Perturbation of fetal hematopoiesis in a mouse model of Down Syndrome's Transient Myeloproliferative Disorder

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Short title: ERG and GATA1s in TMD and fetal erythropoiesis

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Key points

- Transient expansion of fetal Mega-Erythroid progenitors in ERG/Gata1s mouse is biologically similar to Down Syndrome TMD

- The N-terminal domain of GATA1 and the downregulation of ERG expression are essential for normal fetal erythropoiesis

Abstract

Children with Down syndrome (DS) develop a unique congenital clonal megakaryocytic proliferation disorder (transient myeloproliferative disorder -TMD). It is caused by an expansion of fetal megakaryocyte-erythroid progenitors (MEP) triggered by trisomy of chromosome 21 and further enhanced by the somatic acquisition of a mutation in GATA1. These mutations result in the expression of a short isoform GATA1s lacking the N-terminal domain. To examine the hypothesis that the Hsa21 ETS transcription factor ERG cooperates with GATA1s in this process, we generated double transgenic mice expressing hERG and Gata1s. We show that increased expression of ERG by itself is sufficient to induce expansion of MEPs in fetal livers. Gata1s expression synergizes with ERG in enhancing the expansion of fetal MEPs and megakaryocytic precursors, resulting in hepatic fibrosis, transient postnatal thrombocytosis, anemia, a gene expression profile that is similar of human TMD and progression to progenitor myeloid leukemia by 3 month of age. This ERG/Gata1s transgenic mouse model also uncovers an essential role for the N-terminus of Gata1 in erythropoiesis and the antagonistic role of ERG in fetal erythroid differentiation and survival. The human relevance of this finding is underscored by the recent discovery of similar mutations in GATA1 in patients with Diamond-Blackfan anemia.
Introduction

Neonates with Down syndrome (DS) have hematological abnormalities resulting from inappropriate fetal hematopoiesis\(^1\), characterized expansion of fetal liver megakaryocyte-erythroid progenitors (MEP) in second trimester fetuses\(^2,3\). In approximately 5% of children with DS this expansion is dramatically enhanced by an acquired mutation in the X-chromosome transcription factor \textit{GATA1} resulting in a congenital transient clonal myeloproliferative disorder (TMD)\(^4-6\).

\textit{GATA1} regulates the megakaryocytic-erythroid lineages\(^7,8\). Inherited mutations in the N-terminal Zinc finger domains cause X-linked dyserythropoietic anemia and thrombocytopenia\(^9\). The \textit{GATA1} mutations found in DS-TMD cause the expression of a shorter truncated isoform (GATA1s) lacking the N-terminal transactivation domain. This protein is normally co-expressed with full length GATA1 due to alternative translation initiation site or alternative splicing\(^10\). Mice expressing only Gata1s exhibit transient early fetal megakaryocytic hyperproliferation without perinatal abnormalities\(^11\). This hyperproliferative was recently attributed to removal of an inhibitory activity of the Gata1 N-terminal domain on E2F\(^12\).

The unique TMD of DS suggests a specific collaboration between the expansion of fetal MEPs caused by trisomy 21 and the differentiation arrest and hyperproliferation conferred by Gata1s\(^13\). Several chromosome 21 gene products have been suggested to cooperate with GATA1s in the generation of DS-TMD\(^2,6,12,14-18\). Since a trisomy causes overexpression of many genes\(^19\), it is possible that cooperation between several genes on the trisomic chromosome generate the hematopoietic phenotype.
The role of the chromosome 21 ETS transcription factor ERG\textsuperscript{20} in hematopoiesis\textsuperscript{21} and leukemia\textsuperscript{22} places it as a potential regulator of MEP expansion in DS fetuses and a collaborator with GATA1s in TMD. ERG is expressed in megakaryocytic and erythroid progenitors and is downregulated upon their differentiation\textsuperscript{6}. ERG is essential for maintenance of fetal hematopoietic stem cells (HSC)\textsuperscript{21, 23}. It is one of a heptad of transcription factors, bound in combinatorial interactions to hematopoietic stem cell enhancers\textsuperscript{24}. Developmental cooperation between ERG and GATA1 has been suggested by demonstrating their co-binding to regulatory elements of key hematopoietic genes such as Scl/Tal1\textsuperscript{24}. ERG is also a known oncogene involved in chromosomal translocations in leukemia, Ewing sarcoma and prostate cancer\textsuperscript{25, 26}. We demonstrated in-vitro and in-vivo that ERG is a megakaryocytic oncogene that cooperating with Gata1s in transforming mouse megakaryocytic progenitors\textsuperscript{5, 16, 17}. Further support to the hematopoietic role of ERG was recently given by an experiment in which reversing ERG trisomy to functional disomy in the Ts65Dn mouse model of DS corrected the myeloproliferative syndrome observed in these adult mice\textsuperscript{27}. Yet, unlike human DS fetuses, no prenatal hematopoietic abnormalities were reported in Ts65Dn mice.

