Melk-Like Kinase Plays a Role in Hematopoiesis in the Zebra Fish

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A serine/threonine kinase, *Melk*, was initially cloned in mouse oocytes as a maternal gene, but whose function was unknown. In adult mice, *Melk* was strongly expressed in the thymus and bone marrow, suggesting a role for Melk in hematopoiesis. We cloned a *Melk*-like gene from zebra fish (*zMelk*). *zMelk*-like gene was expressed in the brain and lateral mesoderm at 12 hours postfertilization (hpf) and in several tissues of adult fish, including the kidney and spleen, both of which are known to be hematopoietic tissues in zebra fish. Abrogation of *zMelk*-like gene function by *zMelk*-like gene-specific Morpholino (MO) resulted in abnormal swelling around the tectum region. In addition, the start of blood circulation was severely delayed but, in contrast, the vessel formation seemed normal. Expression of *scl*, *gata-1*, and *lmo-2* was down regulated at 12 to 14 hpf in the *zMelk*-like gene MO-injected embryos, and the coexpression of gata-1 rescued the anemic phenotype induced by *zMelk*-like gene MO. Expression of the *zMelk*-like gene in embryos enhanced *gata-1* promoter-dependent enhanced green fluorescent protein expression, suggesting that the *zMelk*-like gene affects *gata-1* expression at the transcriptional level. Taken together, our data suggest that the *zMelk*-like gene may play a role in primitive hematopoiesis by affecting the expression of genes critical for hematopoiesis.

By using the PCR-based differential display technique on the eyes and brains of mice of various stages, we cloned a large number of mouse genes and screened them in terms of their primary sequences and expression patterns. The clone described in this work, DD29, is unique, since it encodes a protein kinase having a zinc finger domain. The full-length DD29 turned out to be identical with the previously reported gene *Melk* (19), which is a member of the sucrose nonfermenting protein kinase (SNF1)/AMPK serine/threonine kinase family (16). *Melk* was originally identified by differential display to isolate maternal cDNA from unfertilized eggs and preimplantation embryos (19). The expression pattern of Melk during the maturation of oocytes and preimplantation development was typical of that described for maternally expressed genes, including β-actin and E-cadherin (19). Therefore, Melk is surmised to play a role in transducing signals in embryonic cells during preimplantation, but its exact roles in development were not revealed (18). On the other hand, a *Melk* equivalent gene, *Eg3*, was cloned from a murine teratocarcinoma cell line (12), and predominant expression of *Melk* in hematopoietic organs such as the thymus and spleen and several hematopoietic cell lines was reported (12). However, the function of Melk in hematopoiesis has remained unknown. Induction of *Melk* transcription by T-cell activation was reported (11). So far, five members belonging to the SNF1/AMPK family of mammalian kinases have been reported. SNF1 was originally identified in *Saccharomyces cerevisiae* and is thought to play a role in the expression of glucose-repressible genes (4). Subsequent studies revealed that the mammalian members of this family also play

a role in protecting cells from environmental stress, hypoxia, heat shock, and ischemia (16, 34).

To examine the biological functions of Melk, we took the strategy of using the zebra fish (*Danio rerio*; *z*), for this fish is an excellent model vertebrate for the analysis of central nervous system development for many practical reasons, including the high similarity of its genetic and structural organization with that of other vertebrates, including mammals. In addition, another advantage of using the zebra fish is that this animal can be used in Morpholino (MO) antisense oligo-based knockdown experiments (17). In fact, in our present study, we found zebra fish embryos to develop severe anemia when they were injected with MO against the *zMelk*-like gene. The zebra fish is assumed to be a great tool for understanding normal and disease-related hematopoiesis. Hematopoiesis occurs in sequential waves in vertebrates including zebra fish (38), and striking similarities between zebra fish mutant (mt) phenotypes and human diseases have been reported (1). Furthermore, genes critical for normal mammalian hematopoiesis have been found, and they play a similar role in zebra fish.

In this work, we cloned the zebra fish *Melk*-like gene. *zMelk*-like gene was expressed in neural and other tissues, which was similar to that of the mouse *Melk* (*mMelk*) expression pattern. We did functional analysis of Melk by using the zebra fish. Unexpectedly, knockdown of the *zMelk*-like gene by MO oligonucleotides did not affect early embryogenesis, as previously surmised from the expression pattern of mammalian *Melk* (18). However, we found that disruption of the *zMelk*-like gene function did affect blood cell development.

MATERIALS AND METHODS

Animals. Zebra fish (*Danio rerio*) were purchased from a local pet shop and maintained under a 14-h day, 10-h night cycle at 28.5°C. Embryos were staged according to hours postfertilization (hpf) and morphological criteria (22).

Cloning, construction of DNA and RT-PCR assay. The *zMelk*-like gene was cloned by reverse transcription-PCR (RT-PCR) using primers designed based on the sequences of GenBank expressed sequence tag (EST) fragments (BM859334,

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AW165111), which are homologous to the *mMelk* sequence. Full-length *zMelk*-like gene (AB108827) was subcloned from pGEM-T easy vector into pCS2+ vector by using BamHI and EcoRI sites. A mutant *zMelk*-like gene having alternate codon sequences, *zMelk*-like 5′mt, was constructed by PCR mutagenesis. A total of 7 nucleotides were substituted as follows: in the original sequence, TTC AGT *ATG* CCA GTA GAC AGC ACT TCT; in the mutant sequence (mutated nucleotides are underlined), TTC AG<u>C</u> *ATG* CC<u>T</u> GT<u>C</u> GA<u>T</u> <u>TCT</u> ACT TCT. The initiation codon is expressed in italics. Then, the *mt zMelk*-like gene was subcloned into pCS2+ vector and subjected to RNA synthesis. *zMelk*-like genes having a point mutation at threonine 169 (T169A and T169E) were made by PCR mutagenesis. One or two nucleotides were substituted as follows: in the original sequence, ACA (threonine); in mutant sequences (mutated nucleotides are underlined), G<u>C</u>A (alanine, T169A) and G<u>AA</u> (glutamic acid, T169E). Then, the *zMelk*-like*T169A* and *zMelk*-like*T169E* genes were subcloned into pCS2+ vector and subjected to RNA synthesis. Semiquantitative RT-PCR to examine the expression pattern of the *zMelk*-like gene was done by using mRNA or total RNA extracted from various zebra fish tissues. The amplified transcripts, primer sequences (upstream and downstream), and the length of amplified fragments were as follows: zebra fish *Melk*-like gene, 5′-TAACAACG TAACCGATTGTAT-3′ and 5′-TACTGTACTACCTTCCTGCA-3′, and 2,130 bp. The conditions for PCR of zebra fish *Melk* were as follows: 94°C for 1 min, 56°C for 1 min, and 72°C for 3 min for 35 cycles. Full-length zebra fish *scl* and *lmo-2* for injection were cloned by RT-PCR according to the published sequence and subcloned into pCS2+ vector and subjected to RNA synthesis. Full-length zebra fish *gata-1* in pCS2+ vector and zebra fish *gata-1* promoter enhanced green fluorescent protein (EGFP) were provided by Kobayashi and Yamamoto (Tsukuba University) (23).

RH and genetic mapping. The genetic map position of the *zMelk*-like gene was determined by using the LN54 collection of the radiation hybrid (RH) panel (21) which was kindly provided by the Ottawa Health Research Institute. The *zMelk*-like gene was mapped onto this panel by PCR using the primers 5′-CCTCAAG CACTATGAGGTTTA-3′ (forward) and 5′-ATGTAGATTTTGCTTGTGGTC T-3′ (reverse).

Whole-mount in situ hybridization. Probes for the *scl*, *gata-1*, *myoD*, *sonic hedgehog* (*shh*), *fli-1*, *lmo-2*, *cdx-4*, *hoxb6b*, *c-myb*, *tie-1*, and *flk-1* genes were cloned by PCR amplification using the primers designed according to their published sequences. PCR products were subcloned into the pGEM-T easy vector, and digoxigenin (DIG)-labeled sense and antisense RNAs were made by using a DIG-RNA labeling kit (SP6/T7; Roche Diagnostics GmbH, Mannheim, Germany). Whole-mount in situ hybridization was done as described previously (24, 25). Briefly, embryos were fixed in 4% paraformaldehyde (PFA) and stored in methanol. Rehydrated embryos were incubated in 10 μg/ml of proteinase K for 5 min and refixed in 4% PFA for 20 min. Then they were treated with cold acetone for 8 min at −20°C, after which hybridization was done overnight. Embryos were blocked with 2% blocking reagent (Roche Diagnostics GmbH, Mannheim, Germany) consisting of 5% lamb serum, 0.1 M maleic acid (pH 7.5), and 150 mM NaCl and then incubated with anti-DIG-alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany). The enzyme activity was visualized with nitroblue tetrazolium 5-bromo-4-chloro-3-indolylphosphate (BCIP) AP substrate solution (Roche Diagnostics GmbH, Mannheim, Germany). Photographs were taken with a Zeiss Axio Cam attached to a Leica (Vienna, Austria) MZFL III dissecting microscope.

