Defects in Hemopoietic Stem Cell Activity in Ikaros Mutant Mice

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Summary

Here we provide evidence that the Ikaros family of DNA binding factors is critical for the activity of hemopoietic stem cells (HSCs) in the mouse. Mice homozygous for an Ikaros null mutation display a >30-fold reduction in long-term repopulation units, whereas mice homozygous for an Ikaros dominant negative mutation have no measurable activity. The defect in HSC activity is also illustrated by the ability of wild-type marrow to repopulate unconditioned Ikaros mutants. A progressive reduction in multipotent CFU-S (colony-forming unit-spleen) progenitors and the earliest erythroid-restricted precursors (BFU-E [burst-forming unit-erythroid]) is also detected in the Ikaros mutant strains consistent with the reduction in HSCs. Nonetheless, the more mature clonogenic erythroid and myeloid precursors are less affected, indicating either the action of a compensatory mechanism to provide more progeny or a negative role of Ikaros at later stages of erythromyeloid differentiation. In Ikaros mutant mice, a decrease in expression of the tyrosine kinase receptors flk-2 and c-kit is observed in the lineage-depleted c-kitSca-1 population that is normally enriched for HSCs and may in part contribute to the early hemopoietic phenotypes manifested in the absence of Ikaros.

Key words: transcription factors • hemopoiesis • flk2 • c-kit regulation

The hemopoietic system is derived from the mesodermal germ layer early in embryogenesis (1–3). Hemopoietic commitment of the elusive hemangioblast, a mesodermally derived progenitor that gives rise to vascular endothelium and hemopoietic cells (4), occurs over a narrow window of time during embryogenesis. Once established, the hemopoietic system supplies the organism with at least 10 different lineages in a highly regulated manner. As the half-life of mature hemopoietic cells varies from several hours to years, a continuous production of end-stage cells from hemopoietic stem cells (HSCs) is required throughout the life span of the organism. Accordingly, HSCs must be capable of self-renewal to maintain the HSC pool and its more mature progeny. Differentiation from the HSC proceeds through a series of progressive restrictions that give rise to multipotent progenitors with short-term repopulating ability, lineage-restricted precursors, and, finally, terminally differentiated cells (5). To increase the number of mature progeny, proliferative expansions take place along various stages in these pathways. The molecular control of hemopoiesis has been the focus of intense study. The role of membrane receptors, underlying signaling pathways, and potential nuclear effectors has been studied in both vertebrates and invertebrates (6–9).

Ikaros is a hemopoietic-specific member of a family of zinc finger transcription factors that is essential for specification in lymphoid lineages (10, 11). Ikaros generates by means of alternate splicing a number of protein isoforms (12, 13) that share a common COOH-terminal zinc finger domain that mediates interactions with self and other family members (14, 15). A subset of the Ikaros isoforms (Ik-1, Ik-2, Ik-3, Ik-4) has a second zinc finger domain located at the NH2-terminal half that mediates sequence-specific DNA interactions (12). The remaining Ikaros isoforms, Ik-5, Ik-6, and Ik-7, do not have a DNA-binding domain yet can negatively regulate the activity of the DNA-binding Ikaros proteins (12, 14). Although functionally distinct, both Ikaros zinc finger domains are essential for high-affinity interactions with DNA and are indispensable for transcriptional activity (reference 14 and Koipally, J., and K. Georgopoulos, unpublished results). The relative expression levels of Ikaros splicing variants do not change significantly during development; the DNA-binding isoforms Ik-1, Ik-2, and

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1Abbreviations used in this paper: AGM, aorta-gonad-mesonephros regions; BM, bone marrow; CFC, colony-forming cell; DN, dominant negative; G, granulocytes; HSCs, hemopoietic stem cells; LTR, long-term repopulation; M, macrophages; RT, reverse transcriptase; YS, yolk sac.
IK-4 are the predominant species in the hematopoietic populations analyzed thus far (12, 15).

Two distinct deletions have been targeted independently in the Ikaros locus in mice (10, 11). Mice homozygous for a deletion in the last translated exon that encodes the COOH-terminal half of the Ikaros proteins are null for any Ikaros protein (11). Ikaros null mice lack both fetal and adult mature B cells as well as the earliest defined B lineage precursors. Fetally derived T cells and their precursors are also absent; however, postnatally, a severely reduced number (10–30-fold reduction) of T cell precursors appears in the thymus (16). These T cell precursors undergo abnormal differentiation along the CD4+ T cell pathway, are unable to differentiate along the NK lineage, and produce severely reduced numbers of γ/δ T cells and thymic dendritic APCs (11, 17). A distinct Ikaros mutation that deletes part of the NH2-terminal zinc finger domain abolishes the DNA binding capability of Ikaros proteins but leaves the COOH-terminal zinc finger interaction domain intact. Proteins are stably produced by this Ikaros mutant allele, which can interfere with the activity of the DNA-binding Ikaros isoforms and with other Ikaros family members (14, 15, 18). Consistent with these in vitro observations, mice homozygous for the Ikaros dominant negative (DN) mutation display more severe lymphoid defects relative to Ikaros null mice. Ikaros DN−/− mice lack all cells of lymphoid origin, including NK cells and thymic and splenic APCs (10, 17).