To study the putative fetal developmental hematopoietic cooperation between ERG and Gata1s we generated double transgenic mice expressing hERG and Gata1s. We show that similar to trisomy 21 in DS, increased expression of ERG by itself is sufficient to induce megakaryopoiesis and expansion of MEPs in fetal livers. We also demonstrate that expression of Gata1s together with ERG further enhances and prolongs hyper-proliferation of fetal MEP and megakaryocytic progenitors generating a human TMD-like gene expression profile and liver pathology. Strikingly most male transgenic ERG/Gata1s did not survive beyond embryonic day 12.5 due to severe anemia, revealing the necessity of the amino terminus of Gata1 and of down-regulation of ERG for fetal erythropoiesis.
Materials and Methods

Generation of double ERG/Gata1s transgenic mouse

ERG transgene was generated as described previously\(^2^8\). In brief, the human \(ERG3\) hematopoietic isoform was cloned into the HS21/45-vav vector replacing hCD4\(^2^\). Double transgenic mice expressing both ERG and Gata1s were generated by crossing Tg\(ERG\) males with homozygous \(Gata1s\) knock-in females\(^1^1\). Animal studies were approved by the institutional animal care and use committee (ACUC) of the Chaim Sheba Medical Center – Tel Hashomer.

Isolation and immunostaining of fetal liver, hematopoietic stem and progenitor cells

FL single cell suspensions were washed in PBS with 0.05M EDTA, 0.1% BSA. Lin-progenitors were isolated using the lineage cell depletion kit (Miltenyi) and cells were stained with PE-Cy7-conjugated C-kit, FITC-conjugated Sca1, APC-conjugated FCgammaR and PE-conjugated CD34 (e-bioscience). Erythroid FL cells were stained with FITC-conjugated Ter119 and APC-conjugated CD71 (e-bioscience). For apoptosis assays cells were stained with APC-conjugated annexin V (Enzo life sciences), washed with PBS, 1%FBS, re-suspended with Propidium iodide (PI) (5\(\mu\)g/ml) and were analyzed by Flow cytometry (FACSCalibur BD Biosciences). Flow cytometry data was analyzed by Flowjo software.

Colony forming assay

FL single cells were cultured in methylcellulose (Stem Cell Technology). For megakaryocytic colonies, cells were plated in Methocult 3231 supplemented with 50 ng/mL thrombopoietin (TPO) (Peprotech) and scored 5-7 days later. For erythroid colonies, cells were plated in Methocult 3334 containing 3U/ml erythropoietin (EPO) and colonies scored 7-10 days later.
RNA Preparation

Total cellular RNA was prepared using TRIzol (Life Technologies). cDNA was generated using Verso cDNA kit (Thermo Scientific). Real-time quantitative PCR was done using SYBER Green and an ABI Prism 7900HT Fast Sequence Detection System (Applied Biosystems).

Gene expression profiling

Experiments were performed using Affymetrix Mouse gene 1.0 ST oligonucleotide arrays (Affymetrix) with total RNA from each FL sample (GSE46481) (supplemental methods).

G1ME and FL cell infection and western blots

G1ME cells\(^{29}\) and FL cells were infected with MIGR1-GATA1, MIGR1-GATA1s, or vector alone as described\(^{30}\). Two days after infection, RNA was extracted and cell lysates were generated and the proteins were separated by gel electrophoresis. Anti-GATA-1 (sc-1234), HA-tag (sc-7392), FOG-1 (sc-9361), GATA-2 (sc-9008), and HSC70 (sc-7298) antibodies were purchased from Santa Cruz Biotechnology. The anti-Myb antibody (05-175) was purchased from Millipore.

Benzidine staining and cytospins

FL cells were stained with benzidine as described\(^{31}\). Cytospins were generated by centrifuging \(5 \times 10^5\) cells at 600rpm for 10 min on glass slides and then Gimsa stained (Sigma-Aldrich).

Reticulin staining

Fetal liver tissues were fixed in 4% neutral buffered formalin, and paraffin-embedded. Reticulin staining was carried out using conventional techniques.
Results

Fetal expansion of Megakaryocytic-Erythroid and megakaryocytic progenitors in ERG/Gata1s mouse model

We generated transgenic mice expressing human ERG3 hematopoietic isoform regulated by vav promoter that is expressed in all hematopoietic lineages starting at embryonic day 9.5 (E9.5). Four transgenic lines were generated with a similar phenotype. ERG expression was detected in yolk sacs (YS) from E10.5 and in fetal livers (FL) (Figure S1). The level of TgERG expression was comparable to the endogenous ERG expression levels in human cord blood CD34+ cells and in AMKL cell lines (Figure S1). To model the fetal hematopoietic events of DS, TgERG male mice were crossed with homozygous Gata1s knock-in females. This cross generates four genotypes (Figure 1): females are heterozygous and males are hemizygous for Gata1s while 50% of the animals express the hERG transgene.

