Zinc Finger Protein, \textit{Hzf}, Is Required for Megakaryocyte Development and Hemostasis

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Abstract

Using an expression gene trapping strategy, we recently identified a novel gene, hematopoietic zinc finger (\textit{Hzf}), which encodes a protein containing three C\textsubscript{2}H\textsubscript{2}-type zinc fingers that is predominantly expressed in megakaryocytes. Here, we have examined the in vivo function of \textit{Hzf} by gene targeting and demonstrated that \textit{Hzf} is essential for megakaryopoiesis and hemostasis in vivo. \textit{Hzf}-deficient mice exhibited a pronounced tendency to rebleed and had reduced \textit{\alpha}-granule substances in both megakaryocytes and platelets. These mice also had large, faintly stained platelets, whereas the numbers of both megakaryocytes and platelets were normal. These results indicate that \textit{Hzf} plays important roles in regulating the synthesis of \textit{\alpha}-granule substances and/or their packing into \textit{\alpha}-granules during the process of megakaryopoiesis.

Key words: \textit{\alpha}-granules • hemorrhage • hemostasis • megakaryopoiesis • thrombopoiesis

Introduction

During hematopoiesis, anucleate platelets are generated from megakaryocytes and have an essential role in maintaining hemostasis in vivo. During megakaryocyte differentiation, megakaryocyte-restricted progenitor cells commence an unusual process, terminal endomitosis, in which DNA replication occurs but neither the nucleus nor the cell undergoes division \textsuperscript{(1)}. Hence, mature megakaryocytes are invariably polyploid, containing 4N to 64N of the normal diploid amount of DNA \textsuperscript{(2)}. Because platelets can produce only limited amounts of proteins, their cytoplasmic structures, including characteristic granules, are mostly derived from megakaryocytes \textsuperscript{(3)}. Morphologically, four distinct categories of granules differing in their internal constituents are produced by maturing megakaryocytes: \textit{\alpha}-granules, dense granules, lysosomal granules, and microperoxidosomal granules \textsuperscript{(3)}. The \textit{\alpha}-granules, which are the most numerous, contain various proteins, including platelet-derived growth factor (PDGF\textsuperscript{*}), platelet factor 4, and von Willebrand factor (vWF). These proteins are synthesized in megakaryocytes and then transported to \textit{\alpha}-granules. These packed \textit{\alpha}-granules are subsequently shed from megakaryocytes \textsuperscript{(4)}. Thus, the process of megakaryocyte differentiation involves a complex series of cellular processes that culminate in the generation of functionally mature platelets.

Genetic approaches involving gene targeting in mice have revealed several genes and their protein products that are essential for megakaryopoiesis. These include various transcription factors, such as p45 NF-E2 \textsuperscript{(5)}, mafG \textsuperscript{(6)}, GATA-1 \textsuperscript{(7)}, and Fli-1 \textsuperscript{(8)}. The transcription factor NF-E2 is a heterodimer of two proteins, p45 and p18, that are members of the Cap’n’Collar protein and Maf subfamilies, respectively \textsuperscript{(9, 10)}. Deficiency of the p45 NF-E2 gene blocks completion of megakaryopoiesis and results in the

\textsuperscript{*}Abbreviations used in this paper: AChE, acetylcholinesterase; ES, embryonic stem; GP, glycoprotein; GPS, gray platelet syndrome; Hzf, hematopoietic zinc finger; IRES, internal ribosome-entry site; MGDF, megakaryocyte growth and development factor; neo, neomycin resistant gene; PDGF, platelet-derived growth factor; TPO, thrombopoietin; vWF, von Willebrand factor.
complete absence of platelets (5), whereas the absence of MafG leads to hyperproliferation of megakaryocytes and mild thrombocytopenia (6). Mice carrying megakaryocyte-specific deletion of the GATA-1 zinc finger protein exhibit severely impaired cytoplasmic maturation of megakaryocytes and reduced platelet numbers (7). Mice carrying a null mutation in Flt-1, a member of the ETS family of winged helix-turn-helix transcription factors, exhibit a block in megakaryocyte differentiation (8).

