

Zebrafish model for allogeneic hematopoietic cell transplantation not requiring preconditioning

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Recent work on vertebrate hematopoiesis has uncovered the presence of deeply rooted similarities between fish and mammals at molecular and cellular levels. Although small animal models such as zebrafish are ideally suited for genetic and chemical screens, the study of cellular aspects of hematopoietic development in lower vertebrates is severely hampered by the complex nature of their histocompatibility-determining genes. Hence, even when hosts are sublethally irradiated before hematopoietic cell transplantation, stable and long-term reconstitution by allogeneic stem cells often fails. Here, we describe the unexpected observation that transplantation and maintenance of allogeneic hematopoietic stem cells in zebrafish homozygous for the *c-myb*^{t25127} allele, carrying a missense mutation (Ile181Asn) in the DNA binding domain can be achieved without prior conditioning. Using this model, we examined several critical parameters of zebrafish hematopoiesis in a near-physiological setting. Limiting dilution analysis suggests that the kidney marrow of adult zebrafish harbors about 10 transplantable hematopoietic stem cells; this tissue also contains thymus-settling precursors that colonize the thymic rudiment within days after transplantation and initiate robust T-cell development. We also demonstrate that *c-myb* mutants can be stably reconstituted with hematopoietic cells carrying specific genetic defects in lymphocyte development, exemplifying one of the many potential uses of this model in experimental hematology.

ENU mutagenesis | chimera

Hematopoietic stem cells (HSCs) are self-renewing progenitor cells capable of multilineage differentiation. In mouse (1) and humans (2), HSCs can be isolated to near homogeneity based on species-specific expression of cell surface molecules. However, a universal pattern for phenotypic and/or molecular markers of HSC has yet to be defined (3); thus, long-term differentiation into all blood lineages after *in vivo* transplantation (4–6) remains the only reliable criterion for assessing HSC function (7), particularly in species other than mouse and humans (8, 9).

The outcome of hematopoietic cell transplantation depends on many variables. A major factor is the availability of specific stromal niches in hematopoietic tissue environments that are capable of supporting the long-term survival and differentiation of HSCs (10). In general, myeloablative conditioning, such as irradiation of the recipient, is a prerequisite for stable engraftment by donor HSCs. Preconditioning of the host serves two purposes: first, it weakens the immune system that would otherwise attack and destroy incoming cells of the donor, and second, it empties hematopoietic niches to allow the settling of donor cells in such supportive environments. Recently, a mouse model has been generated that stably accommodates histo-incompatible HSCs, which renders prior myeloablation of the host unnecessary; this was achieved by generating a mouse lacking T, B, and NK cells as a result of synergistic null mutations in *Rag2* and *Il2rg* genes, in addition to impairing self-renewal of HSCs owing to the combined presence of two hypomorphic alleles of the *c-kit* locus (*Kit*^W/*Kit*^{W-v}) (11). Despite the complexity of its genetic background, the availability of such a “universal” acceptor for hematopoietic cell transplantation studies represents a major step forward and provides unprecedented opportunities for assessing the developmental

potential of HSCs and other types of hematopoietic progenitor cells under near-physiological conditions in the mouse model. However, functionally comparable recipient strains have so far not been described for other species.

Another important factor determining the success of hematopoietic cell transplantation is the degree of immunological compatibility between host and donor tissues. This is determined by the structure of the polymorphic major histocompatibility (MHC) genes; identical MHC haplotypes are generally associated with favorable outcomes (12). In allogeneic situations, that is, when donor and host carry different MHC haplotypes, immunosuppression following transplantation is additionally used to reduce the activity of the immune system of the host and hence to increase the chances of successful long-term engraftment; moreover, the transplantation of highly purified HSC preparations lessens the incidence of graft versus host disease (GvHD) resulting from donor cells attacking host tissues.

