

Downregulation of an AIM-1 Kinase Couples with Megakaryocytic Polyploidization of Human Hematopoietic Cells

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Abstract. During the late phase of megakaryopoiesis, megakaryocytes undergo polyploidization, which is characterized by DNA duplication without concomitant cell division. However, it remains unknown by which mechanisms this process occurs. AIM-1 and STK15 belong to the Aurora/increase-in-ploidy (Ipl)1 serine/threonine kinase family and play key roles in mitosis. In a human interleukin-3–dependent cell line, F-36P, the expressions of AIM-1 and STK15 mRNA were specifically observed at G2/M phase of the cell cycle during proliferation. In contrast, the expressions of AIM-1 and STK15 were continuously repressed during megakaryocytic polyploidization of human erythro/megakaryocytic cell lines (F-36P, K562, and CMK) treated with thrombopoietin, activated ras (H-ras^{G12V}), or phorbol ester. Furthermore, their expressions were suppressed

during thrombopoietin-induced polyploidization of normal human megakaryocytes. Activation of AIM-1 by the induced expression of AIM-1(wild-type) canceled TPA-induced polyploidization of K562 cells significantly, whereas that of STK15 did not. Moreover, suppression of AIM-1 by the induced expression of AIM-1 (K/R, dominant-negative type) led to polyploidization in 25% of K562 cells, whereas STK15(K/R) showed no effect. Also, the induced expression of AIM-1(K/R) in CMK cells provoked polyploidization up to 32N. These results suggested that downregulation of AIM-1 at M phase may be involved in abortive mitosis and polyploid formation of megakaryocytes.

Key words: AIM-1 • STK15 • polyploidization • megakaryocyte • cell cycle

Introduction

Megakaryopoiesis occurs through a series of complex processes that involve proliferation of committed precursor cells and subsequent maturation of their progeny, both of which are required for effective platelet production *in vivo*. During the late phase of megakaryopoiesis, megakaryocytes are known to undergo polyploidization, which is characterized by repeated rounds of DNA replication without concomitant cell division. In hematopoietic cells, polyploidization is a phenomenon unique to megakaryocytic lineage cells, whereas it is also observed in other cell types, including liver, urinary bladder epithelium, trophoblast, and salivary gland (Odel and Jackson, 1968; Brodsky and Uryvaeva, 1977).

It was previously thought that polyploidization of megakaryocytes was a result of skipping mitosis after each round of DNA replication. Datta et al. (1996) reported that cdc2 (cdk1) activities, which are indispensable for entry into mitosis, were severely reduced due to the lack of cdc2 protein during phorbol ester (TPA)¹–induced polyploidization of HEL cells. Also, Garcia and Cales (1996) showed that cdc2 activities decreased to a hardly detectable level, because of downregulation of cdc25C phosphatase during TPA-induced polyploidization of HEL and Meg-01 cells. Also, Zhang et al. reported that cdc2 kinase activities were reduced during polyploidization, because of

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¹Abbreviations used in this paper: dn, dominant negative; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPIIb, glycoprotein IIb; IL, interleukin; Ipl, increase-in-ploidy; LacR, lactose repressor; PPP, platelet-poor plasma; rh, recombinant human; rm, recombinant murine; SCF, stem cell factor; TdR, thymidine; TPA, phorbol ester; TPO, thrombopoietin; WT, wild-type.

ubiquitin-dependent degradation of cyclin B in normal rat megakaryocytes, as well as in the megakaryocytic cell lines MegT and Y10/L8057 (Zhang et al., 1996, 1998). In contrast, Vitrat et al. (1998) showed that both cyclin B expression and H1 histone kinase activities were retained in normal human polyploid megakaryocytes. Furthermore, they showed these megakaryocytes did enter mitosis and proceeded to anaphase during polyploidization. Based on these findings, they speculated that megakaryocytic polyploidization was due to the lack of cytokinesis. Also, Nagata et al. (1997) demonstrated that during thrombopoietin (TPO)-induced polyploidization, normal murine megakaryocytes entered mitosis and progressed up to, at least, anaphase A. In a recent review, Zimmet and Ravid (2000) described that these variant findings on cyclin B expression and cdc2 kinase activity might be attributable to the instinctive character of the cells, i.e., the reduction of cyclin B expression might be necessary for the rapid reentry into S phase during polyploidization of highly proliferative megakaryocytic cell lines, because these cells possess higher levels of cyclin B and cdc2 kinase activities than normal megakaryocytes. However, at least in normal megakaryocytes, polyploidization is now considered to be a result of abortive mitosis. Nevertheless, the molecular mechanisms underlying abortive mitosis and megakaryocytic polyploidization remains largely unknown.

