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Self-Renewal of the Long-Term Reconstituting Subset of Hematopoietic Stem Cells is Regulated by Ikaros

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ABSTRACT

Hematopoietic stem cells (HSCs) are rare, ancestral cells that underlie the development, homeostasis, aging, and regeneration of the blood. Here we show that the chromatin-associated protein Ikaros is a crucial self-renewal regulator of the long-term (LT) reconstituting subset of HSCs. Ikaros, and associated family member proteins, are highly expressed in self-renewing populations of stem cells. Ikaros point mutant mice initially develop LT-HSCs with the surface phenotype cKit+Thy1.1(lo)Lin(-/lo)Sca1+Flk2-CD150+ during fetal ontogeny but are unable to maintain this pool, rapidly losing it within two days of embryonic development. A synchronous loss of megakaryocyte/erythrocyte progenitors results, along with a fatal, fetal anemia. At this time, mutation of Ikaros exerts a differentiation defect upon common lymphoid progenitors that cannot be rescued with an ectopic Notch signal in vitro, with hematopoietic cells preferentially committing to the NK lineage. Although dispensable for the initial embryonic development of blood, Ikaros is clearly needed for maintenance of this tissue. Achieving successful clinical tissue regeneration necessitates understanding degeneration, and these data provide a striking example by a discrete genetic lesion in the cells underpinning tissue integrity during a pivotal timeframe of organogenesis. STEM CELLS *2009;27:3082–3092*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

A vital issue in stem cell biology is to understand the molecular mechanisms that control the self-renewal and differentiation of long-term hematopoietic stem cells (LT-HSCs) [1]. Identifying key molecular pathways has important implications for not only understanding normal organogenesis, tailoring tissue engineering for regenerative medicine, and cellular reprogramming, but also for cancer as a disease of aberrant organogenesis mainly through unbridled cell self-renewal [2]. Indeed, many of the regulatory genes found in HSCs largely also operate in leukemogenesis and, more generally, carcinogenesis [3, 4]. The majority of these genetic studies reporting adult stem cell function frequently require the generation of intricate stage- and tissue-specific conditional null alleles since traditional germline mutations in the stem/progenitor cells that underpin tissue development in a mouse can have highly deleterious effects on the organism [5, 6].

As a means by which to ascribe gene function in adult stem cells, and in particular the identification of critical protein domains, forward genetic phenotype screening of offspring from mice mutagenized with ethylnitrosourea produces highly subtle genetic alleles through DNA point mutation [7, 8]. Such a methodology provides greater specificity for assigning sequence-to-cell function and was recently used to identify DNA break repair enzymes as limiting for HSCs during aging [9]. Also employing this strategy, mutation of Ikaros, a transcription factor largely associated with lymphopoiesis [10], was found to cause a more widespread hematopoietic defect [11]. An analysis of differential isoform [12] and transgenic marker [13] expression in purified subsets

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described Ikaros' role in regulating developmental decisions during early hematopoiesis, while unfractionated mutant bone marrow (BM) [14] and fetal liver (FL) [11] transplants showed that HSC activity was impaired. This defect could have been due to any one of numerous causes including (a) a lack of HSCs, or a defect in (b) HSC migration, (c) the HSC niche, (d) HSC proliferation, (e) HSC differentiation for the preferential choice of some cell fates ahead of others, or (f) HSC self-renewal. Here, we show that Ikaros is required for self-renewal of the LT reconstituting pool of HSCs through a high-resolution analysis of stem and progenitor cells in FL. Compared to adult BM HSCs, FL LT-HSCs boast numerous superior features, such as higher in vivo engraftment levels [15, 16] and broader developmental spectrum [17, 18]. Given the precise timing of these changes, intrinsically-timed genetic switches in LT-HSCs are likely to be tightly coordinated during development, with Ikaros an attractive molecule underlying this ontogenic advantage [19]. Here, we utilize the recently identified SLAM marker CD150 [20, 21] on LT-HSCs with the well established cKit+Thy1.1(lo)Lin(−/lo)Sca1+ (KTLS) phenotype [22] to examine the effects of a site-specific Ikaros point mutation on the self-renewing pool of LT-HSCs.

Materials and Methods

Mice

C57BL/6-Ka, -Thy1.1, and -*Plstc* strains were maintained at Stanford University's Research Animal Facility in accordance with animal ethics guidelines. Mice used were 8-12 weeks old. For FLs, the morning of vaginal plug observation was E0.5.

Flow Cytometry

Before sorting, stem/progenitor cells from FL/BM were prepared by lineage depletion with Dynabeads M-450 (Dynal, Oslo, Norway, www.invitrogen.com/site/us/en/home/brands/Dynal.html) or cKit-enrichment with streptavidin-conjugated magnetic beads (Miltenyi, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com). Unconjugated lineage mAbs were B220 (clone 6B2), CD3 (2C11), CD4 (GK1.5), CD5 (53-7.3/7.8), CD8 (53-6.7), Gr1 (8C5), Mac1 (M1/70), and TER119. Mac1 was only used in the Lin cocktail for BM [15] and IL7Rα (A7R34) included for myeloid progenitors. The Lin− depleted cells were labeled with Tricolor- or PE Texas Red-conjugated goat anti-rat IgG (Caltag, Burlingame, CA, www.invitrogen.com) and stained with stem/progenitor cell markers: Sca1 (E13-161-7), cKit (2B8), Thy1.1 (19XE5), Flk2 (A2F10) (eBioscience, San Diego, CA, http://www. ebioscience.com), CD150 (TC15-12F12.2) (Biolegend, San Diego, CA, http://www.biolegend.com), IL7Rα, CD34 (RAM34) (BD Pharmingen, San Diego, CA, http://www.bdbiosciences.com), and FcγR (CD16/32) (2.4G2) (93) (eBioscience). Immature B cell fractions had the common Lin-B220+IgM-NK1.1− phenotype and CD43+CD19− (Fraction A), CD43+CD19+ (Fraction B), and CD43−CD19+ (Fraction C). Lineage cocktails for sorting non-stem/progenitor cells were as follows: immature B cell: CD3, Gr1, Mac1, TER119; pro T-cell: CD3, CD4, CD8, B220, CD19 (1D3) (BD), GL3 (BD), CD11c (HL3) (BD), Gr1, Mac1; and neutrophils: CD3, B220, TER119, Sca1. NK cells were sorted/analyzed with NK1.1 (PK136) (BD) and CD44 (IM7) (eBioscience), and immature B cells were sorted with CD43 (S7) (BD) and IgM [11–26] (eBioscience). Unless otherwise indicated, all mAbs were prepared in I.L.W. Lab. Cells were analyzed/sorted on an LSRII, FACSAria, or highly-modified FACSVantage cytometer (BD, Mountain View, CA, http://www.bdbiosciences.com). All cells were at least double-sorted. Dead cells were discriminated by high forward scatter and propidium iodide staining. Fluorescence-activated cell sorting (FACS) data was analyzed using FlowJo (Tree Star, Inc., Ashland, OR, http://www.treestar.com).

