Functional Analysis of Human Hematopoietic Stem Cell Gene Expression Using Zebrafish

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Although several reports have characterized the hematopoietic stem cell (HSC) transcriptome, the roles of HSC-specific genes in hematopoiesis remain elusive. To identify candidate regulators of HSC fate decisions, we compared the transcriptome of human umbilical cord blood and bone marrow CD34⁺CD33⁻CD38⁻Rho⁻c-kit⁻ cells, enriched for hematopoietic stem/progenitor cells with CD34⁺CD33⁺CD38⁺Rho⁺c-kit⁺ cells, enriched in committed progenitors. We identified 277 differentially expressed transcripts conserved in these ontogenically distinct cell sources. We next performed a morpholino antisense oligonucleotide (MO)-based functional screen in zebrafish to determine the hematopoietic function of 61 genes that had no previously known function in HSC biology and for which a likely zebrafish ortholog could be identified. MO knockdown of 14/61 (23%) of the differentially expressed transcripts resulted in hematopoietic defects in developing zebrafish embryos, as demonstrated by altered levels of circulating blood cells at 30 and 48 h postfertilization and subsequently confirmed by quantitative RT-PCR for erythroid-specific hbae1 and myeloid-specific lcp1 transcripts. Recapitulating the knockdown phenotype using a second MO of independent sequence, absence of the phenotype using a mismatched MO sequence, and rescue of the phenotype by cDNA-based overexpression of the targeted transcript for zebrafish spry4 confirmed the specificity of MO targeting in this system. Further characterization of the spry4-deficient zebrafish embryos demonstrated that hematopoietic defects were not due to more widespread defects in the mesodermal development, and therefore represented primary defects in HSC specification, proliferation, and/or differentiation. Overall, this high-throughput screen for the functional validation of differentially expressed genes using a zebrafish model of hematopoiesis represents a major step toward obtaining meaningful information from global gene profiling of HSCs.

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Introduction

Hematopoiesis is the process by which hematopoietic stem cells (HSCs) give rise to all hematopoietic lineages during the lifetime of an individual. To sustain lifelong hematopoiesis, HSCs must self-renew to maintain or expand the HSC pool [1], and they must differentiate to form committed hematopoietic progenitor cells (HPCs) that progressively lose self-renewal potential and become increasingly restricted in their lineage potential. A combination of extrinsic and intrinsic signals is thought to converge to regulate HSC differentiation versus self-renewal decisions, but the molecular mechanisms that regulate these processes are poorly understood [2].

A multitude of cytokines have been cloned that affect HSCs and HPCs; however, to date none of these, alone or in combination, can induce the symmetrical, self-renewing HSC cell division in vitro that is required for HSC expansion. Recently, several novel regulators of HSC fate decisions have been identified. For instance, overexpression of Hoxb4 results in expansion of murine and human HSCs with increased competitive repopulation potential [3–5]; novel extrinsic regulators implicated in self-renewal of HSCs include Notch [6], Wnt [7,8], and the morphogens, SHH (sonic hedgehog) [9] and BMP4 (bone morphogenetic protein 4) [9]. While the discovery of these novel regulators provides credence to the hypothesis that extrinsic and intrinsic signals can influence HSC fate, a more global gene and/or protein expression analysis of human HSC should provide additional insight into pathways that support HSC self-renewal.

Our current understanding of the expressed gene profile of HSCs comes primarily from murine HSCs that can be purified to near homogeneity [10–14]. The inability to purified human HSCs to similar degrees of homogeneity makes study of the transcriptome of human HSCs more difficult. Human HSCs and HPCs are CD34 positive, while cells that engraft in severe combined immunodeficiency (SCID) mice are enriched for CD34⁻/CD38⁻/c-kith⁻ cells, enriched for hematopoietic progenitor cells with CD34⁺/CD33⁺/CD38⁺/Rho⁺.
in the CD34+/Lin- CD38- fraction [15]. As fewer than 1/500 CD34+/Lin+ CD38+ cells can repopulate SCID mice [15], the expressed gene profile of CD34+/Lin- CD38- cells is likely only partially enriched for HSC-specific genes [12,16]. We previously demonstrated that the rhodamine (Rho) 123+/c-kit+ subpopulation of CD34+/Lin- CD38- cells (Rho+) cells are highly enriched for primitive HPCs with myeloid-lymphoid initiating cell (ML-IC) capacity relative to CD34+/CD38+ CD33+ Rhohi (Rhohi) cells [17]. Thus, such selection separates CD34+/Lin- CD38- cells into HSC-enriched and HSC-depleted populations.

