Zebrafish etv7 regulates red blood cell development through the cholesterol synthesis pathway

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ABSTRACT
ETV7 is a human oncoprotein that cooperates with Eμ-MYC to promote pre-B-cell leukemia in mice. It is normally expressed in the bone marrow and fetal liver and is upregulated in primary hematopoiesis, suggesting that it is involved in proper hematopoiesis and leukemogenesis. ETV7 has been deleted in most rodents, but is conserved in all other vertebrates, including the zebrafish, Danio rerio. In this report, we characterize the function of the zebrafish etv7 gene during erythropoiesis. Our results demonstrate that etv7 regulates the expression of the zebrafish lanosterol synthase (lss) gene, an essential gene in the cholesterol synthesis pathway. Furthermore, morpholino knockdown of etv7 leads to loss of hemoglobin-containing red blood cells, a phenotype that can be rescued by injection of exogenous cholesterol. We conclude that etv7 is essential for normal red blood cell development through regulation of the lss gene and the cholesterol synthesis pathway.

KEY WORDS: Cholesterol, etv7, Oncogene, Red blood cell

INTRODUCTION
ETV7 is a human oncogene that causes leukemia when expressed in murine bone marrow (Cardone et al., 2005; Carella et al., 2006). It is an E26 transformation specific (ETS) factor that is mostly expressed in the human hematopoietic system. ETV7 was originally identified by three independent groups and is highly homologous to ETV6 (Gu et al., 2001; Poirel et al., 2000; Potter et al., 2000). The ETV6 and ETV7 proteins each belong to the TEL/Yan subclass of ETS transcription factors and have a highly conserved ETS DNA-binding domain (ETS domain) and a pointed (PNT) protein-protein interaction domain (Slupsky et al., 1998).

In humans, ETV7 is primarily expressed in the bone marrow and fetal liver, and has been implicated in the regulation of hematopoiesis. Our laboratory demonstrated that, in the U937 human monocytic cell line, expression of ETV7 decreases upon vitamin-D3-induced differentiation (Kawagoe et al., 2004), suggesting that the expression level of ETV7 is highly regulated during the differentiation process. Moreover, forced expression of ETV7 in murine bone marrow causes a latent myeloproliferative disease that is dependent on the cooperation of secondary mutations (Carella et al., 2006). One example of a secondary mutation capable of cooperating with ETV7 during transformation is the Eμ-MYC allele: it has been established that overexpression of ETV7 in murine bone marrow harboring this allele accelerates pre-B-cell lymphomagenesis (Cardone et al., 2005). Taken together, these data suggest that ETV7 might play an important role during normal hematopoiesis and leukemia.

Although previous work has provided valuable information about the potential oncogenic role of ETV7, they have not addressed the physiological role of endogenous ETV7. In order to determine the physiological role of ETV7, we developed a novel in vivo developmental model using the zebrafish, Danio rerio. This model is unique because most rodents, including mice, have deleted the endogenous ETV7 gene. Using zebrafish to study etv7 function, we show that loss of etv7 leads to a marked reduction in hemoglobinized red blood cells, which is mediated indirectly through the cholesterol synthesis pathway. Here we provide evidence for the efficacy of this new model and for the newly identified role of etv7 in the cholesterol biosynthesis pathway.

RESULTS
The human and zebrafish etv7 genes have overlapping expression patterns
The goals of this work were: (1) to employ an appropriate animal model to study etv7 function, and (2) to determine the function of etv7 during development. Zebrafish provide a unique means of determining etv7 gene function because the gene is highly conserved and loss-of-function studies cannot be done in the mouse because it does not have the gene. Because human ETV7 is expressed in a variety of adult tissues (Gu et al., 2001), we performed semi-quantitative PCR on adult zebrafish tissues and demonstrated relatively high expression in the intestine, testes and liver, whereas all other organs examined had a much lower level of expression (Fig. 1A). Of the tissues examined, only the brain did not express etv7. The low level of expression in most tissues is consistent with other studies (Gu et al., 2001). Previous work also demonstrated that ETV7 is expressed developmentally (Gu et al., 2001; Potter et al., 2000). Quantitative real-time PCR analysis demonstrated that zebrafish etv7 expression increased ~5.8-fold by 5 days post-fertilization (dpf) (Fig. 1B). In addition, in situ hybridization of animals at 1, 2, 3 and 4 dpf confirmed that etv7 was developmentally expressed (supplementary material Fig. S1). These data demonstrate that etv7 is expressed during development and into adulthood.

