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c-myb hyperactivity leads to myeloid and lymphoid malignancies in zebrafish

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Abstract

The c-MYB transcription factor is a key regulator of hematopoietic cell proliferation and differentiation, and dysregulation of c-MYB activity often associates with various hematological disorders. Yet, its pathogenic role remains largely unknown due to lack of suitable animal models.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

WL, MW and ZH designed the research, performed most of the experiments and analyzed the data. JL, JC and TW performed some experiments. AYHL, YL, ZZ, QL, KY, SL and LIZ designed some experiments and revised the manuscript. WL, ZW, YZ and WZ designed the research, analyzed the data and prepared the manuscript.

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Here, we report a detail characterization of a *c-myb-gfp* transgenic zebrafish harboring c-Myb hyperactivity (named *c-myb*^{hyper}). This line exhibits abnormal granulocyte expansion that resembles human myelodysplastic syndrome (MDS) from embryonic stage to adulthood. Strikingly, a small portion of *c-myb*^{hyper} adult fish develops acute myeloid leukemia-like or acute lymphoid leukemia-like disorders with age. The myeloid and lymphoid malignancies in *c-myb*^{hyper} adult fish are likely caused by the hyperactivity of *c-myb*, resulting in the dysregulation of a number of cell-cycle-related genes and hyperproliferation of hematopoietic precursor cells. Finally, treatment with *c-myb* target drug flavopiridol can relieve the MDS-like symptoms in both *c-myb*^{hyper} embryos and adult fish. Our study establishes a zebrafish model for studying the cellular and molecular mechanisms underlying c-Myb-associated leukemogenesis as well as for anti-leukemic drug screening.

INTRODUCTION

The proto-oncogene *c-myb* is a master regulator essential for proliferation and differentiation of hematopoietic cells during normal hematopoiesis.[1,2] Studies of c-MYB-deficient mice and zebrafish have revealed that c-MYB plays essential roles in various stages of hematopoiesis, including hematopoietic stem cell (HSC) development,[3] erythropoiesis,[4] myelopoiesis[5] and lymphopoiesis.[6,7] The importance of c-MYB is exemplified by its involvement in hematological diseases in animals and humans. Aberrant activation or ectopic expression of *c-MYB in vitro* has been shown to contribute to the transformation of hematopoietic progenitors into leukemic cells.[8] In avian and in mice, c-MYB truncated by virus targeting often leads to its aberrant activity, and causes leukemia transformation.[9,10] Conversely, suppression of c-MYB activity in mice inhibits aggressive acute myelogenous leukemia (AML).[11] Clinically, *c-MYB* has been found to be highly expressed in leukemic cells in patients with AML, chronic myeloid leukemia and acute lymphoblastic leukemia (ALL), and it is essential for the proliferation and maintenance of leukemic cells.[2,12,13] Several recent reports have identified genetic lesions, including chromosomal translocation,[14] genomic duplication[15] and mutations,[16] that alter the *c-MYB* activity in human lymphoid and myeloid leukemias. Collectively, these studies have documented that c-MYB plays a key role in human leukemogenesis. However, the molecular basis underlying *c-MYB*-associated leukemogenesis remains undefined and effective treatment for leukemia patients with aberrant *c-MYB* activity is still lacking.

Zebrafish, which share high similarities in blood contents and genetic regulatory networks to mammals,[17] have emerged as an excellent model organism for studying the pathogenesis of some hematological disorders[18] as well as for drug discovery.[19] Despite increasing numbers of zebrafish models with hematologic malignancies have been generated by mutagenesis or by overexpressing key oncogenic proteins in the past several years,[20,21] a zebrafish malignant model associated with *c-MYB* hyperactivity remains unavailable.

Here, we investigated the induction of myeloid and lymphoid leukemia in transgenic zebrafish with *c-myb* hyperactivity. We found that a *c-myb-gfp* transgenic zebrafish line generated previously[22] overexpresses WT c-Myb and a hyperactive fusion c-Myb because of the duplication of the *c-myb* locus. The *c-myb*-overexpressing zebrafish, referred as to *c-*

myb^{hyper} (*c-myb* with hyperactivity), display hematopoietic perturbation at the embryonic stages and can develop myeloid and lymphoid leukemia-like phenotypes in adulthood. Our findings show that c-Myb functions as a driver for leukemogenesis *in vivo* by promoting hematopoietic cell proliferation and the *c-myb*^{hyper} zebrafish may serve as a suitable animal model for anti-leukemia drug screening.

MATERIALS AND METHODS

Zebrafish husbandry

All experiments involving zebrafish were performed in accordance with the guidelines laid down by the Institutional Animal Care and Use Committee of Southern Medical University. Zebrafish (3 days–24 months old) of either sex were maintained as described previously.[23,24] The following strains were used: AB, *c-myb-gfp*,[22] *c-myb*^{hkz3} (a loss-of-function *c-myb* mutant),[25] *rag2-dsRed*[26] and *lyz-dsRed*.[27]

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed with antisense digoxigenin-labeled RNA probes, according to standard protocols.[28]

Sudan black staining

Fixed embryos were incubated in Sudan black B (Sigma-Aldrich, St Louis, MO, USA; 199664) solution and washed as described previously.[29]

Leukemia transplantation

Leukemic cell suspensions were prepared from *c-myb*^{hyper}*/lyz-dsRed* (myelodys-plastic syndrome (MDS)-like), *c-myb*^{hyper} (AML-like) or *c-myb*^{hyper}*/rag2-dsRed* (ALL-like) fish as previously described.[20] Two days after receiving a sublethal dose of radiation (25 Gy), 0.2 million cells were injected intracardially into the irradiated recipients using a glass capillary needle (World Precision Instruments, Sarasota, FL, USA; 1B100-6).

Bromodeoxyuridine labeling

See Supplementary Methods.

Cytological analysis

Peripheral blood (PB) and kidney marrow (KM) were re-suspended in ice-cold phosphate-buffered saline with 5% fetal bovine serum, followed by cytospins at 400 r.p.m. for 3 min. The cells were then stained with Giemsa (Merck, Darmstadt, Germany; 1.09204.0500) and May-Grunwald's eosin methylene blue (Merck; 1.01424.0500) according to the manufacturer's instructions. Blood cells of KM and PB were calculated manually based on their morphologies.[30]

Treatment with chemotherapeutic agents

Embryos were soaked in egg water containing cytarabine (Pfizer, Milano, Italy), quizartinib (LC Laboratories, Woburn, MA, USA) or flavopiridol (Santa Cruz, Dallas, TX, USA;

sc-202157) for drug treatment. Adult fish were intraperitoneally injected with cytarabine (600–2000 mg/kg) and flavopiridol (30–130 mg/kg) once daily for 4 days. The doses for intraperitoneal injection were based on the trials in murine models,[31,32] and 4–20-fold higher doses were applied in adult fish.

Statistical analysis

The differences between categorical variables were analyzed by Fisher's exact tests. Continuous variables were compared by two-tailed Student's *t*-tests. In all graphs, error bars reflect mean ± s.d. A *P*-value less than 0.05 was considered significant. *P*-values were not adjusted for multiple comparisons and the variation was similar between the groups that were compared. No statistical methods were used to predetermine sample size. The sample size was determined based on numbers reported in previous studies and no samples were excluded. Experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment.

