The Role of Zinc Finger Protein 521/Early Hematopoietic Zinc Finger Protein in Erythroid Cell Differentiation

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ZNF521 (zinc finger protein 521) is a transcription factor with an N-terminal transcriptional repressor motif and 30 zinc finger domains. Although a high expression level of ZNF521 in human CD34+ progenitors and hematopoietic malignancies has been demonstrated, the functional role of ZNF521 in hematopoietic cell differentiation has not been clarified. In this study, we analyzed the role of ZNF521 in erythroid cell differentiation using the short hairpin RNA (shRNA)-mediated gene silencing method. Down-regulation of ZNF521 mediated by transient expression of shRNA for ZNF521 resulted in increased synthesis of hemoglobin in K562 and HEL cell lines as compared with control cells. K562-derived clones in which ZNF521 was constitutively silenced by shRNA also showed marked synthesis of hemoglobin and an increased expression level of glycophorin A. Since GATA-1 is the key regulator of erythroid differentiation, the effect of ZNF521 on transcription activity of GATA-1 was analyzed using a luciferase assay. GATA-1 activity was markedly inhibited by ZNF521 in a dose-dependent manner. Deletion analysis of ZNF521 showed that the repressive effect requires an N-terminal repression motif. Furthermore, the direct interaction of ZNF521 with GATA-1 was demonstrated. These results indicate that ZNF521 modulates erythroid cell differentiation through direct binding with GATA-1.

ZNF521 (zinc finger protein 521), formerly designated as EHZF (early hematopoietic zinc finger protein), the human homolog of mouse EVI3/Zfp521, is a transcription factor with 30 Krüppel-like zinc finger (ZF) domains (1). The gene encoding EVI3 was initially identified as a common site of retroviral integration in murine AKXD B-cell lymphomas (2). The integration site of Evi3 is located upstream of the first translated exon and results in up-regulation of Ev3 by a promoter insertion mechanism. Although Ev3 expression in normal B cells is ontogenetically and developmentally regulated, alteration of Ev3 expression induced by retrovirus insertion might be involved in the genesis of murine B-cell lymphoma.

ZNF423/OAZ (olfactory-associated zinc finger protein), another 30-ZF protein that displays highly homology with ZNF521, was identified as a protein interacting with EBF1 (early B-cell factor 1) in a yeast two-hybrid screen (3). EBF1, previously designated as OLF-1, is a basic helix-loop-helix transcription factor required for development of the olfactory epithelium and B-cell lineage commitment (4, 5). ZNF423 inhibits EBF1-mediated trans-activation. Although ZNF423 is not expressed in normal B cells, ZNF423 expressed ectopically at a high level following retroviral integration has been detected in murine AKXD B-cell lymphomas (6). Although direct interaction between ZNF521 and EBF1 has not been demonstrated, ZNF521 also inhibits the transcriptional activity of EBF1. These findings indicate that aberrant expression of ZNF521 and/or ZNF423 results in inhibition of B-cell differentiation and induction of B-cell malignancies by modulating the transcriptional activities of EBF1 (1, 7). Genome-wide analysis has detected frequent monoallelic deletion of EBF1 or PAX5, which is downstream of EBF1, in B-progenitor acute lymphoblastic leukemia (8). In addition, fusion of PAX5 to ZNF521 resulting in formation of PAX5-ZNF521 fusion protein has been found in B-cell acute lymphoblastic leukemia (8).

Both ZNF521 and ZNF423 contain an N-terminal 12-amino acid motif, which is associated with transcriptional repression and conserved among other ZF transcriptional repressors, including FOG-1, FOG-2, BCL11A, and SALL family members (9–11). ZNF423 has also been identified as a cofactor of Smad protein in bone morphogenetic protein (BMP) signaling. ZNF423 binds to Smad1 and Smad4 in response to BMP2 and activates transcription of BMP target genes, such as Xenopus Vent-2 (12) and murine Smad6 (13). On the basis of structural and sequential homology, ZNF521 is speculated to have func-
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Among them, a large amount of ZNF521 transcript is not detected in peripheral blood leukocytes. On the other hand, a significant expression level of ZNF521 mRNA is also expressed in several hematopoietic cell lines derived from acute myelogenous leukemia and chronic myelogenous leukemia at blast crisis. Among them, a large amount of ZNF521 transcript is detected in K562 and HEL cell lines (1), both of which have potential for differentiation to erythroid lineage. These findings strongly suggest that ZNF521 plays an important role in hematopoietic differentiation and leukemogenesis. On the basis of this concept, we investigated the function of ZNF521 in hematopoietic differentiation by using shRNA-mediated silencing of ZNF521 in these cell lines. The data we obtained implicate ZNF521 in the suppression of erythroid differentiation of hematopoietic progenitors via inhibition of the transcriptional activity of GATA-1. Furthermore, it was suggested that ZNF521 interacts directly with GATA-1 via the C-terminal ZF domains and suppresses the transcriptional activity of GATA-1 through the N-terminal suppression domain.

