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ORIGINAL ARTICLE

Gfi1.1 regulates hematopoietic lineage differentiation during zebrafish embryogenesis

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Growth factor independence 1 (GFI1) is important for maturation of mammalian lymphocytes and neutrophils and maintenance of adult hematopoietic stem cells (HSCs). The role of GFI1 in embryonic hematopoiesis is less well characterized. Through an enhancer trap screen and bioinformatics analysis, we identified a zebrafish homolog of *Gfi1* (named *gfi1.1*) and analyzed its function during embryonic development. Expression of both an endogenous *gfi1.1* gene and a *GFP* reporter gene inserted near its genomic locus was detected in hematopoietic cells of zebrafish embryos. Morpholino (MO) knockdown of *gfi1.1* reduced expression of *scl*, *lmo2*, *c-myb*, *mpo*, *rag1*, *gata1* and *hemoglobin alpha embryonic-1* (*hbae1*), as well as the total amount of embryonic hemoglobin, but increased expression of *pu.1* and *l-plastin*. Under the same conditions, MO injection did not affect the markers involved in vascular and pronephric development. Conversely, overexpression of *gfi1.1* via mRNA injection enhanced expression of *gata1* but inhibited expression of *pu.1*. These findings suggest that Gfi1.1 plays a critical role in regulating the balance of embryonic erythroid and myeloid lineage determination, and is also required for the differentiation of lymphocytes and granulocytes during zebrafish embryogenesis.

***Keywords*:** *gfi1.1*, hematopoiesis, zebrafish

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Introduction

Growth factor independence 1 (*Gfi1*) was initially identified in a retroviral insertional mutagenesis screen for tumor cell proliferation that was independent of the interleukin-2 growth factor [1]. GFI1 is widely conserved as its related proteins have been identified from many species including *Drosophila*, *Caenorhabditis elegans*, zebrafish (*Danio rerio*), mice and humans. The human GFI1 protein contains a domain consisting of six C-terminal C2H2 zinc fingers for DNA binding, a 20-amino-acid N-terminal SNAG domain for mediating transcriptional repressor activity and a conserved nuclear localization signal (NLS). These domains are highly conserved, although the middle region of GFI1 protein is more variable among different species [1-3]. Mouse *Gfi1* and human *GFI1* are expressed exclusively in hematopoietic stem cells (HSCs) and the immune system, and mouse GFI1 functions as a transcriptional regulator in lymphoid cells [1, 4]. Previous studies have shown that mouse *Gfi1* can act as an oncogene when overexpressed and can cooperate with other oncoproteins such as PIM-1 and MYC in promoting T-cell lymphomagenesis [5-8]. Mouse GFI1 also regulates IL-4/STAT6-dependent Th2 cell proliferation [9] and IL-6/STAT3-mediated cell proliferation in response to antigenic stimulation [9, 10]. Genetic studies have shown that mouse *Gfi1* is important for neutrophil maturation [11] and that mice lacking *Gfi1* are neutropenic owing to their failure to repress *Ela2* [12, 13]. In these mice, the differentiation of both neutrophils and T lymphocytes is blocked [14]. Recent analyses have established that GFI1 plays critical roles in regulating self-renewal and maintaining adult HSCs [15-17]. Mice deficient in *Gfi1* exhibit altered proliferation of HSCs in their bone marrow, which is probably due to abnormal cell cycle through deregulation of the interaction between

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GFI1B, a protein closely related to GFI1, and $p21^{Cipl/wafl}$ [15, 16]. These studies indicate that GFI1 plays multiple roles during hematopoiesis, including self-renewal, multilineage differentiation, and lineage commitment and terminal differentiation. Despite the progress that has been made by these studies, the role of GFI1 during embryonic hematopoiesis has not been well studied.

We seek to study Gfi1 function in embryogenesis using zebrafish as a model. Zebrafish is ideal for analyzing hematopoiesis owing to its rapid external embryonic development and transparency. Zebrafish hematopoiesis can be visualized in live embryos using transgenic fluorescent protein reporters and embryos can survive for several days without circulating blood cells [18]. Studies in zebrafish have established that many genes critical for mammalian hematopoiesis are conserved, and their tightly regulated spatial-temporal expression leads to the balanced differentiation of different blood cells during zebrafish development [19, 20]. However, it seems that *Gfi1* is not included as one of these genes even though a homolog of *Gfi1* has been identified in zebrafish [21]. The likely reason is that this gene is not the true zebrafish ortholog of mammalian *Gfi1* since its expression is not in hematopoietic cells but appears prominently in neural tissues. Recently we performed an independent large-scale enhancer trap screen based on the *Tol2* transposon in zebrafish with the aim of identifying tissue-specific genes and generating their transgenic fluorescent protein reporter lines. Through this screen we identified more than 30 lines of transgenic zebrafish that exhibit hematopoietic expression of a green or red fluorescent protein reporter gene (our unpublished data). Molecular analyses of these fish revealed that one of the enhancer trap lines carries an insertion near a new *Gfi1* homologous gene.

