Immune-related zinc finger gene ZFAT is an essential transcriptional regulator for hematopoietic differentiation in blood islands

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TAL1 plays pivotal roles in vascular and hematopoietic developments through the complex with LMO2 and GATA1. Hemangioblasts, which have a differentiation potential for both endothelial and hematopoietic lineages, arise in the primitive streak and migrate into the yolk sac to form blood islands, where primitive hematopoiesis occurs. ZFAT (a zinc-finger gene in autoimmune thyroid disease susceptibility region) is an essential transcriptional regulator containing 18 C2H2-type zinc-finger domains and one AT-hook (21). ZFAT is a critical transcriptional regulator in immune-regulation (21) and an antiapoptotic molecule in lymphoblastic leukemia cell line (22). Recently, ZFAT was reported to be associated with IFN-β responsiveness in multiple sclerosis (23). However, developmental roles for ZFAT remain unknown.

In this study, we generated Zfat-deficient (Zfat\textsuperscript{−/−}) mice and found that Zfat-deficiency results in early embryonic lethality, with the reduction in the number of blood islands and impaired differentiation of hematopoietic progenitor cells in blood islands. Furthermore, in vitro and in vivo molecular analyses revealed that ZFAT directly regulates the transcription factors including Tal1, Lmo2, and Gata1 in blood islands. Taken together, these results suggested that ZFAT plays critical roles in the development of hematopoietic system in blood islands.

Results

ZFAT Deficient Mice with Early Embryonic Lethality. To examine developmental roles for ZFAT, we targeted the Zfat allele for disruption by homologous recombination (Fig. L1). In construction of the targeting vector, a 1.4-kb fragment of Zfat genomic DNA containing exon 8 was replaced with neomycin resistance (neo) gene (Fig. L4). Targeted ES cell clones with homologous recombination and heterozygous (Zfat\textsuperscript{+/−}) mice were confirmed by Southern blot analysis (Fig. 1B, Left) and by PCR (Fig. 1B, Right). Then, Zfat\textsuperscript{−/−} mice with the genetic background of C57BL/6 were established and analyzed in this study. Zfat\textsuperscript{−/−} mice were viable, fertile, and phenotypically indistinguishable from wild-type (Zfat\textsuperscript{+/+}) litters. Obvious developmental abnormalities in T or B cells from Zfat\textsuperscript{−/−} mice were not observed in the thymus or spleen, where ZFAT is abundantly expressed (20, 21) (Fig. S1); however, the possibilities of altered immune-responses in peripheral T and B cells of Zfat\textsuperscript{−/−} mice are not excluded and a full understanding of the ZFAT function in the immune system awaits future studies.

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gene. WT, wild-type; HR, homologous external probe (yolk sacs at E8.0 (placentas μ is indispensable for mouse embryonic development. (embryos did mice. The number and ratio of embryos showing To further characterize the abnormality of blood islands at E8.0. The region surrounded by the blood islands were spindle-shaped at both E8.0 and placentas (Fig. 2). Impaired differentiation of hematopoietic progenitor cells in blood islands of Zfat−/− mice. (A) Embryos with yolk sacs from Zfat+− or Zfat−/− mice at E9.5. (Scale bars, 500 μm.) (B) H&E-stained sections of blood islands of Zfat+− and Zfat−/− placentas at E8.0. Region surrounded by the dotted line represents spongiotrophoblast layer. (Scale bars, 100 μm.) (C) H&E-stained sections of blood islands of Zfat+− and Zfat−/− yolk sacs at E8.0 (Upper) and E8.5 (Lower). Region surrounded by the dotted line represents hematopoietic progenitor cells. Arrows, endothelial cells; asterisks, visceral endodermal cells. (Scale bars, 50 μm.) (D) ZFAT protein expression in endothelial and hematopoietic progenitor cells in Zfat−/− blood islands at E8.0. The region surrounded by the dotted line represents hematopoietic progenitor cells. Arrows, endothelial cells. (Scale bars, 10 μm.)
were not significantly different (P > 0.1) (Table 1). Of interest was that the number of blood islands in Zfat−/− yolk sacs and hematopoietic progenitor cells in Zfat−/− blood islands were significantly decreased by 2.3-fold (*P = 0.014; Table 1) and 2.9-fold (**P = 0.004; Table 1), respectively, compared with those of Zfat+/+ mice. Furthermore, the ratios of hematopoietic progenitor cells per endothelial cells in Zfat−/− and Zfat+/+ blood islands were 1.43 and 0.71, respectively, with a statistically significant difference (P = 0.0037). Taken together, these results suggested that proper differentiation in the hematopoietic lineage was impaired in Zfat−/− blood islands.