Recently, a family of protein kinases that profoundly regulate microtubule-based mitotic events has been described. These kinases were identified from mutants of *Saccharomyces cerevisiae* and *Drosophila* showing mitotic defects (Chan and Botstein, 1993; Shevelyov, 1993). Both gene products, named Ipl (increase-in-ploidy)1 and aurora, were shown to be highly homologous to each other and have the conserved serine/threonine kinase domain. Conditional temperature-sensitive mutants of Ipl1 showed severe chromosome nondisjunction at the restrictive temperature, eventually resulting in the accumulation of unbudded polyploid cells (Chan and Botstein, 1993). Also, mutation in aurora effected the generation of monopolar spindles due to the failure in centrosome separation, thereby leading to the production of severely aneuploid cells (Glover et al., 1995). As reviewed and classified by Bischoff and Plowman (2000), at present, several other members of Aurora/Ipl1 family kinases have been identified: *Caenorhabditis elegans* AIR-2, human STK15 (also called BTAK and aurora2), human AIM-3, rat AIM-1 (Aurora and Ipl1-like midbody-associated protein-1), human AIM-1 (also called aurora1), murine STK-1, murine AYK1 (IAK1), and *Xenopus* pEg2 (Niwa et al., 1996; Kimura et al., 1997, 1998, 1999; Yanai et al., 1997; Bischoff et al., 1998; Katayama et al., 1998; Roghi et al., 1998; Schumacher et al., 1998; Terada et al., 1998; Bischoff and Plowman, 2000). Among this family, human STK15, murine AYK1, and *Xenopus* pEg2 seem to constitute a subfamily, because these molecules have the closely related NH₂-terminal domain, as well as the COOH-terminal kinase domain. In addition, AYK1 was supposed to be a murine homologue of human STK15. Also, rat AIM-1, human AIM-1, and murine STK-1 are considered to be homologous, because they show high amino acid sequence identities in their full coding region.

In proliferating cells, expressions of STK15 and AIM-1 are regulated in a cell cycle-dependent manner; both ex-

pressions are low in G1/S, upregulated during G2/M, and reduced rapidly after mitosis (Kimura et al., 1997; Terada et al., 1998). By immunofluorescence analysis, STK15 was found to be localized to the spindle pole during mitosis, especially from prophase through anaphase (Kimura et al., 1997; Zhou et al., 1998). In contrast, AIM-1 was localized at the equator of central spindles during late anaphase and at the midbody during telophase and cytokinesis (Terada et al., 1998). From the localization data, STK15 was supposed to be required for a centrosome function(s), such as sister chromosome segregation and spindle formation, and AIM-1 for proper progression of cytokinesis, respectively. In addition, *C. elegans* AIR-2, which is expressed on metaphase chromosomes, moves to midbody microtubules at anaphase, and then persists at the cytokinesis remnant, was reported to be required for polar body extrusion and cytokinesis (Schumacher et al., 1998). Because loss of function of aurora and Ipl1 have been shown to give rise to polyploid cells (Chan and Botstein, 1993; Glover et al., 1995), these findings led us to speculate that Aurora/Ipl1 family of protein kinases expressed in megakaryocytes might be involved in polyploidization of megakaryocytes.

