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AML1-ETO reprograms hematopoietic cell fate by downregulating *scl* expression

Jing-Ruey J. Yeh[1,2,3,*], Kathleen M. Munson[1,2,3], Yvonne L. Chao[1,2], Quinn P. Peterson[1,2], Calum A. MacRae[1,2] and Randall T. Peterson[1,2,3,*]

AML1-ETO is one of the most common chromosomal translocation products associated with acute myelogenous leukemia (AML). Patients carrying the *AML1-ETO* fusion gene exhibit an accumulation of granulocyte precursors in the bone marrow and the blood. Here, we describe a transgenic zebrafish line that enables inducible expression of the human *AML1-ETO* oncogene. Induced AML1-ETO expression in embryonic zebrafish causes a phenotype that recapitulates some aspects of human AML. Using this highly tractable model, we show that AML1-ETO redirects myeloerythroid progenitor cells that are developmentally programmed to adopt the erythroid cell fate into the granulocytic cell fate. This fate change is characterized by a loss of *gata1* expression and an increase in *pu.1* expression in myeloerythroid progenitor cells. Moreover, we identify *scl* as an early and essential mediator of the effect of AML1-ETO on hematopoietic cell fate. AML1-ETO quickly shuts off *scl* expression, and restoration of *scl* expression rescues the effects of AML1-ETO on myeloerythroid progenitor cell fate. These results demonstrate that *scl* is an important mediator of the ability of AML1-ETO to reprogram hematopoietic cell fate decisions, suggesting that *scl* may be an important contributor to AML1-ETO-associated leukemia. In addition, treatment of AML1-ETO transgenic zebrafish embryos with a histone deacetylase inhibitor, Trichostatin A, restores *scl* and *gata1* expression, and ameliorates the accumulation of granulocytic cells caused by AML1-ETO. Thus, this zebrafish model facilitates in vivo dissection of AML1-ETO-mediated signaling, and will enable large-scale chemical screens to identify suppressors of the in vivo effects of AML1-ETO.

KEY WORDS: Hematopoiesis, Myeloid, Leukemia, Zebrafish

INTRODUCTION

Acute myelogenous leukemia (AML) is the most common form of leukemia. Each year, more than ten thousand new cases of AML are diagnosed in the United States and approximately a quarter of a million new cases are reported in the world (Stone et al., 2004). The (8;21)(q22;q22) chromosomal translocation can be found in 12-15% of AML patients. This translocation joins the *AML1* (also known as *CBFα2*, *RUNX1* and *PEBPαB*) gene and the *ETO* (also known as *MTG8*) gene (Downing, 1999; Peterson and Zhang, 2004). A majority of the patients carrying the *AML1-ETO* fusion gene have the M2 subtype of AML, according to the French-American-British classification, which is characterized by overproduction of granulocytic precursors. Granulocytes, like the cells of all blood lineages, are derived from multipotent hematopoietic stems cells (HSCs) through a series of cell fate decisions. It is thought that many leukemic oncogenes, including *AML1-ETO*, may contribute to the development of AML by affecting the specification and maturation of hematopoietic cells (Tenen, 2003).

AML1 by itself plays an important role in hematopoiesis. Normally, AML1 forms a complex with CBFβ, called the core-binding factor (CBF) complex. This complex binds the enhancer core motif and activates tissue-specific expression of a number of hematopoietic genes (Borregaard et al., 2001; Lutterbach and Hiebert, 2000). By contrast, ETO normally functions by recruiting the nuclear receptor co-repressor (N-CoR)/mSin3/histone deacetylase (HDAC) complex (Licht, 2001). The chromosomal translocation juxtaposes the region encoding the DNA-binding domain of the AML1 protein to the region encoding almost all of the ETO protein. Thus, the AML1-ETO fusion product is thought to antagonize the functions of AML1. In addition, previous studies indicate that this fusion protein has additional activities other than antagonizing AML1 function. For example, AML1-ETO knock-in mouse embryos contain abnormal hematopoietic progenitor cells that are not found in AML1-deficient mouse embryos (Okuda et al., 1998; Yergeau et al., 1997). Moreover, many of the AML1-ETO target genes that have been identified are not affected by AML1 (Shimada et al., 2000). At present, the full range of AML1-ETO target genes and their roles in AML pathogenesis remain poorly understood.

Many lines of evidence indicate that the (8;21) chromosomal translocation is likely to occur in a primitive hematopoietic progenitor cell capable of generating all hematopoietic lineages. For example, transcripts of the *AML1-ETO* fusion gene can be found in hematopoietic cells of non-myeloid lineages in some patients (Miyamoto et al., 2000). Moreover, it has been demonstrated that the cells capable of initiating leukemia are $CD34^{+}CD38^{-}$, a characteristic of non-committed primitive hematopoietic progenitor cells (Bonnet and Dick, 1997). Thus, it is important to know how AML1-ETO affects the specification of various hematopoietic lineages from multipotent progenitor cells. Unfortunately, AML1-ETO knock-in mouse embryos die in early gestation, making it difficult to analyze the effect of AML1-ETO in these models (Okuda et al., 1998; Yergeau et al., 1997). Interestingly, it has been shown that AML1-ETO can promote myelopoiesis in adult mice (de Guzman et al., 2002; Fenske et al., 2004; Schwieger et al., 2002). However, these mouse models manifest phenotypes only after

[1]Developmental Biology Laboratory, Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, MA 02129, USA. [2]Department of Medicine, Harvard Medical School, Boston, MA 02115, USA. [3]The Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA.

*Authors for correspondence (e-mails: jyeh1@partners.org; peterson@cvrc.mgh.harvard.edu)

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several months of latency. Thus, it is difficult to identify the immediate transcriptional and cytological changes that lead to the observed AML1-ETO effects.

During embryonic development of mammals, hematopoiesis starts and continues as two successive waves. The first wave, named primitive hematopoiesis, begins in the blood islands of the yolk sac at embryonic day 7 (E7.0) in the mouse. Although it is generally thought that only erythrocytes and macrophages are produced during primitive hematopoiesis in mammals, multilineage precursors are detected in the later, but still pre-circulation, yolk sac (Palis et al., 2001). The second wave of hematopoiesis, named definitive hematopoiesis, begins at E8.5 in the aorta-gonad-mesonephros (AGM) region. Definitive hematopoiesis produces HSCs that not only give rise to all blood lineages, but also possess self-renewal capabilities. Studies of zebrafish embryonic development have also identified two waves of hematopoiesis, a primitive wave beginning at 12 hours post-fertilization (hpf), and a definitive wave beginning at 24 hpf (Davidson and Zon, 2004). There is some evidence that the myeloerythoid progenitor cells (MPCs) arising from zebrafish primitive hematopoiesis are functionally equivalent to the common myeloid progenitors (CMPs) arising from mammalian definitive hematopoeisis, and many of the pathways governing hematopoietic cell fate decisions may be shared between these cells (Galloway et al., 2005; Rhodes et al., 2005). The MPCs of the primitive wave of hematopoiesis reside in two distinct embryonic locations that appear to influence their ultimate cell fates. MPCs of the rostral blood island (RBI) express the myeloid-specific transcription factor Pu.1 (Spi1 - Zebrafish Information Network) and produce cells of the myeloid lineage including macrophages and granulocytes, whereas MPCs of the intermediate cell mass (ICM) region express the erythroid-specific transcription factor Gata1 and produce erythrocytes (Davidson and Zon, 2004). It has been shown that abrogation of *pu.1* expression in zebrafish embryos results in erythropoiesis in the RBI. Conversely, abrogation of *gata1* expression results in myelopoiesis in the ICM region (Galloway et al., 2005; Rhodes et al., 2005). These results suggest that the primitive hematopoietic cells of the RBI and ICM are multipotent MPCs.

The zebrafish may be a useful model for uncovering the in vivo effects of AML1-ETO expression. Zebrafish MPCs arise in predictable locations and differentiate in synchrony, making it possible to assess the effects of AML1-ETO on reprogramming cell fate decisions. Manipulation of gene expression and tracking of specific cell lineages are straightforward in zebrafish, making it possible to identify the direct effects of AML1-ETO expression in vivo. In addition, zebrafish embryos can be readily adapted for high-throughput chemical screens to identify compounds that modify the effects of AML1-ETO in vivo. Here, we report the generation of an inducible zebrafish AML1-ETO model. Previously, it had been shown that injection of the human *AML1-ETO* cDNA can cause hematopoietic defects in zebrafish embryos (Kalev-Zylinska et al., 2002). However, the effects of *AML1-ETO* expression on cell fate decisions had not been explored, and temporal control of *AML1-ETO* expression was not possible. Using our stable, inducible transgenic line, we demonstrate that *AML1-ETO* expression in zebrafish disrupts both primitive and definitive hematopoiesis. We show that AML1-ETO reprograms erythropoiesis to granulopoiesis, resulting in a robust phenotype that exhibits cytological and transcriptional characteristics similar to those seen in human AML. We also show that the cell fate changes caused by *AML1-ETO* expression are preceded by and dependent upon a rapid downregulation of the hematopoietic stem cell factor Scl (also known as Tal1), and that a small molecule histone deacetylase inhibitor is able to rescue many of the effects of *AML1-ETO* expression on hematopoiesis.

MATERIALS AND METHODS

Zebrafish care and embryo collection

All zebrafish experiments were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. Zebrafish embryos were collected in Petri dishes and kept in a 23.0-28.5°C incubator until reaching the desired stages. The stages (hours post-fertilization) described in this report are based on the developmental stages of normal zebrafish embryos at 28.5°C (Kimmel et al., 1995).

Generation of the Tg(*hsp:AML1-ETO*) zebrafish line

To construct pHSP/AML1-ETO, we first amplified a 1.5-kb zebrafish *hsp70* promoter fragment from pHSP70/4 (Xiao et al., 2003) and cloned it into the *Hin*dIII and *Pst*I sites of the pG1 vector. Subsequently, the GFP fragment in pG1 was removed and replaced with the *Xba*I fragment containing the human *AML1-ETO* gene from pCS2cmv-RUNX1-CBF2T1 (Kalev-Zylinska et al., 2002). The transgenic line is maintained in TL strain background. The zebrafish carrying the transgene were identified by fin clipping and genotyping, using PCR primers AML1-f (5′-GGAAGA-GGGAAAAGCTTCAC-3′) and ETO-r (5′-GAGTAGTTGGGGGAG-GTGG-3′).