Cell Culture

OP9 BM stromal cells expressing the Notch ligand Delta-like1 (OP9-DL1) and OP9-control (gifts from Juan Carlos Zúñiga-Pflücker) were maintained in minimum essential medium α-MEM (InVitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 50 μg/ml gentamycin, and 10% heat-inactivated fetal calf serum in a humidified 5% CO_2 incubator at 37°C. FL cells (E12.5: 10,000/well; E14.5: 2,000/well) were homogenized in α-MEM and plated onto freshly plated OP9-DL1 stromal cells on a 24-well plate. The culture was supplemented with 1 ng/ml Flt3L, 2 ng/ml IL15, 1 ng/ml IL6, and 5 ng/ml IL7.

Gene Expression

Total RNA was isolated using TRIzol (InVitrogen) from equivalent cell numbers, digested with DNase I to remove DNA contamination, and used for reverse transcription (SuperScript II kit, Invitrogen). All reactions were performed in triplicate in an ABI-7000 (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) using SYBR Green (Applied Biosystems) and cDNA equivalent of ~500 cells. Fold expression relative to whole BM was calculated following β-actin transcript normalization. Primer sequences are included in supplemental online Table S1.

Statistics

Data were analyzed for significance between groups using a two-tailed Student's *t*-test. Differences were considered significant at $p < .05$.

Results

Expression of Ikaros Family Members Throughout Hematopoiesis

The Ikaros protein family has been reported to play a critical role in blood cell development, including at the level of the least differentiated cells [11–14]. In particular, the ENU-induced point mutant allele strain *Plastic* (*Plstc*) showed that Ikaros exerts a broader effect on hematolymphoid differentiation, extending well beyond its recognized function in lymphocyte development and homeostasis [7]. *Plstc* homozygotes were strikingly anemic and lethal at E15.5 with a complete failure of FL to engraft hematopoiesis in irradiated recipients [11].

We first sought to garner insight as to how the expression of *Ikaros* and its family member genes fluctuate during adult hematopoiesis starting from LT-HSCs through to mature cells. In total, 25 cell populations were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) and expression levels compared to mean fold change relative to mouse whole BM (Table 1). The hematopoietic stem and multipotent progenitor cell subfractions within the KLS population were sorted with the more well established cell surface markers Thy1.1 [22] and CD34 [23] in conjunction with Flk2 [24, 25]. Within both purified LT-HSC populations, *Ikaros* mRNA (encoded by the gene *Zfpn1a1*) was expressed (~6- and ~4-fold change relative to whole BM for (KLS)Thy1.1(lo)-Flk2− and (KLS)CD34-Flk2− cells, respectively). Relative to all other primitive cell subsets, transcript levels from two other family members, *Helios* (*Zfpn1a2*) [26] (347- and 122-fold change) and *Eos* (*Zfpn1a4*) [27] (2,216- and 2,772-fold change), were observed in both LT-HSC subsets. Interestingly, both *Helios* and *Eos* expression (140- and 19,300-fold

Table 1. Expression levels of Ikaros family member genes during hematopoiesis as measured by quantitative real-time polymerase chain reaction (qRT-PCR)

Population	*Ikaros*	*Aiolos*	*Helios*	*Eos*	*Pegasus*
ES cells	1.3	0.0	140	19,300	110
LT-HSC(KLS Thyl.1(lo)F1k2−)	6.1	0.1	347	2,216	5.6
LT-HSC (KLS CD34−F1k2−)	4.1	0.1	122	2,772	7.4
ST-HSC (KLS CD34+F1k2−)	1.1	0.0	69	350	4.5
MPP (KLS Thyl.1−F1k2+)	1.1	0.0	55	1.5	2.5
MPP (KLS CD34+F1k2+)	8.6	0.0	69	3.1	6.4
CLP	5.8	0.0	0.1	83	3.0
CMP	3.0	0.0	39	0.9	3.2
GMP	0.9	0.1	42	1.4	9.1
MEP	12.7	0.1	104	1.5	9.0
B cell, Fraction A	12.2	0.0	18.3	0.8	3.9
B cell, Fraction B	1.3	2.7	3.7	174	7.0
B cell, Fraction C	2.3	3.6	10.1	3.5	2.1
B cell, B220+IgM+IgD−	3.0	39	0.0	2.6	5.2
B cell, B220+IgM+IgD+	9.2	41	0.1	2.7	3.0
pro Ti (cKit+CD44+CD25−)	5.3	0.1	105	0.9	5.2
pro T2 (cKit+CD44+CD25+)	8.6	0.0	157	1,280	5.4
pro T3 (CD44-CD25+Thyi.i+)	1.9	2.0	510	4,686	8.2
pro T4 (CD44−CD25−Thy1.1+)	20.3	25.3	225	292	5.7
CD3(hi)CD4+CD8+double positive	8.9	22.1	268	2.6	11.9
CD3(hi)CD8+ single positive	5.9	25.7	513	6,920	8.1
CD3(hi)CD4+ single positive	7.1	10.5	255	8,067	9.5
Lin−cKit−Gri+Maci+	0.7	0.0	0.0	0.8	1.5
cKit−TER119+	2.9	0.0	0.2	9.3	52.0
NK1.1+	2.5	5.9	10.4	1.5	1.1

Expression levels in all 25 populations listed above are shown as mean fold change relative to mouse whole BM (expression set to 1.0) for each gene. qRT-PCRs (with 3-actin housekeeping control) were performed using the cDNA equivalent of 500 purified cells with triplicate measurement of at least two independently double- or triple-sorted populations. Stem and progenitor cells were sorted according to the gates described in Figs. 1A, 2A, and 2C. All cells were sourced from adult mouse BM (8-12 weeks old) with the exception of the two mature 1gM-expressing B-cell subsets (spleen), all seven T-cell subsets (thymus), and natural killer cells (spleen). ES cells were mouse D3 cells.

changes, respectively) were also seen in embryonic stem cells, another self-renewing stem cell population, but were dramatically lower in the next differentiation subset immediately downstream of LT-HSCs, which had lost LT self-renewal ability. *Pegasus* (*Zfpn1a5*) [27] showed a similar expression trend, albeit with less striking oscillations across all populations of cell subsets analyzed. By contrast, *Aiolos* (*Zfpn1a3*) was not turned on in any stem/progenitor cell population analyzed and showed the highest mRNA expression levels in mature B (∼40-fold change) and T-cells (10- to 25-fold change) in agreement with its predominant functions in vivo [28]. The most uniform pattern of expression of *Helios* transcripts was in purified T-cell subsets, consistent with its established role in T lymphopoiesis [26]. In agreement with its key function in erythropoiesis [11, 29], the highest progenitor level of *Ikaros* mRNA expression was in megakaryocyte/erythrocyte progenitors (MEPs) (∼13-fold change), with marked expression also in differentiated erythroid cells (cKit-TER119+; ∼3-fold change). *Helios*, *Eos*, and *Pegasus* expression was also observed in the erythroid lineage, with *Helios* expression levels within the three myeloid progenitor subsets the highest of all family members analyzed relative to mouse whole BM. As expected, *Ikaros* expression was also seen in common lymphoid progenitors (CLPs) (∼6-fold change), with concomitant upregulation of *Helios* and *Aiolos* expression in NK lineage cells. Together, these results clearly show that Ikaros and its binding partners are expressed at the population level throughout all stages of hematopoietic development and particularly in self-renewing stem cells and transit-amplifying progenitors.