**ZFAT Expression Does Not Affect Apoptosis or Proliferation in Yolk Sac Blood Islands.** In immunohistochemical analysis using anti-ZFAT monoclonal antibody M16 (Fig. S3), ZFAT signals were evidently detected in endothelial and hematopoietic progenitor cells of Zfat+/+ blood islands at E8.0, whereas ZFAT signals were not observed in endothelial cells or hematopoietic progenitor cells of Zfat−/− blood islands (Fig. 2D), indicating that ZFAT was exactly expressed in endothelial and hematopoietic progenitor cells in blood islands at E8.0. Furthermore, signals of Ki-67 as a proliferation marker were evenly detected in endothelial and hematopoietic progenitor cells in both Zfat+/+ and Zfat−/− blood islands at E8.0, and signals of activated caspase-3 as an apoptosis marker were rarely detected in Zfat+/+ or Zfat−/− blood islands at E8.0 (Fig. S4). Taken together, these results indicate that ZFAT expression in blood islands does not function by inhibiting apoptosis or promoting progenitor cell proliferation, suggesting that ZFAT may instead be involved in promoting hematopoietic progenitor differentiation.

**ZFAT Regulates the Genes Involved in Hematopoietic Differentiation in Blood Islands.** To address a possibility whether ZFAT regulates the genes essential for development of hematopoietic progenitor cells in blood islands, we performed real-time quantitative RT-PCR (qRT-PCR) assay for the hematopoiesis-related genes, including Tal1, Lmo2, Gata1, Gata2 (26) and Kit (1, 27), and Gapdh as a control gene in yolk sacs at E7.5. Expression levels of Tal1, Lmo2, and Gata1 in Zfat−/++ yolk sacs were decreased by 50-, 20-, and 200-fold, respectively, compared with those of Zfat+/++ yolk sacs (Fig. 3A; *, P < 0.001), whereas the expressions of Gata2 and Kit were not different between Zfat+/+ and Zfat−/− yolk sacs (Fig. 3A; P > 0.05). Reduced expressions of Tal1, Lmo2, and Gata1 were consistent with the histological features in blood islands in Zfat−/− mice (Fig. 2), suggesting that ZFAT is an essential regulator for the expression of the hematopoiesis-related genes, including Tal1, Lmo2, and Gata1 in blood islands.

**Direct-Regulation of Tal1, Lmo2, and Gata1 by ZFAT.** We, next, determined whether or not ZFAT directly regulates Tal1, Lmo2, and Gata1 expressions. In luciferase reporter assay using 1-kb probes for the promoter regions of Tal1, Lmo2, and Gata1 genes, the luciferase activities by ZFAT fused with a transcriptional activator-domain (AD-ZFAT) were increased by 2.6-, 5.7-, and 2.8-fold, compared with those by a transcriptional activator-domain construct (AD), respectively (Fig. 3B, P < 0.05). ZFAT binding regions were further narrowed down with 200-bp probes from the 1-kb probes showing the activities. The luciferase activities for the 200-bp probes for Tal1, Lmo2, and Gata1 were increased to 5.5-fold (Tal1-3), 4.3-fold (Lmo2-3), and 3.7-fold (Gata1-5), respectively (Fig. 3B; **P < 0.01).

To address the bindings of ZFAT with these DNA sequences in vivo, ChIP-PCR assays on yolk sacs at E7.5 and on adult kidney as a control tissue, where ZFAT is rarely expressed (21), using anti-ZFAT M16 antibody (Fig. S3) and control IgG, were done for the 200-bp regions with the highest luciferase activity (Tal1-3, Lmo2-3, and Gata1-5) and the promoter region of Kifap3 as a hematopoiesis-unrelated control gene. Differences of ChIP DNA concentrations were semiquantified by 35- and 42-cycle end-point PCR products. Promoter regions for Tal1, Lmo2, and Gata1 in the M16-ChIP DNA from E7.5 yolk sacs were enriched and compared with those of control IgG-ChIP DNA, whereas M16-ChIP DNA for Tal1, Lmo2, and Gata1 in kidney as a control tissue were not enriched (Fig. 3C); taken together, these data are suggestive of the specificity of anti-ZFAT M16 antibody and the bindings of ZFAT with these promoter regions. Furthermore, quantification by real-time qPCR assay for ChIP DNA showed that total amount of promoter regions for Tal1, Lmo2, and Gata1 in the M16-ChIP DNA were 126.4 units, 88.5 units, and 13.2 units, respectively (Fig. 3D, P < 0.05), whereas M16-ChIP DNA on the promoter regions for Cd41, Runx1, and Flk-1—the expressions of which are reported to be regulated by a Tal1-Lmo2-Gata1 transcriptional complex (4, 5, 14, 28-32)—were not enriched in the end-point PCR or ChIP-qPCR assays (Fig. S5), suggesting that ZFAT specifically binds to the promoter regions for Tal1, Lmo2, and Gata1 in yolk sacs at E7.5.