The zebrafish has emerged as a versatile experimental model for studies on vertebrate hematopoiesis (13). This success is mostly due to its unique biological features such as high fecundity and transparency of embryos and larvae; moreover, different methods of genetic manipulation and the amenability to high-throughput genetic and chemical screens greatly increase its practical utility. However, the development of methods for hematopoietic cell transplantation in zebrafish is still in its infancy (8, 9, 14). The situation is further complicated by the lack of robust procedures for the purification of HSCs, the difficulty of generating isogenic lines (15, 16) that gives rise to a high frequency of histoincompatibility between host and donor tissues (14), and the considerable toxicity of the required preconditioning regimens (14). In previous work, immune reactions against donor cells were circumvented by transplantation into immunologically immature hosts (before commencement of lymphoid development) (8); however, only very few cells can be transplanted into recipient embryos, which limits the practical utility of this approach. As an alternative, myeloablative irradiation of adult recipients before transplantation (9) combined with matching of haplotypes at several MHC loci (14) was shown to result in stable multilineage hematopoietic engraftment in a substantial fraction of transplant recipients. Preconditioning is associated with significant levels of toxicity (14); these side effects may also alter the environments supporting the maintenance and differentiation of HSCs, influencing the interpretation of outcomes in this setting.

In the present report, we introduce a zebrafish model for hematopoietic cell transplantation that overcomes the above limitations. We demonstrate that unmanipulated fish homozygous for the *c-myb*^{t25127} allele, carrying a missense mutation (I181N) in the highly conserved DNA binding domain (17), stably accept allo-

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genic hematopoietic cell transplants, thus obviating the need for myeloablation before transplantation. We have exploited this unexpected property of the *c-myb* mutant model to examine key parameters of HSC biology in zebrafish.

Results

Transplantation of Whole Kidney Marrow Leads to Sustained Hematopoietic Activity in *c-myb* Mutants. Fish homozygous for the *c-myb*^{t25127} allele encoding a mutant form of the *c-myb* transcription factor, *c-myb*^{I181N} (hereafter referred to as *c-myb* mutants), lack definitive hematopoiesis (17). The mutant fish are easily distinguishable from their heterozygous or wild-type siblings by their smaller size, developmental retardation, anemia (accompanied by cardiac edema), and lack of sexual maturation. However, these mutants are capable of surviving for several weeks (Fig. 1A). Using retro-orbital injections (18), we transplanted unfractionated whole kidney marrow (WKM) cells ($\sim 4 \times 10^5$ cells per recipient) from

wild-type fish into 6–9-wk-old *c-myb* mutants; this time point was chosen because the fish have by then reached a size large enough to facilitate experimental manipulation yet are still healthy enough to cope with the transplantation procedure. The wild-type donor cells were transgenic for an *ikaros:eGFP* reporter gene, encoding an enhanced green fluorescent protein under the transcriptional control of the *ikaros* gene (19) to enable intravital tracking of the fluorescing transplanted cells. Although procedure-related mortality of about 50% was high, we established that a substantial fraction of transplanted mutant fish survived their untransplanted siblings by several months (Fig. 1B). Strikingly, the transplanted mutants grew rapidly, acquired a reddish complexion, lost their cardiac edema, and eventually exhibited the characteristic signs of sexual dimorphism. As a result, their phenotype was indistinguishable from that of their wild-type siblings about 5 wk after transplantation (Fig. S1A); accordingly, their lifespan was extended to well over 8 mo, the latest time point of our longitudinal