Loss of etv7 causes a reduction in hemoglobinized red blood cells
The low level of etv7 expression in multiple tissues might indicate a fundamental role of etv7 during both development and adulthood.

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**TRANSLATIONAL IMPACT**

**Clinical issue**

The human E26 transformation-specific (ETS) transcription factor ETV7 is normally expressed mainly in bone marrow and fetal liver, and is highly regulated during differentiation. Notably, ETV7 is upregulated in a variety of different human cancers and, when introduced into murine bone marrow, can cooperate with other oncogenes to cause hematopoietic malignancy. Thus, it seems that ETV7 is involved in hematopoiesis and in leukemogenesis. However, although significant progress has been made in establishing ETV7 as an oncogene, little is known about the normal function of this gene in development and adulthood, in part because of the lack of an appropriate model system – Etv7 has been deleted in most rodent lineages, including the mouse.

**Results**

ETV7 is highly conserved in zebrafish, which is an established model for the study of hematopoiesis. In this study, therefore, the authors use zebrafish to study the physiological role of ETV7. The authors show that etv7 is expressed during development and throughout adulthood in zebrafish, which is consistent with previous reports of ETV7 expression in humans. Microarray analysis of embryos transiently overexpressing Etv7 indicates that Etv7 regulates the expression of the zebrafish lanosterol synthase (lss) gene, an essential gene in the cholesterol synthesis pathway. Finally, the authors use a morpholino-based approach to demonstrate that loss of etv7 in zebrafish results in a severe reduction in the number of hemoglobin-containing red blood cells, a phenotype that can be rescued by injection of exogenous cholesterol.

**Implications and future directions**

These findings demonstrate that etv7 is essential for normal red blood cell development in zebrafish and suggest that it acts through regulation of lss and the cholesterol synthesis pathway. Importantly, these findings identify zebrafish as an appropriate animal model in which to study molecular and cellular mechanisms regulated by etv7. Uncovering these mechanisms could lead to the development of new models in which to characterize the role of etv7 during development and disease, and to the discovery of new cancer therapies.

To address the role of etv7 during development, we performed morpholino-oligonucleotide-mediated knockdown and examined the developing embryos. Two independent morpholinos were designed: one that inhibited translation and one that inhibited proper splicing of exon 3, which encodes most of the PNT domain. The efficacy of the translation-blocking morpholino (MT ATG) was assessed with an injection of an additional morpholino inhibiting pre-mRNA splicing at exon 5 (supplementary material Fig. S3A,B) (Gerety and Wilkinson, 2011). These data were independently confirmed providing as a positive loading control. Quantification of the etv7 signals relative to actin is shown below. (B) Quantitative real-time PCR measuring the level of etv7 expression during the first 5 days of development. All values are relative to day 0, which is 4 hpf. d1=day 1 post-fertilization etc. Error bars represent standard deviation.

**Fig. 1. etv7 is expressed in the adult and developing zebrafish.** (A) Semi-quantitative RT-PCR of etv7 mRNA of adult zebrafish tissues. actin is provided as a positive loading control. Quantification of the etv7 signals relative to actin is shown below. (B) Quantitative real-time PCR measuring the level of etv7 expression during the first 5 days of development. All values are relative to day 0, which is 4 hpf. d1=day 1 post-fertilization etc. Error bars represent standard deviation.