Here, we investigated the roles of AIM-1 and STK15 in megakaryocytic polyploidization. In the process of proliferation, both AIM-1 and STK15 were specifically expressed at G2/M phase of the cell cycle in an interleukin (IL)-3-dependent hematopoietic cell line, F-36P. In contrast, during the process of polyploidization, both expressions were continuously downregulated in normal human megakaryocytes, as well as in various human erythro/megakaryocytic cell lines. In addition, ectopically expressed wild-type (WT) AIM-1, but not STK15, significantly inhibited TPA-induced polyploidization of K562 cells. Furthermore, the induced expression of dominant-negative (dn)-AIM-1 led to polyploid formation of K562 and CMK cells, whereas that of dn-STK15 was not effective. Here, we provide unique evidence that sustained downregulation of AIM-1 is required and also sufficient for inducing polyploidization of K562 and CMK cells, and propose the possibility that lack of AIM-1 may be responsible for abortive mitosis and polyploid formation of megakaryocytes.

Materials and Methods

Reagents and Antibodies

Highly purified recombinant human (rh) IL-3, rhTPO, recombinant murine (rm) IL-3, and rm stem cell factor (SCF) were provided by Kirin Brewery Company, Ltd. Murine anti-Flag mAb (M2) was purchased from Sigma-Aldrich. Murine anti-AIM-1 pAb was generated by Dr. M. Tatsuka (Hiroshima University, Hiroshima, Japan). Alexa-conjugated goat anti-mouse IgG antibody, FITC-conjugated murine anti- α -tubulin mAb, and FITC-conjugated rabbit anti-CD41 mAb were purchased from PharMingen. Rabbit anti-cyclin B1 pAb (H-433) was purchased from Santa Cruz Biotechnology, Inc.

Cell Lines and Cultures

A human IL-3-dependent erythroleukemia cell line F-36P and a human erythroleukemia cell line K562 were obtained from RIKEN Cell Bank. CMK, a human megakaryoblastic leukemia cell line, was provided from Otsuka Pharmaceutical Company, Ltd. F-36P/ras and K562/ras were transfected with a Lac-inducible expression vector of activated ras (H-ras^{G12V}), and F-36P-*mpl* was transfected with the TPO receptor *c-mpl*, as described previously (Matsumura et al., 1998, 2000b). K562, K562/ras, and CMK cells were cultured in RPMI 1640 (Nakarai Tesque) supplemented with 10% FCS (Flow) at 37°C. F-36P, F-36P/ras, and F-36P-*mpl*

cells were cultured in RPMI 1640 supplemented with 10% FCS in the presence of rhIL-3 (5 ng/ml).

Preparation and Culture of CD34⁺ Cells from Normal Human Bone Marrow Cells

Human bone marrow cells were obtained from normal volunteers after informed consent was given. CD34⁺ progenitor cells were purified from bone marrow cells by using a magnetic cell sorting system Mini-MACS (Miltenyi Biotec), according to the manufacturer's instruction. The short-term liquid culture of human megakaryocytes was performed as described previously (Debili et al., 1992; Choi et al., 1995), with minor modifications. In short, the enriched CD34⁺ cells were seeded at 5×10^5 cells/ml in Iscove's modified Dulbecco's medium supplemented with rhTPO (20 ng/ml), platelet-poor plasma (PPP) (20%) from the patients with aplastic marrows, and L-asparagine (2 mg/ml), and then cultured in 96-well culture plates for up to 6 d.

Preparation and Culture of Murine Bone Marrow Mononuclear Cells

Bone marrow cells were obtained from femur and tibia of 8–10-wk-old C57BL/6Njcl female mice purchased from CLEA Japan, Inc., and suspended in RPMI 1640 supplemented with 10% FCS. Mononuclear cells were isolated by Ficoll-Hypaque (Nycomed Pharma) density gradient centrifugation, and cultured in RPMI 1640, supplemented with 10% FCS in the presence of rhTPO (100 ng/ml), or rmIL-3 (10 ng/ml) and rmSCF (100 ng/ml), at 37°C.

Double Thymidine (TdR) Blocks

F-36P cells were treated with 2.5 mM of TdR (Sigma-Aldrich) for 20 h, washed with PBS three times, and resuspended in the usual culture medium. After the 16-h culture, the cells were again treated with 2.5 mM of TdR for 20 h. After the second TdR treatment, 92% of F-36P cells were synchronized at a G1/S boundary. Then, the cells were released by washing out TdR, and then subjected to cell cycle and Northern blot analyses.