To avoid conflict in the nomenclature with the previously reported zebrafish *gfi1* gene, we designated this new *Gfi1* homolog as *gfi1.1*. In this study, we showed that zebrafish *gfi1.1* shares high sequence homology and a good genomic synteny with mouse *Gfi1* and human *GFI1*. The embryonic expression of *gfi1.1* is restricted to the hematopoietic tissues in zebrafish. More importantly, *gfi1.1* functions in promoting differentiation of the erythroid while repressing myeloid lineage determination; it is also required for the differentiation of lymphocytes and granulocytes during zebrafish embryogenesis. Our studies therefore established a critical developmental role for *gfi1.1* during hematopoiesis in a non-mammalian vertebrate species.

Results

Identification of zebrafish gfi1.1

Our enhancer trap screen generated approximately 1 700 transgenic zebrafish lines with *GFP* expressed in various organs, including more than 30 lines expressing GFP specifically in hematopoietic tissues (our unpublished data). Sequence analyses of insertion sites by linker-mediated PCR revealed that one of these lines trapped a gene designated as *NM_001020776.1* in GenBank or *zgc:113277* in Ensembl at approximately 20 kb upstream of its putative transcription start site. This gene encodes a protein of 385 amino-acid residues (~47 kDa) which shares significant homology with mouse Gfi1 and human GFI1. Since Dufourcq *et al.* previously already reported a different zebrafish gene as *gfi1* as being expressed in the ganglion cells of the neural retina and in developing hair cells of the ear [21], we named the gene revealed by our enhancer trap screen as *gfi1.1*.

Mammalian GFI1 proteins have an evolutionally conserved SNAG domain at the N-terminus and a cluster of six zinc-finger motifs at the C-terminus. Zebrafish Gfi1.1 protein also contains a SNAG domain with 95% similarity and a six zinc-finger domain with 98% similarity to those in humans (Figure 1A). Phylogenetic analysis using the conserved zinc-finger domain sequences also suggests that Gfi1.1 has a close relationship in evolution with the mammalian GFI1 proteins (Figure 1B). Zebrafish *gfi1.1* is located on chromosome 2, where its syntenic neighboring genes are also conserved in the chromosomal region around human *GFI1* on chromosome 1 and the chromosomal region around mouse *Gfi1* on chromosome 5 (Figure 1C). Interestingly, the previously reported zebrafish *gfi1* gene that is on chromosome 6 also shows similar syntenic conservation to its mammalian homologs (Supplementary information, Figure S1), which suggests that there is a duplication of this chromosome region in the zebrafish genome.

Expression of gfi1.1 and GFP in gfi1.1:GFP transgenic embryos

To detect the transcriptional activities of zebrafish *gfi1.1* during early embryonic development, we first analyzed *gfi1.1* mRNA levels in 0 to 5 days post fertilization (dpf) embryos using real time quantitative PCR (RT-qPCR). Initially, *gfi1.1* showed a high level of maternal expression, which gradually decreased to approximately 10% of the maternal level at 48 hpf (hours post fertilization) (Figure 2A). To further investigate the tissue-specific expression pattern of *gfi1.1*, RNA whole-mount *in situ* hybridization was performed. Consistent with the RT-PCR result, *gfi1.1* mRNA could be detected in the one-cell stage embryo and distributed evenly in the zygote until the late gastrula stage (Figure 2B). At approximately 10 hpf, *gfi1.1* expression was mainly restricted to two bilateral stripes flanking the paraxial mesoderm. Weak expression was also detected in the anterior lateral mesoderm (ALM) (Figure 2B). At 24 hpf, *gfi1.1* expression domains converged into the intermediate cell mass (ICM), the single midline hematopoietic region,