The ZFAT binding regions detected in the Tal1, Lmo2, and Gata1 genes are mapped in the genome, showing that ZFAT binds to the distinct regions from the known regulatory regions including the −187 element in Tal1 (33), the proximal promoter and the −75 enhancer element in Lmo2 (34, 35), and the CACCC motif in Gata1 (36, 37) (Fig. 3E).

**Reduction in Protein Expressions of Tal1, Lmo2, and Gata1 and Tal1-Downstream Genes in Zfat−/− Blood Islands.** Immunohistochemical analysis on Zfat+/+ and Zfat−/− blood islands at E8.0 was performed to confirm the expression levels of Tal1, Lmo2, and Gata1. The signals for Tal1, Lmo2, and Gata1 were observed in Zfat−/− blood islands, especially in hematopoietic progenitor cells, whereas all these expressions were much reduced in Zfat−/− blood islands (Fig. 4), suggesting that ZFAT is indispensable for the proper expressions of Tal1, Lmo2, and Gata1 in hematopoietic progenitor cells in blood islands at E8.0.

Real-time qRT-PCR assay at E7.5 showed that expression levels of Cd41, Runx1, and Flk-1 in Zfat−/− yolk sacs were decreased by 50-, 6.6-, and 4-fold, respectively, compared with Zfat+/+ yolk sacs (Fig. 3A; *, P < 0.001), although these genes were not directly regulated by ZFAT (Fig. S5). Protein expres-

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*P < 0.05; **P < 0.01.
Discussion

In this study, we generated Zfat−/− mice and demonstrated that Zfat-deficiency results in early embryonic lethality with the reduction in the number of blood islands and impaired differentiation of hematopoietic progenitor cells in blood islands (Fig. 1 C and D, Fig. 2, and Table 1) and that ZFAT is a critical transcription factor directly regulating Tal1, Lmo2, and Gata1 expressions in blood islands (Fig. 3). In hematopoietic differentiation, Tal1 is thought to function through a complex with GATA1 and LMO2 in the process of differentiation from hemangioblasts to hemogenic endothelium, and the development of extraembryonic vasculature is cooperatively regulated by the limited members of transcriptional factors (1, 14, 16, 32, 41). The findings of direct regulations of Tal1, Lmo2, and Gata1 by ZFAT in hematopoietic progenitor cells and ZFAT-mediated expressions of Flk-1, Runx1, and Cd41 through the direct regulation of Tal1, Lmo2, and Gata1 expressions may shed light on the transcriptional network in the developmental program of blood islands. However, this study only suggests a role for ZFAT in the differentiation of primitive hematopoietic progenitor cells; thus, the in vitro yolk sac progenitor culture system (42) would be useful to demonstrate the precise roles for ZFAT in the differentiation of primitive hematopoietic cells.

FLI-1 was reported to be an upstream regulator for Tal1 and Lmo2 (43–46) and is also involved in immunological disease (47, 48). Thus, elucidation of the relation between ZFAT and FLI-1 might lead to a better understanding of the transcriptional network not only in hematopoietic differentiation, but also in immune regulation. Genetic variants in ZFAT were recently reported to be associated with height in the Japanese and Korean populations (49, 50), and development of Zfat−/− embryos were impaired by E8.5, suggesting that ZFAT might be involved in development of mesodermal cells; however, molecular functions of ZFAT in embryonic development and mesodermal lineage should await future studies.

