Stable Form of JAB1 Enhances Proliferation and Maintenance of Hematopoietic Progenitors*

Received for publication, June 13, 2008, and in revised form, July 30, 2008. Published, JBC Papers in Press, July 30, 2008, DOI 10.1074/jbc.M804539200

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Overexpression of JAB1 is observed in a variety of human cancers, but how JAB1 is involved in tumor development remained to be investigated. Here we analyzed mice with modified Jab1 expression. Mice ectopically expressing a more stable form of JAB1 protein under the control of a constitutive promoter were rescued from the embryonic lethality caused by the Jab1−/− allele and developed a myeloproliferative disorder in a gene dosage-dependent manner. Hematopoietic cells from the bone marrow of Jab1 transgenic mice had a significantly larger stem cell population and exhibited higher and transplantable proliferative potential. In contrast, Jab1−/− mice, which express ~70% as much JAB1 protein as their wild-type littermates, showed inefficient hematopoiesis. Expression of the tumor suppressor p16INK4a was inversely correlated with that of JAB1, and the oncoprotein SMYD3, a newly identified JAB1 interactor, suppressed transcription of p16 in cooperation with JAB1. Thus, the expression and function of JAB1 are critical for the proliferation and maintenance of hematopoietic progenitors.

JAB1 was identified originally as a protein binding to the transcription factors c-JUN and JUN D (1) and later as the fifth (CSN5) of eight (CSN1–8) subunits of the COP9 signalosome (CSN) complex (2), which is located in the nucleus, well conserved from yeast to humans, and involved in a variety of biological responses (3–6). JAB1 is a multifunctional protein that (i) determines the specificity of transcription factors, such as c-JUN, JUN D, and E2F-1 (1, 7); (ii) mediates the phosphorylation of c-JUN, NFκB, and p53 by CSN-associated kinases (2, 8); (iii) catalyzes the reaction to remove a ubiquitin-like polypeptide, NEDD8, from the Cullin subunit (deneddylation) (9, 10), thereby regulating the activity of Cullin-based ubiquitin ligases, such as the SKP1-Cullin-F box protein complex; and (iv) controls the stability and intracellular distribution of many signaling molecules, including the CDK inhibitor p27 (11), by ill defined mechanisms. However, it remains to be determined whether these different activities were regulated simultaneously or separately, and were specifically associated with the JAB1 subunit or regulated by the entire CSN complex.

JAB1 seems to play an essential role in the control of mammalian cell proliferation and survival. In mice, the level of JAB1 expression is higher in embryos, where vigorous cell proliferation occurs, than in adults (12). Jab1-null embryos die soon after implantation, and Jab1−/− embryonic cells exhibit impaired proliferation and accelerated apoptosis. Jab1+/- mice, which express only ~70% as much Jab1 protein as their wild-type littermates, were viable and fertile but smaller in size, and Jab1+/- mouse embryonic fibroblast cells exhibited an inefficient down-regulation of the CDK inhibitor p27 during G1 and delayed cell cycle progression (13).

Recently, high levels of JAB1 have been found in human cancers. Although a certain level of JAB1 is expressed in normally proliferating and in nonproliferating cells, an increase in JAB1 expression of several-fold is observed in a variety of human cancer cells, such as breast cancer (14, 15), malignant lymphoma (16), ovarian tumor (17), melanoma (18), non-small cell lung cancer (19, 20), laryngeal squamous cell carcinoma (21), and thyroid medullary carcinoma (22). In hepatocellular carcinoma (23), a high level of JAB1 is associated with an increase in the genomic DNA copy number of the Jab1 locus. In chronic myeloid leukemia (24), JAB1 functions downstream of BCR-ABL kinase through the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways and facilitates cell cycle progression. A high level of JAB1 is often correlated with a poor prognosis and sometimes related to a low level of the CDK inhibitor p27. Furthermore, in cases of breast cancer, coordinated amplification of the MYC and CSN5 (JAB1) genes was observed (25). However, the physiological significance of JAB1 overexpression in cancer cells and the molecular mechanism by which JAB1 is involved in the proliferation of cancer cells remain to be examined. In this study, we analyzed mice with a modified Jab1 expression. We demonstrated that the level of Jab1 expression is a critical determinant of the proliferation and maintenance of hematopoietic progenitor cells in vivo, which may explain how the overexpression of JAB1 contributes to tumor development.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Colony Formation Assay—NIH3T3 mouse fibroblasts and 293T human embryonic kidney cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum.
serum. GFP2 fusion expression vectors were generated based on pMSCV-IRES-puro (Clontech), in which cDNAs were inserted in frame with a GFP sequence (26). The vectors for RNA interference were constructed with the pSilencer expression vector system (Ambion) according to the manufacturer’s instructions. Transfection was carried out using a modified calcium phosphate-DNA precipitation method (27). Recombinant retroviruses were produced by transfecting 293T cells with MSCV-based expression vectors, together with a plasmid encoding a ψ\textsubscript{2} helper virus. The supernatants were harvested 48–72 h post-transfection and immediately used for infection. Bone marrow cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum, 5% WEHI conditioned medium, mouse stem cell factor (50 ng/ml), mouse IL-3 (6 ng/ml), and human IL-6 (10 ng/ml) and infected with culture supernatants twice according to the spin infection procedure in the presence of Polybrevine (4 μg/ml). For colony formation assays, cells isolated from bone marrow, spleen, and peripheral blood were plated (1.5–2.0 × 10⁴ cells/35-mm dish) on a methylcellulose medium (MethoCult GF M3434; StemCell Technologies Inc.). After 7–12 days, colonies containing more than 100 cells were enumerated. For the second and third plating, cells were collected from the methylcellulose medium and replated (1.0 × 10⁴ cells/35-mm dish) in fresh medium. Some colonies were cytencrifuged onto glass slides, stained with a May–Grunwald Giemsa solution, and viewed by phase-contrast microscopy in order to evaluate lineage.