The process of megakaryocyte differentiation also requires the interaction between thrombopoietin (TPO), also known as megakaryocyte growth and development factor (MGDF), and its cognate receptor c-Mpl (11–18). The ligand TPO/MGDF increases megakaryocyte ploidy at low concentrations while the final stage of platelet release is not dependent on TPO/MGDF (19). c-mpl–deficient mice exhibit thrombocytopenia and impaired megakaryopoiesis (20, 21). In addition, TPO/MGDF null mutant mice have decreased number of both platelets and megakaryocyte progenitors, and lower ploidy levels of megakaryocytes (22). Interestingly, both c-Mpl and TPO/MGDF null mice do not exhibit a bleeding disorder, suggesting that platelets produced from the abnormal megakaryocytes in these mice can function normally.

Using an approach that we have termed ‘expression gene trapping,’ we have previously identified and mutated in embryonic stem (ES) cells a number of genes that are expressed in hematopoietic and/or endothelial cells in vitro and in vivo (23, 24). One of these genes, hematopoietic zinc finger (Hzf), encodes a novel zinc finger protein which is predominantly expressed in megakaryocytes (24). Mice homozygous for the gene trapped allele of Hzf (Hzf<sup>−/−</sup>) exhibit no obvious phenotype (24). Transcripts corresponding to the Hzf gene were detected in Hzf<sup>+/+</sup> mice, consistent with the possibility that the trapped Hzf allele generated in our previous study was not a null allele. Therefore, to investigate the in vivo role of Hzf, we generated mice with a null Hzf mutation by gene targeting. Here we describe the essential function of Hzf in α-granule formation and megakaryopoiesis.

Materials and Methods

**Generation of Hzf<sup>−/−</sup> Mutant Mice.** DNA fragments corresponding to the murine Hzf gene fragments were cloned from a 129/Sv genomic DNA library using a mouse Hzf cDNA probe (24). 17 overlapping phage genomic clones containing exons encoding three zinc finger domains were isolated. A targeting vector was designed to replace a 5.5-kb genomic fragment including the exons encoding three zinc finger domains with an internal ribosome-entry site (IRES) LaZ and a neomycin resistant gene (neo). IRES LaZ and neo were inserted in the pPNT targeting vector in the sense orientation to Hzf transcription, such that IRES LaZ was flanked on the 3′ side by 1.6 kb of genomic DNA and that neo was flanked on the 5′ side by 4.5 kb of genomic DNA. The targeting vector was linearized with NotI and electroporated into R1 ES cells. After positive-negative selection with gancyclovir and G418, 800 surviving clones were picked and screened by Southern blot analysis. Three out of six homologous recombinant ES clones were aggregated into CD1 blastomeres and transferred to foster mothers to generate chimeras. Chimeric mice were mated with CD1 females, and germline transmission of the mutant allele was verified by PCR and Southern blot analysis of ear punched tissues and tail DNA from F1 offspring. A primer “a” and “b” specific for the Hzf gene (5′-GGACCTGTACAAAAAGCTGT-3′ and 5′-GCTTGGTC-TACAGATGATT-3′, respectively) and a primer “c” specific for the IRES gene (5′-GGAGGGAGAGGGCCGAATT-3′) were used in PCR analysis. Germline transmission of the targeted Hzf allele was achieved for three independent ES clones. F2 offspring from heterozygous intercrosses were genotyped by Southern blotting. Mutant mice derived from these targeted ES cells showed the identical phenotype.

**Histological Analysis.** Organs were isolated in ice-cold PBS 3 wk after birth, fixed overnight in 4% paraformaldehyde at 4°C for brains, fixed at least 1 wk in 4% paraformaldehyde at 4°C, dehydrated, and embedded in paraffin. Sections 5-μm thick were cut and stained with hematoxylin and eosin.

**Hematological Analysis.** 4-wk-old mice were anesthetized with methoxyflurane. Peripheral blood was collected by heparinized capillary puncture of the retroorbital venous plexus into a tube containing 5 μl of 0.5 M EDTA, pH 8.0 (Becton Dickinson). Peripheral blood cell counts were determined with MASCOT automated hematology system (CDC Technologies). Blood smears and bone marrow smears were stained with Wright-Giemsa stain.

The preparation of platelets was performed essentially as described previously (25). Peripheral blood, which was prepared as described above, was centrifuged at 300 X g for 10 min at room temperature to obtain platelet-rich plasma. The platelets were washed three times with PBS by centrifugation at 1,300 X g for 10 min and were used immediately for Western immunoblot analysis.