RESULTS

Two different *c-myb* transcripts, a wild-type and a truncated form, are produced from the *c-myb* PAC in the *c-myb-gfp* transgenic line

North *et al.*[22] have described the generation of a *c-myb-gfp* transgenic zebrafish line for labeling *c-myb*-positive hematopoietic stem cells. Unexpectedly, when examined the phenotype of *c-myb*$^{hkz3-/-}$; *c-myb-gfp*$^{+/-}$ embryos, we found that the *c-myb*$^{hkz3-/-}$ mutant phenotypes were completely rescued (data not shown). This result prompted us to speculate that a functional c-Myb must be produced in the *c-myb-gfp* transgenic line. Consistent with this idea, whole-mount *in situ* hybridization staining showed that *c-myb* expression in the aorta-gonad-mesoderm, caudal hematopoietic tissue and kidney was significantly increased in *c-myb-gfp* transgenic line compared with that in WT control fish (Figure 1a), suggesting *c-myb* might be overproduced in this line. To prove this is indeed the case, we sequenced the *c-myb* PAC in *c-myb-gfp* transgenic fish and found two duplicated sequences in the modified *c-myb* PAC: a 485-bp sequence (including a 315-bp core promoter region and a 170-bp 5′ UTR of *c-myb*) inserted before the translation start site (Figure 1b and Supplementary Figure S1A); and a large region from pWSMK-T vector to *c-myb* intron 10 inserted into the breakpoint within intron 10 (Figure 1b and Supplementary Figure S1B). 5′ and 3′-rapid amplification of cDNA ends analysis revealed that two different *c-myb* transcripts were produced from the *c-myb* PAC in the *c-myb-gfp* transgenic fish (Figure 1c and Supplementary Figure S1C). One transcript *c-myb*-WT was produced from the second *c-myb* core promoter and was identical to the endogenous *c-myb* (Figure 1c). The other transcript *c-myb*-T1 (4.3 kb), generated from the first *c-myb* core promoter, comprised of a truncated *c-myb* (from exon 1 to exon 10) followed by a near full-length *c-myb* (from exon 2 to exon 15) (Figure 1c). The *c-myb*-T1 transcript is predicted to produce a large fusion protein, in which a truncated c-Myb lacking the majority of negative-regulatory region[33] is fused with a nearly full-length c-Myb (lacking the first 8 aa) (Figure 1c and Supplementary Figure S1D). Quantification analysis indicated that the ratio of endogenous *c-myb,* transgenic *c-myb*-WT and *c-myb*-T1 in the KM of adult *c-myb-gfp* transgenic fish was 1:5:2 (Figure 1d), confirming that *c-myb* transcripts are indeed overproduced in the *c-myb-gfp*

transgenic line. As c-Myb-T1 fusion protein contains a truncated c-Myb lacking the inhibitory domain, we reasoned that the c-Myb-T1 had a higher transactivation activity as previously reported in mammals.[33] Indeed, luciferase reporter assay confirmed that c-Myb-T1 displayed a higher transactivation activity in activating the *lect2l* gene promoter (the zebrafish ortholog of the chicken c-Myb target gene *min-1*[34]) than c-Myb-WT (Supplementary Figure S1E). Thus, the *c-myb-gfp* transgenic fish appears to produce higher level of c-Myb-WT and a hyperactive truncated c-Myb-T1 and it is therefore referred as to *c-myb*^{hyper}.

Aberrant *c-myb* hyperactivity results in accumulation of abnormal granulocytes in early development

It has been shown that the appropriate levels of c-Myb are important for regulating the distinct differentiation steps during hematopoietic cell development,[35] and aberrant c-MYB activities have been known to associate with human myeloid leukemia.[36,37] We therefore explored the effects of aberrant c-Myb production on myeloid cell development in *c-myb*^{hyper} embryos and larvae using lineage-specific markers. We found that early myeloid markers, *c/epba* (CCAAT/enhancer-binding protein a)[38] and *lcp* (lymphocyte cytosolic protein 1),[39] were increased in *c-myb*^{hyper} embryos (Supplementary Figures S2A and B). Likewise, the expression of granulocytic markers, *lyz* (lysozyme C)[40] and *mpx* (myeloid-specific peroxidase),[41] was also elevated (Figure 2a and Supplementary Figure S2C). In contrast, the macrophage lineage markers, *mfap4* (microfibrillar-associated protein 4)[42] and c-*fms* (colony-stimulating factor-1 receptor),[43] did not show obvious increase in *c-myb*^{hyper} embryos (Supplementary Figures S2D and E). These data suggest a robust expansion of granulocytic lineage in *c-myb*^{hyper} embryos. This was further supported by the increased Sudan black (SB) staining, which preferentially marks granulocytes in zebrafish,[44] in the caudal hematopoietic tissue and KM in *c-myb*^{hyper} larvae (Figures 2b–d). Notably, the SB^{+} granulocytes in *c-myb*^{hyper} appeared to be larger in size and darker in SB staining (Figures 2b and c), compared with their sibling controls, indicating that the increased granulocytic cells perhaps as a result of early developmental defect.

To determine the myeloid cell expansion is attributed to single transcripts generated from the PAC or caused by combination effects of both products, we compared the granulocytic lineage expansion ability of the two forms by ectopically expressing *c-myb*-WT and *c-myb*-T1 driven by the same promoter in WT embryos. Consistent with the observation that c-Myb-T1 is hyperactive, we found that under the control of the *c-myb* mini promoter (Supplementary Figure S1A), ectopically expressing *c-myb*-T1 caused a modest increase of SB^{+} granulocytes, while *c-myb*-WT failed to do so (Figures 2e and f), though the expression of both transcripts is comparable (Figure 2g). However, overexpressing either *c-myb*-WT or *c-myb*-T1 under a stronger ubiquitous promoter (elongation factor-1 alpha promoter, *ef1a*) was sufficient to induce granulocytic lineage expansion as indicated by SB staining, though the induction by *c-myb*-T1 was more profound (Supplementary Figures S2F–H). These data indicate that the myeloid accumulation in *c-myb*^{hyper} fish is likely to be attributed to additive effects of *c-myb*-WT and *c-myb*-T1.

c-myb^{hyper} adult fish display abnormal myeloid cell expansion resembled MDS-like phenotypes mainly caused by proliferation perturbation

MYB hyperactivity has been shown to be main genetic mutations in several types of human lymphoid and myeloid leukemias.[2,14,15] To examine the possible development of leukemia-like hematological disorders in *c-myb*^{hyper} adult fish, KM and PB cells were randomly collected from 3-month-old and 1-year-old *c-myb*^{hyper} fish or siblings and subjected to cytological and blood cell count analyses. No significant alterations were observed in PB constituents in *c-myb*^{hyper} fish at 3 months or 1 year (Supplementary Figures S3A and B and Figures 3a and b). However, analysis of KM cells indicates a significant expansion of the myeloid cell population in 3-month-old *c-myb*^{hyper} fish, along with a corresponding reduction in hematopoietic precursors (Supplementary Figures S3C and D). By 1-year old, the myeloid population had almost doubled, accompanied by obvious reductions in other blood constituents in *c-myb*^{hyper} fish KM (Figures 3c and d), consistent with myeloid hyperplasia. In accordance with the cytological results, flow cytometry analysis of KM also showed a twofold increase in the myeloid cell population in 1-year-old adult *c-myb*^{hyper} fish (Supplementary Figures S3E and F). These results indicate marked myelodysplasia in *c-myb*^{hyper} fish characterized by elevated cell numbers, enlarged cell size and increased cell granularity, resembled the symptoms of human MDS, composed by a heterogeneous group of clonal hematological disorders that are usually diagnosed based on findings in PB, and especially the bone marrow and characterized by ineffective hematopoiesis, showing dysplastic feature in at least one lineage in the bone marrow.[45] To further determine if their internal organs were affected, *c-myb*^{hyper} and control fish were dissected and observed under the microscope. The kidneys in 1-year-old *c-myb*^{hyper} zebrafish appeared swollen, with an enlarged kidney area and almost fourfold increase in kidney weight compared with siblings (Figures 3e–g). Livers in *c-myb*^{hyper} fish were also heavier, with enrichment of invaded SB^{+} granulocytes compared with controls (Figures 3h and i). These defects resembled the effects on organs and invasion observed in myelogenous hematological malignancies. Overall, the blood phenotypes and organ infiltrations in *c-myb*^{hyper} fish resembled the symptoms of MDS.