EXPERIMENTAL PROCEDURES

Cells and Cell Lines—The K562 and HEL cell lines were cultured in RPMI1640 medium supplemented with 10% fetal calf serum, and the 293T and NIH3T3 cell lines were cultured in RPMI1640 medium supplemented with 10% fetal calf serum in a 5% CO2 incubator at 37 °C.

Plasmids—Two segments of ZNF521 cDNA (start codon to the XhoI site and the XhoI site to the terminal codon) were generated by reverse transcription-PCR. Total RNA was isolated from 2 × 10^7 K562 cells using an RNeasy minikit (Qiagen, Valencia, CA), and cDNA was generated using an Omniscript reverse transcriptase kit (Qiagen). Reverse transcription-PCR was performed with PrimeSTAR HS DNA polymerase (TaKaRa) reverse transcriptase kit (Qiagen). Reverse transcription-PCR was performed with PrimeSTAR HS DNA polymerase (TaKaRa, Tokyo, Japan) for 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min. The following primers were used for PCR: ZNF521 5’ fragment, forward (5’-TGCAGCGAGATGTC-TGCAGGAAGCAAG-3’) and reverse (5’-TTGCTTGTTT-ACTCGAGGCCAGCTCATG-3’); ZNF521 3’ fragment, forward (5’-CTGGTCTCCCTGAGTAAAAGGCTCAAAAG-3’) and reverse (5’-GGGCGGCGGCTACTGTGCTGTGTCGCTG-3’). The PCR products were cloned into the pUC 118 vector using a Mighty Cloning Kit (TaKaRa) and sequenced.

The full-length ZNF521 cDNA was constructed by ligation of the cDNA fragments and cloned into the pcDNA3 vector (Invitrogen) or p3FLAG-CMV-7.1 expression vector (Sigma). The pCDNA3 3’×FLAG/ZNF521 was constructed by PCR using the primers to generate a 5’-cDNA fragment encoding the FLAG epitope fused to the N terminus of ZNF521 protein.

pGL3-MaP-Luc, a plasmid in which the 3×GATA-1 binding site is inserted just upstream of the JunB promoter in JunB-MP-Luc (15), was kindly provided by Dr. I. Matsumura. Human GATA-1 cDNA was kindly provided by Dr. S. Takahashi. The pcDNA3 myc/GATA-1 was constructed by PCR using the primers to generate a 5’ cDNA fragment encoding the Myc epitope fused to the N terminus of GATA-1 protein.

For the establishment of stable cell lines, K562 cells were transferred to culture medium containing G418 (Invitrogen) at a concentration of 2 mg/ml 48 h after transfection. Resistant clones were then isolated and expanded over several weeks. The levels of GATA-1 expression in established clone cells were examined by Western blotting.

shRNA Plasmids and Transfection—Two kinds of shRNA for ZNF521, GGGACAAGAAGTACCACTG and GGAAGAGCATATTAGACAG, were designed, and their expression vectors were constructed using pcPURU6β vector (TaKaRa). These vectors were transfected into K562 cells using a Nucleofector device (Amaxa Biosystems GmbH, Cologne, Germany).

For the establishment of stable cell lines, K562 cells were transferred to culture medium containing puromycin (Invitrogen) at a concentration of 5 μg/ml 48 h after transfection. Resistant clones were then isolated and expanded over several weeks. The expression levels of ZNF521 mRNA were examined by reverse transcription-PCR and confirmed by Northern blot analysis and real-time PCR.

Quantitative Real Time PCR—Quantitative real-time PCR of ZNF521 mRNA (Hs01031325_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (4326317E) as an internal control was performed using the TaqMan Gene Expression assay (Applied Biosystems, Foster City, CA) in accordance with the manufacturer’s instructions using an Applied Biosystems 7500 real-time PCR system (Applied Biosystems). The expression level of ZNF521 mRNA was corrected by reference to that of GAPDH mRNA, and the relative amount of ZNF521 mRNA in each sample was calculated by comparative ΔCt method.