We hypothesized that comparison of the transcriptome of Rhohi and Rholow cells from umbilical cord blood (UCB) and bone marrow (BM) should identify conserved genes and gene pathways that define the human HSC. Because of the inherent limitations of using gene expression data to infer biological function, we also assessed the hematopoietic role of these genes in a high-throughput in vivo functional genomics screen in the zebrafish. Using this strategy we have not only identified a series of genes that may represent novel regulators of human HSC fate decisions, but this work also represents, to our knowledge, the first example of a functional genetic screening strategy that is a critical step toward obtaining biologically relevant functional data from global gene-profiling studies.

**Results/Discussion**

**ML-ICs Are Highly Enriched in Rhohi Compared to Rholow Cells**

The study of human HSCs has been limited since the CD34+/Lin- CD38- fraction of hematopoietic cells, commonly used as an HSC-enriched population, contains fewer than 0.2% SCID-repopulating cells [15], suggesting considerable heterogeneity. We have shown that ML-ICs, single hematopoietic cells that can generate several daughter cells capable of reinitiating long-term myeloid and long-term lymphoid cultures, are highly enriched by selecting the Rhohi fraction of CD34+/Lin- CD38- cells. While the Rhohi population still only contains 15%–25% ML-ICs and therefore remains heterogeneous, the enrichment factor is 5- to 10-fold greater than CD34+/Lin- CD38- cells [17]. Similar to our previous studies, the ML-IC frequency was greater than or equal to 10-fold higher in UCB Rhohi compared to Rholow cells (Figure S1).

**Genes Differentially Expressed between Rhohi and Rholow Cells from Both UCB and BM**

We hypothesized that comparing genes differentially expressed between Rhohi and Rholow cells from ontogenically distinct sources would identify conserved genes and gene pathways that govern self-renewal and differentiation of human HSCs. The experimental design used is illustrated in Figure 1. We defined differentially expressed probe sets as those with \( p < 0.05 \), using a paired Student’s \( t \)-test. By taking into account the variability present in primary cell populations, this provides a more accurate analysis of differential gene expression compared with the commonly used fold change cutoff.

We identified 2,707 and 4,667 probe sets differentially expressed between Rhohi and Rholow cells from UCB and BM, respectively (see Tables S1 and S2). The fidelity of our microarray results was confirmed using quantitative RT-PCR (Q-RT-PCR) (see Figure S2). We focused our further analysis on 277 unique transcripts, represented by 304 probe sets that were differentially expressed between Rhohi and Rholow cells from both UCB and BM with a fold change greater than 1.5 in either UCB or BM (Table S3).

Among the conserved genes enriched in Rhohi cells, many have been implicated in early hematopoiesis, including CDKN1A, a cell cycle regulator required for maintenance of murine HSCs [18], and ABCB1, the ABC-transporter family member responsible for the Rhohi phenotype [17]. Several transcription factors (TFs) known to play a role in early hematopoiesis or leukemogenesis were also identified, including HLF, involved in leukemogenic chromosomal translocations [19] and EVII, a TF associated with myeloid leukemias [20]. Other TFs without a known role in hematopoiesis were also more highly expressed in Rhohi cells, including HMGA2, a high-mobility group gene, and the zinc finger TFs ZNF165, ZNF331, and KLF5. All Rhohi-enriched genes are listed in Table S3. As demonstrated in previous HSC gene-profiling studies [12–14], more than 40% of genes enriched in Rhohi cells lack a functional annotation, are hypothetical proteins, or are expressed sequence tags, and thus may represent currently uncharacterized regulators of HSC fate decisions (Figure S3).
Some genes with well-established roles in HSC self-renewal and early differentiation are not present in the Rho<sup>lo</sup> enriched gene list. However, most of these were differentially expressed in both datasets, but differences did not reach statistical significance. For instance, LMO2 [21] and GATA2 [22], known to be involved in HSC development and self-renewal, were expressed significantly higher in BM Rho<sup>lo</sup> than in Rho<sup>hi</sup> cells. Although similar trends were seen in UCB Rho<sup>lo</sup> cells, these differences were not statistically significant (Table S4). Conversely, HOXB4 [4] expression was significantly higher in UCB Rho<sup>lo</sup> than in Rho<sup>hi</sup> cells, but this difference was not statistically significant in BM. Although our stringent criteria for differential expression likely contribute to the omission of some genes that might be differentially expressed, another explanation might be that expression of these genes is maintained when cells differentiate from a Rho<sup>lo</sup> to a Rho<sup>hi</sup> stage. The latter is consistent with the fact that most known HSC-associated genes were expressed at much higher levels than the normalized average microarray expression level in Rho<sup>lo</sup> and in Rho<sup>hi</sup> cells from both UCB and BM (Table S4).