Microinjection of MO or RNA into zebra fish embryos. Two MO antisense oligonucleotides (GENE-TOOLS, LLC, Philomath, OR) were designed against 25 bases around the AUG translation initiation codon of the *zMelk*-like gene. The MO sequences were 5′-T<u>A</u>TGCCAGTAGACAGCACTTCTGAA-3′ (start codon is underlined) and 5′-CATCATTGGAGCACAGTTGGTGTAT-3′. The MO was diluted to 0.5 mM with 1× Danieau buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO$_4$, 0.6 mM Ca(NO$_3$)$_2$, 5.0 mM HEPES; pH 7.6], and 0.05% phenol red was included as a microinjection control. Fertilized eggs from wild-type zebra fish at the one-cell stage were injected with approximately 1 to 3 nl of DNA solution by using a microinjector (IM-300; Narishige, Tokyo, Japan). Standard control MO, which was available from the same manufacturer, was used as an injection control and had no effect on embryonic development under our experimental conditions. The microinjected embryos were examined under a Leica MZFL III microscope. For RNA injection, capped sense RNAs were synthesized by using an mMessage mMachine in vitro transcription kit (Ambion, Austin, TX) according to the manufacturer's instructions. The synthesized RNAs were diluted to an appropriate concentration with RNase-free water and injected into one- or two-cell-stage embryos.

Light microscopy. Embryos of appropriate stages were dechorionated and fixed overnight at 4°C in a solution containing 1% glutaraldehyde, 2% formal-

dehyde, and 0.1 M sodium phosphate (pH 7.4). The samples were washed in 4% sucrose and then fixed in 1% osmium tetroxide in the same buffer for 1 h on ice. They were then dehydrated in a graded series of ethanol and embedded in Epon 812 resin mixture (TAAB Laboratories Equipment Ltd., Berks, United Kingdom). Each block was cut into 0.7-μm-thick sections, which were then stained with toluidine blue and observed under a Zeiss axioplan microscope.

Immunohistochemistry. Embryos of appropriate stages in frozen OCT were sectioned at a 10-μm thickness and fixed with 4% PFA for 30 min at room temperature. Then the sections were blocked with 2% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature and incubated with histone H3 antibody (Molecular Probes) in blocking solution overnight at 4°C. Antibody was visualized by using a Vectastain Elite ABC mouse immunoglobulin G kit (Vector Laboratories, Inc.) and 3,3′-diaminobenzidine tetrahydrochloride (DAB).

Microangiography, hemoglobin staining, and heart rate. Microangiography was done as described previously (29). Fluorescein isothiocyanate (FITC)-dextran with a molecular mass of 2,000 kDa (Sigma, St. Louis, MO) was suspended in 1× Danieau buffer at 2 mg/ml. About 3 nl of the prepared solution was injected into the sinus venosa/cardinal vein of the anesthetized embryos at various stages (24, 32, 48, and 54 hpf). Photographs were taken with a MZFL III microscope using a standard FITC filter set. Hemoglobin staining was done as follows. Embryos were treated for 15 min in 40% ethanol containing 0.6 mg/ml *o*-dianisidine (ICN), 0.01 M sodium acetate (pH 4.5), and 0.65% hydrogen at room temperature in the dark. Then, the reaction was stopped and the embryos were dehydrated by being washed twice with 40% ethanol. The heart rates were counted upon visual inspection of unanesthetized embryos at room temperature (25°C).

RESULTS

Isolation of the zebra fish *Melk*-like gene with an expression pattern similar to that of *mMelk*. By differential display using mouse neural tissues, we cloned a serine/threonine kinase, *Melk*, as a gene expressed in mouse embryonic neural tissues. *mMelk* was originally cloned in oocytes as a maternal gene, but whose function was unknown (12, 19). To examine its functions using zebra fish (*Danio rerio*) as a model system, we then searched an adult zebra fish EST database (GenBank, National Center for Biotechnology Information) for sequences homologous with the *mMelk* sequence. Two EST sequences, BM859334 and AW165111, which had strong similarities with the respective N and C termini of *mMelk*, were found. A full-length zebra fish *Melk*-like clone (AB108827) was obtained from adult zebra fish ovary-derived mRNA by RT-PCR with primers designed based on these EST fragments. RH mapping assigned the *zMelk*-like gene to linkage group 1, and conserved syntenic genes were not found by comparative analysis with regions of *mMelk*, which maps to 4B1, and of human *Melk* (*hMelk*), which maps to 9p13.1. Thus, we could not conclude from this information whether the *zMelk*-like gene is a zebra fish orthologue of mammalian *Melk* or not.

The deduced amino acid sequence of the *zMelk*-like gene encoded 677 amino acids, and C-terminal and N-terminal regions had more than 70% homology at the nucleotide level with those regions of *mMelk* and *hMelk*. In addition, the putative *Xenopus* egg-specific protein kinase gene *Eg3* (31), which was identified by differential screening of genes expressed in the unfertilized eggs, also had significant homology with the *zMelk*-like gene in both C and N termini (Fig. 1A). Protein sequence alignment between the zMelk-like gene and mMelk showed conserved sequences of the N-terminal kinase domain and C-terminal region at the amino acid level (Fig. 1B). We could not find any significant homology with other genes or motifs in the central region of the *zMelk*-like gene by database searching. The N-terminal region (residues 13 to 268) con-

tained the putative catalytic domain of protein serine threo-nine kinases (13, 14). The catalytic domain consisted of 12 subdomains typical of serine/threonine kinase, and this region was highly conserved among mMelk, the zMelk-like gene, Eg3, and hMelk. A leucine zipper motif consisting of a periodic repetition of leucine every seventh residue was found within subdomain VIII of the catalytic domain of mMelk and hMelk, but only an incomplete leucine zipper was found in the *zMelk-like* gene and in Eg3 (19). In the mMelk and zMelk-like ki-nases, there was a conserved threonine residue (Thr-167 in mMelk and Thr-169 in the zMelk-like gene) within kinase subdomain VIII. These threonines seem to be equivalent to Thr-197 of protein kinase A, which is known to be an auto-phosphorylation site (15, 33), suggesting Thr-167 and Thr-169 to be potential autophosphorylation sites of mMelk and zMelk-like genes, respectively. In addition, the periodic repeat of Q (glutamine) every 8 or 9 amino acids was found in the C-terminal region in both the zMelk-like gene and mMelk.

To examine the tissue distribution of *zMelk*-like gene tran-scripts, we then performed RT-PCR using total RNAs isolated from various tissues of the adult zebra fish (Fig. 1C). The *zMelk*-like gene transcripts were strongly expressed in the eye, gill, kidney, spleen, muscle, ovary, and testis and weakly in the heart, liver, and gut in the adult zebra fish. No signal was detected in the adult brain, but a strong signal was observed in the eye, which was different from the case of *mMelk* (data not shown). In adult fish, the kidney contains hematopoietic cells and is assumed to be equivalent to mammalian bone marrow in terms of hematopoietic function (35). Thus, the expression of Melk in hematopoietic tissues is a common feature in zebra fish and mice. We next examined the spatial distribution of *zMelk*-like gene mRNA in developing embryos by whole-mount in situ hybridization using DIG-labeled RNA as probes. *zMelk*-like gene mRNA was strongly expressed in the entire embryonic region of 3-hpf eggs (Fig. 1D). In contrast, in the shield stage embryos (around 6 hpf), only a very weak signal was found (Fig. 1D). Since zygotic gene expression in zebra fish is known to start around 4 hpf (22), the temporal transition of *zMelk*-like gene expression was assumed to be typical of the expression of maternal mRNA and its degradation and of newly synthesized zygotic transcription products. RT-PCR analysis of *zMelk*-like gene mRNA expression from different stages of embryos supported this idea (Fig. 1D). By the six-somite stage (at around 12 hpf), signals appeared in the entire embryo, but slightly stronger expression was observed in the eye primordium and lateral mesoderm of the midtrunk region than elsewhere (Fig. 1D, arrows). A dorsal view and flat-mounted samples of a 12-hpf embryo showed *zMelk*-like gene

expression as bilateral stripes (Fig. 1D, arrows). Later, these stripes spread both rostrally and caudally. By 24 hpf, the signals had disappeared from the caudal half of the embryos and weak expression was observed in the entire anterior half of the embryo except for the brain, where relatively strong signals were found in the ventricular region of the midbrain and tel-encephalon and in the retina (Fig. 1D).