Mice—Jab1⁺⁻/ mice and ES cells were generated as described (13). Mouse Jab1 cDNA was combined with a tandem affinity purification (TAP)-tagged sequence, cloned into a pCAG-IRES-puro expression vector (26), introduced into Jab1⁺⁻/RF8 mouse ES cells by electroporation, and selected in 5 μg/ml puromycin. Two TAP-JAB1-positive Jab1⁺⁻/ ES cell lines were microinjected into C57BL/6 embryos, and chimeric males were crossed with C57BL/6 females. The transgene was determined as described (13). All mice were maintained in the Nara Institute of Science and Technology Animal Facility in accordance with the Nara Institute of Science and Technology guidelines. Blood samples were routinely analyzed with an automated cell counter (F-820 analyzer; Sysmex) and also by inspecting blood smears after May–Grunwald Giemsa staining (Merck). For myelosuppressive treatment, mice were injected with 5-fluorouracil (5-FU) (300 mg/kg of body weight; Kyowa Hakko Kogyo) intravenously, and hematopoietic recovery was monitored in peripheral blood. At specific points in time, mice were sacrificed, femurs were fixed in 4% formaldehyde, and BM sections were stained with hematoxylin and eosin. For the bone marrow transplantation experiment, cells were injected into the veins of NOD.CB17-Prkdcscid/J (NOD-SCID) mice (Jackson Laboratory), and peripheral blood was routinely monitored.

Flow Cytometric Analysis—PharM Lyse (BD Bioscience) was used to remove red blood cells, and FcγIII/II receptors were blocked with a specific antibody (2.4G2; BD Bioscience). Cells were stained for 30 min at 4 °C with a mixture of phycoerythrin-Cy5-conjugated antibodies to CD3ε (145-2C11), B220 (RA3-6B2), TER-119 (TER-119), MAC-1 (M1/70), and GR-1 (RB6-8C5); allophycocyanin-conjugated antibody to c-Kit (ACK2); phycoerythrin-conjugated antibody to Sca-1 (E13-161.7); and fluorescein isothiocyanate-conjugated antibody to CD34 (RAM34/49E8) (all from eBioscience) and analyzed with FACSVantage (BD Biosciences). Spleen cells were stained with fluorescein isothiocyanate-conjugated antibody to MAC-1 (M1/70) and phycoerythrin-conjugated antibody to GR-1 (RB6-8C5). For the cell cycle analysis, cells were stained with a 1-ml solution of 0.1% sodium citrate and 0.1% Triton X-100 containing 50 μg/ml propidium iodide and treated with 1 μg/ml RNase for 30 min at room temperature. Fluorescence from the propidium iodide-DNA complex was measured with a FACScan flow cytometer (BD Biosciences), and the percentages of cells in phases G₁, S, and G₂/M of the cell cycle were determined with ModFit cell cycle software.

Histological Methods—Tissues were fixed in 4% formaldehyde, embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin. For blood smears, blood was obtained from a tail vein onto glass slides and stained with May–Grunwald Giemsa solution. Blood counts were determined using an automated cell counter. For cytopsins, 1.0 × 10⁵ cells were cytocentrifuged (500 rpm for 3 min) onto glass slides and stained for 30 min with May–Grunwald Giemsa solution. Myeloperoxidase activity was detected with the DAB staining kit (Muto Pure Chemicals) according to the manufacturer’s instructions. Chloroacetate esterase staining was performed with assistance from Narabayuri Research.

Protein Analyses—Cell lysis, immunoprecipitation, SDS-PAGE, and immunoblotting were performed as described (11, 13, 24, 26). Rabbit polyclonal antibodies to Jab1 and SMYD3 were generated against bacterially produced recombinant polypeptides. A rabbit polyclonal antibody to GFP (BD Living Colors) and mouse monoclonal antibodies to γ-tubulin (GYU-88) and an HA epitope (clone 12CA5) were obtained from BD Biosciences, Sigma, and Roche Applied Science, respectively.

Quantitative RT-PCR Analysis—Total RNA was isolated and converted to cDNA using ISOGEN reagent (Nippon Gene) and RNase-free Superscript reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The polymerase chain reaction was performed within a linear range described previously (26, 28), and the data were normalized to the expression level of β-actin for each sample. We confirmed that the reactions were quantitatively performed within a linear range in several control experiments. The following oligonucleotide primer pairs were used: Jab1, 5′-GGA CGG CAG AGT ATC GAT GAA A-3′ and 5′-GCC AAT GCT GAG ATT TTG-3′.
CA-3′; γ-tubulin, 5′-ATG AGT GAC GTG GTG GTC CAA-3′ and 5′-CGG TCT GTG GCT ATC AGG TTC A-3′; β-actin, 5′-TCA GCA AGC AGG AGT ACG ATG A-3′ and 5′-CTG CGC AAG TTA GGT TTT GTC A-3′. Primer pairs for Ink4a, Arf, and Cip1 were reported previously. Real time PCR was performed with an ABI7700 sequence detection system using a quantitative RT-PCR kit with SYBR Green (Invitrogen) according to the manufacturer’s instructions. PCR products were confirmed by agarose gel electrophoresis. All experiments were performed in triplicate.

