fetal liver with retrovirus expressing the *ERG* hematopoietic isoform. We found that progenitor cells expressing *ERG* could not be serially replated in the presence of thrombopoietin and SCF, which synergize to enhance growth of murine megakaryocyte colonies (ref. 20; Supplementary Fig. S2*A*). However, we found that *ERG*-transduced HPCs could be serially replated in the presence of thrombopoietin, SCF, and IL-3 (Fig. 1*A*). IL-3 supports megakaryocytic colony formation and synergizes with thrombopoietin to

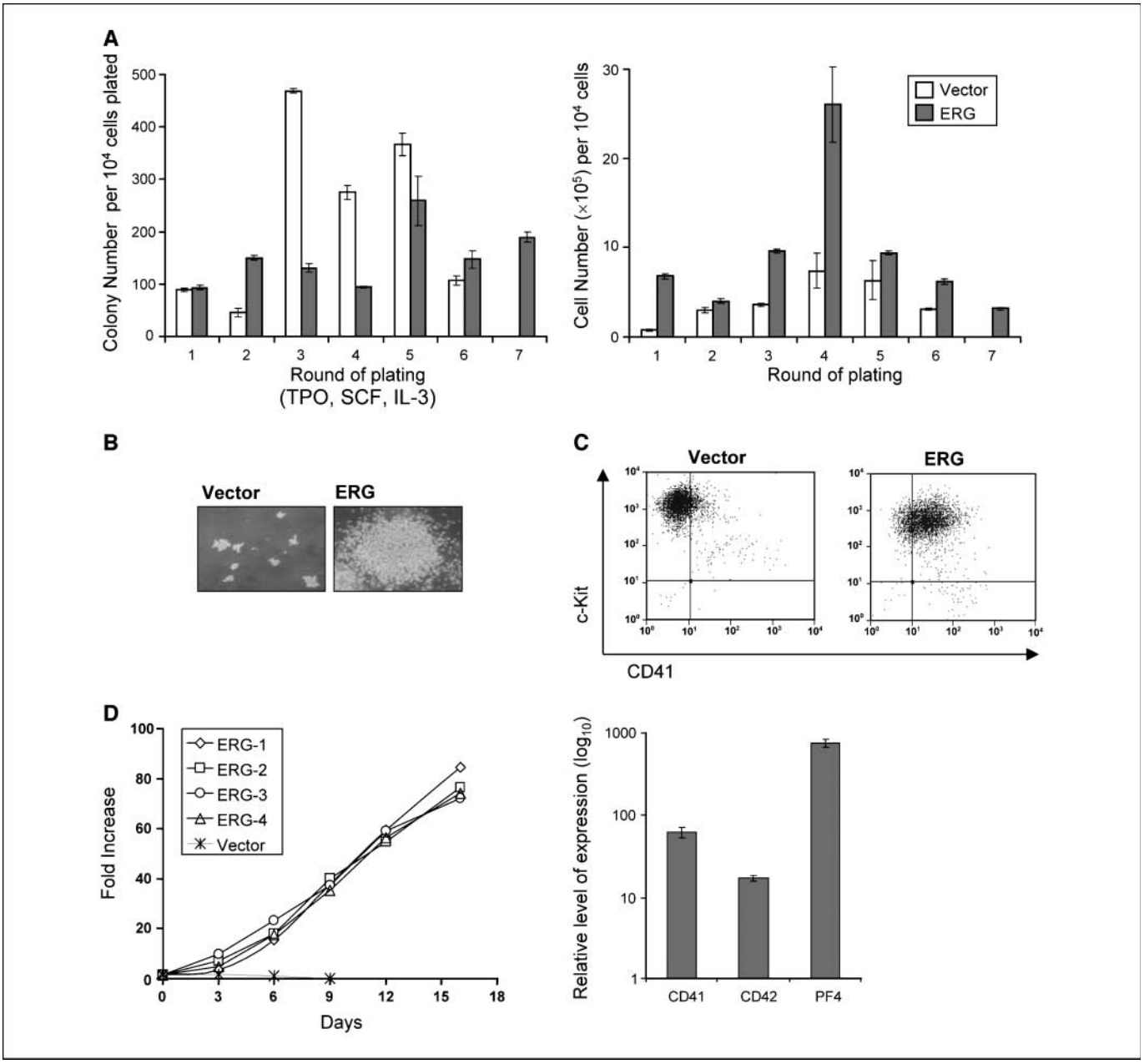

Figure 1. *ERG* promotes megakaryopoiesis in fetal-derived progenitor and immortalizes HPCs in the presence of IL-3. *A,* number of colonies formed (*left*) and number of cells (*right*) at each round of plating following retroviral infection of HPCs. Cells were cultured in methylcellulose in the presence of thrombopoietin (*TPO*), SCF, and IL-3. *White* and *gray columns,* cells transduced with empty vector or *ERG* retrovirus, respectively. Mean ± SD of duplicate cultures. *B,* typical morphology of colonies formed by cells transduced with vector or *ERG* retrovirus. Original magnification, ×100. *C,* flow cytometry analysis of cells transduced with vector or *ERG* retrovirus from the third round of plating. Expression levels of c-Kit and CD41 on the transduced cells are shown in a dot plot. *D,* generation of *ERG* immortalized cell lines. The fold accumulation in cell number of ERG-1 (◇), ERG-2 (□), ERG-3 (△), and ERG-4 (○) cell lines. Single colonies were grown in liquid culture in the presence of thrombopoietin, SCF, and IL-3. Cells transduced with vector alone did not survive in liquid culture. Relative level of megakaryocytic mRNA expression (CD41, CD42, and PF4) measured by real-time PCR in an ERG immortalized cell line. Expression levels of CD41, CD42, and PF4 were normalized to glyceraldehyde-3-phosphate dehydrogenase expression. *Columns,* mean of quadruplet measurement; *bars,* SD.

stimulate megakaryocyte production and colony-forming units-megakaryocyte (21). Cells transduced with MSCV vector could form colonies up to the sixth round of plating (Fig. 1*A*). However, there was a dramatic difference in the morphology of the colonies formed. *ERG*-expressing cells formed large, hyperproliferative colonies, whereas vector control cells formed small, intact colonies at each round of plating (Fig. 1*B*). Furthermore, *ERG*-transduced cells were mostly c-Kit$^+$CD41$^+$ (CD41 is a megakaryocytic differentiation marker) compared with control cells that expressed the early hematopoietic progenitor cell marker c-Kit alone (Fig. 1*C*). Individual colonies were picked and propagated in liquid culture and only *ERG*-transduced colonies grew (Fig. 1*D*). These cell lines could be maintained in culture for up to 10 weeks and were found to be IL-3 and SCF dependent (Supplementary Fig. S2*B*). Real-time PCR analysis of the *ERG*-immortalized cells showed expression of the genes associated with megakaryocytic

differentiation such as CD41, CD42 (a marker of more mature megakaryocytes), and PF4. The relative mRNA expression was normalized to untransduced HPCs. These data show that *ERG* can promote megakaryopoiesis and immortalize early HPCs.