The myeloid cell accumulation in *c-myb*^{hyper} fish from embryonic stage to adult could be caused by accelerated proliferation or reduced apoptosis. To clarify the cellular mechanisms responsible for myeloid cell expansion in *c-myb*^{hyper} fish, we monitored cell proliferation and cell death in neutrophils by bromodeoxyuridine incorporation and terminal dexynucleotidyl transferase (TdT)-mediated dUTP nick end labeling assay, respectively. Myeloid cell apoptosis in *c-myb*^{hyper} embryos and adult KM was comparable to those in sibling control (Supplementary Figures S3G–J), suggesting that the expansion of myeloid cells in *c-myb*^{hyper} is not caused by reduced apoptosis. On the other hand, myeloid cell bromodeoxyuridine incorporation was significantly increased in *c-myb*^{hyper} embryos and adult KM (Figures S3j–m), indicating that the expansion of myeloid cells in *c-myb*^{hyper} fish is the result of increased proliferation.

To further unveil how *c-myb* hyperactivity caused proliferation perturbation, we examined the expression of 12 cell-cycle-related genes including *ccna1,*[46] *ccnb1,*[47] *ccne,*[47] *cdk1,*[48] *pcna*[49] and *cdkn1c,*[50] which are known to be or predicted to be the direct targets of *c-myb*.

As shown in Table 1, the expression of the cell-cycle-promoting genes *ccna1, ccnb1, ccnd1, ccne, ccnh, cdk1, cdk2, cdk7, cdc20* and *pcna* were upregulated in *c-myb*^{hyper} MDS KM cells, while the cell-cycle inhibition genes *cdkn1c* and *cdkn2d* were downregulated in *c-myb*^{hyper} MDS KM cells (Table 1). Collectively, these results indicate that *c-myb* hyperactivity affects the expression of a cluster of cell-cycle-related genes, resulting in myeloid cell proliferation perturbation.

c-myb^{hyper} MDS fish can progress to AML and ALL

MDS is the most common hematologic malignancy in the elderly, and approximately one-third of MDS patients will eventually progress to acute leukemia.[51] We were therefore keen to test if adult *c-myb*^{hyper} fish developed AML or ALL (with myeloblasts or lymphoblasts increased) by analyzing blood cell counts or FACS of a large number of 10- 24-month-old adult *c-myb*^{hyper} fish and siblings. As expected, acute leukemia were never found in 324 of 10–24 months sibling fish. However, 18 AML and 18 ALL were found among 852 of 10-24-month-old *c-myb*^{hyper} fish (Table 2).

The AML-like *c-myb*^{hyper} fish showed >25% increase in precursors with myeloblasts morphology in the PB or >40% increase of precursors in KM (Figure 4a and Table 2), and some of them showed pathologic external features, including abnormal body contour, exophthalmos, bleeding and abdominal mass (Supplementary Figures S4C–F). Moreover, examination of tissue sections of AML-like *c-myb*^{hyper} fish showed obvious infiltrations of leukemic cells with myeloblast morphology in many non-hematopoietic tissues including liver, muscles and gills (Figure 4b and Supplementary Figure S5A). The typical granulocytic morphology of the myeloid cells accumulated in the KM (Figure 4a) and infiltrated in non-hematopoietic tissues (Figure 4b and Supplementary Figure S5A) indicates that *c-myb*^{hyper} fish could progress to AML with granulocytic origin.

As anticipated, the ALL-like *c-myb*^{hyper} fish showed >80% increase in lymphocytes or >60% increase in precursors with lymphoblasts morphology in the PB, and a >40% increase in lymphocytes in the KM (Figure 4c and Table 2), and likewise, some of them showed pathologic external features, including cachexia, exophthalmos, bleeding and curvature (Supplementary Figures S4G–J). Histological analysis revealed that many non-hematopoietic tissues including the gill, muscle, liver, eye and central nervous system were invaded by lymphocytic cells in the ALL-like *c-myb*^{hyper} fish (Figure 4d and Supplementary Figure S5B), which are similar to lymphocyte infiltration in ALL human patients. This was further confirmed by using the lymphoid-specific transgenic line *rag2-dsRed*,[26] in which *rag2*$^{+}$ cells were found in the head and the anterior half of the body (Supplementary Figure S5C). Examination of T-cell (*tcrα*, the T-cell receptor) and B-cell (*igu* and *iglc3* for immunoglobins) markers in the PB derived from 10 individual ALL-like *c-myb*^{hyper} fish revealed that six of the fish expressed a high level of T-cell receptor alpha (*tcrα*) but not B-cell marker, immunoglobins *igu* and *iglc3* (Figure 4e), whereas the remaining four fish were enriched in the expression of immunoglobins *igu* and *iglc3* but not T-cell marker *tcra* (Figure 4f). These results indicate that aberrant *c-myb* hyperactivity can lead to both T-cell and B-cell ALL.

Collectively, these data demonstrate that *c-myb*^{hyper} fish (10–24 months) can progress to AML and ALL with the incidence of ~ 2.1%, respectively. The occurrence of AML and ALL appears to be age-dependent as the incidence is much lower in 3- to 10-month-old young adult *c-myb*^{hyper} fish (~0.5%, only 1 out of 203 fish).

c-myb hyperactivity induced zebrafish leukemias are transplantable

To determine the aggressiveness of leukemia induced by c-Myb hyperactivity, whole KM cells from *c-myb*^{hyper} fish were transplanted into γ-irradiated WT adult hosts and tested whether the MDS, AML and ALL phenotype developed in *c-myb*^{hyper} fish could be transplanted into WT fish. For MDS, KM cells were harvested from 1-year-old *c-myb*^{hyper}*/lyz-dsRed* MDS-like donors or *lyz-dsRed* controls, in which granulocytes are marked by red fluorescence. Each of the irradiated WT fish received 0.2 million KM cells from 10 independent MDS donors and then raised under normal condition. All survived 21 recipients developed MDS within 2–8 weeks after transplantation as indicated by the infiltration of dsRed^{+} granulocytes in the periphery (Figure 5a and Supplementary Figure S6A) and the robust expansion of dsRed^{+} myeloid cells in the KM (with ~ 45% increase of myelomonocytes) (Figures 5b and c and Supplementary Figures S6B and C). In contrast, none of control fish showed any sign of MDS-like phenotype (Figures 5a–c and Supplementary Figures S6B and C). For AML, similar protocol was employed with the exception that the donor KM cells were collected from AML-like *c-myb*^{hyper} fish (fluorescence labeled *c-myb*^{hyper} AML fish are unavailable) and WT fish as control. As expected, all three survived recipients received cells from two independent AML donors displayed a significant increase of myeloblasts in the KM within 4–6 weeks after transplantation, whereas controls exhibited normal blood composition (Figures 5d and e). For ALL transplantation, KM cells were collected from 1-year-old *c-myb*^{hyper}*/rag2-dsRed* ALL-like donors or *rag2-dsRed* controls, in which lymphocytes were marked by dsRed. Within 6–9 weeks after transplantation, all six survived recipients received cells from three different ALL donors displayed ALL-like phenotype as indicated by the robust infiltration of *rag2*$^{+}$ lymphoblasts, whereas none of controls developed ALL-like phenotype (Figure 5f). These data demonstrate that MDS, AML- and ALL-like leukemic cells in *c-myb*^{hyper} are transplantable.

c-myb^{hyper} leukemic model responds to chemotherapeutic drug treatment

Growing evidences have demonstrated that zebrafish is a promising animal model for drug discovery.[19] We therefore evaluated the effects of widely used anti-leukemic agent in *c-myb*^{hyper} fish. For drug treatment, the tolerance of chemotherapeutic was tested and the maximum dose was used (Supplementary Figure S7A). Cytarabine[52] is commonly used to treat AML by inhibiting the cell cycle. *c-myb*^{hyper} embryos and their siblings were incubated with 10^{3} mg/l cytarabine at 1 dpf, and the numbers of SB^{+} granulocytic cells were calculated in the caudal hematopoietic tissue at 6 dpf (Figures 6a, b and f). Results showed that cytarabine treatment significantly reduced the number of SB^{+} granulocytes in *c-myb*^{hyper} embryos (Figures 6b and f), compared with the untreated controls (Figures 6a and f). However, when we performed cytarabine treatment in adult *c-myb*^{hyper} fish with a 6–20-fold higher than the dose used in mice,[31] no curative effect was observed (Supplementary Figure

S7B). These results indicate that the cell-cycle-specific antiproliferation drug cytarabine can relieve neoplastic proliferation of granulocytes in *c-myb*^{hyper} embryos but not in adult fish.

