c-Myb Is an Evolutionary Conserved miR-150 Target and miR-150/c-Myb Interaction Is Important for Embryonic Development

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Human c-Myb proto-oncogene is highly expressed in hematopoietic progenitors as well as leukemia and certain solid tumor. However, the regulatory mechanisms of its expression and biological functions remain largely unclear. Recently, c-Myb has been shown to be targeted by microRNA-150 (miR-150) which thereby controls B cell differentiation in mice. In this study, we demonstrated that c-Myb is an evolutionary conserved target of miR-150 in human and zebrafish, using reporter assays. Ectopic expression of miR-150 in breast cancer and leukemic cells repressed endogenous c-Myb at both messenger RNA (mRNA) and protein levels. Among several leukemia cell lines, primary leukemia cells, and normal lymphocytes, expression levels of miR-150 inversely correlated with c-Myb. The miR-150 overexpression or c-Myb silencing in zebrafish zygotes led to similar and serious phenotypic defects in zebrafish, and the phenotypic aberrations induced by miR-150 could be reversed by coinjection of c-Myb mRNA. Our findings suggest that c-Myb is an evolutionarily conserved target of miR-150 and miR-150/c-Myb interaction is important for embryonic development and possibly oncogenesis.

Introduction

MicroRNAs (miRNAs) are a family of ~22-nt small endogenous RNAs that function as posttranscriptional regulators of gene expression and play important roles in multiple biological and metabolic processes of eukaryotic organisms (for reviews, Ambros 2004; Bartel 2004; Esquela-Kerscher and Slack 2006). In general, miRNA negatively regulates the target gene expression by translation inhibition in animal cells and messenger RNA (mRNA) degradation in plants cells (Bartel 2004). Recent studies have demonstrated the association between miRNAs and tumorigenesis (Calin et al. 2002, 2004; Cimmino et al. 2005; He et al. 2005; Lu et al. 2005).

The c-Myb proto-oncogene encodes a transcription factor involved in proliferation, differentiation, and survival of hematopoietic cells (Oh and Reddy 1999). The c-Myb gene is highly expressed in hematopoietic progenitor cells and declines as they differentiate (Westin et al. 1982; Gonda and Metcalf 1984). Elevated expression of c-Myb has been reported in many cases of acute myeloid and lymphoid leukemia, colon cancer, and breast cancer (Mavilio et al. 1986; Slamon et al. 1986; Torelli et al. 1987; Gueirin et al. 1990; Tesch et al. 1992; Birocchio et al. 2001), suggesting that c-Myb plays a role in tumorigenesis. However, except for rarely reported amplification (Pelici et al. 1984), gene truncation (Tomita et al. 1998), and mutation (Lutwyche et al. 2001), the mechanisms of c-Myb activation in cancer remain unclear.

MicroRNA-150 (miR-150) is selectively expressed in mature B and T cells (Monticelli et al. 2005), and premature overexpression of miR-150 blocks early B cell development (Zhou et al. 2007). Recently, miR-150 has been verified to control B cell differentiation in mice by targeting c-Myb (Xiao et al. 2007). Whether miR-150/c-Myb partnership is operative in biological systems other than B cells, and whether such partnership is conserved through evolution (Lin et al. 2007) remains to be elucidated. In this study, we addressed both issues in human cancer and zebrafish development.

Materials and Methods

Human Leukemia Samples Engrafted in Mice and Healthy Lymphocytes Preparations

Heparinized bone marrow or peripheral blood samples were obtained at diagnosis from patients with B-precursor ALL at the University of California, San Diego, and patients with T-acute lymphoid leukemia (T-ALL) enrolled in Pediatric Oncology Group Protocols #9000 and #9400 (ALL Biology Study). Samples are fully encoded to protect patient confidentiality and conform to the Health Insurance Portability and Accountability Act standards and are utilized under a University of California, San Diego-approved Institutional Review Board protocol (#041429). Tumor cell content was determined to be 80% or more in all samples by flow cytometry. Leukemic cells were engrafted in mice by intravenous injection into irradiated preconditioned NOD-SCID mice (Dialynas et al. 2001). Preconditioning with fetal cord blood facilitates engraftment of primary childhood T-cell acute lymphoblastic leukemia in immunodeficient mice (Dialynas et al. 2001). Mononuclear cells from leukemia samples or peripheral blood samples were isolated by isopycnic sedimentation through Ficoll-Hypaque (specific gravity 1.077 g/ml; Pharmacia, Piscataway, NJ) at 400 × g for 30 min followed by 2 washes with RPMI 1640 medium. Lymphocytes were isolated from the peripheral blood mononuclear cells (PBMC) which were treated with the human CD14 microBeads (Miltenyi Biotec Inc., Auburn, CA) to remove monocytes.

