Genome-wide analysis of the zebrafish Klf family identifies two genes important for erythroid maturation

Yuanyuan Xue 1, Shuai Gao 1, Feng Liu * 1,2

State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

A R T I C L E   I N F O

Article history:
Received 17 January 2015
Received in revised form 17 May 2015
Accepted 18 May 2015
Available online 23 May 2015

Keywords:
Klf family
Klf3
Klf6a
erythropoiesis
zebrafish

A B S T R A C T

Krüppel-like transcription factors (KlfTs), each of which contains a CACCC-box binding domain, have been investigated in a variety of developmental processes, such as angiogenesis, neurogenesis and somatic-cell reprogramming. However, the function and molecular mechanism by which the Klf family acts during developmental hematopoiesis remain elusive. Here, we report identification of 24 Klf family genes in zebrafish using bioinformatics. Gene expression profiling shows that 6 of these genes are expressed in blood and/or vascular endothelial cells during embryogenesis. Loss of function of 2 factors (klf3 or klf6a) leads to a decreased number of mature erythrocytes. Molecular studies indicate that both Klf3 and Klf6a are essential for erythroid cell differentiation and maturation but that these two proteins function in distinct manners. We find that Klf3 inhibits the expression of ferric-chelate reductase 1b (frrs1b), thereby promoting the maturation of erythroid cells, whereas Klf6a controls the erythroid cell cycle by negatively regulating cdkn1a expression to determine the rate of red blood cell proliferation. Taken together, our study provides a global view of the Klf family members that contribute to hematopoiesis in zebrafish and sheds new light on the function and molecular mechanism by which Klf3 and Klf6a act during erythropoiesis in vertebrates.

© 2015 Elsevier Inc. All rights reserved.

Introduction

Zebrafish has been recognized as an ideal genetic and developmental biology model to study vertebrate hematopoiesis owing to its special characteristics. The physiological and molecular mechanisms underlying hematopoiesis are well conserved from zebrafish to higher vertebrates, including mammals. There are two distinct waves of hematopoiesis in vertebrates: primitive hematopoiesis and definitive hematopoiesis (Davidson and Zon, 2004). Primitive erythropoiesis and myelopoiesis are initiated during the primitive wave; however, hematopoietic stem cells (HSCs) are produced only during the definitive wave. Many genes participate in the regulation of the complex process of hematopoiesis, such as klf genes during erythropoiesis; ets genes during hemangioblast differentiation, angiogenesis and HSC development; and runx1 and cmyb during HSC emergence (Dzierzak and Speck, 2008; Liu and Patient, 2008; North et al., 2002; Oates et al., 2001; Wang et al., 2013). However, our understanding of Klf’s role during erythropoiesis is still incomplete.

The Krüppel-like transcription factors (KlfTs) are well-known master regulators that are involved in various biological processes during embryogenesis. Members of the Klf family are characterized by Cys2-His2 zinc finger motifs at the C-terminus that bind to the sequence CACCC or to GC-rich elements of DNA, whereas the variable N-terminus is able to recruit different cofactors to function as activators or repressors, such as Klf5, Klf6, Klf7 and Klf16 (Conkright et al., 1999; Kaczynski et al., 2002; Koritschoner et al., 1997; Matsumoto et al., 1998; Nagai et al., 2009). Such cooperation is highly conserved in zebrafish and mammals (Miller and Bieker, 1993; Shields and Yang, 1998). Additionally, other factors influence Klf function in vivo, such as epigenetic modification (Chen and Bieker, 2004; Ma et al., 2013) and cellular context (Botella et al., 2002; Eaton et al., 2008; Fischer et al., 2001; Matsumoto et al., 2006; Porcu et al., 2011; Stadhouders et al., 2012; Xue et al., 2014). The functions of several Klf factors during development have been investigated. Klf1, which is also known as eklf, is highly expressed in erythroid cells in both the yolk sac and the fetal liver (Miller and Bieker, 1993). By directly binding to the promoter of the beta-globin gene, Klf1 regulates the globin switch to guide the differentiation of erythrocytes (Donze et al., 1995). In addition, klf4 functions as a tumor suppressor in many organs (Guan et al., 2010; Zammarchi et al., 2011) and is now well known as one of the Yamanka factors for the generation of induced pluripotent stem cells (iPSCs) from mouse fibroblasts in vitro (Takahashi and Yamanka, 2006). klf2a is expressed in the heart tube and...
vasculature and is involved in blood vessel remodeling, heart valve development (Lee et al., 2006; Vermot et al., 2009) and primitive hematopoiesis (Alhashem et al., 2011; Basu et al., 2007; Basu et al., 2005; Pang et al., 2012). We have previously demonstrated that blood flow-dependent klf2a expression regulates HSC development by mediating NO signaling in zebrafish embryos (Wang et al., 2011).

Although first identified as a weak activator (Crossley et al., 1996), Klf3 also acts as a transcriptional repressor. By binding to the cofactors (such as CtBP) (Crossley, 1998) that recruit other factors (such as chromatin modifying enzyme (Shi et al., 2003)), Klf3 represses transcription during several significant biological processes, including adipogenesis (Sue et al., 2008), B cell development (Turchinovich et al., 2011), erythropoiesis (Crossley et al., 1996), muscle cell development (Haldar et al., 2007) and cardiovascular development (Kelsey et al., 2013). During erythropoiesis, the expression of klf3 is directly regulated by Klf1; Klf3 then represses the expression of non-erythroid genes (Funnell et al., 2012), which might partially contribute to erythroid progenitor differentiation. Nevertheless, the downstream mechanism of action of Klf3 has not yet been illustrated.

Klf6 was originally identified as a tumor suppressor that inhibited cancer cell proliferation in prostate cancer (Narla et al., 2001). Mutations or alternative splicing variants of klf6 increase the risk of cancers such as lung cancer, ovarian cancer, human hepatocellular carcinoma and glioblastoma (DiFeo et al., 2008; DiFeo et al., 2006; Narla et al., 2007; Tchirkov et al., 2010). However, during organogenesis, Klf6 induces cellular proliferation and promotes the growth of internal organs. Studies have shown that hepatocyte proliferation was decreased after klf6a (the zebrafish homolog of the mouse klf6) was knocked down in zebrafish (Zhao et al., 2010). klf6 is an important regulator of yolk sac hematopoiesis in mice (Matsumoto et al., 2006) and is also essential for the processes of nerve regeneration and vascular remodeling (Botella et al., 2002; Garrido-Martín et al., 2013; Veldman et al., 2007). However, whether klf6 functions in primitive erythropoiesis in other vertebrates remains unknown.