Heat treatments

Initially, zebrafish embryos were heat shocked in a 37-42°C incubator for 1 hour. The heat treatment was repeated three to four times every 12 hours from 4 hpf (Fig. 1, Fig. 2B,C, Figs 3, 4; Fig. S1 in the supplementary material). Later on, we found that incubation at 38°C for 1 hour once between 14 to 19.5 hpf is enough to reproducibly induce the AML1-ETO phenotypes, and, thus, this regimen was used in all subsequent experiments (Fig. 2A, Figs 5-9; Fig. S2, Figs S3-S6, Table S1 in the supplementary material).

Morpholino oligonucleotides and microinjection

The morpholino antisense oligonucleotides hAML1-MO (5′-CTGGC-ATCTACGGGGATACGCATCA-3′) and pu.1-MO (5′-GATGATGTACC-CCTCCATTCTGTAC-3′) were obtained from Gene-Tools, LLC. The Gata1-MO was purchased from Open Biosystems. For microinjection, 500 μM hAML1-MO, 200 μM pu.1-MO or 250 μM Gata1-MO in 0.3×Danieau's buffer were prepared and injected as described (Nasevicius and Ekker, 2000). For *scl* mRNA injection, *scl* mRNA was transcribed from *Danio rerio* full-length IMAGE cDNA clone (catalog number MDR1734, Open Biosystems) using SP6 mMESSAGE mMACHINE (Ambion), subjected to a Poly (A) tailing reaction (Ambion), and was then injected at 100-200 ng/μl concentrations.

Fluorescence microangiography

Fluorescence microangiography was performed as described (Weinstein et al., 1995).

Isolation of hematopoietic cells from zebrafish embryos

For embryos older than 26 hpf, hematopoietic cells were isolated as follows. Anesthetized embryos were transferred into phosphate buffered saline (PBS) containing 50 U/ml heparin, 1% bovine serum albumin and 0.006% tricaine. Tails were excised posterior to the yolk extension using a scalpel, and blood cells were extruded from the site of excision with the scalpel and collected using a micropipette.

For embryos younger than 26 hpf, hematopoietic cells were isolated as follows. Homozygous *gata1-DsRed* transgenic fish were crossed with wild-type or Tg(*hsp:AML1-ETO*) fish. Embryos were dechorionated and deyolked as described (Westerfield, 2000). The embryos were then rinsed in calcium-free Ringer's solution (116 mM NaCl, 2.9 mM KCl and 5 mM HEPES, pH 7.2), and incubated in 0.05% Trypsin-EDTA solution (GIBCO) at 28.5°C for 30 minutes. Finally, dissociated cells were rinsed with PBS and filtered through 40 μm meshes to obtain a single cell suspension. The cell suspension was processed with a flow cytometer and fluorescent cells were collected at the Massachusetts General Hospital Flow Cytometry Core.

Cytology

For cytological analyses, blood cells collected from the zebrafish embryos were transferred onto glass slides by cytospin and stained by Protocol® Wright-Giemsa stain (Fisher Diagnostics) following the manufacturer's instructions.

Microarray analysis

For hematopoietic cells collected from the flow cytometer, total RNA was isolated with Trizol (Invitrogen) from 5×10^4 cells. The samples were then processed and hybridized to Zebrafish Oligo Microarrays (Agilent Technologies) at the Whitehead Institute Center for Microarray Technology. For blood cells extracted from 38- to 40-hour-old zebrafish embryos, total RNA was isolated with RNAqueous®-Micro (Ambion) and was used to synthesize fluorescent probes using the Agilent Low RNA Input Fluorescent Linear Amplification kit. The probes were hybridized to Zebrafish Oligo Microarrays (Agilent Technologies) following the manufacturer's processing protocol. After washing and drying steps, the microarrays were scanned in an Agilent DNA Microarray Scanner and the images were processed using Feature Extraction software.

In situ hybridization

Digoxigenin-labeled antisense riboprobes for *gata1*, *mpo*, *l-plastin*, *scl*, *flk1*, *aml1*, *cmyb* and *c/ebpα* were made according to previous publications (Bennett et al., 2001; Burns et al., 2005; Lyons et al., 2001; Thompson et al., 1998). Whole-mount in situ hybridization was performed as described (Ransom et al., 1996).

RESULTS

Induced expression of *AML1-ETO* causes a rapid accumulation of hematopoietic cells and a concomitant loss of circulating blood cells in zebrafish embryos

To create a zebrafish model of *AML1-ETO* expression, we generated an inducible transgenic zebrafish line Tg(*hsp:AML1-ETO*) in which the human *AML1-ETO* transgene is under the control of the zebrafish *hsp70* promoter (Fig. 1A). To test the effect of *AML1-ETO* expression in zebrafish, we first crossed homozygous Tg(*hsp:AML1-ETO*) fish with wild-type fish and induced transgene expression at various times (see Materials and methods for heat-shock conditions). We then screened the embryos for any visible phenotypes at 24-40 hours post-fertilization (hpf). Wild-type embryos that have been subjected to the heat treatments do not show any abnormality and establish blood circulation (Fig. 1B,D). By contrast, Tg(*hsp:AML1-ETO*) embryos have no circulating blood cells even though their hearts are beating. Moreover, the majority of the blood cells in these embryos accumulate in a region that lies along the trunk ventral to the dorsal aorta but above the yolk extension, and in a region posterior to the yolk extension (Fig. 1C,E). The observed phenotype is consistent with the previously reported phenotype caused by injections of *AML1-ETO* DNA into zebrafish embryos (Kalev-Zylinska et al., 2002).

Using an antisense morpholino oligonucleotide against AML1-ETO, we have confirmed that this phenotype is dependent on the induced expression of *AML1-ETO* (see Fig. S1 in the supplementary material). In addition, we find that inducing *AML1-ETO* expression after 21 hpf greatly diminishes its effect (see Fig. S2 in the supplementary material).

AML1-ETO transgenic zebrafish possess a functional cardiovascular system despite a lack of circulating cells

In order to determine whether the loss-of-circulation and blood cell accumulation phenotypes are caused by cardiovascular defects in these embryos, we first analyzed the expression of the endothelial lineage marker *flk1* at 21 and 36 hpf. We found that, at 21 hpf, *flk1* expression is slightly decreased in Tg(*hsp:AML1-ETO*) embryos (Fig. 2A). However, at 36 hpf, there are no significant differences between the expression levels and patterns of *flk1* in wild-type embryos and those in Tg(*hsp:AML1-ETO*) embryos (Fig. 2A). We also crossed Tg(*hsp:AML1-ETO*) fish with an endothelial reporter line carrying the *fli1-EGFP* transgene (Lawson and Weinstein,

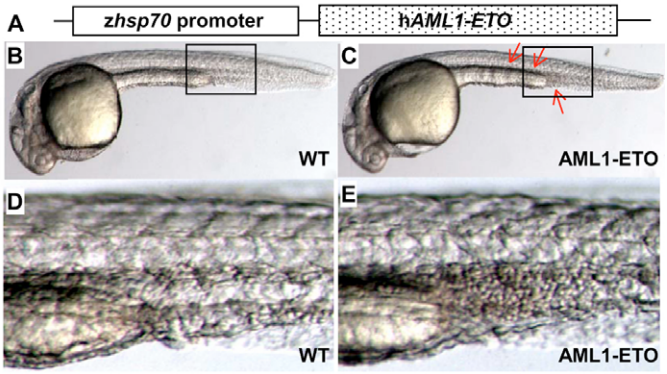

Fig. 1. Expression of *AML1-ETO* in zebrafish embryos causes an accumulation of hematopoietic cells. (**A**) Schematic diagram of the DNA fragment used to generate the Tg(*hsp:AML1-ETO*) zebrafish line. (**B**,**C**) Bright-light images of 1-dpf embryos that have been subjected to heat treatment. The accumulated hematopoietic cells in Tg(*hsp:AML1-ETO*) embryos are indicated with red arrows (C). The areas in the boxes are shown at a higher magnification in **D** and **E**.

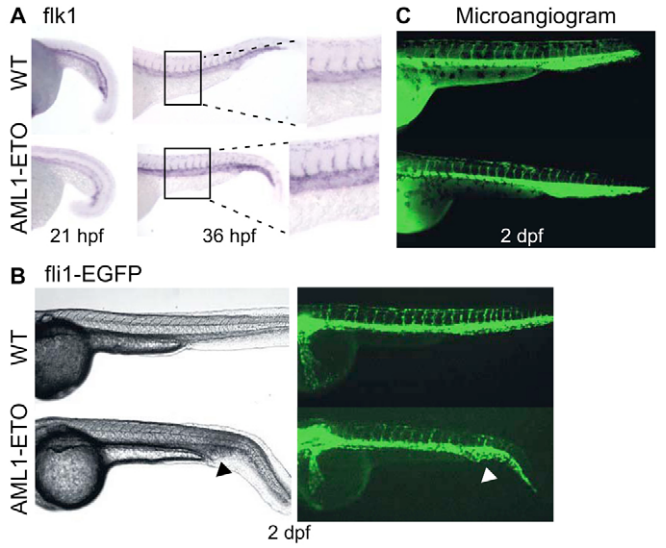

Fig. 2. *AML1-ETO* transgenic zebrafish possess functional cardiovascular systems. (**A**) *flk1* in situ hybridization of heat-treated wild-type and Tg(*hsp:AML1-ETO*) embryos harvested at designated stages as indicated. (**B**) Bright-light and fluorescent images of *fli1-EGFP* transgenic embryos in wild-type or Tg(*hsp:AML1-ETO*) background. These embryos have been subjected to the same heat treatment. The arrowhead indicates the site of the accumulated blood cells. (**C**) Fluorescent microangiography shows that the vasculature is continuous and the site of the accumulated blood cells is connected to the rest of the circulatory system.