**etv7 morphants maintain beta-globin mRNA expression during development**

The absence of hemoglobinized red blood cells could result from the inability to express beta-globin. To determine whether the loss of hemoglobinized red blood cells results from defects in beta-globin mRNA expression, in situ hybridization at 23, 24, 33 and 48 hours post-fertilization (hpf) was performed on embryos injected with the standard control morpholino or MT ATG. We found that beta-globin (hbbe1.1) mRNA was equivalently expressed in control and morphant
animals at each time point examined (Fig. 4A). These data suggest that a loss of hemoglobinized red blood cells is not due to the inability to express beta-globin mRNA. Furthermore, our analysis demonstrated that beta-globin-positive cells are localized to the correct regions before and after the onset of circulation, ruling out the possibility that etv7 knockdown results in circulation defects (Fig. 4A, 23 and 24 hpf). Semi-quantitative PCR at 24 hpf confirmed that beta-globin expression did not change in morphant animals (Fig. 4B). Furthermore, a reduction in hemoglobinized red blood cells was evident as early as 30 hpf in morphant animals (Fig. 2A). Defects in proper specification of red blood cells could result in an absence of mature red blood cells. To begin to address this possibility, in situ hybridization detecting gata1 mRNA expression was performed at 23, 24, 33 and 48 hpf. Prior to the onset of circulation, gata1 expression was located in the PBI (peripheral blood island) and ICM (intermediate cell mass) of both morphants and control animals. At the onset of circulation, ~24 hpf, gata1 expression was observed in the PBI and on the yolk sac with no significant difference between morphant and control animals. At 33 hpf, after the onset of definitive hematopoiesis, gata1 expression was observed on the yolk sac of both control and morphant animals. However, at 48 hpf, gata1 expression was retained on the yolk sac of morphants (n=40), whereas there was no detectable gata1 expression in the embryos injected with control standard morpholinos (n=29) (Fig. 5). Approximately 72% of the embryos analyzed demonstrated this abnormal pattern of gata1 expression relative to control (P<0.0001).

Etv7 regulates red blood cell development through the cholesterol synthesis pathway

Etv7 is a transcription factor and therefore it is plausible that Etv7 regulates red blood cell development indirectly via downstream target genes. To test this idea, microarray analysis on embryos transiently overexpressing Etv7 was performed in order to identify possible downstream target genes. This analysis revealed that Etv7 regulates expression of the zebrafish lanosterol synthase (lss) gene (supplementary material Table S1). Morpholino-mediated loss of etv7 resulted in a significant decrease of lss mRNA (Fig. 6A). Furthermore, drug inhibition of Lss enzyme activity with Ro 48-8071, an Lss-specific inhibitor, resulted in a loss of hemoglobinized red blood cells (Fig. 6B; Table 1). LSS regulates the final step in the cholesterol synthesis pathway and has been shown to regulate the self-renewal of chicken erythrocyte progenitors (Mejia-Pous et al., 2011). If Etv7 regulates red blood cell development through the cholesterol synthesis pathway, then we predicted that administration of exogenous cholesterol to etv7 morphants should rescue the observed phenotype. Indeed, injection of exogenous cholesterol restored wild-type levels of hemoglobinized red blood cells in the etv7 morphants (Fig. 6B). This result indicated that exogenous cholesterol compensates for loss of endogenous cholesterol synthesis due to the reduction in lss expression and adequately rescues the phenotype associated with loss of etv7. Furthermore, we found that lss was expressed in a similar subset of tissues as was etv7 (supplementary material Fig. S4). Taken together, these data provide evidence that loss of etv7 leads to reduced expression of lss, which in turn affects red blood cell development.

DISCUSSION

ETV7 has been shown to promote tumorigenesis in mice and previous work has characterized the effects of ETV7 overexpression in murine bone marrow. However, a comprehensive understanding
of the mechanisms via which \textit{ETV7} mediates tumorigenesis is lacking and has been impeded by the lack of an appropriate animal model. Part of the rodent lineage has deleted the \textit{Etv7} gene; however, all other vertebrates, including zebrafish, have retained the gene. Here we used zebrafish as an appropriate animal model to study \textit{ETV7} function and provide evidence that \textit{etv7} regulates red blood cell development indirectly through the cholesterol synthesis pathway.