In humans, constitutional trisomy 21 causes a marked expansion of fetal MEPs preceding occurrence of GATA1s mutation. We therefore examined if increased ERG expression affects fetal hematopoietic progenitors. Lin− cells isolated from E14.5 FLs were immunostained for c-Kit and Sca1 to distinguish between HSCs (Lin−,Sca1+,c-Kit+) and hematopoietic progenitor cells (HPCs) (Lin−,Sca1−,c-Kit+)33. HPCs but not HSCs were significantly expanded in FLs from TgERG embryos compared to Wt littermates (Figure 2A B). ERG over expression in FLs of E14.5 embryos caused a nearly two fold expansion of MEPs (FcRgamma−,CD34−)33 (Figure 2A,C) compared to Wt embryos and a smaller increase in CMPs (FcRgamma−,CD34+). MEPs expansion was on the expense of GMPs (FcRgamma+,CD34+) as a significant decrease in these progenitors was observed in TgERG embryos. Hence, increased expression of ERG during fetal hematopoiesis resulted in significant increase in MEPs, similar to the expansion observed in DS fetuses2, 3.
To study the consequences of ERG and Gata1s interactions on expansion and distribution of E14.5 FL progenitors, these immunostainings were repeated on Lin⁻ cells isolated from E14.5 FLs of Wt/Gata1s and ERG/Gata1s fetuses. Similar to the TgERG phenotype, ERG/Gata1s FL exhibited extensive expansion of MEPs (Figure 2D, E). The magnitude of the expansion was higher in ERG/Gata1s fetuses than those expressing ERG alone (compare the scales in Figure 2 B, C to D, E; p<0.05 for progenitors and p<0.06 for MEPs), demonstrating an additive effect of the two transcription factors.

Both ERG and GATA1s are implicated in megakaryopoiesis⁶,⁷,¹⁶,¹⁷,²¹. As TMD is a perinatal megakaryocytic proliferation syndrome we next examined their effect on fetal megakaryopoiesis. FL cells were isolated from TgERG and Wt littermates and from Wt/Gata1s and ERG/Gata1s mice and cultured in methylcellulose supplemented with thrombopoietin (TPO) for growth of megakaryocytic colonies (CFU-MK) (Figure 3A-D). Consistent with previous reports, we observed increased formation of CFU-MK colonies in E12.5 FL cells expressing Gata1s, as males (which are hemizygous for Gata1s) formed twice the number of colonies compare to females that express Gata1 Wt isoform (Figure 3B). At E12.5 the presence of the ERG transgene lead to a slight increase in MK colonies on a Gata1 wt background (Figure 3A) and caused no further enhancement of the effect of Gata1s (Figure 3B). This may result from the low level of ERG expression at E12.5 (Figure S1). In contrast, at E14.5 ERG significantly enhanced the number and size of Gata1s CFU-MK (Figure 3C, D). These observations indicate a synergistic effect of the two proteins in promoting fetal megakaryocytic proliferation.

The transient prenatal proliferation of megakaryoblasts in DS newborns represents a later stage of the progenitor expansion observed in DS FL²³,³⁴. Therefore, we hypothesized that expanded fetal progenitors in ERG/Gata1s mice may have similar biological properties to human DS-TMD. To test this hypothesis we profiled gene expression (Figure S2A) of FL
RNA derived from E12.5 and E14.5 Wt, TgERG, Gata1s and ERG/Gata1s pooled embryos. E14.5 FL cells from ERG/Gata1s mice demonstrated significantly increased expression of megekaryocytic genes (Supplemental Table 1) including Mpl gene and of Pecam1 in addition to Mycn, that were previously associated with DS-TMD\textsuperscript{35} (Figure 3E). Gene Set Enrichment Analysis (GSEA) demonstrated significant enrichment of ERG/Gata1s gene signature in DS-AMKL compared to non DS-AMKL\textsuperscript{14} (Figure S2B) and in TMD\textsuperscript{35} versus DS-AMKL (Figure 3F,G) which was higher than the enrichment observed with Gata1s alone (Figure S2C).

Hepatic fibrosis is a major complication of human DS-TMD\textsuperscript{36}. Accordingly liver fibrosis was demonstrated by Reticulin stain in ERG/Gata1s FL (Figure 3H). Postnatally, the transient feature of this fetal megakaryocytic progenitor expansion is evident by a transient increase in platelet counts in 3 week old TgERG and ERG/Gata1s mice that was reduced to normal levels by 7 weeks (Figure 3I). Together these phenotypic and genomic studies suggest that the co-expression of ERG and Gata1s cause a prenatal syndrome that is biologically similar to the TMD associated with DS.

**Constitutive ERG expression uncovers a vital role for the N-terminal domain of GATA1 in fetal erythropoiesis**

Genotyping offsprings born to TgERG males and Gata1s homozygous females (Figure 1A) revealed a dramatic decrease in ERG/Gata1s live males (7%, expected 25%, P<0.01 (Figure 1B). Gross examination of FL at E14.5 revealed that the ERG/Gata1s males suffer from severe anemia; ERG/GATA1s FLs were considerably smaller than littermate FLs (Figure 4A -
arrow) and exhibited a paler cellular suspension (Figure 4A). Genotyping confirmed early embryonic death of ERG/Gata1s males between E12.5 and E14.5 (Figure 4B).

We therefore studied early fetal erythropoiesis in the transgenic embryos. E12.5 and E14.5 FL cells were isolated from females and males of Wt, TgERG, Wt/Gata1s and ERG/Gata1s embryos and cultured in methylcellulose supplemented with EPO to promote erythroid colonies (BFU-E). In E12.5 Gata1s FLs, regardless of ERG expression, the number of erythroid colonies (BFU-E) was significantly lower in male FL cells, that are hemizygous for Gata1s (Figure 4D). The number of erythroid colonies (BFU-E) that were formed with E12.5 TgERG FLs was lower compared to Wt FL (Figure 4C). In E14.5, Gata1s and constitutive ERG expression synergized to inhibit BFU-E formation (Figure 4E); There was a significant drop in BFU-E colony number in FLs that express both Gata1s and ERG compared to the other genotypes.