**Bleeding Time Assays.** Bleeding times were measured as described previously (26, 27) using 4-wk-old mice. 2 mm of the tip at the tail was cut with a sharp scalpel blade, the tail was placed in a solution of saline at 37°C, and the time for the flow of blood to cease was recorded. Genotyping of the animals took place after this procedure so that tails were intact for bleeding-time measurements. If bleeding restarted within 1 min, this result was recorded as a rebleed and taken to indicate an unstable hemostatic event.

**Measurements of Megakaryocyte Frequency and DNA Content.** DNA distribution of megakaryocytes in unfractionated bone marrow cells was determined as described previously (2, 28). Bone marrow cells were harvested from Hzf mutant and control mice.

**CFU-Mk Assay.** Megakaryocyte progenitor cells, in the bone marrow from Hzf mutant and control mice, were assayed in collagen-based media (StemCell Technologies) supplemented with recombinant human TPO (50 ng/ml), IL-6 (20 ng/ml), IL-11 (50 ng/ml), and recombinant mouse IL-3 (10 ng/ml). Colonies were stained for acetylcholinesterase (AChE) activity counted after 6 d incubation.

**Ultrastructural Studies.** Freshly excised spleens obtained from 3–5-wk-old Hzf<sup>−/−</sup> and control mice were fixed in 2% glutaraldehyde (Canemco, Inc.) in 0.1 M sodium cacodylate buffer, pH 7.3, (Canemco, Inc.). Samples were washed with 0.1 M sodium cacodylate buffer, pH 7.3, postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.3, dehydrated in graded ethanol, and embedded in spur epoxy resin (Canemco, Inc.). Embedded tissues were sectioned with an RMC 6000 ultramicrotome, (RMC-EM), stained with uranyl acetate, lead citrate,
and examined in a Philips CM 100 electron microscope (Philips Electron Optics).

For platelet analysis, blood was collected into a syringe with prewarmed 2% glutaraldehyde (Canemco, Inc.) in 0.1 M sodium cacodylate buffer, pH 7.3 (Canemco, Inc.), from the inferior vena cava of anesthetized mice. Immediately, the syringe was inverted several times and was set at room temperature for 2 h. Platelet-rich plasma was prepared as described above and then analyzed, as described previously (29).

For quantitative estimation of vacant α-granules in platelets, dense and vacant α-granules were counted in platelets from Hzf−/− and +/+ mice by observers blinded to the experiment. The frequency of vacant α-granules was calculated from the number of detected vacant α-granules divided by that of dense α-granules.

To estimate the levels of PDGF-A in megakaryocytes, freshly excised spleens obtained from 3–5-wk-old Hzf−/− and control mice were fixed in 0.1 M sodium cacodylate buffer, pH 7.3 containing a combination of 0.1% glutaraldehyde (Canemco, Inc.) and 4% paraformaldehyde (Canemco, Inc.). The samples were washed with the same buffer, dehydrated with graded ethanol, infiltrated in lowcryl (Canemco, Inc.), and polymerized under UV at –20°C overnight. The sections were rinsed with PBS containing 0.15% glycine and 0.5% BSA, washed with 0.5% BSA in PBS, and then reacted with anti–PDGF-A antibody (Santa Cruz Biotechnology, Inc.) for 1 h at 22°C. After washing with 0.5% BSA in PBS, the sections were treated with anti–rabbit IgG conjugated with 10-nm gold (Amersham Pharmacia Biotech). After washing with 0.5% BSA in PBS, PBS, and H2O, the sections were stained with uranyl acetate, lead citrate, and examined in a Philips CM 100 electron microscope (Philips Electron Optics).

Electron Optics).

To determine the stage of neonatal development affected by the Hzf mutation, neonatal genotyping was performed. Of 300 offspring derived from heterozygous matings, 1-wk-old Hzf−/− mice were fertile and were intercrossed to generate homozygous Hzf−/− mice (Fig. 1 B). The null mutation of Hzf was confirmed by the absence of Hzf expression, as determined by Northern blot analysis of RNA extracted from brains of Hzf mutant mice at 3 wk after birth (Fig. 1 C).