Analysis of the expression patterns in zebrafish demonstrated that \textit{etv7} is expressed both developmentally and throughout adulthood. The expression level of \textit{etv7} was low across most adult tissues, an observation that is consistent with previously published work using human tissues (Gu et al., 2001). To examine the role of \textit{etv7} during development, we used a morpholino-based approach. The most obvious phenotype of \textit{etv7} knockdown was a marked reduction in the number of hemoglobinized red blood cells. A hematopoietic defect is not an unanticipated result because human \textit{ETV7} is expressed in the bone marrow and fetal liver (Potter et al., 2000). Here we have focused entirely on red blood cell development, given that preliminary analysis of markers associated with other lineages such as \textit{PU.1} and \textit{scl} did not indicate additional blood cell defects. In the future, a more robust analysis of other cell types will need to be performed to potentially uncover a wider role of \textit{etv7} during zebrafish hematopoiesis.

Although little is known about the endogenous role of \textit{ETV7}, an animal model for the highly related \textit{ETV6} gene has been characterized. Loss of \textit{Etv6} in the mouse causes defects in hematopoiesis (Wang et al., 1998), whereas overexpression of the \textit{Etv6} gene in erythroid cells leads to enhanced proliferation and increased hemoglobin synthesis (Eguchi-Ishimae et al., 2009). Given the phenotypes associated with the gain or loss of function of both \textit{ETV6} and \textit{ETV7}, which can physically interact (Potter et al., 2000), it is plausible that these two proteins have some overlapping functions. However, \textit{ETV6} and \textit{ETV7} do not completely overlap in functionality because \textit{ETV6} inhibits cell proliferation and transformation (Kawagoe et al., 2004), whereas \textit{ETV7} is a known oncogene (Carella et al., 2006).

This study provides evidence that \textit{etv7} directly or indirectly regulates the expression of \textit{lss}, a gene involved in cholesterol biosynthesis. Recent work has demonstrated that LSS is essential for the self-renewal of chicken erythroid precursor cells (Mejia-Pous et al., 2011). In this study, the authors demonstrated that LSS was important for maintaining the self-renewal capacity of red blood cells, but LSS inhibition by Ro 48-8.071 did not affect the function of fully differentiated cells. Furthermore, maintenance of self-renewal was directly dependent upon the presence of cholesterol. LSS is highly conserved between chicken and zebrafish. The enzymatically active squalene cyclase domain (measuring 639 amino acids) is 77% identical and 85% homologous between the two species. Thus, there is little doubt that the Ro 48-8.071-induced
of specific oncogenes in cancer development and it has been shown to regulate cholesterol biosynthesis in other cell types and during tumorigenesis. Zebrafish have been used in the past to study the role of etv7 in red blood cell development (Mohandas and Gallagher, 2008). Our combined analysis of etv7 morpholinos (MT ATG) and inhibition of Lss enzymatic activity phenocopies knockdown of etv7. Wild-type embryos were treated with the Lss inhibitor Ro 48-8.071 (50 nM) and stained with o-dianisidine at 2 dpf. Injection of etv7 morpholinos was performed simultaneously and compared with embryos treated with Ro 48-8.071. Rescue experiments were performed by injecting cholesterol into the yolk of embryos at 1 dpf. o-Dianisidine staining was used to visualize the presence or absence of hemoglobinized red blood cells at 2 dpf (Std n=26, Std with cholesterol n=32, MT ATG n=20, MT ATG with cholesterol n=15). Refer to Table 1 for P-values and percent affected in each category.

Fig. 6. etv7 regulates red blood cells through the cholesterol synthesis pathway. (A) Real-time PCR analysis of lss expression in embryos injected with either 8.2 ng of standard control morpholino (Std) or 8.2 ng of etv7 morpholinos (MT ATG). Error bars represent standard deviation. (B) Inhibition of Lss enzymatic activity phenocopies knockdown of etv7. Wild-type embryos were treated with the Lss inhibitor Ro 48-8.071 (50 nM) and stained with o-dianisidine at 2 dpf. Injection of etv7 morpholinos was performed simultaneously and compared with embryos treated with Ro 48-8.071. Rescue experiments were performed by injecting cholesterol into the yolk of embryos at 1 dpf. o-Dianisidine staining was used to visualize the presence or absence of hemoglobinized red blood cells at 2 dpf (Std n=26, Std with cholesterol n=32, MT ATG n=20, MT ATG with cholesterol n=15). Refer to Table 1 for P-values and percent affected in each category.