Benzidine Staining—The hemoglobinization of K562 cells was analyzed by benzidine staining 2, 4, 6, and 8 days after transient transfection with shRNA for ZNF521. In brief, cells were collected and washed twice with cold phosphate-buffered saline and then stained with benzidine solution containing 3% H2O2 for 5 min, and 200 cells were counted in each analysis under a light microscope. Cells with blue-brown-stained cytoplasm were counted as benzidine-staining-positive cells. The experiments were repeated three times. Statistical analysis between the two groups was performed using a Tukey-Kramer test.

Northern Blot Analysis—Northern blotting was performed by using a 32P-random-labeled ZNF521 cDNA fragment generated by PCR using the primers 5’-AGACAGTTTCACTG-TGACCT-3’ and 5’-TGGAGACACAGTGTGGT-3’. A β-actin control probe was also labeled by random labeling.

Flow Cytometric Analysis—Expression of cell surface glycoporphin A was examined by flow cytometric analysis using PE-
conjugated anti-glycophorin A monoclonal antibody (BD Biosciences). Cells to be used as unstained controls for glycophorin A were incubated with control PE-conjugated mouse monoclonal IgG (Pharmingen).

Luciferase Assays—Four micrograms of the plasmid DNAs (cDNA for GATA-1, ZNF521, and/or empty vector), 1.1 μg of the GATA-1 reporter gene pGL3-MoP-Luc (15), and 0.1 μg of the sea pansy luciferase expression plasmid pRL-SV40 (Toyo B-Net, Tokyo, Japan) as an internal control were transfected into NIH3T3 cells (2 × 10⁵ cells seeded in a 60-mm dish) using Fugene 6 (Roche Applied Science). After 16 h, the cells were harvested, and luciferase activity and sea pansy luciferase activity were assayed using the Pico Gene Dual sea pansy luminescence kit (Toyo B-Net) and a luminometer in accordance with the manufacturer’s instructions. Results are expressed as the ratios of luciferase activity to sea pansy luciferase activity. Statistical analysis of data between the two groups was performed using a paired sample t test. Five micrograms of the GATA-1 reporter gene pGL3-MoP-Luc and 0.05 μg of the sea pansy luciferase expression plasmid pRL-SV40 were transfected into K562 cells (1 × 10⁶ cells) by electroporation. After 36 h, the cells were lysed, and dual luciferase assays were performed.

Glutathione S-Transferase (GST) Fusion Protein Pull-down Assay—Five ZNF521 cDNA fragments were constructed by PCR and sequenced. They were then cloned into the pEUE01-His vector (CellFree Sciences Co. Ltd., Matsuyama, Japan) containing the His⁶ tag region and SP6 promoter. The full-length GATA-1 cDNA and ZF parts of GATA-1 generated by PCR were cloned into the pEUE01-GST vector (CellFree Sciences Co. Ltd.) containing a GST tag region and SP6 promoter. The His₆-ZNF521 proteins and GST-GATA-1 fusion protein were synthesized by use of the wheat cell-free protein synthesis system (16). His₆-ZNF521 fusion proteins were purified using nickel-Sepharose high performance (GE Healthcare). GST-GATA-1 fusion protein was purified using glutathione-Sepharose 4B (GE Healthcare). Expression of the fusion proteins was confirmed by SDS-PAGE following Coomassie Blue staining and by Western blotting analysis. GST-GATA-1 fusion protein immobilized on beads and various amounts of His₆-ZNF521 proteins were mixed in 1 ml of lysis buffer (20 mM sodium phosphate, pH 7.0, 250 mM NaCl, 30 mM sodium pyrophosphate, 0.1% Nonidet P-40, 10 mM NaF, 0.1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride) and incubated for 4 h at 4 °C. The beads were washed five times with lysis buffer and analyzed by SDS-PAGE followed by Western blotting using anti-His antibody (Takara).