Morphologic Analysis

The morphologic characteristics of the cultured cells were determined by staining the cytospin preparations (Shandon, Inc.) with May-Grunwald-Giemsa.

Immunostaining

The cells were attached to sylan-coated slide glasses (Dako) by cytospin centrifugation. The cytospin preparations were pretreated with microtubule stabilizing buffer (80 mM potassium Pipes, pH 6.8, 5 mM EGTA, 1 mM MgCl₂) and then fixed with methanol at -20°C for 10 min. The fixed cells were washed with a solution of 0.1 M Pipes, pH 7.2, 1 mM MgSO₄, 1 mM EGTA, 1.83% L-lysine, 1% BSA, and 0.1% sodium azide, and then they were incubated with murine anti-Flag mAb (M2) (2 μg/ml) or murine anti-AIM-1 (1:100 dilution) antibody for 1 h. Next, the cells were incubated with Alexa-conjugated goat anti-mouse IgG antibody. After rinsing with PBS containing 0.2 μg/ml of DAPI three times, the cytospin preparations were incubated with FITC-conjugated murine anti-α-tubulin antibody (4 μg/ml) or FITC-conjugated rabbit anti-CD41 antibody (0.5 μg/ml). The stained cells were observed under a fluorescent microscope.

Flow Cytometry

DNA content of cultured cells was quantitated by staining with propidium iodide and analyzed on FACSort[®] (Becton Dickinson), as described previously (Matsumura et al., 1999).

Northern Blot Analysis

The isolation of total cellular RNA and the method for Northern blot analysis were described previously (Matsumura et al., 1999).

Plasmid Constructs and cDNAs

Flag-tagged WT and point mutated (K/R type) AIM-1 and STK15 cDNAs were subcloned into the KpnI/XbaI site of a Lac-inducible expression vector, pOPRSVI. Both the K/R type of AIM-1 and STK15 harbor amino acid substitutions, from lysine (K) to arginine (R), at a putative ATP-

binding site (AIM-1, codon 109; STK15, codon 151), and act as dn mutants over the respective endogenous protein, as reported previously (Terada et al., 1998). Human cyclin A, cyclin B, and cyclin E cDNAs were a gift from Dr. H. Kiyokawa (University of Illinois, Cancer Center, Chicago, IL).

Lac-inducible System

To express a target cDNA, we used a LacSwitch[®] II-inducible expression system (Stratagene). In short, K562 cells were initially transfected with an expression vector of lactose repressor (LacR), pCMV-LacI, by electroporation (250 V, 960 μFD) (Bio-Rad Laboratories). The transfected cells were screened by selecting with hygromycin (Sigma-Aldrich) at 0.5 mg/ml. Out of several hygromycin-resistant clones, one clone, in which LacR was most intensely expressed, was selected and designated K562-LacR. K562-LacR cells were further transfected with pOPRSVI, each containing Flag-tagged AIM-1(WT), AIM-1(K/R), STK15(WT), and STK15(K/R). The expression vector of pOPRSVI contains the RSV promoter linked to the *Escherichia Coli* lactose operon, and the expression of the target cDNA is suppressed by LacR through the lactose operon. When IPTG was added to the culture medium, LacR was released from the lactose operon and the transcription of the target cDNA was initiated. After selection with G418 (1.5 mg/ml; GIBCO BRL), the induction levels of the target proteins in each clone were examined before and after the 0.5 mM IPTG treatment by Northern and Western blot analyses. Several clones, in which the target protein was effectively induced by the IPTG treatment, were prepared from each transfectant and designated K562/AIM-1(WT), K562/AIM-1(K/R), K562/STK15(WT), and K562/STK15(K/R), respectively. To induce expression of AIM-1(K/R) in CMK cells, we transfected pOPRSVI containing Flag-tagged AIM-1(K/R) together with pCMV-LacI. After the selection with G418, we selected several clones in which AIM-1(K/R) was efficiently induced by the IPTG treatment.

