hGATA1 Under the Control of a μLCR/β-Globin Promoter Rescues the Erythroid but Not the Megakaryocytic Phenotype Induced by the Gata1low Mutation in Mice

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The phenotype of mice carrying the Gata1low mutation that decreases expression of Gata1 in erythroid cells and megakaryocytes, includes anemia, thrombocytopenia, hematopoietic failure in bone marrow and development of extramedullary hematopoiesis in spleen. With age, these mice develop myelofibrosis, a disease sustained by alterations in stem/progenitor cells and megakaryocytes. This study analyzed the capacity of hGATA1 driven by a μLCR/β-globin promoter to rescue the phenotype induced by the Gata1low mutation in mice. Double hGATA1/Gata1low/0 mice were viable at birth with hematocrits greater than those of their Gata1low/0 littermates but platelet counts remained lower than normal. hGATA1 mRNA was expressed by progenitor and erythroid cells from double mutant mice but not by megakaryocytes analyzed in parallel. The erythroid cells from hGATA1/Gata1low/0 mice expressed greater levels of GATA1 protein and of α- and β-globin mRNA than cells from Gata1low/0 littermates and a reduced number of them was in apoptosis. By contrast, hGATA1/Gata1low/0 megakaryocytes expressed barely detectable levels of GATA1 and their expression of acetylcholinesterase, Von Willebrand factor and platelet factor 4 as well as their morphology remained altered. In comparison with Gata1+/0 littermates, Gata1low/0 mice contained significantly lower total and progenitor cell numbers in bone marrow while the number of these cells in spleen was greater than normal. The presence of hGATA1 greatly increased the total cell number in the bone marrow of Gata1low/0 mice and, although did not affect the total cell number of the spleen which remained greater than normal, it reduced the frequency of progenitor cells in this organ. The ability of hGATA1 to rescue the hematopoietic functions of the bone marrow of the double mutants was confirmed by the observation that these mice survive well splenectomy and did not develop myelofibrosis with age. These results indicate that hGATA1 under the control of μLCR/β-globin promoter is expressed in adult progenitors.
INTRODUCTION

GATA1 is a member of the GATA family of transcription factors that ensures appropriate differentiation in hematopoietic cells of multiple lineages including erythroid (Tsai et al., 1989), megakaryocytic (Martin et al., 1990; Romeo et al., 1990), eosinophil (Yu et al., 2002a) and mast cells (Migliaccio et al., 2003). The lineages most stringently controlled by GATA1 are the erythroid and megakaryocytic ones (Orkin and Zon, 2008). In fact, genetically engineered alterations in mice and spontaneous mutations in humans are often associated with X-linked inherited disorders with erythroid and/or megakaryocytic phenotypes (Crispino and Weiss, 2004; Crispino and Horwitz, 2017). In mice, loss of GATA1 blocks the maturation of erythroid cells at the pro-erythroblast stage inducing the cells into apoptosis (Fujiiwara et al., 2002) while selective loss of GATA1 expression in megakaryocytes (MK) arrests their terminal maturation and retains the cells in proliferation (Shviddasani et al., 1997; Vyas et al., 1999). Therefore, GATA1 deficient mice die of severe anemia between day 10.5 and 11.5 of gestation with sign of intraembryonic hemorrhage (McDevitt et al., 1997). In human, both inherited and acquired GATA1 mutations have been described. Point mutations suppressing the binding of the amino-terminal zinc-finger domain of the protein with either the DNA or the nuclear protein FOG1 are found in inherited disorders with erythroid or megakaryocyte deficiency (Nichols et al., 2000; Freson et al., 2001; Mehaffey et al., 2001; Yu et al., 2002b; Di Pierro et al., 2015; Iolascon et al., 2020). The good agreement between the phenotype of the patients and that of mice carrying the corresponding mutations (Campbell et al., 2013; Russo et al., 2017), indicates that these mutations are indeed responsible for sustaining the clinical traits. Based on extensive information indicating that in mice the ratio between the GATA1 and GATA2 content (Gata2/Gata1 switch) controls the proliferation of murine stem/progenitor cells (Bresnick et al., 2010), it is not surprising that acquired frame shift and point mutations impairing GATA1 functions induce leukemia in humans. Mutations resulting in the expression of a shorter amino-truncated protein (GATA1s) are associated with human Acute Megakaryocytic Leukaemia in Down syndrome (DS-AMKL) (Wechsler et al., 2002), transient myeloproliferative syndromes in newborns (Ahmed et al., 2004) and, in rare cases, with adult megakaryocytic leukemia (Harigae et al., 2004). In addition, ribosomopathies affecting the efficiency of translation of GATA1 mRNA are associated, in addition to Diamond Blackfan Anemia (Ludwig et al., 2014), with primary myelofibrosis, the most severe of the Philadelphia-negative myeloproliferative disorders (Vannucchi et al., 2005; Gilles et al., 2017).

The GATA family genes encode proteins very similar in structure and extremely conserved across species (Trainor et al., 1990). The specificity of their functions is therefore not dictated by the protein structure but rather by their accurate spatial-temporal expression during development and cytogenesis ensured by specific regulatory sequences (Ferreira et al., 2007). As an example, in mice loss of Gata1 function is rescued by gain of function of either Gata2 or Gata3, providing that their expression is driven by the regulatory sequence of Gata1 (Ferreira et al., 2005). The regulatory sequences of Gata1 include three hypersensitive sites which represent putative enhancers (Trainor et al., 1990; Ferreira et al., 2007). Their deletions are defined hypomorphic mutations because they reduce expression and protein content of Gata1 in a lineage-specific fashion. The most studied of these deletions are the Gata1β5 developed by the Yamamoto laboratory, that induce a transmissible erythroid leukemia in mice (Shimizu and Yamamoto, 2016), and the Gata1low mutation developed by the Orkin laboratory, that induce a lethal anemia and thrombocytopenia at birth in the C57BL6 strain (McDevitt et al., 1997; Vyas et al., 1999) and thrombocytopenia with myelofibrosis in CD1 mice (Vannucchi et al., 2002; Martelli et al., 2005). The phenotype induced by the Gata1low mutation in the CD1 background is particularly intriguing. Indeed, these mice are viable at birth because their anemia is rescued within 1 month by recruiting the spleen as extramedullary hematopoietic site (Migliaccio et al., 2009). In this strain, bone marrow hematopoiesis is ineffective as demonstrated by low hematopoietic stem cell content and by the fact that, when splenectomised, these mice die of profound anemia within 15 days (Migliaccio et al., 2009; Spangrude et al., 2016).