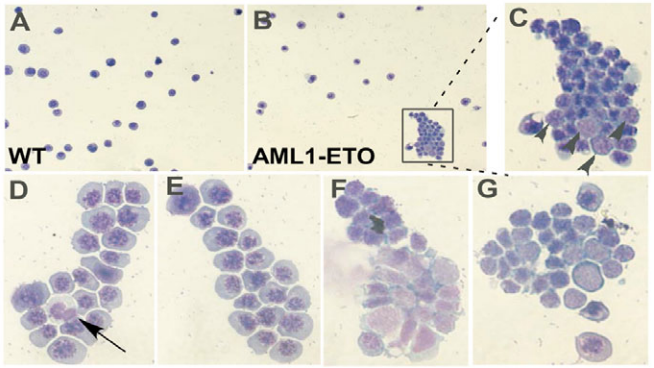

Fig. 3. The accumulated hematopoietic cells in Tg(*hsp:AML1-ETO*) embryos are enriched for immature blast cells. (**A-G**) Cytology of hematopoietic cells from wild-type (A,D,E) and Tg(*hsp:AML1-ETO*) (B,C,F,G) embryos at 40 hpf. Low magnification (A,B); high magnification (C-G). Arrowheads, blast-like cells (C); black arrow, a bi-nucleated heterophil/neutrophil (D).

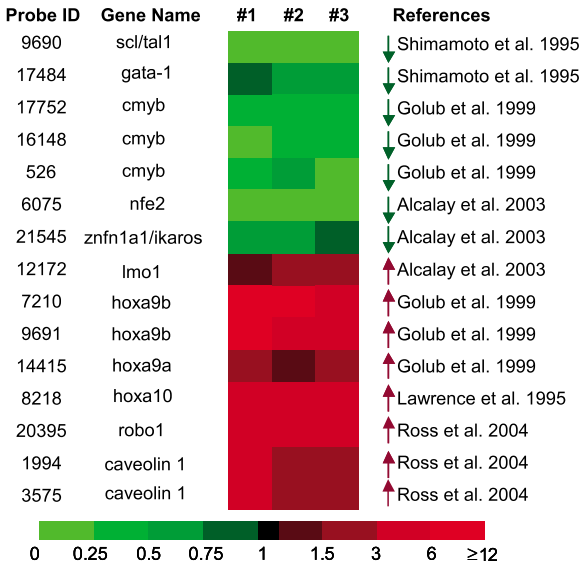

Fig. 4. The transcriptional changes in hematopoietic cells of Tg(*hsp:AML1-ETO*) embryos correlate with the transcriptional changes caused by *AML1-ETO* expression in human patients and in human cell lines. The embryos used for array #1 were heat shocked two times at 40 and 42°C for one hour each time at 16 and 24 hpf respectively. The blood cells were extracted at 38 hpf. The embryos for arrays #2 and #3 were heat-shocked three times at 40, 42 and 42°C for one hour each time at 16, 24 and 38 hpf respectively. The blood cells were extracted at 40 hpf. The ratios between the signals from Tg(*hsp:AML1-ETO*) and wild-type samples are shown in the heat map. The corresponding changes in the human studies are indicated in the References column.

2002) and found that, in the double transgenic embryos, *fli1-EGFP* expression is largely normal, except for a mildly reduced expression in the intersomitic vessels and an expansion in the ventral tail region (Fig. 2B). These results indicate that, if vascular development is affected in Tg(*hsp:AML1-ETO*) embryos, the defect is subtle. Finally, we employed fluorescent microangiography to test cardiovascular structure and function, and showed that fluorescein-coupled latex beads injected into the inflow tract of the atrium are able to perfuse the whole vascular system of Tg(*hsp:AML1-ETO*) embryos (Fig. 2C). These data indicate that Tg(*hsp:AML1-ETO*) embryos possess functional hearts, as well as lumenized and patterned circulatory systems. In contrast to our results, Kalev-Zylinska et al. have shown that injections of DNA containing the *AML1-ETO* cDNA elicit a vascular patterning defect (Kalev-Zylinska et al., 2002). The discrepancy between our results and those previously reported may be due to differences in the timing and duration of *AML1-ETO* expression in these two models.

AML1-ETO expression leads to an accumulation of immature hematopoietic blast cells

To investigate the blood phenotype induced by AML1-ETO expression, we extracted blood cells at 40 hpf from wild-type and *Tg*(*hsp:AML1-ETO*) embryos that had been subjected to the same heat treatment. Using cytology, we determined that the accumulated blood cells in *Tg*(*hsp:AML1-ETO*) embryos are dramatically enriched for immature blast-like cells. As shown in Fig. 3, blood from both wild-type and transgenic fish contains a mixture of individual cells and clusters of cells, although cell clusters are more prevalent in samples from the transgenic fish than in samples from wild-type fish. The blood cells from wild-type fish are predominantly erythrocytes, with myeloid cells only occasionally observed (Fig. 3A,D,E). By contrast, the blood cells from the transgenic fish contain abundant blast-like cells, which are larger than the erythrocytes and have high nuclear to cytoplasmic ratios (Fig. 3B,C,F,G). Surprisingly, these blast-like cells remained in the immature state even 4 days after the heat treatment (see Fig. S3 in the supplementary material). Therefore, *AML-ETO* expression results in an accumulation of immature hematopoietic blast cells in zebrafish embryos.

Transcriptional changes in the blood of Tg(*hsp:AML1-ETO*) embryos parallel those observed in human AML

To test whether transcriptional profiles in the zebrafish AML1-ETO model are similar to expression signatures of human AML, we performed microarray analysis using blood cells from wild-type and Tg(*hsp:AML1-ETO*) embryos isolated at 38-40 hpf. We found that the *scl*, *gata1*, *cmyb*, *nfe2* and *znfn1a1* genes are downregulated in Tg(*hsp:AML1-ETO*) zebrafish blood (Fig. 4). The *scl*, *gata1* and *cmyb* genes are all expressed in primitive erythroid cells. The reduction of their expression is likely to reflect a reduction of erythropoiesis in Tg(*hsp:AML1-ETO*) zebrafish. Decreases in *SCL* expression have also been shown in leukemia samples harboring the t(8;21) translocation (Shimamoto et al., 1995). In addition, it has been shown that *MYB* expression is downregulated in human AML patients but upregulated in acute lymphoblastic leukemia (ALL) patients (Golub et al., 1999). The *NFE2* and *ZNFN1A1* (also called *Ikaros*) genes, both involved in hematopoietic differentiation, are downregulated by AML1-ETO in a human mononuclear cell line (Alcalay et al., 2003).

The *lmo1*, *hoxa9*, *hoxa10*, *robo1* and *caveolin 1* genes are upregulated in Tg(*hsp:AML1-ETO*) zebrafish blood (Fig. 4). *lmo1* upregulation by AML1-ETO has been shown in a cell culture study (Alcalay et al., 2003). In addition, it has been shown that both *HOXA9* and *HOXA10* genes are strongly upregulated in myeloid but not lymphoid leukemias (Golub et al., 1999; Lawrence et al., 1995). Moreover, the upregulation of

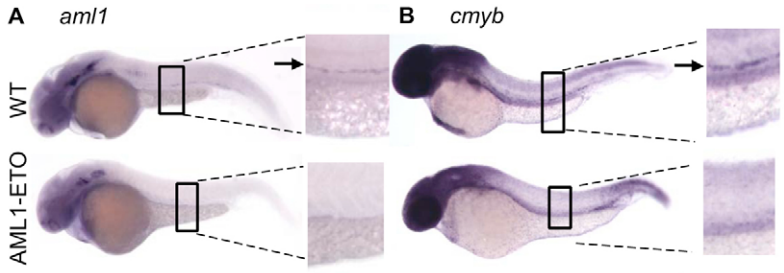

Fig. 5. AML1-ETO disrupts definitive hematopoiesis. (**A**,**B**) In situ hybridization of (A) *aml1* and (B) *cmyb* at 33 hpf indicates that definitive hematopoiesis is blocked in Tg(*hsp:AML1-ETO*) embryos.

ROBO1 and *Caveolin 1* have been demonstrated as expression signatures of pediatric AML associated with AML1-ETO (Ross et al., 2004).

In all, these results show that in addition to the cytological similarities between our model and human AML, AML1-ETO exerts comparable transcriptional changes in key hematopoietic genes in zebrafish and in humans.

AML1-ETO disrupts definitive hematopoiesis

In zebrafish, definitive hematopoiesis begins between 24-48 hpf, as the hematopoietic stem cell markers *aml1* and *cmyb* begin to be expressed in the ventral wall of the dorsal aorta. It has been shown that AML1-ETO disrupts definitive hematopoiesis in mice (Okuda et al., 1998; Yergeau et al., 1997). Thus, we examined whether AML1-ETO also affects definitive hematopoiesis in zebrafish. We incubated wild-type and Tg(*hsp:AML1-ETO*) embryos at 38°C for 1 hour at 18 hpf, and fixed the embryos at 33 hpf for *aml1* and *cmyb* in situ hybridization. Although both *aml1* and *cmyb* are expressed in wild-type embryos, they fail to be expressed in Tg(*hsp:AML1-ETO*) embryos (Fig. 5). These results indicate that AML1-ETO disrupts definitive hematopoiesis in zebrafish embryos.

AML1-ETO rapidly shuts down *gata1* expression and converts erythropoiesis to granulopoiesis in the myeloerythroid progenitor cells

Next, we sought to understand how AML1-ETO exerts the observed hematopoietic phenotype. Interestingly, the blood accumulation site in Tg(*hsp:AML1-ETO*) embryos corresponds to the posterior blood island, or intermediate cell mass (ICM) of zebrafish embryos. The ICM contains multipotent MPCs capable of producing cells of the erythroid and myeloid lineages. However, for reasons still to be identified, MPCs in the ICM homogeneously express Gata1, a transcription factor essential for erythropoiesis, and are developmentally programmed to adopt erythroid cell fates (Galloway et al., 2005; Rhodes et al., 2005). By in situ hybridization, we found that *gata1* expression is completely abolished in the ICM region of Tg(*hsp:AML1-ETO*) embryos one hour after AML1-ETO induction (Fig. 6A). The reduction in *gata1* expression suggests that AML1-ETO inhibits the normal erythropoietic process (Fig. 6B). In addition, three hours after we observed the downregulation of *gata1*, we detected increased *pu.1* expression in the ICM region of Tg(*hsp:AML1-ETO*) embryos, as shown by an increase in the number and intensity of the fluorescent cells in *AML1-ETO* and *zpu.1-EGFP* double transgenic embryos (Hsu et al., 2004) (Fig. 6A).