Cell Extract Preparation and Immunoblotting

The protein expression levels of cyclin B1 were examined by Western blot analysis on the total cellular lysates, as described previously (Matsumura et al., 2000a). Since both AIM-1 and STK15 proteins are insoluble in the usual lysis buffer, the preparation of the cellular lysates for Western blot analysis was performed according to the following procedures. The cultured cells were lysed with the buffer (10 mM Tris-HCl, pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 4 mg/ml leupeptin), followed by centrifugation at 10,000 g for 5 min. For immunoprecipitation, the precleared lysates, which were obtained from 10⁷ cells, were incubated with 1 μg of anti-Flag mAb and then by the addition of protein G-Sepharose beads. The immunoprecipitates were subjected to SDS-PAGE and electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore). After blocking the residual binding site on the filter, immunoblotting was performed with anti-Flag mAb. Immunoreactive proteins were visualized with the enhanced chemiluminescence detection system (DuPont).

Semiquantitative Reverse Transcription (RT)-PCR Analyses

Total cellular RNA was extracted from a pellet containing ~10⁴ cells, and was reverse transcribed into cDNA with oligo (dT) primers (Amersham Pharmacia Biotech) by using SuperScript reverse transcriptase (GIBCO BRL) in 20 μl of reaction buffer, according to the manufacturer's instruction. The cDNA product (1 μl) was resuspended in 20 μl of PCR reaction buffer containing 0.5 U of TaqGold DNA polymerase (Perkin Elmer), 2 mM MgCl₂, 200 μM dNTP mix, 30 μCi (α-³²P) dCTP, and 15 pmol of forward and reverse primers. The primer sets used to amplify AIM-1, STK15, glycoprotein IIb (GPIIb), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: AIM-1, a sense primer (5'-AGATC-GAAATCCAGGCCAC-3', nucleotides 286–305) and a reverse primer (5'-GACAGATTGAAGGGCAGA-3', nucleotides 1,069–1,086); STK15, a sense primer (5'-TGAGGAGGGAACTGGCATCAA-3', nucleotides 498–517) and a reverse primer (5'-GACCACCCAAAATCTGCAAT-3', nucleotides 979–999); GPIIb, a sense primer (5'-TGAGGAGGAACTG-CATCAA-3', nucleotides 498–517) and a reverse primer (5'-AAG-AACTCCAAGGCTCCAGA-3', nucleotides 1,101–1,120); and GAPDH, a sense primer (5'-CCACCCATGGCAAATTCATGGCA-3', nucleotides 146–169) and a reverse primer (5'-TCTAGACGGCAGGTCAG-GTCCACC-3', nucleotides 720–743). The samples were denatured at 94°C for 10 min, followed by 16–35 cycles of amplification (94°C, 30 s for denaturation; 55°C, 30 s for annealing; 72°C, 45 s for extension). At first, we adjusted the amounts of cDNA product in each sample. In brief, the al-

iquots (5 μ l) of GAPDH products obtained after 16, 20, and 24 PCR cycles from each sample were size fractionated on 3.5% polyacrylamide gels, dried, and autoradiographed. Because GAPDH was amplified exponentially during 16–24 PCR cycles in all of the samples (at day 0, 2, 4, and 6), we quantified the amounts of cDNA by measuring the intensities of GAPDH products obtained after 20 PCR cycles with a densitometry. After normalization according to the amounts of GAPDH products, equal amounts of cDNA products from each sample were subjected to the PCR reaction. The amounts of AIM-1, STK15, and GPIIb were evaluated after appropriate PCR cycles indicated which PCR products from all of the samples were exponentially amplified.