Here, we used zebrafish as a model to obtain a global view of the expression of the Klf family members, focusing on the Klf factors involved in hematopoiesis in vivo. Our studies represent a comprehensive analysis of the Klf family during embryogenesis in vertebrates and provide insights into the functions and mechanisms of action of klf3 and klf6a in hematopoiesis. Loss of klf3, klf6a or both led to more erythroid progenitors and fewer mature erythrocytes, indicating a significant role for Klf factors during the initial progression of erythroleukemia and/or anemia.

Materials and methods

Zebrafish

Zebrafish embryos were maintained via the natural spawning of adult zebrafish. Zebrafish embryos were raised at 28.5 °C in system water and staged as described previously (Kimmel et al., 1995). The fkh1:GFP/gata1:dsRed transgenic line (Jin et al., 2007) and the eistpβ mutant (Pham et al., 2007) were kindly provided by Stefan Schulte-Merker (Hubrecht Institute, Utrecht, The Netherlands) and Brant Weinstein (NIH), respectively. The cloche mutant (Stainier et al., 1995), the moonshine mutant (Ransom et al., 2004) and the p53 mutant (Tao et al., 2013) were kindly provided by Anming Meng (Tsinghua University, Beijing, China). This study was approved by the Ethical Review Committee of the Institute of Zoology, Chinese Academy of Sciences, China.

Bioinformatics analysis

To identify klf genes, the amino acid sequences of the human and mouse Klf family members were used to query the zebrafish genome (http://www.ncbi.nlm.nih.gov). The full-length amino acid sequences of 24 Klf proteins from UniProt were aligned via the CLUSTALW program, and the phylogenetic tree was generated using the Construct/Test UPGMA Tree program with MEGA5 software. Chromosomal locations of klf genes were obtained from the zebrafish model organism database (ZFIN), whereas the protein structures of the Klf family members were analyzed by integrating the information from the Ensembl zebrafish database (http://asia.ensembl.org/Danio_rerio/Info/Index).

Morpholinos, mRNA and microinjection

The antisense morpholinos (MOs) including klf3-utmMO, klf3-misMO, frrs1b-atgMO, klf6a-atgMO, p53MO, cdkn1aMO and standard MOs were purchased from GeneTools. The sequences of specific MOs are described as follows.

klf3-utmMO (5′-AGCATGCGCTGCTTCAGTGAATTT-3′), klf3-misMO (5′-AGGATCGCTCCCTCCACTGAAATT-3′), frrs1b-atgMO (5′-GAAGCCAGCAGTACAATCCACAT-3′), klf6a-atgMO (5′-CACATTGATGACACATTGCAA-3′) (Veldman et al., 2007), p53MO (5′-CGCCATTGCTTTGCAAGAATT-3′), cdkn1a-spliceMO (5′-TAATAGAGGCTCTGACCCGTGATG-3′) (Sidi et al., 2008).

Stock solutions at 1 mM in dH2O were prepared, and 4–8 ng MOs were injected into 1-cell stage embryos at the yolk/blastomere boundary. The pCS2+ plasmid was used to generate the overexpression constructs of klf3-myc, frrs1b, klf6a-myc and cdkn1a (primers were provided in Supplemental Table S1). The mRNAs were synthesized using mMessage mMACHINE SP6 kit (Ambion). For overexpression or rescue experiments, 100–200 pg of mRNAs were injected into 1-cell stage embryos. A partial sequence of klf3 gene containing the utr-MO recognition site was fused with GFP in frame to generate the klf3–GFP fusion construct for validation of klf3MO efficiency. For rescue experiments, to avoid the klf6a atgMO recognition, the klf6a full length cDNA was amplified with forward primer containing five silent mutations and the same reverse primer (Supplemental Table S1), using the klf6a-myc construct as template. Then the PCR product was cloned into pCS2+ plasmid for mRNA synthesis. Since the target sequence of klf3 utrMO is located in the upstream 5′-UTR region of klf3 gene, there was no need to modify the klf3 full length mRNA for rescue experiments.

Whole-mount in situ hybridization

Whole-mount in situ hybridization of zebrafish embryos was performed using a ZF-A4 in situ hybridization machine (Zfind, China) with probes targeting genes of the Klf family, gata1, scl, bel1-globin, pit1, cdkn1a, mcl1, bcl2-l10, wee1, ank1, ragd, cripl, acp5a, acp5b, unc13d, frrs1a, frrs1b, bag1, aco9t1, aco9t2, anxa5a, anxa5b, capg, ctmdl, ghpr2, pql3 and aka2 as previously described (Wang et al., 2011). The primers used in probes synthesis are provided in Supplemental Table S2.

O-dianisidine staining

To detect the hemoglobin level, zebrafish embryos were stained with o-dianisidine staining solution for 15–60 min in the dark as previously reported (Detrich et al., 1995).

Whole-mount in situ hybridization of zebrafish embryos was performed using a ZF-A4 in situ hybridization machine (Zfind, China) with probes targeting genes of the Klf family, gata1, scl, bel1-globin, pit1, cdkn1a, mcl1, bcl2-l10, wee1, ank1, ragd, cripl, acp5a, acp5b, unc13d, frrs1a, frrs1b, bag1, aco9t1, aco9t2, anxa5a, anxa5b, capg, ctmdl, ghpr2, pql3 and aka2 as previously described (Wang et al., 2011). The primers used in probes synthesis are provided in Supplemental Table S2.