To obtain better curative effects, we turned to the *c-MYB*-targeting drug flavopiridol.[53] Maximum tolerance dose (MTD) (0.5 μM) of flavopiridol significantly reduced the numbers of SB^{+} granulocytes in *c-myb*^{hyper} embryos compared with the untreated controls (Figures 6c, d and f and Supplementary Figure S7A). As expected, *c-myb* RNA levels were also significantly reduced in *c-myb*^{hyper} embryos upon flavopiridol treatment compared with untreated fish (Figure 6g). On the other hand, Flt3-targeting drug quizartinib[54] was included as a control and treatment with maximum tolerance dose (0.5 μm) of quizartinib had little effect on the number of SB^{+} granulocytes and the *c-myb* RNA levels in *c-myb*^{hyper} embryos (Figures 6c, e and f and Supplementary Figure S7A). These results indicated that clinically used *c-MYB*-targeting drug can specifically and effectively reduced the expanded myeloid population in *c-myb*^{hyper} embryos. To further evaluate the anti-leukemic activity of flavopiridol in adulthood, MDS-like *c-myb*^{hyper} adult fish were intraperitoneal injected with flavopiridol and phosphate-buffered saline control (once per day) and the number of myeloid cells was quantified 4 days later. Results showed that a low dose (30 mg/kg/day) of flavopiridol was able to significantly reduce the number of myeloid cells in *c-myb*^{hyper} KM, and a higher dose (130 mg/kg/day) produced a much profound effect (Figure 6h). Likewise, the *c-myb* level was also significantly reduced in *c-myb*^{hyper} KM after flavopiridol treatment (Figure 6i). Notably, flavopiridol treatment (both low and high concentrations) had no obvious effect on the number of myeloid cells in control adult fish (Figure 6h). Taken together, the above results demonstrate that the *c-MYB*-targeting drug flavopiridol can effectively relieve MDS in *c-myb*^{hyper} embryos and adult fish.

DISCUSSION

For decades, high expression of *c-MYB* has been found to associate with oncogenic activity and poor prognosis in human AMLs,[55] colorectal tumors[56] and adenoid cystic carcinomas.[57] *c-MYB* duplication was also identified in 8.4–15% human T-ALL.[14,15] The C-terminal truncated *c-MYB* lacking the negative-regulatory region region was also identified in human angiocentric glioma,[58] AML[59] and a leukemic cell line established from a chronic myeloid leukemia patient in T-cell blast crisis.[16] In this study, we investigated a zebrafish leukemia model with aberrant *c-myb* hyperactivity. This *c-myb*^{hyper} model display MDS, AML and ALL phenotypes that resemble the situation of human patients. The neoplastic abnormalities of blood cells in *c-myb*^{hyper} fish are likely caused by the combined contribution of the duplicated c-Myb-WT and the hyperactive c-Myb-T1, which leads to the upregulation of the same sets of downstream genes including many well-known c-Myb targets involved in cell-cycle progression shown in Table 1, resulting in the development of neoplastic phenotypes. However, we cannot exclude the possibility that the hyperactive c-Myb-T1 may regulate additional sets of the downstream targets distinctive from those of c-Myb-WT and further investigation is required to clarify this issue. Nonetheless, our study demonstrates that *c-myb* plays a crucial role in leukemogenesis *in vivo*, and the effective response of this model to chemotherapeutic agents will facilitate future drug evaluation and screening.

MDS is the most common hematologic malignancy affecting the elderly, and patients with MDS often progress to AML or ALL.[51] However the mechanisms responsible for the progression from early MDS to ALL or AML remain unclear. The acquisition of additional mutations acts as a factor driving this transition.[60] *c-myb*^{hyper} zebrafish exhibited MDS-like pathological phenotypes from the embryonic stages to adulthood with the subsequent development of AML- or ALL-like symptoms in a small portion of adult fish, with a long latency, similar to the clinical observation of MDS progression to AML or ALL in human patients. MDS phenotypes were found in almost all *c-myb*^{hyper} fish progeny, and ectopic expression of *c-myb*-WT and *c-myb*-T1 contributed to myeloid expansion in WT embryos, suggesting the c-Myb alone is sufficient for the development of MDS; however, the subsequent transformation to an acute leukemia-like phenotype only occurred in some individuals, suggesting c-Myb may be an initiating event for this progression, and clonal evolution or other events were responsible for this progression. The *c-myb* model thus provides an opportunity to investigate additional risk factors for myeloid and lymphoid leukemias.

Flavopiridol is a *MYB*-targeting drug that inhibits *myb* transcriptional elongation.[53] Although flavopiridol has been used for the treatment of lymphocytic leukemia in the clinic, its efficacy in relation to myeloid leukemia remains unclear. Some reports have indicated that flavopiridol, combined with other chemotherapeutic drugs, could be used to treat high-risk or drug-resistant patients with myeloid leukemia.[61] In our study, overaccumulated granulocytes in the *c-myb*^{hyper} were largely restored after treatment with flavopiridol, suggesting that it may be effective for myeloid leukemias with aberrant *c-myb* hyperactivity. These results thus provide new evidence for the clinical application of flavopiridol, as well as confirming the use of the *c-myb*^{hyper} zebrafish disease model as an *in vivo* system for investigating new chemotherapeutic drugs.

In summary, this study demonstrated that *c-myb* hyperactivity in zebrafish leads to MDS, AML and ALL, and that this leukemia animal model responds effectively to clinically used chemotherapeutic drugs. These results suggest that c-Myb may be an attractive therapeutic target in certain types of leukemia, and *c-myb*^{hyper} zebrafish may provide a valuable *in vivo* drug-screening model for anti-leukemia drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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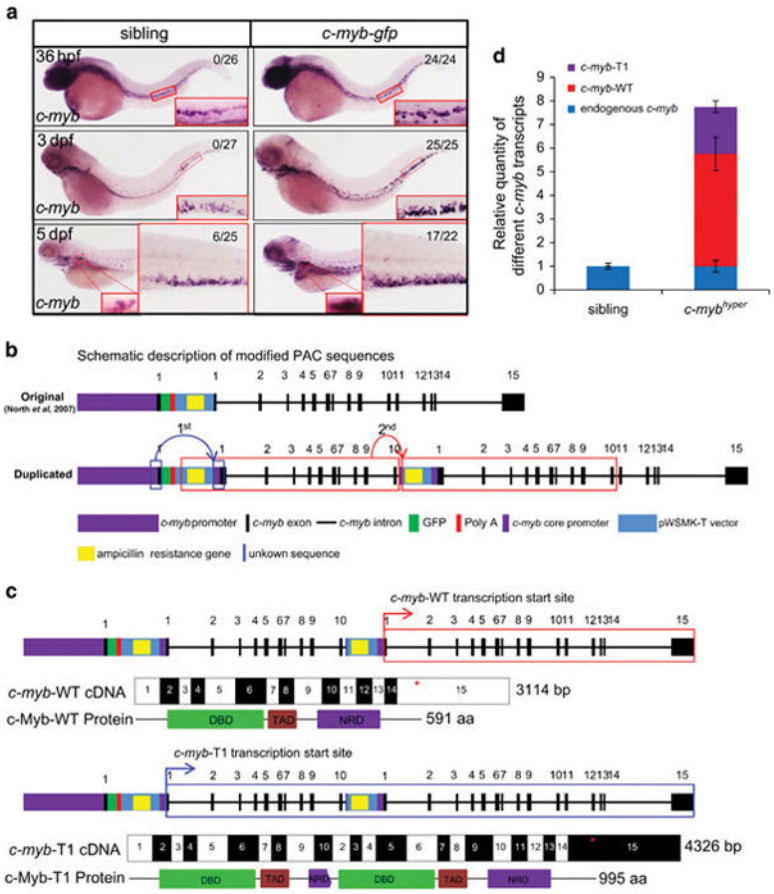