In addition, mice heterozygous for the Ikaros DN mutation develop T cell leukemias and lymphomas with 100% penetrance within the first 4 mo of their lives (19). The more severe effects on development and homeostasis of the lymphoid lineage caused by the Ikaros DN mutation suggest that Ikaros DN proteins interfere with the activity of other family members during lymphopoiesis. One of the Ikaros homologues, Aiolos, is expressed in lymphoid precursors and mature lymphocytes and interacts both physically and functionally with Ikaros (reference 15 and Cortez, M., and K. Georgopoulos, unpublished data). Two additional family members, Helios and Dedalos, also form complexes with Ikaros and colocalize within higher order nuclear structures (18, 20, and Morgan, B., manuscript in preparation). Both Helios and Dedalos are expressed in populations enriched for HSCs and in early thymic T cell precursors (reference 18 and Morgan, B., manuscript in preparation). We have recently identified Ikaros and family members in two distinct higher order chromatin remodeling complexes having nuclear compartmentalization and gene targeting that relies on the DNA binding activity shared by Ikaros and its family members (21). Thus, the more severe phenotypes manifested in Ikaros DN mutants may be due to interference with the Aiolos, Helios, and Dedalos protein activity and inappropriate targeting of the Ikaros-associated chromatin remodeling complexes.

In this study, we delineate the effects on HSC activity manifested by the Ikaros null and DN mutations. The effects of the Ikaros mutations are apparent in long-term re-population (LTR) activity, which is reduced by 20–40-fold in Ikaros null mutants and is undetectable in Ikaros DN homozygotes. The more committed multipotent CFU-S14 (colony-forming unit-spleen) progenitors and the earliest erythroid-restricted precursors (BFU-E [burst-forming unit-erythroid]) are also reduced in Ikaros mutant mice to an extent that correlates with the reduction in HSC activity. Nevertheless, the more mature clonogenic precursors, including CFU-E, are less affected. Ikaros null HSCs and DN−/− hematopoietic progenitors lack expression of the tyrosine kinase receptor fik-2 at the mRNA level and express progressively reduced levels of the tyrosine kinase receptor c-kit at the cell surface.

Materials and Methods

Mice. Ikaros null and DN mutant mice as well as wild-type control littermates on a mixed C57BL/6J × 129Sv background were bred and maintained under sterile conditions in a pathogen-free animal facility at Massachusetts General Hospital. Due to the high morbidity of the Ikaros DN mutant mice, all animals were kept on oral antibiotics. Mice used for the different studies were between 2 and 5 wk old. The genotypes of mice were determined by PCR analysis of the Ikaros locus with primers and conditions described previously (10). C57BL/6J–Ly5a−/− congenic mice used as transplant recipients or donors were obtained from The Jackson Laboratory and bred in the animal facility.

Hematopoietic Tissue Preparation. Bone marrow (BM) was prepared by crushing femora and tibiae with a mortar and pestle and then passing the suspension through a 70-μm cell strainer to remove bone debris. Spleen cell suspensions and day 14 fetal liver cells were obtained by disrupting the tissue in PBS (plus 5% dialyzed FBS) and passing it through a 70-μm cell strainer. The aorta-gonad-mesonephros regions (AGM) and yolk sacs (YS) from 11 d postcoitum embryos were prepared as described previously (22). In brief, the AGM and YS were dissected in PBS/5% FCS and digested for 1 h at 37°C in 0.125% collagenase (Sigma Chemical Co.). Viable cell counts were based on trypan blue exclusion.

mAbs. The mAbs used for immunofluorescent labeling and the fluorochromes employed are specified elsewhere (17). In brief, three-color flow cytometry was performed using anti-Ly5b (AL1-4A2) to identify donor-derived cells and antibodies against B220 (RA3-6B2), CD4 (RM4-5), CD8 (53-6.7), TCR-α/β (H57-597), M-cer-1 (M1/70), Gr-1 (RB6-8C5), and TER-119. All antibodies were purchased from PharMingen.

In Vitro Colony-forming Cell Assay. Marrow and spleen cells were harvested from neonate and 2–5-wk-old mice. Single-cell suspensions from each tissue were prepared in PBS (plus 5% dialyzed FBS), counted, and cultured in 1% DMEM containing 1% methyl cellulose, 15% FBS, 0.5% BSA fraction V, transferrin, insulin, lipids, α-thioglycollate, 15 U/ml IL-3, 2 IU/ml erythropoietin, and 50 ng/ml kit ligand. Colonies were scored after 2–3 d for CFU-E and after 7–10 d for all other colony types. Erythroid colonies containing at least two other lineages were attributed to colony-forming cell (CFC)-multi. Pure erythroid colonies on day 7–8 were attributed to BFU-E, and colonies containing at least 500 granulocytes (G) and/or macrophages (M) were attributed to CFC-G/M. Purified murine kit ligand was provided by Genetics Institute.