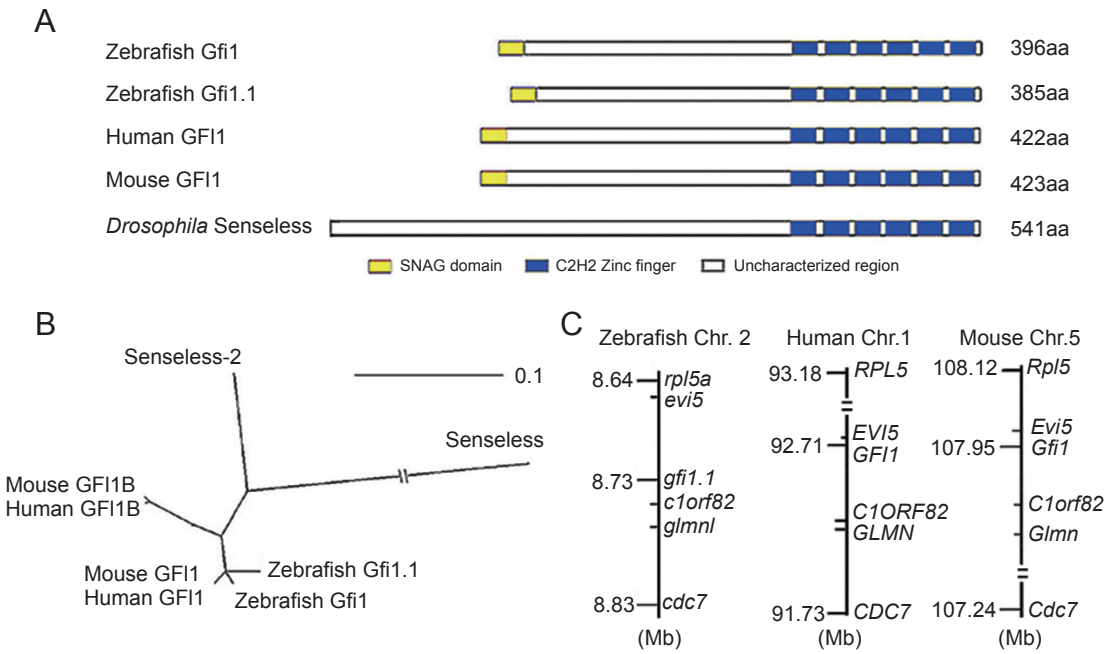

Figure 1 Identification of *gfi1.1* in zebrafish. **(A)** Protein structure comparison of Gfi1 homologs from zebrafish, human, mouse and *Drosophila* (Senseless). **(B)** Phylogenetic tree showing evolutionary correlations among some Gfi1 family members from zebrafish, human, mouse and *Drosophila* (Senseless and Senseless-2). **(C)** Syntenic arrangement around the *gfi1.1* locus on zebrafish chromosome 2 shows remarkable similarity to the locus of its human ortholog on chromosome 1 and its mouse ortholog on chromosome 5. The relative distance between genes (in Mb) is shown to scale. Mb, megabase.

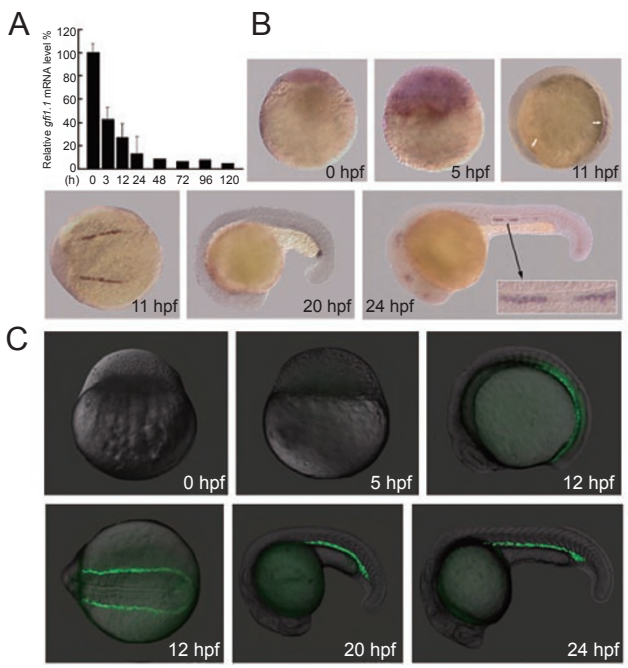

Figure 2 Expression of *gfi1.1* during zebrafish embryo development. **(A)** Quantitative real-time PCR analysis of *gfi1.1* mRNA at different stages of zebrafish embryo development. **(B)** Whole-mount *in situ* hybridization of the *gfi1.1* gene at the first 24 h of development. **(C)** Green fluorescent signal showing the *GFP* expression pattern in *gfi1.1:GFP* transgenic embryos.

and the ears (Figure 2B). Bilateral and ICM expression is characteristic of hematopoietic marker genes such as *gata1* and *lmo2*.

Compared with the endogenous expression of *gfi1.1*, the transgenic line *gfi1.1:GFP* lacks maternal expression of *GFP* (Figure 2C). At 12 hpf, GFP fluorescence was visible as two stripes that correspond to the regions of *gfi1.1* expression detected using RNA *in situ* hybridization. Later, at 24 hpf, GFP was similarly detected in the ICM and ears. The delay in appearance of the GFP fluorescence signal compared with *gfi1.1* mRNA might reflect the time needed to accumulate enough GFP protein. Nonetheless, *gfi1.1:GFP* faithfully recapitulated zygotic expression of the endogenous *gfi1.1* during embryonic development of zebrafish. In addition, the GFP pattern of *gfi1.1:GFP* is very similar to that of *gata1:GFP* transgenic zebrafish, a well-characterized line that expresses GFP in early hematopoietic cells.

Functional analysis of gfi1.1 in embryonic hematopoiesis

Mice deficient in *Gfi1* are viable for 2-3 months [14-16] and it is unclear whether *Gfi1* has any function in embryonic hematopoiesis during early development. Given that the expression of *gfi1.1* is high in blood cells during the embryonic development of zebrafish and that *Gfi1* has been

found to have multiple functions in mammalian hematopoiesis, we have focused on studying the function of *gfi1.1* in embryonic hematopoiesis using zebrafish as a model.

