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GATA-2 Plays Two Functionally Distinct Roles during the Ontogeny of Hematopoietic Stem Cells

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Abstract

GATA-2 is an essential transcription factor in the hematopoietic system that is expressed in hematopoietic stem cells (HSCs) and progenitors. Complete deficiency of GATA-2 in the mouse leads to severe anemia and embryonic lethality. The role of GATA-2 and dosage effects of this transcription factor in HSC development within the embryo and adult are largely unexplored. Here we examined the effects of GATA-2 gene dosage on the generation and expansion of HSCs in several hematopoietic sites throughout mouse development. We show that a haploid dose of GATA-2 severely reduces production and expansion of HSCs specifically in the aorta-gonad-mesonephros region (which autonomously generates the first HSCs), whereas quantitative reduction of HSCs is minimal or unchanged in yolk sac, fetal liver, and adult bone marrow. However, HSCs in all these ontogenically distinct anatomical sites are qualitatively defective in serial or competitive transplantation assays. Also, cytotoxic drug-induced regeneration studies show a clear GATA-2 dose–related proliferation defect in adult bone marrow. Thus, GATA-2 plays at least two functionally distinct roles during ontogeny of HSCs: the production and expansion of HSCs in the aorta-gonad-mesonephros and the proliferation of HSCs in the adult bone marrow.

Key words: GATA-2 • hematopoietic stem cells • AGM • haploinsufficiency • gene dosage

Introduction

Hematopoietic stem cells (HSCs) at the foundation of the adult hematopoietic differentiation hierarchy have the ability to self-renew and produce all the distinct blood cell lineages (1, 2). HSCs capable of complete long-term hematopoietic repopulation of irradiated adult recipients are first generated in the aorta-gonads-mesonephros (AGM) region at mid-embryonic day (E)10.5 (3, 4) and localize to the dorsal aorta, vitelline, and umbilical arteries (5). Subsequently, at mid/late E11, HSC activity is also found in the yolk sac (YS) and fetal liver (FL). HSC activity increases significantly in E11 AGM and E12 YS when they are cultured as whole tissue explants for 3 d (3, 6). Although the spatial and temporal appearance of HSCs during development has been described and quantitated, the molecular mechanisms underlying HSC generation, expansion, and maintenance are not well explored.

One molecule important during hematopoietic ontogeny is GATA-2, a member of the GATA family of zinc finger transcription factors (7, 8). RT-PCR analysis shows high expression of GATA-2 in adult hematopoietic progenitor cells and HSCs (9, 10). Furthermore, immunohistochemistry, in situ hybridization, and transgenic analyses show GATA-2 expression as early as E8 in the para-aortic splanchnopleura (precursor tissue to AGM) and subsequently in the AGM (11, 12). In the E11.5 AGM, GATA-2 is expressed in the aortic endothelium and neighboring mesenchymal cells, which are both considered putative hemogenic cell populations. Embryos lacking GATA-2 are anemic, have moderately reduced numbers of primitive erythroid cells and he-
matopoietic progenitor cells (13), and die at E10.5, the time of HSC induction and expansion. Due to this lethal-
ity, the role of GATA-2 has been examined primarily by in
vitro colony-forming assays of cells from early embryonic
tissues and hematopoietic cultures of GATA-2−/− embryo-
monic stem (ES) cells. In both cases, hematopoietic progeni-
tor numbers are severely reduced. Further studies in chi-
meric mice produced with GATA-2−/− ES cells show no
contribution of the mutant cells to any hematopoietic tis-
ues. Together, these data indicate that GATA-2 is crucial
for the maintenance, proliferation, and/or survival of im-
mature hematopoietic progenitors (13).

The function of GATA-2 had also been studied through
enforced overexpression achieved by retroviral transduc-
tion or transfection of genes encoding either a wild-type
GATA-2 or an inducible GATA-2–estrogen receptor (ER)
fusion protein (12, 14–19). Irrespective of these attempts, a
simple conclusion on the function of GATA-2 in the
HSCs or progenitor cells is difficult. On one hand, expres-
sion of GATA-2 in murine BM cells blocked
progenitor-derived colony formation (14). The opposing
result may be due to the cell types chosen for these exper-
iments. However, it is also suggested that the biochemical
behavior of the artificially generated GATA-2–ER fusion
protein may not be the same as the wild-type unmodified
GATA-2 protein (18). Thus, the most relevant data on
GATA-2 dose effects on hematopoiesis may be best ob-
tained within the physiological context of the whole or-
ganism wherein GATA-2 is expressed under the endoge-
nous regulatory machinery in the appropriate cell types.

The study of transcription factor dose and function at the
earliest stages of hematopoietic development is of particular
interest for an understanding of HSC generation. Studies on
the runx1 transcription factor have shown that a haploid
dose results in changes in HSC induction, expansion, and
distribution in the midgestation mouse embryo (20). More-
ever, haploinsufficiency of human Runx1 (AML-1) results
in thrombocytopenia and a propensity to develop myeloid
leukemia (21). Recently, a correlation between a reduction
in GATA-2 expression and aplastic anemia (22) has been
demonstrated. Hence, to further understand the role of
GATA-2 in the ontogeny of HSCs we examined the effects
of GATA-2 haploinsufficiency on induction and expansion of
HSCs during development by in vivo hematopoietic transplanta-
tion assays and phenotypic analysis of compound
transgenic embryos (GATA-2−/−-Ly-6A GFP) (23).