Spleen Colony-forming Assay (CFU-S14). The CFU-S14 content in the BM and spleens of Ikaros mutant and wild-type littermates 2–5 wk after birth was determined by injection of 5 × 104 nucleated BM cells or 2.5 × 106 spleen cells into lethally irradiated (9.5 Gy, 137Cs single dose) wild-type recipients or donors were obtained from The Jackson Laboratory and bred in the animal facility.
mice. Mice were killed 14 d after the injection, and their spleens were fixed in Bouin’s solution for macroscopic examination and weighing. Absolute numbers of CFU-S per organ were calculated based on the frequency measurement and the cellularity of the spleen and the BM, assuming that one femur and one tibia represent 10 and 5%, respectively, of total BM. To determine the lineage composition of spleen colonies, single colonies were dissected before fixation, erythrocytes were lysed in 0.4 M ammonium chloride buffer, and cells were stained with a subset of antibodies described previously and subjected to FACS™ analysis. For PCR genotyping, colonies were lysed in DNA lysis buffer, and DNA was prepared as previously described (10). Irradiated mice injected with PBS alone were included as controls in all experiments.

R adioprotection and Competitiv e R epopulation A ssay s. Congenic C57BL/6–Ly5a–Pepb mice were irradiated with a single lethal dose of 9.5 Gy from a 137Cs radiation source (gamma irradiator; J.L. Shepherd) at 0.95 Gy/min. Cell suspensions containing 105 wild-type or 6 × 105 mutant BM cells from 2–5-wk-old mice in a final volume of 200 μl PBS were injected intravenously into the lateral tail vein. Recipient mice were maintained on 1.1 g/liter neomycin sulfate (Sigma Chemical Co.) and 105 U/liter polymyxin B sulfate (Sigma Chemical Co.) in their drinking water for the duration of the assay. Mice were monitored for survival daily for 35 d. For the competitive assay, lethally irradiated Ly5a mice (9.5 Gy of gamma irradiation) were injected with a fixed amount of 108 Ly5a autologous BM cells along with 105–106 Ikaros mutant or wild-type BM cells (Ly5b). Donor-derived (Ly5b) hematopoietic contribution was measured from the peripheral blood at different time points starting on day 19 after transplant and from the spleen and the BM, assuming that one femur and one tibia of 2–5-wk-old mice. Cells were layered at 2.5 g/cm3 over 3 ml of sodium metrizoate (Nycodenz; Accurate Scientific) so-
than that expected given the lack of B cells and impaired T cell development (11, 17). Ikaros DN−/− mice, which lack both B and T lymphocytes, also show a reduction to only 57% of the cellularity of the wild-type organ (Fig. 1 A). Taken together, these results reveal a significant increase in myeloid (Mac-1+) cells in the spleens of Ikaros mutants. Such disproportionate changes in hemopoietic populations between BM and spleen are indicative of extramedullary hemopoiesis.

We have previously shown (11) that Ikaros null mice have normal hematocrits throughout their life spans. Ikaros DN−/− mice, however, display a drop in hematocrit with age (Fig. 1 B). The hematocrits of 2–3-wk-old Ikaros DN−/− mice are similar to those of wild-type littermates but soon after drop and by 6 wk of age reach a value of <50% of wild-type levels. During this period, Ikaros DN−/− mice also develop extensive infections from opportunistic microorganisms and die (10). Therefore, the cause of death in these mice cannot be unequivocally ascribed to the lack of an immune system or to hemopoietic failure but rather may be due to both. Thus, in addition to lymphoid defects, Ikaros deficiency has other effects on hemopoiesis.

Radioprotection Capabilities of Ikaros Null and DN−/− BM. Mice exposed to high doses of whole body irradiation die within 9–18 d from hemopoietic failure unless they are transplanted with hemopoietic progenitors and progenitors that provide radioprotection and short-term reconstitution. The radioprotective quality of Ikaros null and DN−/− BM was asayed in strains of mice congenic for the panleukocyte marker Ly5. Both Ikaros null and DN−/− donor mice expressed the Ly5b variant, whereas the transplant recipients expressed the Ly5a allele.

Ikaros null and wild type BM cells, when given at a dose of 105 cells, radioprotected 100% of the lethally irradiated (900 rads) recipients for at least 30 d after transplant (Fig. 2 A). Animals receiving 106 Ikaros null BM had almost 100% donor contribution to the myeloid (Mac-1+) lineage 7 mo after transplant (Fig. 2 A, left). Ikaros DN−/− BM cells provided at doses of 1–6 × 106 were capable of only short-term radioprotection at first (Fig. 2 A), with a steady decrease in donor contribution observed between 3.5 and 5 wk after transplant (Fig. 2 B, right). The hematocrits of Ikaros DN−/− BM recipients also decreased during this time period. 3 wk after transplant, hematocrits of Ikaros DN−/− BM recipients were <50% of wild-type BM recipients, and by day 35 they were down to one-third of wild-type values (Fig. 2 C). All recipients of Ikaros DN−/− BM died by day 35 after transplant from severe anemia.

Given the extramedullary hemopoiesis manifested in the spleens of Ikaros DN−/− mice, we examined whether hemopoietic progenitors developed in this secondary hemopoietic site. Splenocytes from these mutants were unable to radioprotect even when provided at a dose of 1.2 × 107 cells (data not shown). This observation suggests that there is no shift in the production or expansion of hemopoietic progenitors from the BM to the spleen and that extramedullary hemopoiesis at this site is due to the differentiation of more committed and short-lived erythromyeloid precursors.