O-dianisidine staining

To detect the hemoglobin level, zebrafish embryos were stained with o-dianisidine staining solution for 15–60 min in the dark as previously reported (Detrich et al., 1995).
Real-time PCR

Total RNA used for quantitative RT-PCR (qPCR) was collected from the trunk region dissected from control and morphant embryos at 24 and 36 hours post fertilization (hpf), and at 2 days post fertilization (dpf). Total RNA was extracted by TRIzol reagent and was reversely transcribed. The CDNs were all diluted 4 times to be used as templates. The qPCR primers used in this work were summarized in Supplemental Table S3. The detailed protocols were described previously (Wang et al., 2011). Three independent experiments were performed. The qPCR results were analyzed using SPSS software.

Western blotting

Protein level of Klf3, Frrs1b, Cdkn1a and Klfl6a was evaluated using Western blotting as described previously (Wang et al., 2011), to verify the efficiency of overexpression of klf3 mRNA, klfl6a mRNA, frrs1b mRNA, cdkn1a mRNA and klfl6aMO knockdown. About 50–100 control embryos, frrs1b mRNA or klfl6aMO injected embryos were collected for protein extraction. The following antibodies were used: Klfl6 (sc-7158, Santa Cruz Biotechnology), c-Myc (9e10, Santa Cruz Biotechnology), and Flag (F7425, Sigma).

Cell sorting

Embryos were prepared for FACS as described by Covassin et al. (2006). The dsRed-positive cells were sorted from flk1:GFP/gata1: dsRed transgenic embryos using MoFlo XDP (Beckman).

Blood-smear and Giemsa staining

Zebrafish blood cells were collected from the heart region or vessels using an oil pressure injector and attached to slides by centrifugation using a cytospin. After the slides were air dried overnight, Wright–Giemsa staining was carried out to label erythrocytes according to the standard protocol. The images were acquired using a Nikon ECLIPSE 80i (100 ×).

TUNEL, BrdU labeling and pH3 staining

TUNEL and BrdU assay were performed as described previously (Wang et al., 2011). For anti-phosphohistone H3 (pH3) antibody staining, the Tg (gata1:dsRed) embryos were collected at 2 hpf and fixed in 4% paraformaldehyde (PFA). The fixed embryos were dehydrated with methanol and kept at −20 °C overnight. After rehydration, the embryos were treated with Proteinase K (10 µg/ml) for 20 min at room temperature (RT) and refixed in 4% PFA for 20 min. After washing with PBST, the embryos were blocked in 10% goat serum for 2 hpf at RT and incubated with primary rabbit anti-phosphohistone H3 antibody (Cell Signaling) at 4 °C overnight. After washing with PBST, the embryos were incubated with anti-rabbit-IgG-fluorescein (1:500) at 4 °C overnight.

Promoter constructs and reporter assay

To generate firefly luciferase reporter construct, a 1.5 kb (−4080 bp to −2510 bp) fragment of cdkn1a promoter, and a 2.3 kb (−2274 bp to +91 bp) fragment of frrs1b promoter were amplified from zebrafish genomic DNA and inserted into the pGL3.0 expression vector. To generate Klf3 and Klfl6a overexpression constructs, the full length of klf3 and klfl6a coding sequences were amplified from zebrafish cDNA and inserted into pcDNA3.1(+) expression vector. Reporter assay was performed as previously described (Wang et al., 2013).

ChIP assay

Chromatin immunoprecipitation (ChIP) assay was carried out with 24 hpf or 36 hpf embryos injected with klf3-myc or klfl6a-myc mRNA respectively, and the eluted DNA (precipitated by Myc antibody) was assayed by PCR as previously described (Wang et al., 2013). The primers specific and nonspecific to the Klf3 binding site within the promoter regions of frrs1b or Klfl6a binding site within the promoter regions of cdkn1a were designed according to the conserved binding sites. The primers were summarized in Supplemental Table S1. Nonspecific primers and rabbit purified immunoglobulin G were included as controls.

Confocal imaging and Statistical analysis

Confocal images were acquired by a Zeiss LSM 510 META confocal laser microscope, and Zeiss LSM software (Carl Zeiss) was used to generate 3D projections. Student’s unpaired 2-tailed t-test was applied for comparisons unless otherwise indicated.

Results

Phylogenetic analysis and expression patterns of Klf family members in zebrafish

Bioinformatics analysis identified a total of 24 Klf family members in zebrafish (based on the latest update of the Zv9 database), each of which has a mammalian homolog. The chromosomal locations and genomic IDs of the proteins were acquired from ZFIN and NCBI (Supplemental Table S4). The full-length amino acid sequences of the zebrafish Klf proteins were obtained from NCBI and Uniprot and used to generate a phylogenetic tree (Fig. 1A).

Phylogenetic analysis of the Klf family identified 4 Klf subgroups in zebrafish, which is consistent with the classification of their mammalian counterparts (Nagai et al., 2009) (Fig. 1A). Klf family members share a common conserved C-terminal and have highly varied N-terminal domains, which were used to group the Klf proteins (Fig. 1B). Members in both group 1 (Klf1d, Klf2a, Klf2b, Klf4, and Klf17) (Evans et al., 2007; SenBanerjee et al., 2004; Zhang and Bieker, 1998) and group 2 (Klf5a, Klf5b, Klf6a, Klf7a, Klf7b, and Klf18) (Li et al., 2005; Matsumoto et al., 1998; Zhang and Teng, 2003) interact with co-activators such as the histone acetyltransferases CBP and p300 and share a basic R/K-rich region N-terminal to the zinc finger domains that might function as a nuclear localization signal (NLS). The group 1 proteins contain a conserved Asp/Glu-rich region in their N-terminals, whereas group 2 contains more Asp/Leu-rich residues, indicating that the two groups utilize different cofactors to act as transcriptional activators. Group 3 members including Klf3, Klf8, Klf12a, and Klf12b can bind to CtBP, a co-repressor, to recruit HDACs to form a transcriptional repressor complex (Schuierer et al., 2001; Turner and Crossley, 1998; van Vliet et al., 2000). Members of group 4 (Klf9, Klf10, Klf11a, Klf11b, Klf13, and Klf16) (Brubaker et al., 2000; Fernandez-Zapico et al., 2003; Gowri et al., 2003; Kaczynski et al., 2001; Kaczynski et al., 2002; Zhang et al., 2001) interact with the Sin3A protein through a conserved N-terminal Sin3A-binding motif, which in turn recruits chromatin remodeling and histone modification enzymes, thus facilitating gene transcriptional activation or repression. Our results are consistent with a previous report (Nagai et al., 2009) indicating that the Klf family is highly conserved between zebrafish and mammals.