Ablation of *zMelk*-like gene function by MO antisense oli-gonucleotides resulted in severe anemia and slight retardation of eye development. We then analyzed the roles of the zMelk-like gene in the development of these tissues by using MO antisense oligonucleotides. When MO was injected into zebra fish embryos at the one- or two-cell stage, no apparent effect was observed until 12 hpf (data not shown). Although MO is known to suppress the translation of maternal transcripts, it is notable that the ablation of Melk function in early embryogen-esis did not show any obvious effects. At 24 hpf, abnormal swelling in the tectum region of the brain and small eyes were found (Fig. 2D, asterisk). These phenotypes were seen in nearly 90% (171/192) of the embryos when 6 ng of *zMelk*-like gene-first MO was injected (Table 1). In addition, curly and thickened tails and yolk sac extensions were observed (Fig. 2D). However, these tail and yolk sac abnormalities seemed to be nonspecific, since we observed similar effects with other unrelated MO (data not shown). At 48 hpf, the tectum region was still swollen and the eyes were slightly smaller in compar-ison with those of the control MO-injected embryos (Fig. 2E and F, asterisks).

Blood circulation of zebra fish embryo starts to be visible by around 24 to 26 hpf (22, 32). In *zMelk*-like gene MO-injected embryos, the blood circulation was not apparent at 24 hpf (in 91% of them treated with 6 ng of first MO). It became visible about 29 hpf, but the number of blood cells was far smaller than that for the control MO-injected embryos. Even at 48 (Fig. 2E and F, arrowheads) and 72 (Fig. 3A and B, arrow-heads) hpf, only a trace amount of blood cells could be seen.

To confirm the specificity of these effects, we used a second MO synthesized to be specific against a different region of the *zMelk*-like gene mRNA. As shown in Table 1, this second MO showed similar effects on zebra fish embryo development, i.e., swelling of the tectum and small eyes. We then examined the dose requirement for the second MO and found that a higher amount (9 ng) of it was required to obtain a similar incidence of the phenotype caused by the first MO (6 ng).

We next examined whether coinjection of the *zMelk*-like gene RNA could rescue embryos from the MO phenotypes or not. We constructed a mutant *zMelk*-like gene (*zMelk*-like 5′mt) having alternate codon sequences at 7 nucleotides and

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FIG. 1. Expression of *zMelk*-like gene during zebra fish embryonic development. (A) Schema showing similarity at the nucleotide level among *mMelk*, *zMelk*-like gene, and Xenopus *EG3*. (B) Comparison of *zMelk*-like gene and *mMelk* in amino acid sequences. Schema showing similarity at the amino acid among *hMelk*, *mMelk*, and *zMelk*-like gene. (C) Semiquantitative RT-PCR analysis of *zMelk*-like gene mRNA expression. Total RNA was isolated from various tissues of adult zebra fish (upper panel), and various developmental stages of whole embryos (lower panel). *zMelk*-like gene transcripts were amplified by using specific primers. *G3PDH* was used as a control in both panels. (D) Whole-mount in situ hybridization patterns of *zMelk*-like gene expression during zebra fish embryonic development. Zebra fish embryos at various stages were hybridized with antisense or sense probes. Upper panels are expression of *zMelk*-like gene at 3, 6 hpf (lateral view, animal pole to top), 12 hpf (lateral view, dorsal to right), and 24 hpf (lateral view, anterior to left): and bottom panels, 12 and 14 hpf (dorsal view, anterior at the top), 12 and 24 hpf (flat-mounted with anterior to left) stages. lm, lateral mesoderm; tel, telencephalon; mb, midbrain; op, optic primordium. Scale bars, 100 μm.
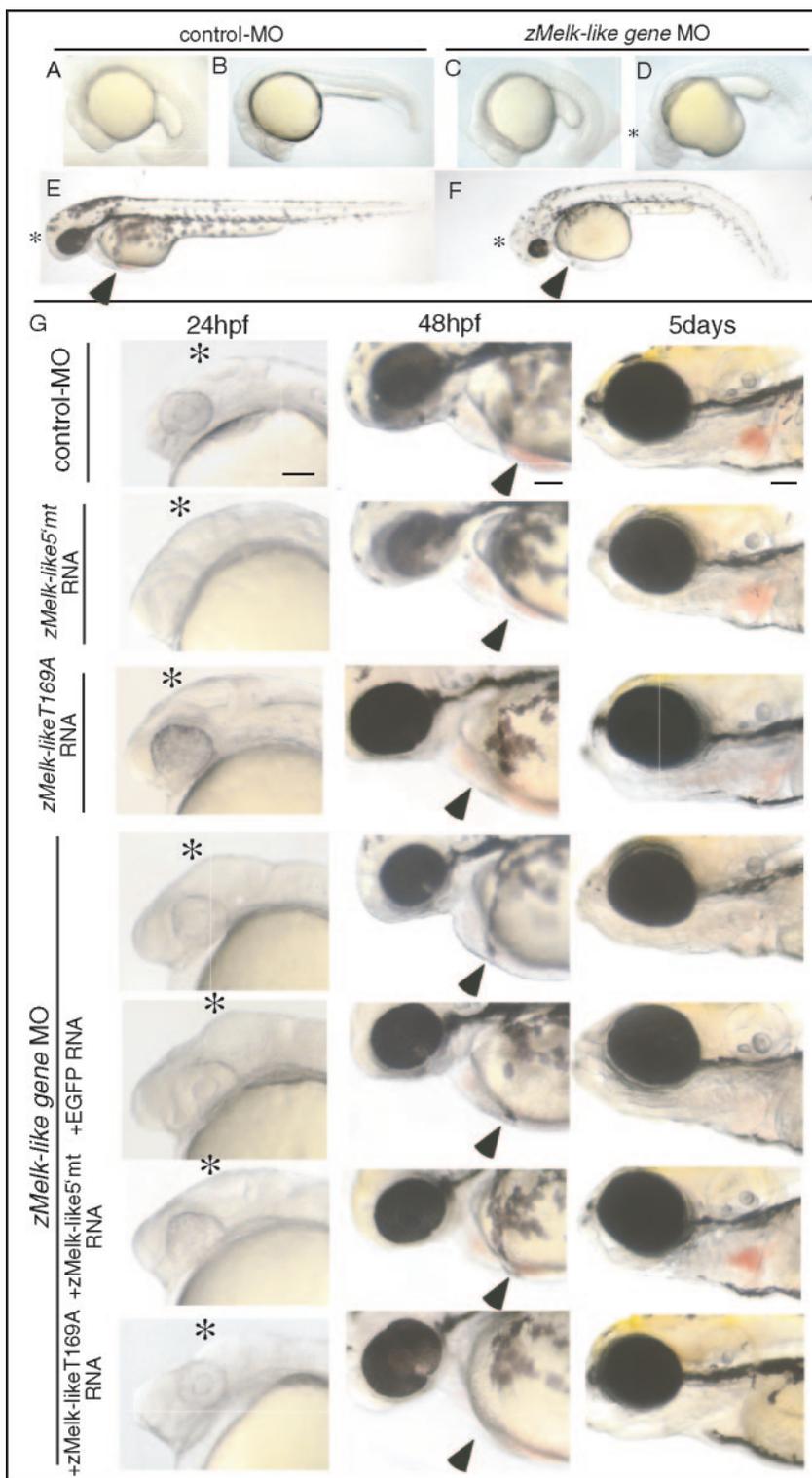
FIG. 2. Abrogation of *zMelk*-like gene function by MO antisense oligonucleotide. Lateral views (anterior to left) of 21 hpf (A and C), 24 hpf (B and D), and 48 hpf (E and F) zebra fish embryos injected with control MO (A, B, and E) or *zMelk*-like gene MO (C, D, and F) oligonucleotides. (G) Lateral views (anterior to left) of embryos injected with either control MO, *zMelk*-like 5′mt RNA, *zMelk*-likeT169A RNA or *zMelk*-like gene MO are shown. Coinjection of *zMelk*-like gene MO and either EGFP RNA, *zMelk*-like 5′mt RNA, *zMelk*-likeT169A RNA was done and views of these embryos at 24 and 48 hpf and 5 days are shown. Scale bars, 100 μm.