Antisense morpholinos (MOs) are effective in inhibiting gene function in zebrafish through blocking either protein translation or mRNA splicing [22]. In this study, two MOs to repress translation (MO1, which targets the ATG region, and MO2, which targets the 5′ UTR region) and one MO to block splicing (MO I1E2, which targets the junction between first intron and second exon) were used to knockdown *gfi1.1* expression. First, several assays were carried out to validate these MOs. To test whether MO1 and MO2 could block translation of *gfi1.1* mRNA, we performed Western blot analysis using a *Gfi1.1* antibody on protein extracts from embryos injected with either 4 ng MO1 or 8 ng MO2. The results showed that MO1, when injected at 4 ng per embryo, efficiently reduced the amount of Gfi1.1 protein to approximately 10% of that from uninjected controls (Supplementary information, Figure S2A, left panel), while MO2 at 8 ng per embryo led to a 60% reduction (Supplementary information, Figure S2B). Additionally, injection of MO1 or a mixture of MO1 or MO2 with 100 pg *gfi1.1*-N1 plasmid showed that translation of the *gfi1.1-GFP* fusion gene could be effectively blocked (supplementary information, Figure S2A, right panel). Finally, the efficacy of MO I1E2 was demonstrated by a significant reduction of *gfi1.1* mRNA in the injected embryos, as shown through RT-PCR (Supplementary information, Figure S2C).

Injection of the three MOs at a defined dose (4-8 ng for MO1, 8 ng for MO2, or 10 ng for MO I1E2) did not cause gross developmental abnormality in the embryos (embryonic survival rate: MO1>90%, MO2>50%, MO I1E2>90%) and all gave similar phenotypes. Given that MO1 was most efficient in repressing expression of the *gfi1.1-GFP* fusion gene, we used MO1 in most of our experiments.

In zebrafish, embryonic hematopoiesis begins at the 1- to 2-somite stage (about 10 hpf) and blood circulation becomes visible at about 24 hpf [19, 20, 23]. Many studies have shown that hematopoiesis in zebrafish is controlled by conserved transcriptional regulators, which leads to progressive blood cell differentiation [19, 20, 23]. These factors include Cdx4, which is important for the initial determination of hematopoietic progenitors from the posterior lateral mesoderm (PLM) [24]; Scl and Lmo2, which are critical for the specification of all blood lineages [25-27]; C-myb, which is important for the development of both primitive and definitive erythroid cells [28]; Gata1, which promotes erythroid cell development and suppresses myeloid cell differentiation in zebrafish [29]; and Pu.1, which promotes myeloid cell development [30, 31]. Since GFI1 was identified as a transcriptional regulator that modulates many genes such as those that are critical for blood cell differentiation in mammals, we first analyzed the effects of *gfi1.1* downregulation on the expression of the transcription factors that are critical for blood cell development.

Whole-mount *in situ* hybridization results showed that the injection of MO1 reduced the expression of *scl* (83%, *N*=149) and *lmo2* (81%, *N*=97). This reduction occurred primarily in the region around PLM that contains hematopoietic progenitor cells, and affected both the amount of expression and the size of the domain where the expression occurred (Figure 3A and 3B). In the ALM, the amount of *scl* and *lmo2* expression was also reduced but the size of the expression domain was not affected (Figure 3A and 3B). Furthermore, the expression of *c-myb* (83%, *N*=69) and *gata1* (97%, *N*=191) was also reduced, showing much weaker staining of the bilateral hematopoietic cells that flank the paraxial mesoderm (Figure 3C and 3D). The effect of *gfi1.1* MO1 on *gata1* expression was also demonstrated by analyzing live *gata1:GFP* transgenic zebrafish embryos (96%, *N*=213, Figure 3E). On the other hand, the expression of *pu.1* was increased (89%, *N*=78). The number of *pu.1* positive cells in *gfi1.1* morphants is about 3 times more than that from control embryos as calculated using Image-Pro Plus 5.1 software (Figure 3F). However, *gfi1.1* MO1 had no effect on *cdx4* expression (*N*=61, Figure 3G), which suggests a downstream role for *gfi1.1* in relation to *cdx4*.

The repressive effects of *gfi1.1* on the expression of *scl, lmo2, gata1, c-myb* and *pu.1* would probably lead to changes in the expression of their downstream genes and blood cell differentiation. We then investigated whether *gfi1.1* would affect lineage-specific marker gene expression at later developmental stages. These markers include *l-plastin* for macrophages of the embryonic myeloid lineage [32], *mpo* (*myeloperoxidase*) for granulocytes of the embryonic myeloid lineage [33], *ikaros* for lymphoid progenitors [34], hemoglobin alpha embryonic-1 (*hbae1*) for erythroid cells [35] and *rag1* for T lymphocytes [34]. Consistent with the effect that *gfi1.1* has on the expression of blood-specific transcription factors at about 12 hpf, injection of *gfi1.1* MO1 downregulated the expression of *hbae1* (85%, *N*=121, Figure 4A–4C), upregulated *l-plastin* (92%, *N*=153, Figure 4D), and did not affect *ikaros* (*N*=90, Figure 4E). Interestingly, decreased expression of *mpo* (90%, *N*=114, Figure 4F) and *rag1* (80%, *N*=91, Figure 4G) was also detected in *gfi1.1* MO embryos. Since zebrafish contains multiple embryonic globin genes and *hbae1* is just one of them, we further investigated the total amount of hemoglobin using *o*-dianisidine staining. As expected, the staining for hemoglobin was noticeably reduced in *gfi1.1* MO1-injected embryos at 84 hpf (94%, *N*=157, Figure 4H). To elucidate the specificity of *gfi1.1* function, we tested the expression of *etsrp, flk* and *fli1a* as markers for blood vessels and *pax2.1* as a marker for pronephric cells. *In situ* hybridization re-