**GST Pulldown Assay**—cDNA fragments containing the CSN6 and SMYD3 coding sequences were inserted into the pGEX vector (Amersham Biosciences) in frame with GST. GST-fused proteins were expressed in bacteria and purified as described (11). Crude cell extracts containing HA-JAB1 protein were prepared from COS7 cells transfected with the HA-JAB1 expression vector in an EBC buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, and 1 mM EDTA) containing 5 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM NaF, 0.1 mM NaVO₄, and 1 mM dithiothreitol. Binding was performed in the EBC buffer, the protein complexes were washed in the same buffer, and the bound protein was detected by immunoblotting. The same amounts of beads used for the binding assay were subjected to immunoblotting with antibodies to GST to quantitatively examine the GST-fused proteins.

**Transactivation Assay**—Fragments containing ~2 kb of DNA upstream of exon 1α of the Ink4a locus were inserted into the pGL3-Basic and pGL3-Enhancer vectors (Promega) to generate luciferase reporter constructs. Cells were transfected with the p16 reporter and the cytomegalovirus-β-galactosidase plasmid, together with additional SMYD3 and JAB1 expression plasmids. Luciferase and β-galactosidase activities were determined using reporter gene assay kits (Roche Applied Science) according to the manufacturer’s instructions. The luciferase activity was normalized to that of β-galactosidase for each sample.

**Chromatin Immunoprecipitation (ChIP) Assay**—Cells were cross-linked in 1% formaldehyde for 10 min, and chromosomal DNA was fragmented by sonication. The fixed chromatin samples were subjected to immunoprecipitation using antibodies specific to SMYD3 and JAB1. The sets of primers used for the ChIP assay were mouse Ink4a (5′-GTT GAT ATA ATA GTA CCT GAG GCT GCT CTC TCT CTT TTG TTA TAT GGC CAC AGC TAT TCC 3′) and human Ink4a (5′-ACT CTT GAT GTA CAA CAC TCG TCA GAG CCC CAC CGA GAA TCG AAA TC-3′).

**RESULTS**

**Ectopic Overexpression of Stable Form of JAB1 Rescues jab1 Knock-out Phenotype**—In order to analyze the effect of the ectopic expression of JAB1 in a wide range of cell lineages, we constitutively expressed ectopic JAB1 protein in the whole body of mice. In a previous experiment, we were successful in transiently overexpressing JAB1 protein in a variety of cell types (11). However, after selection with a drug marker, few stable cell lines that overexpressed ectopic JAB1 protein were established. Following exhaustive efforts using a variety of expression constructs (HA- and FLAG-tagged JAB1 as well as an intact JAB1), cell lines, and transfection methods, we finally ended up with a TAP-tagged JAB1 (TAP-JAB1) vector, with which we established a number of human and mouse cell lines overexpressing ectopic JAB1 protein. This is partially due to the stabilization of JAB1 protein by fusion with the TAP tag. Pulse-chase experiments in the presence of cycloheximide revealed that the half-life of endogenous JAB1 was ~5.1 h, whereas that of TAP-JAB1 was about 46 h. Encouraged by this result, we introduced TAP-JAB1 into mouse ES cells and successfully established 11 independent ES clones, which stably express ectopic JAB1 protein (Fig. 1A). We picked two independent lines and used them in assays giving basically the same results. The level of TAP-JAB1 in ES cells was ~4-fold more than that of endogenous JAB1 protein normally expressed in ES cells. The morphology of ES cells expressing TAP-JAB1 was indistinguishable from that of parental cells, suggesting that TAP-JAB1 expression did not alter the multipotency of ES cells. In fact, after the injection of ES cells expressing TAP-JAB1 into mouse embryos, mice expressing TAP-JAB1 were born. TAP-JAB1 transgenic (Tg) mice appeared normal and healthy at birth and were fertile. The expression of TAP-JAB1 was stably maintained in all organs and cell types tested, which included kidney, lung, spleen, bone marrow, and embryonic fibroblasts (examples of kidney and lung are shown in Fig. 1B). Ectopic TAP-JAB1 expression increased the amount of the COP9 signalosome complex without increasing the amount of endogenous JAB1 protein, suggesting that TAP-JAB1 was incorporated into the COP9 complex in addition to enhancing the small complex (Fig. 1C), which makes a clear distinction from that of HA-tagged JAB1 protein. HA-JAB1 was preferably incorporated into the small complex (24).

We previously found that jab1 knockout mice die in the embryonic stage (they die before embryonic day 6.5–7.5) (13). To investigate whether ectopic TAP-JAB1 can rescue jab1+/− mice, we first expressed TAP-JAB1 in mice with the Jab1+/− genetic background. After intercrossing TAP-JAB1 /Jab1+/− mice, we found that TAP-JAB1 /Jab1+/− mice were born alive (Fig. 1D), with a frequency (18.5%, n = 27) close to that expected from the Mendelian rule. The appearance of these mice was normal and healthy, indicating that the TAP-JAB1 transgene can substitute for the endogenous jab1 gene. Therefore, together with the result in Fig. 1C, we concluded that TAP tagging did not interfere with the normal function of JAB1 protein.

**JAB1-Tg Mice Develop a Myeloproliferative Disorder**—TAP-JAB1 transgenic (JAB1-Tg) mice appeared normal at birth and showed little difference in size, behavior, and reproductive ability from their wild-type littermates. After 6 months, however, they started to exhibit abnormal peripheral blood (PB) leukocyte numbers (Fig. 2A) and anemia (Fig. 2, B and C) and developed a myeloproliferative disease, the population being dominated by granulocytes (Fig. 2D). Interestingly, this occurrence (more than 2 × 10⁴ leukocytes/mm³ in PB) was correlated with the integrity of the endogenous jab1 locus in a gene dosage-dependent manner (Fig. 2D), because both TAP-JAB1 jab1+/+ and TAP-JAB1 jab1−/− mice developed this disease, whereas TAP-JAB1 jab1−/− mice did not, and the frequency was slightly lower than that of TAP-JAB1 jab1+/− mice (Fig. 2D). These mice showed little difference in size, behavior, and reproductive ability from their wild-type littermates.