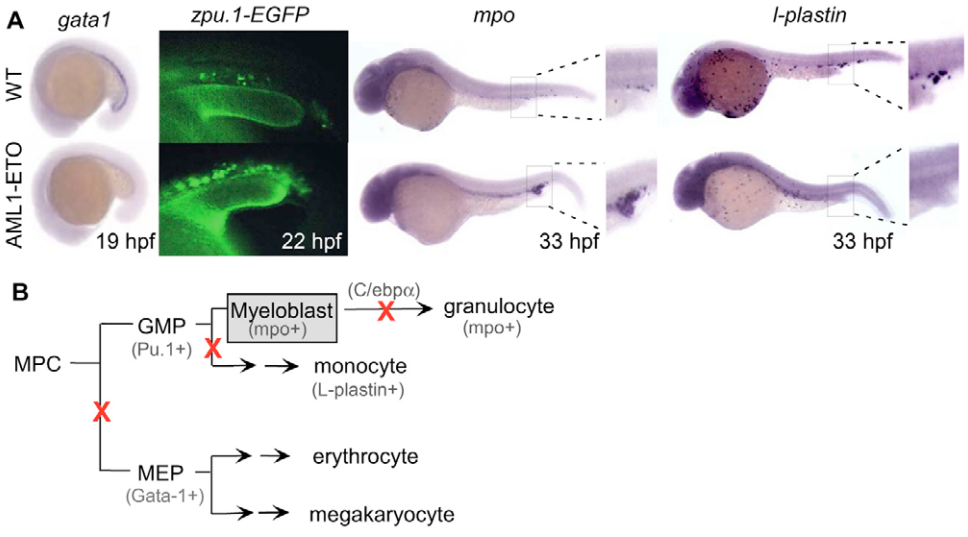

Fig. 6. AML1-ETO reprograms hematopoietic cell fate, converting erythropoiesis to granulopoiesis. (**A**) In situ hybridization of *gata1*, fluorescent images of *zpu.1-EGFP* transgenic fish, and in situ hybridization of *mpo* and *l-plastin*. *AML1-ETO* expression results in *gata1* downregulation. Subsequently, *pu.1* expression is increased. Finally, the accumulated blood cells express the granulocytic cell marker *mpo* but not the monocytic cell marker *l-plastin*. All embryos were subjected to the heat treatment and then were collected at designated stages as indicated. (**B**) Proposed effects of AML1-ETO in hematopoietic progenitor cells. MPC, myeloerythroid progenitor cell; GMP, granulocyte/monocyte progenitor; MEP, megakaryocyte/erythroid progenitor. The red crosses indicate the steps suppressed by AML1-ETO. The parentheses indicate the markers for each cell type or the genes involved in the processes. These data suggest that AML1-ETO reprograms hematopoietic cell fate, resulting in an enrichment of myeloblasts that express *mpo* (boxed and shaded).

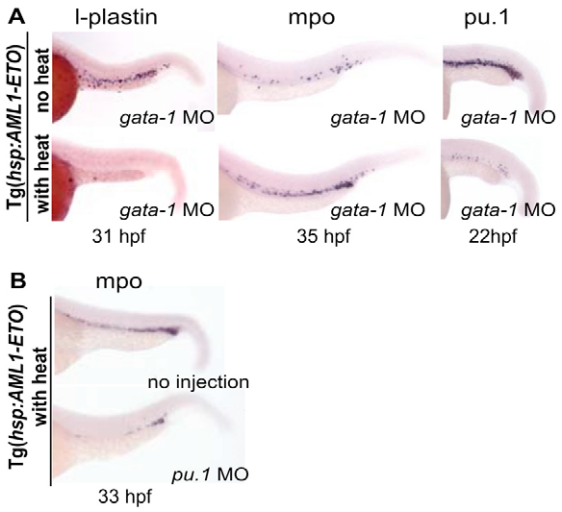

Fig. 7. AML1-ETO suppresses the monocytic cell fate. (**A**) In situ hybridization of *l-plastin*, *mpo* and *pu.1*. Tg(*hsp:AML1-ETO*) embryos were injected with *gata1* morpholino (MO). Half of the injected embryos were heat-treated to induce *AML1-ETO* expression. AML1-ETO suppresses the expression of *pu.1* and *l-plastin* but promotes the expression of *mpo* in *gata1* morphants. (**B**) In situ hybridization of *mpo*. Injections of *pu.1* MO decreased the expression of m*po* in *AML1-ETO*-expressing embryos, inicating that Pu.1 is essential for the specification of granulocytes.

Pu.1 is a master regulator and marker of myeloid cells. Thus, these results suggest that AML1-ETO reprograms the cell fate decision of many of the multipotent hematopoietic progenitor cells, converting the erythroid cell fate to the myeloid cell fate (Fig. 6B).

We then sought to determine whether the pu.1$^+$ myeloid cells arising in the ICM after *AML1-ETO* induction would go on to adopt the granulocytic and/or monocytic cell fates. After allowing embryogenesis to proceed to 33 hpf, we looked for the presence of granulocytes, which express the myeloid-specific peroxidase (Mpo), and monocytes, which express l-plastin. While both Mpo$^+$ and l-plastin$^+$ cells were observed in wild-type embryos, Tg(*hsp:AML1-ETO*) embryos exhibited a dramatic expansion in Mpo$^+$ cells and a complete loss of l-plastin$^+$ cells in the trunk region (Fig. 6A). We have confirmed that these Mpo$^+$ cells are unlikely to be immature erythroid progenitors based on their lack of hemoglobin expression (see Fig. S4 in the supplementary material). Because there is no circulation in Tg(*hsp:AML1-ETO*) embryos, the abundant Mpo$^+$ cells detected in the trunk region are likely to be derived from MPCs in the ICM of these embryos. In addition, the loss of the monocytic lineage appears specific to the ICM, because l-plastin$^+$ cells are seen in the RBI, and some of them migrate into various tissues in Tg(*hsp:AML1-ETO*) embryos (Fig. 6A). These data suggest that *AML1-ETO* expression directs primitive hematopoietic progenitor cells to the granulocytic cell fate at the expense of erythropoietic and monocytic cell fates (Fig. 6B).

Moreover, it has been shown that the CCAAT/enhancer-binding protein α (C/ebpα) plays a crucial role in the maturation of granulocytes, and that human t(8;21) blasts express very low levels of *CEBPA* (Pabst et al., 2001a; Pabst et al., 2001b). We find that hematopoietic cells in the trunk region of Tg(*hsp:AML1-ETO*) embryos do not express C/ebpα (see Fig. S5 in the supplementary material). Thus, the loss of *cebpa* expression may cause a defect in the maturation of myeloblasts (Fig. 6B), resulting in the accumulation of hematopoietic blasts observed in Tg(*hsp:AML1-ETO*) embryos (Fig. 3).

AML1-ETO suppresses the monocytic cell fate

It has been shown that, in zebrafish embryos, injections of *gata1* antisense morpholino oligonucleotides result in increased expression of *pu.1* and myelopoiesis in the ICM (Galloway et al., 2005; Rhodes et al., 2005). These results suggest that *pu.1* expression in the ICM is normally inhibited by Gata1. Subsequently, the hematopoietic cells in the ICM of *gata1* morpholino-injected embryos (morphants) express both the granulocytic cell marker *mpo* and the monocytic cell marker *l-plastin* (Galloway et al., 2005; Rhodes et al., 2005). We have shown that AML1-ETO also inhibits *gata1* expression, leading to increased expression of *pu.1* (Fig. 6A). However, in Tg(*hsp:AML1-ETO*) embryos, the accumulated hematopoietic cells in the ICM express only *mpo* and not *l-plastin*. Thus, it is tempting to hypothesize that AML1-ETO actively suppresses the specification of the monocytic cell fate in hematopoietic progenitor cells. To test this hypothesis, we injected *gata1* morpholinos into Tg(*hsp:AML1-ETO*) embryos and heat shocked half of the injected embryos only to induce *AML1-ETO* expression. We find that induced expression of *AML1-ETO* not only reduces *l-plastin* expression in the *gata1* morphants, but also enhances *mpo* expression in these embryos (Fig. 7A). These effects can be seen in all of the embryos treated. In addition, it has been shown that injections of *chordin* antisense morpholino oligonucleotides result in an expanded pool of blood cells consisting of mostly erythrocytes and some monocytes in the ICM (Leung et al., 2005). We find that induced expression of *AML1-ETO* in the *chordin* morphants also results in downregulation of *gata1* and *l-plastin*, but upregulation of *mpo* (see Fig. S6 in the supplementary material). These data strongly suggest that AML1-ETO suppresses the monocytic cell fate and promotes the granulocytic cell fate.

Interestingly, when we examined *pu.1* expression, we found that *gata1* morphants express very high levels of *pu.1*, but induced expression of *AML1-ETO* reduces the extent of *pu.1* induction in *gata1* morphants (Fig. 7A). Thus, *pu.1* is kept at a moderate level of induction in the ICM of Tg(*hsp:AML1-ETO*) embryos compared

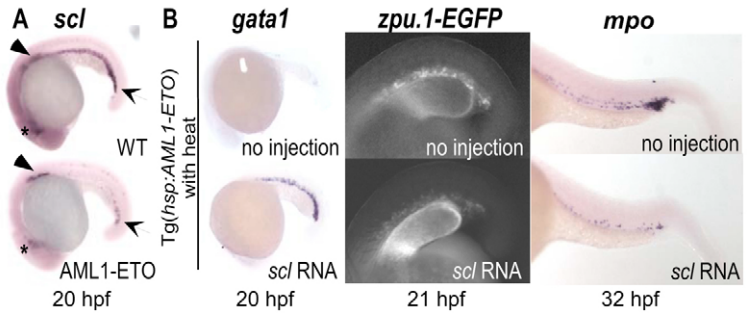

Fig. 8. AML1-ETO downregulates *scl*, leading to the early effects of *AML1-ETO* expression in primitive hematopoietic cells. (**A**) In situ hybridization of *scl*. *AML1-ETO* expression results in *scl* downregulation specifically in hematopoietic cells. The expression of *scl* in the head (asterisk), in the endothelial primordium (arrowhead), and in the posterior tail region (arrow) was not affected. (**B**) In situ hybridization of *gata1*, *mpo* and fluorescent images of *zpu.1-EGFP* transgenic zebrafish. Injections of *scl* mRNA restored *gata1* expression and reversed *pu.1* and *mpo* induction in Tg(*hsp:AML1-ETO*) embryos.