However, the mice remain thrombocytopenic in spite of the high number of megakaryocytes present in their bone marrow and spleen, and with age develop a syndrome similar to human primary myelofibrosis characterized by fibrosis and hematopoietic failure in bone marrow, and development of hematopoiesis in extramedullary sites (Vannucchi et al., 2002). To define the function of human GATA1 (hgGATA1), two transgenic mice lines have been created carrying either the entire hgGATA1 gene or its cDNA under the control of a μLCR coupled with the promoter of the β-globin gene (Peterson et al., 1993; Li et al., 2002). The line carrying the genomic hgGATA1 dies between 10.5 and 12.5 days of gestation and, in spite of high expression of the transgene in erythroid cells, is anemic. By contrast, the line containing the μLCR/β-globin promoter coupled with hgGATA1 cDNA (hereinafter referred to as hGATA1) is viable at birth (Li et al., 2002). This observation allowed the generation of hGATA1/β-YAC doubly hemizygous transgenic mice that were instrumental to demonstrate that hGATA1 is a specific repressor of the human ε gene in vivo but is dispensable for the expression of the human γ and β globin genes (Peterson et al., 1993). However, this demonstration was confounded by the fact that in these mice the hGATA1 cDNA was expressed at high levels in embryonic and fetal hematopoietic tissues but not in adult and erythroid cells but not in megakaryocytes rescuing the erythroid but not the megakaryocyte defect induced by the Gata1low/0 mutation.

Keywords: GATA1, μLCR, erythroid cells, megakaryocytes, hematopoietic progenitor cells
tissues (Li et al., 2002). The fact that a following study was capable to detect expression of a LacZ reporter driven by the μLCR/β-globin promoter in erythroid cells from adult transgenic mice (Papayannopoulou et al., 2000), raises the possibility that high levels of GATA1 protein, as a consequence of expression of both the hGATA1 transgene and the endogenous gene, may induce erythroid cells into apoptosis, providing a negative pressure which selects against cells expressing hGATA1. This possibility suggested us the hypothesis that the spectrum of cells in which hGATA1 is expressed is best identified in a genetic contest in which the expression of the endogenous gene is reduced. To test this hypothesis, we analyzed whether hGATA1 is expressed in hematopoietic cells from adult mice hypomorphic at the Gata1 locus, which contain low levels of the endogenous protein in the progenitor, erythroid and megakaryocyte cell compartments. The results obtained indicate that in the context of low GATA1 content, hGATA1 is expressed in adult erythroid progenitor and precursor cells at levels sufficient to rescue their defective phenotype. By contrast, hGATA1 was not expressed in cells of the closely related megakaryocyte lineage of these mutants, which remained abnormal, providing evidence that even in the context of reduced expression of the endogenous gene, the μLCR/β-globin promoter is erythroid specific.

### MATERIALS AND METHODS

#### Generation of Double hGATA1/Gata1low/0 Mutants

Transgenic hGATA1 (hGATA1/Gata1+/0) males generated as previously described (Li et al., 2002) were crossed with either CD1 (wild type at the Gata1 locus) or Gata1low/low females and their progeny genotyped by polymerase chain reaction (PCR) at birth, as previously described (Li et al., 2002; Vannucchi et al., 2002) (Table 1). Since Gata1 is on the X chromosome while μLCR/hGATA1 is autosomal, the offspring of these matings are all heterozygous for hGATA1 and heterozygous for Gata1low when females or hemizygous Gata1low/0 (wild type) when males. All the experiments were conducted on Gata1low/0 and Gata1low males containing or not the hGATA1 transgene at 3–4 months of age, unless otherwise indicated. Mice were housed under good animal care practice conditions in the animal facilities of Istituto Superiore Sanità and the experiments were performed according to the protocol n. 419/2015-PR approved by the Italian Ministry of Health and according to the directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes.

#### Splenectomy

The spleen was removed from mice anaesthetized with xylazine (10 mg/kg; Bayer) and ketamine (200 mg/kg; Gellini Pharmaceutics) by double ligation of the splenic artery and vein, as described in the animal protocol n. 419/2015-PR. See reference (Migliaccio et al., 2009) for further details.

#### Hematological Parameters

Mice were topically anesthetized with lidocaine (one drop/eye) and then blood was collected from the retro-orbital plexus into ethylen-diamino-tetracetic acid-coated microcapillary tubes (20–40 μL/sampling). Hematocrit (Hct) and platelet counts (Ptl) were determined manually.