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Jab1 Controls Hematopoietic Progenitors

**A**

|          | TAP-Jab1 | TAP-Jab1 | Jab1 | Jab1 |
|----------|----------|----------|------|------|
|          |          |          |      |      |
|          |          |          |      |      |
|          |          |          |      |      |

|          |          |
|----------|----------|
| Kidney   | Lung     |
| TAP-Jab1 | Jab1     |
|          | γTub.    |

**B**

|          |          |          |          |          |          |
|----------|----------|----------|----------|----------|----------|
|          |          |          |          |          |          |
|          |          |          |          |          |          |
|          |          |          |          |          |          |

**C**

|          |          |          |          |          |          |
|----------|----------|----------|----------|----------|----------|
|          |          |          |          |          |          |
|          |          |          |          |          |          |
|          |          |          |          |          |          |

**D**

|          |          |          |          |          |
|----------|----------|----------|----------|----------|
| KO - WT  | +/+      | +/-      | -/-      |
|          | -2464 bp | 2047 bp  | 2047 bp  |

**FIGURE 1.** Generation of JAB1 transgenic mice. A, lysates of Jab1++/− ES cells transfected with (+) and without (−) the TAP-JAB1 expression construct were immunoblotted with antibody to JAB1 (top) and γ-tubulin (bottom; serving as a loading control). B, lysates from kidneys and lungs of 3-month-old control (WT) and TAP-JAB1 transgenic (Tg) mice were subjected to Western blotting with antibodies to JAB1 (top) and γ-tubulin (bottom; serving as a loading control). C, the same amount of lysate from spleens and BMs of 3-month-old control (WT) and TAP-JAB1 transgenic mice were separated by SDS-PAGE (top) and native PAGE (bottom) and analyzed by Western blotting with an antibody to JAB1 (top). D, genomic DNA extracted from the tail of 4-week-old mice was subjected to a PCR-based analysis. The positions of WT (2047 bp) and mutant (KO; 2464 bp) amplification products are indicated. Representative results are shown.

higher and the timing of occurrence was a little earlier for TAP-JAB1 Jab1++/+ mice than for TAP-JAB1 Jab1++/− animals. (Note that Jab1++/− alleles express ~70% as much JAB1 protein as Jab1++/+ alleles, so that the difference is subtle between Jab1++/+ and Jab1++/+ cells, whereas Jab1++/− cells contain no endogenous JAB1 protein.) These results suggest that the TAP-JAB1 transgene was cooperating with the endogenous JAB1 to induce the abnormality. However, once mice developed a myeloproliferative disorder, there was no significant difference in the counts of white blood cells and red blood cells (Fig. 2, A and B) and the type of cells (granulocytes) that dominated in PB (Fig. 2E) between TAP-JAB1 Jab1++/+ and TAP-JAB1 Jab1++/− animals.

The mice were sacrificed for autopsy when they developed anemia, less than 8 × 10⁶ RBC/mm³, or leukocytosis, over 2 × 10⁶ cells/mm³, or when they apparently became sick. JAB1-Tg mice frequently showed a massive enlargement of the spleen (Fig. 2, F and G), and JAB1-Tg BM appeared pale and hypercellular. Flow cytometric analysis revealed that the c-Kit+Sca-1+Lin− (KSL) fraction, which was enriched for the hematopoietic stem cell/progenitor population, was expanded in JAB1-Tg bone marrow (5.27 ± 0.53 × 10⁶ to 1.67 ± 0.42 × 10⁶ cells/bone marrow, p < 0.01, n = 5). Fig. 2H shows one example of such an analysis, in which the percentages of the KSL population among all bone marrow cells are shown (0.04% versus 0.25% for those of wild type and JAB1-Tg mice, respectively). Furthermore, the CD34−/low population was enriched in the KSL fraction isolated from JAB1-Tg mice (Fig. 2H, 9.0 versus 19%), indicating that JAB1-Tg mice contained a larger pool of hematopoietic stem cell/progenitor cells than wild type animals.

FACS analysis of spleen cells revealed that the number of Gr-1hi/Mac-1hi granulocytes increased in spleen (2.30 ± 0.96 × 10⁶ to 1.79 ± 0.38 × 10⁷, p < 0.002, n = 5) of JAB1-Tg mice. Fig. 2I represents one such analysis, in which percentages of the Gr-1hi/Mac-1hi population among total spleen cells are shown (1.9 versus 5.7% for wild type and JAB1-Tg mice, respectively). Fig. 2I also shows that the single anti-Mac1 staining revealed a marked difference between the two (8.2 versus 23%). These data show that granulocytes and myeloid progenitor cells were accumulated in the spleen of JAB1-Tg mice. The cytological investigation of the May-Grunwald Giemsa-stained cytospins also confirmed the results, indicating that JAB1-Tg mice developed a progressive myeloid hyperplasia with overproduction of granulocytes. In contrast to this, we observed less influence on lymphopoiesis and erythropoiesis.