To characterize the expression pattern of Klf family genes in zebrafish, we synthesized RNA probes of 24 klf genes and examined their expression at two critical hematopoietic stages (24
and 36 hpf) using whole-mount in situ hybridization (WISH) (Fig. S1A and B). Expression profiling indicated overlapping or distinct expression patterns among the Klf members. For example, klf2b, klf4 and klf15 were highly expressed in epidermal cells, although klf15 expression was also detected in myotomes. klf7a was reported to be expressed in the diencephalon of zebrafish (Li et al., 2010), and here, we detected expression of klf7a in the dorsal thalamus and the olfactory bulb and the expression of klf7b in the cranial ganglion. There were also several newly isolated klf factors that showed a specific expression pattern in zebrafish. For example, klf13 was found to be expressed in the spinal cord, klf6b and klf12b were present in the metencephalon, and klf12a was expressed in the olfactory placode.

Several Klf family members have been implicated in hematopoiesis in zebrafish. klf6d plays an important role in erythropoiesis by promoting the expression of the embryonic alpha-globin gene (Fu et al., 2009), and klf17 is essential for erythroid differentiation by regulating gata1 and beta-globin expression (Gardiner et al., 2007; Kawahara and Dawid, 2001). Additionally, klf2a is required for the maintenance of HSC programming (Wang et al., 2011). Our data showed that klf6d, klf2a, and klf17 were expressed in hematopoietic cells (Fig. S1A and B), which is consistent with previous studies (Oates et al., 2001). To gain more insights into the functions of the klf genes in hematopoiesis, we focused on the genes specifically expressed in the intermediate cell mass (ICM) and the neural plate and the notochord at the 5 somite (s) stage (14 hpf) and in the ICM region at 24 hpf (Fig. 2A). To further validate the expression of klf3 in hematopoietic and/or endothelial cells, we tested whether expression of klf3 was affected in hematopoietic mutants such as cloche, etsrp, and moonshine. cloche mutants exhibit the earliest hematopoietic and endothelial lineage defects, etsrp mutants have defective blood vessels, and moonshine mutants lack primitive red blood cell (Pham et al., 2007; Ransom et al., 2004; Stainier et al., 1995). The expression of klf3 was completely absent in cloche embryos at 26 hpf (Fig. 2A), suggesting that klf3 is expressed in hematopoietic and/or endothelial cells. In etsrp mutants, the expression of klf3 did not change compared with wild type, whereas expression of klf3 was hardly detected in moonshine mutants. These data indicate that klf3 is expressed in primitive hematopoietic cells.

The zebrafish klf6a gene is a homolog of mammalian klf6 (Supplemental Table S4). WISH results showed that klf6a was expressed in the neural plate and the notochord at the 5–10 somite stages, klf6a was expressed in the central nervous system and blood vessels, including in the head and caudal region, at 24 and 36 hpf (Fig. S2B). However, klf6a expression was enriched in the gut and swim bladder at later stages. To verify the hematopoietic and/or vascular expression of klf6a, we tested the expression of the gene in the three aforementioned mutants. Interestingly, compared with control embryos, klf6a expression was markedly decreased in cloche mutants and etsrp mutants but was only slightly altered in moonshine mutants at 30 hpf (Fig. 2B). These data indicate that klf6a might be associated with vascular and/or hematopoietic development. Furthermore, klf3 and klf6a were enriched in gata1 positive cells isolated from gata1:dsRed transgenic line (Fig. S2C), supporting their potential roles in primitive erythroid cells.

**Knockdown of klf3 affects the maturation, but not the initiation, of primitive erythrocytes**

To explore whether klf3 is required for primitive hematopoiesis,
Fig. 2. The expression of klf3 and klf6a in cloche, etsrp, and moonshine mutants. (A) The expression of klf3 was lost in cloche and moonshine mutants, whereas no change was observed in etsrp mutants at 26 hpf. The arrowheads mark red blood cells in the ICM. (B) klf6a expression in cloche and etsrp mutants was greatly decreased, but only slightly reduced in moonshine mutants at 30 hpf. The arrowheads mark klf6a expression in the posterior ICM region. Anterior is shown to the left, with dorsal to the top.