Figure 2. Short-term radioprotection and repopulation ability is unaffected by the Ikaros null mutation but severely compromised by the Ikaros DN mutation. (A) Short-term radioprotective ability of wild-type and Ikaros mutant BM. Ly5a congenic mice were lethally irradiated (9.5 Gy) and injected with 105 wild-type BM (■), 106 Ikaros null−/− BM (□), 106 Ikaros DN−/− BM (▲), or PBS alone (○). The numbers of mice injected per donor group are given in the legend, and their age was 3–4 wk. (B) Donor contribution in the myeloid lineage after transplant with wild-type and Ikaros mutant BM. Ly5a congenic mice were lethally irradiated and injected with 106 wild-type BM (left panel, hatched bars), or 6 × 105 Ikaros DN−/− BM (right panel, hatched bars). At the indicated time points after transplant, recipients were bled and assayed for donor contribution to the myeloid (Mac-1+) lineage by FACS™ analysis. A pool of four wild-type or mutant mice was used as a donor population. The experiment was repeated twice, and data shown are the analysis of one representative mouse out of eight recipients. (C) Development of lethal anemia in recipients of Ikaros DN−/− BM. 5 × 106 Ikaros DN−/− (●) or wild-type (□) BM cells were transplanted into lethally irradiated recipients, and recipient hematocrits were monitored over 5 wk. R recipients of Ikaros DN−/− BM (●) eventually died of anemia 5 wk after transplant. Numbers of mice bled were as follows: +/- recipients, n = 12; and Ikaros DN−/− recipients, n = 28. Donors were 2–4 wk old.

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Competitive Repopulating Units Are Reduced among Ikaros Mutant Hemopoietic Cells. The LTR potential of Ikaros null and DN⁻/⁻ BM was analyzed in a competitive repopulation assay (23, 24). Ikaros mutant Ly5b BM cells were injected into lethally irradiated Ly5a recipients along with a constant competitor dose of 10⁵ Ly5a marrow. When Ikaros null BM (10⁵) was transplanted with an equal amount of wild-type competitor marrow, it failed to contribute to myeloid (Mac-1⁺) cells in the BM or the periphery of recipients (Fig. 3 A, top right panel). In sharp contrast, wild-type BM contributed to 44% of the BM myeloid (Mac-1⁺) populations (Fig. 3 A, top left panel). Ikaros null BM injected in 10-fold excess (10⁶) over wild-type competitor gave 17% contribution to the myeloid (Mac-1⁺) lineage in the BM (Fig. 3 A, center panels). No significant contribution to the T cell lineage was observed in the thymus or the spleen (data not shown). In comparison, wild-type BM coinjected at a similar dose contributed to >99% of the myeloid (Fig. 3 A, center panels) and lymphoid (data not shown) populations in the BM and periphery. When 7.5 × 10⁶ Ikaros null BM cells were injected alongside 10⁵ competitor BM cells, donor-derived contribution to the myeloid lineage increased to 75.3% (Fig. 3 A, bottom panels). This analysis indicates that there is a 30–40-fold decrease in LTR activity in the Ikaros null BM.

In a previous study (17), we reported short-term myeloid lineage contribution by Ikaros DN⁻/⁻ BM when injected at a ratio of 100:1 over competitor BM. At this ratio, Ikaros DN⁻/⁻ BM contributed to 52% of myeloid (Mac-1⁺) cells by 19 d after transplant, but by day 27, this contribution declined to 6% and by day 48 to 3% (Fig. 3 B). No donor-derived myeloid contribution was detected 120 and 260 d after transplant (Fig. 3 B). PCR analysis of DNA prepared from the blood, spleens, and BM of recipients also failed to detect donor-derived cells, confirming the lack of LTR activity in Ikaros DN⁻/⁻ BM (data not shown).

Thus, in the absence of Ikaros, LTR activity as estimated in competitive repopulation assays is reduced by 30–40-fold. However, a >100-fold reduction in LTR activity is observed in mice homozygous for the DN Ikaros mutation.

BM Engraftment into Unconditioned Ikaros Mutant Recipients. Lethal irradiation of normal murine recipients before BM

![Figure 3](image-url)
transplant is required to ensure engraftment and detection of donor-derived hematopoietic cells. However, if the hematopoietic system of the recipient is already compromised, then donor-derived hematopoiesis is detectable even in the absence of prior conditioning of the recipient. This phenomenon has been observed with c-kit receptor (W) mutant mice (25). Given the depletion in hematopoietic progenitors resulting from the null and DN Ikaros mutations, we attempted to repopulate unconditioned Ikaros mutants with wild-type BM.

A transplant of 10^6 wild-type Ly5a BM cells into unconditioned Ikaros null mice completely repopulated the B cell lineage, consistent with the total lack of B cells and their precursors in Ikaros mutants (Fig. 4 A, Ly5b^+/B220^+ cells). Donor repopulation of T cells and some myelocytes was also observed, albeit at low levels, possibly due to competition with endogenous precursor populations (Fig. 4 A, Ly5b^-/TCR-^a^+/^b^- and Ly5b^-/Mac-1^-/Gr-1^-).