Ikaros Is a Genetic Regulator of KTLS(CD150+) Cells

Having observed high *Ikaros* expression in the LT subset of HSCs that was rapidly extinguished in the daughter short-term (ST)-HSC population, we next sought to directly analyze the stem cell phenotype in *Plstc* mice. We chose to use two independent positive LT-HSC markers (Thy1.1 and CD150) and so first backcrossed *Plstc*/+ heterozygotes expressing the Thy1.2 genotype to C57BL/6-Thy1.1 mice. A preliminary assessment of HSCs via the KLS compartment at E14.5-15.5 suggested the *Plstc* mutation caused the stem cell pool to significantly expand with *Plstc*/+ heterozygotes and *Plstc*/*Plstc* homozygotes, boasting a 2- and ∼4-fold increase in KLS cells, respectively (Fig. 1A, 1C). This step-wise expansion in the stem cell-containing pool intimated that the *Plstc* allele positively exerted a gene dosage effect on HSCs and potentially acted as a neomorphic allele in these cells. A more detailed examination revealed a similar stepwise increase in all frequency subsets within the KLS subfraction, including KTLS LT-HSCs (Fig. 1D). These results were surprising since they were completely at odds with the established notion of loss of *Ikaros* function exerting a deleterious effect on the HSC pool [11, 14]. Incorporating CD150 to further dissect the KTLS compartment of LT-HSCs showed there was a stepwise and significant reduction in a clear KTLS(CD150+) cellular population: these cells normally comprised 18% of the KLS compartment (wild-type) but were 9% in *Plstc*/+ heterozygotes and ∼0% in *Plstc*/*Plstc* homozygotes (Fig. 1A). As numbers, the KTLS(CD150+) subfraction normally comprised

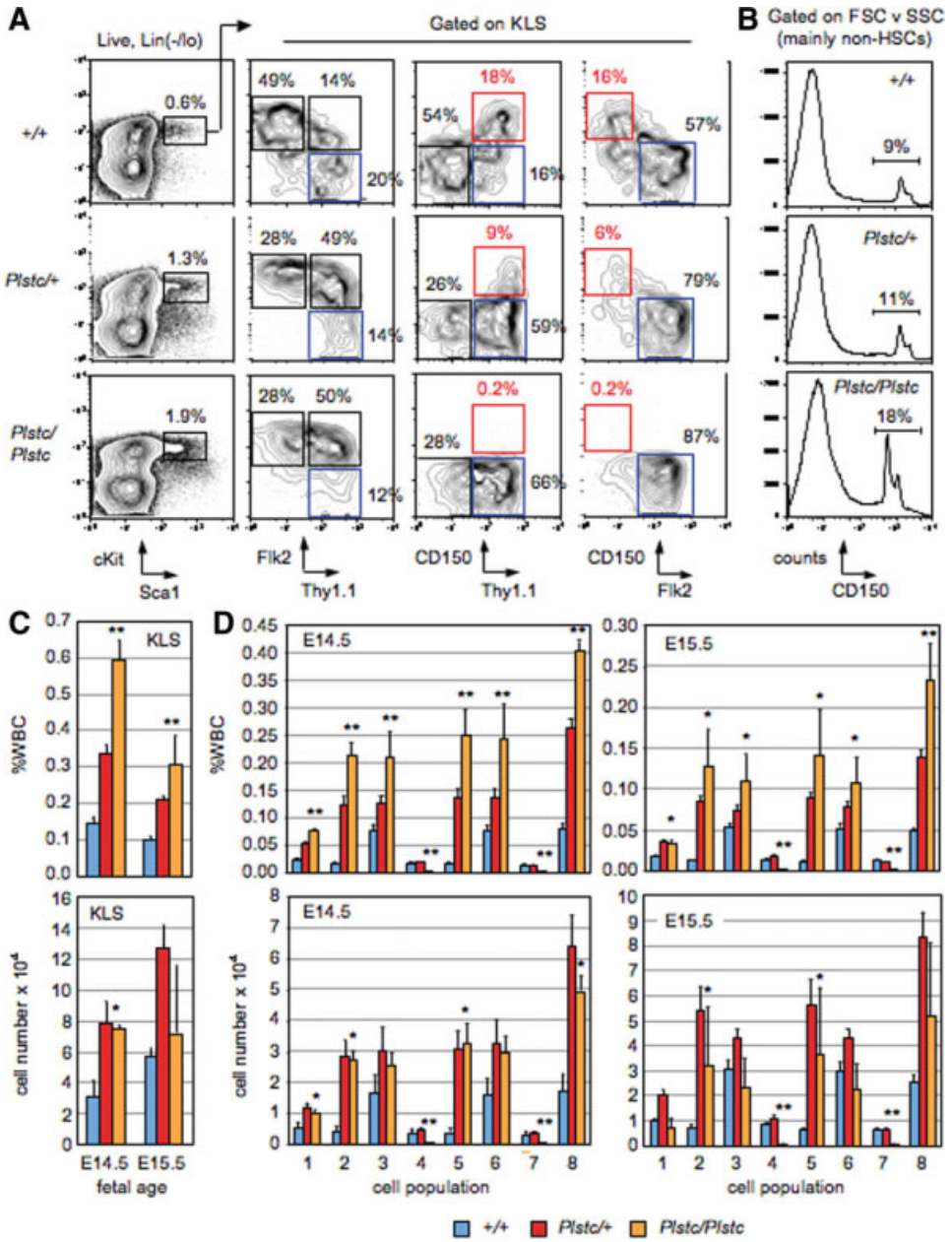

Figure 1. *Plastic* homozygotes selectively lack cKit+Thy1.1(lo)Lin(-/lo)Sca1+ (KTLS)(CD150+) long-term (LT)-HSCs, but all other KLS subsets are expanded. **(A):** Expression of HSC markers cKit, Sca, Thy1.1, Flk2, and CD150 in wild-type +/+, *Plstc*/+, and *Plstc*/*Plstc* embryos. The percentage of KLS cells within the gates is shown, with the exception of the Live, Lin(−/lo) plot (left column), which shows percentage of total white blood cells (WBCs). A representative E14.5 fetal liver (FL) is shown. **(B):** Expression of CD150 on FL cells gated only according to FSC vs SSC. Although this fraction contains HSCs, it is predominantly non-HSCs. Percentage total WBCs is shown. A representative E14.5 FL is shown. **(C):** Quantitative analysis (mean ± s.e.m.) of KLS cells in E14.5-15.5 FL shown as percentage total WBCs and cell number. All data are from more than eight *Plstc*/*Plstc* mutants and their sibling controls from three separate experiments. Statistically significant differences ($p < .05$) between *Plstc*/*Plstc* and +/+ groups are indicated by an asterisk (*); double asterisk (**) is significance between *Plstc*/*Plstc* and both +/+ and *Plstc*/+ groups. **(D):** Quantitative analysis (mean ± s.e.m.) of cellular subfractions within the KLS compartment in E14.5-15.5 FL shown as percentage total WBCs and cell number. Populations shown within the KLS subcompartment are: (1) Thy1.1(lo)Flk2-; (2) Thy1.1(lo)Flk2+; (3) Thy1.1-Flk2+; (4) Thy1.1(lo)CD150+; (5) Thy1.1(lo)CD150-; (6) Thy1.1-CD150-; (7) Flk2-CD150+; and (8) Flk2+CD150-. Abbreviations: FSC, forward scatter; HSC, hematopoietic stem cell; SSC, side scatter.