ERG induces megakaryoblastic leukemia in mice. Having established cell lines with a megakaryocytic phenotype, we examined the leukemogenic potential of *ERG in vivo*. HPCs transduced with *ERG* or vector control were transplanted into sublethally irradiated syngeneic mice 48 h after transduction. The transduction efficiency of the HPCs was ~90% as assessed by human CD2 expression (Fig. 2*A*) because ERG and CD2 were coexpressed by retroviral vector (Supplementary Fig. S1*A*). Mice transplanted with control cells remained healthy, but all recipient mice transplanted with cells expressing *ERG* succumbed to leukemia within 100 days, the majority in <50 days (Fig. 2*A*). The *ERG* mice exhibited profound splenomegaly and histologic examination showed disrupted spleen architecture and heavy infiltration of organs such as spleen, bone marrow, and liver by leukemic cells (Fig. 2*B*). Because the transduced HPCs harbored both *ERG* and human *CD2*, the $CD2^+$ cells from the enlarged spleens were assayed for c-Kit and CD41 expression. $CD2^+$ spleen cells from the *ERG* mice predominantly expressed CD41 and very low levels of c-Kit, indicating a megakaryoblastic phenotype (Fig. 2*C*). Wright-Giemsa staining of leukemic cells from bone

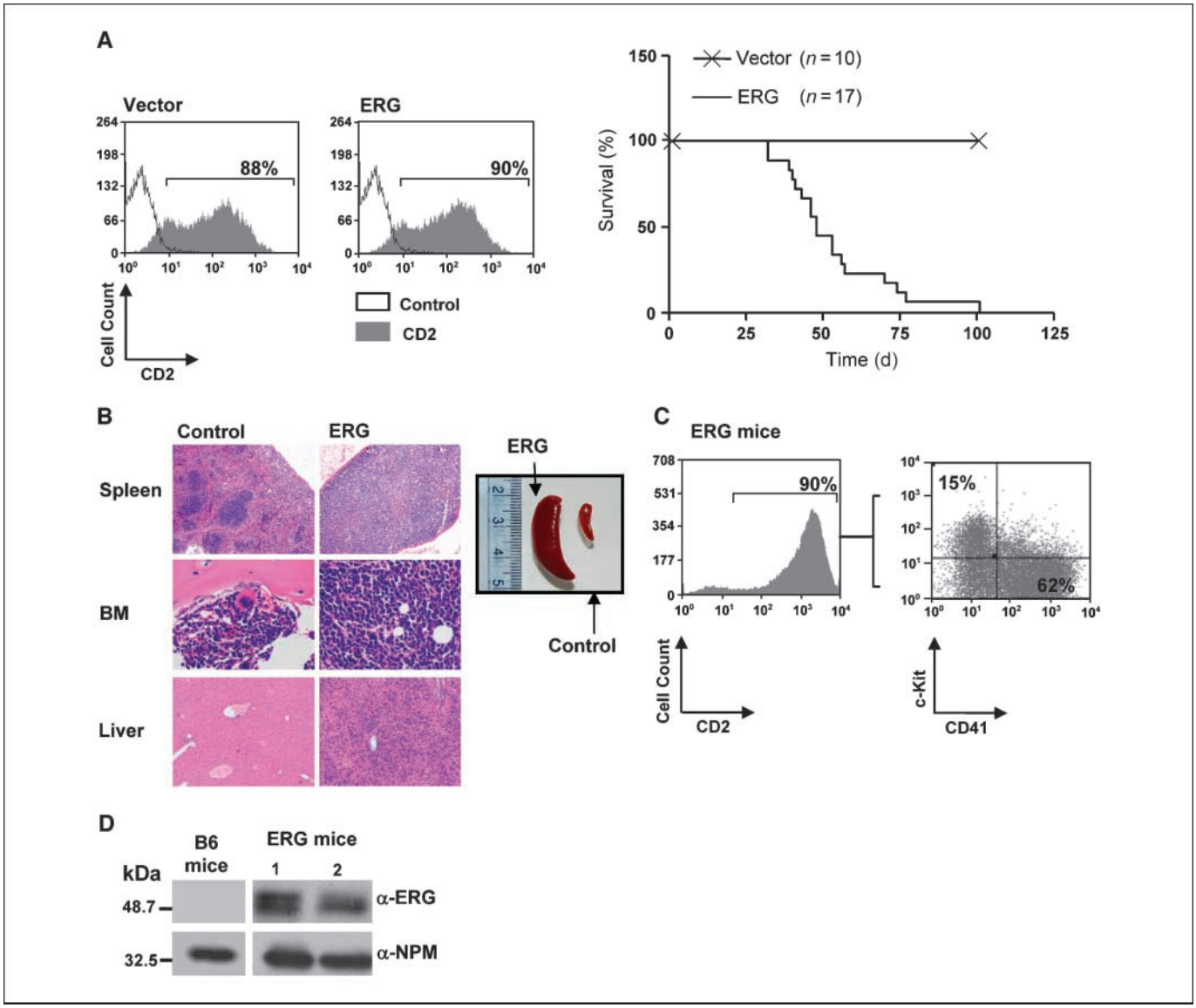

Figure 2. Cells transduced with *ERG* alone induce leukemia when transplanted into sublethally irradiated mice. *A,* expression level of hCD2 (*gray histograms*) against an isotype control (*black line*). Infection efficiencies were determined by analyzing the expression of hCD2 48 h post-transduction. *B,* survival curve for cohorts of mice injected with HPCs transduced with indicated viruses (n = 10 for vector control and n = 17 for *ERG*). Leukemic cell infiltration into different organs [spleen, bone marrow (*BM*), and liver] was determined by H&E staining. Original