Here we present data showing that the numbers of he-
atopoietic progenitors in GATA-2−/− embryos are re-
duced. More importantly, we observe a dramatic quantita-
tive reduction in HSC activity specifically in GATA-2−/−
AGMs and a further reduction in the serial repopulating
ability of these HSCs. In contrast, GATA-2−/− HSC num-
bers appear quantitatively normal in the adult BM but are
qualitatively defective in the setting of competitive trans-
plantation. In addition, GATA-2−/− HSCs exhibit a delay
in regeneration of the hematopoietic system after cytox-
ous drug challenge, suggesting that GATA-2 levels play a role
in HSC proliferation. Thus, GATA-2 plays functionally
distinct roles in the production of HSCs in the AGM re-
region and the proliferation of HSCs throughout ontogeny.

Materials and Methods

GATA-2 Mutant Mice and Embryos. GATA-2 mutant mice
(13) were backcrossed onto the C57BL/6 background for over
10 generations and were housed in the Erasmus Medical Center
Animal unit according to the institution guidelines with food and
water provided ad libitum. The day of vaginal plug discovery
from overnight matings (GATA-2−/− male × C57BL/6 GATA-
2−/− or GATA-2−/+ female) was counted as day 0. Pregnant
dams were killed and embryos isolated from the uterus as de-
scribed previously (24). Embryos (E10–E11) were staged by
counting somite pairs (25). Genotyping was performed by
PCR as described previously (13). Compound transgenic
embryos were obtained by mating Ly-6A GFP hemizygous (23) and
GATA-2−/− mice.

Dissection, Explant Culture, Cell Preparations, and In Vivo Trans-
plantation. Dissections, tissue explants, and cell preparation were
performed as described previously (24). Recipient mice (C57BL/6
or [129sv × C57BL/6] F1 females, 8–16 wk old) received a split
dose of 1,000 rad (for colony-forming unit–spleen [CFU-S])
900 rad (for HSCs), or 640 rad (for competitive repopulation as-
say) at a 3-h interval from a 137Cs source on the day of donor cell
injection. Cells were injected i.v. into the tail veins. Except for
CFU-S, serial, and competitive transplantation assays, 2 × 107
female spleen cells from the recipient strain were coinjected to pro-
vide short-term survival. Secondary transplantations were per-
dformed with 3 × 109 BM cells from the primary recipients. Cell
dose for competitive repopulation assays was 3 × 105–3 × 107.
Injected animals were provided with 0.16% Neomycin (Sigma-
Aldrich)–supplemented water. For CFU-S, recipients were killed
at 11 d posttransplantation by cervical dislocation, spleens iso-
lated, and microscopic colonies scored after fixing with Teley-
nesnicky’s solution overnight.

Semiquantitative PCR for Donor Contribution. Blood, tissue, or
specific cell lineage DNA (100 ng) was used for semiquantitative
PCR to detect the donor HSC contribution to the recipient. For
male-derived donor cells, YMT-specific PCR (350-bp product)
was used together with myogenin-specific PCR (250 bp) for
DNA normalization. The detection of GATA-2 mutant–derived
donor cells was performed with GATA-2/NEO (950 bp) and
GATA-2 wild-type (600 bp) PCR. Primers and PCR conditions
were as previously described (4, 13).

Cell Sorting and Flow Cytometry Analysis. FACS was per-
formed on a FACS Vantage SE (Becton Dickinson) (23), and
flow cytometric analyses were performed on a FACSCaliber dual
laser instrument (Becton Dickinson) with CellQuest software
(BD Bioscience). Staining of embryonic tissue cell suspensions
was performed in PBS supplemented with 10% FCS, and 2 μg/ml
7AAD (Molecular Probes) was used for dead cell exclusion.
Staining of adult HSCs was performed in PBS supplemented with
0.5% BSA. Biotin–conjugated anti-Gr-1 (ER–MP20) was a gift
from Dr. P.J.M. Leenen (Erasmus MC, Rotterdam, The Nether-
lands). All other antibodies were obtained from BD Bioscience
including APC–conjugated anti-c-kit (clone 2B8), PE–conju-
gated anti–Sca-1 (clone D7), PerCP-Cy5.5–conjugated anti-
CD8a (clone 53–6.7), anti-B220 (clone RA3-6B2), anti-CD19
Results

GATA-2+/- AGM and YS Explants Contain Fewer CFU-S_{11}. To investigate if GATA-2 gene dosage affects the production of hematopoietic progenitors during development, colony-forming unit spleen activity was assayed at 11 d postinjection (CFU-S_{11}) so as to measure the more immature erythroid-megakaryoid progenitors. AGM, YS, and FL were isolated from GATA-2+/- and GATA-2+/- embryos at E10.5–E12, explant cultured, and BM cells were transplanted into irradiated adult recipients (Fig. 1). At all time points tested, CFU-S_{11} activity was detected both in GATA-2+/- and GATA-2+/- AGM, YS, and FL explants (Fig. 2). As expected from previous data (3), high numbers of E10.5 CFU-S_{11} are first detected in the GATA-2+/- AGM explants, they increase at E11, and thereafter decline in number. In GATA-2+/- AGM and YS explants, CFU-S_{11} were reduced by three- to ninefold and one- to fourfold, respectively, compared with wild-type tissues. In contrast, CFU-S_{11} activity in GATA-2+/- FL explants was normal. No reductions in FL CFU-S_{11} numbers were observed at any time. Therefore, GATA-2 gene dosage affects the generation and/or proliferation of immature hematopoietic progenitor cells in the YS and AGM of the midgestation embryo.