TABLE 1. Abnormal phenotypes caused by *zMelk*-like gene MO injection and rescue from them by *zMelk*-like gene mRNA, *zMelk*-like 5′mt RNA, or *zMelk*-like*T169A* gene mRNA

| Material(s) injected | No. of embryos examined[a] | No. of embryos with normal or abnormal phenotypes (%) | | | |
| | | Normal | Abnormal[b] | | Lethal |
			Tectum swelling	Blood circulation	
Control MO (6 ng)	173	170 (98%)	0 (0%)	0 (0%)	3 (2%)
Control MO (9 ng)	53	52 (98%)	0 (0%)	0 (0%)	1 (2%)
zMelk-like gene RNA (300 pg)	182	178 (98%)	0 (0%)	0 (0%)	4 (2%)
zMelk-like gene 5′mt RNA (300 pg)	68	67 (99%)	0 (0%)	0 (0%)	1 (1%)
zMelk-like gene*T169A RNA* (300 pg)	32	30 (94%)	0 (0%)	0 (0%)	2 (6%)
zMelk-like gene first MO (6 ng)					
zMelk-like gene first MO (6 ng)	192	14 (7%)	171 (89%)	174 (91%)	4 (2%)
zMelk-like gene first MO (6 ng) + EGFP RNA (300 pg)	120	9 (8%)	107 (89%)	108 (90%)	3 (2%)
zMelk-like gene first MO (6 ng) + *zMelk*-like geneRNA (300 pg)	75	53 (71%)	21 (28%)	21 (28%)	1 (1%)
zMelk-like gene first MO (6 ng) + *zMelk*-like gene 5′mtRNA (300 pg)	88	62 (70%)	24 (27%)	25 (29%)	1 (1%)
zMelk-like gene first MO (6 ng) + *zMelk*-like gene*T169A* RNA (300 pg)	58	4 (7%)	48 (83%)	51 (88%)	3 (5%)
zMelk-like gene second MO (6 ng)					
zMelk-like gene second MO (6 ng)	167	69 (41%)	66 (40%)	95 (57%)	3 (2%)
zMelk-like gene second MO (6 ng) + EGFP RNA (300 pg)	111	43 (39%)	44 (40%)	66 (59%)	2 (2%)
zMelk-like gene second MO (6 ng) + *zMelk*-like geneRNA (300 pg)	70	58 (83%)	11 (16%)	12 (17%)	0 (0%)
zMelk-like gene second MO (9 ng)					
zMelk-like gene second MO (9 ng)	154	14 (9%)	66 (43%)	137 (89%)	3 (2%)
zMelk-like gene second MO (9 ng) + EGFP RNA (300 pg)	102	10 (10%)	47 (46%)	90 (88%)	2 (2%)
zMelk-like gene second MO (9 ng) + *zMelk*-like gene RNA (300 pg)	70	52 (74%)	11 (16%)	18 (26%)	0 (0%)

[a] One- or two-cell-stage embryos were injected with the materials indicated.
[b] Number (%) of embryos with abnormal phenotypes, i.e., disintegration of the tectum region due to abnormal swelling or abnormal blood circulation.

used RNA transcribed from the mutant to reverse the effects of the *zMelk*-like gene MO. Injection of *zMelk*-like 5′mt mRNA alone (300 pg) did not cause any morphological defect, at least at 24 or 48 hpf or 5 days (Fig. 2G). Furthermore, we did not observe ectopic or early-onset blood circulation (data not shown). Then, coinjection of 6 ng of first MO together with either the *zMelk*-like 5′mt mRNA or EGFP mRNA was done (Table 1). Recovery from tectum swelling at 24 hpf (Fig. 2G, left panels, asterisks) and blood circulation at 48 hpf and 5 days (Fig. 2G, middle and right panels, arrowheads) were clearly observed by coinjection with *zMelk*-like 5′mt RNA but not by that with *EGFP* RNA. We also examined the effect of coinjection of the wild-type *zMelk*-like gene mRNA with the MO. Wild-type *zMelk*-like gene mRNA reduced the incidence of the MO-induced phenotype of tectal swelling and blood circulation from 91% to 28% (Table 1). Coinjection with wild-type *zMelk*-like gene mRNA also neutralized the effects of the second MO (Table 1). This significant rescue achieved by coinjection with the *zMelk*-like 5′mt RNA or wild-type *zMelk*-like gene RNA suggests strongly that the *zMelk*-like gene MO phenotypes were caused by the specific inhibition of the zMelk-like gene functions.

Since the zMelk-like gene contains a putative serine/threonine kinase domain, we next examined the role of the kinase domain by using a mutant zMelk-like gene having a point mutation within a critical amino acid in the putative ATP binding domain. One of the mutant zMelk-like genes which has substitution of threonine 169 with alanine (T169A) was expected to be destroyed with its kinase activity. We injected *zMelk*-like geneT169A mRNA with *zMelk*-like gene MO and found that the phenotype was indistinguishable from that of embryos injected with the *zMelk*-like gene MO alone (Fig. 2G), suggesting the essential role of the kinase domain in zMelk-like gene function. We also constructed a mutant *zMelk*-like gene having a substitution of threonine 169 with glutamic acid (T169E) which was expected to make its kinase constitutively active. The T169E mutant rescued the effect of *zMelk*-like gene MO (data not shown). We tried to confirm whether the T169E is constitutively active or not by different experiments; however, we could not obtain clear evidence suggesting the activity of the T169A (data not shown).