Conserved genes enriched in the Rho<sup>hi</sup> cells included LEF1, an effector of Wnt signaling expressed in pre-B and T cells [23], and NOTCH2, involved in hematopoietic differentiation cell-fate decision [24]. Several TFs known to play a role in hematopoietic cell differentiation were more highly expressed in Rho<sup>hi</sup> compared to Rho<sup>lo</sup> cells, including HELLS and MAFB [25,26]. Additional TFs with no known role in hematopoietic development were also enriched in Rho<sup>hi</sup> cells, such as the zinc finger homeobox gene, ZFHX1B and the polycomb genes, EZH2 and SUZ12, the latter required for germ cell development [27]. Globin (Hb) gene family members were also more highly expressed in UCB and BM Rho<sup>lo</sup> than in Rho<sup>lo</sup> cells. Consistent with the ontogenic expression patterns of fetal versus adult Hb genes, Hb<sub>f</sub> genes were more highly expressed in perinatal UCB Rho<sup>hi</sup> cells, while Hb<sub>a</sub> genes were more highly expressed in adult BM Rho<sup>hi</sup> cells. Additional genes enriched in Rho<sup>hi</sup> cells are listed in Table S3.

Because of functional redundancy among gene families, we examined the data for common differentially expressed gene family members. The Id family of transcriptional repressors [28] was enriched in the Rho<sup>lo</sup> fraction, but was represented by different family members in UCB (ID4) and BM (ID1, ID2, and ID3). Similarly, various H1 and H2 histone genes were enriched in the Rho<sup>lo</sup> fraction in both datasets, but were represented by distinct family members.

We also evaluated whether common differentially expressed genes were concentrated on specific chromosomes. We found that genes were not only concentrated on certain chromosomes, but at specific g-band addresses. Of the genes enriched in Rho<sup>lo</sup> cells, 9% reside at 6p21, a region involved in recurrent chromosomal translocations in myeloid [29] and lymphoid [30] leukemias, and home to the PIM1 oncogene [31]. Six members of the H2B and one member of the H1 histone family, as well as CDKN1A, more highly expressed in Rho<sup>lo</sup> than Rho<sup>hi</sup> cells, reside at 6p21. The remaining Rho<sup>lo</sup>-enriched genes at 6p21 consist of six class II major histocompatibility complex (MHC) family members and a putative testis-specific zinc finger TF, ZNF165. H1 and H2 histone gene family members [11,13], class II MHC antigens [12,13], and CDKN1A [13] were also found among the genes identified in studies characterizing the transcriptome of murine HSC. The differential expression of such a large number of genes located at this chromosomal address suggests that, like CDKN1A, other genes located at 6p21 with as yet unknown hematopoietic function may play a role in HSC proliferation or differentiation.