In 4 of 4 sick JAB1-Tg mice examined, histological and cytological analyses revealed that a massive number of leukemic cells had infiltrated nonhematopoietic organs, such as the lung, liver, and kidney (examples of liver and lung are shown in Fig. 2, J and K, respectively). Examinations of cell morphology at higher magnification (Fig. 2L for the example of the liver) revealed that the infiltrates were blood cells, some of which were positive for myeloid markers, such as chloroacetate esterase and myeloperoxidase (data not shown).

In contrast, the wild-type or TAP-GFP control mice, which constitutively express ectopic TAP-tagged GFP protein throughout the body, showed little evidence of increased myelopoiesis for up to 13 months (Fig. 2D). Thus, these results indicate that JAB1-Tg mice develop a progressive myeloid hyperplasia with overproduction of granulocytes, which resem-
**FIGURE 2.** JAB1 transgenic mice develop a myeloproliferative disorder. **A–C**, hemograms of 6–13-month-old wild-type (Jab1+/+, n = 9), JAB1 heterogeneous (Jab1+/−, n = 10), and TAP-JAB1 transgenic (n = 5, 8, and 10 for TAP-JAB1 Jab1+/−, TAP-JAB1 Jab1+/−, and TAP-JAB1 Jab1+/−, respectively) littermates. The counts of leukocytes (A), red blood cells (B), and hemoglobin (C) are shown. **D**, disease-free survival curves of JAB1 transgenic mice with wild-type (red, n = 28), heterozygous (blue, n = 24), and nullizygous (black, n = 5) Jab1 alleles. The result for transgenic mice expressing TAP-tagged GFP (black, n = 4) is also shown. **E**, May-Grunwald Giemsa-stained peripheral blood cells from representative JAB1 transgenic mice (6 months old; see A for the actual number). **F**, massive enlargement of spleen from a 6-month-old JAB1 transgenic mouse (top) compared with an age-matched wild-type control (bottom). **G**, spleen weights of individual mice (n = 9 and 10 for WT and JAB1-Tg mice, respectively) are shown. **H**, increase in the number of hematopoietic progenitor/stem cells in bone marrow of JAB1 transgenic mice. Single cell suspensions from BM of Jab1 transgenic mouse (TAP-Jab1) and their WT littermates were stained with the indicated combinations of antibodies (see “Experimental Procedures”) and analyzed by flow cytometer. The numbers indicate the percentages of KSL cells among all cells (left) and KSL CD34low cells among KSL cells (middle and right). Experiments were repeated (WT (n = 6) and TAP-JAB1 (n = 7)), and similar results were obtained. Representative FACS profiles (left and middle) and the averages of the percentages of KSL CD34low cells among KSL cells (right) are shown. **I**, increase in the number of Gr-1high Mac-1high cells in spleen of JAB1 transgenic mice. Single cell suspensions from spleen of JAB1 transgenic mice (TAP-JAB1) and their WT littermates were stained with the indicated antibodies and analyzed by flow cytometer. The numbers indicate the percentages of Gr-1high Mac-1high cells (left) and Mac-1-positive cells (right) among all cells. Experiments were repeated (WT, n = 6; TAP-JAB1, n = 7), and similar results were obtained. Representative FACS profiles are shown. J–L, leukemic infiltration of the lung (J) and liver (K) of 6-month-old JAB1 transgenic mice. Higher magnification of the infiltrated area in the liver is shown (L).
bles human CML in the chronic phase, and this phenotype is due to the enhanced activity or function of JAB1 protein.

**Increased Proliferation and Extended Survival of JAB1-Tg Hematopoietic Progenitors**—To investigate the potential for growth and differentiation of the hematopoietic progenitors expanded in JAB1-Tg mice, cells isolated from bone marrow, spleen, and peripheral blood of wild-type and JAB1-Tg mice were quantitatively analyzed by a colony formation assay in a methylcellulose medium supplemented with a combination of cytokines (IL-6, IL-3, mouse stem cell factor, and erythropoietin) (Table 1). The cells from BM formed colonies of erythroids, granulocytes, macrophages, granulocyte/macrophages, and all mixed lineages, and colony-forming frequencies of all lineages were increased for cells from JAB1-Tg mice compared with those of wild-type mice (~1.8-fold increase in total) (Table 1, first and second rows). Cells from control spleen and PB barely formed any colonies, whereas JAB1-Tg spleen and PB cells formed colonies of all lineages (Table 1, fifth and sixth rows). The formation of colonies predominantly consisted of either erythroids, granulocytes, or macrophages in addition to the mixed type. We isolated bone marrow cells from the wild-type and JAB1-Tg mice with the retrovirus containing GFP and GFP-JAB1 fusion genes and cultured them in a liquid medium in the presence of combined cytokines. We found that the introduction of JAB1 markedly improved the proliferation and survival of hematopoietic cells compared with the empty vector-infected control cells in an in vitro culture system (data not shown). Encouraged with this result, we further analyzed the potential of the hematopoietic progenitors by conducting a transplantation experiment (Fig. 3). We isolated bone marrow cells from the wild-type and JAB1-Tg mice and injected them into the veins of recipient mice. For this purpose, we used NOD-severe combined immune deficiency (SCID) mice as the host, because they are deficient in B and T cell function and an ideal model for cell transfer experiments. The injection of wild-type BM cells increased white blood cell counts in peripheral blood, and JAB1-Tg BM cells had an even greater effect (Fig. 3A, top). Notably, the CD3-negative T cells, which were virtually absent in NOD-SCID PB, increased in proportion from 0.4 to 2.5% and 5.3% for wild type and JAB1-Tg BM, respectively, indicating that BM cells were successfully transplanted and that the ability