Figure 1.

c-myb-WT and *c-myb*-T1 transcripts were produced from the *c-myb* PAC in *c-myb-gfp*. (**a**) *c-myb* mRNA is overproduced in *c-myb-gfp* transgenic zebrafish. Whole-mount *in situ* hybridization of *c-myb* expression at 36 hpf, 3 dpf and 5 dpf. Enlarged details of the aorta-gonad-mesoderm, caudal hematopoietic tissue (CHT) and kidney are boxed in red (×20). Numbers in each panel indicate the number of embryos with elevated expression of markers out of total number of fish (Fisher's exact tests, $P<0.01$). (**b**) Two duplications were found in the modified PAC in *c-myb-gfp* transgenic zebrafish. The first repeat (1st, 485-bp repetitive mini promoter) is indicated by blue box, and the second repeat (2nd, pWSMK-T to *c-myb* intron 10 region) is indicated by red box. The inserting sites of repetitive sequence are indicated by arrows. Exons of *c-myb* (black bar); pWSMK-T (light blue bar) including GFP (green bar), SV40 polyA signal (red bar) and the reversed ampicillin resistance gene (AmpR, yellow bar); 77-bp additional unknown sequence is indicated (blue bar). (**c**) Transcripts and proteins of *c-myb*-WT and *c-myb*-T1. The transcription start sites of *c-myb*-WT and *c-myb*-T1 are indicated by red arrows (upper panels) and blue arrows (lower panels), respectively. Stop codons are marked by asterisk. The DNA-binding domain (DBD), transactivation domain (TAD) and negative-regulatory domain (NRD) of c-Myb protein are indicated. (**d**) Relative expression of different *c-myb* transcripts in 1-year adult kidney marrow (sibling and *c-myb*^{hyper}, $n=15$ and $n=12$, respectively).

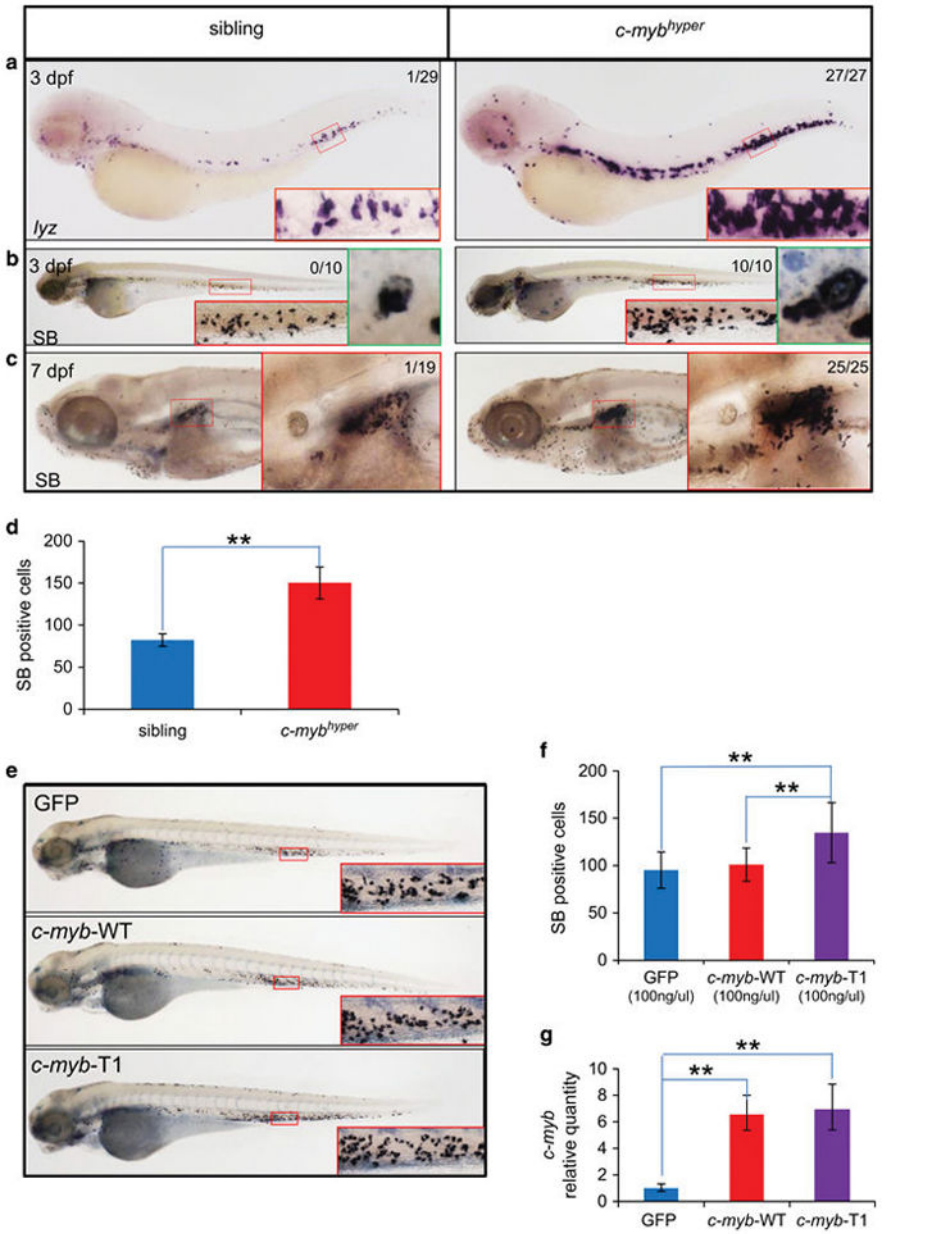

Figure 2.

Abnormal myeloid cell expansion in *c-myb-gfp* embryos and larvae. (**a**–**d**) Accumulation of neutrophils in *c-myb-gfp* embryos and larvae. Whole-mount *in situ* hybridization of *lyz* (**a**) expression at 3 dpf in *c-myb-gfp* fish and non-transgenic siblings. SB staining in 3 dpf (**b**) and 7 dpf (**c**) larvae. The caudal hematopoietic tissue (CHT) and kidney regions were enlarged in red box (×20). Green boxes (**b**) show further enlarged region focusing on a single cell (×100). Numbers in each panel indicate the number of embryos with elevated expression of markers out of total number of fish (Fisher's exact tests, $P<0.01$). SB^+ cell counts in 3 dpf (**d**) (*t*-test, $n=10$; mean ±s.d.; $*P<0.05$, $**P<0.01$). (**e**–**g**) Ectopically expressed *c-myb*-WT and *c-myb*-T1 under the *c-myb* mini promoter. cDNAs of *c-myb*-WT-*gfp*, *c-myb*-T1-*gfp* and *gfp* control driven by *c-myb* mini promoter were injected into one-

cell stage WT embryos, respectively. Representative pictures of SB-positive cell counts in 3 dpf embryos (**e**). SB^+ myeloid cell counts (**f**) and *c-myb* transcript levels (**g**) after injection were calculated and compared at 3 dpf (*t*-test; *gfp, c-myb*-WT-*gfp*, *c-myb*-T1-*gfp*, n =21, n =24 and n =23, respectively; mean ±s.d.; *P<0.05, **P<0.01).