Ikaros DN homozygous mutants and heterozygous littermates were injected with 10^6 wild-type Ly5a BM cells at 12 d of age and subsequently analyzed at 4, 12, and 22 wk after transplant. No detectable donor contribution in any hematopoietic lineages was observed after transplant of wild-type Ly5a BM into unconditioned Ly5b Ikaros DN heterozygotes (Fig. 4 B, top panels, Ly5a^-/lin^+ cells). In striking contrast, complete donor repopulation of B and T lymphocytes and myeloid cells was observed in unconditioned Ikaros DN^-/- mice receiving 10^6 wild-type BM cells (Fig. 4 B, bottom panels, Ly5a^+/B220^+, Ly5a^-/TCR-^a^-/^b^+, and Ly5a^-/Mac-1^-/Gr-1^-), which persisted for the full duration of the experiment. To detect potential hematopoietic contribution by endogenous Ikaros DN^-/- mutant cells at levels that may fall below the detection limit of FACS™, PCR analysis of DNA prepared from various hematopoietic tissues was employed (Fig. 4 C). Given a PCR DNA detection limit of 1:1,000 mutant/wild-type DNA (Fig. 4 C), we conclude that host-derived hematopoietic cells were at least 1,000-fold less frequent in unconditioned Ikaros DN^-/- recipients after transplant of wild-type BM.

Thus, the hematopoietic system of Ikaros null and DN^-/- mice is readily repopulated by wild-type HSCs without the need for prior myeloablative conditioning. This repopulation of unconditioned Ikaros mutant mice by wild-type HSCs reflects a severe depletion in the endogenous pool.

Reduction in CFU-S in Ikaros Mutant Mice. The more
committed myeloid precursor content of Ikaros mutant mice was investigated in a spleen colony-forming assay (CFU-S14) (26). CFU-S14 levels in Ikaros null and DN−/− BM were reduced 9.2- and 81-fold relative to wild type (Fig. 5 A). Splenic CFU-S14 was also reduced by three- and fivefold in Ikaros null and DN−/− mice relative to wild type (Fig. 5 A). The combined limb BM and splenic CFU-S14 contents of Ikaros null and DN−/− mice were reduced 7.7- and 34-fold relative to wild type. Although these data assume similar spleen seeding efficiencies by wild-type and Ikaros mutant CFU-S, we have not tested this hypothesis.

Qualitative effects of the Ikaros mutations on the size and lineage composition of day 14 spleen colonies were also observed. First, spleen colonies derived from Ikaros mutant cells were smaller than those derived from wild-type populations (Fig. 5 A, bottom panels, arrows). Second, Ikaros mutant colonies contained 20–40% of nucleated erythroid (TER-119+ ) cells, whereas wild-type colonies consisted mainly (76–91%) of more mature erythroid cells (data not shown). The donor origin of the CFU-S14 colonies was confirmed by PCR (data not shown). Interestingly, a small number of endogenous colonies was detected in recipients of Ikaros mutant BM and spleen but not in recipients of wild-type populations or in PBS controls. A possible facilitating effect is suggested, perhaps in the form of growth factors produced by the Ikaros mutant cells that promote expansion and differentiation of endogenous progenitors.

The CFU-S14 content of day 10 YS and AGM and day 14 fetal livers of Ikaros DN−/− mutant and wild-type embryos was also measured. The CFU-S14 content in these late embryonic and early fetal sites of hemopoiesis was drastically reduced in Ikaros DN−/− embryos compared with wild type (Fig. 5 C). Thus, the lack of Ikaros, coupled with interference toward the activity of other family members, affects the production of both fetal and adult hemopoietic progenitors.

Effects of the Ikaros mutations on In Vitro Clonogenic Precursors. BM and splenic hemopoietic populations were analyzed for their content of myeloid-restricted in vitro CFCs. The absolute number of precursors giving rise to multilineage colonies (CFC-multi) and G- and/or M-restricted colonies (CFC-G/M) in Ikaros null BM was within the range of

![Figure 5](image-url)
wild-type values (Fig. 6 A). In contrast, the absolute number of mature erythroid-restricted clonogenic precursors (CFU-E) was reduced to 30% of wild-type levels (Fig. 6 A). Furthermore, the most immature erythroid-restricted precursors (BFU-E) were reduced to 5% of wild-type numbers.

In the spleens of Ikaros null mice, all CFC classes, including CFU-E, BFU-E, CFC-G/M, and CFC-multi, were reduced in absolute number (Fig. 6 A). However, the frequencies of all CFCs, with the exception of BFU-E, were higher than in wild type (Fig. 6 B) due to the observed decrease in spleen (and BM) cellularity in Ikaros mutants.

A more severe reduction in the absolute number of all CFCs was detected in Ikaros DN−/− mice. BFU-E cells were the most drastically affected in BM and spleen, with absolute numbers <1% of wild type (Fig. 6 A). CFU-E cells were also reduced, but to a lesser extent than BFU-E. A CFU-E reduction to 5% of wild type was noted in the BM and to 25% of wild type in the spleen. In the BM, the absolute number of CFC-G/M cells was reduced to 15% of wild-type levels, reflecting the reduction in BM cellularity. Thus, the CFC-G/M frequency was similar to that of wild-type BM. However, in the Ikaros DN−/− spleen, CFC-G/M numbers were greatly increased (greater than twofold), indicating a dramatic increase in granulomonocytic precursors in this normally lymphoid organ (Fig. 6 A). Nonetheless, no increase in splenic erythroid precursors (BFU-E and CFU-E) was detected, suggesting specific effects of the Ikaros mutation on the expansion of myeloid versus erythroid precursors (see Fig. 8).