To further study the effect of the two transcription factors on erythropoiesis, E12.5 FL cells were immunostained for expression of the erythroid marker Ter119 (Figure 4F,G). Gata1s expressing FLs had significantly reduced Ter119 expressing cells compare to their level in Wt FLs. The decrease in Ter119 cells was larger in hemizygous males (lacking a Wt Gata1 protein). While the presence of ERG with a Wt Gata1 background had no effect on Ter119 cells, it added to the effect of Gata1s, leading to an almost complete absence of erythroid cells at E12.5 ERG/Gata1s males with only 1.4% of Ter119 expressing cells compare to 2.8% in Wt/Gata1s males (p=0.0145) (Figure 4G). Furthermore, about a third of Ter119 cells in ERG/Gata1s male FL were apoptotic compared with 2% apoptosis found in the absence of the ERG transgene (p=0.017) (Figure 4H). These phenotypes were reflected in gene expression profiles of E12.5 FL cells displaying reduction in expression of erythroid genes.
(Supplemental tables 2,3), key erythroid transcription factors (Klf1, Ldb1), erythropoietin receptor (Epor) and the antiapoptotic gene Mcl1 (Figure 4I).

Together, these observations demonstrate that the lack of the amino-terminal of Gata1 coupled with the constitutive expression of ERG block fetal erythropoiesis resulting in severe anemia below the survival threshold for most ERG/Gata1s male embryos.

**ERG synergizes with GATA1s to attenuate terminal erythroid differentiation**

We next studied erythropoiesis in embryos surviving the initial block in erythropoiesis. Similar to E12.5 embryos, in E14.5 FL cells the generation of BFU colonies and the number of Ter119 positive cells were lower in Gata1s mice and further reduced by the expression of ERG (Figure 4E and Figure S3A). These observations were accompanied by decrease in the expression of erythroid genes (Figure S3B).

The combined expression of TER119 and CD71 can discriminate early and late differentiated erythroid cells (Figure 5A). The ratio between early (TER119+/CD71+) and late (TER119+/CD71-) erythroid cells was significantly higher in E14.5 ERG/Gata1s male FL cells (Figure 5B). Benzidine staining that measures hemoglobin carrying cells also showed that male ERG/Gata1s FL cells contain less differentiated erythrocytes as fewer than 10% of the cells were positive for benzidine staining compared to 20% observed in ERG/Gata1s FL female cells (p=0.0004) (Figure 5C,D). Accordingly, cytospins show that ERG/Gata1s male FL cells contain mainly immature pro-erythroblasts (Figure 5E). Furthermore, the expression of hemoglobin major beta chain, Hbb-b1 was reduced by ten-fold in the FLs of ERG and Gata1s expressing animals compared to Wt FLs (Figure 5F). The block in fetal erythroid differentiation was manifested by transient reduction in RBC and HGB levels in peripheral blood of 3 weeks old TgERG and ERG/Gata1s mice compared to Wt and Wt/Gata1s mice.
(Figure S4). Consistently, GSEA analysis using gene expression of human erythroid cells at different stages of differentiation\textsuperscript{37} shows enrichment of \textit{Wt/Gata1s and more} significantly more \textit{ERG/Gata1s E14.5 FL gene signature} in early erythroid cells (Figure 6A,B). Taken together, these results show that constitutive expression of ERG throughout erythroid differentiation and expression of Gata1s blocks terminal erythroid maturation.

**Altered expression of the early erythroid genes GATA2 and Myb in ERG/Gata1s FL cells**

Using the GSEA described above for \textit{ERG/Gata1s FL} (Figure 6A, right panel), we identified \textit{Gata2} among the top 20 core enrichment genes (Figure 6B). \textit{Gata2} expression levels, as measured by expression array and confirmed by real time PCR on separate embryos, was elevated in \textit{ERG/Gata1s E14.5 FLs} (Figure 6C). \textit{GATA2 expression} is directly activated by ERG and repressed by GATA1\textsuperscript{23, 38}. In G1ME cells\textsuperscript{29}, Gata2 expression was reduced by an average of 3 folds upon expression of Gata1 compare to Gata1s expressing G1ME cells (Figure 6D,E). In E14.5 Wt/Gata1s FL cells transduced with GATA1, GATA2 expression was reduced by an average of 1.7 folds compare to its expression in E14.5 FL cells that expressed only GATA1s (Figure 6F). Both in G1ME and in the FL cells GATA2 expression was significantly reduced when GATA1 was expressed, despite the much higher expression levels of GATA1s (Figure 6D,E,F). This implies that repression by GATA1s is less efficient than repression by GATA1 and that even small amount of GATA1 is sufficient to repress GATA2.

Another potential cause of the persistent high levels of Gata2 and the fetal anemia of \textit{ERG/Gata1s FL cells} is the repression of c-myb by ERG. c-myb suppresses GATA2 during erythroid differentiation\textsuperscript{37}. Low levels of c-myb cause anemia and enhance megakaryopoiesis\textsuperscript{39, 40}. ERG binds to c-myb promoter\textsuperscript{24} and represses c-myb expression in
transduced primary fetal liver hematopoietic cells regardless of GATA1 expression\textsuperscript{17}. Consistent with this data we observed a 4 fold reduction in c-myb expression in TgERG FL cells (Figure 6C). Together these results suggest that persistent expression of Gata2 and suppressed expression of c-myb contribute to the block in erythroid differentiation in ERG/Gata1s fetal liver.