magnification, ×400. Splenomegaly in mice injected with cells expressing *ERG*. *Arrows,* representative example of spleen size from leukemic and control mice. *C,* flow cytometry analysis of leukemic splenocytes of representative animals injected with *ERG*-tranduced HPCs. The histogram shows the percentage of $hCD2^+$ cells in the spleen and the dot plot shows c-Kit and CD41 expression within the $hCD2^+$ population. The numbers in the plot represent the percentage of c-Kit-positive and $CD41^+$ cells. *D,* Western blot analysis of splenocytes from two independent mice that were transplanted with *ERG*-transduced cells. The presence of ERG protein in the splenocytes of transplanted mice was detected by using an anti-ERG antibody and an anti-NPM antibody was used for loading control.

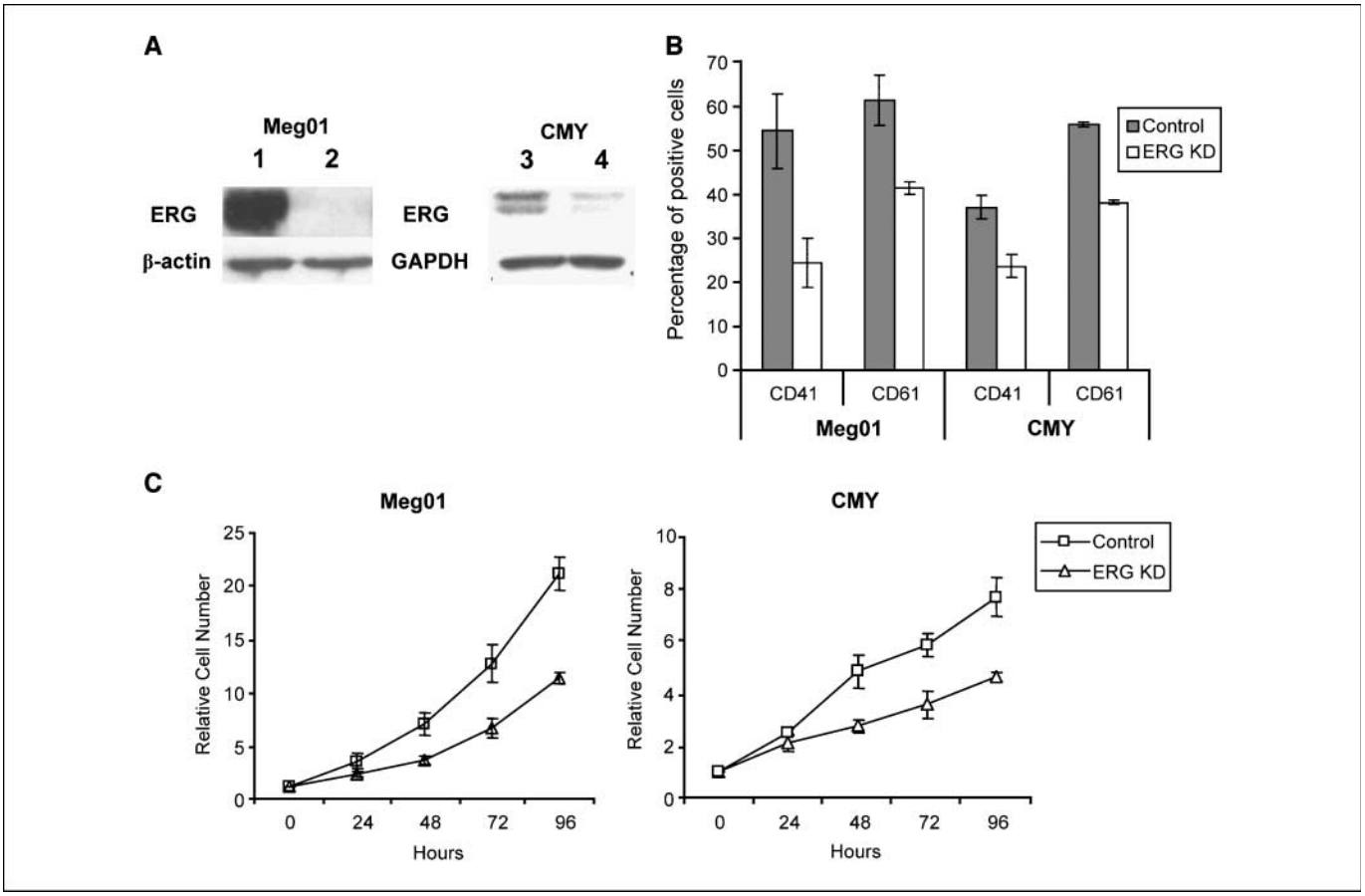

Figure 3. ERG is required for megakaryocytic differentiation and proliferation of megakaryoblastic cell lines, Meg01 and CMY. *A,* Western blot analysis of Meg01 and CMY cells transduced with control or *ERG* knockdown retrovirus. ERG knockdown was confirmed after transduction using an anti-ERG antibody and anti-actin or anti-GAPDH was used as loading control. *B,* summary of flow cytometry analysis showing the percentage of cells expressing CD41 and CD61 after transduction with control (*gray column*) or ERG knockdown (*KD*) retrovirus (*white column*). *C,* growth curves of cells transduced with either control or *ERG* knockdown retrovirus. Cells were counted by trypan blue exclusion. Summary of three independent experiments.

marrow and spleen showed the presence of cells with characteristic blast-like phenotype (Supplementary Fig. S3). Immunoblotting of spleen cells from ERG mice confirmed the expression of ERG (Fig. 2*D*). The data show that *ERG* can act as a potent oncogene and induces megakaryoblastic leukemia *in vivo*.