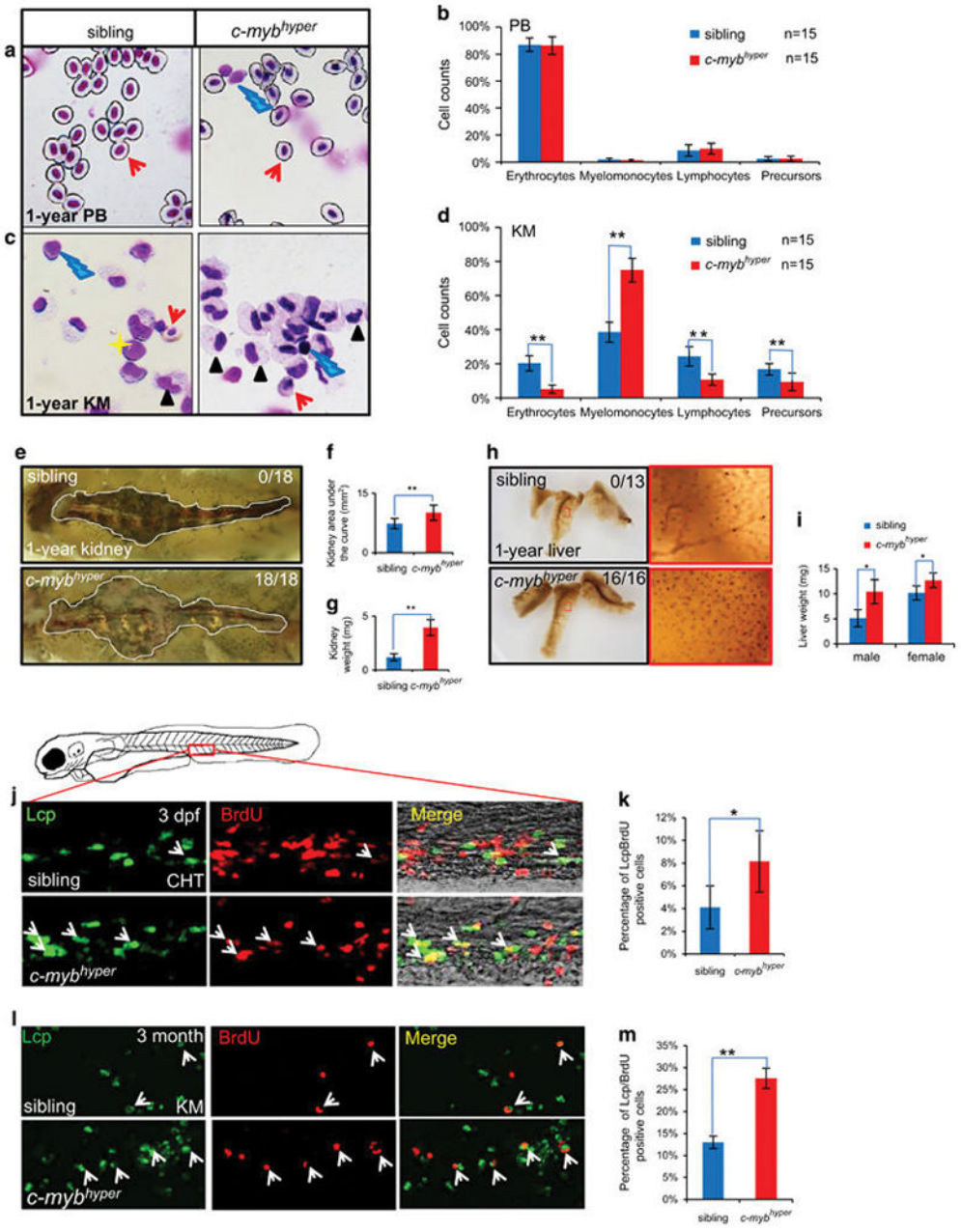

Figure 3.

c-myb^{hyper} adult fish exhibit MDS-like phenotypes with abnormal myeloid cell expansion in the kidney. (**a**–**d**) Peripheral blood (PB) cells (**a**) and whole kidney marrow (KM) blood cells (**c**) in 1-year-old *c-myb*^{hyper} and sibling fish stained with May-Grunwald/Giemsa. Blood cell counts of PB (**b**) and KM (**d**) in siblings (blue bars) and *c-myb*^{hyper} fish (red bars) were calculated manually based on their morphology. The black asterisks indicate statistical difference (*t*-test) ($n = 15$; mean ±s.d.; *$P<0.05$, **$P<0.01$). Red arrows, yellow asterisks, black arrowheads and blue lightnings indicate erythrocytes, precursors, myelomonocytes and lymphocytes, respectively. (**e**–**i**) Nephromegaly and hepatomegaly occurred in *c-myb*^{hyper} fish. Kidneys from 1-year transgenic fish were enlarged (**e**) to 1.4-fold in area (**f**) and 3.3-

fold in weight (**g**) compared with siblings. (**h**) SB staining in livers from 1-year transgenic fish showed more SB^+ cells than that of siblings. The right red columns show enlarged details of the left red-boxed region. (**i**) Liver weights in *c-myb*^{hyper} fish were increased compared with siblings (*t*-test, sibling and *c-myb*^{hyper}, $n=10$ and $n=12$, respectively; mean ±s.d.; *$P<0.05$, **$P<0.01$). Numbers in each panel indicate the number of liver with elevated SB-positive cells out of total number of liver (Fisher's exact tests, $P<0.01$) (**j**–**m**) Aberrant *c-myb* activity caused increased proliferation of myeloid cells in embryos (**j**, **k**) and KM of adults (**l**, **m**). Double staining of bromodeoxyuridine (BrdU)/Lcp (**j**, **l**) show BrdU incorporation of caudal hematopoietic tissue (CHT)/KM Lcp^+ cells in 3 dpf/3-month *c-myb*^{hyper} and siblings. Arrows indicate Lcp/BrdU double-positive cells. Percentage of the CHT and KM localized Lcp^+ myeloid cells that incorporate BrdU (**k**, **m**) in Lcp^+ myeloid cells (*t*-test, sibling and *c-myb*^{hyper}, $n=15$ and $n=13$, respectively; mean ±s.d.; *$P<0.05$, **$P<0.01$).

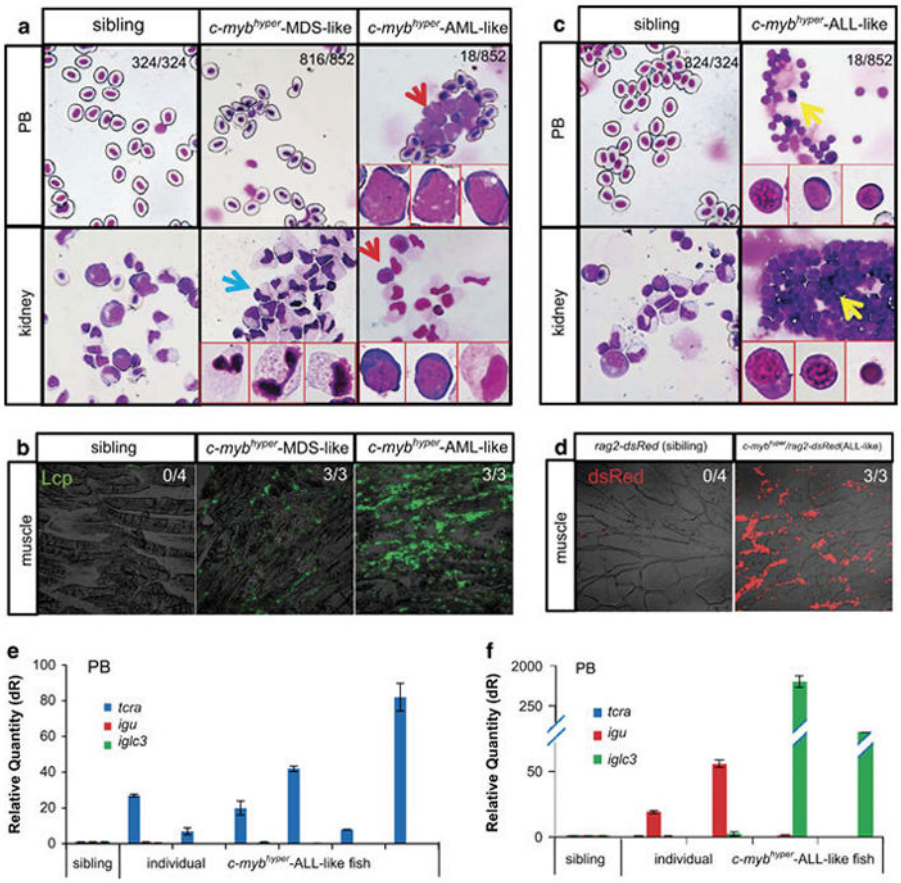

Figure 4.