**Discussion**

We present here a detailed analysis of the developmental interactions between ERG and Gata1s during fetal hematopoiesis. The collaboration between these two mutated genes results in marked suppression of the generation of fetal erythroid cells, their differentiation and their survival, coupled with expansion of megaerythroid and megakaryocytic progenitors similar to those observed in DS-TMD.

DS-TMD is characterized by the transient expansion of fetal megakaryoblasts that continues in the first three months of life. While often asymptomatic some of the patients develop liver failure caused by liver fibrosis secondary to the infiltration of pathological fetal megakaryoblats. Up to 30\% of the DS-neonates recovering from the TMD develop AMKL before the age of 4yrs. The syndrome we observed in ERG/Gata1s transgenic mice is similar in the phenotype and gene expression of the expanded fetal cells, in its transient nature, in its association with liver fibrosis and in the progression to mega-erythroid progenitor leukemia (Figure S5). Yet it differs in the timing of the TMD which is *prenatal* in the mice and *perinatal* in humans.

The current model for DS-TMD assumes two cooperating processes\textsuperscript{13}. First trisomy 21 causes expansion of fetal MEPs. Then GATA1s, created by a somatic mutation in \textit{GATA1},
blocks their terminal megakaryocytic differentiation and enhances their proliferation leading to
the perinatal TMD. Here we show that increased expression of a single chromosome 21
gene, ERG, can cooperate with Gata1s to create a TMD like fetal hematopoietic state in a
mouse model.

ERG was recently demonstrated to be required for maintenance of the fetal liver HSCs\textsuperscript{23}. We
show here for the first time that constitutive hematopoietic expression of human ERG causes
expansion of FL MEP in a mouse model with no other genetic aberrations related to DS.

Although Gata1s enhanced early fetal megakaryopoiesis, as previously reported\textsuperscript{11}, it did not
affect the percentage of MEPs. These results support previous observations in human fetal
livers\textsuperscript{2, 3, 34}, that the expansion of fetal MEPs is caused by trisomy 21 preceding the "second
hit" of the mutations in GATA1.

 Constitutive transgenic expression of hERG, to a similar level observed in normal human
hematopoietic precursors (Figure S1) cooperated with Gata1s to further expand the same
fetal megakaryocytic precursor observed in human TMD as demonstrated by gene
expression profiling. However, in humans it is likely that the same effect is achieved by
cooperation of several Hsa21 genes, such as ERG, ETS2\textsuperscript{17, 41}, DYRK1A\textsuperscript{18} or miR-125-b2\textsuperscript{12}.
Yet consistent with our results, Ng et al reported that the addition of one copy of Erg was
both necessary and sufficient for the postnatal myeloproliferation in a mouse model of DS.

The gene expression profile of Gata1s/ERG fetal cells was similar to both human TMD
and DS AMKL, consistent with the development of DS leukemias from the same cells
causing TMD. Specifically, we saw increased expression of Mycn in FLs of the double
transgene ERG/Gata1s (Figure 3E). Mycn is highly expressed in DS-TMD\textsuperscript{35} and was
suggested to participates in the elimination of Gata1 mediated cell cycle arrest via Cdkn2c\textsuperscript{42}.
Therefore, elevated Mycn levels may contribute to the hyperproliferation phenotype by
repressing Cdkn2, similar to what have been previously shown for cMyc\textsuperscript{42}. Indeed, we see about a twofold decrease in Cdkn2c expression in Gata1\textsubscript{s} mice compared to animals expressing Wt Gata1, with the highest repression observed in ERG/Gata1\textsubscript{s} male FLs (Figure S6).

GATA1 transcription factor plays a key role in differentiation and maturation of erythroid cells by regulating numerous erythroid genes\textsuperscript{43-45}. However, the role of the N-terminal "transactivation" domain in erythropoiesis is unclear. The initial report of the Gata1\textsubscript{s} "knock in" mouse, used in our study, reported reduced fetal E12.5 erythropoiesis without any detailed analysis\textsuperscript{11}. We show here that the expression of ERG dramatically enhances the fetal erythropoiesis defect caused by the elimination of the N-terminus of Gata1. Embryos that express both ERG and Gata1\textsubscript{s} suffer from severe anemia as demonstrated by low cellularity in their FLs (Figure 4A) and almost complete absence of erythroid cells (Figure 4G), leading to death of ERG/Gata1\textsubscript{s} males at embryonic day E13.5 (Figure 4B). Our detailed analysis reveals a block in all stages of erythropoiesis; Decreased number and survival of erythroid progenitors coupled with arrest in erythroid differentiation. The erythroid phenomenon is not observed in children with DS since the GATA mutation in DS is somatic, occurring only in the TMD cells and not in the germline as in Gata1\textsubscript{s} mouse.