#### Optical, Immunofluorescence and Electron Microscopy Observations

Histological (Hematoxylin and eosin, and Gomori) and Transmission Electron Microscopy (TEM) observations were performed as previously described (Vannucchi et al., 2002; Centurione et al., 2004). For immunofluorescence analysis, spleens were fixed in 10% (v/v) phosphate-buffered formalin, paraffin embedded and cut into 2.5–3 μM sections. Sections were incubated first with an anti-GATA1 monoclonal antibody (M-20, sc-1234, Santa Cruz Biotechnology, Santa Cruz, CA) and then with a fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG antibody (Santa Cruz Biotechnology). After several washes, slides were mounted in glycerol-DABCO containing 4-6-diamidine-2-phenylindole (DAPI, 5 μg/ml) (Sigma-Aldrich, Saint Louis, MO) to counterstain the nuclei. Cells not incubated with the primary antibody served as negative controls. In selected experiments, GATA1 content was evaluated by immune-histochemistry with the same GATA1 antibody followed by peroxidase staining with

### Table 1

Summary of the offsprings of the mating of CD1 or Gata1low/low mutants with the hGATA1 transgenic mice. hGATA1/Gata1low/0 males were crossed with either CD1 (Gata1+/0) or Gata1low/0 females and their progeny genotyped. All the experiments described in the paper were carried out on Gata1low/0 and Gata1+/0 males, containing or not the hGATA1 transgene, highlighted in pink.

| Litters | Offsprings | Genotype of the OFFSPRINGS (%) |
|---------|------------|---------------------------------|
|         |            | Gata1+/0 | hGATA1/Gata1low/0 | Gata1low/0 | hGATA1/Gata1low/0 | Gata1+/0 | hGATA1/Gata1low/0 |
| 9CD1 X hGATA1/Gata1+/0 | 10 | 66 | 23 | 23 | 31 | 23 |
| 9Gata1low/low X hGATA1/Gata1+/0 | 20 | 99 | 23 | 38 | 20 | 20 |
the DAB CHROMOGEN kit (Bio Optica, Milan, Italy) followed by Hematoxylin staining. Consecutive sections, were instead processed for the terminal deoxy transferase uridine triphosphate nick end-labelling (TUNEL) reaction with the "In Situ Cell Death Detection Kit" (Boehringer Mannheim, Mannheim, Germany), as described by the manufacturer. At the end, slides were counter-stained with propidium iodide (Sigma-Aldrich). Samples were analyzed with a light microscope (Leica) equipped with a Coolsnap video camera for computerized images (RS Photometrics, Tucson, AZ, United States).

**Flow Cytometry and Cell Sorting**

Mononuclear cells obtained from liver and marrow and light density (p< 1.080) spleen cells separated over standard Ficoll gradient (Sigma-Aldrich) were suspended in Ca++ Mg++-free phosphate buffered saline supplemented with 1% (v/v) bovine serum albumin, 2 mM EDTA, 0.1% NaN3 and incubated for 30 min on ice with 1 µg/10⁶ cells of phycoerythrin (PE)-conjugated CD117 (anti-cKit), CD71 and CD61, and fluorescein isothiocyanate (FITC)-conjugated anti-CD34, TER119 and CD41 (all from PharMingen, San Diego, CA). Apoptotic cells were detected by double staining with FITC-Annexin V (PharMingen) and propidium iodide (5 µg/ml). In selected experiments, erythroid cells (TER119⁺/CD71⁺), megakaryocytes (CD41⁺/CD61⁺), hematopoietic stem cell (LSK, lineage negative/CD117⁺/Sca1⁺) and erythroid-megakaryocyte (MEP, CD117⁺/CD34⁶⁻⁸) restricted progenitor cells were enumerated and eventually prospectively isolated as previously described (Akashi et al., 2000; Ghinassi et al., 2007). The sorted populations were 80–90% pure, upon re-analyses. Cell analysis and sorting were performed using the FACSArray cell sorter (Becton Dickinson, Franklin Lakes, NJ) with three lasers (488-nm argon laser, 599-nm dye laser and ultraviolet laser). Cells labeled with fluorophore-conjugated isotype antibodies (PharMingen) were used to gate non-specific fluorescence signals, while dead cells were excluded on the basis of propidium iodide (5 µg/ml, Sigma) staining.

**Liquid Cultures Under Conditions of Limiting Dilution of Prospectively Isolated Progenitor Cells**

MEP cells were cultured under conditions of limiting dilution (3 cells/mL) in 96-well plates containing Iscove modified Dulbecco medium (IMDM; Gibco, Invitrogen, Carlsbad, CA) supplemented with fetal calf serum (FCS, 10% v/v; Sigma-Aldrich), 7.5 × 10⁻⁵ M β-mercaptoethanol (Sigma-Aldrich), penicillin, and streptomycin sulfate (50 U/mL, Gibco), and glutamine (2 mM, Gibco). Cultures were stimulated with a cocktail of growth factors contained rat SCF (100 ng/ml; Amgen, Thousand Oaks, CA), murine IL-3 (10 ng/ml, PeproTech, London, United Kingdom) and GM-CSF (10 ng/ml; PeproTech), human FLT3-ligand (10 ng/ml; PeproTech), IL-11 (10 ng/ml; PeproTech) in combination with either thrombopoietin (TPO, 50 ng/ml; PeproTech) (megakaryocyte-permissive conditions) or erythropoietin (EPO, 3 U/mL; Hoffman-La Roche, Basel, Switzerland) (erythroid-permissive conditions), as previously described (Ghinassi et al., 2007). The cultures were incubated at 37°C in a humidified incubator containing 5% CO₂ in air. Cell growth and phenotype (erythroid: CD71⁺/TER119⁺, megakaryocyte: CD117⁺/CD34⁻⁸⁻, and mast cells: CD117⁺/FeR⁺ by flow cytometry) were analyzed 7 days after the beginning of the culture, as described (Ghinassi et al., 2007).

**Ribo Nucleic Acid Isolation and Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis**

Total RNA was prepared using a commercial guanidine thiocyanate/phenol method (TRIzol, Gibco BRL, Paisley, UK) as described by the manufacturer. Glycogen (20 µg, Roche Ltd., Basel, CH) was added to each sample as a carrier. cDNA was synthesized from 1 µg of total RNA using random primers and SuperScript III (Invitrogen, Life Technologies, Bethesda, MD). Quantitative PCR was carried out as recommended by the manufacture with TaqMan PCR kits specific for the human or the murine Gata1 gene, or additional erythroid (Hba-a1 and Hbb-b1) or megakaryocytic (Ache, vWF and Pf4) specific genes (Applied Biosystems, Foster City, CA), with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). For each gene (X) quantitative values were obtained from the threshold cycle number (Cₓ), and expressed as 2⁻ΔCₓ, were ΔCₓ is the difference between the average Cₓ of the target X gene from that of the housekeeping gene (Gapdh).