c-myb^{hyper} zebrafish develop AML and ALL in adulthood. (**a**, **b**) *c-myb*^{hyper} zebrafish develop AML in adulthood. (**a**) May-Grunwald/Giemsa staining of PB cells (upper panels) and KM blood cells (lower panels) were obtained from siblings (left panels) and *c-myb*^{hyper} zebrafish (middle and right panels). Blue arrows indicate accumulated neutrophils in MDS-like fish KM. Red arrows indicate myeloid blasts in AML-like fish. Red boxes show enlarged details of myeloid and blast cells (×60). (**b**) Immunofluorescent staining of the myeloid-specific marker Lcp in paraffin-embedded sections confirmed myeloid cells infiltration of the skeletal musculature in *c-myb*^{hyper} MDS and AML. (**c**–**f**) *c-myb*^{hyper} zebrafish develop ALL in adulthood. (**c**) May-Grunwald/Giemsa staining of PB cells (upper panels) and KM blood cells (lower panels) obtained from siblings (the left column) and *c-myb*^{hyper} zebrafish (the right column). Yellow arrows indicate lymphoid blasts in ALL-like fish. Red boxes show enlarged details of lymphoid and blast cells (×60). (**d**) Immunofluorescent staining of sections confirmed lymphocytes infiltration of the skeletal musculature in *c-myb*^{hyper} ALL. Expression of T-cell marker *tcra* (**e**) or B-cell markers *igu* and *iglc3* (**f**) in individual siblings and *c-myb*^{hyper} fish. Numbers in (**b**) and (**d**) indicate the number of fish with leukemia infiltration out of total number of fish (Fisher's exact tests, $P<0.05$).

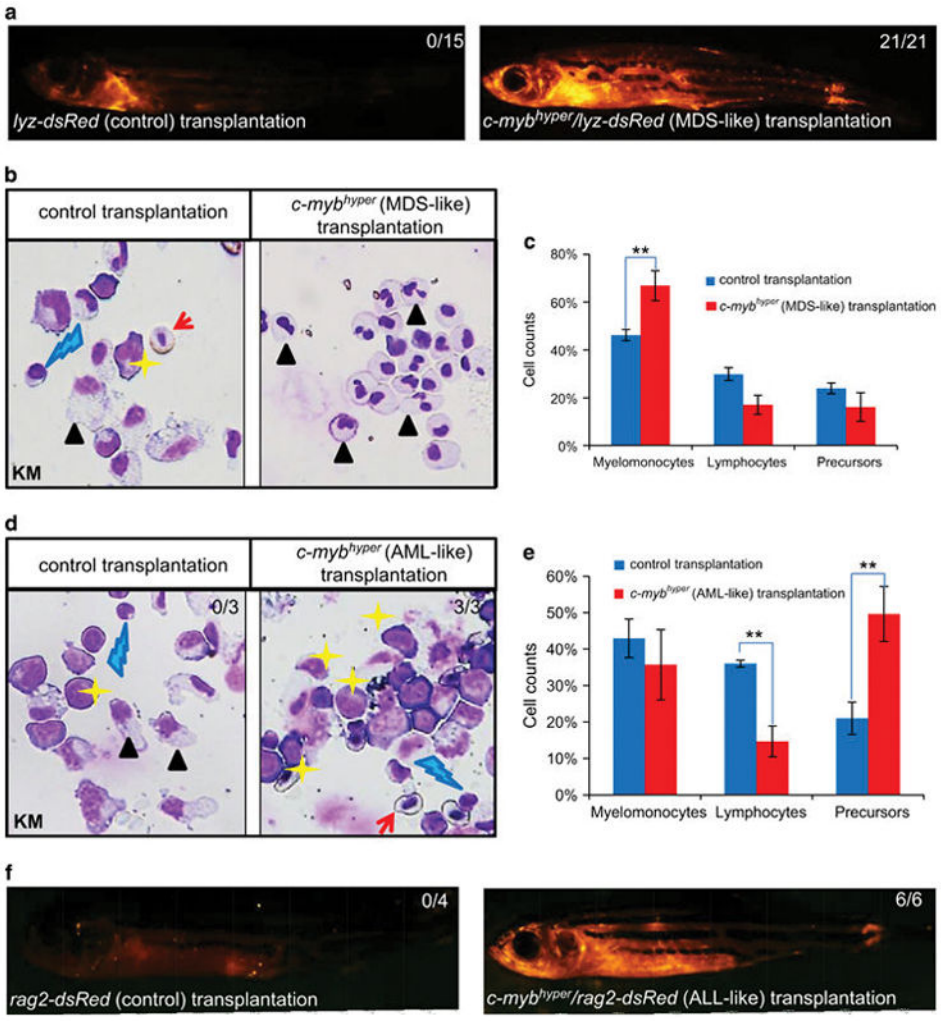

Figure 5.

MDS, AML and ALL-like leukemic cells in *c-myb*^{hyper} zebrafish are transplantable. (**a**–**c**) Twenty-one and 15 survived (of 102 and 80) recipients transplanted with MDS-like *c-myb*^{hyper}*/lyz-dsRed* and *lyz-dsRed* control KM cells, respectively. dsRed positive cells repopulated in all survived recipients within 4–8 weeks after transplantation (**a**). KM cells in fish transplanted with *c-myb*^{hyper}*/lyz-dsRed* MDS cells (left panel) and *lyz-dsRed* KM cells (right panel) were stained by May-Grunwald/Giemsa (**b**). Blood cell counts of KM were calculated manually based on their morphology (**c**). (**d**, **e**) Three and 3 survived (of 20 and 21) recipients transplanted with ALL-like *c-myb*^{hyper} and siblings control KM cells, respectively. All survived recipients were stained by May-Grunwald/Giemsa (**d**). Blood cell counts of KM (**e**) were calculated manually based on their morphology (KM in fish transplanted with sibling and AML-like *c-myb*^{hyper}, $n=3$ and $n=3$, respectively). (**f**) Six and 4 survived (of 56 and 25) recipients transplanted with *c-myb*^{hyper}(ALL-like)/*rag2-dsRed* and *rag2-dsRed* control KM cells. dsRed-positive cells repopulation in all survived recipients within 4–8 weeks after transplanted. Numbers in each panel indicate the number of fish with leukemia-like phenotype out of total number of fish (Fisher's exact tests, $P<0.05$). The black asterisks indicate statistical differences (t-test; mean ±s.d.; *$P<0.05$, **$P<0.01$). Red arrows,

yellow asterisks, black arrowheads and blue lightnings indicate erythrocytes, precursors, myelomonocytes and lymphocytes, respectively.