ERG is required for megakaryocytic differentiation and growth of AMKL-derived cell lines. We have previously shown the expression of ERG in both Down syndrome and non-Down syndrome AMKLs (2). To determine the role of "endogenous" ERG in AMKL, we generated an *ERG* knockdown retroviral construct targeting the 3′-untranslated region of all the *ERG* isoforms. Knockdown of ERG was evident in protein analysis of leukemic cell lines derived from non-Down syndrome AMKL (Meg01; harboring wild-type *GATA1*) and Down syndrome AMKL (CMY; harboring the *GATA1s* mutation) following transduction with a retrovirus expressing small interfering RNA that targets *ERG* (Fig. 3*A*). Real-time PCR analysis revealed a reduction in the expression of megakaryocytic differentiation genes *GP2B* (CD41) and *GP3A* (CD61) in both Meg01 and CMY cells (Supplementary Fig. S4). Similarly, CD41 and CD61 expression levels were significantly reduced in cells when *ERG* expression was knocked down compared with cells transduced by retrovirus expressing a nonspecific small interfering RNA (Fig. 3*B*). Furthermore, *ERG* knockdown led to a significant growth reduction in both AMKL cell lines (Fig. 3*C*; $P < 0.001$). These results show that endogenous expression of ERG in both Down syndrome and non-Down syndrome AMKL cell lines enhances growth and the expression of genes that define megakaryocytic differentiation.

ERG collaborates with GATA1s to immortalize murine HPCs. As *ERG* is located on chromosome 21 and after having established that *ERG* is a megakaryoblastic oncogene, we hypothesized that ERG might cooperate with the GATA1s mutated protein, which has a clear initiating role in Down syndrome AMKL. This hypothesis is supported by the effects of knocking down ERG in the Down syndrome AMKL CMY cell line. To determine if coexpression of *GATA1s* and *ERG* would be sufficient to immortalize HPCs, we decided to apply a highly stringent criterion for megakaryocytic colony-forming assay using only thrombopoietin and SCF, which in themselves were insufficient to support hematopoietic progenitor cell immortalization by *ERG* alone (Supplementary Fig. S2*A*). Based on the study by Li and colleagues. (16), we hypothesized that GATA1s has a dominant activity even when expressed on a background of wild-type GATA1. Data from the same study showed that embryonic day 12.5 fetal liver contains a significant fraction of GATA1s-responsive hyperproliferative megakaryocytic precursors, which diminishes very rapidly at later days in embryonic development (16). Therefore, we set up colony-forming assays using retrovirally transduced embryonic day 12.5

fetal liver HPCs in the presence of thrombopoietin and SCF. HPCs transduced with *GATA1s* or *ERG* individually produced only small numbers of colonies and very few cells. Cells cotransduced by *ERG* and the full-length *GATA1* failed to divide after the second round of plating. However, HPCs coexpressing *ERG* and *GATA1s* produced large numbers of colonies, which could be continuously replated in methylcellulose (Fig. 4*A*). The increased number of colonies formed by cells expressing *ERG* and *GATA1s* was shown by staining of cultures with *p*-iodonitrotetrazolium at each round of plating (Fig. 4*B*). The ability of leukemia-associated oncogenes to immortalize HPCs is directly assessed using colony-forming cell assays in methylcellulose (22). Single colonies of cells coexpressing *ERG* and *GATA1s* were transferred from methylcellulose into liquid culture containing thrombopoietin and SCF and these cells expanded and divided continuously over an extended time course (Fig. 4*C*). Flow cytometry showed that the ERG and GATA1s immortalized cells had a characteristic megakaryoblastic phenotype expressing high levels of c-Kit and CD41 (Fig. 4*D*). By contrast, cells transduced with vector alone did not express the megakaryocytic marker CD41 (data not shown). ERG and GATA1s