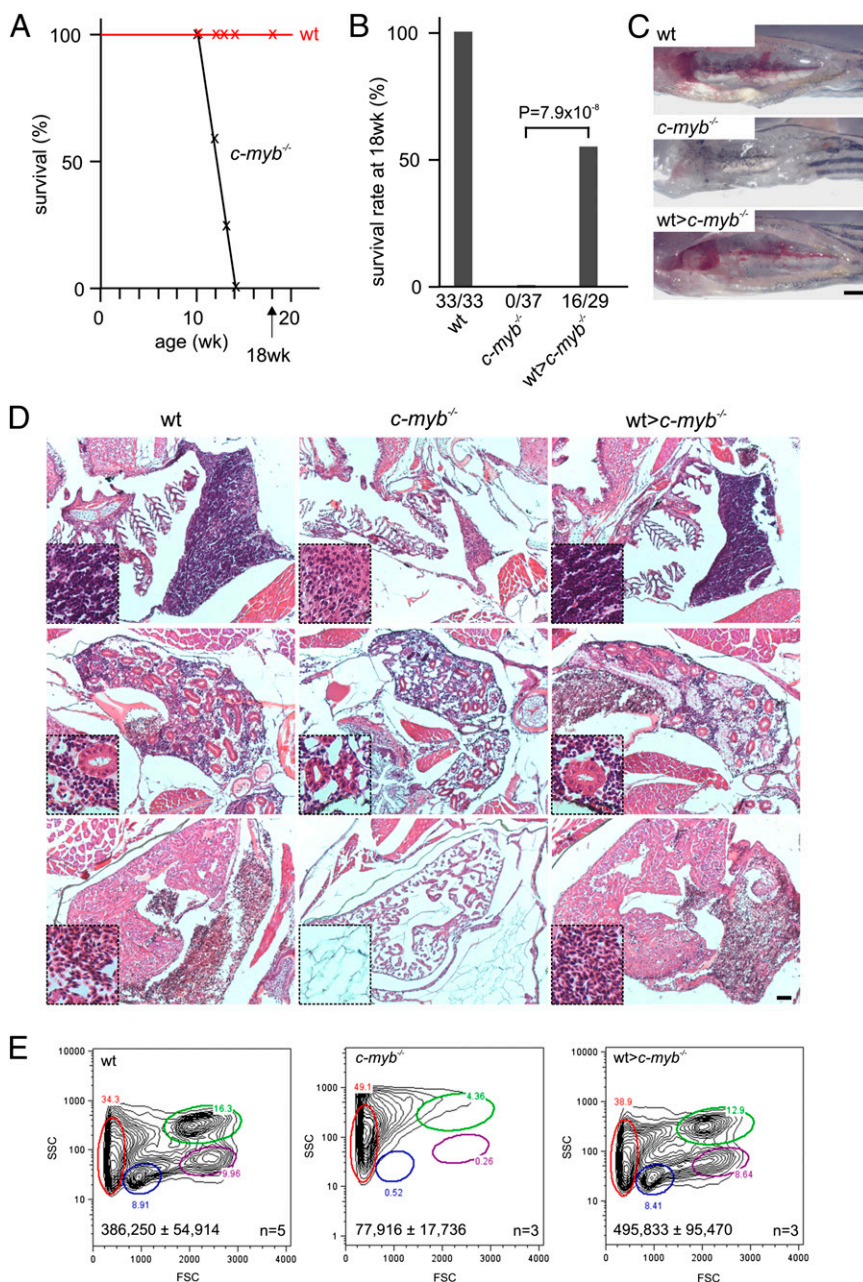


Fig. 1. Reconstitution of the *c-myb*^{-/-} mutant phenotype. (A) Survival of wild-type ($n = 33$) and *c-myb*^{-/-} mutant ($n = 37$) fish; mutants do not survive longer than 14 wk of age. (B) Survival rates of wild-type, *c-myb*^{-/-} mutants and mutant recipients transplanted with wild-type WKM (*wt*>*c-myb*^{-/-}) transgenic for an *ikaros:eGFP* reporter gene assessed at 18 wk of age. All deaths occurring within the first 24 h after transplantation were considered to be procedural and thus not used for the calculation of survival rates. (C) Macroscopic appearance of head kidneys in wild-type, *c-myb*^{-/-} mutants and transplanted mutant recipients; note that mutant kidney shows a complete lack of hematopoietic cells (17). (Scale bar, 1 mm.) (D) Hematoxylin/eosin staining of sections through the region of the thymus (Top), kidney (Middle), and heart (Bottom) of wild-type, *c-myb*^{-/-} mutants and transplanted recipients at 9 wk of age (i.e., 3 wk after transplantation); insets are at fourfold higher magnifications. Note the lack of hematopoietic cells in the mutants and complete hematopoietic reconstitution in transplanted recipients. (Scale bar, 50 μ m.) (E) Flow cytometric analyses of WKM cells of wild-type, *c-myb*^{-/-} mutants and transplanted recipients as in D. FSC, forward light scatter; SSC, side light scatter. Circles denote different blood cell populations in adult wild-type fish: red, erythrocytes; blue, lymphocytes; magenta, precursors; green, myelomonocytes. Total cell numbers (mean \pm SD) in WKM preparations are indicated.