**Statistical Analysis**

Statistical analysis was performed by one-way analysis of variance Dunnett’s Multiple Comparison and one-way analysis of variance Tukey Multiple Comparison Test, as indicated. The analyses were performed with GraphPad Prism 5 software for Windows (GraphPad Software, San Diego, California United States).

**RESULTS**

**Mendelian Inheritance of the hGATA1 Transgene**

Males wild type at the Gata1 locus (Gata1⁺/⁺) carrying the hGATA1 transgene (hGATA1/Gata1⁺/⁺) were crossed with either CD1 (wild-type at the Gata1 locus) or Gata1low/low females. For each crossing, 10 and 20 separate matings, respectively, were performed. These crossing generated a total of 66 and 99 pups with an average number of pups per mating of 6.6 and 4.9 for CD1 and Gata1low/low mating, respectively (Table 1). The genotype of the offspring was distributed according to the expected Mendelian ratios. The slight prevalences of wild-type females not carrying hGATA1 and of hGATA1/Gata1low/low males are not statistically significant. The Mendelian inheritance of the transgene was associated with a normal phenotype of the embryos (Figure 1). In fact, at both E11.5 and E13.5, Gata1low embryos were reported to be pale with
clear evidence of anemia (McDevitt et al., 1997). Consistently with this observation, by E11.5 Gata1low/0 embryos mice were pale, an indication of insufficient red blood cell production (Figure 1A) while E11.5 hGATA1/Gata1low/0 embryos had a normal morphology, which included a red appearance of the fetal liver (Figure 1B).

The hGATA1 Transgene Rescues the Red Cell Counts of the Blood From Adult Gata1low Mice

The hematocrit (Hct) and platelet counts of the Gata1+/+, Gata1low/0, hGATA1/Gata1+/+ and hGATA1/Gata1low/0 mice are compared in Figure 2A. As expected (McDevitt et al., 1997; Vannucchi et al., 2001), Gata1low/0 males expressed levels of Hct and platelet counts significantly lower than normal. The presence of the hGATA1 transgene restored the Hct of the Gata1low/0 mutants up to normal values but had no effects on platelet counts that remained low. This platelet deficiency was confirmed by morphological evaluation that showed reduced number of platelets with size greater than normal (megathrombocytes) in blood smears not only as expected in Gata1low/0 mice (McDevitt et al., 1997) but also in their hGATA1/Gata1low/0 littermates (Figures 2B,C).

These results indicate that the presence of hGATA1 rescues the anemia but not the thrombocytopenia induced by the Gata1low mutation.

hGATA1 is Expressed in Adult Hematopoietic Progenitor and Precursor Cells.

By quantitative RT-PCR, we compared the levels of hGATA1 and of the endogenous murine Gata1 (mGata1) gene expressed by adult hematopoietic progenitor and precursors cells prospectively isolated from the bone marrow of adult Gata1+/+, Gata1low/0 and hGATA1/Gata1low/0 mice (Figure 3). Human erythroblasts (CD235pos/CD36pos cells), expanded in vitro as previously described (Federici et al., 2019), were analyzed in parallel as positive control for hGATA1 expression. We measured only the total level of Gata1 mRNA because in humans the ribosomopathy induced by the driver mutations found in myelofibrosis equally reduces the megakaryocyte content of the full-length and short isoform of the GATA1 protein (Vannucchi et al., 2005; Gilles et al., 2017).

As expected (Ling et al., 2018), CMP (by ~25-fold), MEP (by ~2.5-fold), erythroid precursors (by ~20-fold) and megakaryocytes (by ~10-fold) from Gata1low/0 mice expressed significantly lower levels of mGata1 mRNA than the corresponding cells from their Gata1+/0 littermates. The presence of hGATA1 did not affect expression of the endogenous gene in these populations purified from hGATA1/Gata1low/0 mice, which remained lower than normal (Figure 3).

Unsurprisingly, hGATA1 was not detected in cells prospectively isolated from Gata1+/+ or Gata1low/0 littermates but it was expressed already at the level of progenitor cells (both CMP and MEP) prospectively isolated from hGATA1/Gata1low/0 mice. In these mice, expression of hGATA1 persisted at the level of the erythroid precursors while it was barely detectable in megakaryocytes (Figure 3).

These results indicate that in the Gata1low/0 genetic contest, the expression of hGATA1 driven by the μLCR coupled with the β-globin gene promoter is restricted to progenitor and erythroid cells and that its expression does not affect the levels of the endogenous gene which remain lower than normal.

hGATA1 Rescues the Defective Phenotype of Erythroid Cells but not That of Megakaryocytes from Gata1low/0 Mice.

In accordance to the Gata1low levels detected by RT-PCR (Figure 3), by immunofluorescence, erythroid cells in the spleen from Gata1low/0 mice contain low levels of GATA1 protein (Figure 4A). By contrast, the erythroid cells in the spleen from mice containing the hGATA1 transgene were more intensively green fluorescent indicating a higher GATA1 content. The GATA1 protein is synthesized in the cytoplasm and translocate in the nucleus as part of the NuRSEY complex formed by HADC5, GATA1, EKLF and ERK in response to factors that phosphorylate ERK (Varricchio et al., 2014). It is not surprising, then, that the GATA1 fluorescent signal was detected both in the cytoplasm and in the nucleus of the cells (see also Supplementary

![Figure 1](https://example.com/figure1.png) | Fetuses from GATA1low/0 mice carrying the hGATA1 transgene are not anemic. Representative photograph of 11.5 days fetuses from the same litter. The genotype of the fetuses is indicated on the top. Compared to age-matched Gata1low/0 mice (A), the double mutant hGATA1/Gata1low/0 mice (B) are not anemic. Results are representative of those observed with 5 fetuses per genotype.
Of note, the fluorescence intensity is particularly evident in the nuclei of the hGATA1/Gata1low/0 mutants. The antibody used to detect GATA1 does not discriminate between the murine and the human protein. Since the levels of the endogenous mGata1 expressed by erythroid cells from Gata1low/0 and hGATA1/Gata1low/0 mice is the same (Figure 3), we believe that the greater levels of GATA1 protein detected in the erythroid cells from the double mutants is the result of hGATA1 expression.

