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...
maintained in RPMI containing 10% FCS, 2 mM 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- gene. The pMSCV-ERG retroviral construct was generated by cloning the human ERG-3 (hematopoietic isoform of ERG) cDNA into EcoRI sites of the pMSCV-IRESC2 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-IRESC2 vector (gift from Dr. M.J. Bevan, Department of Immunology, University of Washington; Supplementary Fig. S1A). The expression of each protein from this vector was confirmed by immunoblotting of LinXFe-transfected cells (Supplementary Fig. S1B).

**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 (Mm00553151_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 used 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 ATGCCGATCTCTTCTTCTTG, and as control, a nontargeting sequence TCACACAGCACTCACA 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 appropriate retroviral constructs. 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). The viral supernatant was used to transduce either Meg01 or CMY cells in the presence of 10% FCS, 2 mmol/L-glutamine, 100 units/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, t-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, 50 mg/mL thrombopoietin, and 10 ng/mL IL-3.

**In vivo leukemogenesis assay.** HPCs (2 × 10^5^–3 × 10^6^) transduced with empty vector, ERG alone, GATA1s alone, or ERG + GATA1s retroviruses were intravenously injected into sublethally irradiated (6 Gy) C57BL/6J 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 a polyvinylidene difluoride membrane (Immobilon-P; Millipore) using one transfer buffer (N-cyclohexyl-3-amino-panesulfonic acid; pH 11.0) for 5 h at 100 V. Following blocking for 6 h at 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/Reg2; Abcam), CD2 (LFA2; BD Pharmingen), CD61 (F803; 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-G42 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. S24). However, we found that ERG-transduced HPCs could be serially replated in the presence of thrombopoietin, SCF, and IL-3 (Fig. 1A). IL-3 supports megakaryocytic colony formation and synergizes with thrombopoietin to...
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. 1A). 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. 1B). 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. 1C). Individual colonies were picked and propagated in liquid culture and only ERG-transduced colonies grew (Fig. 1D). 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). Real-time PCR analysis of the ERG-immortalized cells showed expression of the genes associated with megakaryocytic

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.
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. 2A) because ERG and CD2 were coexpressed by retroviral vector (Supplementary Fig. S1A). 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. 2A). 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. 2B). 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. 2C). Wright-Giemsa staining of leukemic cells from bone

![Figure 2.](image)

*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-transduced 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.
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. 2D). 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. 3A). 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. 3B). Furthermore, ERG knockdown led to a significant growth reduction in both AMKL cell lines (Fig. 3C; 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. S2A). 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

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.
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. 4A). 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. 4B). 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. 4C). Flow cytometry showed that the ERG and GATA1s immortalized cells had a characteristic megakaryoblastic phenotype expressing high levels of c-Kit and CD41 (Fig. 4D). 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 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. 5A), 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. S5A) and histology examination showed a marked disruption in the normal architecture of the spleen and extensive infiltration into bone marrow and liver (Supplementary Fig. S5B). Immunoblotting of spleen cells from ERG + GATA1s mice shows high-level coexpression of both retrovirally transduced ERG and FLAG-tagged GATA1s (Fig. 5B). 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. 5C). 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. 6A) but no significant difference in expression levels of c-Kit (Fig. 6B). 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. 6C). 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.

**Discussion**

ERG involvement in cancer has been generally characterized by fusion translocations of a NH2-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/TALI expression in HPCs and SCLI/TALI 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 progeni-
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. 3B). 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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14. Hasle H, Clemmensen IH, Mikkelsen M. Risks of patients with 18 U.S.C. Section 1734 solely to indicate this fact. This article must therefore be hereby marked advertisement. This work was done in partial fulfillment of the requirements for the Ph.D. degree for Gil Smooha and Liat Rainis, Sackler Faculty of Medicine, Tel Aviv University.

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