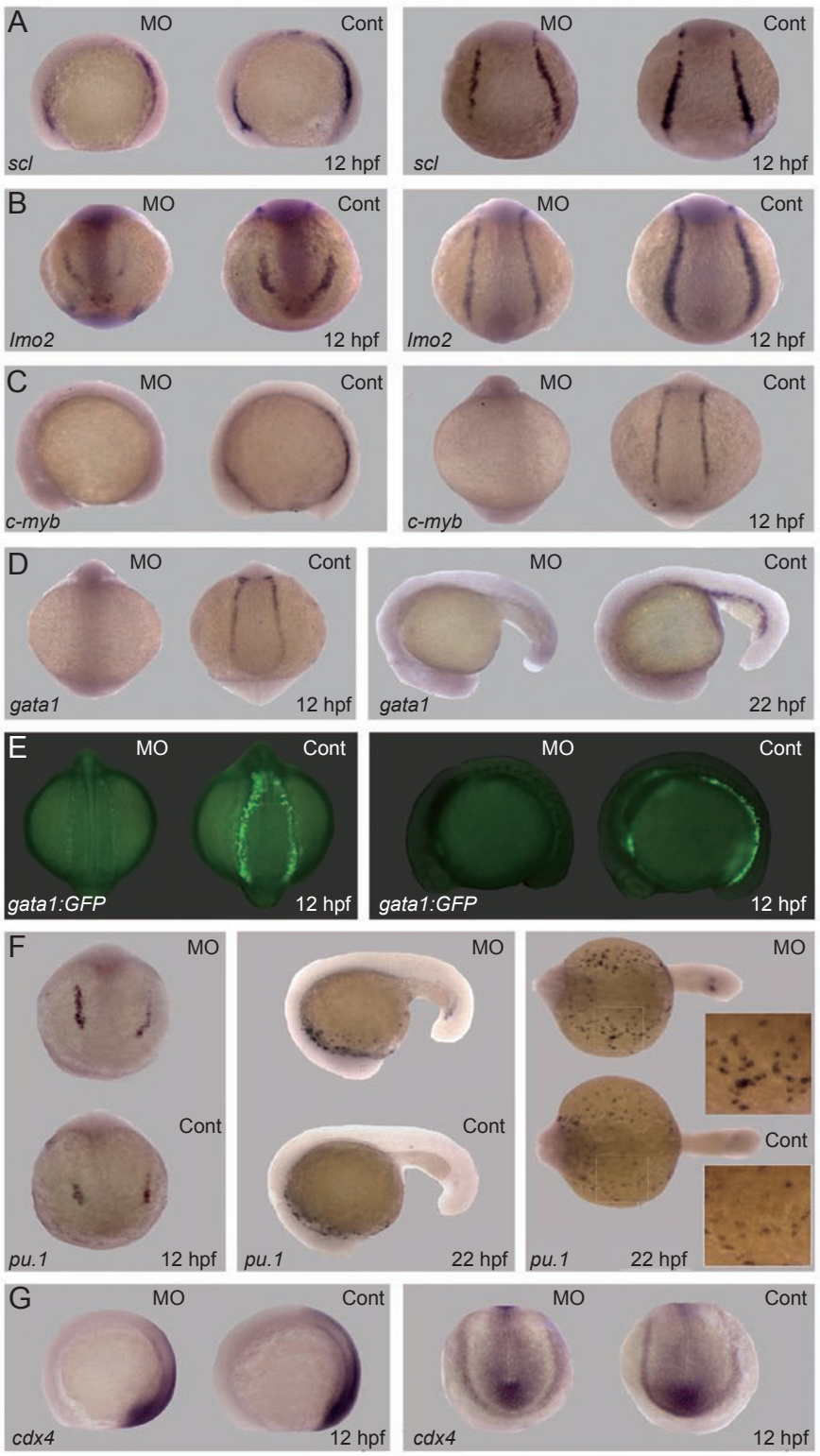

Figure 3 The effect of *gfi1.1* knockdown on embryonic hematopoiesis. **(A-F)** Whole-mount *in situ* hybridization of *scl* **(A)**, *lmo2* **(B)**, *c-myb* **(C)** and *gata1* **(D)** at the indicated developmental stages of zebrafish embryos injected with 4 ng MO1 (MO) or the corresponding uninjected controls (Cont). **(E)** The effect of *gfi1.1* MO1 (8 ng) on *GFP* expression in *gata1:GFP* transgenic fish. Whole-mount *in situ* hybridization of the *pu.1* **(F)** and *cdx4* **(G)** genes at the indicated developmental stages of zebrafish embryos injected with 4 ng MO1 (MO) or the corresponding uninjected controls (Cont).