observations. The macroscopic appearance of the head kidneys in transplanted *c-myb* mutants indicated the presence of hematopoiesis (Fig. 1C); likewise, productive thymus colonization was evident from the strong green fluorescence emanating from *ikaros*-expressing thymocytes (Fig. S1B). The characteristic features of ongoing hematopoietic activity were also observed in histological sections of thymus and kidney: the thymus was densely populated by lymphoid cells; the kidney of transplanted mutant fish exhibited the cellular pleomorphy of normal hematopoietic tissues (Fig. 1D). Furthermore, blood vessels were replete with mature erythrocytes and lymphoid and myeloid cells (Fig. 1D). In accordance with the histological appearance of kidney sections, flow cytometric analysis of WKM cell preparations indicated that the major cell populations, distinguishable by their light scatter characteristics (8), are present in normal proportions, thus supporting the notion of multilineage engraftment (Fig. 1E). The phenotypic normalization of hematopoietic tissue morphology is supported by the expression of genes indicative of restored hematopoietic activity (*c-myb*), including lymphoid (*rag1*) and myeloid (*mpx*) differentiation (Fig. S1C and D). Collectively, these qualitative results indicated that the transplantation of WKM cells reestablished normal hematopoietic activity in *c-myb* mutants; this also holds true in quantitative terms, as the numbers of hematopoietic cells in the WKM cell preparations reach wild-type levels (Fig. 1E). No indication of the presence of GvHD, such as edema, ascites, and flaring of scales (14), was observed in any of the transplanted fish. To exclude the possibility that *ikaros:eGFP* transgenic wild-type hematopoietic cells stimulate hematopoietic activity of nontransgenic mutant recipient tissues, we examined the fraction of green cells in the WKM of donor, primary, and secondary (see below) transplant recipients; the percentage of green cells does not change in transplanted fish, indicating that nonfluorescent host cells do not contribute to hematopoietic recovery (Fig. S24).

The above observations suggest that at least some of the non-hematopoietic phenotypes in *c-myb*^{t25127} homozygous fish, including early lethality, are not caused by the lack of wild-type *c-myb* activity but were an indirect consequence of failing definitive hematopoiesis. We examined this issue directly by assessing the fertility of successfully transplanted sexually dimorphic *c-myb* mutants (Fig. S1A). To this end, we mated hematopoietically rescued male and female *c-myb* mutant fish and examined the genotypes of their offspring. Whereas an incross (IC) of *c-myb*^{t25127} heterozygous fish yielded the expected wild-type, heterozygous, and homozygous mutant genotypes, all progeny of mutant matings were homozygous for the *c-myb* mutation (Fig. 2A) and exhibited the characteristic phenotypic abnormalities (Fig. 2B–D). The transplantation of wild-type hematopoietic cells restores immune function to *c-myb* mutants; whereas all mutants die from overwhelming infection within 2–3 d of clipping their fins, the transplanted fish survive this insult (Fig. S2B).