around 3,000-4,000 cells in wild-type FL at E14.5 and 6,000-8,000 cells at E15.5, but in *Plstc* homozygotes, this was significantly reduced to a mere 400-500 cells, representing a 15- to 20-fold reduction in number ($p = .0003$ at E14.5) (Fig. 1D). By contrast, the KTLS(CD150-) ($p = .001$ at E14.5) and KTLS(Flk2+) ($p = .00003$ at E14.5) populations were significantly expanded in mutant FLs, although their absolute cell numbers were comparable. Overall, our quantitative analyses revealed that most mutant subpopulations of cells within the KLS fraction were expanded, with *Plstc*/+ heterozygotes giving intermediate reading. Strikingly, the only subset that was an exception to this trend of accumulated stem/multipotent progenitor compartments was those KTLS cells expressing CD150 (Fig. 1D). Although the CD150 promoter was found

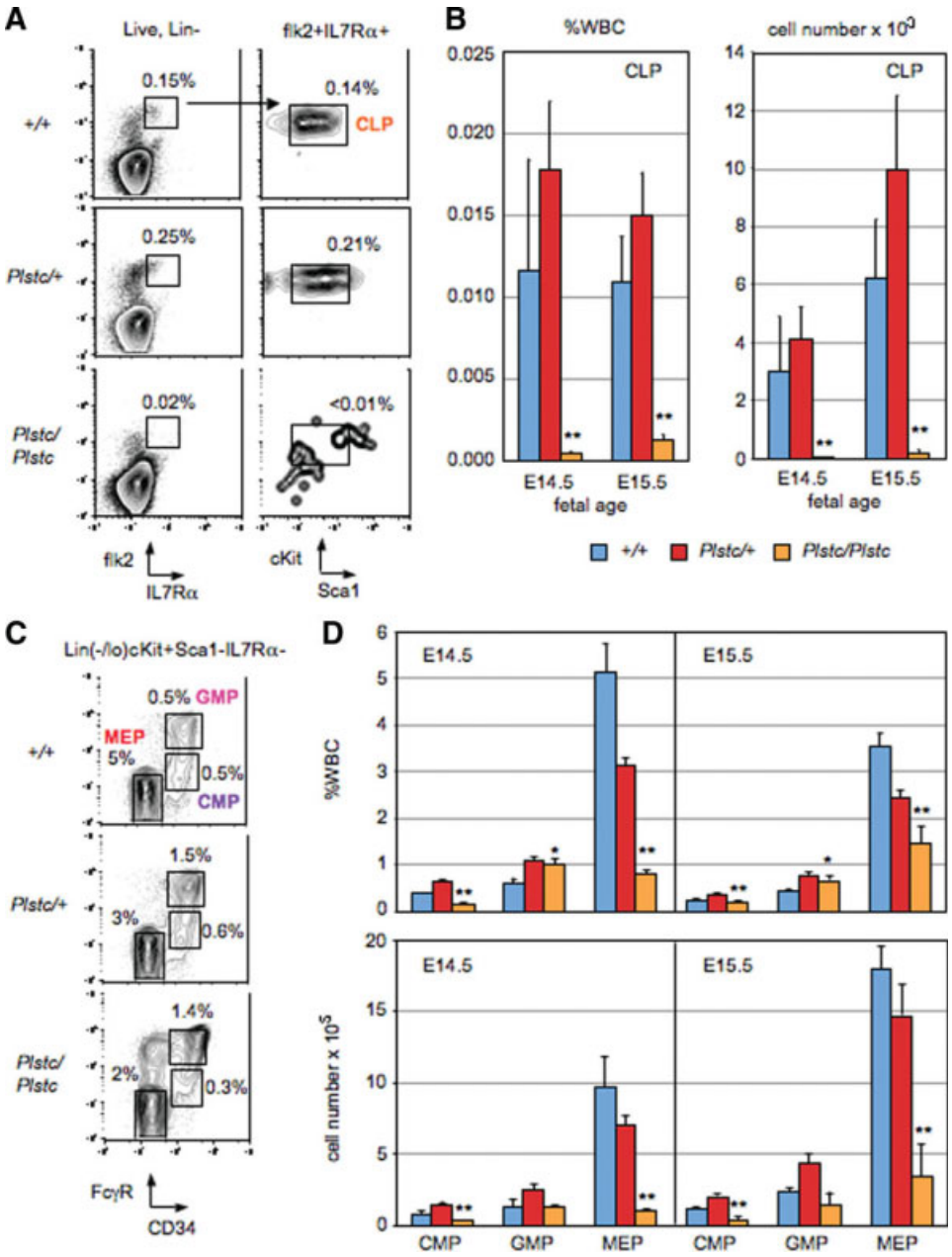

Figure 2. The *Plastic* point mutation most significantly affects CLP and MEP populations. **(A):** CLPs were gated as Lin-IL7Rα+Flk2+cKit(lo)-Sca1(lo) cells. Percentage total white blood cells (WBCs) within gates is shown. A representative E14.5 fetal liver (FL) is shown. **(B):** Quantitative analysis (mean ± s.e.m.) of CLPs in E14.5-15.5 FL shown as percentage total WBCs and cell number. Data are from more than eight *Plstc*/*Plstc* mutants and their sibling controls from three separate experiments. Statistically significant differences ($p < .05$) between *Plstc*/*Plstc* and +/+ groups are indicated by an asterisk (*); double asterisk (**) is significance between *Plstc*/*Plstc* and both +/+ and *Plstc*/+ groups. **(C):** Three populations of myeloid progenitors—common myeloid progenitors, granulocyte/macrophage progenitors, and megakaryocyte/erythrocyte progenitors—were gated according to their expression of FcγR and CD34 within the Lin(-/lo)cKit+Sca1-IL7Rα- subfraction. Percentage total WBCs within gates is shown. A representative E14.5 FL is shown. **(D):** Quantitative analysis (mean ± s.e.m.) of myeloid progenitors in E14.5-15.5 FL shown as percentage total WBCs and cell number. Abbreviations: CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; MEP, megakaryocyte/erythrocyte progenitor.

to contain several *Ikaros* consensus binding sites (data not shown), expression of CD150 was observed at normal levels (Fig. 1B) and numbers (data not shown) in other non-stem cell hematopoietic populations of *Plstc* cells. This indicated that, rather than acting as a general regulator of this signaling lymphocyte activation molecule (SLAM) family member, the point mutation in *Ikaros* was highly selective in its effect for only CD150 cells within the five-parameter KTLS(Flk2-) HSC fraction. This was a particularly unanticipated result since independent qRT-PCR experiments found *Ikaros* mRNA was not differentially expressed in purified KTLS(CD150+) and KTLS(CD150-) subsets (data not shown).