| JAB1 genotype | Source of cells | Without TAP-JAB1 | With TAP-JAB1 |
|---------------|----------------|-----------------|--------------|
|               | Colony number/10,000 cells |                  |              |
|               | E | GM | Mix | E | GM | Mix |
| +/+           | BM | 6.33 ± 1.86 | 58.2 ± 13.3 | 1.00 ± 0.63 | 16.3 ± 13.2 | 122 ± 73.7 | 6.50 ± 5.39 |
| +/+           | BM | 2.50 ± 1.00 | 24.0 ± 8.70 | 0.75 ± 0.96 | 3.75 ± 1.71 | 67.0 ± 10.0 | 5.75 ± 0.96 |
| +/+           | BM 2nd* | 6.00 ± 1.41 | 17.5 ± 0.71 | 0.75 ± 0.96 | 10.5 ± 4.95 | 36.5 ± 2.12 | 1.00 ± 1.41 |
| +/+           | BM 3rd* | 0 | 12.0 ± 5.66 | 0 | 0 | 120 ± 7.78 | 0 |
| +/+           | Sp | 1.00 ± 0.89 | 1.67 ± 2.34 | 0.33 ± 0.82 | 3.17 ± 1.72 | 5.00 ± 2.97 | 0.83 ± 0.75 |
| +/+           | PB | 0 | 0 | 0 | 2.00 ± 3.37 | 3.50 ± 3.70 | 0.50 ± 0.58 |
| +/+           | BM 4th* | 0.58 | 0.75 | 0.96 | 5.39 |

* Replacing for the second and third rounds.

**TABLE 2**

Increased colony formation of JAB1-Tg KSL cells *in vitro*

All colonies formed were the mixed type containing erythroids, granulocytes, and macrophages. Note that the number of cells plated was one-tenth of that in Table 1. Results are the means ± S.D. of three independent experiments.

| JAB1 genotype | Colony number/10,000 cells | Colony number/10,000 cells |
|---------------|----------------|----------------|
|               | KSL CD34* | KSL CD34--/low |
| +/+           | 53.7 ± 5.16 | 94.0 ± 5.66 |
| +/+           | 75.8 ± 1.98 | 114 ± 14.1 |
| +/+           | 40.5 ± 19.0 | 62.0 ± 2.83 |
| +/+           | 72.0 ± 2.83 | 91.3 ± 1.77 |

JAB1 Controls Hematopoietic Progenitors
to produce blood cells was greater for JAB1-Tg BM cells than WT BM cells. At 6 months post-transplantation, the mice were sacrificed and analyzed. Consistent with the results for PB, the spleen was enlarged, and the degree of change was again much more evident for JAB1-Tg BM cells than wild-type cells (Fig. 3A, bottom). Unexpectedly, the KSL population of BM cells from mice transplanted with WT BM cells was only marginally changed, whereas the transplantation of JAB1-Tg BM cells markedly increased the number of KSL cells in BM (Fig. 3, A (middle) and B), consistent with the results of the donor mouse analysis (Fig. 2). Further analysis showed that the majority of JAB1-Tg BM KSL cells expressed low levels of CD34 antigen (Fig. 3C). Genomic PCR analysis confirmed that JAB1-Tg cells were maintained in spleen and bone marrow of transplanted NOD-SCID mice (Fig. 3D).

The average lifespan of typical NOD-SCID mice is about 257–269 days, largely due to the development of lymphomas. Wild-type BM transplantation, although it improved the development of lymphoid lineage cells at least in a temporal manner, in the end did not rescue the hematopoietic deficiency of NOD-SCID animals (average life span was ~250 days). In contrast, NOD-SCID mice that successfully received a transplantation of JAB1-Tg BM cells showed a recovery of the lymphoid lineage in PB and survived much longer (~400 days) than average NOD-SCID animals, indicating that wild-type BM can temporarily reconstitute blood cells but JAB1-Tg BM has a more profound effect, presumably due to the reconstitution of HSCs, which was absent for WT BM transplantation. Thus, these results imply that the enhanced proliferation and prolonged survival of BM cells in JAB1-Tg mice are due to the ectopic expression of JAB1 in hematopoietic progenitor cells and probably not the result of altered properties in the niche or other types of cells in transgenic animals.

**Requirement of JAB1 for the Proliferation and Maintenance of Normal Hematopoietic Progenitors**—To examine whether JAB1 functions in normal hematopoiesis, we analyzed JAB1 heterozygous (Jab1+/−) mice, which express ~70% of the JAB1 protein found in their wild-type littermates (13). An examination of bone marrow cells by flow cytometric analysis revealed that Jab1+/− BM is less capable of hematopoiesis due to a poor maintenance of HSCs.

To further explore the potential of HSCs in Jab1+/− mice, we analyzed the hematopoietic recovery after treatment with 5-FU, which induces the apoptosis of actively cycling cells but not quiescent stem cells. To measure the kinetics of recovery, a

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**FIGURE 3.** Enhanced potential of hematopoietic progenitors in Jab1-Tg mice is cell-autonomous. Single cell suspensions from bone marrow of JAB1-Tg mice (n = 4) and their WT littermates (n = 3) were injected into the veins of NOD-SCID mice. Cells in peripheral blood were routinely monitored and 6 months after the bone marrow transplantation (BMT), and mice were sacrificed and analyzed. A, the counts of leukocytes (top), percentages of KSL cells (middle), and spleen weights (bottom) are shown. B, representative FACS profiles of KSL cells. The numbers indicate the percentages of KSL cells among all cells. C, representative FACS profiles of KSL CD34−/low cells. The numbers indicate the percentages of KSL CD34−/low cells among KSL cells. D, PCR analysis of genomic DNA extracted from spleen and bone marrow cells of mice indicated using sets of primers specific to endogenous JAB1 (endo-Jab1) and TAP-JAB1.
Jab1 Controls Hematopoietic Progenitors