Primitive hematopoiesis was suppressed, but vasculogenesis seemed to occur normally in *zMelk*-like gene MO-injected embryos. Although severe anemia was observed until at least 72 h after MO was injected into embryos (Fig. 3A and B), blood circulation and the number of blood cells at about 6 days postfertilization (dpf) were similar in both *zMelk*-like gene MO-injected and control embryos (data not shown). Since definitive erythropoiesis starts around 4 dpf (1), there are two possibilities, i.e., that definitive hematopoiesis as seen in control embryos had occurred in *zMelk*-like gene MO-injected embryos at around 6 dpf or that the effects of MO disappeared around this time. In addition to the defect in blood cells, the beating of the heart tube was slower than that of the control MO-injected embryos. At 32 hpf, the heart rate of control MO-injected embryos was 100 ± 3 beats per min, which is comparable to previously reported values (2, 20), whereas the rate for the *zMelk*-like gene MO-injected embryos was 81 ± 4 beats per min. At 48 hpf, the heart rate of the former was 150
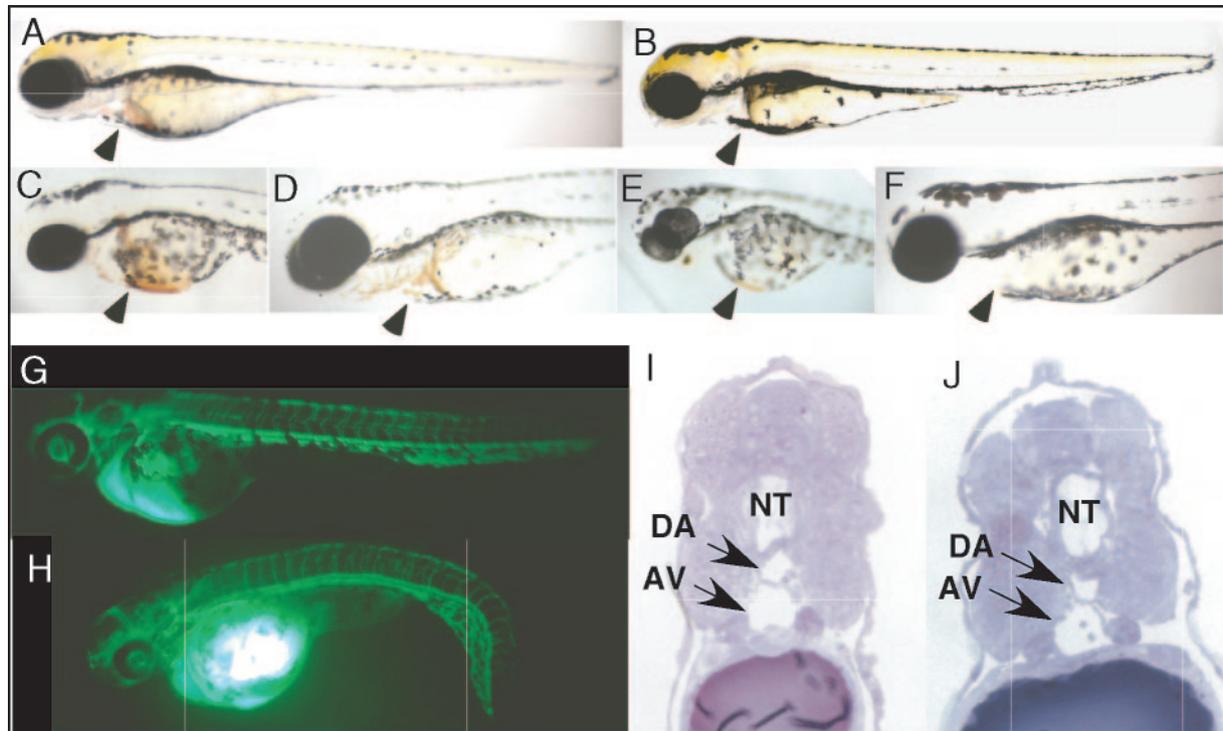
FIG. 3. Hematopoiesis and vasculogenesis of MO-injected embryos. (A and B) Lateral views of 72-hpf zebra fish embryos injected with control MO (A) or *zMelk*-like gene MO (B). (C through F) Hemoglobin staining using *o*-dianisidine for 58-hpf (C and E) and 72-hpf (D and F) zebra fish embryos injected with the control MO (C and D) or the *zMelk*-like gene MO (E and F). (G and H) Microangiography by injecting FITC-dextran into control MO (G) or *zMelk*-like gene MO (H) -injected zebra fish embryos at 48 hpf. (I and J) Plastic, thin, transverse sections of the mid-trunk region of 24-hpf zebra fish embryos injected with control MO (I) or *zMelk*-like gene MO (J). The sections were stained with toluidine blue. NT, neural tube; DA, dorsal aorta; and AV, axial vein.

± 3 beats per min and that of the latter was 121 ± 4 beats per min.

The *zMelk*-like gene was expressed relatively strongly in the lateral mesoderm. Since this mesoderm is a place where the differentiation of primitive erythroid progenitors is initiated, we next analyzed the effect of *zMelk*-like gene MO on hematopoiesis directly by hemoglobin staining with *o*-dianisidine (7). Blood cell pools could be seen in the duct of Cuvier of control MO-injected embryos at 36 hpf, but they were absent in the *zMelk*-like gene MO-injected embryos (data not shown). Even at 48 hpf, stain deposits in the *zMelk*-like gene MO-injected embryos were not detectable (Fig. 3C and E, arrowheads). By 72 hpf, blood cells circulated through the heart and branchial vessels in control embryos, but *zMelk*-like gene MO-injected embryos did not express detectable levels of hemoglobin at that time (Fig. 3D and F, arrowheads). There was no evidence of blood pooling or hemorrhage. To examine the possibility that the apoptosis occurs with blood cells, we examined apoptotic cells by staining embryos with acridine orange at 48 and 72 hpf, but no difference in the staining pattern between control- and *zMelk*-like gene-MO injected embryos was observed (data not shown).

Next, we examined whether the ablation of zMelk-like gene functions would affect vascular-network formation. FITC-dextran was used to visualize vasculogenesis in zebra fish embryos. FITC-dextran was injected into the sinus venosus/cardinal vein at various stages (30, 48, 56, and 72 hpf) of development, and

blood vessels were examined 10 min after the injection. At 48 hpf, there was no significant difference in the pattern of FITC signals between the *zMelk*-like gene MO-injected embryos and the control MO-injected ones (Fig. 3G and H). Similarly, in other stages (30, 56, and 72 hpf), no significant abnormality of the vascular network in the *zMelk*-like gene MO-injected embryos was observed (date not shown). Furthermore, observation of transverse sections of the midtrunk of 48-hpf embryos revealed that the dorsal aorta and axial vein formation occurred at the appropriate region (Fig. 3I and J, arrows), supporting the idea that the *zMelk*-like gene is not necessary for the proper formation of blood vessels.

scl, gata-1, and lmo-2 are critical transcription factors for vertebrate embryonic hematopoiesis (10, 38). So next we analyzed the expression pattern of these genes in *zMelk*-like gene MO- or control MO-injected embryos by whole-mount in situ hybridization. During zebra fish embryogenesis, *gata-1* mRNA is reported to be initially detected at the two-somite stage in putative progenitors that reside in two stripes flanking the paraxial mesoderm (7). As shown in Fig. 4A, at 10 hpf, two *gata-1*-positive stripes were observed in both control MO-injected embryos and *zMelk*-like gene MO-injected embryos, but the length of the stripes in the latter was far shorter and the signals significantly weaker than those in the former. When we looked at 12-hpf embryos, a similar tendency was observed (Fig. 4A). The length of the area with *gata-1*-positive signals was about half of that of the control MO-injected embryos, and
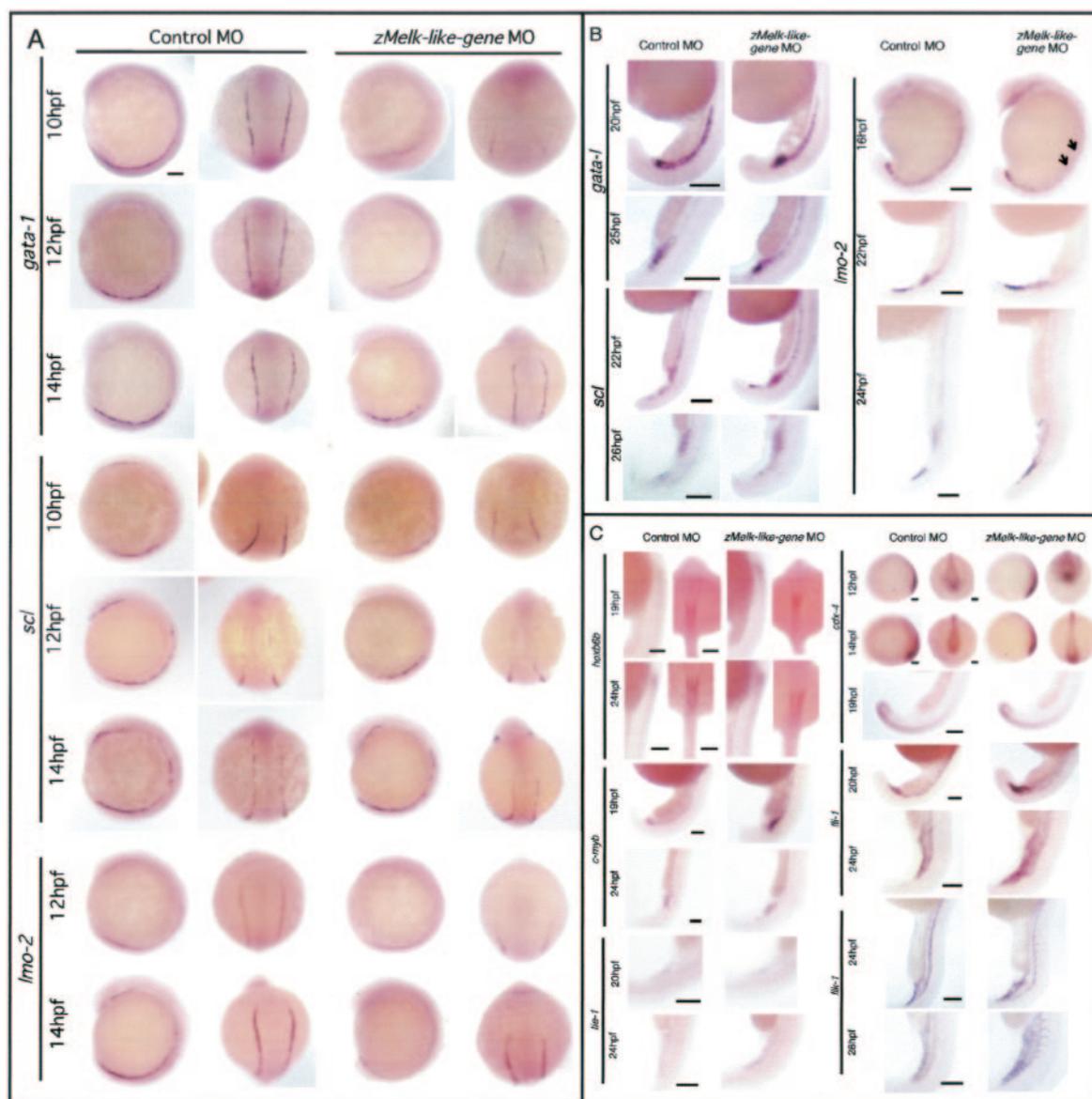
FIG. 4. Whole-mount in situ analysis of various hematopoietic and vascular markers. Expression of various hematopoietic genes in control and in *zMelk*-like gene MO-injected embryos. (A) Whole-mount in situ hybridization of *gata-1*, *scl*, and *lmo-2*. Lateral view with anterior at the top (first and third columns from the left) and dorsal view with anterior at the top (second and fourth column from the left) of probe-hybridized zebra fish embryos injected with either control MO or *zMelk*-like gene MO are shown. Scale bars, 100 μm. (B) Lateral views of whole-mount control MO- or *zMelk*-like gene MO-injected embryos at 16 to 26 hpf in situ hybridized for *gata-1*, *scl*, and *lmo-2* mRNA. Scale bars, 150 μm. (C) Embryos injected with control MO or *zMelk*-like gene MO were harvested at the indicated stages, and whole-mount in situ hybridization was done by using *hoxb6b*, *c-myb*, *tie-1*, *cdx-4*, *fli-1*, and *flk-1* probes. Expression patterns of *hoxb6b* at 19 and 24 hpf (lateral view with dorsal to right and dorsal view with anterior at the top), *c-myb* at 19 and 24 hpf (lateral view of ICM region), *tie-1* at 20 and 24 hpf (lateral view of ICM region), *cdx-4* at 12 hpf, 14 hpf (lateral view with anterior to left and posterior view with dorsal at the top), *cdx-4* at 19 hpf (lateral view of ICM region), *fli-1* at 20 and 24 hpf (lateral views of ICM region) and *flk-1* at 24 and 28 hpf (lateral view of ICM region) are shown. Results of antisense probe hybridization are shown in the figure. Sense probe control experiments were done for all of the probes, and no significant signals were detected. Scale bars, 100 μm.