Cell Culture and Plasmids

Human breast cancer MCF-7 cells were cultured in modified Eagle medium supplemented with 10% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, and 10 µg/ml bovine recombinant insulin in 5% CO2. Human erythroleukemia cell line derived from chronic myeloid leukemia, K562 and T-cell acute lymphoblastic leukemia...
Jurkat and CCRF-CEM cell lines were cultured in RPMI1640 medium with 10% FBS in 5% CO₂. Human embryonic kidney 293T (HEK293T) cells were cultured in Dulbecco’s Modified Eagles Medium with 10% FBS in 5% CO₂. All primer sequences are listed in supplementary table S1 (Supplementary Material online). To construct luciferase reporter plasmids, various target fragments were inserted at Xhol site, downstream of the luciferase gene in the pHRG-TK vector (Promega, Madison, WI) as described previously (Kiriakidou et al. 2004; Boutz et al. 2007; Lin et al. 2007). The 3’ untranslated region (UTR) fragment (nt3001 to nt3168) of human c-Myb cDNA (GenBank accession number NM_005375), which contained 2 putative target sites for miR-150 (nt3012 to nt3033 and nt3076 to nt3097), was amplified by polymerase chain reaction (PCR) with myb3001F and myb3168R primers from the human genomic DNA of PBMC. PCR was performed by using Phusion DNA polymerase (Finnzymes, Oy, Espoo, Finland) following manufacturer’s instruction, and the product was cloned into phRG-TK vector to construct the MYB wild-type (WT) reporter. MYB mutant reporters, which contained mutations to mismatch to the “seed region” of miR-150, were generated by QuickChange II XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) with the primers, hMYBmt1F, hMYBmt1R, hMYBmt2F, and hMYBmt2R, according to the instruction. The pmiR150AS reporter plasmid, which contained the antisense sequence of miR-150, was generated by inserting the ds-oligo, which was annealed from 150ASF and 150ASR, into pHRG-TK vector. The pGL-3 control reporter plasmids (Promega) were cotransfected with the indicated plasmids and were used for assessment of transfection efficiency. A 211-bp 3’ UTR common region containing 2 putative miR-150 target sites of both long and short transcripts of zebrafish c-Myb orthologues (GenBank accession number BC059803 and NM_131266), which represented nt1952 to nt2162 of the long transcript, was amplified from genomic DNA using dremyb1952F and dremyb2162R primers and then cloned into pHRG-TK to construct zebrafish MYB WT reporter. Zebrafish MYB mutant reporters, which contained mutations to mismatch to the seed region of miR-150, were generated by QuickChange II XL Site-directed Mutagenesis Kit (Stratagene) with the primers, dMYBmt1F, dMYBmt1R, dMYBmt2F, and dMYBmt2R, according to the instruction. To express miR-150, we constructed pmiR150-GFP plasmid by cloning a 340-bp genomic fragment containing precursor miR-150 from the human genomic DNA prepared from PBMC, which was amplified by PCR with miR-150F and miR-150R primers, into the Nhel and Xhol sites of pIRE-S2-EGFP vector (BD Biosciences Clontech, Palo Alto, CA) (supplementary fig. S3, Supplementary Material online). The pmiR150-GFPmrt, which contained a mutated seed region of miR-150, was generated by QuickChange II XL Site-directed Mutagenesis Kit (Stratagene) with miR-150mtF and miR-150mtR primers.