Transfer of Genetically Impaired Hematopoietic Systems to *c-myb* Mutant Recipients. The success of hematopoietic cell transplantations prompted us to examine the possibility of transferring genetically impaired hematopoietic tissues to allogeneic hosts. To this end, we chose two well-characterized genotypes for further study, *rag1*- (20) and *jak3*- (21) deficiencies. Phenotypically, these mutant genotypes rescued the characteristic anemia of *c-myb* mutants, as expected, but recapitulated the distinct lymphocyte lineage-specific defects; in particular, no somatic assembly of *tcr* and *ig* genes could be detected in *rag1*^{-/-} fish and in *jak3*-deficient hematopoietic chimeras, T-cell development was affected to a greater extent than B-cell development (Fig. 2E).

Thymus-Settling Cells in Adult WKM. Under physiological conditions, T-cell development in the thymus is initiated by importation of T-cell precursors (22). Alymphoid *c-myb* mutants represent a unique opportunity to examine the presence of

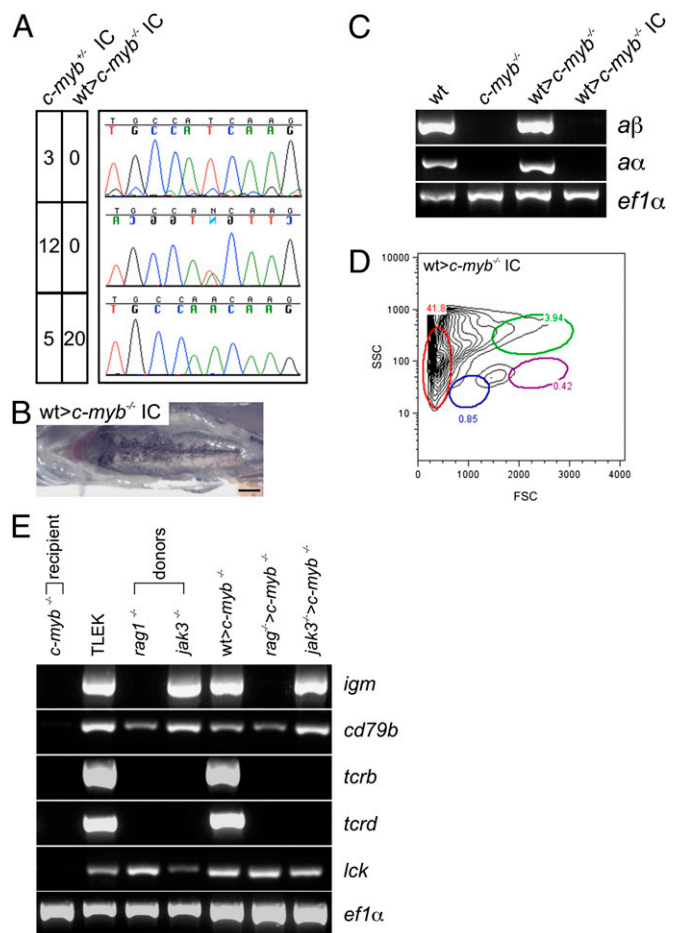


Fig. 2. Characterization of reconstituted *c-myb*^{-/-} mutants. (A) Genotypes of offspring embryos resulting from in-crosses of *c-myb*^{t25127} heterozygous fish and reconstituted female and male *c-myb*^{-/-} mutant fish, respectively. The fecundity of reconstituted transplant recipients is indistinguishable from wild-type fish. The number of wild-type (Top panels), heterozygous (Middle panels), and homozygous mutant (Bottom panels) embryos are indicated as are representative sequence traces. (B) Macroscopic phenotype of the head kidney in the offspring of reconstituted *c-myb*^{-/-} mutants. (Scale bar, 1 mm.) (C) Expression of adult hemoglobin alpha (α) and beta (β) genes in WKM cells of wild-type (wt), *c-myb*^{-/-} mutants; reconstituted mutants (wt>*c-myb*^{-/-}); and offspring of reconstituted mutants (wt>*c-myb*^{-/-} IC) as determined by RT-PCR at 11–12 wk of age; amplification with *ef1α*-specific primers serves as a control for cDNA integrity and amount. (D) Flow cytometry analysis of WKM cells from offspring of reconstituted mutant fish (see Fig. 1E legend for explanation of indicated cell populations); profile representative of three fish. (E) Expression of T- and B-cell-specific marker genes in TLEK fish (wild-type strain), *c-myb*^{-/-} mutants, *rag1*^{-/-} and *jak3*^{-/-} deficient donor fish, and *c-myb*^{-/-} recipients transplanted with wild-type (transgenic for the *ikaros:eGFP* reporter), *rag1*^{-/-}, and *jak3*^{-/-} deficient WKM cells. *cd79b*, gene encoding the $\text{ig}\beta$ adaptor protein, constituting part of the B-cell receptor in B cells; *ig*m, Ig μ heavy chain gene expressed in B cells; *lck*, gene encoding a receptor-associated tyrosine kinase expressed in T cells; *tcrb*, T-cell receptor β chain gene expressed in T cells; *tcrd*, T-cell receptor δ chain gene expressed in T cells.