the intensity was far weaker in the *zMelk*-like gene MO-injected embryos. Once the embryos had reached 14 hpf, signals in the *zMelk*-like gene MO-injected embryos were still weaker than those in the control ones, but the difference in the expression pattern had become more subtle. We next examined the expression of *scl* by in situ analysis. In normal development, *scl* is known to be expressed at a slightly earlier stage than *gata-1*, as two stripes situated in the lateral margins of the

mesoderm (9), and at 10 to 14 hpf, we detected *scl* transcripts in the anterior and posterior lateral plate mesoderms. However, in the 10- and 12-hpf *zMelk*-like gene MO-injected embryos, expression of *scl* in the anterior lateral plate was hardly detected. The length of the plate expressing *scl* and the intensity of *scl* signals in the posterior lateral plate were shorter and weaker, respectively, in *zMelk*-like gene MO-injected embryos than in the control MO-injected ones (Fig. 4A). However, at 14

hpf, the pattern of *scl* expression between the two groups was similar. Therefore, in later stages, the expression pattern of both *gata-1* and *scl* became indistinguishable between *zMelk*-like gene MO-injected embryos and control MO-injected ones.

The LIM domain transcription factor lmo-2 interacts with scl and is essential for primitive and definitive hematopoiesis (37). Like *scl*, *lmo-2* is expressed in endothelial and hematopoietic progenitors and in cells of the erythroid and megakaryocytic lineage (10). In zebra fish embryos, *lmo-2* is expressed in the two stripes of the ventral mesoderm, as well as in the two anterior stripes, by 12 hpf (35). The signals of *lmo-2* in *zMelk*-like gene MO-injected embryos were faint in both the anterior and posterior at 12 hpf and at 14 hpf, and intensity of the signals was still weaker than that observed with control MO-injected embryos (Fig. 4A). Then, by 16 hpf, expression of *lmo-2* in the lateral mesoderm of *zMelk*-like gene MO-injected embryos was clearly observed in the most posterior region but not in the midtrunk (Fig. 4B, arrows). In zebra fish, by 18 hpf, the ventral mesodermal cells converge to form the intermediate cell mass (ICM) (1). Around 22 to 24 hpf, *lmo-2* was strongly expressed in the posterior ICM in both control and *zMelk*-like gene MO-injected embryos with a similar pattern (Fig. 4B). Similarly, although *scl* and *gata-1* expression was suppressed in the lateral mesoderm around 12 to 14 hpf (Fig. 4A), their expression was recovered around 20 to 22 hpf in the ICM in *zMelk*-like gene MO-injected embryos, and the patterns and intensity of the signals were indistinguishable from those of the control MO-injected embryos (Fig. 4B). We then analyzed yet other hematopoietic markers by whole-mount in situ analysis of control MO- and *zMelk*-like gene MO-injected embryos (Fig. 4C). Cdx-4, a homeobox transcription factor, was recently identified as the locus mutated in *kugelig* (*kgg*), which is a zebra fish mutant with an early defect in hematopoiesis (6). *cdx-4* was expressed similarly in terms of both pattern and intensity in *zMelk*-like gene MO- and control MO-injected embryos (Fig. 4C). It is notable that the *cdx-4* expression in early embryos such as at 12 hpf and 14 hpf was also not perturbed by injection of the *zMelk*-like gene MO. Hoxb6b is one of the *hox* genes downstream of cdx-4 (6). We found that the *hoxb6b* mRNA was expressed similarly in *zMelk*-like gene MO-injected embryos at both 19 hpf and 24 hpf (Fig. 4C). As also shown in Fig. 4C, other hematopoietic genes such as *fli-1*, *flk-1*, *c-myb*, and *tie-1* showed no apparent difference in their expression pattern or the strength of signals between the two groups. Since the *zMelk*-like gene was not expressed in the ICM, its effects on hematopoiesis in this region may not be due to the direct regulation of ICM function. These results indicate that suppression of the *zMelk*-like gene affected the expression of *gata-1* and *scl* in the early stage but was not involved in subsequent stages.

We also examined the expression pattern of *en-2* (18 hpf, 24 hpf, 28hpf), *islet*-1 (22 hpf, 24 hpf, 28 hpf), *dlx-2* (22 hpf, 24 hpf), *wnt*-1 (20 hpf, 24 hpf), *hlx-1* (20 hpf, 24 hpf, 28 hpf), *shh* (20 hpf, 24 hpf), and *myo*D (20 hpf, 24 hpf), but again, no significant difference in the expression patterns between the two groups of embryos was observed (data not shown).

The expression of gata-1 rescued the effects of the *zMelk*-like gene MO in embryos. Since suppression of the expression of *gata-1*, *scl*, and *lmo-2* was observed by the injection of *zMelk*-like gene MO, we next examined whether the coexpression of

these genes with *zMelk*-like gene MO rescued the MO-induced phenotype or not. We first examined effects of the expression of gata-1 in embryos. Various doses of mRNA of full-length *gata-1* were injected into zebra fish embryos, and embryos were examined at 24 hpf, 48 hpf, and 5 days after injection. As shown in Fig. 5A, injection of 50 pg of *gata-1* mRNA did not show apparent abnormal morphological defects and the onset of blood circulation was normal (data not shown). However, 100 pg of *gata-1* mRNA resulted in the appearance of significant population of the lethal phenotype (Table 2). Using the mRNA doses which did not induce abnormal embryos, we next examined whether coexpression of the gata-1 rescued the phenotype induced by *zMelk*-like gene MO. Embryos injected with *zMelk*-like gene MO and *gata-1* mRNA (50 pg) were examined at 24 hpf, 48 hpf, and 5 days after injection (Fig. 5A). The expression of gata-1 reversed the phenotype induced by *zMelk*-like gene MO. We had done exactly the same set of experiments using full-length mRNA for *scl* and *lmo-2*; however, we could not observe rescue of the phenotype induced by *zMelk*-like gene MO by the injection of *scl* or *lmo-2* mRNA.