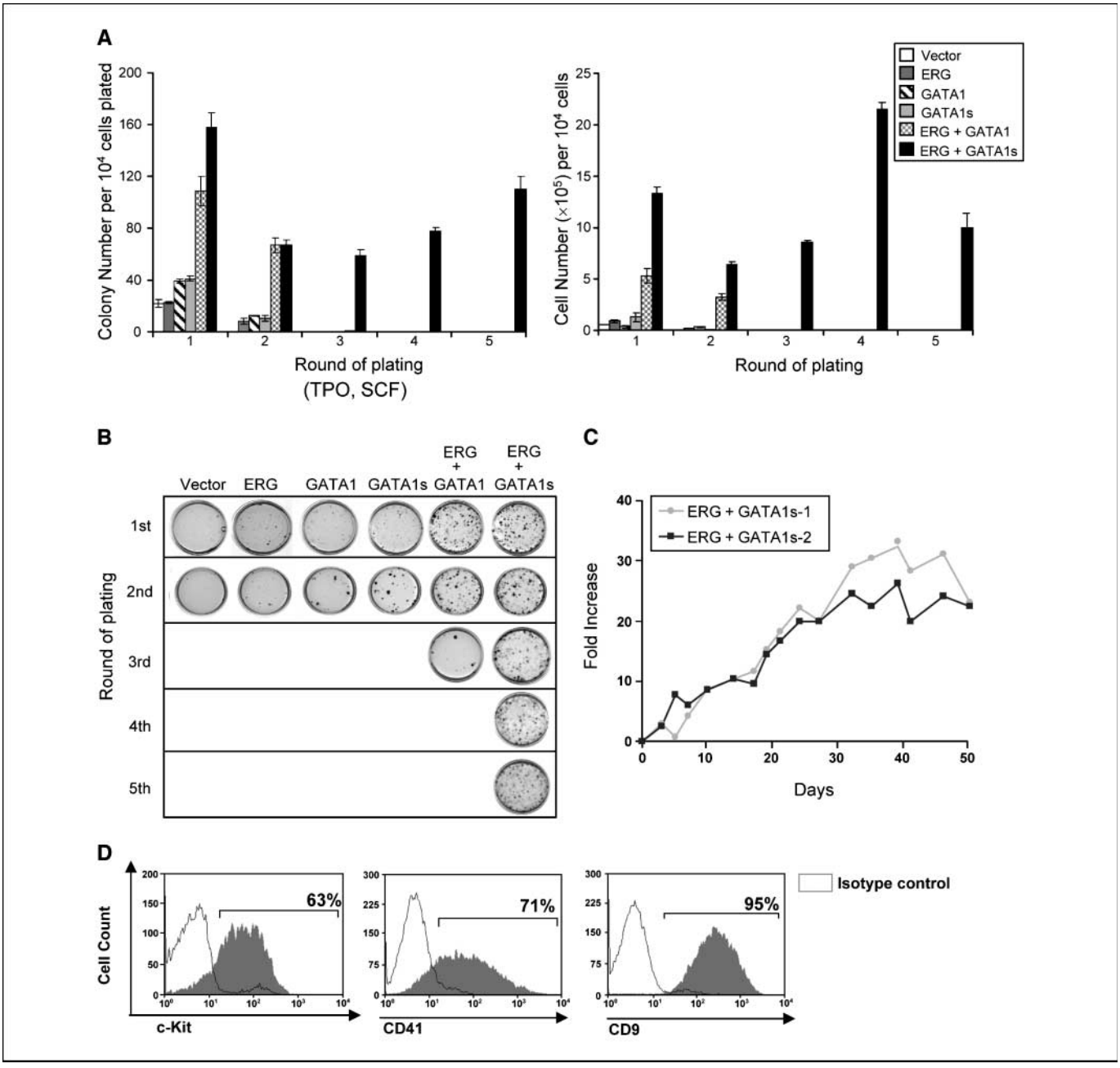

Figure 4. GATA1s collaborates with ERG to immortalize HPCs in the presence of thrombopoietin and SCF alone. *A,* number of colonies formed (*left*) and number of cells (*right*) at each round of plating following retroviral infection of HPCs. Transduced cells were cultured in methylcellulose in the presence of thrombopoietin and SCF. Mean ± SD of duplicate cultures. *B, p*-iodonitrotetrazolium stains of cells at each round of methylcellulose cultures of transduced HPCs. *C,* fold increase in cell number of two different ERG + GATA1s immortalized cell lines from the same experiment. *D,* representative flow cytometry analysis of ERG + GATA1s immortalized cell lines. Expression of c-Kit-positive, $CD41^+$, and $CD9^+$ cells (*gray histogram*) and the relevant isotype controls (*black line*). Numbers represent the percentage of cells positive for each cell surface antigen.

immortalized cells also express high levels of CD9, an early-stage megakaryoblast marker (23). The data suggest a classic oncogenic cooperativity (24, 25) between GATA1s and ERG that immortalizes megakaryoblasts analogous to the megakaryoblastic leukemia cells found in Down syndrome AMKL.

To determine the consequences of ERG and GATA1s cooperativity *in vivo*, we transplanted HPCs cotransduced with both *ERG*- and *GATA1s*-expressing retroviruses into sublethally irradiated syngeneic mice. HPCs purified from embryonic day 12.5 fetal liver were transduced with *GATA1s* alone, *ERG* and *GATA1s*, or empty vector and cells were transplanted into syngeneic mice 48 h post-transduction (data not shown). Mice transplanted with HPCs expressing *GATA1s* alone or empty vector remained healthy. Cells expressing *ERG* + *GATA1s* induced leukemia (Fig. 5*A*), with similar latency to that found for mice transplanted with HPCs expressing *ERG* alone. Similarly to the *ERG* mice, the ERG + GATA1s mice had severe splenomegaly (Supplementary Fig. S5*A*) and histology examination showed a marked disruption in the normal architecture of the spleen and extensive infiltration into bone marrow and liver (Supplementary Fig. S5*B*). Immunoblotting of spleen cells from ERG + GATA1s mice shows high-level coexpression of both retrovirally transduced ERG and FLAG-tagged GATA1s (Fig. 5*B*). As above, each leukemic cell harbored the *ERG* retrovirus and therefore coexpressed hCD2. The c-Kit and CD41 expression within the $CD2^+$ population of ERG + GATA1s leukemias was assayed (Fig. 5*C*). These leukemias consisted of heterogeneous populations of cells, with most mice exhibiting cells that are predominantly c-Kit positive. In comparison with cells from ERG mice, the $CD2^+$ leukemic cells in ERG + GATA1s mice had significantly reduced expression of CD41 (Fig. 6*A*) but no significant difference in expression levels of c-Kit (Fig. 6*B*). Furthermore, real-time PCR analysis of leukemic spleens from *ERG* versus *ERG* + *GATA1s* mice showed a significant decrease in expression of the CD41, CD42, and PF4 genes associated with megakaryocytic maturation (Fig. 6*C*). Southern blotting showed that all the leukemias harbored multiple integrations and were oligoclonal. These data suggest that the *ERG* and *GATA1s* leukemias appear less differentiated than those induced by *ERG* alone.