Ikaros in Progenitors

A recent study reported Ikaros was not required for the initial segregation of erythro- and lympho-myeloid progenitors but

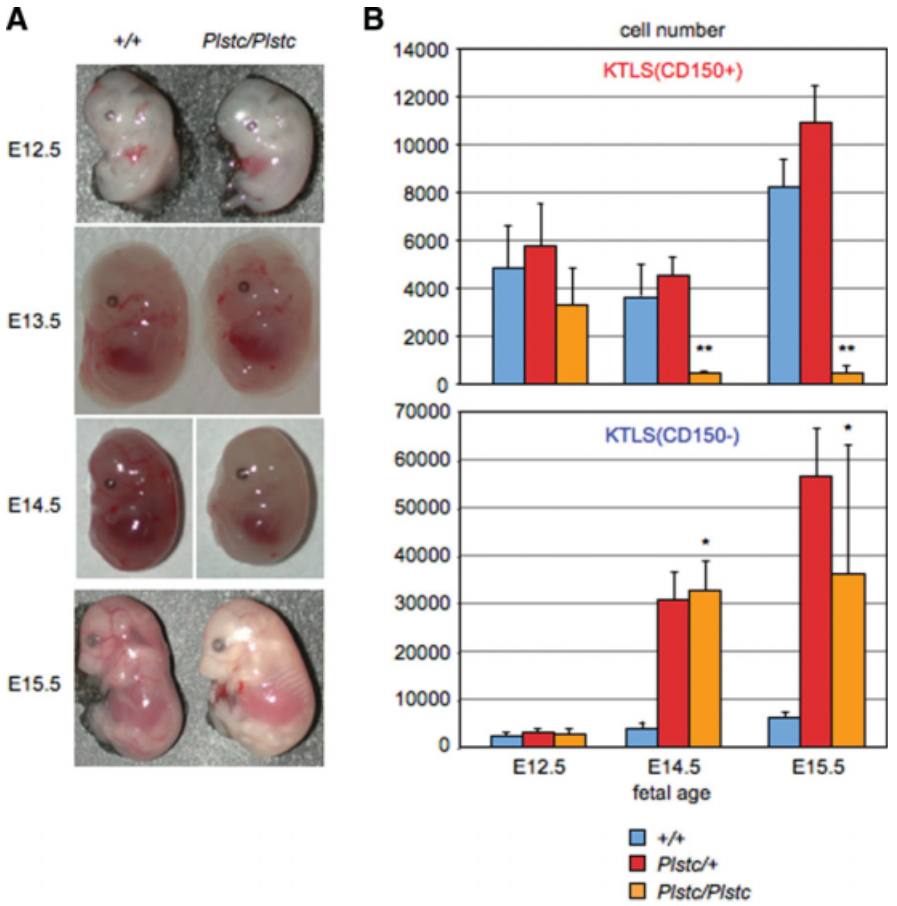

Figure 3. *Plastic* homozygotes initially appear phenotypically normal and have normal numbers of KTLS(CD150+) long-term hematopoietic stem cells that they selectively lose within two developmental days, becoming severely anemic and leaving accumulated numbers of KTLS(CD150-) cells. **(A):** Phenotype of *Plstc*/*Plstc* embryo and wild-type sibling at E12.5-15.5. **(B):** Quantitative analysis (mean ± s.e.m.) of KTLS(CD150+) and KTLS(CD150-) populations in E12.5, E14.5, and E15.5 FL shown as cell number. E14.5 and E15.5 data are replicated from Figure 1D. Data are from at least three *Plstc*/*Plstc* mutants and their sibling controls from at least two separate experiments. Statistically significant differences ($p < .05$) between *Plstc*/*Plstc* and +/+ groups are indicated by an asterisk (*); double asterisk (**) is significance between *Plstc*/*Plstc* and both +/+ and *Plstc*/+ groups. Abbreviations: KTLS, cKit+Thy1.1(lo)Lin(-/lo)Sca1+.

that it was essential for their subsequent cell fate choice [13]. To further elucidate Ikaros' role at these checkpoints during differentiation, we performed an analysis of lymphoid and myeloid progenitors in *Plstc* mutants. As anticipated, Flk2+ CLPs were significantly reduced with a barely detectable population at E14.5–15.5 compared to wild-type FLs ($p = .01$ at E14.5) (Fig. 2A, 2B). By contrast, the only myeloid progenitors to show comparable levels of cellular attrition were *Plstc*/*Plstc* MEPs (~20% of wild-type numbers) (Fig. 2C, 2D). Like HSC and multipotent progenitor (MPP) subsets, *Plstc*/+ mutants again had median cell numbers, further implicating a gene dosage effect. The common myeloid progenitor (CMP) fraction was also significantly decreased in *Plstc* homozygotes ($p = .02$ at E14.5). This was milder than, but consistent with, a defect along the megakaryoid/erythroid differentiation pathway ($p = .0001$ at E14.5) through which the CMP has been hierarchically modeled to act as a conduit. Unlike other precursors, percentages of granulocyte/macrophage progenitors (GMPs) were significantly increased in *Plstc* homozygotes ($p = .01$ at E14.5), consistent with prior analyses of Ikaros mutants [11, 13]. As with previous erythroid assays in vitro [11], methylcellulose colony assays also revealed largely normal colony-forming unit (CFU)-granulocyte/macrophage, CFU-granulocyte, and CFU-macrophage colonies produced from 100 FACS-purified wild-type or *Plstc*/*Plstc* mutant myeloid progenitors in the presence of stem cell factor, Flt3 ligand (Flt3L), IL3, IL11, and GM-CSF (supplemental online Figs. 1 and 2). The FcγR(hi)CD34− cellular subfraction, which formed a distinct and abundant cellular population in the *Plstc*/*Plstc* mutants, produced predominantly CFU-GM, CFU-G, and CFU-M colonies with a total plating efficiency of ~25%, consistent with commitment to the myeloid lineage (supplemental online Fig. 2). Therefore, this novel population appears to be GMP-like, showing decreased levels of CD34 expression based on its in vitro function. By morphology (stained cytospins of sorted FcγR(hi)CD34− cells), they appear undifferentiated and are indistinguishable from GMPs but also from other myeloerythroid progenitors (data not shown). Since CD34 can be cell cycle regulated and there seems to be a deregulation of myeloid cells in the mutant *Plastic* mice [11], we believe the FcγR(hi)CD34− population consists mainly of GMP-like cells. Together, these experiments indicate an active role for Ikaros in myeloid cell fate decisions, in particular along the erythroid pathway, and are consistent with earlier studies and the fatal fetal anemia to which *Plstc* homozygotes succumb during late gestation.