Gata-1 promoter activity was modulated by the expression of the *zMelk*-like gene. Since the reversed phenotype of *zMelk*-like gene MO by gata-1 expression was observed, we next examined the mechanism of the effect of *zMelk*-like gene in *gata-1* expression by using *gata-1* promoter EGFP. The *gata-1* 8.1-kb 5′ promoter region contains a sufficient genomic region to direct proper expression of the *gata-1* gene (23). We first confirmed the expression of *gata-1* promoter EGFP in zebra fish embryos by our hand. As shown in Fig. 5B, *gata-1* promoter-directed EGFP was expressed in the lateral mesoderm at 14 hpf and ICM at 18 hpf, both of which were proper expression regions of endogenous *gata-1*. We then examined the effect of coexpressed *zMelk*-like gene mRNA with *gata-1* promoter EGFP (Fig. 5B; Table 3). Coinjection of wild-type *zMelk*-like gene mRNA enhanced the expression of *gata-1* promoter EGFP expression at 14 hpf, suggesting that the *zMelk*-like gene positively affects *gata-1* promoter activity (Fig. 5B). However, the *zMelk*-like*T169A* gene did not affect the expression, indicating the essential role of the kinase domain of the *zMelk*-like gene. At 18 hpf, we did not observe the clear enhancement of EGFP expression in the presence of the wild-type *zMelk*-like gene. We used full-length *gata-1* mRNA as a positive control in the experiments, and the expression of gata-1 enhanced *gata-1* promoter EGFP (Table 3), as expected from previous paper (23). Ectopic expression of EGFP was observed with gata-1 expression; however, ectopic expression was never observed with *zMelk*-like gene expression, suggesting that expression of the *zMelk*-like gene alone was not sufficient to promote *gata-1* promoter activation. We also examined the coinjection of *gata-1* mRNA and *zMelk*-like gene mRNA in addition to *gata-1* promoter EGFP, but we could not observe any apparent enhancement of the expression level of EGFP in comparison with that of the embryos injected with *gata-1* mRNA and *gata-1* promoter EGFP.

DISCUSSION

In this report, we identified the *zMelk*-like gene, which showed an expression similar to that of *mMelk*, by taking advantage of the strong sequence similarity with *mMelk* at the C-
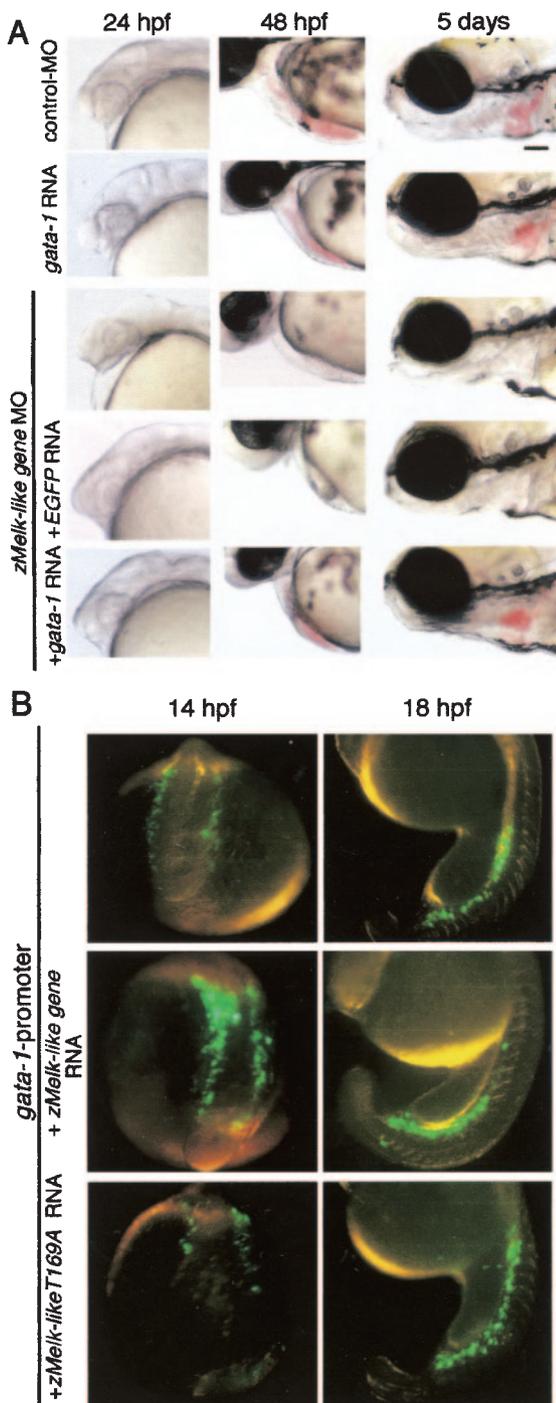
FIG. 5. Effects of gata-1 gene expressions for phenotype induced by the injection of *zMelk*-like gene MO and regulation of *gata-1* promoter by a *Melk*-like gene. (A) Lateral views (anterior to left) of embryos injected with either control MO, *gata-1* RNA or *zMelk*-like gene MO are shown. Coinjection of *zMelk*-like gene MO and either *EGFP* RNA, or *gata-1* RNA was done and views of these embryos at 24 and 48 hpf and 5 days (lateral view with anterior to left) are shown. Scale bars, 100 μm. (B) Effects of zMelk-like gene for activation of zebra fish *gata-1* promoter in the embryos. Zebra fish *gata-1* promoter EGFP and mRNA for either control, *zMelk*-like gene, or zMlke-likeT169A were coinjected into fertilized eggs and the expression of EGFP was examined under fluorescence dissection microscope. Dorsal views of embryos at 14 hpf and lateral views with anterior to left embryos at 18 hpf are shown.

and N-terminal regions. Knockdown of the *zMelk*-like gene product indicated that this product may play a role in primitive hematopoiesis in the zebra fish.

Although the *zMelk*-like gene does not have similarity with *mMelk* at the middle part of the molecule, several features of expression patterns were similar between the *zMelk*-like gene and *mMelk*. For example, both genes were expressed in embryonic brain and retina, especially in the ventricular zone. Furthermore, both were expressed in hematopoietic tissues in adult animals. We tried to find mouse gene(s) having similarity with the middle region of the *zMelk*-like gene by database searching, but no such gene or fragment was found. Vice versa, our attempt to find sequences in the zebra fish database that were homologous with the middle region of mMelk was not successful. Taken together, our data suggest that the *zMelk*-like gene may be a zebra fish orthologue of *mMelk*.

Primitive hematopoiesis in the zebra fish embryo occurs in the region located between the notochord and endoderm of the trunk called the ICM (1). The earliest expression of hematopoietic genes *gata-1*, *scl*, and *lmo-2* commences in bilateral stripes within the lateral plate mesoderm at approximately the five-somite stage and 11 hpf, respectively (10, 26). We found that in the *zMelk*-like gene MO-injected embryos, the expression of *gata-1*, *scl*, and *lmo-2* around 12 to 14 hpf was severely suppressed but that it seemed relatively normal in the later stage, suggesting that the *zMelk*-like gene may be involved in the regulation of these genes' expression only in the early stage. Recently, *cdx-4*, a homeobox transcription factor, was identified as the locus mutated in *kugelig (kgg)*, which is a zebra fish mutant with a defect in the early stage of hematopoiesis (6). In *kgg* mutants, the number of *scl*-positive hematopoietic precursor cells and *gata-1*-positive erythroid precursor cells are decreased (6). Since the expression of *cdx-4* was not altered by the injection of *zMelk*-like gene MO, we surmise that the *zMelk*-like gene plays a role downstream of *cdx-4*. *hox* genes are candidate targets of *cdx-4*, and in fact, rescue from the *kgg* phenotype by the overexpression of some *hox* genes has been reported (6). The fact that we did not observe a reduction in the expression of *hoxb6b*, one of the *hox* genes whose expression is suppressed in *kgg* (10), suggests that the target of the *zMelk*-like gene lies in between *hox* and *scl*. Although redundant activities of multiple *hox* genes were suggested (6), hoxb6b alone is not sufficient to reverse the *kgg* phenotype completely. Thus, there is another possibility, that the *zMelk*-like gene regulates the expression or function of *hox* members other than *hoxb6b*. The *zMelk*-like gene expression pattern at about 12 to 14 hpf did not overlap with that of *gata-1*, *scl*, and *lmo-2*, but weak expression of the *zMelk*-like gene was observed within most of the lateral mesoderm area. Thus, we could not rule out the possibility that the *zMelk*-like gene directly regulates *gata-1* or *scl* expression in this region. Enhancement of the *gata-1* promoter by expression of the *zMelk*-like gene supported this idea. Positive feedback of the *gata-1* promoter by gata-1 itself was reported (23). Gata-1 is known to be phosphorylated at their serine residues (5), and the involvement of mitogen-activation protein kinase in the phosphorylation of gata-1 was recently suggested (36). Requirement of the kinase domain of the zMelk-like gene for enhancement of the *gata-1* promoter was evident; however, we need further experiments to conclude whether direct the phosphorylation of