thymus-settling cells in zebrafish WKM. To this end, we transplanted WKM from adult wild-type fish transgenic for an *ikaros:eGFP* reporter gene (19) to enable noninvasive monitoring of the process of thymus colonization by fluorescence microscopy. Thymus colonization, as detected by the presence of eGFP-positive cells in the thymus rudiment, takes place in less than 7 d after transplantation (Fig. 3A). These results also demonstrate that the thymic epithelium remains receptive for progenitors for an extended period; in previous experiments using *ikaros*-

c-myb mutants, as measured by the number of cells in WKM preparations. Irrespective of the numbers of cells in the original inoculum, cellular yields from WKM preparations were always the same (Fig. 3D), with normal proportions of hematopoietic cell lineages (Fig. 3E). These results indicate that *c-myb* mutants provide appropriate niches conducive to the survival, self-renewal, and differentiation of exogenous HSCs that are contained in WKM cell preparations and suggest that transferred HSCs proliferate to occupy all available niches in the *c-myb* mutant recipient. Stable engraftment of hematopoietic cells was also observed in secondary *c-myb* mutant recipients, indicating that stem cells capable of contributing to all hematopoietic lineages had colonized the primary transplant recipients (Fig. 3D–F).

Allogeneic Hematopoietic Cell Transplantation. Two major factors impede the use of hematopoietic cell transplantation in fish biology. First, isogenic lines as a means of establishing histocompatibility between host and donor are exceedingly difficult to maintain owing to severe in-breeding depression (15, 16). Second, unlike mammals in which the MHC genes are encoded at a single chromosomal locus, fish possess several MHC I and MHC II loci that are situated on different chromosomes (24). Because of the fragmented nature of the MHC locus, MHC genotypes resulting from genetic crosses in fish species are difficult to predict. Although initial attempts at resolving the relative biological contribution of different MHC loci to the outcome of hematopoietic cell transplantation have been reported (14), transplantations across histocompatibility barriers remain a major challenge. Intriguingly, the experiments described above collectively suggested the possibility of successful allogeneic transplantations using *c-myb*^{-/-} recipients, although the host–donor pairs were initially not examined for congruency at the MHC loci. To examine this issue more specifically, we surveyed the diversity of some *mhcl* and *mhclII* genes; these experiments revealed the presence of considerable allelic polymorphism (Fig. 4A; Figs. S2 and S3). For instance, when WKM cells from the Assam strain, a recently established wild-type zebrafish line (25), were transferred into *c-myb* mutants, successful hematopoietic reconstitution could be easily achieved, despite substantial allelic differences between MHC alleles of different strains (Fig. 4B; Figs. S2 and S3). Collectively, these analyses support the notion that unfractionated allogeneic WKM cells containing hematopoietic progenitor cells can be successfully transferred into unmanipulated *c-myb* mutants to achieve stable hematopoietic reconstitution.