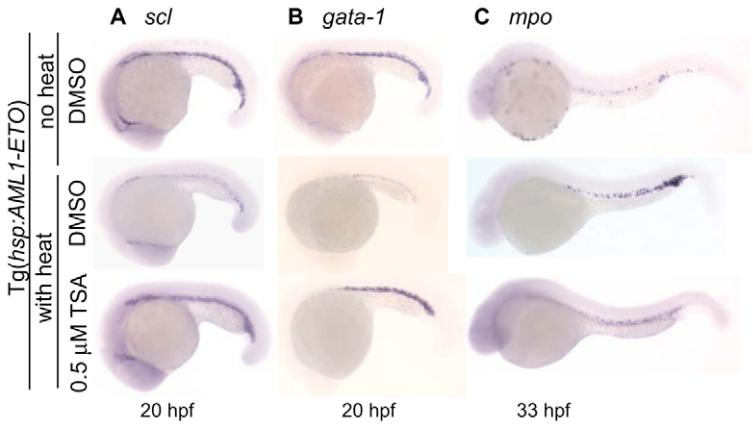

Fig. 9. Trichostatin A suppresses the effects of AML1-ETO in zebrafish embryos. (**A-C**) DMSO or 0.5 μM TSA was added to Tg(*hsp:AML1-ETO*) embryos 2 hours before (A,B) or at the end of a 1-hour heat treatment (C) at 38°C at 18 hpf. Tg(*hsp:AML1-ETO*) embryos that had not been subjected to the heat treatment were used as a control. TSA treatment rescues *scl* and *gata1* downregulation and blocks the accumulation of Mpo$^+$ cells caused by *AML1-ETO* expression.

with that of *gata1* morphants. It has been shown that *Pu.1* is expressed at a higher level in monocytic cells than its level in granulocytic cells (Dahl et al., 2003). Thus, a moderate level of *pu.1* induction in Tg(*hsp:AML1-ETO*) embryos may not be able to support the monocytic cell specification in these embryos. To test whether this moderate level of *pu.1* induction is required for the MPCs to adopt the granulocytic cell fate in Tg(*hsp:AML1-ETO*) embryos, we injected *pu.1* morpholinos into Tg(*hsp:AML1-ETO*) embryos. We found that expression of the granulocytic lineage marker *mpo* in the ICM is reduced in *pu.1* morphants, suggesting that *pu.1* expression is required for granulocytic cell specification in Tg(*hsp:AML1-ETO*) embryos (Fig. 7B).

AML1-ETO downregulates *scl* expression in primitive hematopoietic cells

To identify the immediate downstream targets of AML1-ETO, we examined the transcriptional profiles of hematopoietic cells only two hours after the heat treatment. To isolate blood cells in the early embryos (~22 hpf), we crossed wild-type and Tg(*hsp:AML1-ETO*) fish to the *gata1-DsRed* transgenic zebrafish line (Traver et al., 2003). The *gata1-DsRed* transgene is expressed transiently in all hematopoietic cells in the ICM before the heat treatment. Because of the stability of DsRed protein, the hematopoietic cells remain fluorescent after the heat treatment. We incubated embryos at 38°C for 1 hour at 19 hpf and isolated fluorescent cells using a flow cytometer. The experiments were done twice and the transcription profiles of these samples were analyzed and compared using DNA microarrays. We found that the most strongly downregulated gene in Tg(*hsp:AML1-ETO*) embryos is the hematopoietic stem cell gene *scl*, which is reduced 11-fold (see Table S1 in the supplementary material).

Overexpression of *scl* reverses the effects of AML1-ETO induction

The *scl* gene encodes a basic helix-loop-helix (bHLH) transcription factor involved in the specification of both hematopoietic and endothelial cells (Gering et al., 1998; Liao et al., 1998). It has also been shown that Scl plays important roles in both primitive and definitive hematopoiesis in mice and in zebrafish (Begley and Green, 1999). The rapid and dramatic downregulation of *scl* observed in our microarray analyses prompted us to investigate whether it mediates some of the early effects of *AML1-ETO* expression. To confirm that *scl* is downstream of AML1-ETO, we heat-shocked wild-type and Tg(*hsp:AML1-ETO*) embryos at 18 hpf for 1 hour and harvested the embryos 1 hour after the heat treatment for *scl* in situ hybridization. As shown in Fig. 8A, *scl* expression is rapidly downregulated in Tg(*hsp:AML1-ETO*) embryos. Interestingly, this downregulation seems to be specific to hematopoietic cells in the ICM region, as *scl* expression in the head (Fig. 8A, asterisk), which will contribute to both cranial vasculature and primitive macrophages, in the trunk above the yolk (Fig. 8A, arrowhead), which is likely to be the precursors of the endothelium of the ducts of Cuvier, and in the posterior tail region (Fig. 8A, arrow) is not affected (Zhang and Rodaway, 2007).

To investigate whether *scl* downregulation may mediate some of the early effects of *AML1-ETO* expression that we observed, we injected *scl* mRNA into Tg(*hsp:AML1-ETO*) embryos and induced *AML1-ETO* expression in these embryos. We found that injections of *scl* mRNA restores *gata1* expression (in 47 out of 52 embryos injected; Fig. 8B). Next, we tested if *scl* expression could also rescue the effect of AML1-ETO on *pu.1* and *mpo* expression. As shown in Fig. 8B,C, we found that injections of *scl* mRNA can abolish the upregulation of *pu.1* and *mpo* in the presence of *AML1-ETO* expression (Fig. 8B, 39 out of 46 embryos injected; Fig. 8C, 20 out of 56 embryos injected). These data indicate that *AML1-ETO* leads to the downregulation of *gata1* and the upregulation of *pu.1* and *mpo* through *scl*, and that *scl* is an important mediator of the effect of AML1-ETO on the specification of multipotent hematopoietic progenitors.

Trichostatin A (TSA) suppresses the effect of AML1-ETO in zebrafish embryos

The recruitment of histone deacetylase (HDAC) by the ETO domain of AML1-ETO is believed to play an important role in AML1-ETO-mediated pathogenesis (Wang et al., 1999). To test whether the observed AML1-ETO effects can be suppressed pharmacologically, we added TSA, an HDAC inhibitor, to the embryo media two hours before the heat treatment. We found that 0.5 μM TSA blocks AML1-ETO-mediated downregulation of both *scl* and *gata1*, as shown by in situ hybridization (Fig. 9A,B). In addition, adding 0.5 μM TSA to embryos before (data not shown) or after 1-hour heat treatment also reverses the ability of AML1-ETO to induce the accumulation of Mpo$^+$ cells (Fig. 9C). These results indicate that the zebrafish AML1-ETO phenotype may be dependent on HDAC activity. Moreover, the zebrafish AML1-ETO phenotype can be reversed by treatment with small molecules and may enable facile identification by high-throughput screens of novel compounds that suppress the effect of AML1-ETO in vivo.

DISCUSSION

We report here the generation of a highly tractable in vivo model of *AML1-ETO* expression. We show that induced expression of *AML1-ETO* in zebrafish embryos results in rapid manifestation of a robust phenotype that exhibits cytological and transcriptional hallmarks of human AML, suggesting that AML1-ETO signaling pathways are likely to be conserved between human and zebrafish. Most importantly, using the zebrafish model of *AML1-ETO* expression enabled us to track the molecular changes that take place well before morphological phenotypes can be detected, and to determine the roles of candidate AML1-ETO target genes. We demonstrate that AML1-ETO regulates *scl* and several lineage-specific transcription factors, reprogramming hematopoietic cell fate in vivo.

Induced expression of *AML1-ETO* in zebrafish results in an accumulation of non-circulating hematopoietic cells. Our results suggest that the loss-of-circulation phenotype is likely to be due to intrinsic defects in hematopoietic cells rather than to a general disruption of cardiovascular function. By histological analyses, we know that at least some hematopoietic cells are able to leave their niche and enter the vasculature (data not shown). In addition, we occasionally observe some circulating cells bypassing a pool of non-circulating blood cells. Interestingly, granulocytic sarcomas (chloromas or myeloblastomas) are a common clinical manifestation of t(8;21) AML (Schwyzer et al., 1998; Tallman et al., 1993). Granulocytic sarcoma is a solid tumor composed of myeloblasts. In this situation, the myeloblasts are very adhesive and have a high tendency to form aggregates. Whether the blood cells in Tg(*hsp:AML1-ETO*) embryos are more adhesive than those in wild-type embryos is not clear at present.

We show that induced expression of *AML1-ETO* results in *gata1* downregulation and *pu.1* upregulation in multipotent hematopoietic progenitor cells, suppressing erythropoiesis and promoting myelopoiesis. These results corroborate the finding that AML1-ETO inhibits erythropoiesis of purified human hematopoietic progenitor cells (Choi et al., 2006), and the finding that it suppresses erythropoiesis and stimulates granulopoiesis in mice (Schwieger et al., 2002). Our results indicate that the level of *pu.1* expression is determined by the ability of AML1-ETO to regulate both *gata1* and *pu.1*. AML1-ETO downregulates *gata1*. As a result, *pu.1* is induced. Conversely, AML1-ETO may directly suppress *pu.1* expression. It has been shown that AML1-ETO can bind to Pu.1 and inhibit its function (Vangala et al., 2003). Such interaction may pose an inhibitory effect on the autoregulation of the *pu.1* gene (Chen et al., 1995). The ability of AML1-ETO to fine tune the level of *pu.1* induction is likely to be very important for its leukemogenic effect. Supporting this idea, it has been shown that the hypomorphic allele, but not the null allele, of the *Pu.1* gene in mice leads to AML-like phenotypes, suggesting the importance of *Pu.1* gene dose in leukemogenesis (Rosenbauer et al., 2005).