**Results**

**Generation of Hzf−/− Mutant Mice.** The Hzf gene was disrupted in murine ES cells using a targeting vector in which the exons encoding three zinc finger motifs were deleted (Materials and Methods, and Fig. 1 A). The targeting vector was electroporated into R1 ES cells. After positive-negative selection and genotyping by Southern blot analysis as described in Materials and Methods, six independent clones were identified as heterozygous for the targeted mutation at the Hzf locus. Three out of the six heterozygous mutant ES clones were used to generate chimeric mice. Chimeras were backcrossed to CD1 mice to generate mice heterozygous for the Hzf mutation. Heterozygous Hzf−/+ mice were fertile and were intercrossed to generate homozygous Hzf−/− mice (Fig. 1 B). The null mutation of Hzf was confirmed by the absence of Hzf expression, as determined by Northern blot analysis of RNA extracted from brains of Hzf mutant mice at 3 wk after birth (Fig. 1 C).

**Hemorrhage and Neonatal Lethality of Hzf−/− Mice.** Initial examination of the progeny from Hzf−/+ parents revealed a reduced frequency of Hzf−/− mice at 3 wk of age. To determine the stage of neonatal development affected by the Hzf mutation, neonatal genotyping was performed. Of 300 offspring derived from heterozygous matings, 1-wk-old Hzf−/− pups were viable, usually of normal size, and were present in the expected Mendelian frequency. However, thereafter, especially between 2–3 wk after birth, the percentage of homozygotes decreased (Table I).

As shown in Fig. 2 A, the size of 4-wk-old Hzf−/− mice were clearly distinguishable from their wild-type littermates. Moreover, there was marked internal hemorrhaging evident primarily in the brains and gastrointestinal tracts (Fig. 2 B, and data not shown) in mutant neonates. This hemorrhaging was the likely cause of neonatal lethality. The surviving Hzf−/− mice of either sex were fertile (data not shown).

**Unstable Hemostatic Plug Formation and Abnormal Platelet Morphology in Hzf−/− Mice.** To determine whether the hemorrhaging phenotype was related to defective hemostasis in Hzf−/− mice, we compared the bleeding times and rebleeding occurrences of Hzf−/+, Hzf−/−, and Hzf−/− mice. There were no significant differences in bleeding
The Essential Function of Hzf in α-Granule Formation and Megakaryopoiesis

Times when Hzf<sup>−/−</sup> mice were compared with wild-type and Hzf<sup>+/−</sup> heterozygous mice (Fig. 3 A). However, Hzf<sup>−/−</sup> mice rebled after transient hemostasis relative to the rebleeding frequency in wild-type and Hzf<sup>+/−</sup> mice (Fig. 3 B). These results indicate that Hzf-deficiency leads to unstable hemostatic plug formation in vivo.

The defects in hemostasis in Hzf<sup>−/−</sup> mice led us to analyze hematologic parameters in Hzf<sup>−/−</sup> mice. Surprisingly, complete hematologic profiles revealed no significant differences in platelet counts and differential counts between Hzf<sup>−/−</sup> mice and their control littermates (data not shown).

To address the defect of hemostasis in detail, we next examined the morphology of blood cells in peripheral blood smears from Hzf<sup>−/−</sup> mice. Normal platelet sizes from control mice were observed by comparison with the diameter of erythrocytes (Fig. 3 C). Large faint-colored platelets were occasionally observed in blood smears from Hzf<sup>−/−</sup> mice (Fig. 3 D). Although the frequency of these pale ghostlike platelets was low, they were never observed in peripheral blood smears from Hzf<sup>+/−</sup> mice. To examine these morphological differences more closely, we performed ultrastructural analysis of peripheral platelets from Hzf<sup>−/−</sup> and control mice. As shown in Fig. 3 E, platelets from wild-type mice had easily identified dense α-granules. In contrast, Hzf<sup>−/−</sup> platelets had significantly reduced numbers of α-granules, with many vacuoles (Fig. 3 F; vacant frequency of α-granules, 9.4 ± 15.4 [controls] vs. 80.6 ± 12.3 [Hzf<sup>−/−</sup>] n = 11, P < 0.0001).