Transfection, Microporation, and Luciferase Reporter Assay

Precursor miRNA oligos for hsa-miR-150 (pre-miR-150), for hsa-miR-198 (pre-miR-198), and for negative control #1 (pre-ctrl) were purchased from Ambion (Austin, TX). MCF-7 cells were seeded in 12-well plates 24 h before transfection and were transfected at 70% confluency with indicated amounts of precursor miRNA oligo and 2 µl of Lipofectamine2000 reagent (Invitrogen, Carlsbad, CA) following manufacturer’s manual. Leukemia cells were transfected with plasmids and pre-miRNA oligo by an electroporation method, microporation (Digital Bio Technology Co., Ltd., Kyungki-do, Korea), according to the manufacturer’s protocol. The pulse conditions used in K562, Jurkat, and CCRF-CEM cells were 1,450 V/10 ms/3 pulses, 1,380 V/30 ms/1 pulse, and 1,350 V/30 ms/1 pulse, respectively. The luciferase reporter assays were performed as described previously with modification (Kiriakidou et al. 2004; Boutz et al. 2007). Briefly, 2 × 10⁵ CEM cells in 10 µl of buffer R were cotransfected with 1 µg of effector plasmid (pmiR150-GFP or pIRES2-EGFP vector), 0.1 µg of MYB reporter (or pHRG-TK vector), and 0.1 µg of pGL3-control reporter by microporation. After electroporation, cells were cultured in 12-well plates with 1 ml of medium. HEK293T cells were seeded in 12-well plates 24 h before transfection. Cells were transfected with indicated amounts of effector and reporter plasmids in the presence of Lipofectamine2000 reagent (Invitrogen) following manufacturer’s manual. Two days after transfection, luciferase activity was determined using Dual-luciferase assay system (Promega) according to the manufacturer’s instruction. The Renilla luciferase activity was divided by the firefly luciferase activity of the same transfection, the normalized luciferase activity of the transfectant with pre-ctrl oligo or pIRES2-EGFP vector control in each reporter was set to 100%, and all others were expressed relative to it. The result represents the average of 3 independent experiments with standard deviations.

miRNAs and c-Myb mRNA Quantification

Total RNA was extracted by the Trizol reagent (Invitrogen). For quantification of miR-150, miR-198, and U6 RNA (as an internal control RNA), quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) was performed using the TaqMan Real-time PCR kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instruction. For c-Myb mRNA quantification, 25 µl of quantitative PCR contained cDNA reverse transcribed from 4 ng of RNA using SuperScriptII First-strand Synthesis System for RT-PCR kit (Invitrogen) and 1 × TaqMan primer/probe sets (Applied Biosystems) and 1 × TaqMan universal PCR master mix (Applied Biosystems). The TaqMan primer/probe sets for c-Myb and glyceraldehyde 3-phosphate dehydrogenase (as an internal control RNA) were TaqMan gene expression assay number Hs00193527_m1 and Hs99999905_m1, respectively. All quantitative PCRs were performed using ABI Prism 7000 sequence detection system (Applied Biosystems), and the raw data were analyzed by ABI Prism 7000 SDS software (Applied Biosystems) following the manufacturer’s instruction. The cycle threshold, Ct, of each sample was generated with the default setting. The target RNA (miRNA or mRNA) expression level of each sample was
expressed as \( \Delta Ct \), \(-\left(\text{Ct of target RNA} - \text{Ct of internal control}\right)\), which higher value means higher target RNA expression.

Western Blot Analysis

Two days after transfection, cells were lysed in the lysis buffer (10 mM Tris–HCl [pH 7.4], 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, and 1× protease inhibitor cocktail [Roche, Indianapolis, IN]). Protein contents were determined against a standardized control using Bio-Rad, Hercules, CA protein assay kit (Bio-Rad). Indicated amounts of cell lysates were resolved in the NuPAGE Novex 4–12% Bis-Tris gel (Invitrogen) for western blot analysis of c-Myb, \( \alpha \)-tubulin (tubulin), and heat shock protein 72/73 (HSP72). For detecting specific proteins, the primary antibodies used were Anti-Myb clone 1-1 mouse monoclonal antibody (1:1,000 dilution; Upstate, Lake Placid, NY) (Salomoni et al. 1997), monoclonal anti-\( \alpha \)-tubulin antibody (clone B-5-1-2; 1:50,000; Sigma, St Louis, MO), and monoclonal anti-Hsp72/73 (Ab1) mouse antibody (clone W27; Calbiochem-EMD Biosciences, Inc., La Jolla, CA). HSP72 serves an alternative internal control in PBMC and lymphocytes (Birocchio et al. 2001) but not suitable for ALL samples because ALL had been reported to express low level of HSP72/73 (Fujita et al. 2001). Anti-mouse IgG (H + L) AP-conjugated antibody (1:10,000 dilution; Promega) was used as the secondary antibody. The signal of protein bands was visualized using ECF western blotting kit (Amersham Biosciences, Piscataway, NJ) and detected by Typhoon 9400 imager (Amersham Biosciences). The signal intensity of each protein was quantitatively evaluated using ImageQuant software and normalized to the tubulin of the same transfectant. The normalized protein signal of the sample without transfection was set to 1.0, and the values of all others were calculated accordingly.