In this study, we demonstrated that zebrafish provide a unique model to study etv7 gene function. We addressed the function of etv7 during development, but we have not addressed the role of etv7 during tumorigenesis. It will be interesting to determine whether etv7 regulates cholesterol biosynthesis in other cell types and during tumorigenesis. Zebrafish have been used in the past to study the role of specific oncogenes in cancer development and it has been shown that zebrafish develop histologically similar cancer phenotypes as mice and humans. For example, AML1:ETO, TEL1:JAK2A and TEL1:AML1 transgenic zebrafish have been generated, which closely phenocopy the results obtained in mouse models (reviewed in Quintana and Grosfeld, 2011). There is now a need to gain a more complete understanding for the effects of etv7 overexpression and the potential role of this gene in a zebrafish cancer model system.

MATERIALS AND METHODS

Zebrafish and maintenance
Zebrafish (Danio rerio) were maintained at St Jude Children’s Research Hospital according to the Institutional Animal Care and Use Committee (IACUC) guidelines. For all experiments, zebrafish embryos [Tupfel long fin (TL) or tp53zdf1] were maintained in egg water consisting of 0.03% Instant Ocean (Aquarium Systems, Inc., Mentor, OH) in R.O. water at 28.5°C.

RNA isolation and PCR analysis
For RNA isolation, larvae were manually dechorionated, lysed in Trizol (Invitrogen, Grand Island, NY) (200 μl), and processed according to the manufacturer’s recommendations. RNA (3 μg) was converted into cDNA with Superscript III Reverse Strand Synthesis system (Invitrogen, Grand Island, NY) (200 μl) according to the manufacturer’s instructions. For RNA isolation, larvae were manually dechorionated, lysed in Trizol (Invitrogen, Grand Island, NY) according to manufacturer’s protocol with random hexamer primers. Semi-quantitative PCR was performed with GoTaq mastermix (Promega, Madison, WI). Sequences of primers are listed in supplementary material Table S2.

In vitro knockdown analysis and western blot
For in vitro transcription/translation, 360 ng pGEM-etv7 DNA was used to program the TNT SP6 Quick Coupled Transcription/Translation system (Promega, Madison, WI) according to the manufacturer’s instructions. Western blots were probed with anti-HA antibody according to manufacturer’s protocol (Cell Signaling Technology, Danvers, MA). Western blots were developed with SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL).

Morpholino injection and analysis
Tupfel long fin (TL) or tp53zdf1 larvae were injected with 8.2 ng of standard control morpholino (5′-CCTTTCACCTCGTTACAATTTTATA-3′), 8.2 ng of translational blocking morpholino (5′-GTGAAAGGCGTCATCATGTTCT-3′), 4.1 ng of etv7 splice site exon 3 morpholino (5′-GATGGCCTGCAATTTACATC-3′) or 8.2 ng of etv7 splice site exon 5 (5′-GACCTGCAAAACCAATTATTG-3′) (Gene Tools, LLC, Philomath, OR). A range of concentrations from 2.0 to 16.4 ng of standard control

| Sample                  | Number of embryos | Percent affected (reduced heme) | P-value  |
|-------------------------|-------------------|---------------------------------|----------|
| Std                     | 26                | 7.7                             | NA       |
| Std + cholesterol       | 32                | 15.6                            | NS       |
| MT ATG                  | 20                | 80                              | <0.0005* |
| MT ATG + cholesterol    | 15                | 20                              | 0.001**  |
| Lss inhibitor           | 46                | 82.6                            | <0.0001* |
| Inhibitor + cholesterol | 43                | 27.9                            | 0.0001** |

*Statistical analysis was performed using a Fisher’s exact t-test comparing embryos treated with the MT ATG or the Lss inhibitor to embryos injected with standard control morpholino. Std, standard control morpholino; MT ATG, etv7 translational-inhibiting morpholino; Lss inhibitor, embryos treated with 50 nM Lss inhibitor. **Analysis was performed using Fisher’s exact t-test comparing MT ATG with cholesterol or inhibitor with cholesterol to Std with cholesterol. NS, not significant and determines the statistical difference between the Std and the Std with cholesterol.
morpholino was injected to determine that the standard control morpholino did not cause any blood cell defects. For subsequent experiments an equal concentration of standard control and etv7-specific morpholinos was used and is specified in the manuscript figure legends. α-dianisidine (Sigma, St Louis, MO) staining was performed as previously described (Paffett-Lugassy and Zon, 2004). Statistical analysis was performed according to a Fisher’s exact t-test with the online software calculator from Graphpad Prism.