HSC Activity Is Severely Reduced in the GATA-2+/- AGMs. To investigate if GATA-2 dose also affects midgestation HSC development, we performed the most stringent functional HSC test: the long-term, high level, multilineage repopulation of irradiated adult mouse recipients. E11 and E12 GATA-2+/- and GATA-2+/- AGM, YS, and FL cells were transplanted directly into irradiated adult recipients (Fig. 1). Engraftment by GATA-2+/- and GATA-2+/- cells was assayed by semiquantitative PCR of the male Y chromosome–specific marker Ymt and the GATA-2/NEO mutant allele (respectively) in recipient peripheral blood DNA at 4 mo posttransplantation. Only those recipients showing >10% engraftment with donor-marked cells were considered positive for high level repopulation. PCR results of one representative experiment are shown in Fig. 3. Briefly, each recipient received one-third of the cells obtained from an individually prepared E12 tissue (0.33 tissue equivalents). At 4 mo postinjection, progeny of transplanted GATA-2+/- AGM cells were found in the peripheral blood of five out of eight recipients (Ymt
ever, no reduction in HSC activity was found in explants in comparison to the of injected AGM explants: E10.5
pendent experiments were performed with 0.2–4.5 tissue equivalents injected per recipient. GATA-2
PCR). In contrast, reduced HSC activity was found in E12
larly, recipients transplanted with YS cells revealed some
phocytes, erythroid and myeloid cells) (not depicted).
ment within all hematopoietic tissues (blood, thymus, LNs, BM, and spleen) and subsets tested (splenic T and B lymphocytes, erythroid and myeloid cells) (not depicted).
The cumulative results of all transplantation experiments are shown in Table I and reveal that at both E11 and E12, HSC activity is severely reduced in GATA-2+/- AGMs. The percentage of recipients repopulated with E11 GATA-2+/- AGM cells is only 6%, whereas 25% of recipients are repopulated with E11 GATA-2+/- AGM cells. This represents a greater than fourfold decrease in HSC activity in E11 GATA-2+/- AGMs. Furthermore, at E12 GATA-2+/- AGMs are ninefold reduced in HSC activity compared with GATA-2+/- AGMs. Reductions in the HSC activity of GATA-2+/- YS and FL tissues are less severe and stage dependent. The percentage of mice repopulated by E11 GATA-2+/- YS (23%) is comparable to that of GATA-2+/- YS (28%), and the FL at this stage contains only limited HSC activity. However, at E12 slight reductions in HSC

Figure 2. CFU-S11 activity in GATA-2 mutant tissue explants. CFU-S11 were assayed from AGM, YS, and FL explants (E10.5–E12.0) after 3 d of culture. Each point represents the average CFU-S11 number per embryo tissue equivalent ± SEM detected in the corresponding tissue and genotype. 3–14 independent experiments were performed with 0.2–4.5 tissue equivalents injected per recipient. +/+; GATA-2+/-; +/-, GATA-2-/-.

The cumulative results of all transplantation experiments were the recipient considered to be positive.

Figure 3. Detection of donor hematopoietic cell contribution in transplantation recipients by peripheral blood DNA PCR analysis. A representative PCR analysis for donor cell contribution to the peripheral blood of transplantation recipients. DNA was isolated from the corresponding recipients (at >4 mo posttransplantation) of GATA-2+/-, GATA-2-/-, and GATA-2-/- AGM, YS, and FL. Lanes 1–8 and 9–14 are blood DNA samples isolated from recipients receiving cells from E12 GATA-2+/- and +/- tissues, respectively. Each sample was analyzed with primers specific for Y chromosome (ymt), and GATA-2 (GATA-2/NEO for targeted allele). DNA samples were normalized by PCR with two endogenous gene controls (myo, myogenin; GATA-2, wild-type allele). Control DNA: 0, 1, 10, and 100% represents percentage of the male GATA-2+/- DNA mixed with female DNA. Only when the donor marker-specific PCR product was >10%, compared with controls, was the recipient considered to be positive.
Explant equivalents for E11.0, E11.5, and E12.0.

Five tissue explant equivalents were transplanted per recipient. 1 tissue explant equivalent was transplanted per recipient for E10.5 and 0.33 tissue explant equivalents were transplanted for E11.0 and E12.0, respectively. 2 independent experiments, respectively, were performed for E11 (41–47 somite pairs) and E12 tissues (60 sp).

Since it was shown previously that HSC activity generated in the AGM can be amplified (either by induction or proliferation) when whole tissues are cultured for 3 d (3), we examined the effects of gene dosage on HSCs in such explant cultures of AGM, YS, and FL from GATA-2 tissues. Thus, two copies of the GATA-2 gene are required for the normal generation, expansion, and/or survival of HSCs in the AGM region.