Immunoprecipitation and Western Blot Analysis—The p3×FLAG-ZNF521 CMV-7.1 plasmid and the pcDNA3 Myc/GATA-1 plasmid were transfected into 293T cells using FuGENE HD (Roche Applied Science). After 48 h, the cells were lysed at 4 °C in 1 ml of lysis buffer. After centrifugation, the supernatants were mixed with EZview red anti-FLAG M2 affinity gel or anti-Myc affinity gel (Sigma) and incubated for 2 h at 4 °C. The beads were then washed five times with lysis buffer. The bound protein was released from the beads by boiling for 5 min in 10 μl of sample buffer and analyzed by SDS-PAGE followed by Western blot analysis using anti-Myc antibody (Sigma) and anti-FLAG antibody (Sigma). Total cellular lysate was isolated from K562/3×FLAG-ZNF521 cell lines, and immunoprecipitation was performed with anti-FLAG M2 affinity gel. The protein was analyzed by SDS-PAGE followed by Western blot analysis using anti-GATA-1 antibody (N6; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed with a Quik ChIP kit (IMGENEX, San Diego, CA). K562/3×FLAG-ZNF521 cells (5 × 10⁶ cells) were fixed with 1% of formaldehyde for 10 min at 37 °C and the reactions were stopped by adding 125 mM glycine. Chromatin was sonicated with 20 pulses, 30-s pulses followed by 20 s of rest on ice, using Bioruptor (Cosmo Bio, Tokyo, Japan). After sonication, immunoprecipitation was performed with EZview red anti-FLAG M2 affinity gel (Sigma) and normal mouse IgG (Santa Cruz Biotechnology). The immunoprecipitated DNA samples and input samples were eluted and reverse cross-linked and purified using phenol/chloroform extraction. The primers used were as follows. GATA-1-binding sites on the β-globin locus were 5′-GGAGAGATCAGATAAGTTGAC-3′/5′-TGGAAA-TCTGCGTTATGGAG-3′. PCR was performed with HotStarTaq DNA polymerase (Qiagen) for 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The PCR products were electrophoresed on 1.5% agarose gel and visualized by UV illumination following ethidium bromide staining.

RESULTS

Inhibition of ZNF521 Expression Promotes Erythroid Maturation in K562 and HEL Cells—It is well known that K562 and HEL cells are able to differentiate toward erythroid lineage when they are exposed to chemical inducers (17–19). Because both K562 and HEL cells constitutively express ZNF521, we investigated whether down-regulation of this ZF protein influences the differentiation of these cells. To silence ZNF521 gene expression, two kinds of shRNA corresponding to a sequence of the ZNF521 coding region were cloned into the expression vector and transfected transiently into K562 and HEL cells by electroporation. The erythroid differentiation was monitored every other day by benzidine staining. A green fluorescent protein-expressing plasmid was used to quantify the transduction efficiency after electroporation. Flow cytometric analysis revealed that more than 60% of both K562 and HEL cells appeared to be green fluorescent protein-positive after 24 h (data not shown). The expression levels of ZNF521 mRNA were decreased in shRNA-mediated cell lines as compared with those in control cell lines. As shown in Fig. 1, A and B, from day 4 to day 8 after transfection of shRNA plasmids for ZNF521, the number of benzidine staining-positive cells increased significantly as compared with control cells in both the K562 and HEL cell lines.