FIGURE 4. Jab1 is required for normal hematopoiesis. A and B, flow cytometric analysis of the KSL population in bone marrow of WT and Jab1 heterozygous (Jab1+/−) mice. Representative FACS profiles (A) and the results for individual mice (B) are shown. C and D, hematopoietic recovery after treatment with 5-FU. WT (black line) and Jab1 heterozygous (Jab1+/−) (red line) mice were intravenously injected with a single dose of 5-FU (300 mg/kg body weight), and the peripheral blood count was monitored. C, white blood cell (WBC) counts were monitored. WT (black line) and Jab1+/− mice were sacrificed, and bone marrow sections were stained with hematoxylin and eosin.

FIGURE 5. Down-regulation of p16INK4a transcription in bone marrow of Jab1 transgenic mice. A and B, total RNA was isolated from bone marrow cells of WT, Jab1-Tg, and Jab1 heterozygous (Jab1+/−) mice and subjected to a quantitative RT-PCR analysis (A). The reactions were performed within a linear range, and the results were confirmed with the real time PCR technique. Quantified results are the means ± S.D. of three independent experiments (B). The relative amount normalized to the expression level of β-actin in wild-type cells was set to 1.0. C, total RNA was isolated from KSL cells, which were sorted from bone marrow of WT and Jab1-Tg mice by flow cytometer and subjected to a quantitative RT-PCR analysis. Total Jab1 mRNA was measured as a control.

A single dose of 5-FU was administered, and peripheral blood counts were monitored. Jab1+/− mice exhibited a more severe leukopenia and slower recovery than wild-type mice (Fig. 4C). Consistent with the white blood cell count, wild-type bone marrow was depleted at day 7 after the injection of 5-FU and almost fully recovered at day 10, whereas the effect of myelosuppression on Jab1+/− mice was more severe, and the recovery was hampered (Fig. 4D). Thus, the reduction of Jab1 expression in the heterozygous mice impaired hematopoiesis, which resulted in the death of 33% (2 of 6) of the 5-FU-treated Jab1+/− mice, whereas all of the wild-type mice (5 of 5) survived under these conditions.

JAB1 regulates p16INK4a transcription in cooperation with a novel JAB1-interacting protein, SMYD3. To obtain an insight into how JAB1 promotes the expansion and maintenance of the hematopoietic progenitor cell population, we analyzed the expression and function of various cell cycle and signal transduction regulators, finding the decreased expression or the functional inactivation of several tumor suppressor proteins in JAB1-Tg cells. Among these, we focused on p16INK4a, because recent reports show that p16INK4a plays a critical role in the regulation of the hematopoietic stem cell pool’s size and in tumorigenesis (29–32). A quantitative RT-PCR analysis of RNA isolated from BM cells of wild-type, Jab1-Tg, and Jab1+/− mice (Fig. 5, A and B) showed that the level of the p16INK4a transcript inversely correlated with that of the Jab1 transcript, whereas the levels of other transcripts exhibited marginal change (p15INK4b and little difference (ARF and p21)). Similar results were obtained when we used RNA isolated from KSL cells (Fig. 5C). A quantitative genomic PCR analysis proved that the INK4a gene locus in Jab1-Tg cells did not contain extensive deletions (data not shown), suggesting that p16INK4a is transcriptionally regulated by JAB1.

JAB1 is known to regulate the transcription of many genes through various transcription factors (3, 33), but none of the known JAB1-binding proteins have been reported to control the transcription ofINK4a. In order to explore the molecular mechanism by which JAB1 controls INK4a gene transcription and subsequent cell proliferation and tumorigenesis, we searched for novel JAB1-interacting molecules by yeast two-hybrid screening. After screening 5 × 10⁶ clones of the cDNA library generated from K562 mRNA, we identified several genes whose products interact with JAB1 in yeast cells. One such gene encoded SMYD3, a histone methyltransferase, overexpressed in human colorectal and hepatocellular carcinomas, directly binding to DNA by specifically recognizing the DNA sequence 5′-CCCTCC-3′ or 5′-GGAGGG-3′, and thereby regulating transcription of the target genes (34). GST pulldown assays showed that SMYD3 is capable of directly binding to JAB1 in vitro, although much less extensively than other known JAB1 interactors, such as CSN6 (Fig. 6A). Co-immunoprecipitation assays using cell lysate revealed that endogenous SMYD3 specifically bound to endogenous JAB1 in living cells (Fig. 6B). However, we again found that only a fraction of SMYD3 was in a complex with JAB1 in vivo.

Searching for the SMYD3-binding motif in the vicinity of the INK4a gene locus, we found the consensus sequence scattered...
throughout the locus but particularly clustered in front of exon 1α of the p16\(^{INK4a}\) coding sequence (Fig. 6C), where the p16 promoter is expected to reside. To test whether SMYD3 cooperates with JAB1 to regulate the p16 promoter activity, we cloned a 2-kb genomic DNA fragment from upstream of exon 1α of p16\(^{INK4a}\) into the luciferase reporter plasmid in the presence and absence of the enhancer sequence derived from the SV40 genome. Introduction of the reporter construct into the NIH3T3 mouse fibroblastic cell line, which sustains an extensive deletion around the \(\text{INK4a}\) gene locus, showed that the 2-kb fragment upstream of exon 1α exhibited the promoter activity, and the additional enhancer sequence markedly increased the activity (Fig. 6D). Interestingly, co-transfection of JAB1 and SMYD3 significantly suppressed the promoter activity regardless of the enhancer sequence (Fig. 6D). SMYD3 alone had only a marginal effect, and ectopic expression of JAB1 had a moderate effect. However, the co-expression of JAB1 and SMYD3 was most effective, having a synergistic effect on the suppression of \(\text{INK4a}\) gene transcription (Fig. 6E).