Results

Both AIM-1 and STK15 Are Specifically Expressed at G2/M Phase of the Cell Cycle during IL-3–induced Proliferation of F-36P Cells

Since it has been shown that AIM-1 and STK15 are specifically expressed at G2/M phase of the cell cycle in nonhematopoietic cells, we initially examined expression patterns of these molecules in a human IL-3–dependent hematopoietic cell line F-36P synchronized by double TdR blocks. After the second culture with TdR, 92% of the cultured cells were synchronized at a G1/S boundary. Then, the cells were released and subjected to cell cycle (Fig. 1 A) and Northern blot analyses (Fig. 1 B), at the times indicated. After the release, 66% of the cells entered S phase at 4 h, and then progressed to G2/M phase from 12 to 20 h; the proportion of cells in G2/M phase increased maximally (37%) at 16 h. Consistent with the findings on cell cycle analysis, expression of cyclin A and cyclin B gradually in-

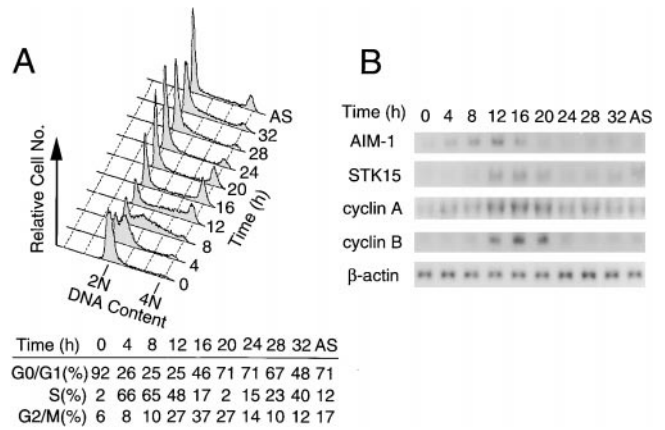


Figure 1. (A) DNA content analysis by flow cytometry. F-36P cells were synchronized by double TdR blocks. After release, the cells were cultured with rhIL-3 for the times indicated. The DNA content of cultured cells was quantitated by staining with propidium iodide and analyzing on a FACSort[®] with Modfit LT 2.0. The results are summarized in the table. AS, asynchronous cells. (B) Changes in expression of AIM-1, STK15, cyclin A, and cyclin B mRNA during IL-3–induced cell cycle progression. Total cellular RNA was isolated at the times indicated and subjected to Northern blot analysis. The filters were hybridized with ³²P-labeled probes as indicated.

creased, reached a peak at 16 h, and decreased at 24 h. The expression of AIM-1 appeared ~4 h, peaked at 12 h, and disappeared at 20 h. Also, the expression of STK15 was observed from 12 to 20 h. Because both expression patterns