Numerous procoagulant substances are packed in platelet α-granules, and the release of granule contents is important for platelet function (33). vWF, one of the procoagulant substances contained in platelet α-granules, is associated

Table 1. Genotype Analysis of the Progeny from Hzf Heterozygous Intercrosses

| Stage | Number of mice of each genotype | Percentage |
|-------|---------------------------------|------------|
|       | +/+    | +/−   | −/−   | Total  | −/−   |          |
| 1 wk  | 10     | 22    | 12    | 44     | 27    |
| 2 wk  | 6      | 22    | 8     | 36     | 22    |
| 3 wk  | 32     | 90    | 24    | 146    | 16    |
| 4 wk  | 16     | 27    | 10    | 53     | 19    |
| Total | 295    |       |       |        |       |

Breeding pairs were set up between Hzf<sup>+/−</sup> males and females (129 × CD1 mixed background). Tails of newborn and weaning pups were collected at the time of 1, 2, 3, and 4 wk after birth. The genotypes were determined by PCR analyses, as described in Materials and Methods. Each genotyping was confirmed by Southern blot analyses.

Figure 1. Targeted disruption of the Hzf locus. (A) A portion of the mouse 129/Sv Hzf wild-type locus (top) showing exons (open boxes) and a 6.5-kb PstI fragment in the wild-type allele. The targeting vector (middle) was designed to replace exons encoding three zinc finger domains with an IRES <sup>Lac</sup>Z (closed box) and a neo (hatched box). The mutated Hzf locus (bottom) contains a 4.5-kb PstI fragment. The positions of the 5′ flanking and 3′ flanking probes used for Southern blot analysis are shown. Positions of PCR primers for the wild-type allele (a and b) and the mutant allele (c) are also shown. P, Ks, Rf, Kp, C, X, and B represent PstI, KspI, EcoRI, KpnI, Clal, Xhol, and BamHI sites, respectively. (B) Genomic DNA was isolated from Hzf<sup>−/−</sup>, Hzf<sup>+/−</sup>, and Hzf<sup>+/−</sup> mice, digested with PstI and analyzed by Southern blot analysis. Wild-type (6.5-kb) and mutant (4.5-kb) bands are indicated. (C) Northern blot analysis of Hzf expression. Total RNA extracted from the brains (40 μg per lane) was analyzed for the expression of transcripts corresponding to the Hzf gene.
with attachment of platelets onto damaged blood vessels, thereby reinforcing the stability of the plug, and activating the coagulation pathway that leads to blood clot formation (33). To investigate whether $\alpha$-granules in $Hzf^{-/-}$ platelets contain $\alpha$-granule substances, we examined the protein levels of platelet-vWF, a key GP involved in coagulation. As shown in Fig. 3 G, the levels of vWF were dramatically reduced in $Hzf^{-/-}$ platelets compared with that observed in platelets from wild-type mice.

Number and DNA Ploidy Levels of Megakaryocytes and Megakaryocyte Progenitors from $Hzf^{-/-}$ Mice. To explore in greater detail the nature of the platelet defects described above, we characterized megakaryocytes from $Hzf^{-/-}$ mutant mice. No differences in the numbers of megakaryocytes were observed in the spleens and bone marrows of $Hzf^{-/-}$ mutant mice, as determined morphologically and by flow cytometric analysis (Fig. 4 A–E).

Megakaryocyte development initiates with proliferation of precursor cells. These precursor cells then undergo endomitosis, followed by cytoplasmic maturation and organization, culminating in platelet release (1). To determine whether the process of endomitosis was affected by the absence of $Hzf$, we performed DNA ploidy analysis on bone marrow megakaryocytes. As shown in Fig. 4 F, no significant differences in DNA ploidy patterns were observed between wild-type, heterozygous, and homozygous mutant mice. Next examined the levels of megakaryocyte progenitor cells (CFU-Mk) in the bone marrow of $Hzf$ wild-type and mutant mice by plating bone marrow cells in semi-solid media in the presence of TPO/MGDF. Normal numbers of megakaryocyte progenitors were observed in $Hzf$-deficient mice (data not shown).