Fig. 3. Maturation of erythrocytes was blocked in klf3 morphants. (A) The expressions of gata1 and be1-globin did not change in klf3 morphants at 24 hpf. However, the expressions of be1-globin and be3-globin were remarkably decreased during the differentiation and maturation of blood red cells at 36 hpf. The arrowheads mark RBCs in CHT. (B) O-dianisidine staining of hemoglobin during erythropoiesis. At 36 hpf and 2 dpf, the staining in klf3 morphants was significantly reduced, indicating that the maturation of RBCs was blocked. The RBCs in the heart region are indicated by arrowheads. (C) Blood-smear and Giemsa staining. At 2.5 dpf, the size and nucleo-cytoplasmic ratio of RBCs from klf3 morphants were increased compared with those of controls, indicating the arrest of erythroid cell maturation. The arrowhead marks the delayed erythroid progenitor. (D) Quantification of the nucleo-cytoplasmic ratio between controls and klf3 morphants (mean ± SD, ***P < 0.001, n = 15). The nucleo-cytoplasmic ratio was calculated as follows: the diameter of nucleus (D₁) and whole cell (D₂) were measured by Photoshop, and the nucleo-cytoplasmic ratio (NCR) was calculated using formula NCR = 4π(D₁)² / 4π(D₂)² - 1. Each sample contains 15 cells in controls and morphants.
we used an antisense utr-MO (MO) to knock down endogenous klf3 expression in zebrafish. To verify the MO efficiency, a KLF3–GFP fusion construct was generated in which a partial sequence of the klf3 gene containing the utr-MO recognition site was fused with GFP in frame, and this construct was co-injected with klf3MO or klf3-utr-misMO (misMO). The results showed that GFP expression was markedly abolished by klf3MO injection but not by klf3-misMO, indicating the high efficiency of this utr-MO (Fig. S3A). We next examined the efficiency of primitive hematopoietic markers in klf3 morphants with WISH. At 24 hpf, the expression of erythroid markers gata1 and bet1-globin was normal (Fig. 3A), and the expression of the vascular endothelial marker flkl, the early myeloid marker pui.1 and the early hematopoietic marker scl did not change compared with the control embryos (Fig. S3B). The erythromyeloid progenitor markers lmo2, gata1 and l-plastin seemed normal at 30 hpf in klf3 morphants (Fig. S3C). These data demonstrated that klf3 was not involved in the initiation of primitive hematopoiesis. However, at later stages, the expression of bet1-globin and be3-globin was decreased at 36 hpf (Fig. 3A). The expression of mature erythroid cells markers, such as act1-globin, transferrin (tf) and transferrin receptor (tfr), was downregulated at 2 dpf (Fig. S3D) in klf3 morphants, indicating that the differentiation of erythroid cells was affected. To verify this effect, we performed o-dianisidine staining to label mature red blood cells (RBCs). O-dianisidine staining revealed that the number of RBCs in klf3 morphants was dramatically reduced at 36 hpf and 2 dpf (Fig. 3B), whereas no obvious changes were observed when the same dose of klf3-misMO was injected (Fig. S3A lower panels), indicating that the differentiation of primitive erythrocytes was affected by knockdown of klf3. To further demonstrate that the phenotype was specific to klf3 knockdown, we injected klf3 mRNA (with sequence that does not overlap with MO) into klf3 morphants. The result showed that the expression of bet1-globin was rescued (Fig. S3E and F). To further support the observed erythroid maturation defect, RBCs were collected from 2.5 dpf embryos and subjected to Wright–Giemsa staining, which defines the different developmental stages of the erythroid lineage, ranging from erythroblasts to erythrocytes, in zebrafish (Qian et al., 2007). Compared with the control, the nucleo-cytoplasmic ratio of RBCs was increased in klf3 morphants (Fig. 3C and D), suggesting that the maturation of RBCs was blocked at the early stages of erythropoiesis. To exclude the possibility that the reduced RBCs were due to apoptosis, we performed TUNEL assay. As expected, there was no obvious difference in klf3 morphants compared to controls (Fig. S3G). Taking these results together, we conclude that klf3 plays a critical role in erythroid maturation during primitive hematopoiesis.

**Frrs1b is directly regulated by klf3 during erythropoiesis in vivo**

To gain insight into the mechanism by which klf3 regulates the maturation of erythrocytes, we took advantage of a bioinformatics approach to analyze databases of published literature (Funnell et al., 2012; Tallack et al., 2010; Turchinovich et al., 2011). Because KLF3 acts as a transcriptional repressor, we would expect upregulated expression of its direct target genes when KLF3 was depleted. After screening, our analysis generated a list of 17 genes that were highly expressed in the developing erythrocytes of klf3−/− mutant mice (Funnell et al., 2012). We subsequently evaluated the expression of these 17 genes (20 genes in the zebrafish genome due to gene duplication) in zebrafish klf3 morphants at 24 and 36 hpf by WISH, and found that the expression of 5 genes (frrs1b, glipr2, klf8, capg, and bag1) was significantly increased in klf3 morphants compared with controls (Fig. 4A upper panels; data not shown). qPCR analysis was also consistent with the WISH results (Fig. S4A). Among these genes, the expression pattern of frrs1b was similar to that of klf3, as double fluorescence in situ hybridization demonstrated that frrs1b co-localized with klf3 in the ICM region at 24 hpf (Fig. S4B). In addition, the expression of frrs1b was reduced when klf3 mRNA was injected (Fig. 4A middle panels), and qPCR analysis was also consistent with the WISH result (Fig. 4B). Hence, we propose that frrs1b might be a direct downstream target of KLF3. To determine whether upregulation of frrs1b expression is responsible for the klf3 knockdown phenotype, frrs1b mRNA was synthesized and injected into 1-cell stage wildtype embryos. Strikingly, as shown in Fig. 4A lower panels, the expression of bet1-globin was remarkably attenuated, which was consistent with the phenotype in klf3 morphants, and qPCR analysis confirmed the WISH result (Fig. 4B). To determine whether the upregulation of frrs1b was responsible for the RBC maturation defects observed in klf3 morphants, we employed a double-knockdown approach. Importantly, double knockdown achieved by co-injecting klf3MO and frrs1bMO partially rescued the expression of bet1-globin at 36 hpf and 2 dpf (Fig. S4C and D). Moreover, the intensity of o-dianisidine staining was rescued by double knockdown of klf3 and frrs1b at 2 dpf (Fig. 4C), suggesting that frrs1b is required for klf3-regulated RBC maturation. To test whether KLF3 directly regulates frrs1b expression by binding to its promoter, ChIP analysis was performed. We analyzed the frrs1b upstream regulatory region and found a conserved KLF binding motif (CACCC) in this region (Fig. 4D and E). ChIP-PCR showed that frrs1b was specifically enriched in the KLF3-myc Ab pulldown compared with that of IgG (Fig. 4F). We then cloned the frrs1b upstream regulatory region containing the conserved KLF binding motif (CACCC) and performed a reporter assay. Western blots showed that KLF3 was expressed in vitro after the transfection of pcDNA-FLAG-KIF3 into HEK293T cells (Fig. 4G). Subsequent reporter assay showed that luciferase activity was strongly repressed in a dose-dependent manner when pcDNA-FLAG-KIF3 was co-transfected with the PC3-frrs1b promoter but not when it was co-expressed with a mutant promoter (Fig. 4H and I). Together, we conclude that frrs1b is a direct downstream target that is repressed by KLF3 during erythropoiesis in vivo.