**Ex Vivo Expansion and Maintenance of AGM HSC Activity Is Sensitive to GATA-2 Dose.** Since it was shown previously that HSC activity generated in the AGM can be amplified (either by induction or proliferation) when whole tissues are cultured for 3 d (3), we examined the effects of GATA-2 gene dosage on HSCs in such explant cultures of AGM, YS, and FL from GATA-2+/+ and GATA-2+/- embryos. Tissues (E10.5–E12) were dissected, cultured as AGM explants. As seen in the AGM explants, is completely absent from GATA-2+/- AGMs do generate functional HSCs, albeit at much reduced levels. At later developmental time points (E11, E11.5, and E12), HSC activity, although increasing in GATA-2+/- AGM explants, is completely absent from GATA-2+/- AGM explants. As seen in the GATA-2+/- AGM explants, HSC generation and expansion occurs from E11 and E12 AGM, YS, and FL tissues were made into a single cell suspension and injected into irradiated adult recipients. Each result represents the percentage of repopulated recipients. Only when the donor cells represented >10% was the recipient considered to be positive. Three and two independent experiments, respectively, were performed for E11 (41–47 somite pairs) and E12 tissues (60 sp). 1 and 0.33 tissue equivalents transplanted for E11.0 and E12.0, respectively. 2×E12, secondary transplantation with 3×10^6 BM cells isolated from high level repopulated primary recipients that received cells from E12 tissues (two independent experiments). +/+, GATA-2+/-; +/-, GATA-2+/-.

### Table I. HSC Activity in GATA-2 Mutant Embryonic Tissues

| Stage | AGM | | YS | | FL |
|-------|-----|-----|-----|-----|-----|
|       |      |     |     |     |     |
| E11.0 | 1+/4b (25)* | 1/16 (6) | 2/7 (28) | 4/17 (23) | 0/6 (0) | 1/19 (5) |
| E12.0 | 11/16 (69) | 1/13 (8) | 11/15 (73) | 6/13 (46) | 19/19 (100) | 11/14 (78) |
| 2×E12 | 6/6 (100) | 0/3 (0) | 6/6 (100) | 4/9 (44) | 6/6 (100) | 4/9 (44) |

**Table II. HSC Activity in GATA-2 Mutant Tissues after Explant Culture**

| Stage | AGM explants | | YS explants | | FL explants |
|-------|--------------|-----|--------------|-----|----------|
|       | +/+ | +/− | +/+ | +/− | +/+ | +/− |
| E10.5 | 2+/3b (66)* | 1/12 (8) | 0/3 (0) | 0/11 (0) | ND | 0/2 (0) |
| E11.0 | 1/2 (50) | 0/4 (0) | 1/25 (25) | 1/5 (20) | 0/3 (0) | 0/5 (0) |
| E11.5 | 11/12 (50) | 0/27 (0) | 7/25 (28) | 7/29 (24) | 6/25 (24) | 3/28 (11) |
| E12.0 | 7/12 (58) | 0/13 (0) | 4/16 (25) | 2/14 (14) | 11/12 (92) | 10/19 (53) |
| E10.0 | 0/7 (0) | 0/8 (0) | 0/3 (0) | 0/7 (0) | 0/8 (0) | 0/3 (0) |

E10 to E12 AGM, YS, and FL explants were cultured for 3 d, made into a single cell suspension, and injected into irradiated adult recipients. Each result represents the number of recipient mice showing donor cells in peripheral blood (DNA) isolated at >4 mo posttransplantation, the total number of mice transplanted, and the percentage of repopulated recipients. Results are summarized in Table II. Compared with the results of the direct transplantation experiments (Table I), GATA-2+/- AGM explants were even more severely reduced in the HSC activity. At E10.5, only 8% of recipients receiving GATA-2+/- AGM cells were repopulated, representing an eightfold decrease in HSC activity from GATA-2+/- AGM cells. The GATA-2+/- cell contribution to the various hematopoietic organs (thymus, spleen, LN, and BM) and purified cell lineages (B and T lymphocytes, myeloid and erythroid) was tested and found to be multipotent, thus demonstrating that GATA-2+/- AGMs do generate functional HSCs, albeit at much reduced levels. At later developmental time points (E11, E11.5, and E12), HSC activity, although increasing in GATA-2+/- AGM explants, is completely absent from GATA-2+/- AGM explants. As seen in the GATA-2+/- AGM explants, HSC generation and expansion occurs from HSC activity are observed for both GATA-2+/- YS and FL (1.6- and 1.3-fold, respectively) compared with GATA-2+/- tissues. Thus, two copies of the GATA-2 gene are required for the normal generation, expansion, and/or survival of HSCs in the AGM region.
As shown in Table II, although high tissue equivalents (up to five) of cells from GATA-2 mutant (+/-) and YS explants were injected, HSC activity was not detected in any of the recipients. Also, GATA-2<sup>−/−</sup> E10.5 YS and E10.5 and E11 FL explants showed no HSC activity. However, HSC activity initiates normally in GATA-2<sup>−/−</sup> AGM explants at E10.5 at the same stage as in the GATA-2<sup>+/−</sup> AGM. HSCs also appear at normal time points in GATA-2<sup>−/−</sup> YS and FL explants (E11 and E11.5, respectively). Therefore, we conclude that HSC induction initiates on schedule and that there is no early onset of HSC activity in GATA-2<sup>−/−</sup> AGM, YS, or FL.