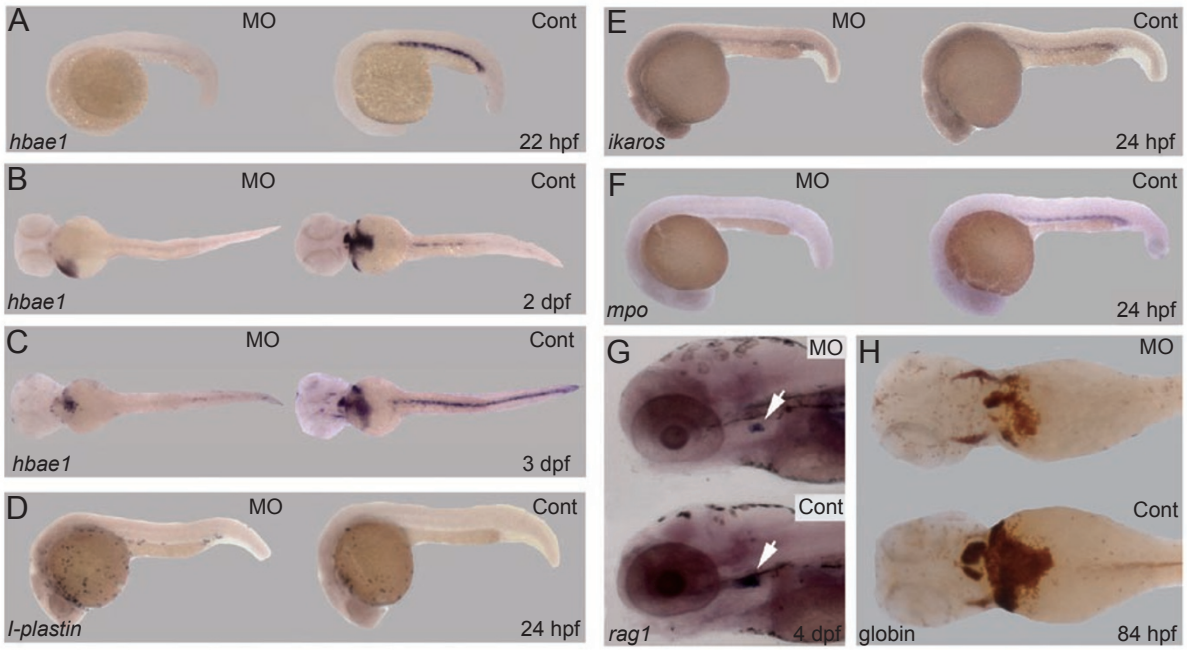

Figure 4 The effect of *gfi1.1* knockdown on the differentiation of blood cells. Whole-mount *in situ* hybridization of *hbae1* **(A-C)**, *l-plastin* **(D)**, *ikaros* **(E)**, *mpo* **(F)** and *rag1* **(G)** at the indicated developmental stages of zebrafish embryos injected with 4 ng MO1 (MO) or the corresponding uninjected controls (Cont). **(H)** Hemoglobin staining by *o*-dianisidine showing the erythrocytes that were affected by the application of *gfi1.1* MO1 (4 ng).

sults showed no apparent differences in expression of these markers between *gfi1.1* MO1-injected and control embryos (Supplementary information Figure S3), which suggests that *gfi1.1* is specifically required for hematopoiesis because it promotes embryonic erythroid differentiation and represses myeloid differentiation. To further confirm our observation, we overexpressed *gfi1.1* by injecting 20 pg of full-length mRNA per embryo; we detected increased *gata1* expression (84%, *N*=90, Figure 5A) but decreased *pu.1* expression (90%, *N*=69, Figure 5B).

Discussion

In this study, we have identified the zebrafish *gfi1.1* gene and have characterized its expression and biological function during embryonic hematopoiesis. Based on three lines of evidences, we believe that *gfi1.1* represents the zebrafish ortholog of mammalian *Gfi1*. First, zebrafish Gfi1.1 protein has a conserved SNAG and six zinc-finger domains that are 95% and 98% similar to human GFI1, respectively. Second, there is a high similarity in syntenic arrangement of genes around the zebrafish *gfi1.1* genomic locus compared with those around the human and mouse *Gfi1* loci, where they share several homologous neighboring genes. Third, *gfi1.1* is specifically expressed in hematopoietic tissues during embryonic development, which is consistent with the expression patterns seen in human and mouse *Gfi1*.