Despite extramedullary hematopoiesis in the spleen corrects erythropoiesis of Gata1low0 mice (Migliaccio et al., 2009), these animals remain anemic due to the increased apoptotic rates of their erythroid cells, detected by TUNEL staining of this organ (Vannucchi et al., 2001). In order to assess whether the greater GATA1 content of the erythroid cells from the double mutants rescued their increased apoptosis rates, TUNEL staining was performed. As expected, TUNEL staining was limited in the spleen from wild type mice but greatly increased in the erythroblasts from the spleen of Gata1low0 mutants (Figure 4B). Since the localization of the TUNEL staining is expected to be perinuclear (Vannucchi et al., 2001 and Supplementary Figure S1B), it is not surprising that at the magnification presented in Figure 4B the Tunnel signal is observed in the cytoplasm.

By contrast, the presence of the hGATA1 transgene drastically reduced the frequency of TUNEL-positive cells present in spleen sections from the double mutant mice (Figure 3B). These results were confirmed at the bone marrow level by flow cytometry determinations showing that erythroid cells from double mutant mice, by contrast with those from their Gata1low0 counterpart, are barely stained by Annexin V. In fact, the frequency of Annexinpos Ter119pos cells in the bone marrow was 25.58 ± 13.8 (n = 4) in Gata1low0 mice vs. 4.17 ± 5.22 in hGATA1/Gata1low0 mice (n = 6) (p = 0.0078 by ANOVA) while the frequency of Annexinpos Ter119pos cells in the bone marrow from two Gata1+/0 mice analysed in parallel was 0.5 and 0.7.

In agreement with the observation that hGATA1 mRNA was similarly undetectable in megakaryocytes from Gata1low0 and hGATA1/Gata1low0 littermates (Figure 3), immunofluorescence determinations indicated that the GATA1 protein was barely detectable in the megakaryocytes from the spleen of these
To confirm that hGATA1 rescues erythropoiesis in the bone marrow of Gata1low/0 mice, the expression of specific erythroid and megakaryocytic genes was assessed in erythroid cells and megakaryocytes prospectively isolated from the bone marrow of Gata1+/0, Gata1low/0 and hGATA1/Gata1low/0 mice (Ghinassi et al., 2007). Compared to wild type mice, erythroid cells from Gata1low/0 mice expressed significantly lower levels of α- and β-globins, that were restored up to normal levels in cells from the double mutant (Figure 3C). By contrast, in megakaryocytes the presence of hGATA1 transgene did not affect the expression of acetylcholinesterase, Von Willebrand factor, and platelet factor 4 in hGATA1/Gata1low/0 mice which remained similar to that detected in cells from Gata1low/0 mice and abnormally greater than that of cells from Gata1+/0 littermates (Figure 3D).

In conclusion, the observation that erythroid cells but not the megakaryocytes from the double mutant mice contain levels of...
GATA1 protein similar to normal provides a mechanistic insight to explain why the presence of hGATA1 rescues the abnormal maturation of erythroid cells but not that of megakaryocytes induced by the Gata1low mutation.

The hGATA1 Transgene Induces Extramedullary Hematopoiesis in Both Spleen and Liver

In Gata1low mice hematopoiesis fails in the bone marrow and is active in the spleen which contains most of the hematopoietic stem cells of these animals (Spangrude et al., 2016). To assess the effect of the hGATA1 transgene on the active site of hematopoiesis, total cell number and frequency of hematopoietic stem/progenitor and precursors cells in bone marrow, spleen and liver from Gata1+/0, hGATA1/Gata1+/0, Gata1low/0 and hGATA1/Gata1low/0 mice were compared (Figure 5). Hematopoietic stem/progenitor cells are defined as Lin−/CD117pos/Sca1pos cells (LSK) and divided by CD34 staining into bipotent erythro-megakaryocytic (MEP, CD34neg) and common myeloid/bipotent granulo-monocytic (CMP/GMP, CD34pos) progenitor cells (Akashi et al., 2000; Ghinassi et al., 2007).
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Bone marrow. As previously reported (Vannucchi et al., 2002), the bone marrow from Gata1low/0 mice contained statistically significant lower numbers of cells than that from wild type mice (Figure 5). Significantly lower than normal were also the frequencies of GMP/CMP while the slightly lower frequency of MEP was not statistically significant. At the precursor level, the
frequency of erythroid cells was within normal values while that of megakaryocytes, as expected (Vannucchi et al., 2002), was greater than normal. The presence of hGATA1 had a small but significant impact on the cellularity of the femur from Gata1low/0 animals. However, it significantly decreased the frequency of erythroid precursors in this organ (a sign of increased maturation) and had no effect on the frequency of progenitor cells and megakaryocytes that remained, respectively, lower and higher than normal (Figure 5).

Spleen. As reported (Vannucchi et al., 2002), the total cell number of the spleen from Gata1low/0 mice was about 0.4-fold greater than normal (Figure 5). However, due to great variability in this organ size among individual mice, there was insufficient power to demonstrate statistically significant differences with the low number (three) of mice analyzed per group. The spleen from these mice contained greater frequencies of progenitor and precursor cells than that of hemizygote Gata1+/0 mice in which these cell populations were barely detectable. The presence of hGATA1 dramatically increased the frequency of LSK and progenitor cells and that of erythroid precursors in the spleen from Gata1+/0 mice. However, although the frequency of these cell populations in the spleen from hGATA1/Gata1+/0 mice remained greater than that of wild type mice, it was significantly lower when compared to that of hGATA1/Gata1+/0 mice (Figure 5).