ERG expression is normally decreased with erythroid differentiation\textsuperscript{6} (Figure S7). However, in this transgenic mouse model, ERG constitutive expression is controlled by the hematopoietic vav promoter. We demonstrate here that the differential regulation of ERG expression along erythroid differentiation is critical, and that lack of its normal down regulation causes a block in final erythroid maturation (Figure 7).
Consisting with these findings, we observed significant enrichment in early erythroid genes in E14.5 ERG/Gata1s FLs (Figure 6A, right panel). Gata2 which was among the top core enrichment genes (Figure 6B), is a key regulator of early erythropoiesis, and promotes the survival and proliferation of immature cells. Gata2 downregulation is required for erythroid differentiation. In K562 cells over-expressing Gata2 a reduced growth rate and inhibition of erythroid differentiation was observed. More significantly, primary adult erythroid progenitor cells that highly expressed Gata2 showed reduced growth, decrease in the number and proportion of mature erythroid cells and reduced hemoglobin A and F levels in a dose dependent manner with increasing amounts of GATA2 expression, suggesting that the blockage in erythroid maturation is due to increased expression of GATA2.

The regulation of erythroid gene expression and erythroid differentiation is governed by the interplay between GATA1 and GATA2, that share a common DNA binding motif. GATA2 directly activates GATA1 expression in early erythroid progenitors, and positively regulates its own expression, whereas GATA1 accelerates its own expression and represses GATA2 allowing occupancy of regulated erythroid genes by GATA1 during erythroid maturation (Figure 7). The absence of the transactivation domain in Gata1s alleviates the repression of Gata2 by Gata1; Gata2 levels were higher in Gata1s FL cells compared to those that express wt Gata1 (Figure 6C). Moreover, induction of GATA1 but not GATA1s in G1ME cells and in GATA1s FLs result in GATA2 repression (Figure 6D,E,F). These results indicate a specific role for the N-terminal domain of Gata1 in Gata2 transcriptional regulation and thus in regulating the transcriptional network in early erythropoiesis.

As shown by chromatin immunoprecipitation studies, Gata2 is a direct target of ERG, that positively regulates its expression in fetal mega- erythroid progenitors. The constitutive
expression of vav driven transgenic hERG throughout erythropoiesis may further explain the consistent high levels of Gata2.

The upregulation of Gata2 in ERG/Gata1s transgenic FL cells may also be explained by the repression of c-myb in these mice. c-Myb is expressed in early erythroid differentiated cells and is downregulated with differentiation by Gata1 in association with the transcription co-repressor FOG148. It was proposed that c-Myb may also function as a Gata2 repressor, since Gata2 expression levels were increased by 5 fold in c-MybKD/KD E14.5 FL cells, inducing an immature erythroid phenotype39. Furthermore ERG is a direct repressor of c-myb as previously shown by ChiP-Seq and gene expression studies17, 24. Thus we suggest that persistent expression of Gata2 coupled with the repression of c-myb play a central role in the fetal erythropoiesis defects observed in ERG/Gata1s embryos.

In summary we present here an ERG/Gata1s double transgenic mouse model of TMD. We show that moderate increased expression of ERG was sufficient to generate expansion of MEP compartment in FLs and that expression of Gata1s together with ERG enhances this expansion. We also demonstrated the requirement of ERG down regulation and of the N-terminal domain of Gata1 for embryonic and fetal erythropoiesis. The relevance of these findings to human diseases is highlighted by a previous case report of anemia in humans with GATA1s germline mutation49 and the recent discovery of GATA1s mutations in some patients with the congenital Diamond-Blackfan anemia50.
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Author Contribution
YB designed and performed experiments, analyzed data and wrote the paper, LG performed experiments analyzed data and helped write the paper, TMC, BG and GS performed experiments, IM provided research assistance, JJH performed array experiment and analysis, GR provided guidance, JDC assisted in manuscript preparation, SI designed the study, analyzed data and wrote the paper.

There is no conflict of interest.
References


Figures legends

**Figure 1: Generation of ERG/Gata1s mice.** (A) TgERG males were mated with Gata1s knock-in homozygous females to generate animals that express the hERG transgene and the short isoform of GATA1, GATA1s. As GATA1 is located on the X chromosome, male offspring from this cross are all hemizygous and females are all heterozygous for GATA1s. Half of the animals carry the ERG transgene. (B) A table representing the observed versus the expected born mice from the different genotypes. Statistical significance was tested using Fisher’s Exact Test ($p<0.01$ for ERG/Gata1s males and $p=0.09$ for ERG/Gata1s females. n(total) =137) and chi square test ($p=0.0047$).

**Figure 2: Expansion of megakaryocyte / erythroid progenitors (MEPs) in TgERG embryo fetal livers (FL) is enhanced by Gata1s.** (A) Representative flow cytometry analysis showing immunostaining of lineage negative (Lin-) cells. Hematopoietic Stem Cells (HSC) are c-Kit + and Sca1+ and hematopoietic progenitors (HPCs) are c-Kit+ and Sca1-. HPCs were subclassified into Granulocyte/Monocyte Progenitors (GMPs, FcgammaR+ and CD34+), Common Myeloid Progenitors (CMPs, FcgammaR- and CD34+ ) and Megakaryocyte/Erythrocyte Progenitors (MEPs, FcgammaR- and CD34- ) respectively . B,D. Relative progenitor and stem cell populations in wild type and TgERG (B), and in wt/Gata1s and ERG/Gata1s (D) E14.5 FLs. C,E. Distribution of myeloid progenitors in wild type and TgERG (C), and in Wt/Gata1s and ERG/Gata1s (E). The bar graphs represent the average of at least three independent experiments with n>10 embryos for each genotype. Statistical significant differences (t-test for pairs and ANOVA for groups) are detailed in the figure.