Given the BM hypoplasia and abnormal splenic hematopoiesis manifested in both Ikaros mutants, the combined total BM and spleen content of hematopoietic precursors was calculated per mouse (Fig. 6 C). CFU-E cells are reduced 10-fold in the Ikaros null and 30-fold in Ikaros DN−/− mice (Fig. 6 C). These BFU-E reductions are in line with the reductions seen in the most primitive HSC population.

However, the total number of the later erythroid precursors (CFU-E) was reduced only three- and sixfold in Ikaros null and DN−/− mice, respectively. Furthermore, in both Ikaros mutants, CFC-G/M cells were reduced to 40–75% of wild-type levels, a less severe decrease than that observed for other CFC classes.

Although the numbers of myeloid precursors in the spleens of the Ikaros null and DN−/− mice (with the exception of CFC-G/M) are decreased, the number of terminally differentiated erythroid (TER-119+) and myelomonocytic (Mac-1+/Gr-1−) cells is increased in this organ (10, 11). This suggests that the splenic microenvironment is conducive to the rapid differentiation of myeloid precursors to their mature progeny.

Lack of BM cells with the Lin−c-kit+Sca-1+ stem cell phenotype in Ikaros DN−/− mice. Hematopoietic cells that lack expression of mature lineage markers (lin−) and that coexpress Sca-1+Ly6A and the tyrosine kinase receptor c-kit on their cell surfaces (lin−c-kit+Sca-1+) are highly enriched in HSC activity in normal mice (27, 28). The HSC-enriched lin−c-kit+Sca-1+ population was detected in Ikaros null BM (Fig. 7 A, center). In sharp contrast, no c-kit+/Sca-1+ cells were present among the lin− BM cells of Ikaros DN−/− mice (Fig. 7 A, right). The lack of BM cells with a lin−c-kit+Sca-1+ surface phenotype in Ikaros DN−/− mice is consistent with the dramatic depletion in HSC activity in these mutant mice.

Analysis of lin− BM cells from Ikaros null and DN mice revealed a progressive decrease in the cell surface expression level of c-kit (Fig. 7 B). Given the importance of the steel factor/c-kit signaling pathway in the expansion and differentiation of hematopoietic progenitors (29), we predict that the decreased expression of c-kit in Ikaros mutant hematopoietic cells may underlie some of the progenitor defects.

Molecular analysis of Ikaros mutant hematopoietic progenitors. To address the molecular basis of the hemolymphoid deficiencies in Ikaros mutant mice, we analyzed expression of growth factor receptors, signaling molecules, and transcription factors known to be important in the production, maintenance, and differentiation of hematopoietic cells.

Figure 6. Dynamics in myeloid differentiation in Ikaros mutant mice. (A) Proportion of absolute CFCs recovered from BM and spleens (Sp) of Ikaros null and DN−/− mutants relative to wild type (dashed line) is shown. The data represent the average ±SEM from a total of eight animals per genotype. Precursor classes are defined in Materials and Methods. (B) Frequency of CFCs (i.e., number per 10⁵ cells) in Ikaros and wild-type BM and spleens is shown. Data are standardized to wild-type frequencies, represented by the dashed line. (C) The combined CFC content of BM and spleen relative to wild type is shown. CFC content for the entire body BM compartment is included in the calculation by assuming that one femur represents 10% of total BM.
Ikaros null and wild-type populations but at a somewhat reduced level compared with wild type (Fig. 7C, Flk1). The mRNA level of the tyrosine phosphatase Shp-1, a downstream effector of the c-kit signaling pathway, was also determined and found not to be significantly different between Ikaros mutant and wild-type populations (Fig. 7C, Shp1).

Expression of several transcription factors known to play key roles in the development of the hemopoietic system was also determined. Ikaros mutant lin- cells were analyzed for the presence of SCL, GATA-1, GATA-2, GATA-3, and PU.1 transcripts. A small decrease in SCL levels was seen in the Ikaros null c-kit hi and c-kit loSca-1+ populations (Fig. 7C, Scl). Small changes in GATA-2 but not GATA-1 levels were also seen among the Ikaros null and DN-/- hemopoietic populations, the most dramatic being an increase in GATA-2 levels in the c-kit hi cells in the Ikaros DN-/- BM. These possibly reflect changes in hemopoietic progenitor composition in the lin- compartment. GATA-3 is expressed among the lin- hemopoietic cells, possibly in progenitors undergoing specification along the T cell lineage. GATA-3 was expressed within lin- progenitor populations of Ikaros null mice, which generate T cell precursors, however, it was not detected in Ikaros DN-/- lin- populations lacking T cell differentiation potential, reflecting either lack of the relevant precursor population or lack of expression of the GATA-3 factor in these cells. Finally, levels of PU.1 are reduced in the c-kit hi population of the Ikaros null mutants but elevated in the same population in Ikaros DN-/- BM. The Ikaros mutant lin- hemopoietic populations were also analyzed for expression of mRNAs encoding hemopoietic growth factors and receptors. In the c-kit lo populations of Ikaros null and in the c-kit lo- populations of Ikaros DN-/- BM, levels of GM-CSF receptor were significantly elevated relative to wild type, which could be the cause or effect of the increase in GM precursors observed in clonogenic assays.