Ikaros Is a Bona Fide Self-Renewal Regulator of the Pool of KTLS(CD150+) LT-HSCs

The primitive wave of hematopoiesis generates differentiating erythrocytes very early to supply the developing embryonic circulation before the onset of the definitive hematopoietic wave including the LT reconstituting HSCs, which persist to adulthood [30, 31]. This left open the question of how *Plstc* homozygotes could boast a blood cell compartment containing downstream hematopoietic short-term stem/progenitor cells at E15.5 if they lacked the KTLS(CD150+) LT-HSC pool. We characterized this population, and not the KTLS(CD150-) subfraction, as containing the most robustly-engrafting, LT-reconstituting, self-renewing HSCs in a corresponding study using wild-type animals [32]. To investigate this question, we

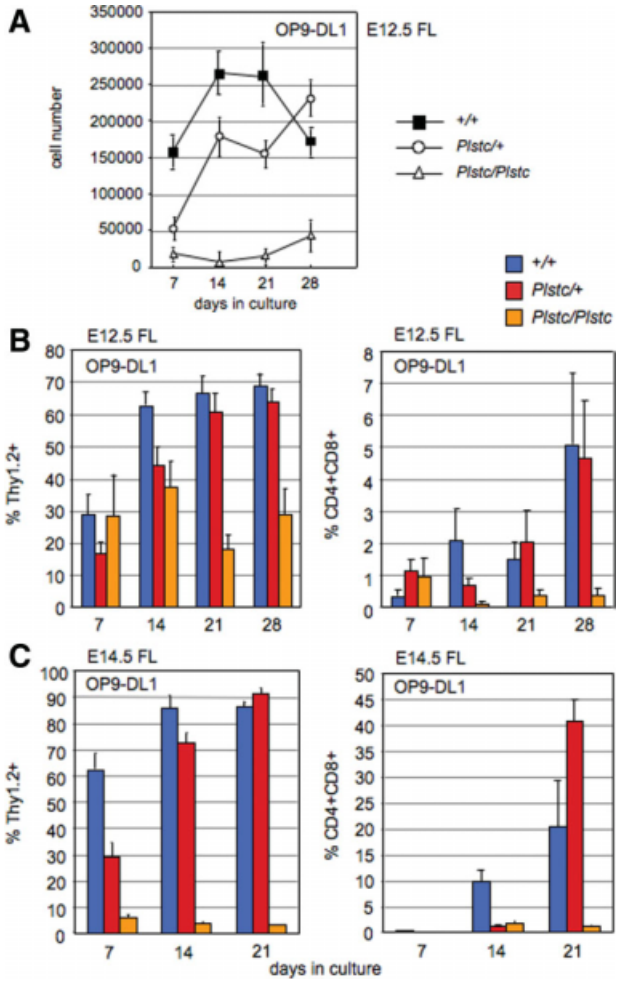

Figure 4. Composition of OP9-DL1 in vitro cultures for T lineage cells formed from wild-type and mutant E12.5 and E14.5 fetal livers (FLs). **(A):** Quantitative (mean ± s.e.m.) and kinetic analysis of total white blood cell (WBC) number of OP9-DL1 cultures from E12.5 FLs. **(B,C):** Quantitative (mean ± s.e.m.) and kinetic analysis of the lineage potential of E12.5 **(B)** and E14.5 **(C)** FLs showing the percentage of Thy1.2+ cells (of total WBCs) and CD4+CD8+ double-positive (of Thy1.2+ cells) after culture on OP9-DL1 stromal cells. Data are from 15 +/+. 28 *Plstc*/+ and nine *Plstc*/*Plstc* E12.5 mutants and 10 +/+. 13 *Plstc*/+ and three *Plstc*/*Plstc* E14.5 mutants from four separate experiments.

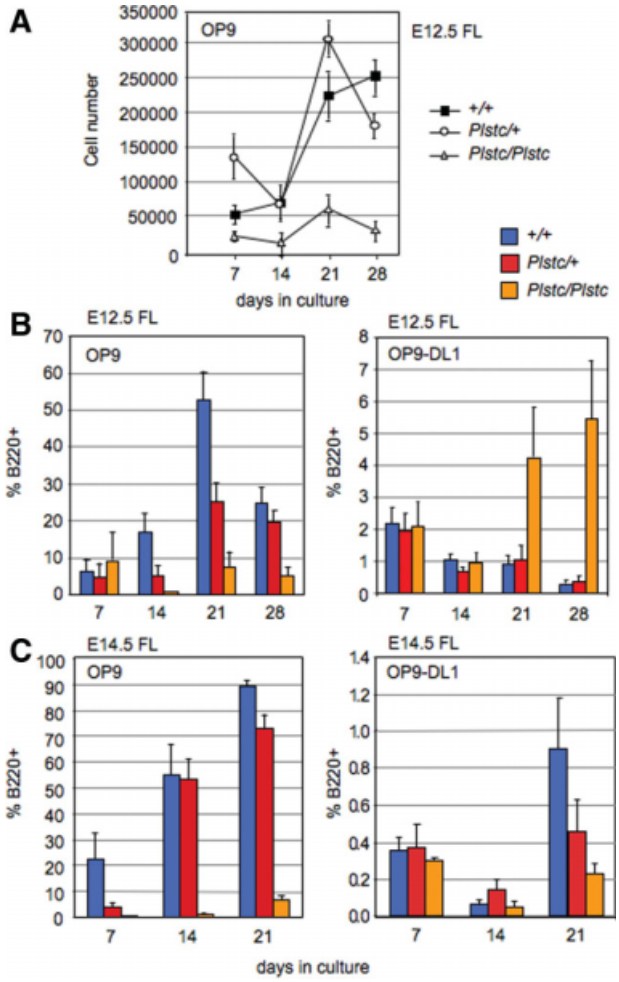

Figure 5. Composition of OP9 and OP9-DL1 in vitro cultures for B lineage cells formed from wild-type and mutant E12.5 and E14.5 fetal livers (FLs). **(A):** Quantitative (mean ± s.e.m.) and kinetic analysis of total white blood cell (WBC) number of OP9 cultures from E12.5 FLs. **(B,C):** Quantitative (mean ± s.e.m.) and kinetic analysis of the lineage potential of E12.5 **(B)** and E14.5 **(C)** FLs showing the percentage of B220+ cells (of total WBCs) after culture on either OP9 or OP9-DL1 stromal cells.

examined *Plstc*/*Plstc* embryos at earlier developmental timepoints and quantified their KTLS(CD150+) and KTLS(CD150-) subsets. The anemia associated with homozygosity of the *Plstc* allele only becomes apparent at E14.5-15.5 when fetuses rapidly appear pale and with a marked deficit of red cells in the FL and vitelline and umbilical circulations (Fig. 3A, supplemental online Fig. 3) [11]; if these embryos had a defect in primitive hematopoiesis, they would have died at a much earlier embryonic time. At E14.5–15.5, the KTLS(CD150+) subfraction is significantly reduced 15- to 20-fold in cell number, whereas KTLS(CD150−) cells and all other non-LT self-renewing populations are significantly expanded (Fig. 1D). By contrast, E12.5 *Plstc*/*Plstc* FLs were found to contain normal numbers of both KTLS(CD150+) and KTLS(CD150−) cells with no marked differences between mutants and wild-type (Fig. 3B). There was a small but statistically non-significant ($p = .28$) decrease in the number of *Plstc*/*Plstc* KTLS(CD150+) cells, potentially indicative of this self-renewing cellular compartment's impending depletion, but otherwise numbers were normal (~3,000-5,000 cells). Given that homozygous *Plastic* mutants contained a normal-sized pool of KTLS(CD150+) LT-HSCs at E12.5, which disappeared by E14.5-15.5 and left accumulated numbers of all non-LT self-renewing cellular subsets, including KTLS(CD150−) cells, this indicated that Ikaros has a crucial role in the molecular self-renewal machinery for the pool of the LT-reconstituting subset of HSCs (supplemental online Fig. 5). The phenotypic consequences of mutation were a rapid and fatal fetal anemia as a result of exhausted maintenance of the LT self-renewing HSC, leading to dysregulated erythroid cell differentiation and proliferation from reduced progenitors.