**Differentiation and maturation of erythroid progenitors are blocked in klf6a morphants**

Previous studies reported that klf6−/− mice exhibited embryonic anemia and early lethality (Matsumoto et al., 2006). To determine the function of klf6a during blood cell generation and vasculogenesis in zebrafish, we used klf6a-atg MO to specifically knock down klf6a,
which was verified by Western blotting (Fig. S5A). WISH analysis indicated that there were no detectable differences in the expression of \( \text{flk1} \) and early hematopoietic markers (\( \text{scl}, \text{pu.1} \) and \( \text{gata1} \)) at 24 hpf between control embryos and \( \text{klf6a} \) morphants (Fig. 5A upper panels; Fig. S3B). The erythromyeloid progenitor (EMP) markers were not changed at 30 hpf in \( \text{klf6a} \) morphants (Fig. S3C). We also performed microscopy to observe blood circulation and found that it was normal in \( \text{klf6a} \) morphants (Movie S1). However, \( \text{be1-globin} \) and \( \text{be3-globin} \) expressions were decreased in \( \text{klf6a} \) morphants at 36 hpf and 2 dpf (Fig. 5A middle and lower panels). Moreover, o-dianisidine staining analysis showed that the morphants possessed fewer mature erythrocytes at 40 hpf and 2 dpf. The real time PCR results displayed that \( \text{ae1-globin} \) and \( \text{be1-globin} \) were downregulated in \( \text{klf6a} \) morphants at 36 hpf and 2 dpf (mean ± SD, *\( P < 0.05 \), **\( P < 0.01 \), \( n = 3 \)). (D) Giemsa staining of erythroid cells from the blood of controls and \( \text{klf6a} \) morphants were noticeably larger than those of controls. The arrowhead marks the delayed erythroid progenitor. (E) Quantification of the nucleo-cytoplasmic ratio between controls and \( \text{klf6a} \) morphants (mean ± SD, **\( P < 0.01 \), \( n = 19 \)).

Compared with control embryos, there were more naïve erythroid progenitors with larger nuclei (erythroblasts) in \( \text{klf6a} \) morphants (Fig. 5 D and E). To determine the phenotype induced by \( \text{klf6a} \) morpholino was specific, we co-injected \( \text{klf6a} \) morpholino and \( \text{klf6a} \) mRNA modified to avoid binding by \( \text{klf6a} \) MO. qPCR and o-dianisidine staining results showed that \( \text{be1-globin} \) expression was rescued by co-injection (Fig. S5C and D). TUNEL assay also showed that there was no obvious difference in \( \text{klf6a} \) morphants compared to control embryos (Fig. S3G). Together, these data demonstrate that primitive erythroid differentiation and maturation are arrested in \( \text{klf6a} \) morphants.

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2015.05.015.

**Figure 5.** \( \text{klf6a} \) knockdown induces a blockage of erythroid cell differentiation and maturation. (A) WISH results showed that \( \text{gata1} \) expression was comparable between controls and \( \text{klf6a} \) morphants from 24 hpf. The expression of \( \text{be1-globin} \) and \( \text{be3-globin} \) was decreased in \( \text{klf6a} \) morphants, compared with controls at 36 hpf and 2 dpf. (B) The number of o-dianisidine-positive cells (marked by arrowheads) decreased dramatically in \( \text{klf6a} \) morphants between 40 hpf and 2 dpf. (C) The real time PCR results displayed that \( \text{ae1-globin} \) and \( \text{be1-globin} \) were downregulated in \( \text{klf6a} \) morphants at 36 hpf and 2 dpf (mean ± SD, *\( P < 0.05 \), **\( P < 0.01 \), \( n = 3 \)). (D) Giemsa staining of erythroid cells from the blood of controls and \( \text{klf6a} \) morphants at 2.5 dpf. The size and nucleo-cytoplasmic ratio of red blood cells in \( \text{klf6a} \) morphants were noticeably larger than those of controls. The arrowhead marks the delayed erythroid progenitor. (E) Quantification of the nucleo-cytoplasmic ratio between controls and \( \text{klf6a} \) morphants (mean ± SD, **\( P < 0.01 \), \( n = 19 \)).
Fig. 6. Cdkn1a acts downstream of klf6a to negatively regulate erythroid cell maturation by inducing cell cycle arrest. (A) Scheme of cell sorting by FACS. The gata1:dsRed-positive cells were sorted from the transgenic line flk1:GFP/gata1:dsRed at 2 dpf. (B) qPCR showed cdkn1a upregulation in the sorted cell population of klf6a morphants (mean ± SD, t-test, *P < 0.05, n = 3). (C) qPCR analysis showed that cdkn1a was decreased in klf6a mRNA-injected embryos at 36 hpf (mean ± SD, t-test, **P < 0.01, *P < 0.05, n = 3). (D) O-dianisidine staining showed that the number of mature RBCs was reduced in klf6a morphants and cdkn1a mRNA-injected embryos, and the defect in klf6a morphants was rescued by co-injection of cdkn1aMO. The arrowheads mark o-dianisidine-positive cells. (E) Analysis of Klf6a binding to the cdkn1a promoter by ChIP assay. The schematic of the cdkn1a promoter structure that was used in the reporter and ChIP assays. (F) ChIP-PCR. cdkn1a-NF/NR were negative control primers that were used to amplify the cdkn1a promoter region without the conserved Klf6a binding site. The cdkn1a-1F/1R primers were used to amplify the promoter region containing the conserved Klf6a binding site. (G) Reporter assay. Western blot analysis of overexpression of zebrafish Klf6a in HEK293T cells. (H) HEK293T cells were co-transfected with the promoter construct of cdkn1a containing the conserved Klf6 binding sites (GGCCGG) or mutated sites (TTTAAA) and the pCDNA-Klf6a plasmid. (I) BrdU and pH3 staining indicated the gata1:dsRed positive cells in S and M phases in CHT region of 2 dpf Tg(gata1:dsRed) embryos. The yellow arrowheads mark the erythrocytes in S/M phase. (J) The statistic graph showed the percentages of BrdU “gata1:dsRed” and pH3 “gata1:dsRed” in the CHT region (mean ± SD, t-test, *P < 0.05, n = 5).
erythropoiesis by WISH that were predicted to be targets of Klf6 in mice by bioinformatics. There was no obvious difference in the expression of bcl2-L10, mcl1b, and NF-kB8A-b between control and klf6a morphants (Fig. S5E). Among the predicted target genes, we only detected a dramatic upregulation of cdkn1a expression in klf6a morphants, especially at 25 and 36 hpf. To understand the mechanism underlying the erythroid maturation defects in klf6a morphants, we sorted gata1:dsRed positive cells from flk1:GFP/gata1:dsRed transgenic embryos by flow cytometry (Fig. 6A). Our real-time PCR analysis showed that cdkn1a expression was upregulated in sorted gata1:dsRed positive erythroid lineage cells in klf6a morphants (Fig. 6B) but decreased in embryos overexpressing klf6a (Fig. 6C). These results indicated that cdkn1a was a downstream target gene of klf6a in zebrafish. Furthermore, overexpression of cdkn1a mimicked the phenotype of klf6a morphants, and co-injecting cdkn1AMO with klf6AMO rescued the reduced o-dianisidine staining (Fig. 6D), and be1-globin expression level which was detected by WISH (Fig. S6A) and qPCR (Fig. S6B). To determine whether Klf6a directly regulates cdkn1a expression, we analyzed the cdkn1a upstream regulatory region and found a conserved Klf6a binding motif (GC-box) in this region (Fig. 6E). ChIP assays using 36 hpf zebrafish embryos demonstrated that Klf6a bound to the promoter of cdkn1a in vivo (Fig. 6F). For reporter assay, we generated a promoter construct of cdkn1a that contained the conserved Klf6a binding sites or mutated binding sites (Fig. 6G). Klf6a protein overexpression in vitro was observed by Western blot only in HEK293T cells that were transfected with pCDNA-Klf6a (Fig. 6G). We found that Klf6a repressed cdkn1a promoter activity in a dose-dependent manner but did not affect promoter activity when the Klf6a binding sites were mutated (Fig. 6H). cdkn1a (also called p21) encodes a cyclin-dependent kinase inhibitor that plays an important role in regulating the cell cycle. Increased expression of cdkn1a induced cell cycle arrest at S and G2/M phases (Wu et al., 2013). To further determine the cell cycle status, we performed a BrdU assay to monitor the cycling cells in S phase in the Tg (GATA1:dsRed) line. In morphant embryos, there were more gata1:dsRed-positive cells labeled with BrdU (Fig. 6I) and the percentage of BrdU⁺gata1:dsRed⁺ cells in klf6a morphants increased to 27.8% compared with 20.4% in control embryos (Fig. 6J). We then performed anti-phospho-histone H3 (pH3) antibody staining to estimate the mitotic number of cells and the percentage of gata1:dsRed cells labeled by pH3 staining was approximately 6% in control embryos, but the ratio in the klf6a morphants was decreased to 4% (Fig. 6I and J). The opposite pattern occurred in S and M phases in klf6a morphants, indicating that there was S phase arrest but not active cell proliferation. Taken together, these results indicate that Klf6a directly represses cdkn1a expression to regulate the normal cell cycle during erythroid progenitor maturation.