We further examined the effect of ZNF521 down-regulation on erythroid differentiation of hematopoietic cells by establishing K562-derived clones in which ZNF521 mRNA is stably disrupted. After transfection of two kinds of ZNF521 shRNA expression vectors and an empty vector into K562 cells, stable clones were isolated by limiting dilution following selection with puromycin. We also established K562 clones overexpressing GATA-1 to compare the phenotype of the ZNF521-silenced K562 clones. As shown in Fig. 1C, the expression levels of ZNF521 mRNA in these clone cells determined by Northern
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Inhibition of ZNF521 expression by ZNF521 shRNA induces erythroid differentiation in K562 and HEL cell lines. A, K562 cells were transfected with either one of the expression plasmids for ZNF521 shRNA (gray bars, shRNA1; black bars, shRNA2) or a noncoding vector (white bars) by electroporation. Benzidine staining-positive cells were counted every other day. Each value represents the mean ± S.E. of two independent experiments conducted in triplicate. Quantitative real time PCR of ZNF521 mRNA and GAPDH mRNA was performed. The expression levels of ZNF521 mRNA were corrected by reference to that of GAPDH mRNA. The amounts of ZNF521 mRNA in shRNA1-mediated lines relative to that in control lines were indicated. B, similarly, HEL cells were transfected with either one of the ZNF521 shRNA expression plasmids (gray bars, shRNA1; black bars, shRNA2) or a noncoding vector (white bars) by electroporation. Benzidine staining-positive cells were counted every other day. C, Northern blotting for expression of ZNF521 and ß-actin mRNAs was performed. Total RNAs were isolated from a control clone and ZNF521-silenced clones and GATA-1-overexpressing clones. Twenty micrograms of total RNAs was analyzed by Northern blot hybridization with a probe for ZNF521 or ß-actin. Quantitative real time PCR of ZNF521 mRNA and GAPDH mRNA was performed. The expression level of ZNF521 mRNA was corrected by reference to that of GAPDH mRNA. D, the percentages of benzidine staining-positive cells among parental K562 cells, the control clone, ZNF521-silenced clones, and GATA-1-overexpressing clones are shown. E, microscopic observations of benzidine staining in parental K562 cells (A, K562), a control clone (B, K562/vector), ZNF521-silenced clones (C, clone 1; D, clone 2 and clone 3), and GATA-1-overexpressing clone (F, K562/GATA-1). F, appearance of the pellets of parental K562 cells (A, K562), a control clone (B, K562/vector), ZNF521-silenced clones (C, clone 1; D, clone 2 and clone 3), and GATA-1-overexpressing clone (F, K562/GATA-1). G, expression of glycophorin A on the cell surface of parent K562 cells, ZNF521-silenced clones, and the GATA-1-overexpressing clone examined by flow cytometric analysis.

blot analysis, and real-time PCR appeared to be dramatically decreased. We compared the erythroid differentiation of ZNF521-silenced clones and GATA-1-overexpressing clones with the control clones by benzidine staining. As shown in Fig. 1, D and E, ZNF521-silenced clones showed a marked increase of benzidine staining-positive cells. The increased hemoglobinization in the ZNF521-silenced clones and GATA-1-overexpressing clones was apparently visible (Fig. 1F). Next, we examined the expression of glycophorin A, an erythroid lineage-associated cell surface marker, using flow cytometric analysis. As shown in Fig. 1G, the expression level of glycophorin A on the cell surface was significantly increased in ZNF521-silenced clones as compared with the parental K562 cells. Similarly, an increased level of glycophorin A expression was observed in the GATA-1-overexpressing clones. We also compared the gene expression patterns in a ZNF521-silenced clone and a control clone comprehensively using Human Genome Survey Microarray version 2.0, covering more than 30000 genes (Applied Biosystems, Foster City, CA). Consequently, expression levels of erythroid lineage-related genes, including globin...
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ZNF521 Inhibits GATA-1 Transcriptional Activity—Since GATA-1 is the key regulator of erythroid differentiation that plays a central role in erythroid lineage-associated gene expression (20), we speculated that ZNF521 might influence the transcriptional activity of GATA-1. To verify this possibility, we performed a luciferase assay with a reporter gene for GATA-1, pGL3-MaP-Luc, which contains three tandem repeats of the GATA-1-responsive element just upstream of the JunB promoter. We used NIH3T3 cells for reporter gene assays, because this cell line does not express endogenous GATA-1. Although transient transfection of the GATA-1 reporter with GATA-1 cDNA alone increased the promoter activity, co-transfection of ZNF521 cDNA with GATA-1 cDNA exerted a significant repressive effect on GATA-1 activity (Fig. 2A). There was no significant effect of ZNF521 cDNA on the basal activity of the JunB promoter, indicating that the inhibitory effect of ZNF521 was specific for the GATA-1-induced transcriptional activity. Next, we co-transfected the GATA-1 reporter with GATA-1 cDNA and various amounts of ZNF521 cDNA into NIH3T3 cells and examined the reporter activity. Consequently, ZNF521 cDNA appeared to inhibit the GATA-1 activity in a dose-dependent manner (Fig. 2B). Next, we compared the GATA-1 promoter activities in various K562 cell lines. The GATA-1 promoter activities and GATA-1 protein levels were increased in the ZNF521-silenced clone and the GATA-1-overexpressing clone (Fig. 2C).