An Ectopic Notch Signal Fails to Rescue the Ikaros Lymphoid Developmental Defect in Primitive Hematopoietic Cells

There is compelling evidence of the interaction between Ikaros and Notch family members in the self-renewal of

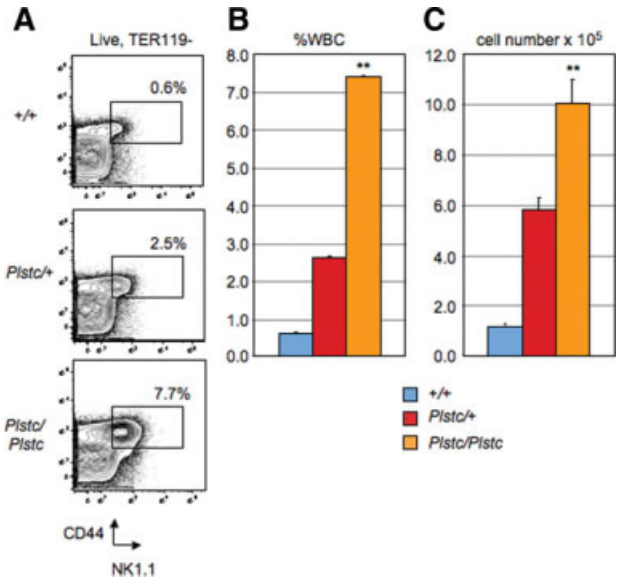

Figure 6. *Plastic* mutants have expanded NK lineage commitment. **(A):** Expression of NK1.1 and CD44 in Live, TER119- cells from E14.5 fetal livers (FLs) showing the percentage of total white blood cells (WBCs). A representative E14.5 FL is shown. **(B,C):** Quantitative analysis (mean ± s.e.m.) of NK1.1+CD44+ cells in E14.5 FL shown as percentage total WBCs (**B**) and cell number (**C**). Data are from five *Plstc*/*Plstc* mutants and their sibling controls from two separate experiments. Statistically significant differences ($p < .05$) between *Plstc*/*Plstc* and +/+ groups are indicated by an asterisk (*); double asterisk (**) is significance between *Plstc*/*Plstc* and both +/+ and *Plstc*/+ groups.

leukemic T-cells [33]. Our phenotypic analyses of *Plastic* showed that Ikaros regulates HSC self-renewal, while independent gain-of-function experiments have implicated Notch signaling as necessary for HSC maintenance [34]. Since the most pivotal developmental pathway from undifferentiated hematopoietic cells that requires a persistent Notch receptor-ligand interaction is T-cell development, we examined whether *Plastic* FLs containing HSCs and progenitors could respond to a Notch signal provided ectopically by the OP9-DL1 cell line [35]. Compared to wild-type and *Plstc*/+ littermates, E12.5 *Plstc*/*Plstc* FLs (containing predominantly HSCs) were severely crippled in their proliferative response to the Notch ligand DL1 with a five-fold reduction in total cell numbers generated after 28 days culture (Fig. 4A). Analysis of the small fraction of viable cells derived from the E12.5 *Plstc*/*Plstc* HSCs revealed that ~30% were arrested at the earliest CD4−CD8−CD44+CD25− (pro T1) stage of development (data not shown). Consistent with the subsequent depletion of the self-renewing HSC pool, culturing E14.5 *Plstc*/*Plstc* FLs with OP9-DL1 produced <5% Thy1.2+ cells present 21 days compared to 85--90% derived from wild-type or heterozygous HSCs/progenitors (Fig. 4C and supplemental online Fig. 4). *Plstc*/*Plstc* FL cells also showed a differentiation block when cultured on OP9 cells normally facilitating B-cell differentiation, consistent with the previously defined role for Ikaros in both T- and B-cell lineage differentiation (Fig. 5A). However, consistent with in vivo findings in thymii [11], only E12.5 *Plstc*/*Plstc* FLs diverted hematopoietic development to form B220+ cells at the expense of the Thy1.2+ cells in vitro in response to an ectopic Notch signal (Fig. 5B, 5C).

Ikaros Positively Regulates NK Lineage Commitment from Hematopoietic Progenitors

Our in vitro findings utilizing OP9-DL1 and OP9 stromal cell cultures supplemented with numerous cytokines including IL15 compelled us to finally examine in vivo whether primitive *Plastic* hematopoietic cells were preferentially developing into NK lineage cells instead of definitive lymphoid cells. Compared to wild-type littermate controls, *Plstc*/*Plstc* FLs showed a 10-fold expansion in the subset of NK1.1+CD44+ cells [36] at E14.5 according to both cell frequency (Fig. 6A, 6B) and number (Fig. 6C). *Plastic* heterozygotes showed an intermediate, stepwise phenotype, intimating that, as with the effect of Ikaros on HSCs (Fig. 1), the mutant *Plstc* allele positively exerted a gene dosage effect on NK cells.

Discussion

This study began with the question of what Ikaros' role was in regulating developmental decisions during early hematopoiesis; we knew that mutant Ikaros HSC activity in vivo was impaired [11, 14], and that Ikaros null mutants lacked the cKit+Lin(-/lo)Sca1+ (KLS) pool which contained the LT-HSC subfraction [14]. Here, we have shown Ikaros is a molecular regulator of the HSC pool and that it clearly distinguishes LT-HSC self-renewal from progenitor proliferation. Ikaros is most highly expressed in self-renewing fractions of stem cells, and point mutant mice selectively lack the six-parameter cKit+Thy.1.1(lo)Lin(−/lo)Sca1+CD150+Flk2− phenotype of LT-HSCs, which they fail to maintain and expand, while all non-self-renewing fractions of cells accumulate in number. An ectopic Notch signal could not rescue the Ikaros developmental defect in primitive hematopoietic cells, including self-renewing LT-HSCs, and these cells preferentially committed to the NK lineage in lieu of B and T lymphopoiesis.

A vast majority of HSC analyses focus upon the three-color KLS population. Our initial cursory observation of an accumulated KLS fraction in *Plastic* homozygotes at E14.5–15.5 was surprising given earlier reports that Ikaros was essential for maintenance of the LT-HSC pool. This data suggested that a point mutation in Ikaros had in fact caused an increase in HSCs even although previous experiments reported the lack of these cells. It is well established that the KLS fraction represents a heterogeneous cellular population containing LT- and ST-HSCs as well as MPPs. A depleted/expanded KLS fraction may thus represent depleted/expanded progenitors rather than a HSC-specific effect. In order to analyze LT-HSCs, this necessitates the use of more than three flow cytometric parameters. Beyond the KLS phenotype, our experiments with *Plastic* found the highly-characterized cKit+Thy1.1(lo)Lin(−/lo)Sca1+ (KTLS) fraction of cells is further heterogeneous for LT-HSCs, since these mutants had a population of KTLS cells at E14.5--15.5 that did not express CD150. Before utilizing CD150 in conjunction with the *Plastic* mutant, our observation of a population of cells characteristic as KTLS LT-HSCs was, as with the finding of an expanded KLS subfraction, irreconcilable with Ikaros' status as a stem cell regulator. The point mutation in *Ikaros*, along with wild-type in vivo assays, allowed us to positively identify the bona fide LT-HSC population. Our experiences with *Plastic* shows the value of genetic mutants in helping clarify lineage relationships [32] and underscores the importance of analyzing the complete stem cell phenotype.