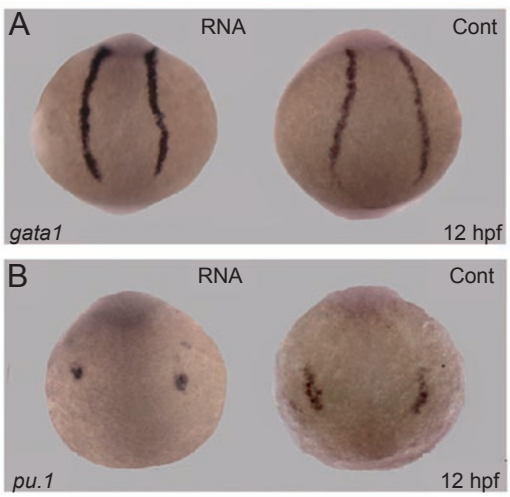

Figure 5 The effect of *gfi1.1* overexpression on *gata1* and *pu.1* expression. Whole-mount *in situ* hybridization of *gata1* **(A)** and *pu.1* **(B)** in the embryos injected with *gfi1.1* mRNA (RNA) or the uninjected controls (Cont).

Our further studies have demonstrated that zebrafish Gfi1.1 functions in promoting embryonic erythroid and repressing myeloid development. This finding is consistent with a recent report by Dahl *et al.* [36], which shows that mouse GFI1 antagonizes PU.1 activity during differentiation of hematopoietic progenitors. In zebrafish, we have

shown in this study that the expression of transcription factors that are important for the erythroid lineage, including *gata1*, was reduced by knockdown of *gfi1.1*. However, we observed partial recovery of circulating red blood cells after 3 days of development, which suggests that the reduction did not lead to a complete block of erythroid cell development. This might be due to the functional redundancy of *gfi* genes in zebrafish. The mammalian GFI protein family includes three members: GFI1, GFI1B and GFI2. Both GFI1 and GFI1B contain an N-terminal SNAG domain and a C-terminal six zinc-finger domain and are mostly involved in hematopoiesis. Zebrafish seems to have two *Gfi1*-like genes: *gfi1.1*, which is described in this study, and the previously reported *gfi1*, which is mainly expressed in the ganglion cells of the neural retina and in developing hair cells of the ear. In addition, we have identified partial sequences of yet another gene that is related to the *gfi1* family from the zebrafish genome and were able to detect its hematopoiesis-specific expression (unpublished data). It is possible that a more dramatic block of erythropoiesis may occur after a combined knockdown of all related hematopoiesis-specific *gfi1* family genes in zebrafish.

Despite the fact that *gfi1.1* expression is detected throughout various stages from the zygote to 5 dpf, and that Gfi1 regulates many spatiotemporally expressed transcription factors that are critical for blood cell development, we propose that this gene is required in multiple hematopoietic processes. Zygotic expression of *gfi1.1* suggests that it represents one of the earliest hematopoiesis-specific genes and starts earlier than or at the same time as most of these transcription factors. In *gfi1.1* knockdown embryos, expression of the genes that are involved in the lineage differentiation of embryonic red blood cells, such as *gata1*, and of myeloid cells, such as *pu.1*, were affected. This indicates that during the early stages *gfi1.1* functions upstream of *gata1* and *pu.1* but downstream of *cdx4*, as *cdx4* expression was not affected in *gfi1.1* MO-injected embryos. Interestingly, decreased expression of *mpo*, which is a marker for granulocytes that have differentiated from the embryonic myeloid lineage, was detected in *gfi1.1* morphants. This result is consistent with the findings that *Gfi1*-deficient mice appeared to be severely neutropenic and to have elevated immature monocytes in their blood and bone marrow [16], and suggests that *gfi1.1* is also required for the differentiation of myeloid progenitor cells into granulocytes. Furthermore, *gfi1.1* knockdown also resulted in impaired expression of *rag1*, a marker gene for T lymphocytes, in 4 dpf embryos, although the expression of *ikaros*, a marker for lymphoid progenitor cells, was not affected in 24 hpf embryos. We reasoned that *gfi1.1* is most probably involved in lymphocyte differentiation by acting downstream of *ikaros*. The effects of *gfi1.1* on hematopoiesis seem to be specific as marker genes that are expressed in the closely related vascular and pronephric cells were not affected.

Mouse GFI1 carries out most of its functions through its transcriptional regulation activity and interacts with different targets in different cells [37]. In this study, *gfi1.1* knockdown affected the expression of several transcription factors that are critical for hematopoiesis, as well as some markers for differentiated blood cells. Gfi1.1 may interact with these targets directly or indirectly. In *gfi1.1*-deficient embryos decreased *gata1* but increased *pu.1* transcription was detected, which is consistent with the notion that *pu.1* and *gata1* have antagonizing functions in the fate determination of hematopoietic cells between embryonic myeloid and erythroid cell lineages. This also suggests that Gfi1.1 is an upstream regulator of the process. Zebrafish *gata1* is likely one of the direct targets of gfi1.1 since the *gata1* promoter region contains several putative conserved binding sites for Gfi1 family transcription regulators as predicated using ModelInspector software (data not shown). However, impaired *gata1* expression in *gfi1.1* MO-injected embryos may also be an indirect effect from the upregulation of *pu.1*. Thus, it would be interesting to identify more direct targets, isolate Gfi1-interacting partners and determine how repression or activation is differentially achieved in different cells.