Although we demonstrate that AML1-ETO causes cell fate changes, converting erythropoiesis to myelopoiesis, there are clearly still some erythrocytes in Tg(*hsp:AML1-ETO*) embryos (Fig. 3). We have observed that both induced expression of *AML1-ETO* and the loss of *gata1* expression last only for a few hours after the heat shock (data not shown). Thus, some hematopoietic cells may eventually commit to the erythroid cell fate as a result of the restoration of *gata1* expression.

During primitive hematopoiesis, zebrafish *mpo* expression initiates between 18 and 20 hpf, first in the ICM and then in the RBI. At 24 hpf, myeloblasts can be identified morphologically only in the RBI and not in the ICM, although granulocytes are reliably found in the circulation by 48 hpf (Bennett et al., 2001; Lieschke et al., 2001). The source of these circulating granulocytes is not totally clear at this moment. For example, it is not known whether the Mpo^+ cells observed in the ICM at 20 hpf can become mature in situ. In our model, induced expression of AML1-ETO causes an enrichment of Mpo^+ cells and morphologically immature hematopoietic blasts in the ICM. The accumulation of hematopoietic blasts is only partially reversed as *AML1-ETO* expression ceases (see Fig. S3 in the supplementary material). What causes the long-lasting effect of transient induction of *AML1-ETO* on the blockade of maturation of these cells is not clear. It could be that these cells cannot migrate to the environment that supports their maturation, that the environmental signals that induce the maturation of these cells no longer exist, or that a factor that is required for maturation, such as C/ebpα, can no longer be expressed after AML1-ETO expression diminishes.

We were able to identify several early downstream targets of AML1-ETO by isolating hematopoietic cells only two hours after the induction of *AML1-ETO* expression. We show that induced expression of AML1-ETO rapidly downregulates *scl* expression. Even though decreased *scl* expression has been shown in t(8;21) leukemia samples, a direct link between *AML1-ETO* and *scl* expression had not been established previously (Shimamoto et al., 1995). We have not been able to identify potential Aml1 binding sites upstream of the coding region of *scl*, indicating that AML1-ETO may inhibit *scl* expression through binding with other factors. Interestingly, the downregulation of *scl* is seen only in hematopoietic progenitor cells and not in cells that will give rise to the endothelial lineage in Tg(*hsp:AML1-ETO*) embryos. It has been shown that the heat shock promoter used to drive the human *AML1-ETO* gene results in ubiquitous expression upon induction (Xiao et al., 2003). Thus, these results suggest that other hematopoietic-specific cofactors may be required for AML1-ETO function.

Overexpression of *scl* can block the ability of AML1-ETO to reprogram hematopoietic cell fate, as shown by the reversal of *gata1*, *pu.1* and *mpo* dysregulation in Tg(*hsp:AML1-ETO*) embryos. However, Scl-mediated rescue of the early effects of AML1-ETO on hematopoiesis does not completely rescue the circulation defect in Tg(*hsp:AML1-ETO*) embryos. This may be because of additional defects caused by ubiquitous *scl* overexpression, or by non-Scl-mediated effects of AML1-ETO at later stages of development. Nevertheless, these results indicate that *scl* is an important effector that mediates the earliest observable effects of AML1-ETO in hematopoietic progenitor cells.

Induced expression of AML1-ETO in zebrafish embryos before 21 hpf disrupts definitive hematopoiesis but also enables us to study the in vivo effects of AML1-ETO in MPCs generated during primitive hematopoiesis. The transcriptional profile of Tg(*hsp:AML1-ETO*) embryo blood at 40 hpf presents expressional signatures of human AML, suggesting that the regulatory mechanisms of cell specification and maturation in these cells resemble those in progenitors of adult human blood. However, MPCs in zebrafish embryos may not have the self-renewal capability that is also essential for leukemogenesis. Thus, the effects of AML1-ETO on the self-renewal capability of hematopoietic progenitor cells may not be accessible using this model. Additionally, in humans and mice, AML1-ETO is not sufficient to induce leukemia in the absence of secondary mutations, so the blast cells observed in AML1-ETO-expressing fish are unlikely to possess full leukemogenic potential. In future studies, it will be interesting to combine AML1-ETO expression with known collaborating mutations, and to characterize the effects of *AML1-ETO* expression in hematopoietic stem cells by inducing *AML1-ETO* expression at later stages of development, or in adults.

Understanding the molecular mechanisms by which AML1-ETO exerts its influence on hematopoietic progenitor cells may help us develop targeted therapeutics. For example, compounds that block *SCL* or *GATA1* downregulation in the presence of AML1-ETO expression may prove useful in treating AML associated with t(8;21) translocation. It has been shown that compounds that enhance *Cebpa* (C/ebpα) transcriptional activity induce differentiation of AML cell lines (Jiang et al., 2005). Moreover, high-throughput screening is feasible in the zebrafish (Yeh and Crews, 2003; Zon and Peterson, 2005). We have shown that some of the effects of AML1-ETO can be suppressed by a histone deacetylase inhibitor Trichostatin A. Therefore, in addition to the fundamental insights into the mechanism of AML1-ETO function already provided by this model, it may ultimately provide a unique opportunity to conduct whole-organism chemical suppressor screens to identify compounds that can reverse AML1-ETO function in vivo.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/2/401/DC1

References

Alcalay, M., Meani, N., Gelmetti, V., Fantozzi, A., Fagioli, M., Orleth, A., Riganelli, D., Sebastiani, C., Cappelli, E., Casciari, C. et al. (2003). Acute myeloid leukemia fusion proteins deregulate genes involved in stem cell maintenance and DNA repair. *J. Clin. Invest.* **112**, 1751-1761.

Begley, C. G. and Green, A. R. (1999). The SCL gene: from case report to critical hematopoietic regulator. *Blood* **93**, 2760-2770.

Bennett, C. M., Kanki, J. P., Rhodes, J., Liu, T. X., Paw, B. H., Kieran, M. W., Langenau, D. M., Delahaye-Brown, A., Zon, L. I., Fleming, M. D. et al. (2001). Myelopoiesis in the zebrafish, Danio rerio. *Blood* **98**, 643-651.

Bonnet, D. and Dick, J. E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat. Med.* **3**, 730-737.

Borregaard, N., Theilgaard-Monch, K., Sorensen, O. E. and Cowland, J. B. (2001). Regulation of human neutrophil granule protein expression. *Curr. Opin. Hematol.* **8**, 23-27.

Burns, C. E., Traver, D., Mayhall, E., Shepard, J. L. and Zon, L. I. (2005). Hematopoietic stem cell fate is established by the Notch-Runx pathway. *Genes Dev.* **19**, 2331-2342.

Chen, H., Ray-Gallet, D., Zhang, P., Hetherington, C. J., Gonzalez, D. A., Zhang, D. E., Moreau-Gachelin, F. and Tenen, D. G. (1995). PU.1 (Spi-1) autoregulates its expression in myeloid cells. *Oncogene* **11**, 1549-1560.

Choi, Y., Elagib, K. E., Delehanty, L. L. and Goldfarb, A. N. (2006). Erythroid inhibition by the leukemic fusion AML1-ETO is associated with impaired acetylation of the major erythroid transcription factor GATA-1. *Cancer Res.* **66**, 2990-2996.

Dahl, R., Walsh, J. C., Lancki, D., Laslo, P., Iyer, S. R., Singh, H. and Simon, M. C. (2003). Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. *Nat. Immunol.* **4**, 1029-1036.

Davidson, A. J. and Zon, L. I. (2004). The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis. *Oncogene* **23**, 7233-7246.

de Guzman, C. G., Warren, A. J., Zhang, Z., Gartland, L., Erickson, P., Drabkin, H., Hiebert, S. W. and Klug, C. A. (2002). Hematopoietic stem cell expansion and distinct myeloid developmental abnormalities in a murine model of the AML1-ETO translocation. *Mol. Cell. Biol.* **22**, 5506-5517.

Downing, J. R. (1999). The AML1-ETO chimaeric transcription factor in acute myeloid leukaemia: biology and clinical significance. *Br. J. Haematol.* **106**, 296-308.

Fenske, T. S., Pengue, G., Mathews, V., Hanson, P. T., Hamm, S. E., Riaz, N. and Graubert, T. A. (2004). Stem cell expression of the AML1/ETO fusion protein induces a myeloproliferative disorder in mice. *Proc. Natl. Acad. Sci. USA* **101**, 15184-15189.

Galloway, J. L., Wingert, R. A., Thisse, C., Thisse, B. and Zon, L. I. (2005). Loss of gata1 but not gata2 converts erythropoiesis to myelopoiesis in zebrafish embryos. *Dev. Cell* **8**, 109-116.

Gering, M., Rodaway, A. R., Gottgens, B., Patient, R. K. and Green, A. R. (1998). The SCL gene specifies haemangioblast development from early mesoderm. *EMBO J.* **17**, 4029-4045.

Golub, T. R., Slonim, D. K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J. P., Coller, H., Loh, M. L., Downing, J. R., Caligiuri, M. A. et al. (1999). Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* **286**, 531-537.

Hsu, K., Traver, D., Kutok, J. L., Hagen, A., Liu, T. X., Paw, B. H., Rhodes, J., Berman, J. N., Zon, L. I., Kanki, J. P. et al. (2004). The pu.1 promoter drives myeloid gene expression in zebrafish. *Blood* **104**, 1291-1297.

Jiang, Y., Xue, Z. H., Shen, W. Z., Du, K. M., Yan, H., Yu, Y., Peng, Z. G., Song, M. G., Tong, J. H., Chen, Z. et al. (2005). Desferrioxamine induces leukemic cell differentiation potentially by hypoxia-inducible factor-1 alpha that augments transcriptional activity of CCAAT/enhancer-binding protein-alpha. *Leukemia* **19**, 1239-1247.