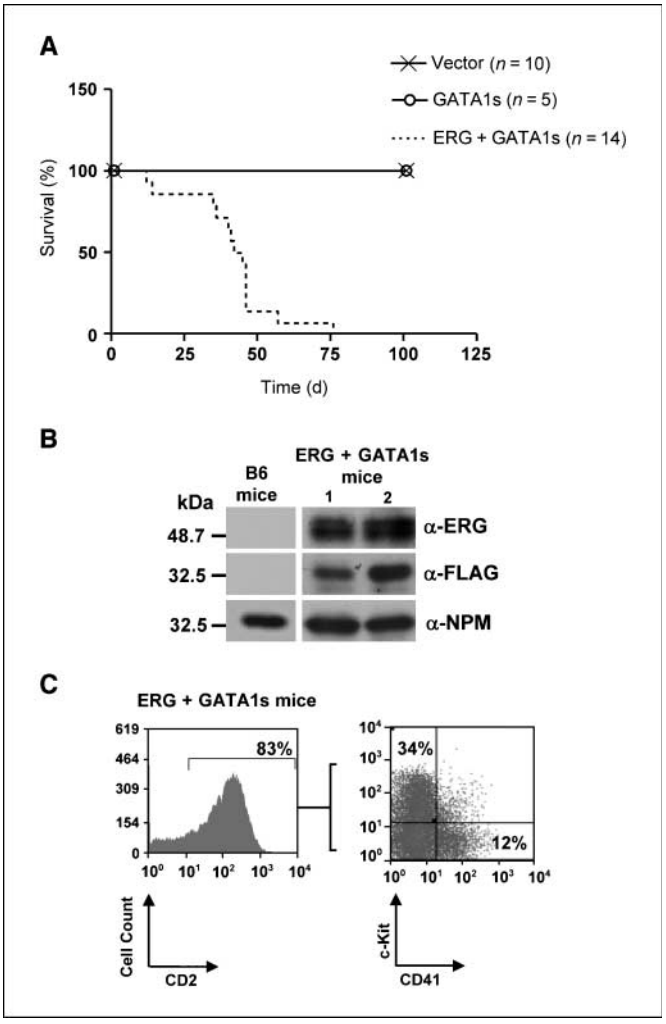

Figure 5. Cells transduced with *ERG* + *GATA1s* induce leukemia when transplanted into sublethally irradiated mice. *A,* survival curve shown for cohorts of mice injected with HPCs transduced with indicated viruses (n = 10 for vector control, n = 5 for GATA1s, and n = 14 for ERG + GATA1s). *B,* Western blot analysis of splenocytes from two independent mice that were transplanted with cells transduced with ERG-3 + FLAG-tagged GATA1s. The presence of ERG-3 and GATA1s protein in the splenocytes of transplanted mice was detected by using an anti-ERG and an anti-FLAG antibody, respectively. An anti-NPM antibody was used for loading control. *C,* flow cytometry analysis of leukemic splenocytes of a representative animal injected with cells coexpressing ERG + GATA1s. The histograms show the percentage of $hCD2^+$ cells in the spleen and dot plots show c-Kit and CD41 expression within the $hCD2^+$ population. The numbers in the plot represent the percentage of c-Kit-positive and $CD41^+$ cells.

Discussion

ERG involvement in cancer has been generally characterized by fusion translocations of a NH_2-terminal truncated ERG (5, 6, 26, 27). Increased expression of full-length *ERG* has been observed in patients with AMKL (2, 28) and found to be associated with poor prognosis of AML (10, 11). However, it has been unclear if this simply represents the differentiation stage of the leukemic blasts or if ERG has a direct leukemogenic activity. Our data show for the first time that increased expression of the full-length ERG protein causes aggressive megakaryoblastic leukemia in mice. Consistent with this, we find that reduction in the expression of endogenous ERG in AMKL leads to decreased expression of megakaryocytic markers and a decrease in growth rate despite the presence of additional oncogenic proteins, that is, BCR/ABL in Meg01 cells and GATA1s and likely other abnormalities in CMY cells. Therefore, ERG is a bona fide megakaryocytic oncogene.

Many leukemia-associated transcription factors are normally involved in hematopoietic differentiation (29–31). We show that *ERG* expression within HPCs is a strong inducer of megakaryopoiesis. Previously, we have shown that ERG binds the *SCL*+19 enhancer (2) that regulates *SCL/TAL1* expression in HPCs and *SCL1/TAL1* overexpression is known to force progenitor cells toward the megakaryocytic lineage (32, 33). Our studies combined with the recently published loss of function experiments (4) suggest that *ERG* may act through gene dosage effect on processes such as megakaryocytic commitment, proliferation, and differentiation.

Recent studies (34, 35) show that germ-line trisomy 21 markedly enhances fetal liver megakaryopoiesis. Because *ERG* is a positive regulator of megakaryopoiesis, it is one of several candidate genes that could cooperate with the somatically acquired *GATA1s* mutation in the initiation of Down syndrome AMKL. Our data show that *ERG* cooperates strongly with the *GATA1s* mutation to immortalize fetal megakaryocytic progen-