**Figure 3: ERG synergizes with Gata1s to promote transient megakaryocytic proliferation presenting a human TMD expression profile.** (A,B,C) FL cells were isolated from E12.5 (A,B) and E14.5 (C) wild type, TgERG embryos and males and females of
Wt/Gata1s and ERG/Gata1s embryos. The cells were plated on methylcellulose supplemented with TPO to promote the growth of megakaryocytic colonies (CFU-MK). MK colonies were counted 5-7 days following plating. Each bar graph represents the average of at least 3 independent experiments. (D) Representative figure of megakaryocytic colonies from TgERG (Top), Wt/Gata1s (middle) and ERG/Gata1s (Bottom) FLs. (E) Real time PCR of common human TMD genes (11). *- Statistical significance was tested using t test p=0.028 for Mpl p=0.009 for Pecam1 and p=0.0 for Mycn. (F) Gene Set Enrichment Analysis (GSEA) using gene expression of E14.5 ERG/Gata1s FL cells (GSE46481) compared to the respective Wt control shows significant enrichment of genes that were up regulated in human TMD (GSE4119). NES (normalized enrichment score) and FDR (false detection rate) q values are shown. (G) Heat map showing the core enrichment genes from the GSEA presented in F. (H) Hepatic fibrosis in ERG/Gata1s male FL. Reticulin staining (arrow heads) of Wt/Gata1s (left) and ERG/Gata1s (right) fetal liver tissues (magnification x400). (I) Transient thrombocytosis in TgERG and ERG/Gata1s mice. Platelet counts were retrieved from males and females TgERG and ERG/Gata1s and their Wt and Wt/Gata1s littermates at 3 and 7 weeks of age. n= at least 6 for each group. A significant difference was found between TgERG and Wt and between ERG/Gata1s and Wt/Gata1s (t-test p<0.017, p<0.0031, respectively).

**Figure 4: Requirement of the amino terminal of GATA1 in fetal erythropoiesis. (A)**  
Representative pictures of E14.5 embryos. The FL of ERG/Gata1s males is smaller and paler (arrow). 1x10^7 FL cells were re-suspended in PBS. The light color of the ERG/Gata1s FL cells point to the decrease number in mature erythrocytes in those embryos. (B) ERG/Gata1s males die between embryonic day 12.5 and 14.5. Females and males generated from crossing heterozygous TgERG males with homozygous Gata1s KI females were tested for
the presence of ERG transgene on embryonic day 12.5, 14.5 and adults. (n=85, 98 and 137 for E12.5, E14.5 and adults respectively). A significant difference in TgERG male population was found between E12.5 and E14.5, t test p<0.014. (C-E) ERG and Gata1s inhibit erythroid colony formation. FL cells were isolated from E12.5 TgERG embryos and Wt littermates (C) and from E12.5 and E14.5 embryos of ERG/Gata1s embryos and Wt/Gata1s littermates (D, E) and were plated on methylcellulose supplemented with EPO to promote growth of erythroid colonies (BFU-E). Erythroid colonies (BFU-E) were counted 7-10 days following plating. Each bar graph represents the average of at least 3 independent experiments. Statistical significance was tested using t test. (F) Representative figures of flow cytometry analysis of male E12.5 FL cells using the erythroid markers Ter119 and CD71. (G) Expression of Gata1s impairs erythropoiesis. Expression of Ter119 erythroid marker as measured by flow cytometry analysis in FL cells generated from E12.5 females and males of Wt, TgERG, Gata1s and ERG/Gata1s animals. (H) Increase apoptosis in ERG/Gata1s males. Annexin V levels were measured by flow cytometry in Ter119 positive FL cells for the detection of apoptosis. FL cells were isolated from E12.5 Wt, TgERG, Wt/Gata1s and ERG/Gata1s males. Significant increase in apoptosis in ERG/Gata1s males compared to Wt/Gata1s males was measured using t test p=0.017. (I) Decreased expression of erythroid and anti-apoptotic genes in ERG/Gata1s males. Expression level of the different genes was obtained from the Affymetrix mouse gene 1.0 ST chip array and confirmed by real time PCR on at least two additional samples for each genotype.

**Figure 5: Erythroid differentiation arrest in ERG/GATA1s mice.** (A) Schematic representation of erythroid differentiation indicating early erythrocytes as Ter119+/CD71+ and late differentiated erythrocytes as Ter119+/CD71-. (B) Increase in early erythroblast population compared to late differentiated erythrocytes in ERG/Gata1s males. FL cells were isolated from E14.5 Wild type, TgERG, Wt/Gata1s and ERG/Gata1s male embryos and
stained with Ter119 and CD71. Statistical significance between Wt embryos and the remaining genotypes was measured using t-test. (C,D) Reduced benzidine stained cells in ERG/Gata1s males. Representative images of benzidine stained FL cells (C) and a graph averaging the ratio of benzidine positive cells in FLs isolated from E14.5 Wt, TgERG, Wt/Gata1s and ERG/Gata1s male and female embryos (D). The bar graph represents the average of at least 3 experiments. (E) Giemsa stained of cytospines of E14.5 FL cells show a decrease in maturing erythroblasts and increase in immature proerythroblasts in ERG/Gata1s males compare to Wt/Gata1s males. (F) Expression of hemoglobin. Adult hemoglobin beta major chain (Hbb-b1) expression was measured by real time PCR in E14.5 FL cells from Wt, TgERG, Wt/Gata1s and ERG/Gata1s males. (*) Significant difference from Wt hemoglobin expression was measured using t-test p=0.00037 for TgERG p<0.000001 for Wt/Gata1s and p<0.00001 for ERG/Gata1s.