**Discussion**

We have previously shown that Ikaros is an essential regulator of lymphoid lineage specification in the fetal and adult hemopoietic system. Ikaros is, however, expressed early during ontogeny of the hemopoietic system and is detected at high levels in mesodermal progenitors in the splanchopleura of the day 8 embryo and within the blood islands of the YS (reference 18 and Georgopoulos, K., unpublished results). In addition, in adult hemopoietic sites, Ikaros is expressed within cell populations that are highly enriched in HSCs (15, 32). A detailed analysis of the entire hemopoietic hierarchy in Ikaros mutant mice is presented here, revealing a role for Ikaros in the production or activity of the self-renewing HSC. These studies also provide evidence for a functional interplay between Ikaros and other nuclear factors, including family members during differentiation of the most primitive of hemopoietic progenitors.

Mice homozygous for an Ikaros null or DN mutation show a decrease in BM cellularity that cannot be ascribed solely to the lack of B cell differentiation. In addition, an
increase in erythromyeloid precursors is detected in the spleens of Ikaros mutant mice, an observation not made in other strains of mice depleted of lymphocytes. The progressive development of anemia in mice homozygous for the Ikaros DN/2 mutation, manifested as a progressive drop in hematocrit, is also indicative of a failure of the hematopoietic system to supply mature erythrocytes at normal levels.

BM progenitors from Ikaros null mice provide short- and long-term contribution to most of the hematopoietic lineages, apart from the lymphoid, when transferred alone into lethally irradiated recipients. However, when measured against wild-type BM in a competitive assay, a severe depletion in both short- and long-term repopulating activities is revealed. Ikaros null BM is unable to contribute to any hematopoietic lineages when competed against wild-type congenic BM at a ratio below 10:1. Even when present in a >10-fold excess, only limited hematopoietic contribution is observed from Ikaros null BM, suggesting a reduced number of HSCs in these mutants. In contrast, wild-type BM, when used at an excess of 10:1 over competitor, repopulates 100% of all hematopoietic lineages tested. Thus, there is a quantitative reduction estimated to be 30–40-fold in the number of HSCs in Ikaros null mice (Fig. 8). A greater reduction in HSC numbers is detected in mice homozygous for a DN Ikaros mutation. Ikaros DN/2 BM is unable to provide LTR in a competitive assay, even when used at 100-fold excess to wild-type congenic BM, indicating a severe depletion in stem cell activity below assayable levels. A limited short-term hematopoietic contribution from the Ikaros DN/2 BM was detected in both competitive and radioprotective assays, suggesting a transient expansion in short-term repopulating cells. Wild-type BM cells sorted according to the absence of cell surface lineage-specific markers and expression of c-kit and Sca-1/Ly6A are enriched for HSC activity (28). This population of cells was absent from the BM of Ikaros DN/2 mice, consistent with the depletion in both short- and long-term repopulating activity in these mutants.

The defect in hematopoietic progenitors within Ikaros mutants was also illustrated by the ability of wild-type BM to provide hematopoietic reconstitution to these animals without their prior conditioning. Transplant of wild-type BM cells into unirradiated Ikaros null mice resulted in chimeric animals in which some hematopoietic lineages, such as T, Mac-1+, and Gr-1 cells, all of which are unaffected by the Ikaros mutation, derive from both wild-type and Ikaros mutant precursors. B lineage cells and Gr-1 cells, neither of which are produced from Ikaros mutant progenitors, are derived exclusively from wild-type precursors. In Ikaros DN/2 mutants, reconstitution by wild-type BM without the need for prior conditioning is observed for all lineages. The ability of normal BM to function in Ikaros mutants suggests that the Ikaros defects are manifest within hematopoietic populations rather than in nonhemopoietic accessory cells of the BM and spleen.

Figure 8. Effects of Ikaros deficiency on the hematopoietic progenitor/precursor hierarchy. Commitment of a mesodermal precursor to the HSC fate, self-renewal (curved arrow), and subsequent progression through intermediate steps that involve long- and short-term repopulating progenitors and precursors to terminally differentiated progeny is illustrated. The mesodermal precursor commitment step as a possible target for the Ikaros deficiencies and a cause for the reduction in HSCs is indicated as the perpendicular intersection of two broken lines, which also indicates a possible defect in HSC renewal. Complete blocks in the differentiation of Ikaros-deficient precursors toward the B cell and NK lineages are shown as the perpendicular intersection of two solid lines. The increase in the differentiation output of these mutant precursor populations toward the erythromyeloid lineage is depicted with green arrows.
Evaluation of the more committed hemopoietic precursor compartment in Ikaros mutant mice reveals amelioration of their phenotypes. CFU-S₃₄ clonogenic assays were used as a measure of the multilineage erythromyeloid-restricted progenitors. An eightfold reduction in CFU-S₃₄ activity was detected in Ikaros null mice, whereas the reduction in Ikaros DN⁻/⁻ mice was 34-fold. In vitro clonogenic assays show that BFU-E, the earliest erythroid-restricted precursor, is reduced by 10- and 30-fold in Ikaros null and Ikaros DN⁻/⁻ mice, respectively. This reduction in BFU-E may in part explain the decrease in CFU-S₃₄, particularly as wild-type spleen colonies are comprised mainly of erythroid lineage cells. However, later stage erythroid precursors (i.e., BFU-E) are not as severely compromised, with only three- and sixfold reductions detected in Ikaros null and DN⁻/⁻ mice. Lack of Ikaros has an even lesser effect (25 and 50% decrease) on the number of myelomonocytic precursors, indicating their possible preferential expansion relative to BFU-E (Fig. 8). The decrease in CFU-S₃₄ and BFU-E activity in Ikaros mutant mouse strains is smaller than the estimated 30-100-fold decrease in LTR cells, suggesting the existence of partial compensatory mechanisms after HSC commitment. Alternatively, Ikaros may normally function in early hemopoietic progenitors to limit differentiation along the myeloid lineages, a constraint that is removed upon Ikaros inactivation (Fig. 8).