**Serial Transplantation Potential of Midgestation HSCs Is Severely Reduced.** HSC self-renewal can be tested by serial transplantation of HSCs from primary to secondary recipients. Since we found that GATA-2<sup>−/−</sup> AGM HSCs are severely reduced in their expansion, we examined whether GATA-2<sup>−/−</sup> embryo-derived HSCs are as potent in their serial repopulation ability as wild-type HSCs. Whole BM cells from primary recipient mice showing high donor contribution from transplanted E12 GATA-2<sup>+/+</sup> or GATA-2<sup>−/−</sup> AGM, YS, and FL cells were injected into irradiated secondary adult recipients. Consistent with previous published results, GATA-2<sup>−/−</sup> AGM, YS, and FL-derived HSCs can successfully reconstitute secondary recipients; 100% of secondary recipients were repopulated with HSCs from primary recipients of these midgestation tissues (Table I). In contrast, HSCs from a primary GATA-2<sup>−/−</sup> AGM recipient failed to repopulate any of the secondary recipients analyzed (0%; zero out of three). Reduced HSC activity was also observed in the secondary recipients receiving BM cells from GATA-2<sup>−/−</sup> YS and FL primary recipients (44% compared with 100% recipient repopulation with GATA-2<sup>−/−</sup> primary BM cells). These results demonstrate that GATA-2<sup>−/−</sup> dose affects HSC serial repopulation ability and suggests a defect in HSC self-renewal.

**GATA-2<sup>−/−</sup> BM HSCs Are at a Competitive Disadvantage.** The decreased serial repopulation ability of embryo-derived HSCs prompted us to investigate if adult BM HSCs are also affected by a reduction in GATA-2 dose. Initially, we injected limiting doses of GATA-2<sup>−/+</sup> and GATA-2<sup>−/−</sup> BM cells into lethally irradiated adult recipients but found
no quantitative differences in repopulation. Hence, we performed reciprocal competitive transplantations in which different concentrations of unmanipulated GATA-2+/+ and GATA-2+/- BM cells were injected into sublethally irradiated GATA-2+/+ and GATA-2+/- adult recipients. When 3 × 10^5 whole GATA-2+/- BM cells were transplanted into GATA-2+/- adult recipients, long-term high level donor contribution was found in two out of five (40%) recipients, whereas GATA-2-/- cells at this dose provided no repopulation (zero out of five recipients; 0%) (Fig. 4). Only at a 10-fold higher cell dose were the GATA-2+/- cells able to repopulate four out of seven (57%) recipients. A dose of 3 × 10^6 GATA-2+/- cells repopulated almost all recipients (six out of seven; 86%), whereas 3 × 10^7 GATA-2+/- cells were required for repopulation of all recipients (five out of five; 100%). To further examine the competitive abilities of GATA-2+/- cells, GATA-2+/- cells were injected into sublethally irradiated GATA-2+/- recipients. Only 3 × 10^5 (or fewer) wild-type cells were required to fully out-compete all the GATA-2+/- HSCs in the recipients. Thus, GATA-2+/- HSCs compete more effectively against GATA-2+/- HSCs than do GATA-2-/- HSCs, demonstrating that GATA-2+/- adult BM contains fewer HSCs or that these HSCs are qualitatively less potent.

**GATA-2 Dose Affects the Number of Phenotypically Defined HSCs in the Embryo But Not the Adult.** Our in vivo transplantation results clearly show that GATA-2+/- HSC activity is affected throughout development. To more specifically investigate the cell types that are affected in the GATA-2+/- mice, we crossed the GATA-2 mutant allele into Ly-6A GFP transgenic mice, in which HSCs can be detected by the expression of the green fluorescent protein (GFP) reporter under the transcriptional control of Ly-6A regulatory sequences (26). Ly-6A encodes the Sca-1 surface glycoprotein that is expressed on HSCs. Previously, we have shown that all AGM, FL, and adult BM HSCs express the Ly-6A GFP transgene and that GFP expression is highly restricted in the AGM region to a few aortic endothelial cells and hematopoietic clusters (23, 27).

To determine whether GATA-2 dose affects these cells, we examined transverse sections through the E11 dorsal aorta from compound transgenics (Ly-6A GFP–GATA-2+/- and Ly-6A GFP–GATA-2-/-). As shown in representative sections in Fig. 5 A, GFP-positive cells are decreased in number in the GATA-2-/- aorta compared with the GATA-2+/- aorta. Quantitations were performed by counting GFP-positive and CD34-positive cells in 37 aorta sections from each genotype (CD34 immunostaining of endothelial cells provided a normalization control). GFP-positive cells were present but decreased by a factor of 10 or more in the GATA-2-/- aortas. Hence, GATA-2 haploinsufficiency leads to a significant decrease in HSCs and/or HSC precursors in the AGM.

Flow cytometric analyses were performed to verify these results and to examine if the phenotypic HSC content of the other hematopoietic tissues was also changed. As shown in Fig. 5 B, in such compound transgenic embryos a sixfold decrease in GFP-positive cells in the E11 GATA-2-/- aorta was found compared with GATA-2+/- aorta. E11 GATA-2+/- YS showed a 1.9-fold decrease in GFP-positive cells. However, no decrease was found in E11 GATA-2+/- mice.
in 2 mice, no defect in hematopoietic differentiation was observed in GATA-2−/− mice when treated with flow cytometry for evidence of hematopoietic regeneration. As shown in Fig. 6 A, both GATA-2+/+ and GATA-2−/− mice were examined (Fig. 5 C). Together, these phenotypic data support the transplantation data in showing that HSCs increase to normal numbers in the FL and adult BM.