We also compared the genes expressed more highly in Rho<sup>lo</sup> versus Rho<sup>hi</sup> cells with published gene expression data.
Comparison with the study by Ivanova et al. [12] that compared human CD34\(^+\)/Lin\(^-\)CD38\(^-\) with CD34\(^+\)/Lin\(^-\)CD38\(^-\) cells, yielded only seven genes in common: ARMCX2, CRYGD, HLF, KIAA1102, RBPMS, SLCO3A1, and SSBP2. The lack of overlap may not be that surprising as Rho\(^{-}\) and Rho\(^{+}\) cells are subpopulations of the CD34\(^+\)/Lin\(^-\)CD38\(^-\) population used by Ivanova et al. Comparison of genes expressed more highly in Rho\(^{-}\) versus Rho\(^{+}\) cells with genes expressed more highly in murine side population/KLS/CD34\(^-\) compared to total BM cells published by Ramalho-Santos et al. [13] identified 16 likely orthologs and 38 common gene family members (Table S5), suggesting that HSC-specific genes are conserved across species.

In vivo functional genomics screen in zebrafish. Because gene profiling per se does not prove functional importance, we developed an in vivo functional genomics screen in zebrafish (Figure 2). The zebrafish, *Danio rerio*, is an ideal organism for high-throughput genetic screens [32] as organogenesis is highly conserved from zebrafish to man [33]. There is abundant evidence that hematopoiesis in zebrafish occurs via a highly conserved genetic program. As in mammals, hematopoiesis in zebrafish occurs via specification of mesoderm to a hemangioblast stage that subsequently commits to either HSC or angioblasts [34], and genes and signals involved in specification (BMP signaling) and commitment (vascular endothelial growth factor signaling; flk1, also known as kdr; lmo2; scl, also known as tal1; gata2; gata1) are conserved from fish to man [35]. This high degree of homology in the genetic control of zebrafish and human hematopoetic development makes genetic screens in zebrafish a powerful tool to elucidate the role of genes in hematopoiesis. Additionally, rapid reverse genetic screens can be accomplished using morpholino antisense oligonucleotides (MOs) to knock down gene expression in the developing zebrafish embryo [36].

From the 277 unique transcripts that were differentially expressed between Rho\(^{-}\) and Rho\(^{+}\) cells of both UCB and BM (Table S3), we eliminated genes with known function in hematopoiesis, MHC genes, histones, and genes that are known to play a role in glucose and protein metabolism and RNA and DNA synthesis, resulting in a final list of 158 genes. Of these, we identified a putative zebrafish ortholog for 86, and designed MOs against 61 (Table S6). The 61 MOs were injected in one- to two-cell zebrafish embryos and assayed for effects on blood development. Initial dosing studies identified 16/61 MOs that reduce blood cell production without confounding toxicities (Table 1). The 16 MOs induced a blood defect in more than 70% of embryos in two or more independent injections. Blood defects identified by *gata1*:DsRed transgenic (Tg) fluorescence microscopy were confirmed by Q-RT-PCR of the erythroid-specific *hbae1* and myeloid-specific *lep1* transcripts in MO-targeted embryos compared to uninjected controls in three or more independent experiments of n = 5 embryos per experiment. A greater than 2-fold reduction in both erythroid and myeloid gene expression levels were seen for five of seven MOs analyzed (see Table 1; Figures 3 and 4). Thus, the addition of Q-RT-PCR to the screening process provides an independent confirmation and quantitation of the observed phenotypes, thereby limiting the false-positive rate, while maintaining the high-throughput nature of the screen. Additionally, the observed reduction of both erythroid and myeloid gene expression following knockdown of candidate genes is consistent with their presumed roles in HSC fate decisions prior to specification of the common myeloid progenitor. The validity of the Q-RT-PCR analysis was corroborated by analysis of *hbae1* and *lep1* transcript levels in *gata1* MO-targeted embryos, in which there was a virtually complete loss of *hbae1* expression and an almost 2-fold increase in myeloid-specific *lep1* transcripts (Figure 4) consistent with the published expression patterns for these genes following loss of *gata1* expression [37]. Analysis of vascular development by