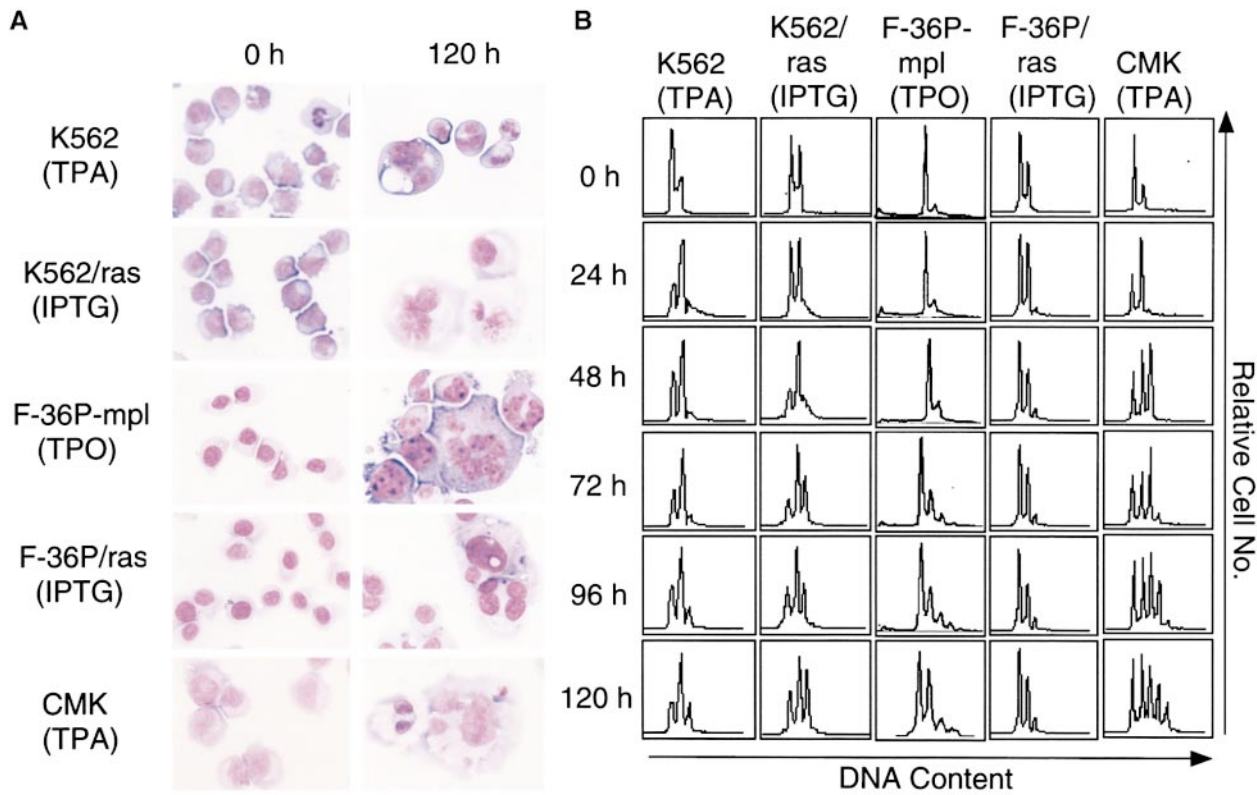


Figure 2. Morphologic and DNA content analyses of various types of leukemia cell lines before and after the induction of megakaryocytic differentiation. The cells were cultured with TPA (1 nM), IPTG (1 mM), and TPO (30 ng/ml) for 120 h. (A) Light micrographs of cells. A cytocentrifugation preparation from each culture was stained with May-Grunwald-Giemsa. Bar, 10 μ m. (B) DNA content of the cultured cells was analyzed at the times indicated by flow cytometry.

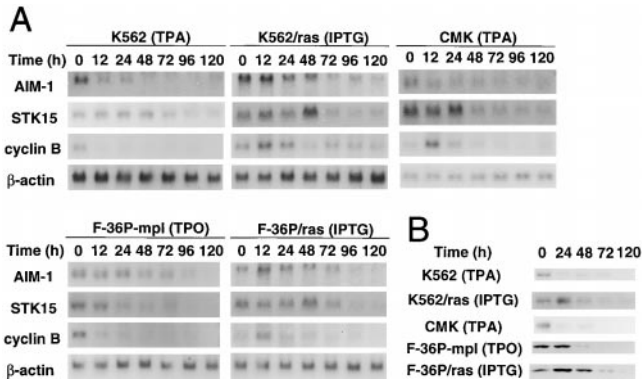


Figure 3. Changes in expression of AIM-1, STK15, and cyclin B during megakaryocytic differentiation of various leukemia cell lines. (A) Northern blot analysis of AIM-1, STK15, and cyclin B mRNA expression. Total cellular RNA was isolated from the cells in Fig. 2, and subjected to Northern blot analysis. The filters were hybridized with 32 P-labeled probes for AIM-1, STK15, cyclin B, and β -actin. (B) Western blot analysis of cyclin B1 expression. Total cell lysates were prepared from the cells in Fig. 2, and then subjected to immunoblot analysis with the anti-cyclin B1 antibody H-433.

were roughly coincident with those of cyclin A and cyclin B, AIM-1 and STK15 are supposed to be expressed specifically at G2/M phase in proliferating hematopoietic cells.