To elucidate the role of SMYD3 in the JAB1-mediated effect, we designed an siRNA expression vector specific to mouse SMYD3 and cultured a semisolid medium supplemented with cytokines as in Table 1 and 2. Colonies containing more than 100 cells were enumerated after 7–12 days. Results are the means ± S.D. of three independent experiments. H, HEK293T cells were transfected with the expression vectors for SMYD3 and Jab1 as indicated at the top, and the fixed chromatin was analyzed by a ChIP assay using an antibody specific to SMYD3 and sets of primers recognizing the 5′-flanking regions of the human \(\text{INK4a}\) and Nkx2.8 genes. I, fixed DNA isolated from Jab1-Tg bone marrow cells was immunoprecipitated with antibodies to SMYD3 and Jab1 and analyzed by a ChIP assay using primers specific to mouse \(\text{INK4a}\).

FIGURE 6. JAB1 and SMYD3 cooperate to regulate p16\(^{INK4a}\) transcription. A, immobilized recombinant GST proteins (shown at the top of the panels) were incubated with lysates from COS7 cells transfected with the HA-JAB1 expression vector. Bound proteins were detected by immunoblotting using antibody to JAB1. B, endogenous SMYD3 protein was immunoprecipitated from the 293T cell lysate and analyzed by immunoblotting with antibodies to JAB1 (top) and SMYD3 (bottom). C, the positions of putative SMYD3-binding motifs within the \(\text{INK4a}\) locus. D and E, NIH3T3 cells were transfected with 1 μg of luciferase reporter construct (P-B, pGL3-Basic; P-E, pGL3-Enhancer; P16-B, pGL3-Basic containing a 2-kb fragment upstream of exon 1α; P16-E, pGL3-Enhancer containing a 2kb fragment upstream of exon 1α) and 1 μg of the cytomegalovirus promoter-driven expression vectors for SMYD3 and GFP-JAB1 together with the cytomegalovirus-β-galactosidase plasmid. The luciferase activity was normalized to that of β-galactosidase for each sample. F, NIH3T3 cells were transfected with the siRNA expression vector specifically designed for mouse SMYD3, selected in puromycin, and analyzed by immunoblotting using antibody to SMYD3. γ-Tubulin was used as a loading control. SMYD3 expression was reduced to ~30% of that in control cells. We used three independent RNA interference constructs with similar results. Representative results are shown. G, JAB1 transgenic bone marrow cells were infected with retroviruses expressing control and SMYD3-specific siRNA (siSMYD3) and cultured in a semisolid medium supplemented with cytokines as in Tables 1 and 2. Colonies containing more than 100 cells were enumerated after 7–12 days. Results are the means ± S.D. of three independent experiments. H, HEK293T cells were transfected with the expression vectors for SMYD3 and Jab1 as indicated at the top, and the fixed chromatin was analyzed by a ChIP assay using an antibody specific to SMYD3 and sets of primers recognizing the 5′-flanking regions of the human \(\text{INK4a}\) and Nkx2.8 genes. I, fixed DNA isolated from Jab1-Tg bone marrow cells was immunoprecipitated with antibodies to SMYD3 and Jab1 and analyzed by a ChIP assay using primers specific to mouse \(\text{INK4a}\).
Jab1 Controls Hematopoietic Progenitors

1000 cells, p < 0.005) (Fig. 6G) to the level of wild-type BM cells, suggesting that SMYD3 is required for JAB1-mediated enhancement of BM cells.

To examine whether SMYD3 interacts with the INK4a promoter, we performed a ChIP assay using an antibody specific to SMYD3 (Fig. 6, H and I). In human embryonic kidney-derived (HEK293T) cells transfected with the SMYD3 expression vector, we found SMYD3 to be associated with the INK4a promoter region containing clustered SMYD3-binding sites, and JAB1 coexpression did not enhance or reduce the binding (Fig. 6H). Furthermore, we detected the binding of endogenous SMYD3 to the INK4a promoter region in JAB1-Tg BM cells (Fig. 6I). Because SMYD3 possesses histone methyltransferase activity, we performed ChIP assays using antibodies to methylated (Lys4 and Lys9) histones H3 but detected little difference of histone methylation in the INK4a region (data not shown). This could mean that the SMYD3-JAB1 complex has substrates other than histones, but this hypothesis should be tested further. Taken together, SMYD3 interacts with JAB1, regulates transcription of the INK4a gene, and controls hematopoietic cells.

DISCUSSION

Overexpression of JAB1 in human cancers has been reported (14–24, 35), and knockdown of JAB1 in cancer cell lines inhibited tumor cell growth (36). However, the functional significance of JAB1 overexpression during the development of tumors was largely unclear. The present study provides a model by which JAB1 participates in tumor development. JAB1 modulates the transcription of p16

 Acknowledgments—We thank Dr. J. Fujisawa for the plasmid, Dr. S. Yamanaka for assistance in generating transgenic mice and TAP-tagged constructs, Dr. K. Tomoda for the initial attempt at the animal experiment, Drs. M. Tsutsui and A. Fukumoto for assistance with the pathological analysis of mice, Dr. R. Farese, Jr., for RF8 cells, I. Nakamae for excellent technical assistance, and M. Kamimoto for assistance with the animal experiment.
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