miR-150 Misexpression and c-Myb Silencing in Zebrafish Models

For miRNA misexpression, 4.6 nl of 40 \( \mu \)M MiRIDIAN miRNA mimics of hsa-miR-150 or negative control (C-300124-01-0005 and CN-002000-01-05; Dharmaco, Lafayette, CO) were microinjected into 1-cell stage of zebrafish embryos. Morpholino knockdown experiments were carried out to silence c-Myb expression. Morpholinos are chemically modified oligonucleotides (Nasevicius and Ekker 2000), which have been widely used in zebrafish and frog studies to specifically knockdown gene expression by blocking translation. We injected 9.2 ng of c-Myb morpholino or control morpholino into the yolk region at the 1-cell stage of zebrafish embryos. The c-Myb morpholino (5′-GCGCCCTGCATCCCGCTTTCG-3′) and nonspecific control morpholino (5′-CCTTTACCTAGTTAC-AATTATA-3′) were purchased from Open Biosystems (Huntsville, AL) and Gene Tools (Philomath, OR), respectively. For each injection, \( \sim 100 \) eggs were injected, and the abnormal embryos were counted and photos taken under the dissection microscope 48-h postinjection. To quantify zebrafish c-Myb and \( \beta \)-actin mRNAs, 24-h postinjection, miR-150 mimic oligo-injected embryos with abnormal phenotypes (\( n = 12 \)) or negative control-injected embryos (\( n = 15 \)) were randomly collected and pooled for total RNA isolation, which were used for quantitative RT-PCRs by High-Capacity cDNA Reverse Transcription Kit and Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s instruction. The primers for c-Myb (dreMYBF and dreMYBR) and \( \beta \)-actin (dreActinF and dreActinR) were listed in supplementary table S1 (Supplementary Material online). The c-Myb expression level of each sample was normalized to the expression level of \( \beta \)-actin in the same sample by the formula: c-Myb/\( \beta \)-actin = 2\(^{\text{-}\Delta Ct \text{ c-Myb-ct of } \beta \text{-actin}}\). The c-Myb/\( \beta \)-actin ratio of the negative control was set to 1.0, and the values of miR-150 oligo injection relative to it were calculated accordingly. The result represents the average of 3 independent experiments with standard deviations.

Eye size was determined by photographing lateral views of anaesthetized larvae 48-h postinjection. For each experiment, eye size was normalized to the average eye size of age-matched control fish (mimic control, \( n = 15 \); miR-150 mimic, \( n = 12 \); c-Myb morpholino, \( n = 12 \)).

Zebrafish c-Myb mRNA was in vitro transcribed from Zebrafish c-Myb plasmid by T3 RNA polymerase. For the c-Myb mRNA and miR-150 coinjection experiment, 33 \( \mu \)M of mimic oligo and 200 pg of in vitro transcribed RNA (in 4.6 nl) were cojected into zebrafish embryos. The c-Myb expression was detected by western blotting with anti-flag M2 antibody (Sigma) 12-h postinjection. The rates of reduced eyes embryos were counted 48-h postinjection from 3 independent experiments. For each experiment, at least 100 eggs were injected. To determine c-Myb and miR-150 expressions (fig. 5), total RNAs of randomly selected 30 injected embryos in each group were collected at 24-h postinjection for quantitative RT-PCR. Because there is no suitable zebrafish internal control in TaqMan qPCR assay, miR-18a which is almost identical to hsa-miR-18a in its mature sequence and is expressed comparably in each injection was chosen as an internal control.

Results and Discussion

The 3′ UTRs of c-Myb Genes of Both Human and Zebrafish Contain the miR-150 Target Sites