**Etv7 overexpression and DNA microarray analysis**

DNA (200 pg/embryo) was injected into single-cell embryos. At 1 dpf, embryos (10) were harvested and total RNA was isolated as described under the ‘RNA isolation and PCR analysis’ heading. Total RNA was hybridized to the Zebrafish Gene Expression Microarray (Agilent Technologies, Santa Clara, CA). Each experiment was performed with biological duplicates. All microarray experiments and analyses were performed by the Hartwell Center for Bioinformatics and Biotechnology core facility at St Jude Children’s Research Hospital.

**Tissue isolation and RNA analysis**

Adult zebrafish were sacrificed in 0.04% tricaine solution and various organs and tissues were dissected out. Organs were lysed in Trizol (Invitrogen, Grand Island, NY) (200 μl) and processed according to the manufacturer’s recommendations. RNA was converted into cDNA with Superscript III Reverse Strand Synthesis system (Invitrogen, Grand Island, NY) with oligo dT primers according to manufacturer’s protocol. Semi-quantitative PCR was performed with GoTaq mastermix (Promega, Madison, WI) and sequences of primers are listed in supplementary material Table S2.

**In situ hybridization**

All in situ hybridization was performed as previously described (Thisse and Thisse, 2008). Briefly, embryos were harvested at the indicated time point and fixed in 4% paraformaldehyde (Sigma, St Louis, MO) overnight at 4°C. Each sample was washed in PBS with 0.1% Tween 20 (Sigma, St Louis, MO) (PBT) and permeabilized with proteinase K for varying amounts of time according to developmental stage. All probes were hybridized overnight, except the etv7 probe, which was hybridized for 72 hours, and washed in wash solution containing 50% formamide (Sigma, St Louis, MO). Each sample was washed in PBS with 0.1% Tween 20 (Sigma, St Louis, MO) and fixed in 4% paraformaldehyde (Sigma, St Louis, MO) overnight at 4°C. Thisse, 2008). Briefly, embryos were harvested at the indicated time point and then incubated with anti-DIG Fab fragments (Roche, Indianapolis, IN) overnight. All samples were developed with the BM Purple AP substrate (Roche, Indianapolis, IN) and imaged by conventional microscopy.

**Lss inhibition and cholesterol injection**

Lss was inhibited with 50 nM Ro 48-8.071 by addition directly to egg water at 4 hpf, followed by incubation until 2 dpf. Cholesterol (Sigma, St Louis, MO) was injected at 2 μg/μl into the yolk sac of embryos at 1 dpf. For rescue experiments, embryos were injected with cholesterol after 24 hours incubation with 50 nM Ro 48-8.071 (Sigma, St Louis, MO). After injection, embryos were incubated an additional 24 hours in 50 nM Ro 48-8.071. To rescue the etv7-specific phenotype, embryos were injected with morpholinos at the single-cell stage and then injected with cholesterol at 1 dpf. All embryos were analyzed at 2 dpf with α-dianisidine according to the protocol described by Paffett-Lugassy and Zon (Paffett-Lugassy and Zon, 2004).

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

A.M.O. conceived the project, performed data analysis, developed morpholinos, characterized phenotype, performed some in situ hybridizations and wrote portions of the manuscript. F.P. performed in situ hybridization for etv7, cloned constructs and analyzed tissue-specific expression in adult fish. R.I.K.G. developed original in situ hybridization probes and characterized expression during development. A.M.G., M.R.T. and G.C.G. developed the project, performed data analysis and wrote portions of the manuscript.

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**Supplementary material**

Supplementary material available online at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.012526/-/DC1

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