**Table 1. Genes Differentially Expressed between Human Rho\(^{-}\) and Rho\(^{+}\) Cell Populations That Have a Functional Role in Zebrafish Hematopoietic Development**

| Human Gene | Region Targeted                  | Dose  | Hematopoietic Phenotype       | Expression     | Q-RT-PCR |
|------------|---------------------------------|-------|------------------------------|----------------|---------|
| C12orf2    | 5’ UTR ATG combination          | 4.5 ng| 90% with blood defect        | Rho\(^{-}\)    | N/D     |
|            |                                 | 4.5 ng| 95% with blood defect        |                |         |
| CCR7       | ATG                             | 3 ng  | 95% with blood defect        | Rho\(^{+}\)    | N/D     |
| FLJ14917   | ATG                             | 2 ng  | 90% with blood defect        | Rho\(^{+}\)    | N/D     |
| FOXM1      | ATG                             | 7 ng  | 80% with blood defect        | Rho\(^{+}\)    | N/D     |
| HDHD2      | ATG                             | 3 ng  | 70% with blood defect        | Rho\(^{+}\)    | N/D     |
| HSPC039    | 5’ UTR                          | 3 ng  | 70% with blood defect        | Rho\(^{+}\)    | N/D     |
| IRAK3      | 5’ UTR                          | 2 ng  | 70% with blood defect        | Rho\(^{+}\)    | N/D     |
| SUZ12      | ATG                             | 3 ng  | 70% with blood defect        | Rho\(^{+}\)    | N/D     |
| MAFB       | ATG                             | 4 ng  | 70% with blood defect        | Rho\(^{+}\)    | N/D     |
| MGC15875   | ATG                             | 4 ng  | 80% with blood defect        | Rho\(^{+}\)    | N/D     |
| MRPS56     | 5’ UTR                          | 4.5 ng| 70% with blood defect        | Rho\(^{+}\)    | N/D     |
| PRKCH      | 5’ UTR                          | 4.5 ng| 70% with blood defect        | Rho\(^{+}\)    | N/D     |
| SLC40A1    | ATG                             | 6 ng  | 80% with blood defect        | Rho\(^{+}\)    | N/D     |
| SNX5       | ATG                             | 5 ng  | 80% with blood defect        | Rho\(^{+}\)    | N/D     |
| SPARC      | ATG                             | 7.5 ng| 70% with blood defect        | Rho\(^{+}\)    | N/D     |
| SPRY1      | ATG 5’ UTR combination          | 3 ng  | 80% with blood defect        | Rho\(^{+}\)    | N/D     |
|            |                                 | 4.5 ng| 65% with blood defect        |                |         |
|            |                                 | 2 ng + 3 ng | 70% with blood defect      |                |         |

ATG, start codon; N/D, not done. DOI: 10.1371/journal.pbio.0030254.s001
hpf (greater than or equal to three experiments shown are representative of greater than 70% of injected embryos at 48 zebrafish embryos compared to an uninjected control. Phenotypes display the hematopoietic phenotypes observed for six MO-targeted types seen in a morpholino-based functional screen of the random fashion [38] and the 4% of hematopoietic phenotypes seen by ethynitrosourea mutagenesis screens that mutate genes in a near frequency of hematopoietic phenotypes seen by ethynitrosourea screen compares very favorably with the 0.5%–1%

remaining blood cells, indicating that the hematopoietic genesis or remodeling that precludes the circulation of the demonstrated no major abnormalities in vascular morpho-
entially expressed, an MO against zebrafish spry4 was used, as it is expressed in the region of the lateral plate mesoderm, the first site of zebrafish hematopoiesis [48], and it was the full-length zebrafish Sprouty gene with the greatest protein homology to human SPRY1. Recently the partial sequence of a potential zebrafish spry1 ortholog was predicted by Ensembl's gene prediction software based on genomic sequence information. However, the single exon that was predicted does not contain an ATG start codon, or a conserved splice donor or acceptor site, and the putative zebrafish spry1 sequence only partially covers the human SPRY1 gene. Moreover, the genomic location of Ensembl's putative zebrafish spry1 is currently not known, and therefore it is not possible to use syntenic relationships to determine the most likely zebrafish ortholog for human SPRY1. At present, there is not sufficient sequence data available to design gain- or loss-of-function experiments for the putative zebrafish spry1, thus precluding an analysis of hematopoietic function in the zebrafish model. Therefore, spry4 is currently the best full-length, MO-targetable candidate ortholog for human SPRY1, and based on our results, at the very least zebrafish spry4 and SPRY1 share a conserved function in hematopoiesis.