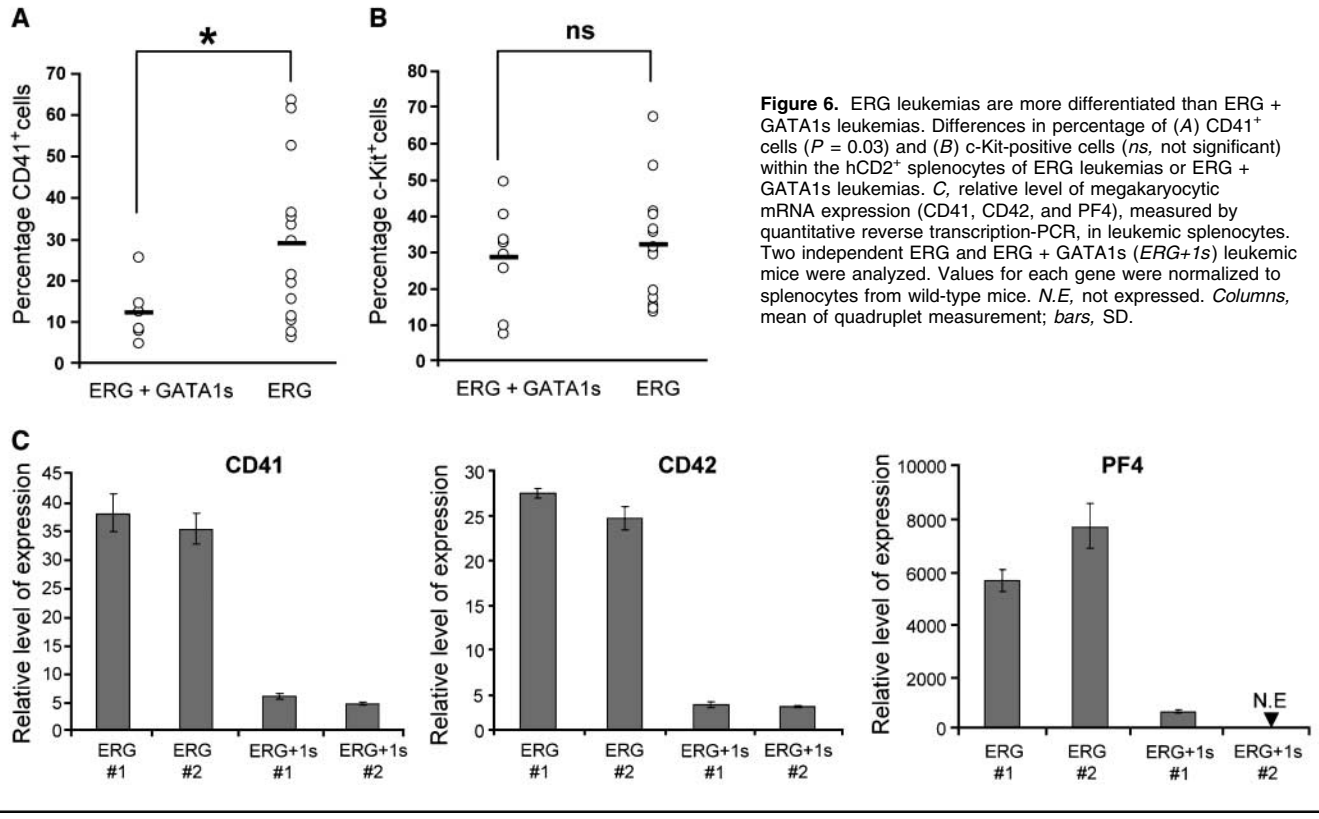

Figure 6. ERG leukemias are more differentiated than ERG + GATA1s leukemias. Differences in percentage of (*A*) $CD41^+$ cells ($P = 0.03$) and (*B*) c-Kit-positive cells (*ns,* not significant) within the $hCD2^+$ splenocytes of ERG leukemias or ERG + GATA1s leukemias. *C,* relative level of megakaryocytic mRNA expression (CD41, CD42, and PF4), measured by quantitative reverse transcription-PCR, in leukemic splenocytes. Two independent ERG and ERG + GATA1s (*ERG+1s*) leukemic mice were analyzed. Values for each gene were normalized to splenocytes from wild-type mice. *N.E,* not expressed. *Columns,* mean of quadruplet measurement; *bars,* SD.

itors in the presence of thrombopoietin and SCF. Under these restrictive conditions, neither GATA1s nor ERG alone could cause immortalization. This phenotype was further confirmed by transferring single colonies from methylcellulose into liquid culture containing thrombopoietin and SCF, where the cells expanded and divided continuously over an extended time course. Immunophenotypic analysis revealed that these ERG + GATA1s immortalized cells had a characteristic immature megakaryoblastic phenotype expressing high levels of c-Kit and CD41. ERG alone could only immortalize HPCs in the presence of IL-3 in addition to SCF. In agreement with this, Stankiewicz and Crispino have also found that ERG induces megakaryopoiesis and it synergizes with GATA1s to enhance colony formation (36).

ERG acts as a potent oncogene *in vivo* rapidly leading to leukemia. The latency of the development of leukemia from HPCs transduced with *ERG* was so short that it was impossible to observe any accelerated effect due to the presence of transduced *GATA1s*. However, the coexpression of ERG and GATA1s *in vivo* did result in leukemias with an immature megakaryocytic phenotype reflected in a profound decrease expression of megakaryocyte differentiation markers such as CD41, CD42, and PF4. This effect may be due to the ability of GATA1s to enhance cell proliferation at the expense of differentiation (37). This finding is also consistent with the reduced megakaryocytic differentiation levels of Down syndrome AMKL (28) as is also evident when comparing CMY with Meg01 cells (Fig. 3*B*). The potential role for ERG in Down syndrome AMKL is suggested by the reduced growth of CMY Down syndrome AMKL cells after knockdown of endogenous ERG. It is likely that the level of ERG expression in human Down syndrome fetal HPCs is lower than its level in our mouse transplantation experiments. Hence, we hypothesize that, in humans, the action of ERG in Down syndrome AMKL would require the presence of GATA1s to initiate leukemogenic expansion of megakaryoblasts observed in congenital Down syndrome TMD. It is highly likely that several Hsa21 genes, besides *ERG*, cooperate with *GATA1s*. Recent work has argued against a requirement for *RUNX1* (38) but has emphasized the possible involvement of fellow *ETS* family member, *ETS2*, in Down syndrome AMKL (36, 39). Therefore, *ERG, ETS2*, and probably other Hsa21 genes overexpressed in early fetal hematopoietic progenitors trisomic for chromosome 21 can enhance megakaryopoiesis as observed in the studies in Down syndrome fetal livers (34, 35).