ZNF521 comprises an N-terminal repression motif and 30 ZFs (Fig. 3A). To determine which parts of ZNF521 inhibit GATA-1 transcriptional activity, we constructed deletion mutants of ZNF521 cDNA and analyzed the effects of these products on GATA-1 transcriptional activity. Two kinds of mutant ZNF521 cDNA, in which the N-terminal repression motif (ΔNT) or C-terminal ZF domains (Δ21–30) were deleted (Fig. 3A), were inserted into the FLAG-tagged expression vector. Production of wild-type and deleted proteins by these cDNAs was confirmed by Western blotting (Fig. 3B). The effects of these mutated ZNF521s on GATA-1 transcriptional activity were compared with that of wild-type ZNF521. As shown in Fig. 3C, the inhibitory effect of wild-type ZNF521 on GATA-1 promoter activity was partially abrogated by deletion of the N-terminal repression motif and C-terminal ZF domains, indicating that these parts are necessary for inhibition of GATA-1 activity. Because deletion mutants of the N-terminal repression motif and C-terminal ZF domains did not completely inhibit GATA-1 activity, another portion(s) of ZNF521 might also be involved in inhibiting GATA-1 transcriptional activity.

ZNF521 Does Not Affect the DNA Binding Activity of GATA-1—We performed an electrophoretic mobility shift assay using K562 clones. The DNA binding activity of GATA-1 was not decreased in ZNF521-silenced clones as compared with that in control clones. These data show that ZNF521 might not affect the DNA binding activity of GATA-1 (supplemental Fig. 1).

ZNF521 Interacts Directly with GATA-1 in Vitro and in Vivo—Since ZNF521 inhibits GATA-1 transcriptional activity as shown above, we next examined the physical interaction between ZNF521 and GATA-1. To verify whether ZNF521 is capable of binding to GATA-1 in vitro, we performed a pull-down assay using a GST fusion protein comprising GATA-1 and three fragments of His6-tagged ZNF521 protein, ZF1–8, ZF9–20, and ZF21–30 (Fig. 4A), which were synthesized in
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**FIGURE 3.** Inhibitory effects of mutant ZNF521s on transcriptional activity of GATA-1. **A**, the diagrams for ZNF521 and the deletion mutants (Δ21–30 and ΔNT) of ZNF521 tested for inhibition of the GATA-1 transcriptional activity. **B**, expression of the wild-type and mutant ZNF521 proteins was detected by Western blotting using anti-FLAG antibody. **C**, NIH3T3 cells were transfected with pGL3-MP-Luc, cDNA for GATA-1, and cDNA for the indicated ZNF521 mutant. After 16 h, cell lysates were measured for luciferase activity.

**FIGURE 4.** Interaction between ZNF521 and GATA-1 in vitro. **A**, diagrams of the His₆-tagged ZNF521 fragments. **B**, production of ZNF521 fragments was detected by Western blotting using anti-His antibody. **C**, purified GST-ZNF521 and GST proteins were mixed with purified ZNF521 fragment proteins. Bound proteins were fractionated by SDS-PAGE and detected by Western blotting using anti-FLAG antibody. Loaded GST and GST-ZNF521 proteins were detected by Coomassie Blue staining.

**FIGURE 5.** Pull-down analysis of GATA-1 and ZNF521 interaction. **A**, diagrams of the His₆-tagged ZNF521 fragments. **B**, production of ZNF521 fragments was detected by Western blotting using anti-His antibody. **C**, purified GST-ZNF521 and GST proteins were mixed with purified ZNF521 fragment proteins. Bound proteins were fractionated by SDS-PAGE and detected by Western blotting using anti-FLAG antibody. Loaded GST and GST-ZNF521 proteins were detected by Coomassie Blue staining.

**FIGURE 6.** Immunoprecipitation of GATA-1 and ZNF521 in vivo. **A**, western blotting of the anti-Myc antibody on the anti-FLAG immunoprecipitates revealed that ZNF521 was co-immunoprecipitated with Myc-tagged GATA-1. **B**, western blotting of the anti-FLAG antibody on the anti-Myc immunoprecipitates revealed that GATA-1 was co-immunoprecipitated with ZNF521. **C**, establishment of K562 cell lines expressing 3XFLAG-ZNF521 to examine endogenous interaction between ZNF521 and GATA-1. Immunoblotting of the anti-FLAG immunoprecipitates with the anti-GATA-1 antibody revealed that ZNF521 was co-immunoprecipitated with endogenous GATA-1 (Fig. 6C). These data confirm that ZNF521 interacts with GATA-1 in vivo.