To confirm the specificity of MO targeting in the spry4MO, a second spry4 MO of independent sequence and a four-base mismatched spry4 MO were injected into zebrafish embryos. Injection of the independent spry4 MOs induced a hematopoietic phenotype in more than 65% of injected embryos, while the four-base mismatched MO did not induce any phenotypic changes (Figure 4). Q-RT-PCR for hbae1 and lcp1 transcripts also did not show changes in expression in four-base mismatched MO embryos. Furthermore, the two independent spry4 MOs acted synergistically when co-injected (Figure 4). In addition to the blood phenotype, a slight facial outgrowth was seen at a low frequency. Also, a weak dorsalization phenotype was seen at 2-fold higher MO doses based on a decreased somite size.

To rule out the possibility that the hematopoietic defect observed in the spry4MO was secondary to a vascular defect, we injected spry4 MO into fli1:EGFP Tg zebrafish. While the resulting embryos exhibited minor defects in cardinal vein remodeling and morphogenesis of intersegmental vessels in...
the posterior tail, there were no major defects in vascular development (Figure 4). The integrity of the vascular network in spry4MO fish was further demonstrated by the unimpeded circulation of the remaining DsRed blood cells in gata1:DsRed Tg spry4MO (data not shown).

This et al. [35] have shown that overexpression of zebrafish spry4 mRNA leads to an expansion of the posterior intermediate cell mass (ICM) [48]. We overexpressed human SPRY1 in gata1:DsRed Tg zebrafish embryos, and observed a similar dose-dependent expansion of DsRed blood cells in the posterior ICM (Figure 4). Q-RT-PCR analysis of embryos overexpressing SPRY1 revealed a 1.7- and 1.4-fold increase hbae1 and let1 mRNA levels, respectively, confirming the observed expansion of blood cells in the ICM. Coinjection of human SPRY1 cDNA with spry4 MOs ameliorated the spry4MO phenotype, yet another confirmation of the specificity of MO targeting (Figure 4). The similar hematopoietic phenotypes observed following the overexpression of either human SPRY1 or zebrafish spry4 indicate that these genes encode proteins with a similar functional potential in blood development. In addition to the blood phenotype, the overexpression often caused a reduction and/or curve in the posterior tail (Figure 4) not seen in the overexpression of zebrafish spry4 [48].

Characterization of hematopoietic gene expression in the spry4MO by whole-mount in situ hybridization revealed a reduction in scl expression at four somites (8/15), and virtually no scl (12/20) or gata1 (18/25) expression at 20 somites (Figure 5), consistent with a defect in mesodermal commitment to HSCs and/or HSC proliferation and differentiation. The few hematopoietic cells that are present in the morphant are hemoglobinized based on o-dianisidine staining (data not shown). To determine whether the hematopoietic defects observed in spry4MO were the result of a defect in mesoderm specification during development, we performed whole-mount in situ hybridization for the vasculature-specific flk1 and muscle-specific myod transcripts. At 10 h postfertilization (hpf) there was a slight defect in myod expression (5/10), while myod expression at 26 hpf was comparable to wild type (21/21) (Figure 5). This suggests that there were no major defects in mesodermal commitment in the spry4 morphants. Considering the nearly absent expression of the early hemangioblast and HSC cell marker scl, these results may suggest that the defect induced in the spry4MO occurs prior to HSC specification. However, the normal expression pattern of other mesodermal genes, such as flk1 and myod at 26 hpf in the spry4MO (Figure 5), indicate that the hematopoietic phenotype is not merely a consequence of defective specification of mesoderm. Finally, the hematopoietic gene emy2, a presumed marker of definitive HSC in zebrafish [49], was absent at 38 hpf (10/12) (data not shown), suggesting that the spry4MO embryos are devoid of definitive HSC.