Ultrastructural Analysis of Megakaryocytic Abnormality. To characterize further the maturation defect in $Hzf^{-/-}$ mice, we performed electron microscopic analysis of megakaryocytes derived from $Hzf^{-/-}$ mice. As expected, megakaryocytes from both $Hzf^{+/+}$ and $Hzf^{-/-}$ mice exhibited hyperlobulated nuclei (Fig. 5 A and B). Megakaryocytes from $Hzf^{+/+}$ mice exhibited well-formed platelet fields, demarcation membrane systems, and numerous $\alpha$-granules (Fig. 5 C); in contrast, $Hzf^{-/-}$ megakaryocytes displayed a dramatic reduction in the number of $\alpha$-granules, with many vacuoles in their cytoplasm (Fig. 5 D). Furthermore, detailed ultrastructural analysis revealed that $\alpha$-granules in megakaryocytes from control mice were dense, readily identifiable, and exhibited distinct zones: dense nucleoid regions and diffuse granular matrixes (Fig. 5 E). In contrast, the $\alpha$-granules in $Hzf^{-/-}$ megakaryocytes were pale and vacant, although membranes of $\alpha$-granules could be identified (Fig. 5 F). In addition, the majority of $Hzf^{-/-}$ $\alpha$-granules were devoid of diffuse granular matrixes, although dense nucleoid regions were observed only in some $\alpha$-granules in $Hzf^{-/-}$ megakaryocytes (Fig. 5 F). Taken together, these data suggest that the process of endomitosis is not affected by $Hzf$-deficiency. However, $Hzf$-deficiency appears to block the process of terminal maturation of megakaryocytes, affecting the assembly of $\alpha$-granule substances in $\alpha$-granules of megakaryocytes.

Reduced Concentrations of $\alpha$-Granule Substances in $Hzf^{-/-}$ Megakaryocytes. Previous biochemical and immunoelectron microscopic analyses have shown that megakaryocytic $\alpha$-granules contain numerous substances essential to the coagulation system (3). To examine the effect of $Hzf$-

![Figure 2. Growth retardation and hemorrhage in $Hzf^{-/-}$ mice. (A) A $Hzf^{-/-}$ mouse compared with a control littermate 4 wk after birth, demonstrating slightly smaller size. (B) Transverse section of the brain of a 3-wk-old $Hzf^{-/-}$ mouse, demonstrating extensive hemorrhage. Original magnification: X100.](image-url)
Figure 3. Unstable plug formation and abnormal platelet morphology in Hzf<sup>-/-</sup> mice. (A) Time taken for initial cessation of bleeding. Each symbol represents a single animal (<sup>Hzf</sup><sup>+/-</sup> mice, open squares, n = 22; <sup>Hzf</sup><sup>-/-</sup> mice, closed triangles, n = 41; <sup>Hzf</sup><sup>-/-</sup> mice, closed squares, n = 19). Bars indicate the average bleeding time for each genotype (<sup>Hzf</sup><sup>+/-</sup> mice, 177.0 s; <sup>Hzf</sup><sup>-/-</sup> mice, 162.8 s; <sup>Hzf</sup><sup>-/-</sup> mice, 290.5 s). Box and whisker graphs indicate the median, first and third quartiles, and the total range of bleeding times for each genotype. (B) Rebleeding occurrences in <sup>Hzf</sup><sup>+/-</sup> (white column), <sup>Hzf</sup><sup>-/-</sup> (gray column), and <sup>Hzf</sup><sup>-/-</sup> (black column) mice. Rebleeding occurred in 68.4% of <sup>Hzf</sup><sup>-/-</sup> mice (n = 19) as compared with 36.4% (n = 22) of <sup>Hzf</sup><sup>+/-</sup> and 36.6% (n = 41) of <sup>Hzf</sup><sup>-/-</sup> mice. (C) Peripheral blood smear from <sup>Hzf</sup><sup>+/-</sup> mouse demonstrates a normal platelet (arrowhead). Original magnification: ×100. (D) Peripheral blood smear from <sup>Hzf</sup><sup>-/-</sup> mouse reveals a large, faintly stained platelet. Representative platelet is highlighted by an arrowhead. Original magnification: ×100. (E) Ultrastructural morphology of platelets of peripheral blood from two individual <sup>Hzf</sup><sup>+/-</sup> mice demonstrates normal α-granules. Scale bars, 500 nm. (F) Ultrastructural analysis of abnormal platelet morphology in <sup>Hzf</sup><sup>-/-</sup> mice. Platelets from two individual <sup>Hzf</sup><sup>-/-</sup> mouse exhibit numerous vacuoles with reduced α-granules. Scale bars, 500 nm. (G) Reduced platelet-vWF in <sup>Hzf</sup><sup>-/-</sup> mice. Whole washed-platelets from control littermates and <sup>Hzf</sup> mutant mice were used to assay protein levels of vWF. <sup>Hzf</sup><sup>-/-</sup> platelets have reduced protein levels of vWF. GPIIb, which is a GP bound to plasma membranes of platelets, was used to verify equivalent sample loading. Molecular size markers are indicated on the left in kilodaltons. This data was representative with comparable results.
Figure 4. The presence of megakaryocytes and normal aspects of megakaryocyte maturation in \(Hzf^{-/-}\) mice. (A and B) Transverse sections through the spleens from \(Hzf^{+/+}\) (A) and \(Hzf^{-/-}\) (B) mice, demonstrating the presence of megakaryocytes in each. A and B, original magnification: \(\times 100\). (C and D) Microscopic examination of bone marrow smears from \(Hzf^{+/+}\) (C) and \(Hzf^{-/-}\) (D) mice, showing the presence of megakaryocytes in each. C and D, original magnification: \(\times 100\). (E) Flow cytometric analysis confirms no significant difference of megakaryocyte frequency between control and \(Hzf^{-/-}\) mice. The proportion of megakaryocytes to bone marrow cells was performed after staining with 4A5 mAb, to murine megakaryocytes. Six mice, which were age- and sex-matched, were analyzed in each genotype with FACScan™. Results are presented as the means \(\pm SD\). (F) Representative megakaryocyte DNA ploidy in \(Hzf^{-/-}\) mice. The proportion of cells in each ploidy class was determined by PI-staining after gated on 4A5-positive cells with FACScan™, demonstrating no effect of \(Hzf\) deficiency on endomitosis. Six mice, which were age- and sex-matched, were analyzed in each genotype. This data was representative with comparable results.
deficiency on production of α-granule substances in megakaryocytes, we analyzed the levels of vWF, fibrinogen, PDGF-A, and PDGF-B by Western immunoblotting. As shown in Fig. 6 A, the levels of adhesive GP, vWF and fibrinogen, were both dramatically reduced in Hzf−/− bone marrow. In addition, Hzf-deficient bone marrow had significantly decreased protein levels of PDGF-A and PDGF-B. To examine further the effect of Hzf deficiency on the levels of α-granule substances in megakaryocytes, we performed immunogold labeling followed by transmission electron microscopy. Using this strategy, we observed concentrated gold particles corresponding to PDGF-A in α-granules and on vesicles in the cytoplasm of Hzf+/+ megakaryocytes (Fig. 6 B; numbers of PDGF-A gold particles in Hzf+/+ α-granules: 19.8 ± 5.7%, n = 5). In contrast, PDGF-A was not detected in α-granules from Hzf−/− megakaryocytes although a few gold particles were present on vesicles (Fig. 6 C; PDGF-A gold particles in Hzf−/− α-granules: 0.8 ± 1.8%, n = 5, P = 0.001).