Elucidation of ZFAT functions in hematopoiesis will lead to a better understanding of transcriptional networks in differentiation.
of the targeting construct were composed of 10.4 and 2.0 kb, respectively. Diphtheria-toxin A fragment cassette (DTA) flanked the 3’ short arm. The targeting vector was linearized with Sall and electroporated into ES cells and targeted ES clones were obtained. The mutant ES cells were microinjected into C57BL/6 blastocysts, as described previously (51), and the resultant male chimeras were mated with C57BL/6 mice. Zfat<sup>−/−</sup> mice were backcrossed six times and maintained in the genetic background of C57BL/6 mice. Heterozygous offspring were intercrossed to obtain Zfat<sup>−/+</sup> mice.

Genotyping. Genotyping was performed by standard PCR using the specific primer set (Dataset S1). PCR was done by GeneAmp PCR System 9700 (Applied Biosystems).

Histopathological Examination. Embryos with yolk sacs were fixed in 3.7% paraformaldehyde and embedded in paraffin. Sections (3 μm) were prepared at every 12-μm intervals throughout the tissues and were stained with H&E. Sections were analyzed using BioRevo BZ-9000 inverted-phase microscope at high-power magnification (×600) (Keyence).

Anti-ZFAT Monoclonal Antibody. Recombinant mouse ZFAT protein (amino acid residues 513–699) was expressed as a GST fusion protein using the pGEX6P-1 vector (GE Healthcare). The fusion protein was soluble in non-denaturing buffer and was purified with glutathione-Sepharose 4 fast flow (GE Healthcare). Clone M16, rat monoclonal antibody against the fusion protein, was established following a general protocol.

Real-Time qRT-PCR. Real-time qRT-PCR was performed as previously described (52). The primer set ID for the assay is listed in Dataset S1. Data were analyzed by the ΔΔCt method as previously described (53, 54).

Luciferase Assay. The pGL3 firefly reporter plasmid and Dual-Luciferase Reporter Assay System (Promega) were used according to the manufacturers’ instructions. The primer sets used are listed in Dataset S1. The probes (1-kb length) used for the assay were selected from the 5-kb upstream or 2-kb downstream region from a transcriptional start site for each gene. ZFAT protein fused with VP16-transcriptional activator-domain (AD-ZFAT) or the VP16-transcriptional activator-domain (AD) as a control was expressed in HEK293 cells in 96-well plates (Thermo Scientific). Luminescence was measured using GloMax 96 Microplate Luminometer (Promega).

ChIP-PCR Assay. ChIP-PCR assays for Tal1, Lmo2, and Gata1 were performed on yolk sacs at E7.5 and adult kidney as a control tissue, where ZFAT is rarely expressed (21). Kifap3 was used as a hematopoiesis-unrelated control gene. As for immunoprecipitation, 100 μg of anti-ZFAT monoclonal antibody M16 or Rat IgG (SM14LE, Acris) as a control were used. End-point PCR assays were performed at 35- and 42-cycled PCR. In ChIP-qPCR assay, the total amount of ChIP DNA was normalized by M16-ChIP DNA for ZFAT, TAL1, LMO2, GATA1, and CD41 were treated with 0.3% hydrogen peroxide in methanol for 30 min. Additionally, sections for CD41-staining were antigen-retrieved by TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Sections for staining of RUNX1 or FLK-1 were treated by citrate buffer (10 mM citric acid, pH 6.0) for the inactivation of endogenous peroxidase and antigen-retrieval. Sections were applied to immunohistochemical analysis using anti-ZFAT antibody (M16), anti-TAL1 antibody (ab75739, Abcam), anti-LMO2 antibody (G-16, Santa Cruz Biotechnology), anti-GATA1 antibody (N6, Santa Cruz Biotechnology), anti-FLK-1 antibody (SSB11, Cell Signaling Technology), anti-RUNX1 antibody (ab35962, Abcam), and anti-CD41 antibody (MwReg30, BD Pharmingen). Signals were detected using HISTOFINE simple stain MAX PO (Nichirei) and DAB substrate (Nichirei). Sections were counterstained with 1% methyl green (Muto Pure Chemicals) for staining of ZFAT, TAL1, LMO2, and GATA1 and with hematoxylin for staining of RUNX1, FLK-1, and CD41. Sections were examined using BioRevo BZ-9000 inverted-phase microscope (Keyence).

Statistical Analysis. Data are presented as means ± SDs of means of triplicate samples. Statistical analyses were performed with an unpaired Student’s t test. Differences at P < 0.05 are considered to be statistically significant.

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