In vertebrates, Sprouty family members act as antagonists for fibroblast growth factor (FGF), vascular endothelial growth factor, and epidermal growth factor signaling, and they may be involved in feedback regulation, as Sprouty gene expression is induced by activation of these signaling pathways [50]. Sprouty genes antagonize receptor tyrosine kinase signaling at the level of the Ras/Raf/mitogen-activated protein kinase pathway; however, they also can serve as positive regulators of these pathways in some cell types [50]. Therefore, our current hypothesis is that SPRY1 may affect HSC by modulating FGF-mediated, perhaps in combination with other receptor tyrosine kinase-mediated, signaling. In fact, three of the 14 genes that induce a hematopoietic defect in the zebrafish screen, SPRY1, MAFB and SPARC, are all involved in FGF signaling [50–52], thus suggesting a role for FGF in hematopoiesis. Studies are ongoing to confirm a role of SPRY1 in mammalian hematopoiesis, by testing the effect of overexpression of SPRY1 on the repopulating ability of human and murine HSCs. Similar definitive studies in zebrafish and subsequent confirmation in mammalian HSC models are underway for genes that were functionally validated in our zebrafish screen. We believe that the sequential genetic screen in zebrafish followed by confirmation in mammalian models as described here will establish a hematopoietic function for genes identified by gene array analysis in a high-throughput and efficient manner.

Materials and Methods

Isolation of RhoLo and RhoHi cell populations from UCB and BM. Human UCB from full-term delivered infants and BM from healthy donors were obtained after informed consent in accordance with guidelines approved by the University of Minnesota Committee on the Use of Human Subjects in Research. Each biologically distinct replicate was comprised of one to four donors for UCB and individual donors for BM samples. CD34+/CD38−/CD33−RhoLo (‘Lo’-CD34+CD38−) and CD34+/CD38+/CD33−RhoHi fractions were selected by sequential Ficoll-Hypaque separation, MACS column depletion, and fluorescence-activated cell sorting as previously described [17]. Postsort analysis
demonstrated that sorted populations contained fewer than 1%–2% contaminating cells from the opposing population (see Figure 1).

**Determination of ML–IC frequencies.** ML–IC frequencies for UCB samples (n = 3) were determined as previously described [17]. An ML–IC was defined as a single cell that gave rise to at least one LTC–IC and one DN–IC. Results are presented as ML–IC frequency ± standard deviation.

**Processing of RNA samples and oligonucleotide microarray analysis.** Total cellular RNA was isolated from UCB (n = 5) and BM (n = 4) Rho<sup>+</sup> and Rho<sup>−</sup> cells using the PicoPure RNA Isolation Kit (Arcturus, Mountain View, California, United States) per the manufacturer’s instructions. Seven to 10,000 Rho<sup>+</sup> and Rho<sup>−</sup> cells were sorted directly into 100 μl extraction buffer (XB) provided with the PicoPure RNA Isolation Kit prior to RNA isolation. Labeled cRNA was generated by one round of IVT-based, linear amplification using the RhoAmp OA RNA Amplification Kit (Arcturus) followed by labeling with the Bioarray HighYield RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, New York, United States) according to manufacturer’s instructions. Samples were hybridized to Affymetrix HG-U133A and B chips (Affymetrix Inc., Santa Clara, California, United States) were washed, and scanned at the University of Minnesota Affymetrix Microarray Core Facility as described in the Affymetrix GeneChip Expression Analysis Technical Manual.

**Oligonucleotide microarray data analysis.** Affymetrix HG-U133 GeneChips were processed using GeneData Refiner software (GeneNet Solutions, Minneapolis, Minnesota, United States) based on Affymetrix NetAffx analysis tool (http://www.affymetrix.com), using human genome sequences. Feature intensities for each chip were condensed into a single intensity value per gene using the Affymetrix Statistical Algorithm (MAS 5.0) with τ = 0.015, x1 = 0.04, x2 = 0.06, and a scaling factor of 500. Expression data was analyzed using GeneData’s Expressionist and Microsoft Excel (Microsoft, Redmond, Washington, United States). Differentially expressed genes were classified according to their respective gene pathways and gene ontologies when available by using the WEB-based Affymetrix NetAffx analysis tool (http://www.affymetrix.com) and the National Institutes of Allergy and Infectious Disease Database for Annotation, Visualization and Integrated Discovery analysis tool (DAVID) (http://david.ncifcrf.gov/daavid).