By surveying the miRBase target database, MIRANDA (http://microrna.sanger.ac.uk; Griffiths-Jones et al. 2006) and PICTAR-VERT (http://pitcar.bio.nyu.edu/; Krek et al. 2005), we found human c-Myb to be the highest ranking predicted target for miR-150. One putative target site in the 3′ UTR of c-Myb mRNA (nt3076 to nt3097) was a perfect match to the mature miR-150 (nt2319 to nt2341). Furthermore, we performed site-directed mutagenesis in order to disable this site (fig. 6). Even though the C-Myb expression was still detectable in miR-150 mimic cojected embryos, it was much lower than in control injected embryos.
match with the seed region (nt2 to nt8 of miR-150) of miR-150 as revealed by MIRANDA (fig. 1A). Using RNAhybrid software (Kruger and Rehmsmeier 2006), we found another putative target site in the 3' UTR of c-Myb (nt3012 to nt3033) that was also a perfect match with the seed region. We cloned these 2 target sites into the downstream of a luciferase reporter gene of pGL3-TK to construct the MYB WT reporter plasmid. MYB mt1, mt2, mt1mt2 reporters, which contained mutations of either or both predicted target sites to mismatch to the seed region of miR-150, were also constructed. (B) The miR-150 repressed the MYB reporter in HEK293T cells. HEK293T cells in 12-well plates were cotransfected with 2 μg of effector plasmid and 0.2 μg of reporter as well as 0.1 μg of pGL3-control by microinjection. Effector plasmids: GFP, pIRES2-EGFP control vector; 150-GFP, pmiR150-GFP; and 150-GFPmt, pmiR150-GFPmt with mutated sequence of the seed region of miR-150. Reporter plasmids: vector, pGL3-TK; MYB WT, MYB WT reporter; MYB mt1, mt1, mt1mt2, MYB mutant reporters; and 150AS, pmiR150AS reporter plasmid with insertion of the antisense sequence of miR-150. The normalized luciferase activity of each reporter transfected with effector control (GFP) was set to 100%, and all other activities of the same reporter were relative to it. The data represent the average of 3 independent experiments with standard deviations (* P < 0.05, Student's t-test).

To express human hsa-miR-150, we constructed pmiR150-GFP plasmid by cloning a 340-bp genomic fragment containing precursor miR-150 into pIRES2-EGFP, which coexpresses both the gene of interest and GFP protein from a single bicistronic mRNA. The control effector plasmid consists of an miR-150 mutant plasmid, pmiR150-GFPmt, with sequence identical to pmiR150-GFP except for a change of the miR-150 seed region to the complementary sequence, which expressed no detectable miR-150 (supplementary fig. S2C, Supplementary Material online). These pmiR150-GFP plasmids and its controls are shown in the supplementary figure S3 (Supplementary Material online). We cotransfected the miR-150-expressing plasmid with different reporter plasmids into HEK293T cells and performed the luciferase assays. As shown in figure 1B, miR-150 repressed neither the empty reporter vector nor the MYB mt1mt2 reporter but effectively repressed MYB WT reporter (to 54.6%) and repressed the positive reporter control p150AS most effectively (to 13.8%). Besides, miR-150 repressed both MYB mt1 (to 75.8%) and mt2 (to 64.7%) reporters, albeit to a lesser degree than MYB mt1mt2, suggesting that both sites are the targets for miR-150. As expected, pmiR150-GFPmt had no effect on MYB or p150AS reporters. These results suggested that 3' UTR of human c-Myb gene contains target sites specific for miR-150.
To confirm the result that miR-150 repressed the MYB reporter, similar reporter assays were performed in T-cell acute lymphoblastic leukemia line, CCRF-CEM. As shown in figure 1C, miR-150 repressed MYB reporter plasmid to 47.6%, suggesting that repression of c-Myb by miR-150 is not cell type specific.

In the miRBase database (http://microrna.sanger.ac.uk; Griffiths-Jones et al. 2006), miR-150 expresses in many vertebrate animals, from zebrafish, mouse, rat, cow, to human, and is predicted in frog. These 6 miR-150s are highly conserved in sequence with identical seed region (supplementary fig. S4, Supplementary Material online). In fact, the sequences of mature miR-150 of human, mouse, and rat are identical. This observation raises the possibility that c-Myb may be an evolutionary conserved target of miR-150. To address this issue, we examined whether miR-150 can repress c-Myb expression in zebrafish model, which is the lowest miR-150-expressing animal in evolution. There were 2 c-Myb orthologues in zebrafish, with high homology in their coding regions that differ in the lengths of 3' UTR (fig. 2A). When the common region of 3' UTRs of these 2 transcripts as well as hsa-miR-150 was entered into RNAhybrid program (Kruger and Rehmsmeier 2006), we found 2 putative target sites, nt1981 to nt1999 and nt2049 to nt2075, of the long transcript of zebrafish c-Myb (fig. 2A). These 2 target sites were also predicted using zebrafish dre-miR-150 and the 3' UTR common region. We cloned a 210-bp fragment (nt1952 to nt2162) containing these 2 putative target sites to construct the zebrafish MYB WT reporter plasmid and subsequently generated mutant MYB reporter plasmids, which were mutated in either or both sites to mismatch to the seed region of miR-150, and 150AS, pmiR150AS reporter plasmid inserted the antisense sequence of miR-150. The normalized luciferase activity of each reporter transfected with effector control (GFP) was set to 100%, and all other activities of the same reporter were relative to it. The data represent the average of 3 independent experiments with standard deviations (*P < 0.05, Student’s t-test).