Cytotoxic Drug Treatment Reveals a Proliferation Defect in GATA-2−/− BM HSCs. To test if the qualitative defect in GATA-2+/+ HSCs is related to proliferation, GATA-2+/+ and GATA-2−/− mice were treated with the cytotoxic drug 5-FU (28). At 0, 4, 8, 12, and 16 d after treatment, BM cells were tested in in vitro assays and analyzed by flow cytometry for evidence of hematopoietic regeneration. As shown in Fig. 6 A, both GATA-2+/+ and GATA-2−/− mice showed similar reductions in total BM cell number at 4 d post-5-FU. Total BM cell numbers increased to starting numbers by 16 d post-5-FU. No significant differences were observed between the number of total GATA-2+/+ and GATA-2−/− BM cells at any time point. In addition, no defect in hematopoietic differentiation was observed in GATA-2+/+ BM cells, as flow cytometric analysis showed the presence of all three lineages, lymphoid, myeloid, and erythroid at similar levels in GATA-2+/+ and GATA-2−/− BM (not depicted).

To investigate whether specific immature hematopoietic progenitors were affected in 5-FU-treated GATA-2+/+ mice, we performed in vitro colony assays for CFU-GM. As shown in Fig. 6, B and C, the starting frequency and number of CFU-GM were the same in GATA-2+/+ and GATA-2−/− BM. At 4 d post-5-FU treatment, frequency and number of CFU-GM reached a similar low point in both GATA-2+/+ and GATA-2−/− BM. However, at 12 d after 5-FU treatment, CFU-GM frequency and number in GATA-2−/− BM reached higher or the same levels as the untreated BM, whereas the GATA-2−/− BM CFU-GM frequency and number remained low (P < 0.008 and 0.002, respectively). Only at day 16 after 5-FU treatment did GATA-2−/− CFU-GM frequency and number reach the same levels as in untreated BM. Thus, the 4 d delay in the regeneration of CFU-GM in GATA-2−/− BM suggests a GATA-2 dose-related proliferation defect acting within these progenitors. Alternatively, a GATA-2 dose-related proliferation defect acts within HSCs and only secondarily influences CFU-GM.

To test this, we analyzed the regeneration of HSCs. We performed flow cytometric analysis for LSK BM cells at 0, 4, 8, 12, and 16 d post-5-FU treatment. In both GATA-2+/+ and GATA-2−/− BM, HSC percentages and absolute numbers were similarly reduced at 4 d post-5-FU treatment (Fig. 6, D and E). Both GATA-2+/+ and GATA-2−/− BM HSC percentages and numbers began recovering at day 8 post-5-FU, when they surpassed the initial percentages and numbers. However, GATA-2−/− BM HSC numbers remained significantly lower than in GATA-2+/+ BM at both day 8.
and 12 post–5-FU treatment (p < 0.04). Furthermore, whereas \textit{GATA-2}\textsuperscript{+/+} HSC numbers peaked at day 12 post-treatment and declined thereafter, \textit{GATA-2}\textsuperscript{+/-} HSC numbers increased slowly up to day 16 post–5-FU treatment. The finding that HSC expansion in 5-FU–treated \textit{GATA-2}\textsuperscript{+/-} mice is delayed by at least 4 d strongly suggests a \textit{GATA-2} dose–dependent proliferation defect in HSCs.

\section*{Discussion}

The data presented here show for the first time that a full dose of \textit{GATA-2} is required during embryonic and adult stages for quantitatively and qualitatively normal HSC activity in vivo. Although HSCs are most likely not produced in \textit{GATA-2}\textsuperscript{+/-} embryos, the effects of \textit{GATA-2} haploinsufficiency had been largely unexplored because such \textit{GATA-2}\textsuperscript{+/-} animals grow normally and present an overtly normal adult hematologic profile. Here we have shown that with only half the dose of \textit{GATA-2}, HSC numbers are severely and specifically reduced in the AGM region, where the first induction and expansion of HSCs is occurring during midgestation. Moreover, AGM HSC quality is compromised. Thereafter, in the other tissues harboring HSCs, quantitative deficiencies in \textit{GATA-2}\textsuperscript{+/-} HSCs appear to be compensated through normal (albeit delayed) expansion of HSCs, but qualitative deficiencies are retained through to adulthood. Therefore, given that only a few HSCs out of the whole cohort of HSCs are actively contributing to the hematopoietic system at any one time (29, 30) and that \textit{GATA-2}\textsuperscript{+/-} cells are not defective in differentiation, no general hematologic defects would be expected in haploinsufficient adults. Only through stringent in vivo transplantations or cytotoxic stress are HSC functional defects observable. The results of these experiments strongly suggest an essential role for \textit{GATA-2} in the induction and expansion of the first HSCs in the AGM and an additional, distinctive role for \textit{GATA-2} in the proliferation of HSCs.