To determine if the hemopoietic defects manifested in Ikaros mutants are the result of a defect in the production or maintenance of HSCs, we examined the hemopoietic precursor content of embryonic and fetal hemopoietic sites. CFU-S₃₄ activities were measured in the AGM and the YS on day 10 and the fetal liver on day 14 of Ikaros DN⁻/⁻ embryos. CFU-S₃₄ activity was depleted in all three sites in Ikaros DN⁻/⁻ embryos. The effects of Ikaros mutation on fetal hemopoietic precursors closely match those seen postnatally in the BM and spleen. Depletion of fetal precursors in the Ikaros mutant embryos supports the idea that Ikaros regulates the production of hemopoietic progenitors during ontogeny of the hemopoietic system.

Molecular analysis of lin⁻ hemopoietic populations in Ikaros mutant mice revealed a severe reduction in expression of two tyrosine kinase receptors important for HSC development. Surface expression of c-kit is progressively reduced from Ikaros null to Ikaros DN⁻/⁻ hemopoietic cells, and mRNA expression of flk-2 is missing in both mutant populations. Flk-2 was originally identified as a tyrosine kinase receptor expressed in fetal liver populations enriched for HSCs (33). It has previously been reported that flk-2 null BM has reduced competitive repopulation activity against wild-type BM, suggesting a potential depletion in HSCs (30). In addition, the number of B cell precursors in the flk-2 null mice is reduced. Both flk-2 hemopoietic phenotypes correlate with those observed in Ikaros mutants. However, hemopoietic progenitors in Ikaros mutant mice appear to be more severely reduced in number, and B cell precursors are absent. It is possible that lack of the flk-2 tyrosine kinase receptor, compounded with a reduction in levels of cell surface c-kit, and possibly other unidentified factors, which are also required at the early stages of B, T, and HSC differentiation, may account for the more severe HSC and B cell phenotypes manifested in Ikaros mutant mice. Indeed, flk-2 and c-kit double mutants display a far more severe hemopoietic phenotype than that manifested by either mutations alone, resulting in early lethality (30). It is also significant that the difference in c-kit expression between Ikaros null and DN⁻/⁻ progenitors directly correlates with the severity in HSC defect manifested in these mutant mice. Expression of the T cell–determining transcription factor, GATA-3, is missing from the Ikaros DN⁻/⁻ mice but is present among the Ikaros null lin⁻ hemopoietic populations. GATA-3 expression, or lack thereof, correlates with the T cell differentiation potential of Ikaros mutants. Interference from the Ikaros DN isoforms toward the activity of other Ikaros-interacting factors expressed in the early hemopoietic progenitor compartment may impair GATA-3 expression in these cells and block their T cell differentiation potential. Finally, increase in expression of the myeloid-specific GM-CSF receptor within Ikaros mutant early hemopoietic progenitors may underlie the apparent expansion of the myeloid precursors and their progeny despite the dramatic reduction in hemopoietic progenitor numbers.

The progressive depletion in long- and short-term hemopoietic progenitors and T cell precursors from the Ikaros null to DN⁻/⁻ mice supports a role for Ikaros and its interacting factors (21) in the development and differentiation of HSCs. Ikaros proteins are all engaged in a higher order complex with family members and components of two distinct chromatin remodeling complexes (21). The proper nuclear compartmentalization and gene targeting of these chromatin remodeling complexes manifested in the presence of Ikaros and its family members are likely to be as critical for differentiation and homeostasis of the hemopoietic system as the Ikaros protein itself. The progressive depletion in long- and short-term hemopoietic progenitors and T cell precursors from the Ikaros null to DN⁻/⁻ mice may reflect progressive misregulation of the remodeling complexes in the nuclei of HSCs in the absence of Ikaros and through DN interference with the activity of its family members, the expression of which is not affected by the Ikaros mutations (data not shown).

Ikaros remodeling complexes may control hemopoietic lineage commitment decisions by potentiating expression of genes that include at least the tyrosine kinase receptors flk-2 and c-kit and possibly the transcription factor GATA-3, required for differentiation along the B and T cell lineages. We have recently shown that Ikaros proteins provide T cell activation thresholds (34), possibly by regulating changes in chromatin structure (21). Ikaros may thus control HSC activation by providing thresholds to signaling pathways for c-kit and other receptors. In the absence of Ikaros, the signaling thresholds for these pathways may be lowered, allowing HSCs and their progeny to enter the cell cycle more readily and possibly deplete the most primitive quiescent stem cell pool. Further studies on the cycling status of Ikaros mutant HSC and the effects of cell cycle–promoting factors on the hemopoietic compartment of Ikaros mutant mice will address this central question of self-renewal.
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