**Figure 6:** **ERG/Gata1s FL cells present an early erythroid expression profile.** (A) Gene Set Enrichment Analysis using gene expression of human erythroid cells at different stages of differentiation\(^\text{37}\) shows enrichment of Wt/Gata1s and ERG/GATA1s E14.5 FL gene signature (GSE46481) in early erythroid cells. (B) Heat map showing the top 20 core enrichment genes from the GSEA presented in A, right panel. (C) Expression level of Gata2 and Myb genes as obtained from the mouse gene 1.0 ST chip array and confirmed by real time PCR on at least two additional samples for each genotype (D) Representative western blot from G1ME cells transduced with MigR1, MIGR1-GATA1 and MIGR1-GATA1s using the indicated antibodies. (E) Average difference in GATA2 expression from three independent G1ME expreriments presented in D. (F) An average repression of GATA2 mRNA by real time PCR in E14.5 GATA1s KI FL cells infected with MIGR1-GATA1 and MIGR1-GATA1s relative to MigR1 infected cells. Relative GATA1 and GATA1s RNA expression levels are indicated below (n=3).
Figure 7: Schematic representation of the main events leading to impaired fetal erythropoiesis. (Top panel) Gata2 which is expressed in erythroid progenitors, positively regulates Gata1 and its own expression (27). Gata1 also positively regulates its own expression but represses Gata2. Progression in erythroid differentiation becomes possible due to Gata1/Gata2 switch on the promoter of erythroid genes resulting in their activation. Gata2 repression is an essential step in erythroid differentiation and is executed by several factors including Gata1 and the decline in ERG expression. (Bottom panel) In the ERG/Gata1s mice several factors block erythroid differentiation. In the absence of the N-terminal domain of Gata1 Gata1s fails to repress Gata2, erythroid genes are not activated and differentiation is blocked. Furthermore, The Gata2 repressor Myb is down regulated by ERG and Gata1s. Since Myb function as a Gata2 repressor, decreased Myb levels result in an increase in Gata2 expression. In the ERG transgenic model ERG expression level fails to decline with erythroid differentiation, thus further maintaining Gata2 expression and preventing erythroid cells to fully differentiate to erythrocytes.
Fig 1

A.

![Genetic diagram showing the inheritance of Tg ERG and Gata1s traits.]

B.

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n=137
Fig 2

A. Wild Type vs. Tg ERG

B. Relative distribution of progenitors and HSC

C. Relative distribution of GMP, CMP, MEP

D. Relative distribution of progenitors and HSC

E. Relative distribution of GMP, CMP, MEP

Legend:
- Wild Type
- ERG Tg
- WT/GATA1 female
- ERG/GATA1 female
- WT/GATA1 male
- ERG/GATA1 male

Statistical significance:
- p<0.02
- p<0.002
- p<0.0003
- p<0.07
- p<0.4
- p<0.53
Fig 3

A. E12.5

B. E13.5

C. E14.5

D. 

E. 

F. 

G. 

H. 

I. 

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A. [Images of samples]

B. [Graph showing ERG female and ERG male]

C. [Bar graph showing Wt/E = 0.02, Wild Type, ERG Tg]

D. [Bar graph showing ERG/Ga t 1s female and ERG/Ga t 1s male]

E. [Bar graph showing ERG/Ga t 1s female and ERG/Ga t 1s male]

F. [Scatter plots showing Wt/E and ERG male]

G. [Bar graph showing % TER119 expressing cells and annexin positive cells]

H. [Bar graph showing expression level of KL F, L B D, E P O R, M c l -1]

I. [Bar graph showing expression level of M L P 1, L D D 1, E P O R, M c l -1]
Erythroid differentiation

A. CD71+ /TER119+ TER119+/CD71+

B. early/late erythrocytes ratio

C. Wt/Gata1s female ERG/Gata1s female

Wt/Gata1s male ERG/Gata1s male

D. % benzidine positive cells

E. Wt/Gata1s ERG/Gata1s

F. Relative Hbb-b1 expression level

**Fig 5**

P = 0.05

P = 0.0004

P = ***
A. Enrichment plot: Up in Gata1s vs wt grp

B. Enrichment plot: most upreg E11.5 ERGGATA1s vs WT grp

C. Relative GATA2 repression

D. Gata1s/Gata1 fold difference

E. GATA2 expression level

F. Relative GATA1/ GATA1s expression

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Wt/Gata1

ERG

GATA2

GATA1

AD

Erythroid genes

KLF1
LDB1
EPO

ERG levels

GATA2

GATA1

Erythroid differentiation

ERG/Gata1s

Elevated ERG in double Tg

GATA2

GATA1s

Consistent ERG expression throughout erythroid differentiation

Erythroid genes

KLF1
LDB1
EPO
Myb

GATA1s

GATA2

Fig 7
Perturbation of fetal hematopoiesis in a mouse model of Down syndrome's transient myeloproliferative disorder

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