Liver. Although the total number of cells in the liver of the various mice groups was not recorded, the liver from transgenic mice containing hGATA1 appeared pale, an indication of reduced vascularization, and with a gummy consistency, an indication of site in these cell populations in the spleen from mice containing various mice groups was not recorded, the liver from transgenic very low, containing the greatest number of progenitor cells, the frequency (Gata1low/0). In a previous publication, we demonstrated that the mutation increases the proliferation potential of MEP which in vitro generate mast cells in culture. MEP making them unable to generate mast cells in culture.

**hGATA1 Rescues the Altered Differentiation Potential Expressed in vitro by Erythroid-Megakaryocyte Carrying the Gata1low Mutation**

In a previous publication, we had shown that Gata1low mice die within 15 days from removal of the spleen, an indication that in these mice erythropoiesis fails in the bone marrow and is sustained mainly by the spleen (Migliaccio et al., 2009). To
assess whether the greater levels of GATA1 content in the erythroid cells from Gata1low/0 mice carrying the hGATA1 transgene rescue the erythropoietic functions of the marrow of these animals, we compared survival rates of untreated hGATA1/Gata1low/0 littermates with those subjected to splenectomy. To take into account that the functions of the bone marrow from...
**Gata1low/0** mice are particularly reduced after 8-months of age when the mutant develop myelofibrosis (Vannucchi et al., 2002), experiments were repeated with 8- and 14-months old mice. Splenectomized **Gata1low/0** mice were analyzed as positive controls. As expected (Migliaccio et al., 2009), all **Gata1low/0** mice die within 15–20 days from splenectomy (Figure 7A), confirming the fundamental role of this organ in sustaining their erythropoiesis. By contrast, splenectomy did not affect survival and Hct values of **hGATA1/Gata1low/0** mice (Figures 7A,B). In addition, no significant differences were detected in the cellularity and progenitor/precursor cell frequency in the bone marrow from **hGATA1/Gata1low/0** splenectomized mice in comparison to that of untreated littermates (Figure 7C).

Collectively, these data provide functional support for the conclusion that **hGATA1** rescues the erythroid failure of the bone marrow of **Gata1low/0** mice.

Although **hGATA1** Does not Rescue the Abnormal Maturation of **Gata1low** Megakaryocytes, it Reduces the Bone Marrow Fibrosis Developed by the **Gata1low** Animal Model

Several lines of evidence in patients (Vannucchi et al., 2005; Gilles et al., 2017) and in mouse models, including the **Gata1low** model (Vannucchi et al., 2002; Malara et al., 2018) suggest that myelofibrosis, a disease associated with hematopoietic failure in the bone marrow and development of hematopoiesis in extramedullary sites, is sustained by proinflammatory cytokines secreted at high levels by immature megakaryocytes, a cellular hallmark of this disorder. However, both in patients (Vainchenker et al., 2019) and in **Gata1low** mice (Ghinassi et al., 2007), the disease is also associated with increased proliferation of the stem/progenitor cells sustained by the driver mutations. The
observation that the presence of hGATA1 rescued erythropoiesis in the bone marrow (Figure 5) and normalized the proliferation potential of the MEP (Figure 6) but did not restore the maturation of the megakaryocytes (Figures 2, 3) suggested to us to assess the relative importance of these two abnormalities for the development of the disease by analyzing whether hGATA1/Gata1low/0 mice develop fibrosis in the bone marrow.

In a first set of experiments we confirmed that hGATA1 does not rescues the megakaryocyte defects induced by the mutation by comparing the frequency of the clusters of megakaryocytes and the ultrastructural morphology of these cells in the spleen and bone marrow from Gata1+/0, Gata1low/0 and hGATA1/Gata1low/0 mice (Figure 8 and data not shown). For reasons linked to technical challenges in performing TEM studies on femurs, ultrastructural studies were performed on spleen only. Previous publications (Vyas et al., 1999; Centurione et al., 2004) had shown that the Gata1low mutation blocks megakaryocytic maturation between stage I and II, resulting in accumulation of
megakaryocytes arranged in clusters and with a cytoplasm partitioned by a rudimental demarcation membrane system and containing mostly granules of light electron-density instead of the heavy electron-density granules that characterize the cytoplasm of normal megakaryocytes. Electron and light microscopy observations indicated that the hGATA1 transgene did not rescue the abnormal morphology of the megakaryocytes from the spleen and marrow of Gata1low mice. In fact, morphological analysis of spleen from Gata1+/0, Gata1low/0 and hGATA1/Gata1low/0 adult mice showed that, in contrast with wild type mice, in both groups megakaryocytes were organized in clusters of small cells with poorly developed platelet territories partitioned by a rudimental demarcation membrane system and light-electron dense granules (Figure 8A). Histological observations of the femur of these mice groups confirmed the presence of megakaryocytes clusters also in the medulla from both hGATA1/Gata1low/0 and Gata1low/0 mice (Figure 8B) while these clusters are absent from the medulla of femurs from wild type animals ([Vannucchi et al., 2002] and data not shown). The conclusion that the megakaryocytes from hGATA1/Gata1low/0 and Gata1low/0 mice are similarly immature is supported also by the observation that the trabecular and the compact bone in both animal groups is thicker than normal (Figure 8B). Increased osteogenesis, in fact, is one of the traits expressed by Gata1low mice that has been more strongly mechanistically linked with the megakaryocyte abnormalities induced by the mutation (Kacena et al., 2004).

Collectively, in agreement with the observation that hGATA1 under the control of μLCR coupled with the β-globin gene promoter is not expressed in megakaryocytes, the presence of this transgene did not rescue the megakaryocyte abnormalities induced by the Gata1low mutation.

To a surprise, however, in spite megakaryocytes of hGATA1/ Gata1low/0 remained immature, the double mutants did not develop fibrosis in bone marrow, providing further indication that the transgene rescues the myelofibrotic trait induced by the Gata1low mutation (Figure 8B).