Discussion

Here, we present a versatile model for studies on the hematopoietic system of zebrafish. We demonstrate that *c-myb* mutant fish readily accept allogeneic hematopoietic cell transplants without prior conditioning, thus providing the best possible physiological setting available to date to study the function of HSCs in a lower vertebrate. Whereas mice deficient for *c-myb* die of anemia in midgestation (26), the unique physiology of fish allows *c-myb* mutants to survive into adulthood in the absence of definitive hematopoiesis (17); this species-specific difference highlights the importance of exploring a wide range of animal models to identify those most suitable for addressing a specific biological problem. Because of its practicability, we expect that the *c-myb* mutant model described here will lend itself to several other applications that would have been essentially impractical under previous experimental schemes owing to the complexity and unclear functional relevance of the many loci collectively defining the MHC genotype. In addition to competitive repopulation assays using cells of different genotypes, *c-myb* mutants might also prove useful in experiments involving transplantations of tumors such as leukemic cells and solid tumors; we also envisage the use of this model in interspecific hematopoietic reconstitutions.

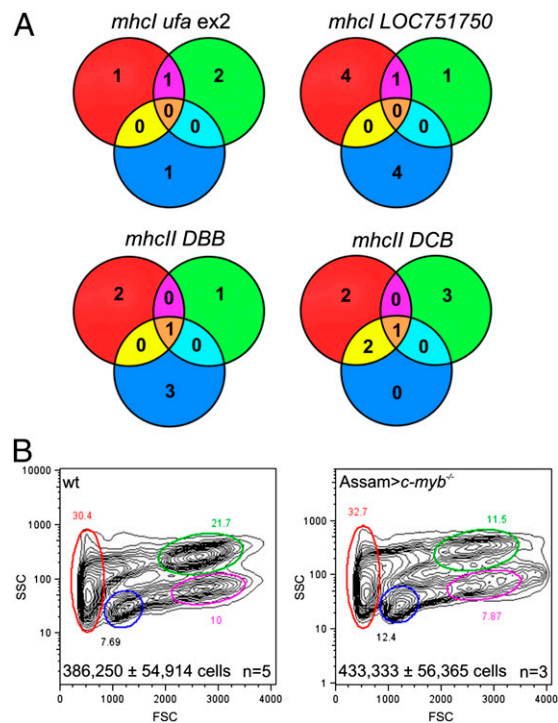


Fig. 4. Allogeneic hematopoietic cell transplantation. (A) Analysis of allelic diversity of *mhcl* and *mhclII* genes in *c-myb*^{-/-} mutants (red circles), *Tg(Ikaros:eGFP)* wild-type fish (green circles), and Assam wild-type fish (blue circles). The Venn diagrams indicate the extent of overlap as determined by nucleotide sequences of the relevant parts of the indicated *mhc* genes (see Table S1 for primer sequences and Fig. S3 for protein sequence comparisons). (B) Flow cytometric analysis of WKM of wild-type and *c-myb*^{-/-} mutants reconstituted with WKM cells of the Assam strain, 9 wk after transplantation (see Fig. 1E legend for explanation of indicated cell populations); representative profiles are shown. Total cell numbers (mean ± SD) in WKM preparations are indicated.

Apart from the practical advantage of working with a single gene deficiency provided by our model system, our results reveal key features of zebrafish HSC biology. The adult kidney marrow contains HSCs, at a concentration of ~1/40,000 cells; considering that leukocytes comprise ~50% of cells in the WKM preparations used for transplantation, this frequency is surprisingly similar to the frequency of HSCs in mouse bone marrow that is estimated to be in the order of 1/10,000 nucleated cells (27). It also suggests that the adult zebrafish kidney contains a total of about 10 transplantable HSCs, a surprisingly low number, but nonetheless compatible with estimates of the number of active HSCs sustaining hematopoiesis in the mouse (4). Collectively, our experiments support the notion that the properties of zebrafish HSCs are similar to those of mammals, further validating the use of the zebrafish hematopoietic system for in-depth genetic and chemical screening (28, 29).