miR-150 Downregulates c-Myb mRNA and Protein

To ascertain whether miR-150 regulates the expression of endogenous c-MYB, 4 c-MYB-expressing cancer
cell lines were transfected with precursor hsa-miR-150 oligo, and the expression levels of endogenous c-Myb protein were determined. These included MCF-7, a breast cancer cell line, and 3 leukemia cell lines, K562, Jurkat, and CCRF-CEM (CEM). As shown in figure 3A, comparing to the precursor negative control oligo (pre-ctrl oligo, lane 1), miR-150 (lane 2) downregulated endogenous c-Myb protein to 22%, 24%, 45%, and 52%, respectively; however, pre-miR-198 oligo (lane 3), which served as a nonspecific miRNA control, also repressed c-Myb protein slightly (7–29% inhibition). This mild repression may arise from nonspecific or indirect effects, not through a direct interaction with the 3’ UTR of c-Myb, which does not contain any predicted target site for miR-150. In a parallel transfection of CCRF-CEM cells, there was a marked increase (~32-fold) of the expression of mature miR-150 in the pre-miR-150 oligo transfectant, but not in the pre-ctrl oligo nor in the pre-miR-198 oligo transfectants (supplementary fig. S2B, Supplementary Material online).

To further confirm the above findings, we transfected CCRF-CEM cells with pmiR150-GFP plasmid or control plasmids, pIRES2-EGFP or pmiR150-GFPpmt, and sorted out the GFP-positive cells in each transfection 2 days later for determination of c-Myb protein. As showed in the supplementary figure S2A (Supplementary Material online), c-Myb protein dropped to 45% in the pmiR150-GFP transfectant but only decreased to 78% in pmiR150-GFPpmt control transfectant, as compared with the pIRES2-EGFP control transfectant. The expression of mature miR-150 was elevated by around 240-fold in the pmiR150-GFP transfectant, but not in the pIRES2-EGFP nor in the pmiR150-GFPpmt transfectants in a parallel transfection experiment (supplementary fig. S2C, Supplementary Material online). Taken together, our transfection studies using pre-miR-150 oligo and miR-150-expressing plasmids indicated that miR-150 is capable of negatively regulating the expression of c-Myb and that this phenomenon is not limited to B lymphocytes (Xiao et al. 2007) because the repressions were demonstrated in at least 3 different types of cancer cells, including breast cancer, T-cell acute lymphoblastic leukemia, and erythroid leukemia.

Although miRNAs in animal system usually repress target genes by inhibition of translation, increasing evidence has shown that certain animal miRNAs also reduce mRNA level (Bagga et al. 2005; Lim et al. 2005; Wu et al. 2006; Fabbri et al. 2007; Lee and Dutta 2007). We thus measured the c-Myb mRNA levels in CCRF-CEM by quantitative RT-PCR in the parallel transfection experiment as in figure 3A. As illustrated in figure 3B, transfection with pre-miR-150 led to a reduction of c-Myb mRNA...
to 73% of the pre-ctrl or pre-miR-198 transfections. This finding suggests that miR-150 represses c-Myb gene expression at both mRNA and translational levels.

Expression of miR-150 Inversely Correlates with Expression of c-Myb Gene in Leukemia Cells and Normal Lymphocytes