**DISCUSSION**

The hypomorphic Gata1low mutation impairs expression of the Gata1 gene already at the levels of hematopoietic progenitor cells and continue to exert its effects in erythroid cells and megakaryocytes (Ling et al., 2018). The consequent reduced content of GATA1 protein results in the following cell specific abnormalities: it increases proliferation of MEP altering their differentiation potential making the cells able to generate mast cells under erythroid-specific conditions (Ghinassi et al., 2007), it increases the apoptotic rates of the erythroblasts (McDevitt et al., 1997; Vannucchi et al., 2001) and blocks megakaryocyte maturation retaining these cells in a proliferative state (Vyas et al., 1999; Vannucchi et al., 2002). As a consequence, the mice are born anemic and thrombocytopenic (McDevitt et al., 1997; Vyas et al., 1999). With age, they recover from their anemia by recruiting the spleen as extramedullary site (Migliaccio et al., 2009; Spangrude et al., 2016) but retain the abnormalities at the level of MEP (Ghinassi et al., 2007) and megakaryocytes (Vyas et al., 1999; Vannucchi et al., 2002) for all their life developing myelofibrosis with age (Vannucchi et al., 2002). In fact, overall, the phenotype of Gata1low mice is similar to that of patients with primary myelofibrosis, the most severe of the Philadelphia-negative myeloproliferative neoplasms which is associated with abnormalities in MEP proliferation and megakaryocyte maturation similar to those observed in these mutants (Zahr et al., 2016).

In this manuscript, we used a standard trans-complementation gene approach with the hGATA1 transgene to address two questions: 1) is expression of hGATA1 erythroid restricted? 2) Since hGATA1 is not expected to be expressed by megakaryocytes, do the double mutants develop myelofibrosis, a disease driven by abnormalities sustained by reduced Gata1 expression in these cells?

To address the first question, we investigated whether the hGATA1 gene under the control of a μLCR coupled with the promoter of the β-globin gene is expressed in hematopoietic cells from adult mice carrying the Gata1low mutation, identified the cells in which the transgene is expressed and analyzed whether the levels of expression sustained by these regulatory sequences are sufficient to rescue the phenotypic abnormalities induced by the mutation. Our results confirmed that embryos carrying the Gata1low/0 mutation are anemic and thrombocytopenic while adult mutants express significantly lower levels of Hct and platelet counts compared to the wild type littermates. We confirmed that in Gata1low/0 mice, the levels of Gata1 transcripts are reduced already in progenitor cells (CMP/GMP and MEP) and not only in erythroid and megakaryocyte precursors. We also confirmed that reduced levels of Gata1 mRNA resulted in lower GATA1 protein content in erythroid cells, which displayed greater apoptotic levels, and in megakaryocytes, that remained morphologically immature. The hGATA1 under the control of μLCR/β-globin regulatory sequences was already expressed at the level of progenitor cells (both CMP/GMP and MEP) and of erythroid cells prospectively isolated from hGATA1/Gata1low/0 littermates. These data are consistent with the observation that the μLCR/β-globin regulatory sequences are already active at the levels of the stem/progenitor cells, which express low but clearly detectable levels of β-globin mRNA, and in erythroid cells but not in cells of other hematopoietic lineages such as megakaryocytes (Beru et al., 1990; Ashihara et al., 1997; McKinney-Freeman et al., 2012). The pattern of cell specificity of the expression of hGATA1 was correlated with the pattern of the traits induced by the Gata1low mutation that were rescued by the presence of the transgene. On one hand, the differentiation potential of MEP prospectively isolated from the double mutants became restricted to cells of erythroid and megakaryocytic lineage having lost their ability to generate mast cells. In addition, erythroid cells expressed nearly normal levels of the erythroid specific genes analyzed and displayed low apoptotic rates. On the other hand, megakaryocytes remain detected in great numbers, an indication of increased proliferation, express altered levels of the megakaryocyte specific genes analyzed and displayed an immature morphology. Consistently with the rescue of the
MEP alterations, erythropoiesis was effective in the bone marrow of the double mutant mice making their extramedullary hematopoiesis in spleen dispensable for survival. Consistently with the rescue of the defective erythroid maturation, the animals were not anemic and, as predicted by the failure of the transgene to rescue the megakaryocyte defects, the mice remained thrombocytopenic and with increased osteogenesis, a trait driven by the abnormal megakaryocytes (Kacena et al., 2004).

The observation that the expression of hGATA1 driven by the μLCRβ-globin regulatory sequences is restricted to the erythroid cells has important implications for gene therapy. In fact, suitable gene therapy approaches require robust evidence not only for the safety of the retroviral vector used, but also for the specificity of its expression, essential to minimize its off-target effects (Demirci et al., 2018; High and Roncarolo, 2019). Based on the strong evidence for erythroid specificity displayed by the μLCRβ-globin regulatory sequences in transgenic mouse models (Li and Stamatoyannopoulos, 1994; Papayannopoulou et al., 2000; Li et al., 2002), these sequences are used to drive the expression of the human β-globin in most of the retroviral vectors currently under investigation of gene therapy of hemoglobinopathies (Wang et al., 2019; Morgan et al., 2020). Our results confirm that these regulatory sequences are indeed erythroid specific (and therefore likely to be safe) because, with the exception of some expression of the transgene at the level of progenitor cells, in which even under normal circumstances these regulatory sequences are active (McKinney-Freeman et al., 2012), they drove expression of the transgene mainly in erythroid cells and were ineffective in the closely related megakaryocytes.