Kalev-Zylinska, M. L., Horsfield, J. A., Flores, M. V., Postlethwait, J. H., Vitas, M. R., Baas, A. M., Crosier, P. S. and Crosier, K. E. (2002). Runx1 is required for zebrafish blood and vessel development and expression of a human RUNX1-CBF2T1 transgene advances a model for studies of leukemogenesis. *Development* **129**, 2015-2030.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

Lawrence, H. J., Sauvageau, G., Ahmadi, N., Lopez, A. R., LeBeau, M. M., Link, M., Humphries, K. and Largman, C. (1995). Stage- and lineage-specific expression of the HOXA10 homeobox gene in normal and leukemic hematopoietic cells. *Exp. Hematol.* **23**, 1160-1166.

Lawson, N. D. and Weinstein, B. M. (2002). In vivo imaging of embryonic vascular development using transgenic zebrafish. *Dev. Biol.* **248**, 307-318.

Leung, A. Y., Mendenhall, E. M., Kwan, T. T., Liang, R., Eckfeldt, C., Chen, E., Hammerschmidt, M., Grindley, S., Ekker, S. C. and Verfaillie, C. M. (2005). Characterization of expanded intermediate cell mass in zebrafish chordin morphant embryos. *Dev. Biol.* **277**, 235-254.

Liao, E. C., Paw, B. H., Oates, A. C., Pratt, S. J., Postlethwait, J. H. and Zon, L. I. (1998). SCL/Tal-1 transcription factor acts downstream of cloche to specify hematopoietic and vascular progenitors in zebrafish. *Genes Dev.* **12**, 621-626.

Licht, J. D. (2001). AML1 and the AML1-ETO fusion protein in the pathogenesis of t (8;21) AML. *Oncogene* **20**, 5660-5679.

Lieschke, G. J., Oates, A. C., Crowhurst, M. O., Ward, A. C. and Layton, J. E. (2001). Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. *Blood* **98**, 3087-3096.

Lutterbach, B. and Hiebert, S. W. (2000). Role of the transcription factor AML-1 in acute leukemia and hematopoietic differentiation. *Gene* **245**, 223-235.

Lyons, S. E., Shue, B. C., Lei, L., Oates, A. C., Zon, L. I. and Liu, P. P. (2001). Molecular cloning, genetic mapping, and expression analysis of four zebrafish *c*/ebp genes. *Gene* **281**, 43-51.

Miyamoto, T., Weissman, I. L. and Akashi, K. (2000). AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation. *Proc. Natl. Acad. Sci. USA* **97**, 7521-7526.

Nasevicius, A. and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**, 216-220.

Okuda, T., Cai, Z., Yang, S., Lenny, N., Lyu, C. J., van Deursen, J. M., Harada, H. and Downing, J. R. (1998). Expression of a knocked-in AML1-ETO leukemia gene inhibits the establishment of normal definitive hematopoiesis and directly generates dysplastic hematopoietic progenitors. *Blood* **91**, 3134-3143.

Pabst, T., Mueller, B. U., Harakawa, N., Schoch, C., Haferlach, T., Behre, G., Hiddemann, W., Zhang, D. E. and Tenen, D. G. (2001a). AML1-ETO downregulates the granulocytic differentiation factor C/EBPalpha in t (8;21) myeloid leukemia. *Nat. Med.* **7**, 444-451.

Pabst, T., Mueller, B. U., Zhang, P., Radomska, H. S., Narravula, S., Schnittger, S., Behre, G., Hiddemann, W. and Tenen, D. G. (2001b). Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat. Genet.* **27**, 263-270.

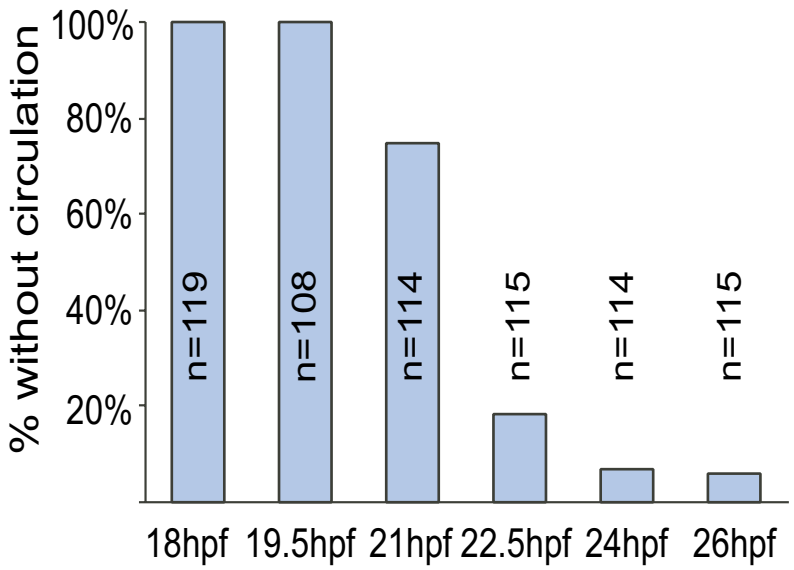
Palis, J., Chan, R. J., Koniski, A., Patel, R., Starr, M. and Yoder, M. C. (2001). Spatial and temporal emergence of high proliferative potential hematopoietic precursors during murine embryogenesis. *Proc. Natl. Acad. Sci. USA* **98**, 4528-4533.

Peterson, L. F. and Zhang, D. E. (2004). The 8;21 translocation in leukemogenesis. *Oncogene* **23**, 4255-4262.

Ransom, D. G., Haffter, P., Odenthal, J., Brownlie, A., Vogelsang, E., Kelsh, R. N., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M. et al. (1996). Characterization of zebrafish mutants with defects in embryonic hematopoiesis. *Development* **123**, 311-319.

Rhodes, J., Hagen, A., Hsu, K., Deng, M., Liu, T. X., Look, A. T. and Kanki, J. P. (2005). Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. *Dev. Cell* **8**, 97-108.
Rosenbauer, F., Koschmieder, S., Steidl, U. and Tenen, D. G. (2005). Effect of transcription-factor concentrations on leukemic stem cells. *Blood* **106**, 1519-1524.
Ross, M. E., Mahfouz, R., Onciu, M., Liu, H. C., Zhou, X., Song, G., Shurtleff, S. A., Pounds, S., Cheng, C., Ma, J. et al. (2004). Gene expression profiling of pediatric acute myelogenous leukemia. *Blood* **104**, 3679-3687.
Schwieger, M., Lohler, J., Friel, J., Scheller, M., Horak, I. and Stocking, C. (2002). AML1-ETO inhibits maturation of multiple lymphohematopoietic lineages and induces myeloblast transformation in synergy with ICSBP deficiency. *J. Exp. Med.* **196**, 1227-1240.
Schwyzer, R., Sherman, G. G., Cohn, R. J., Poole, J. E. and Willem, P. (1998). Granulocytic sarcoma in children with acute myeloblastic leukemia and t (8;21). *Med. Pediatr. Oncol.* **31**, 144-149.
Shimada, H., Ichikawa, H., Nakamura, S., Katsu, R., Iwasa, M., Kitabayashi, I. and Ohki, M. (2000). Analysis of genes under the downstream control of the t (8;21) fusion protein AML1-MTG8: overexpression of the TIS11b (ERF-1, cMG1) gene induces myeloid cell proliferation in response to G-CSF. *Blood* **96**, 655-663.
Shimamoto, T., Ohyashiki, K., Ohyashiki, J. H., Kawakubo, K., Fujimura, T., Iwama, H., Nakazawa, S. and Toyama, K. (1995). The expression pattern of erythrocyte/megakaryocyte-related transcription factors GATA-1 and the stem cell leukemia gene correlates with hematopoietic differentiation and is associated with outcome of acute myeloid leukemia. *Blood* **86**, 3173-3180.
Stone, R. M., O'Donnell, M. R. and Sekeres, M. A. (2004). Acute myeloid leukemia. *Hematology Am. Soc. Hematol. Educ. Program* **2004**, 98-117.
Tallman, M. S., Hakimian, D., Shaw, J. M., Lissner, G. S., Russell, E. J. and Variakojis, D. (1993). Granulocytic sarcoma is associated with the 8;21 translocation in acute myeloid leukemia. *J. Clin. Oncol.* **11**, 690-697.
Tenen, D. G. (2003). Disruption of differentiation in human cancer: AML shows the way. *Nat. Rev. Cancer* **3**, 89-101.
Thompson, M. A., Ransom, D. G., Pratt, S. J., MacLennan, H., Kieran, M. W., Detrich, H. W., 3rd, Vail, B., Huber, T. L., Paw, B., Brownlie, A. J. et al. (1998). The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. *Dev. Biol.* **197**, 248-269.
Traver, D., Paw, B. H., Poss, K. D., Penberthy, W. T., Lin, S. and Zon, L. I. (2003). Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. *Nat. Immunol.* **4**, 1238-1246.
Vangala, R. K., Heiss-Neumann, M. S., Rangatia, J. S., Singh, S. M., Schoch, C., Tenen, D. G., Hiddemann, W. and Behre, G. (2003). The myeloid master regulator transcription factor PU.1 is inactivated by AML1-ETO in t (8;21) myeloid leukemia. *Blood* **101**, 270-277.
Wang, J., Saunthararajah, Y., Redner, R. L. and Liu, J. M. (1999). Inhibitors of histone deacetylase relieve ETO-mediated repression and induce differentiation of AML1-ETO leukemia cells. *Cancer Res.* **59**, 2766-2769.
Weinstein, B. M., Stemple, D. L., Driever, W. and Fishman, M. C. (1995). Gridlock, a localized heritable vascular patterning defect in the zebrafish. *Nat. Med.* **1**, 1143-1147.
Westerfield, M. (2000). *The Zebrafish Book. A Guide for the Laboratory use of Zebrafish (Danio rerio).* Eugene: University of Oregon Press.
Xiao, T., Shoji, W., Zhou, W., Su, F. and Kuwada, J. Y. (2003). Transmembrane sema4E guides branchiomotor axons to their targets in zebrafish. *J. Neurosci.* **23**, 4190-4198.
Yeh, J. R. and Crews, C. M. (2003). Chemical genetics: adding to the developmental biology toolbox. *Dev. Cell* **5**, 11-19.
Yergeau, D. A., Hetherington, C. J., Wang, Q., Zhang, P., Sharpe, A. H., Binder, M., Marin-Padilla, M., Tenen, D. G., Speck, N. A. and Zhang, D. E. (1997). Embryonic lethality and impairment of haematopoiesis in mice heterozygous for an AML1-ETO fusion gene. *Nat. Genet.* **15**, 303-306.
Zhang, X. Y. and Rodaway, A. R. (2007). SCL-GFP transgenic zebrafish: in vivo imaging of blood and endothelial development and identification of the initial site of definitive hematopoiesis. *Dev. Biol.* **307**, 179-194.
Zon, L. I. and Peterson, R. T. (2005). In vivo drug discovery in the zebrafish. *Nat. Rev. Drug Discov.* **4**, 35-44.