**Microarray Q-RT-PCR confirmation.** Reverse transcribed cDNA was reverse transcribed to generate cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, California, United States) according to manufacturer’s instructions. Q-RT-PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, California, United States). Briefly, 3 μg cDNA was amplified by 40 cycles of a two-step PCR reaction (95 °C for 15 s denaturation and 60 °C for 1 min. annealing/elongation) containing 100 nM gene-specific primers (Table S8) and the SYBR Green PCR Master Mix (Applied Biosystems). Gene expression was normalized using a zebrafish gusA gene member as the internal control. Primers used for Q-RT-PCR were designed complimentary to the region of translational frame (ORF) (Open Biosystems, Huntsville, Alabama, United States) were cloned into pENTR1A (Invitrogen) and subsequently transferred into a modified pRM2.1 zebrafish expression vector using the Gateway cloning system (Invitrogen) to create the pRM2.1–SPRY1 vector. pRM2.1–SPRY1 was co-injected with pRM2.1–EGFP at a 5:1 ratio into the yolk/ehicle interface of one-cell gata1:DsRed Tg zebrafish embryos as described for MO injections. Defects in hematopoietic development of MO-injected embryos were analyzed by comparison with control embryos from the same clutch injected with pRM2.1–EGFP alone, using fluorescence microscopy to visualize DsRed<sup>+</sup> blood cells.

**Supporting Information**

**Figure S1.** Frequency of ML–IC in UCB Rho<sup>+</sup> and Rho<sup>−</sup> Cell Populations

ML–ICs are highly enriched in Rho<sup>+</sup> (white bar) compared to Rho<sup>−</sup> (black bar) cells from UCB (n = 3, p < 0.05).

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**Figure S2.** Confirmation of Differential Gene Expression Using Q-RT-PCR

The Rho<sup>−</sup> to Rho<sup>+</sup> fold change for DLK1, ABCB1, BMP6, HELLS, CDC25A, MAFB, and S100A8 was determined using Affymetrix GeneChip analysis (gray bars) and Q-RT-PCR (black bars) to confirm the fidelity of the microarray results for (A) adult BM (n ≥ 2) and (B) UCB (n ≥ 3).

Found at DOI: 10.1371/journal.pbio.0030254.s002 (7.2 MB TIF).

**Figure S3.** Gene Ontology Classifications of Conserved Differentially Expressed Genes

Percentages of Gene Ontology classifications of the genes differentially expressed between Rho<sup>−</sup> and Rho<sup>+</sup> cells from both UCB and adult BM (p < 0.05 and fold change >3 or >1.5 in either UCB or BM).

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**Table S1.** Probe Sets Differentially Expressed between UCB-Derived Rho<sup>+</sup> and Rho<sup>−</sup> Cells (p < 0.05)

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**Table S2.** Probe Sets Differentially Expressed between Adult BM-Derived Rho<sup>+</sup> and Rho<sup>−</sup> Cells (p < 0.05)

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**Table S3.** Conserved Probe Sets Differentially Expressed between Rho<sup>−</sup> and Rho<sup>+</sup> Cells from Both UCB and Adult BM (p < 0.05 and FC > 1.5 in Either UCB or BM)

Found at DOI: 10.1371/journal.pbio.0030254.s006 (75 KB XLS).

**Table S4.** Fold Changes, p-Values, and Average Expression Levels for Genes with Well-Established Roles in HSC Proliferation and Differentiation

Found at DOI: 10.1371/journal.pbio.0030254.s007 (20 KB XLS).

**Table S5.** Comparison of Conserved Rho<sup>−</sup>-Enriched Genes with Ramalho-Santos et al. [13] Dataset

Found at DOI: 10.1371/journal.pbio.0030254.s008 (22 KB XLS).

**Table S6.** Morpholino Sequences for Targeted Genes

Found at DOI: 10.1371/journal.pbio.0030254.s009 (20 KB XLS).
Table S7. Complete Phenotypic Descriptions for the 14 Zebrafish Morphants with Confirmed Blood Defects

Table S8. Q-RT-PCR Primer Sequences

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Competing interests. University of Minnesota and Stephen Ekker are cofounders and shareholders of a small biotechnology company called Discovery Genomics Inc. that has the exclusive license for the commercial use of morpholinos in zebrafish. Dr. Ekker is a consultant to this company.

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