Transcript Levels of Megakaryocyte-specific Genes. To determine whether the structural defects observed in megakaryocytes from Hzf−/− mice were the consequence of an earlier block in megakaryocyte maturation, we examined the expression of two genes associated with megakaryopoiesis (29). In this experiment, bone marrow cells from mutant and wild-type male mice were used as the source of mRNAs for comparison of expression levels of the coadhesive GP, vWF, and the cell surface receptor, GPIIb. As shown in Fig. 7, Hzf−/− megakaryocytes expressed an equivalent level of RNA transcripts corresponding to GPIIb as Hzf+/+ cells. In contrast, transcripts corresponding to vWF gene were significantly reduced in Hzf−/− cells relative to that observed in wild-type cells.

Discussion

We have previously identified a novel gene, Hzf, by a gene trapping strategy in ES cells (24). Hzf encodes a novel protein containing three C2H2-type zinc fingers and is predominantly expressed in megakaryocytes within the hematopoietic system. In this study, we have investigated the in vivo role of Hzf in mice using gene targeting. Hzf deficiency causes a block in the formation of
Megakaryocyte Development and α-Granule Formation in Hzf-deficient Mice. Megakaryocytes in Hzf<sup>−/−</sup> mice were produced in normal number and appeared to undergo complete differentiation based on DNA ploidy analysis. α-granules in megakaryocytes, abnormal platelet morphology, and disruption of hemostasis in vivo. These findings identify Hzf as a novel regulator of megakaryopoiesis and hemostasis.