**DISCUSSION**

In the present study, we found that shRNA-mediated silencing of the ZNF521-encoding gene in K562 and HEL cells resulted in erythroid differentiation. We also showed that ZNF521 inhibits the transcriptional activity of GATA-1. Deletion analysis of ZNF521 showed that the repressive effect of ZNF521 requires the N-terminal repression motif. Furthermore, direct interaction of ZNF521 with GATA-1 through
ZF-(21–26) of ZNF521 and CF of GATA-1 was demonstrated. These findings demonstrate a novel mechanism for differentiation of erythroid cells and maintenance of the “stemness” of hematopoietic progenitors.

GATA-1 is known to interact with a variety of proteins via ZF domains. Among them, GATA-1 NF interacts with FOG-1 (Friend of GATA-1) (21, 22) and c-Myb (23), whereas CF interacts with EKLF (24), Fli-1 (25), PU.1 (26), and EVI1 (27, 28). These interactions play an important role in hematopoiesis, since they lead to transcriptional activation or repression of GATA-1 target genes. On the other hand, ZNF521 contains an N-terminal suppression motif that is conserved among many transcriptional repressors, including FOG-1 and FOG-2 (9). Recently, this portion was shown to be the association site of the nucleosome remodeling and deacetylase corepressor complex (NurD) (10, 11), which is involved in global transcriptional repression. In the present study, we demonstrated that deletion of the N-terminal motif of ZNF521 resulted in reduction of the inhibitory effect of ZNF521 against GATA-1 transcriptional activity. This finding suggests that ZNF521 inhibits GATA-1 transcriptional activity by recruiting NurD.

Brand et al. (29) showed that binding of NurD to the promoter of globin genes was increased in globin-nonproducing immature cells as compared with that in globin-producing erythroid-differentiated cells. This suggests that at an early stage of erythroid differentiation, expression of globin genes is repressed by the NurD complex, whereas at a more mature stage, this repression declines as a result of dissociation from NurD. Taken together with our results, it seems likely that the complex of GATA-1 and ZNF521 inhibits globin gene expression at an early stage of erythroid differentiation through complex formation with NurD.

In the present study, we demonstrated that ZNF521 interacts with GATA-1 through ZF-(21–30). We also demonstrated weak in vitro association of GATA-1 with ZNF521 ZF-(1–8). Since a ZNF521 mutant in which ZF-(21–30) was deleted inhibited GATA-1 activity, the association between GATA-1 and ZF-(1–8) of ZNF521 might also be involved in the repression of GATA-1 transcriptional activity. Fox et al. (30) showed that, in addition to a sixth ZF of FOG-1, ZF1, ZF5, and ZF9 of FOG-1 also interact with the NF of GATA-1 and that each finger contributes to the ability of FOG-1 to modulate GATA-1 activity. Furthermore, they showed that FOG-2 and U-shaped also contain multiple GATA-interacting fingers. These results indicate that GATA-binding proteins including ZNF521 use multiple ZFs to bind GATA-1.

Smad family proteins are essential for BMP and transforming growth factor-β signaling (31, 32). Recent reports have suggested that the association of Smads with other transcription factors is the key for recruitment of Smads to specific promoter elements. BMP4 can increase the proliferation of human hematopoietic progenitors (33, 34), whereas transforming growth factor-β acts as a negative regulator for hematopoietic progenitors in vitro (35, 36). BMP4 induces GATA-2 transcription (37, 38). Whereas GATA-1 regulates terminal differentiation of
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erthroid cells (39), GATA-2 is expressed in hematopoietic progenitors and controls early stages of hematopoiesis (40). ZNF521 has been demonstrated to interact with Smad1/4 in response to BMP signals and to activate BMP-responsive genes, resulting in proliferation of hematopoietic progenitors. Since GATA-2 was demonstrated to be repressed by GATA-1 during erythroid maturation (41), the inhibition of GATA-1 activity by ZNF521 might be important for constitutive expression of GATA-2 in order to maintain hematopoietic progenitor cells. Taken together, ZNF521 might play an important role in maintaining the “stemness” of hematopoietic progenitors.

Interestingly, EVI3, a murine homologue of ZNF521, has been demonstrated to be expressed in the brain, especially the cerebellum, as well as in hematopoietic progenitors (14). In situ hybridization analysis revealed striking enrichment of EVI3 mRNA in the granule layer that hosts granule neural precursors in the postnatal cerebellum (14). These findings suggest that ZNF521 also plays an important role in development of the nervous system. To confirm this, we are now generating mice homozygous for an EVI3 null allele.

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