cdkn1a is also tightly regulated by p53 signaling, and nonspecific MO toxicity often causes p53-dependent cell death in MO-injected embryos (Robu et al., 2007). To further demonstrate that the increased expression of cdkn1a in klf6a morphants was specific to klf6a knockdown and not attributed to nonspecific MO injection effects, we injected klf6aMO into p53 mutant zebrafish embryos. The decrease in the number of red blood cells was still observed in the injected p53 mutant, albeit to a lesser extent (Fig. S6C), p53 is known to be an upstream regulator of cdkn1a; therefore, it is not surprising that p53 mutation or knock down can alleviate the phenotype caused by increased cdkn1a expression in klf6a morphants. qPCR results showed that be1-globin expression was reduced and cdkn1a was still upregulated in klf6aMO and p53MO co-injected embryos, compared to klf6a single knockdown embryos (Fig. S6D), suggesting that cdkn1a might be separately regulated by klf6a and p53. Our results and previous studies have indicated that as a transcription factor, klf6 directly binds to cdkn1a (Navra et al., 2001) and that loss of klf6 also directly results in p53 up-regulation (Sirach et al., 2007). It is possible that negative regulation of cdkn1a by Klf6a involves two pathways: a p53-dependent and a p53-independent pathway.

Double knockdown of klf3 and klf6a causes more severe erythroid maturation defects in zebrafish

To explore the potential functional interaction between klf3 and klf6a, we performed a double knockdown of klf3 and klf6a (Fig. S7). In double-knockdown morphants, the number of mature RBCs was more severely attenuated compared with single knockdown of either klf3 or klf6a (Fig. S7A). Simultaneously, the expression of be1-globin was also remarkably reduced (Fig. S7B), whereas the population of gata1 positive erythroid progenitors increased accordingly (Fig. S7C). Considering the redundancy of the Klf family, the expression of Klf family members involved in hematopoiesis was determined by WISH. Interestingly, the expression of klf3 in the ICM was not affected in klf6a morphants and vice versa (Fig. S8), indicating that their expressions were not reciprocally controlled and that there was no compensatory effect for loss of either gene. Similarly, the other klf genes, including klf4, klf2a and klf17, were not affected by knockdown of klf3 or klf6a in zebrafish (Fig. S8). Taken together, these data suggest a cooperative effect of these two members of the Klf family (klf3 and klf6a) in regulating erythropoiesis.

Discussion

Several Klf family members have been shown to be involved in hematopoiesis in mammals. Nonetheless, our understanding of the roles of the entire Klf family in hematopoiesis is far from complete. To comprehensively explore the Klf family, we performed bioinformatics analysis using zebrafish as a model and identified a total of 24 klf genes in the zebrafish genome. Gene expression profiling showed that 6 genes (klf6d, klf2a, klf3, klf6a, klf8, and klf17) were expressed in hematopoietic and/or endothelial cells during embryogenesis. Among these genes, the roles of klf3 and klf6a in zebrafish hematopoiesis have not been reported to our knowledge. Through functional and mechanistic studies, we demonstrated that both klf3 and klf6a are important for erythroid cell maturation but that they act in distinct manners. Our findings provide novel insights into the molecular mechanisms of klf gene action during vertebrate embryogenesis, particularly during erythropoiesis.