Erythroid commitment and differentiation is sequentially driven by two genes of the GATA transcription factors family, GATA1 and GATA2. GATA2 is expressed at high levels in multipotent progenitors, affecting their expansion and guiding the early stage of erythroid commitment (Tsai et al., 1994). As erythroid commitment progresses, GATA2 activates the expression of GATA1 which is abundantly expressed in late progenitor cells and erythroblasts (Leonard et al., 1993). The switch from GATA2 to GATA1 expression in the control of erythropoiesis is known as the GATA switch and represents the clock which initiates the translation of the erythroid specific genes (Katsumura and Bresnick, 2017; Bresnick et al., 2020). Once activated by GATA2, the expression of Gata1 is self-sustained. In mice, expression of Gata1 is regulated by three DNase hypersensitive sites (HS), two of which (HSI and HSII) lay within 8 Kb upstream of the coding sequences and the third one (HSIII) within the first intron (Kobayashi and Yamamoto, 2007). The Gata1low mutation specifically deletes HSI. The fact that erythroid cells from Gata1low mice express some level of the endogenous gene reflects the positive effects on its translation exerted by GATA2 likely by binding to HSI and/or HSIII. The observation that in the double mutant mice increases in the content of the GATA1 protein due to the contribution of hGATA1 does not increase the expression of the endogenous gene confirms that HSI is an indispensable element of the self-sustained regulatory loop of Gata1 transcription the expression of which remains at the levels driven by GATA2.

To address the second question, we investigated whether hGATA1/Gata1low mice develop myelofibrosis with age. To a surprise, in spite the megakaryocytes of the double mutants remained GATA1 hypomorphic, these mice did not develop hematopoietic failure and fibrosis in their bone marrow, i.e., the two distinctive traits for myelofibrosis. This observation is counterrintuitive given the strong evidence that both in patients and in causative mutation-driven animal models, myelofibrosis is sustained by abnormal GATA1 hypomorph megakaryocytes (Vannucchi et al., 2005; Gilles et al., 2017). In addition, megakaryocyte-restricted expression of the driver mutations is necessary and sufficient to induce myelofibrosis even if the hematopoietic stem cells are normal (Jeremy Wen et al., 2015; Zhan et al., 2016). This apparent contradiction has been recently clarified by new exciting single cell profiling data indicating that the megakaryocytes in the bone marrow are a mixture of three populations, each one exerting a different function (Psaila et al., 2020; Yeung et al., 2020; Migliacci and Hoffman, 2021; Sun et al., 2021; Wang et al., 2021). In fact, in addition to megakaryocytes poised to form platelets, the hematopoietic stem cells also generate megakaryocytes poised to exert immune-functions in the lungs (Pariser et al., 2021) or niche-functions locally, by secreting collagen and other extracellular matrix proteins (Malara et al., 2014). This single cell profiling also indicated that while maturation of platelet-poised and immune-poised megakaryocytes requires upregulation of GATA1 expression, niche-poised megakaryocytes are dependent on low levels of GATA1 (Wang et al., 2021). Data from the Balduini laboratory indicate that megakaryocytes expressing collagen, which are presumably the niche-poised cells, are very rare in the bone marrow from healthy individuals at opposite with myelofibrosis patients where a great proportion of megakaryocytes in the bone marrow express collagen (Abbonante et al., 2016), suggesting that niche poised-megakaryocytes may contribute to deposition of fibrosis in the bone marrow. Overall, these recent results suggest the hypothesis that hypomorphic GATA1 content at the levels of megakaryocytes precursors switches their fate from platelet-poised to niche-poised cells, potentially contributing to fibrosis. Our data are consistent with this refinement of the pathobiological role of megakaryocyte abnormalities in myelofibrosis. In fact, the rescue of the myelofibrotic phenotype of the Gata1low mice is associated with normalization of the abnormal differentiation potential of the stem/progenitor cells (which express hGATA1 and became able to differentiate in the bone marrow) and although expression of hGATA1 is below detection in megakaryocytes prospectively isolated from the bone marrow, it is expressed by megakaryocytes differentiated in culture from the double mutants, the large majority of which has been suggested to represent either niche- or immune-poised MKs (Malara et al., 2014). It is then possible that expression of hGATA1 at the level of the multi-potent megakaryocyte precursors prevented their switch to niche-poised cells, thereby rescuing the myelofibrosis trait. Unfortunately, different populations of megakaryocyte precursors poised to generate megakaryocytes and immune-poised or niche-poised megakaryocytes have all the same morphology (Wang et al., 2021; Migliacci and Hoffman, 2021), thus they cannot be discriminated by morphological observations. This novel pathobiological model may only be tested when cell surface markers to prospectively identify the various cell populations will became available.
Last but not least, our data have implications to design strategies for the cure of primary myelofibrosis, the most severe of the Philadelphia-negative myeloproliferative neoplasms which has presently no cure (Zahr et al., 2016). In fact, drugs targeting the driver mutations displayed by the malignant hematopoietic stem clones, such as the JAK1/2 inhibitor ruxolitinib, are greatly effective in ameliorating the clinical manifestation of the disease but it is still unclear whether they are also effective in halting the progression toward its final stage (Mascarenhas and Hoffman, 2013; Tefferi, 2021). As of today, with exception of bone marrow transplantation which may be offered to a limited number of patients (Tamari et al., 2015), this disease is still an unmet clinical need. Based on the observation that bone marrow transplantation cures the disease by rescuing both the hematopoietic stem cell and the microenvironmental abnormalities displayed by the patient, the consensus has been reached that treatment of the disease requires the use of drug combinations targeting both abnormalities (Eran et al., 2019; Tefferi, 2021). Our data support this consensus and indicate that these combination therapies should not combine drugs targeting the driver mutations (such as ruxolitinib) with those that target the platelet-poised megakaryocytes but rather with those that target the niche-poised cells.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the protocol n. 419/2015-PR approved by the Italian Ministry of Health and according to the directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes.

AUTHOR CONTRIBUTIONS

FM, PV, MZ, MM, and BC performed experiments and analyzed the data. FM, AMV, AL, and GS revised the data and wrote the manuscript. ARM designed the study, interpreted the data and wrote the manuscript. All the authors read the manuscript and concur with its content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.720552/full#supplementary-material

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