% without circulation
20%
40%
60%
80%
100%
control
hAML1-MO
n=133
n=70
*
p<0.0001


% without circulation
100%
80%
60%
40%
20%
n=119
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n=115
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19.5hpf
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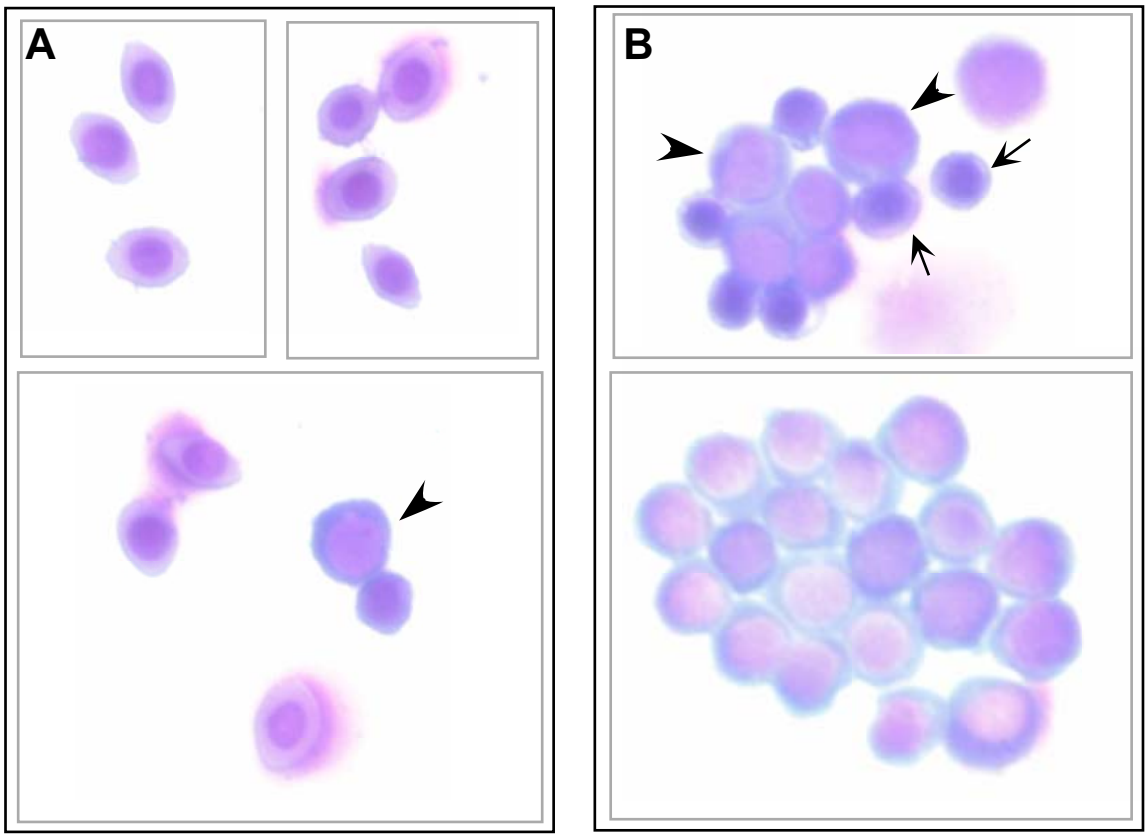
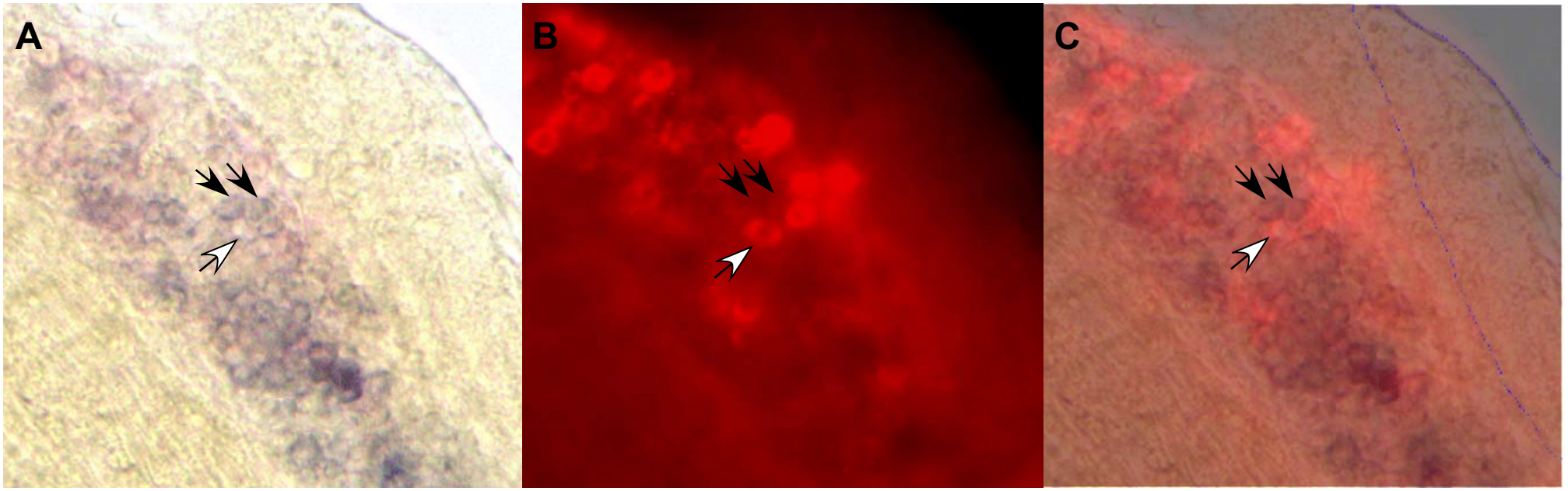
22.5hpf
24hpf
26hpf


A
B


A
B
C


C/ebpα
WT
AML1-ETO
31 hpf

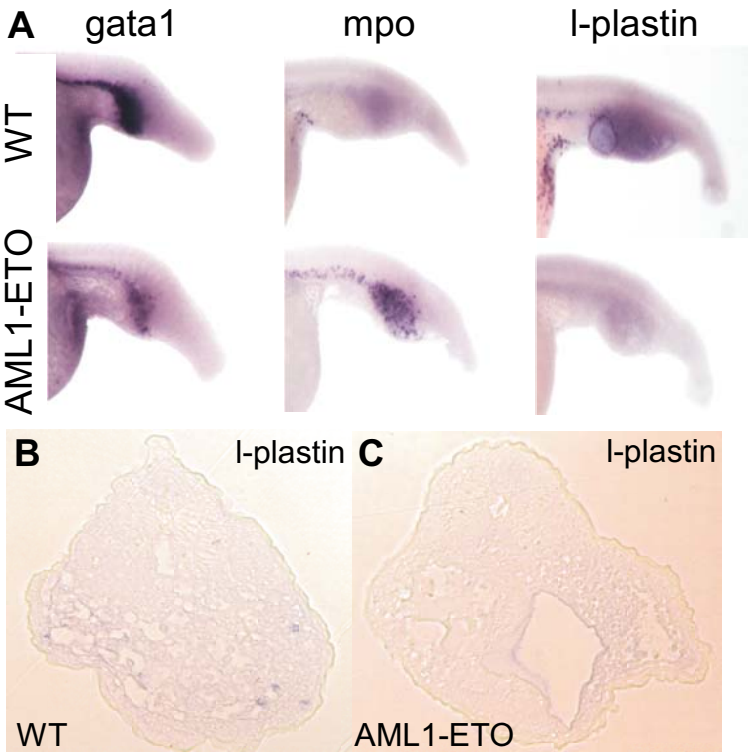

A
gata1
mpo
l-plastin
WT
AML1-ETO
B
l-plastin
WT
C
l-plastin
AML1-ETO

Table S1. Immediate transcriptional changes in the hematopoietic cells caused by AML1-ETO expression in zebrafish

Probe ID	Description	chip #1	chip #2	Function
		⩽0.1*		
9690	Scl/Tal1	0.089	0.086	Transcription factor
		0.1~0.25*		
17484	GATA1	0.225	0.166	Transcription factor
8603	GATA2	0.217	0.177	Transcription factor
526	Myb	0.221	0.258	Transcription factor
16148	Myb	0.104	0.214	Transcription factor
17752	Myb	0.087	0.229	Transcription factor
6075	Nfe2	0.167	0.062	Transcription factor
10258	Cyclin D1	0.219	0.139	Regulation of cell cycle
1074	Zgc:92631	0.245	0.216	Cytochrome c oxidase, electron transport
308	ATP-binding cassette, sub-family B, member 10	0.160	0.134	Mitochondial protein, transporter
6896	Uncoupling protein 2, like	0.211	0.023	Mitochondial protein, transporter
17174	Uncoupling protein 2, like	0.197	0.023	Mitochondial protein, transporter
10696	Zgc:77076	0.199	0.072	Electron transporter
824	Zgc:85890	0.210	0.130	Transporter
10158	Zgc:92505	0.179	0.146	Receptor
13751	Zgc:92301	0.183	0.173	G protein-coupled receptor
7651	BC049429 zgc:56412 (Danio rerio), complete [TC295003]	0.120	0.134	
12217	MGC1842 protein (Fragment), partial (96%) [TC295100]	0.155	0.093	
13269	Zgc:56412	0.200	0.151	
2785	Zgc:66438	0.174	0.113	
5734	Transcribed locus	0.246	0.193	

*Ratio of the signals in Tg(hsp:AML1-ETO) sample to the signals in wild-type sample.