Our results are also pertinent to the question of HSC niche formation and maintenance. We have demonstrated that *c-myb* mutants lack definitive hematopoiesis (17), either as a result of impaired function or complete absence of HSCs. If the former is true, the successful establishment of hematopoietic chimerism described here suggests that mutant HSCs are at a substantial competitive disadvantage when confronted with wild-type HSCs; if the latter holds, the mutant kidney must either contain preformed yet empty niches (generated and maintained in HSC-independent autonomous fashion) or be capable of elaborating such niches in response to incoming HSCs. Our finding that transplantations of WKM cells always result in wild-type levels of hematopoietic cells argues against the presence of normal

numbers of HSCs in the kidney marrow of mutant fish, unless their competitive fitness for niche occupancy is very low. On the other hand, the impaired fitness of mutant HSCs would appear incompatible with their ability to survive the extended periods of time before transplantation takes place in the present experimental scheme. Therefore, we favor the explanation that *c-myb* mutants entirely lack definitive HSCs. It follows that the niches for HSCs either function cell autonomously or that the microenvironment is poised to generate them when required.

The successful hematopoietic transplantations across histocompatibility barriers observed here highlight the role of the recipients' impaired immunocompetence. The use of early embryos as recipients (8) avoids the problem of rejection of transplanted cells; however, there is a limitation in terms of the number of cells that can be transplanted. To overcome this problem, adult fish must be used as recipients, but they in turn require irradiation to destroy the immune system before transplantation (9, 14). The *c-myb* mutants used here as recipients lack cells of the main hematopoietic lineages that are derived from HSCs, namely erythrocytes, myeloid cells, and lymphocytes. Although long-lived myelomonocytic cells that are generated during embryonic stages are still present in the adult fish, these cells do not seem to elicit unwanted graft versus host reactions by donor effector cells. Further study is required to ascertain whether this is due to the low number of embryonic myelomonocytic cells, to their developmentally imprinted inability to function as antigen-presenting cells (APCs), or to their intrinsic *c-myb* deficiency. In any case, the absence of GvHD in transplanted *c-myb* mutants indicates that the newly emerging T-cell compartment derived from donor HSCs—selected by host thymic epithelial cells and donor APCs—becomes tolerant to host tissues.

Methods

Zebrafish Stocks. Zebrafish (*Danio rerio*) wild-type strains (TLEK and Assam—a kind gift of Dr. S. Zala, Konrad Lorenz Institute of Ethology, Vienna, Austria) and the transgenic (Tg) *ikaros:eGFP* (19) and the mutant *c-myb*²⁵¹²⁷ (17) lines are kept at the Max Planck Institute of Immunobiology and Epigenetics. All procedures were conducted in accordance with institutional guidelines under a license from the local government and approved by the Institutional Animal Care and Use Committee at the Max Planck Institute of Immunobiology and Epigenetics.

Transplantation Procedure. WKM cells from adult fish were harvested as described (8) and finally resuspended at 2,000–40,000 cells/ μ L in 0.9 \times PBS supplemented with 5% (vol/vol) FCS; cells were injected retro-orbitally (18) in a volume of 10 μ L.

Flow Cytometry. Flow cytometric analysis of light-scatter characteristics of WKM cells was carried out as described (8); staining with hydroxystilbamidine (Enzo Life Sciences; final concentration 1 μ g/mL) was used to exclude dead cells.

Whole-Mount RNA in Situ Hybridization. Whole-mount RNA in situ hybridization was performed with digoxigenin-labeled RNA riboprobes as described (17).

Gene-Expression Analysis. Primers used for RT-PCR (17, 30, 31) are listed in Table S1.

MHC Genotyping. Seven individuals, each resulting from in-crosses of *c-myb*^{+/-}, Tg(*ikaros:gfp*), and Assam strains, respectively, were genotyped for different *mhcI* and *mhcII* genes by PCR using primers listed in Table S1. The PCR products from two *mhcI* and *mhcII* genes each were cloned into pGEM-T Easy vector (Promega) and sequenced (Fig. S4).

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