Large-scale gene expression studies have varied in their success rate for identifying stem cell regulators. For example, despite strong indications that JamB/Jam2 is robustly expressed in multiple stem cell populations, functional studies of this gene failed to produce any stem cell phenotypes [37]. Differences in input cells and bioinformatics protocols and the complexity of alternatively spliced transcripts in stem cells [38] have been highlighted as underpinning these variable findings. When we analyzed the splice isoform distribution of Ikaros in stem and progenitor cells using earlier cell-sorting protocols, the transition from LT-HSC to ST-HSC/MPP came with the addition of Ikaros isoforms, while the transition to lymphoid precursors and lymphocytes, as well as neutrophils, added another isoform (Ik-6) lacking DNA binding sequences [12]. On the basis solely of the transcriptional repertoire, Ikaros would not have emerged as a candidate stem cell gene: it was robustly expressed in purified LT-HSCs (defined as KLSCD34− Flk2−) in one [39] of two [21] microarray studies by our laboratory. A similar pattern was observed for the polycomb group member Bmi-1, which has a significant role in HSC self-renewal [40, 41]. Furthermore, although frequently overlooked, genes expressed at relatively low levels in stem cells may in fact be the most important regulators of regeneration by virtue of (a) a stem cell's uncommittedness to any particular developmental lineage and (b) their coordinated connectivity within mammalian protein networks and extensive combinatorial assembly to produce phenotypes [7]. Appropriate mutations that selectively alter key protein domains, particularly in stem cells, are pivotal for dissecting links between genome sequence and phenomic diversity. This was recently shown for the non-homologous end-joining DNA Ligase IV enzyme that, through hypomorphic point mutation, was identified as a sensitive regulator of HSC stress over time when all previously generated null mutants were early embryonic lethal [9].

We found that *Plstc*/*Plstc* FLs fail to undergo significant T-cell differentiation in response to an ectopic Notch signal provided by the OP9-DL1 stromal line in vitro, and in vivo preferentially commit to the NK lineage. These findings indicate that Ikaros has potentially two distinct functional roles to play during T/NK-cell lineage commitment from HSCs and progenitors. First, the expression of Ikaros in the BM niche confers to the thymus-seeding progenitor cell an ability to respond to a Notch1 signal that directs the commitment to the T/NK-cell lineage, presumably through promoting the transcription of Notch target genes [42]. Second, at the double-positive T-cell stage, Ikaros is required to repress Notch1 signaling and prepare cells for positive and negative selection [43] (Y.S. and G.F.H., unpublished data, June 26, 2009), and a failure to silence Notch signaling at this checkpoint can facilitate leukemogenesis.

Plastic harbors a point mutation in Ikaros exon four, and the full-length DNA-binding isoform Ik-1 is generated and localizes to its normal cellular niche but fails to bind DNA [11]. This isoform is normally expressed in a broad range of purified populations including LT- and ST-HSC, MPP, CLP, pro-B- and -T-cells, and neutrophils [12]. However, since LT-HSCs specifically expressed only Ik-1 and -2, both of which contain exon 4, while more severely-truncated isoforms appeared in the differentiation subsets downstream of LT-HSCs [12], our in vivo findings corroborate with the *Plastic* allele having its greatest corruptive effect at the LT-HSC level. Ikaros and its family member Aiolos have been shown to operate along with other regulatory factors in a developmentally stage-specific manner during discrete stages of B-cell differentiation [44]. In this instance, the abundance of each factor determined regulatory outcome, with, for example, Ikaros' chromatin structure, and therefore its potential to be expressed at certain concentrations, posited as key to the activation/silencing of genes under its control. In line with reports of less compacted chromatin in undifferentiated stem cells compared with differentiated cells [45], a transcriptionally-permissive environment featuring an interplay of multiple lineage-affiliated expression programs in HSCs [46, 47], and Ikaros' role as a DNA binding moiety of several chromatin remodeling complexes and bivalent role as both an endogenous gene repressor/activator, it seems likely that Ikaros operates via a similar mechanism in HSCs to antagonize other proteins (whether family members or other as yet unidentified regulators) and direct commitment to particular daughter progenitors. On the basis of expression trends and the extensive interaction between Ikaros and its family proteins through heterodimerization, family members such as Helios and Eos would be attractive candidates. A recent study by Georgopoulos and colleagues has shown that Ikaros operates via such a mechanism by, on the one hand, promoting lymphoid priming in HSC and in lymphoid-primed multipotent progenitors (LMPPs) and, on the other hand, preventing the expression of stem cell genes in LMPPs [48]. Red-cell formation is notable in this respect since KTLS(CD150+) LT-HSCs have a highly accessible erythroid-specific *Gata1* locus [30]. Gata1 is essential for formation of the β-globin active chromatin hub [49], and chromosome conformation capture assays have found that Ikaros plays an essential role in the formation of this complex [29]. The development of refined chromatin immuno precipitation (ChIP) assays on low cell numbers including miniChIP [50] means it will be interesting to examine the epigenetic features of KTLS(CD150+) LT-HSCs and progenitors as they stepwise differentiate toward mature erythroid cells from both wild-type and *Plastic* mutants. Similarly, more detailed CD150 promoter studies via ChIP will be valuable to determine whether Ikaros directly regulates the CD150 gene, specifically in only LT-HSCs or more generally within hematopoietic tissues. Given the critical role of Ikaros in HSC self-renewal that we have shown here and the synchronous regulation of the erythroid lineage as revealed by anemia in *Plastic* homozygotes, it will also be important to examine whether Ikaros exerts a concomitant bivalent regulatory mechanism within progenitor cells primed to erythro/megakaryopoiesis in addition to lymphopoiesis. As we previously noted [12], alternate Ikaros isoforms in different hematopoietic subsets might identify and play a role in silencing loci, or possibly act as tags for chromatin opening complexes to identify regulatory regions, similar to bipotential chromatin marks, or both [48]. Other mouse mutants with phenotypes resembling *Plastic* may also be required to connect Ikaros target genes within molecular pathways in HSCs and progenitors.

Conclusion

The data presented here provide clear evidence of a critical and dynamic role for Ikaros throughout the hematopoietic hierarchy, most pertinently in the KTLS(CD150+) LT-HSC subfraction from which definitive blood cells begin developing during embryogenesis. Althoughdispensable for the initial embryonic development of blood, Ikaros is clearly needed for maintenance of this tissue. Elucidating precisely how this factor mechanistically controls self-renewal and cell fate choice remains a vital future area of research, in addition to the identification of new switches and components controlling the genetic circuitry of normal and cancer stem cells.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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