In mammals, there are at least 17 klf genes (Nagai et al., 2009), whereas our bioinformatics analysis identified a total of 24 klf genes in zebrafish. Our phylogenetic tree analysis showed that the zebrafish genome contains 7 pairs of duplicated klf genes, including klf2a and klf2b, klf5a and klf5b, klf6a and klf6b, klf7a and klf7b, klf11a and klf11b, klf12a and klf12b, and klf15 and klf15-like. The extra copies of the zebrafish klf genes appear to arise from a whole-genome duplication during evolution of the teleost (Postlethwait et al., 1998). Interestingly, the duplicated zebrafish klf genes often have distinct expression patterns. For example, klf2a is expressed in blood and endothelial cells, whereas klf2b is ubiquitously expressed at a very low level. Similarly, klf6a is specifically expressed in dorsal ganglia and caudal vascular tissue, whereas klf6b is expressed in the whole embryo. These observations indicate that after genome-wide duplication during evolution, the zebrafish-specific klf genes may have acquired distinct expression patterns and functions to fulfill the complex requirements of the organism.

Klf3, which is known as bklf in mammals, is a master regulator during erythropoiesis. Previous studies in mice have shown that...
klf1 can drive klf3 expression to repress non-erythroid genes during erythrocyte maturation (Eaton et al., 2008; Funnell et al., 2007, 2012). Expression of several downstream target genes including klf8, igals3, and fam132a was significantly increased in klf3 KO mice. Nonetheless, the molecular pathways downstream of Klf3 remained largely unclear. In our study, we identified a novel gene, frrs1b, which is a downstream target gene of Klf3. Ferric-chelate reductase 1 (Frrs1), which is also known as stromal cell-derived receptor 2 (SDR2), is a member of the cytochrome b561 family. Members of this family reduce ferric iron to ferrous iron before transport from the endosome to the cytoplasm (Vargas et al., 2003). frrs1 is highly expressed in the mouse liver, whereas in the hpx mouse (which exhibits an increased absorption of iron), the mRNA level of frrs1 was dramatically reduced. As a member of cytochrome family, frrs1 might be involved in mitochondrion iron metabolism. Mitochondrion is an important site of heme synthesis and plays a critical role in iron metabolism (Richardson et al., 2010). Mitochondrion ferritin (Fmtt), which is located on the mitochondrion membrane, regulates the iron balance between cytoplasm and mitochondrion. WhenFmtt was overexpressed, the level of cytosolic iron was reduced, leading to iron-overload in the mitochondrion (Nie et al., 2005). The similar phenomenon was also shown in Friedreich’s ataxia patients, in which (frataxin-deficiency) the heme synthesis was decreased due to iron overload in the mitochondrion (Li et al., 2008). In our study, the reduced expression of beta-globin in klf3 morphants was possibly due to aberrant expression of frrs1b, which in turn led to abnormal iron metabolism between cytosol and mitochondrion. This was consistent with that the cellular iron homeostasis is important during erythropoiesis (Ponka, 1997). Future work is still needed to illustrate the detailed mechanisms by which frrs1 regulates embryonic hemoglobin maturation of erythroid cells in vertebrates.

Although klf6 was shown to be a regulator of early hematopoietic development in mice (Matsumoto et al., 2006), whether or how klf6a affects hematopoietic lineages in other vertebrates has not been completely determined. Our study demonstrates that klf6a is required for erythropoiesis and that klf6a knockdown leads to erythroid cell maturation defects by up-regulating cdkn1a, which induces cell cycle arrest. Several studies in mice showed that cell cycle arrest by p21 up-regulation is required for terminal erythropoiesis, i.e. from erythroblasts to reticulocytes, then to mature red blood cells (Siatecka et al., 2010; Tallick et al., 2009, 2007). However, during zebrafish erythropoiesis there is no process of enucleation and the mature erythrocytes are still nucleated, so it is possible that the cell cycle arrest might not be necessary for terminal erythroid maturation in zebrafish. Indeed, cell cycle progression is essential for the differentiation of early erythroid precursor/erythroblast in mice (Marinkovic et al., 2007; Pilon et al., 2008), which is consistent with our observation here in that klf6a-mediated p21 downregulation is required for erythroid maturation in zebrafish. Then the decreased number of functional erythrocytes and increased number of erythroid progenitors caused by the blockage of erythroid cell differentiation were strongly associated with a risk of erythroleukemia or anemia. Therefore, klf6a might act as a candidate target gene in the pathogenesis or for the targeted therapy of erythroleukemia or anemia. It had been reported that Klf6 activates cdkn1a to repress tumors in adult tissues such as the prostate in human (Narla et al., 2001). However, in our study, klf6a directly repressed cdkn1a, which appears to be inconsistent with previous results (Narla et al., 2001). We hypothesized that the same gene/regulator might play different roles in different contexts. Various tumor suppressor genes have similar divergent properties between embryonic development and adulthood (Matsumoto et al., 2006). Consistently, knockdown of klf6a decreased hepatocyte proliferation by up-regulating cdkn1a in zebrafish (Zhao et al., 2010). Furthermore, it has been demonstrated that klf6 represses cdkn1a by a transcriptional co-repressor complex (Calderon et al., 2012). Because klf6a is known to be a transcriptional activator or repressor, it might play distinct roles depending on the developmental and tissue-specific contexts. Our studies demonstrate the function of klf6a in primitive erythropoiesis in zebrafish and the potential mechanism by which klf6a negatively regulates cdkn1a expression in erythrocytes.

The similarity between klf gene-deficiency phenotypes in zebrafish and erythroleukemia or anemia, i.e., the blocked maturation of erythroid progenitors, and the downstream target genes (frrs1b and cdkn1a) of klf3 and klf6a identified here might provide new strategies for leukemia diagnosis and treatment in the clinic.

Author contributions

F.L. conceived the project, analyzed the data, and wrote the paper. Y.X. and S.G. performed experiments, and contributed to the writing. All authors read and approved the final manuscript.

Conflict of interest

There are no conflicts of interests.

Acknowledgments

We thank our laboratory members for helpful discussion and critical reading of the manuscript. We thank Drs. Xiangdong Fang, Zhaojun Zhang and Qian Xiong for their support in bioinformatics. This work was supported by Grants from the National Basic Research Program of China (2010CB943502 and 2011CB943904), the National Natural Science Foundation of China (31271570 and 31425016), and the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA01010110).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.05.015.

References


dependent kinase inhibitor CDKN1A gene. J. Biol. Chem. 287, 8662–8674.