\textbf{HSC Quantitative Processes Are Altered in \textit{GATA-2}\textsuperscript{+/-} Mice.} In in vivo transplantation experiments we show quantitatively reduced HSC activity in \textit{GATA-2}\textsuperscript{+/-} embryos. The four- to ninefold decrease in HSC activity in E11/E12 \textit{GATA-2}\textsuperscript{+/-} AGMs compared with \textit{GATA-2}\textsuperscript{+/-} AGMs is the consequence of fewer HSCs, as aorta sections and flow cytometric analysis show a sixfold decrease in phenotypically defined HSCs. Hence, \textit{GATA-2}\textsuperscript{+/-} AGMs can neither expand nor maintain HSCs compared with \textit{GATA-2}\textsuperscript{+/-} AGMs. In contrast, HSCs are expanded and maintained in E11/E12 \textit{GATA-2}\textsuperscript{+/-} YS (at a slightly decreased number), with the fold decrease in phenotypically defined HSCs in the YS corresponding closely to the fold decrease in HSC activity. Considering the fact that HSCs are first detected in the AGM region and then in the YS and FL, the reduced HSC content of the \textit{GATA-2}\textsuperscript{+/-} YS and FL may well be a secondary effect of the reduction in the \textit{GATA-2}\textsuperscript{+/-} AGM region.

Our in vivo analyses for hematopoietic progenitor cells in the AGM region and the YS show that CFU-S\textsubscript{I1} are also \textit{GATA-2} dose dependent. These data are consistent with previous in vitro studies on \textit{GATA-2}\textsuperscript{+/-} YS and ES cells (13, 31), showing much reduced hematopoietic progenitor activity. The reduced CFU-S\textsubscript{I1} activity in \textit{GATA-2}\textsuperscript{+/-} AGMs and YSs could further be a consequence of the reduced HSC activity we detected in these tissues. However, the source of cells providing the CFU-S\textsubscript{I1} activity in the embryo is not clear. Whereas in the adult, hematopoietic progenitor cells are derived from HSCs, in the preE10.5 AGM region and the YS they may be derived from hemangioblasts and/or hemogenic endothelium rather than via a HSC ancestor. Hence, \textit{GATA-2} may act directly on the in vivo generation, survival, and/or expansion of the hematopoietic progenitor cells, HSCs, and/or their direct precursors in the AGM and YS.

\textbf{The YS as a Compensatory Generator of HSCs Independent of \textit{GATA-2} Dose.} For over three decades, the origins of adult HSCs have been a focus of research. The view that the mammalian YS is able to provide hematopoietic cells that migrate and colonize the FL and then the BM during the neonatal/adult stages has been altered by the finding that the first fully functional adult HSCs are autonomously generated in the AGM region. Shortly thereafter, the YS contains HSCs, but due to the experimental constraints of mammalian embryos, it is difficult to definitively demonstrate whether these HSCs are AGM derived or autonomously generated in the YS. Recent data suggest that indeed YS can autonomously generate and expand HSCs (6) and putative pre-HSCs (32). Since we observe a dramatic reduction in HSCs in \textit{GATA-2}\textsuperscript{+/-} AGMs but only a slight reduction in HSCs in \textit{GATA-2}\textsuperscript{+/-} YSs, our transplantation data support the notion of YS HSC generation potential (albeit in a \textit{GATA-2}\textsuperscript{+/-} embryo). However, since HSCs are still generated in the \textit{GATA-2}\textsuperscript{+/-} YS 1 d earlier than in the YS, it remains possible that YS HSCs are AGM derived. Interestingly, the reduced HSC activity in the \textit{GATA-2}\textsuperscript{+/-} YS can be expanded to a magnitude comparable to that of the \textit{GATA-2}\textsuperscript{+/-} YS in explant cultures, suggesting that at least some of the reduced activity in the \textit{GATA-2}\textsuperscript{+/-} YS is a secondary effect of the reduction of HSCs in the AGM. Furthermore, \textit{GATA-2}\textsuperscript{+/-} HSC numbers are compensated to normal levels in the adult, possibly due to HSC generation and expansion in the YS and the further expansion in the FL and BM. Notwithstanding, these data imply that the underlying molecular mechanisms in which the AGM generates, maintains, and expands HSCs are different from that of the YS. The AGM region is exquisitely sensitive to the level of the \textit{GATA-2} dose, whereas the YS is much less sensitive. Hence, the HSC defects in \textit{GATA-2}\textsuperscript{+/-} AGMs do not result in severe anemia in adults since \textit{GATA-2}\textsuperscript{+/-} YS can generate and/or expand sufficient HSCs irrespective of the haploinsufficiency.