We have demonstrated that miR-150 repressed endogenous c-Myb gene in 4 c-Myb-expressing cancer cells (fig. 3A). We next sought to determine if there is an inverse relationship between miR-150 and c-Myb expression in leukemia. The expression levels of endogenous miR-150 and c-Myb were determined in CCRF-CEM and Jurkat leukemia cell lines, 5 leukemia samples engrafted in mice derived from 4 T-ALL patients, and 1 infant B-precursor-ALL with MLL gene rearrangement and peripheral blood mononuclear cells (PBMC) or lymphocytes from 3 normal healthy individuals. By western blot analysis, highest expression levels of c-Myb protein were not detectable in PBMC or lymphocytes from 3 individuals (fig. 3C). When normalized to B-ALL as 100%, the levels of c-Myb protein in CCRF-CEM and Jurkat cell lines were 843.5% and 377.5%, respectively, whereas the levels of c-Myb protein in 4 T-ALL ranged from 43% to 5.3% of B-ALL. The expression of miR-150 (fig. 3C) and c-Myb mRNA (supplementary fig. S5A, Supplementary Material online) as determined by quantitative RT-PCR showed ranking of miR-150 expression in an apparent order of healthy blood cells > leukemia samples > leukemia cell lines. On the other hand, the expression of c-Myb mRNA and protein displayed a reverse trend with the order of healthy blood cells (c-Myb protein undetectable) < leukemia samples < leukemia cell lines (supplementary fig. S5A, Supplementary Material online). The relative c-Myb mRNAs of Lym-2, the healthy control with the highest c-Myb mRNA expression, was only 0.2-fold ([C0-DDCT] = 5/[C0-DDCT] 2.2) of T-ALL-4, the leukemia sample with the lowest c-Myb mRNA expression, and 0.1-fold of Jurkat or CEM ([C0-DDCT] = 3.6 or 4.2) (the relative expression = 2^[C0-DDCT]), suggesting that c-Myb mRNA expressions of normal PBMC and lymphocytes are much lower comparing to leukemia cells. Statistical analysis revealed a highly significant reciprocal relationship between miR-150 and c-Myb protein (Pearson’s correlation coefficient is −0.860) as well as between miR-150 and c-Myb mRNA (Pearson’s correlation coefficient is −0.894), which lent further support that c-Myb expression is negatively regulated by miR-150 in leukemia (supplementary fig. S5B, Supplementary Material online). This result and the results of figure 3 imply that regulation of c-Myb expression by miR-150 may play a role in controlling leukemia growth.

FIG. 4.—The miR-150 misexpression and c-Myb silencing in zebrafish model lead to similar phenotypic aberrations. (A) Representative photos of zebrafish embryos injected with c-Myb morpholino (c-Myb MO) and double-stranded miR-150 mimic oligo (miR-150) or their respective controls 48-h postinjection. (B) The c-Myb mRNA expression in abnormal embryos injected with miR-150 mimic oligo (miR-150) or their controls (ctrl) at 24-h postinjection. The c-Myb mRNA was detected by quantitative RT-PCR. The c-Myb expression level of each sample was normalized to the level of β-actin in the same sample. The c-Myb/β-actin ratio of the control injection was set to 1.0, and the values of miR-150 oligo injection relative to it were calculated accordingly. The result represents the average of 3 independent experiments with standard deviations. (C) The incidence of abnormal embryos in microinjected zebrafish embryos at 48-h postinjection. In all, 40 μM of mimic oligo or 9.2 ng of morpholino in 4.6 nl volume were microinjected into zebrafish embryos. (D) Reduced eye size in embryos with miR-150 misexpression (miR150) and c-Myb silencing (Myb MO). Eye size was determined by photographing lateral views of anaesthetized larvae 48-h postinjection. For each experiment, eye size was normalized to the average eye size of age-matched fish injected with mimic control oligo (ctrl).
miR-150 Misexpression and c-Myb Silencing in Zebrafish Model Led to Similar Phenotypic Aberrations, Which Could Be Rescued by c-Myb mRNA

To further investigate the functions of miR-150 in vivo, double-stranded hsa-miR-150 mimic oligos were injected into zebrafish zygotes. At 24 h after microinjection, some of embryos (<25%) showed slight growth retardation with a reduced c-Myb mRNA level (to 75%) in those retardant embryos as compared with the embryos injected with negative control oligo (fig. 4B). At 48 h after injection, 62% of miR-150-injection embryos (n = 108) showed distinctively abnormal phenotypes with shortened trunk, reduced eye sizes, slow heartbeat, and sluggish blood flow (fig. 4A and C). In contrast, 95% of the embryos injected with negative control oligo showed normal phenotypes (n = 120).

We next silenced the c-Myb expression by microinjecting c-Myb morpholino (MO) into zebrafish zygotes. At 48 h, 85.7% of c-Myb MO-injected embryos were abnormal (n = 84) with abnormal phenotypes similar to or more severe than those injected with miR-150. The most obvious and common phenotypes included shortened trunk and reduced eye sizes (fig. 4A, C, and D).