TABLE 2. Abnormal phenotypes caused by *zMelk*-like gene MO injection and its rescue by *gata-1* RNA[a]

| Material(s) injected | No. of embryos examined[a] | No. of embryos with normal or abnormal phenotypes (%) | | | |
| | | Normal | Abnormal[b] | | Lethal |
			Tectum swelling	Blood circulation	
Control MO (6 ng)	61	59 (97%)	0 (0%)	0 (0%)	2 (3%)
zMelk-like gene first MO (6 pg)	97	3 (3%)	85 (87%)	91 (94%)	3 (3%)
zMelk-like gene first MO (6 ng) + EGFP RNA (50 pg)	96	4 (4%)	83 (86%)	89 (92%)	3 (3%)
zMelk-like gene first MO + *Gata-1* RNA					
zMelk-like gene first MO (6 ng) + *Gata-1* RNA (10 pg)	83	33 (40%)	42 (50%)	48 (58%)	2 (2%)
zMelk-like gene first MO (6 ng) + *Gata-1* RNA (50 pg)	80	56 (70%)	16 (20%)	22 (27%)	2 (3%)
zMelk-like gene first MO (6 ng) + *Gata-1* RNA (100 pg)	80	20 (25%)	4 (5%)	28 (35%)	32 (40%)
Gata-1 RNA					
Gata-1 RNA (10 pg)	41	40 (98%)	0 (0%)	0 (0%)	1 (2%)
Gata-1 RNA (50 pg)	38	36 (95%)	0 (0%)	0 (0%)	2 (5%)
Gata-1 RNA (100 pg)	42	21 (50%)	0 (0%)	4 (10%)	17 (40%)

[a] One- or two-cell-stage embryos were injected with the materials indicated.
[b] Number (%) of embryos with abnormal phenotypes, i.e., disintegration of the abnormal swelling (tectum region) or abnormal blood circulation.

gata-1 by the *zMelk*-like gene occurs or zMelk-like kinase is in the upstream kinase cascade of gata-1 regulation. On the other hand, since the *zMelk*-like gene was not expressed in the anterior or posterior ICM, and because *gata-1* and *scl* expression in this region was not affected in *zMelk*-like gene MO-injected embryos, the suppression of blood circulation by abrogation of products of the *zMelk*-like gene may not occur through the direct regulation of *gata-1* and *scl* expression in the ICM. Currently, we do not have evidence suggesting mechanisms of the anemia phenotype; however, we surmised several possibilities. One is that the underdetection level of zMelk-like gene in ICM affected the proliferation and/or differentiation of hematopoietic cells through regulating the expression of the gene(s), which we did not examine in this work. Alternatively, these unknown genes may be downstream of the gene cascade of *gata-1/scl* expressed in the lateral plate mesoderm. Or, more speculatively, zMelk-like gene expressed in the anterior half of embryos regulating the deriver of soluble factor(s), which is essential for blood circulation, into ICM can be surmised.

We could observe rescue of the phenotype induced by *zMelk*-like gene MO only by the expression of the *gata-1* gene, suggesting the importance of *gata-1* expression in the early phase of embryogenesis for hematopoiesis in zebra fish. *scl* and *lmo-2* are expressed earlier than *gata-1* in the hematopoietic program in vertebrates (1); however, we could not observe recovery of the *zMelk*-like gene MO-induced phenotype by expression of these genes. Our results may suggest that these genes were not sufficient to promote *gata-1* expression. However, we could not rule out that the expression level of *scl* and *lmo-2* was not sufficient for the activity.

There are at least five "bloodless" zebra fish mutants including *vlad tepes* (28), *cloche (clo)*, *spadetail (spt)* (35), and *bloodless (bls)* (26). *bls* is a dominant mutant with phenotypes ranging from complete absence of circulating blood cells to severe anemia (26). Its phenotype is detected with the onset of circulation. In the *bls* mutant, the expression of *gata-1* and *scl* is severely impaired in the lateral mesoderm as well as in the ICM around 24 hpf (26). Because of the intact expression of *gata-1* and *scl* in the ICM in the *zMelk*-like gene MO-injected embryos, different mechanisms for the regulation of hematopoiesis between the *zMelk*-like gene and *bls* are suggested. Both *clo* and *spt* have defects in primitive and definitive hematopoiesis (35). *gata-1* expression in these mutants at 24 hpf is severely impaired. Furthermore, the *clo* mutants also have significantly decreased expression of vascular genes (35). The phenotype of *zMelk*-like gene MO-injected embryos seems to be hematopoietic specific, suggesting that this gene may play a role after the commitment of cells to hematopoietic and vascular endothelial cell lineages.

As part of a large-scale chemical mutagenesis screening of the zebra fish genome, more than 50 mutants that affect hematopoiesis, comprising 25 complementation groups, were isolated (30, 32). The majority of these mutations could be classified into five phenotypic classes based on their hematopoietic phenotype (32). Since hemoglobin staining showed no or an extremely reduced amount of heme in the *zMelk*-like gene MO-injected embryos, impairment of the heme synthesis pathway appears to be another defect in the *zMelk*-like gene MO-injected embryo. There are several mutants grouped as hypochromic anemia, such as *sauternes (sau)* (3) or *chardonnay* (8),

TABLE 3. Zebra fish *gata-1* promoter-dependent EGFP expression in the presence of *zMelk*-like gene or gata-1

| Material(s) injected | No. of embryos examined[a] | No. of EGFP-positive embryos (%) | | | | |
		No expression	Strong level	Weak level	Ectopic	Lethal
gata-1 promoter *EGFP* (50 pg)	65	3 (4%)	5 (7%)	53 (83%)	2 (3%)	2 (3%)
gata-1 promoter *EGFP* (100 pg) + *zMelk*-like gene (300 pg)	72	4 (6%)	33 (45%)	28 (39%)	3 (4%)	4 (6%)
gata-1 promoter *EGFP* (100 pg) + *gata-1* (200 pg)	56	5 (9%)	33 (60%)	7 (12%)	36 (65%)	8 (14%)

[a] Expression levels were arbitrary examined under a fluorescence dissection microscope. Typical examples of strong and weak levels are shown in Fig. 5B.

and they have mutations in the heme synthesis pathway. However, in these cases, a relatively normal number of circulating erythrocytes was observed up to 2 or 3 days after the onset of circulation, thus suggesting that the zMelk-like gene affects more than just the heme synthesis pathway.

Onset of definitive hematopoiesis in the zebra fish starts around 4 dpf (3, 27). Blood circulation was first observed around 6 dpf in *zMelk*-like gene MO-injected embryos, and so, although primitive hematopoiesis was defective in the *zMelk*-like gene product-deleted zebra fish, the definitive hematopoieisis may not have been perturbed.

Since *mMelk* and *zMelk*-like genes are expressed strongly in hematopoietic tissues in adult animals, there is a possibility that Melk also plays a role in adult hematopoiesis and functions in hematopoietic cells. Mouse Melk (MPK38) was reported to be expressed in the cells of the T lineage and macrophage/monocyte lineage (12), implicating a potential function of Melk in early T-cell activation and macrophage functions. In fact, elevation of the expression level of Melk (MPK38) in T-cell activation was reported, but its exact function was not revealed (11). *zMelk*-like gene transcripts were strongly detectable in the thymus of the adult zebra fish, suggesting conserved function of it in T-cell immunology.

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