Figure 6. Reduced concentrations of α-granule substances in megakaryocytes from Hzf<sup>−/−</sup> mice. (A) Western immunoblotting analysis reveals reduced levels of vWF, fibrinogen, PDGF-A, and PDGF-B from bone marrow cells in Hzf<sup>−/−</sup> mice. The AChE, a housekeeping protein expressed specifically in megakaryocytes, was used to verify equivalent sample loading. This data was representative with comparable results. (B and C) Immunogold localization of PDGF-A on thin sections of megakaryocytes in the spleens of Hzf<sup>+/+</sup> (B) and Hzf<sup>−/−</sup> (C) mice. (B) Concentrated gold particles are located in the α-granules (arrowheads) and vesicles (arrows) of cytoplasm in megakaryocytes from Hzf<sup>+/+</sup> mice. Scale bar, 500 nm. (C) A few gold particles are present on the vesicles (arrows) of cytoplasm in megakaryocytes from Hzf<sup>−/−</sup> mice. Partially empty α-granules and vacant α-granules are also observed. Scale bar, 500 nm.
Platelets from HZF-/- mice have dramatically reduced levels of the coadhesive GPs, vWF and fibrinogen. vWF and fibrinogen contribute to hemostasis by producing a platelet plug and then reinforcing the plug by converting fibrinogen to fibrin strands, inducing activation of the coagulation pathway (33, 35). It is also known that GPIbα, which is a heterodimer of GPIbα and GPIbβ, has an important role in the adherence of platelets to subendothelial surfaces with binding to vWF (35–37). Indeed, vWF-/- platelets exhibit delayed adhesion to vessel walls and they failed to form thrombus in arterioles in vivo (38, 39). Thus, the instability of plug formation in HZF-deficient mice may result from a failure of platelet adhesion onto damaged blood vessel walls, a process which is mediated by the binding of vWF to GPIb.

Gray Platelet Syndrome and HZF Deficiency. It is of interest to note that the phenotype of the HZF-/- mice described here resembles that observed in patients with gray platelet syndrome (GPS), a human congenital bleeding disorder (40, 41). Platelets from patients with GPS appear gray and are markedly deficient in morphologically dense α-granule substances. Interestingly, membranes of α-granules were observed in HZF-/- megakaryocytes although a few gold particles were present on vesicles. Interestingly, immunoelectron microscopic analysis shows that vWF is detected on small vesicles. Electronmicroscopic and biochemical analyses revealed the presence of polyploid nuclei and the characteristic demarcation membrane system in megakaryocytes lacking HZF. Together, these findings suggest that HZF-deficiency does not cause a block in the intermediate and late stages of megakaryocyte differentiation. The numbers of megakaryocyte progenitor cells were also not affected by the absence of HZF, suggesting that HZF is not essential for the early stages of megakaryocyte differentiation.

Electronmicroscopic analysis revealed the presence of polyploid nuclei and the characteristic demarcation membrane system in megakaryocytes lacking HZF. Together, these findings suggest that HZF-deficiency does not cause a block in the intermediate and late stages of megakaryocyte differentiation. The numbers of megakaryocyte progenitor cells were also not affected by the absence of HZF, suggesting that HZF is not essential for the early stages of megakaryocyte differentiation.

The electronmicroscopic and biochemical analyses revealed that platelets and megakaryocytes from HZF-/- mice contain numerous vacuoles with reduced amount of α-granule substances. Interestingly, membranes of α-granules were observed in HZF-/- megakaryocytes. In addition, PDGF-A was not detected in the α-granules of HZF-/- megakaryocytes although a few gold particles were present on vesicles. Interestingly, immunoelectron microscopic analysis shows that vWF is detected on small vesicles of human cultured megakaryocytes (34). These findings suggest that HZF is required for the synthesis of α-granule substances and/or the trafficking of these substances into α-granule bags during the process of megakaryocyte maturation.

HZF in Platelet Morphogenesis and Hemostasis. Most HZF-/- mice were smaller than their wild-type littersmates, presumably as a result of sustained hemorrhage during a period of rapid growth. Because the numbers of platelets in HZF mutant mice were within normal range, HZF does not appear to impair the release of platelets from megakaryocytes. Nevertheless, circulating large, faintly stained platelets containing numerous empty vacuoles were present in the peripheral blood of HZF-/- mice.
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