To ascertain whether these similar phenotypic aberrations induced by miR-150 misexpression and c-Myb silencing might be the consequence of c-Myb repression by miR-150 or c-Myb MO, respectively, we explored the possibility of reversing these phenotypic changes by c-Myb mRNA. Flag-tagged zebrafish c-Myb mRNA that contained the open reading frame and did not contain the miR-150 target site was transcribed in vitro and co-injected with miR-150 mimic oligo into zebrafish zygotes. Along with the expression of this flag-c-Myb protein (supplementary fig. S6, Supplementary Material online), co-injection of c-Myb mRNA led to reduced incidence of small eye phenotype elicited by miR-150 misexpression from 65.0% to 42.9% in the GFP control mRNA (fig. 5C). The increased expressions of c-Myb and miR-150 by injection of c-Myb mRNA and miR-150 injection (fig. 5A and B), respectively, were confirmed. In this study, it was noted that miR-150 repressed the expression of c-Myb mRNA only to 88% (group 3, fig. 5A), less than the degree (to 75%) shown in figure 4B. This difference may be influenced by 2 factors. First, RNA was collected from all retardant embryos in figure 4B, whereas RNA was collected from randomly picked embryos in figure 5A. Second, the dosage of miR-150 used was different, 40 μM for figure 4B and 33 μM for figure 5A. These studies in zebrafish reveal that regulation of c-Myb expression by miR-150 is important for embryonic development, especially for eye formation, in addition to its reported role in B cell differentiation in mice (Xiao et al. 2007).

In this study, we have demonstrated that c-Myb genes of both human and zebrafish are negatively regulated by miR-150. Both 3’ UTRs of human and zebrafish c-Myb genes contained target sites of miR-150, mutations of which sequences to mismatch to the seed region of miR-150 were able to reverse the miR-150 repression. This is consistent with the recent report that miR-150 regulates B cell differentiation in mice by targeting c-Myb. Thus, it appears that the miR-150/c-Myb target relationship is conserved through evolution from zebrafish to mice and human. We further showed that ectopic expression of miR-150 in c-Myb-expressing breast cancer and T- and erythroleukemia cell lines resulted in the repression of endogenous c-Myb gene at both mRNA and protein levels. Moreover, we found an inverse correlation of miR-150 expression with c-Myb expression among 10 lymphoid cells of normal and malignant origins. These findings not only confirmed the regulation of endogenous c-Myb by miR-150 but also suggested that the scope of such regulation extends beyond B cell differentiation to include various leukemias and breast cancer. Finally, the demonstration of similar phenotypic abnormalities by misexpressing miR-150 or silencing c-Myb in zebrafish zygotes and the reversal of miR-150-induced abnormal phenotypes by coinjection of c-Myb mRNA provided further support for the regulation of c-Myb by miR-150 and illustrated the importance of miR-150/c-Myb interaction during embryonic development. Taken together, our results establish that c-Myb is an evolutionary conserved target of miR-150 and regulation of c-Myb expression by miR-150 is evident in breast cancer and leukemia in addition to the previously reported B cell development (Xiao et al. 2007) and such regulation plays an important role in embryonic developmental processes.

Elevated expression of c-Myb has been reported in many cases of acute myeloid and lymphoid leukemia, colon cancer, and breast cancer (Mavilio et al. 1986; Slamon et al. 1986; Torelli et al. 1987; Guerin et al. 1990; Tesch et al. 1992; Birocchi et al. 2001), suggesting that c-Myb may...
be involved in tumorigenesis or tumor progression. However, the regulatory mechanisms of c-Myb activation in leukemia are not well understood except for rare cases of c-Myb genomic alterations (Pelicci et al. 1984; Tomita et al. 1998; Lutwyche et al. 2001). Our finding that miR-150 repressed c-Myb gene provides another mechanism of regulation of c-Myb expression in cancer, including T- and B-lineage leukemia, erythroleukemia, and breast cancer. We also observed that leukemia cells expressed lower miR-150 but greater c-Myb as compared with normal lymphocytes, which expressed miR-150 highly but had no detectable c-Myb protein, implying that deregulation of miR-150 expression resulting in activation of c-Myb might be importance in leukemic process. The exact role of c-Myc/miR-150 in tumorigenesis and (or) tumor progress awaits further investigation.

Supplementary Material

Supplementary table S1 and figure S1–S6 are available at Molecular Biology and evolution online (http://www.mbe.oxfordjournals.org/).

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Literature Cited


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