Materials and Methods

Zebrafish gfi1.1 gene identification and cDNA construction

Transgenic zebrafish *gfi1.1:GFP* was identified as a line that exhibits blood-specific expression of *GFP* from a large-scale enhancer trap screen (unpublished data) using a *Tol2* vector [38] containing a *gata2* minimal promoter linked to the *GFP* reporter gene [39]. Genomic sequences flanking this particular insertion were identified through linker-mediated PCR as described previously [40] and then mapped to specific zebrafish chromosomal loci using a blast search of the zebrafish Ensembl database (Jun 2007 Zv6). The *gata1:GFP* transgenic fish line used in this study has been described previously [41]. Zebrafish were maintained at 28.5 °C in a recirculating aquaculture system of the fish facility in Peking University.

For overexpression experiments, the full-length zebrafish *gfi1.1* coding sequence was cloned into the pCS2+ vector containing the Sp6 promoter by reverse transcription-PCR using the following primers:

ATG Sense: 5′-ATG CCG AGG TCA TTT TTG-3′; Antisense: 5′-TTA TTT TAG CCC GTG CTG T-3′.

Gfi1.1 mRNA was produced through *in vitro* transcription following the manufacturer's instructions (Ambion).

The full-length coding sequence of *gfi1.1* was cloned into the *EGFP*-N1 vector (Clontech) to generate a *gfi1.1*-N1 plasmid encoding the Gfi1.1-EGFP fusion protein for detecting the efficiency of *gfi1.1* MO.

Reverse transcription and quantitative real-time PCR of gfi1.1

Total RNA was extracted from 0-5 dpf embryos with TRIzol

(Invitrogen) according to the manufacturer's instructions. Reverse transcription of purified RNA was performed using oligo (dT) priming and Superscript III reverse transcriptase (Invitrogen). The quantification of gene transcripts was done by qPCR, using the Brilliant SYBR Green QPCR Master Mix and Light Cycler apparatus (Stratagene). The primer pairs used were:

gfi1.1: Sense: 5′-AGA AAG TCA AAC CGG ACA GG-3′; Antisense: 5′-AAA CGT ATG GAC GGA AGG AC-3′. *actin*: Sense: 5′-AGC AGA TGT GGA TCA GCA AG-3′; Antisense: 5′-AGT CAA TGC GCC ATA CAG AG-3′.

Microinjection of MO

Two ATG and one splicing MO against *gfi1.1* were ordered from Gene Tools, LLC. The MO sequences used were:

ATG MO1: 5′-GTA AAC ATG CCG AGG TCA TTT TTG G-3′; ATG MO2: 5′-GAG GCA ATA GTC GAA GTC CCA GTG G-3′; Splicing MO I1E2: 5′-GGT TAG ATC GGA CTT TTA CCG TGT T-3′. Control MO: 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′.

The oligos were dissolved in water as a 25 mg/ml stock solution and injected at different dosages (4-10 ng) into 1- or 2-cell-stage embryos. Injected embryos were grown at 28.5 °C and observed under a microscope.

RNA whole-mount in situ hybridization

RNA whole-mount *in situ* hybridization for *gfi1.1*, *scl*, *lmo2*, *gata1*, *pu.1*, *c-myb*, *hbae1*, *l-plastin*, *ikaros*, *mpo*, *rag1*, *etsrp*, *flk-1*, *fli1a* and *pax2.1* was performed as described previously [42]. The cDNA templates of these genes were amplified from total cDNA of 12 or 24 hpf embryos. Digoxigenin-UTP (Roche)-labeled antisense RNA probes were generated by *in vitro* transcription (Promega) and then purified (QIAGEN). All hybridization signals were detected via anti-digoxigenin-AP (Roche) and purple AP substrate (Promega).

Hemoglobin staining

Hemoglobin in zebrafish embryos was analyzed using *o*-dianisidine (Sigma) as described [35].

Gfi1.1 antibody generation and detection by western blot analysis

An antibody against zebrafish Gfi1.1 was raised by immunizing C57/B6 mice with purified Gfi1.1 protein expressed in *Escherichia coli*. Protein bands that were detected using the western blot technique were visualized by enhanced chemiluminescence. The infrared fluorescence image was obtained using the Odyssey infrared imaging system (Li-Cor Bioscience). The antibody against mouse Actin, which cross-reacts with zebrafish actin, was purchased from Sigma.

Image acquisition and analysis

For general examination, GFP-positive embryos or larvae were viewed under an Axioimager Z1 fluorescence microscope (Zeiss), equipped with 5×, 10× and 20× objectives, and a filter set for detection of GFP (excitation: 450-490 nm, barrier: 510 nm, emission: 515-565 nm). Images of *in situ* hybridization results were taken under a Zeiss Stemi 2000-C microscope or an Axioimager A1 microscope (Zeiss).

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(**Supplementary Information** is linked to the online version of the paper on the Cell Research website.)