\textbf{HSC Qualitative Processes Are Altered in \textit{GATA-2}\textsuperscript{+/-} Mice.} The results of adult BM competitive transplantation experiments clearly demonstrate a qualitative difference between the \textit{GATA-2}\textsuperscript{+/-} and \textit{GATA-2}\textsuperscript{+/-} HSCs. The high percentage of \textit{GATA-2}\textsuperscript{+/-} mice engrafted with \textit{GATA-2}\textsuperscript{+/-} HSCs. The high percentage of \textit{GATA-2}\textsuperscript{+/-} mice engrafted with \textit{GATA-2}\textsuperscript{+/-} HSCs. The high percentage of \textit{GATA-2}\textsuperscript{+/-} mice engrafted with \textit{GATA-2}\textsuperscript{+/-} HSCs.
2+/+ cells, even at low donor cell numbers, demonstrate that GATA-2+/+ BM HSCs have a proliferative advantage over the GATA-2+/− BM HSCs. In the reciprocal transplantation in which GATA-2+/− BM cells were transplanted into GATA-2+/+ recipients, high numbers of cells were needed to obtain a high percentage of donor-engrafted recipient mice and thus imply that: (a) the number of HSCs in GATA-2−/− BM is quantitatively reduced; (b) the GATA-2+/− HSCs have a lower proliferative advantage over the GATA-2+/+ HSCs; and/or (c) the homing efficiency is lower for GATA-2+/−−derived HSCs. The fact that no significant difference in the percentage or absolute number of LSK BM cells was found between GATA-2+/− and wild-type BM indicates that the decreased HSC activity is not due to a quantitative decrease in GATA-2+/− BM HSCs. However, the delayed expansion of HSCs in the 5-FU recovery experiments does strongly suggest that the major GATA-2 dose-dependent defect is in HSC proliferation. Although homing of HSCs is not required in this experimental scenario, we cannot exclude an additional GATA-2 dose-dependent defect in homing.

How Does GATA-2 Dose Affect the Quantitative and Qualitative Development of HSCs? We propose here that the GATA-2 dose effects we observe in the AGM act at the level of the hemogenic cells that differentiate into HSCs. Normally, a full dose of GATA-2 is required for the generation, maintenance, and/or expansion of these precursor cells. In the haploinsufficient AGM region, these hemogenic cells fail to differentiate, survive, and/or divide. However, owing to the stochastic nature of gene expression, some hemogenic cells still achieve a threshold level of GATA-2 protein, and therefore, the target genes (which are needed for the differentiation, survival and/or division of the precursor cells) can be activated at some low frequency in the E10.5 AGM. The outcome of GATA-2 haploinsufficiency is then a small production of HSCs followed by an overall reduction in the absolute number of AGM HSCs that we can detect functionally in our transplantation assay and phenotypically in immunostained sections and flow cytometry analysis.

Recent GATA-2 expression data in the AGM support the notion of a role for GATA-2 in hemogenic precursors. Transgenic embryos with a GFP marker under the control of GATA-2 transcriptional regulatory sequences show high levels of GATA-2 expression in CD45− AGM cells with hemogenic potential and a significant decrease in the percentage of CD45+ cells in GATA-2−/− E11.5 AGMs (12). Moreover, during midgestation, at the time of the first induction of HSCs, GATA-2 is expressed in the endothelial cells lining the dorsal aorta and some underlying mesenchymal cells. Hence, high GATA-2 expression in hemogenic cells of the AGM suggests that GATA-2 is acting on the cells just immediately preceding the induction of HSCs.

Since GATA-2 is a transcription factor, its target genes within hemogenic AGM cells are of particular interest. Several markers of AGM HSCs and aortic hemogenic cells have been recently described: the Ly-6A (Sca-1) cell surface glycoprotein (23, 33) and Runx-1 transcription factor (27). These molecules are overlapping with GATA-2 in their expression patterns in hemogenic cells of the dorsal aorta. Targeted mutation of these genes results in qualitative and/or quantitative defects in HSCs. Whereas Ly-6A−/− embryos thrive into adulthood with no or little effect on HSC generation in the embryo, functional analyses of HSCs derived from Ly-6A−/− mutant BM show defects in their self-renewal ability (34), similar to our findings in GATA-2−/− BM. In contrast, Runx-1−/− embryos are completely devoid of HSCs and exhibit FL anemia leading to lethality at E12 (35–37). Moreover, Runx-1 haploinsufficiency leads to a premature extinction of AGM HSCs (20). Hence, the Ly-6A and Runx-1 genes could be targets of GATA-2 or, alternatively, contribute to the activation of the same pathways for HSC self-renewal and/or HSC generation. At present, although many GATA consensus-binding sites appear in the sequences surrounding these genes, there is no in vivo data showing that any of these sites are functional.

Nonetheless, two bona fide target genes of GATA-2 have been proposed. These are SCL/tal-1, an essential early hematopoietic transcription factor and E4bp4, a transcription factor implicated in cell survival. In vivo studies show that GATA-2 forms a multiprotein complex with Fli-1 and Elf-1 that binds the SCL enhancer and activates the expression in HSCs, endothelial cells, and their bipotent progenitor, the hemangioblast (38). However, in vivo mutation analysis on the HSC-specific GATA sites within the SCL locus affects SCL expression not only in the AGM but also in YS and FL (38). Therefore, it is unlikely that the selective defect in the GATA-2−/− AGM HSCs can be attributed to defective SCL expression. Chromatin immunoprecipitation studies on BaF3 cell line stimulated with IL-3 show that GATA-2 binds to a sequence downstream of the transcriptional start site of E4bp4 and is necessary for transcriptional activation of this gene (39). Considering that IL-3 is a survival factor for HSCs, it is plausible that GATA-2 is involved in the activation of this pathway.

In conclusion, GATA-2 dosage is important in regulation of HSC production and expansion. Haploinsufficiency of GATA-2 results in quantitative decreases in HSCs in the AGM and qualitative defects in HSCs in both the embryonic-derived and adult BM HSCs. The pivotal importance of GATA-2 in these processes within HSCs now awaits the identification of the relevant target genes and the functional cascades that GATA-2 activates, most likely in concert with other factors in multiprotein complexes.

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