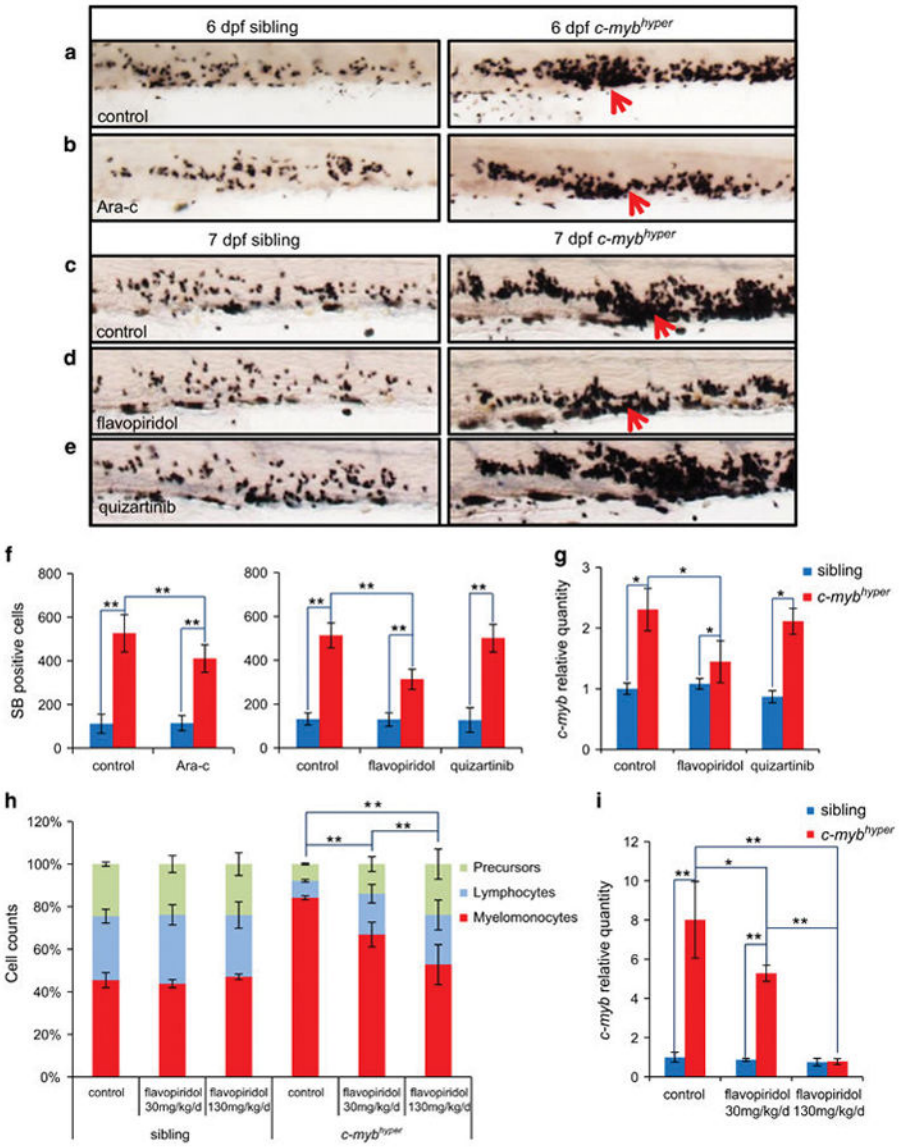

Figure 6.

c-myb^{hyper} MDS-like zebrafish respond to chemotherapy. (**a**–**g**) Drug treatments in embryos. 1 dpf siblings (left panels) and *c-myb*^{hyper} (right panels) larvae treated with phosphate-buffered saline control (**a**) and cytarabine (**b**) for 5 days and stained with SB at 6 dpf. 1 dpf larvae treated with dimethylsulfoxide (DMSO) control (**c**), flavopiridol (**d**) and quizartinib (**e**) for 6 days and stained with SB at 7 dpf. (**f**) Average numbers of SB^{+} cells per larva with drug treatment (*t*-test, sibling and *c-myb*^{hyper}, n =14 and n =10, respectively; mean ±s.d.; **P*<0.05, ***P*<0.01). (**g**) Relative expression of *c-myb* gene in siblings (blue bar) or *c-myb*^{hyper} fish (red bar) treated with flavopiridol or quizartinib were examined by qPCR. (*t*-test, n =30; mean ±s.d.; **P*<0.05, ***P*<0.01). (**h**) Drug treatments in adult fish. Blood cell counts of 1-year siblings and *c-myb*^{hyper} KM after intraperitoneal injection with flavopiridol (30 mg/kg/day or 130 mg/kg once daily for 4 days) or phosphate-buffered saline (*t*-test, n =10; mean ±s.d.; **P*<0.05, ***P*<0.01). (**i**) Relative expression of *c-myb* gene in siblings

(blue bar) or *c-myb*^{hyper} adult KM (red bar) treated with flavopiridol was examined by qPCR. (*t*-test, $n = 30$; mean ±s.d.; $*P<0.05$, $**P<0.01$).

Table 1

Relative expression of 12 cell-cycle-related genes in sibling and *c-myb*^{hyper} MDS-like fish

Gene	Sibling	c-myb^{hyper}
ccna1	1 ±0.10	22.34 ±6.63 **
ccnb1	1 ±0.11	26.25 ±3.94 **
ccne	1 ±0.10	12.81 ±1.13 **
ccnd1	1 ±0.13	14.01 ±1.71 **
ccnh	1 ±0.25	264.88 ±58.91 **
cdk1	1 ±0.24	7.42 ±1.70 **
cdk2	1 ±0.29	39.83 ±14.51 **
cdk7	1 ±0.25	239.9 ±54.34 **
cdc20	1 ±0.25	73.69 ±16.65 **
pcna	1 ±0.24	30.00 ±6.65 **
cdkn1c	1 ±0.05	0.20 ±0.02 **
cdkn2d	1 ±0.06	0.27 ±0.06 **

The total RNA was collected from four KMs of sibling or *c-myb*^{hyper}each time, and the experiments were triplicated, totally. *T*-test, sibling and *c-myb*^{hyper}, n =12 and n =10, respectively; mean ±s.e.m.;

** P<0.01.

Table 2

Hemogram and classification of *c-myb*^{hyper} fish at 10–24 months

Classification	Group	Number	Percentages in PB			Percentages in KM			Leukemic cell types
			Myelomonocytes	Lymphocytes	Precursors	Myelomonocytes	Lymphocytes	Precursors	
Sibling		14	18.5 ±2.6	68.0 ±5.3	14 ±3.9	45.0 ±2.7	32.8 ±2.0	22.3 ±1.7	
c-myb^{hyper}-MDS		47	14.8 ±2.4	76.3 ±2.4	8.9 ±0.8	73.1 ±1.9	16.5 ±1.4	10.5 ±0.9	Neutrophil
c-myb^{hyper}-AML	I	2	8.7 ±4.2	32.8 ±15.9	58.5 ±20.0[a]	23.0 ±13.5	34.5 ±8.5	42.0 ±4.9[a]	Neutrophil
	II	12	12.3 ±4.6	26.1 ±5.1	61.6 ±8.0[a]	77.8 ±2.8	12.5 ±2.3	9.7 ±1.1	Neutrophil
	III	4	15.5 ±1.5	74.2 ±1.4	10.3 ±2.8	36.7 ±1.5	22.2 ±1.0	41.1 ±0.6[a]	Neutrophil
c-myb^{hyper}-ALL	I	13	6.0 ±2.1	89.2 ±3.0[b]	4.9 ±1.7	27.7 ±5.5	60.6 ±6.6[b]	11.5 ±4.3	T-cell
	II	5	0.9 ±0.2	18.8 ±7.1	80.3 ±7.2[c]	29.3 ±10.3	64.4 ±11.1[b]	6.8 ±2.4	B-cell

The percentages were indicated by mean ±s.e.m. Adult *c-myb*^{hyper} fish characterized by >25% precursors with myeloblasts morphology in PB or >40% in KM were divided into AML; and fish characterized by >80% lymphocytes or >60% precursors with lymphoblasts morphology in the PB, and >40% in the KM were divided into ALL. AML-Group I: precursors increased in both PB and KM. AML-Group II: precursors increased in PB. AML-Group III: precursors increased in KM. ALL-Group I: lymphocytes increased in both PB and KM. ALL-Group II: precursors increased in PB and lymphocytes increased in KM (mean ± s.e.m.).

[a]Indicates precursors in PB or KM increased >25% or 40%, respectively, in AML.

[b]Indicates lymphocytes in PB or KM increased >80% or >40%, respectively, in ALL.

[c]Indicates precursors in PB increased >60% in ALL.