The leukemic action of ERG may also be significant for lymphoblastic leukemias. Increased expression of *ERG* in T-cell acute lymphoblastic leukemia is associated with adverse prognosis (40) and a subgroup of pediatric B-cell acute lymphoblastic leukemia shows overexpression of partially deleted *ERG* (9). Now that we have identified the potent effects of ERG on both normal and malignant hematopoiesis, elucidation of the components of the ERG-mediated pathway is clearly an important avenue of future research for both megakaryopoiesis and leukemia in general.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Duterque-Coquillaud M, Niel C, Plaza S, Stehelin D. New human erg isoforms generated by alternative splicing are transcriptional activators. Oncogene 1993; 8:1865–73.
2. Rainis L, Toki T, Pimanda JE, et al. The proto-oncogene ERG in megakaryoblastic leukemias. Cancer Res 2005;65:7596–602.
3. Hart A, Melet F, Grossfeld P, et al. Fli-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia. Immunity 2000;13:167–77.
4. Loughran SJ, Kruse EA, Hacking DF, et al. The transcription factor Erg is essential for definitive hematopoiesis and the function of adult hematopoietic stem cells. Nat Immunol 2008;9:810–9.
5. Sorensen PH, Lessnick SL, Lopez-Terrada D, Liu XF, Triche TJ, Denny CT. A second Ewing's sarcoma translocation, t(21;22), fuses the EWS gene to another ETS-family transcription factor, ERG. Nat Genet 1994;6: 146–51.
6. Tomlins SA, Rhodes DR, Perner S, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 2005;310:644–8.
7. Shimizu K, Ichikawa H, Tojo A, et al. An ets-related gene, ERG, is rearranged in human myeloid leukemia with t(16;21) chromosomal translocation. Proc Natl Acad Sci U S A 1993;90:10280–4.
8. Dastugue N, Lafage-Pochitaloff M, Pagès MP, et al. Cytogenetic profile of childhood and adult megakaryoblastic leukemia (M7): a study of the Groupe Français de Cytogénétique Hématologique (GFCH). Blood 2002;100: 618–26.
9. Mullighan CG, Goorha S, Radtke I, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature 2007;446:758–64.
10. Baldus CD, Liyanarachchi S, Mrózek K, et al. Acute myeloid leukemia with complex karyotypes and abnormal chromosome 21: amplification discloses overexpression of APP, ETS2, and ERG genes. Proc Natl Acad Sci U S A 2004;101:3915–20.
11. Marcucci G, Maharry K, Whitman SP, et al. High expression levels of the ETS-related gene, ERG, predict adverse outcome and improve molecular risk-based classification of cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B Study. J Clin Oncol 2007;25:3337–43.
12. Hasle H, Clemmensen IH, Mikkelsen M. Risks of leukaemia and solid tumours in individuals with Down's syndrome. Lancet 2000;355:165–9.
13. Rainis L, Bercovich D, Strehl S, et al. Mutations in exon 2 of GATA1 are early events in megakaryocytic malignancies associated with trisomy 21. Blood 2003; 102:981–6.
14. Wechsler J, Greene M, McDevitt MA, et al. Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. Nat Genet 2002;32:148–52.
15. Xu G, Nagano M, Kanezaki R, et al. Frequent mutations in the GATA-1 gene in the transient myeloproliferative disorder of Down syndrome. Blood 2003;102:2960–8.
16. Li Z, Godinho FJ, Klusmann JH, Garriga-Canut M, Yu C, Orkin SH. Developmental stage-selective effect of somatically mutated leukemogenic transcription factor GATA1. Nat Genet 2005;37:613–9.
17. Hollanda LM, Lima CS, Cunha AF, et al. An inherited mutation leading to production of only the short isoform of GATA-1 is associated with impaired erythropoiesis. Nat Genet 2006;38:807–12.
18. Izraeli S, Rainis L, Hertzberg L, Smooha G, Birger Y. Trisomy of chromosome 21 in leukemogenesis. Blood Cells Mol Dis 2007;39:156–9.
19. Vyas P, Crispino JD. Molecular insights into Down syndrome-associated leukemia. Curr Opin Pediatr 2007; 19:9–14.
20. Broudy VC, Lin NL, Kaushansky, K. Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy *in vitro*. Blood 1995;85:1719–26.
21. Dolzhanskiy A, Basch RS, Karpatkin S. The development of human megakaryocytes. III. Development of mature megakaryocytes from highly purified committed progenitors in synthetic culture media and inhibition of thrombopoietin-induced polyploidization by interleukin-3. Blood 1997;89:426–34.
22. Lavau C, Szilvassy SJ, Slany R, Cleary ML. Immortalization and leukemic transformation of a myelomonocytic precursor by retrovirally transduced HRX-ENL. EMBO J 1997;16:4226–37.
23. Nakorn TN, Miyamoto T, Weissman IL. Characterization of mouse clonogenic megakaryocyte progenitors. Proc Natl Acad Sci U S A 2003;100:205–10.
24. Land H, Parada LF, Weinberg RA. Cellular oncogenes and multistep carcinogenesis. Science 1983;222:771–8.
25. Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes *in vivo*. Cell 1987;49:465–75.
26. Kong XT, Ida K, Ichikawa H, et al. Consistent detection of TLS/FUS-ERG chimeric transcripts in acute myeloid leukemia with t(16;21)(p11;q22) and identification of a novel transcript. Blood 1997;90:1192–9.
27. Moore SD, Offor O, Ferry JA, Amrein PC, Morton CC, Dal Cin P. ELF4 is fused to ERG in a case of acute myeloid leukemia with a t(X;21)(q25–26;q22). Leuk Res 2006;30:1037–42.
28. Bourquin JP, Subramanian A, Langebrake C, et al. Identification of distinct molecular phenotypes in acute megakaryoblastic leukemia by gene expression profiling. Proc Natl Acad Sci U S A 2006;103:3339–44.
29. Speck NA, Gilliland DG. Core-binding factors in haematopoiesis and leukaemia. Nat Rev Cancer 2002;2: 502–13.
30. Look AT. Oncogenic transcription factors in the human acute leukemias. Science 1997;278:1059–64.
31. Orkin SH, Porcher C, Fujiwara Y, Visvader J, Wang LC. Intersections between blood cell development and leukemia genes. Cancer Res 1999;59:1784–7s; discussion 1788s.
32. Elwood NJ, Zogos H, Pereira DS, Dick JE, Begley CG. Enhanced megakaryocyte and erythroid development from normal human CD34(+) cells: consequence of enforced expression of SCL. Blood 1998;91:3756–65.
33. Valtieri M, Tocci A, Gabbianelli M, et al. Enforced TAL-1 expression stimulates primitive, erythroid and megakaryocytic progenitors but blocks the granulopoietic differentiation program. Cancer Res 1998;58: 562–9.
34. Tunstall-Pedoe O, Roy A, Karadimitris A, et al. Abnormalities in the myeloid progenitor compartment in Down syndrome fetal liver precede acquisition of GATA1 mutations. Blood 2008;112:4507–11.
35. Chou ST, Opalinska JB, Yao Y, et al. Trisomy 21 enhances human fetal erythro-megakaryocytic development. Blood 2008;112:4503–6.
36. Stankiewicz MJ, Crispino JD. ETS2 and ERG promote megakaryopoiesis and synergize with alterations in GATA-1 to immortalize hematopoietic progenitor cells. Blood. Epub 2009 Jan 23.
37. Muntean AG, Crispino JD. Differential requirements for the activation domain and FOG-interaction surface of GATA-1 in megakaryocyte gene expression and development. Blood 2005;106:1223–31.
38. Kirsammer G, Jilani S, Liu H, et al. Highly penetrant myeloproliferative disease in the Ts65Dn mouse model of Down syndrome. Blood 2008;111:767–75.
39. Ge Y, LaFiura KM, Dombkowski AA, et al. The role of the proto-oncogene ETS2 in acute megakaryocytic leukemia biology and therapy. Leukemia 2008; 22:521–9.
40. Baldus CD, Burmeister T, Martus P, et al. High expression of the ETS transcription factor ERG predicts adverse outcome in acute T-lymphoblastic leukemia in adults. J Clin Oncol 2006;24:4714–20.