The Expressions of AIM-1 and STK15 Are Continuously Downregulated during Polyploidization of Megakaryocytic Cells

We previously found that expression of AIM-1 was downregulated during TPA-induced polyploidization of several megakaryocytic cell lines (Katayama et al., 1998). Here, we investigated the changes in expression of AIM-1 and STK15 during various types of megakaryocytic differentiation. Human erythro/megakaryocytic leukemia cell lines

K562 and CMK are known to undergo polyploidization in response to TPA (Tetteroo et al., 1984; Matsumura et al., 1997). In addition, we previously reported that TPO treatment of F-36P-*mpl* yielded highly differentiated polyploid megakaryocytes, and that the induced expression of activated ras (H-ras^{G12V}) provoked polyploidization of F-36P/ras and K562/ras cells (Matsumura et al., 1998, 2000b). Before each treatment, K562, CMK, F-36P-*mpl*, K562/ras, and F-36P/ras cells were exclusively composed of undifferentiated blastoid cells (Fig. 2 A). In contrast, the 120-h treatment with TPA, TPO, or IPTG individually gave rise to mature megakaryocytes with polyploid nucleus (several typical cells are shown in Fig. 2 A). In accordance with the morphologic data, DNA content analyses revealed that polyploid formation was induced by each treatment in all of the clones (Fig. 2 B). When expression levels of AIM-1 and STK15 were examined by Northern blot analysis, both expressions gradually decreased and were retained at a hardly detectable level until 120 h in all of the culture conditions (Fig. 3 A). Because cyclin B is a crucial regulator of mitosis, we also monitored expression levels of cyclin B during these cultures by Northern blot analysis. As shown in Fig. 3 A, the expressions of cyclin B were downregulated in all of the cultures. Consistent with the Northern blot analysis results, immunoblotting showed that the expression levels of cyclin B1 were reduced during polyploidization in all cultures (Fig. 3 B). These findings were largely consistent with previous reports that the expression of cyclin B was downregulated during polyploidization of several megakaryocytic cell lines (Zhang et al., 1996, 1998; Matsumura et al., 2000a), but at variance with the finding that the cyclin B expression was retained in normal polyploidizing megakaryocytes (Vitrat et al., 1998). As reviewed by Zimmet and Ravid (2000), it was speculated that the reduction of cyclin B expression might be necessary for rapid reentry into S phase during polyploidization in highly proliferative megakaryocytic cell

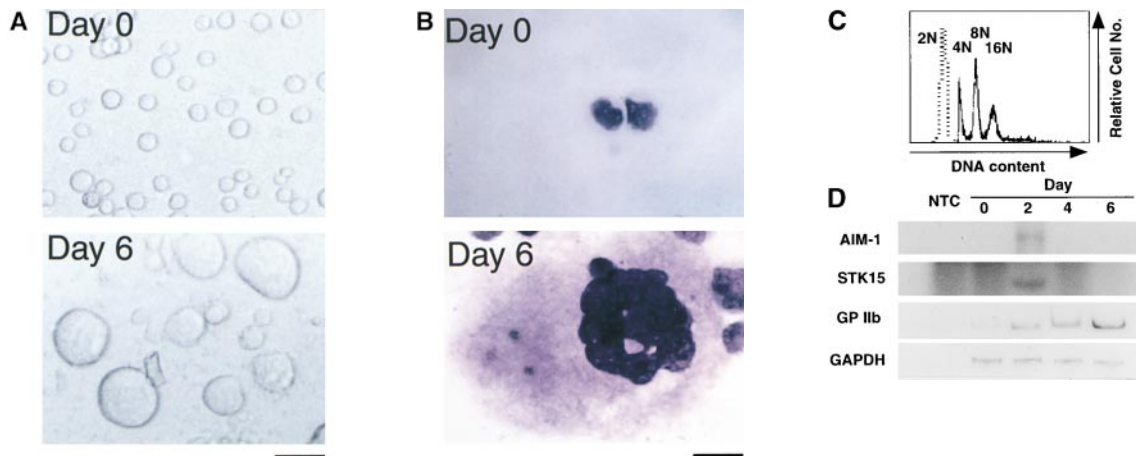


Figure 4. The expressions of AIM-1 and STK15 during the developmental processes of normal megakaryocytes. CD34⁺ cells were purified from normal human bone marrow cells, and then cultured with TPO (20 ng/ml) and PPP (20%) for the indicated times. (A) Phase-contrast micrographs of cells before and after the culture. Bar, 20 μ m. (B) Light micrographs of cells. Cyto-centrifugation preparations of the cells before and after the culture were stained with May-Grunwald-Giemsa. Bar, 10 μ m. (C) DNA content of cultured cells was analyzed by flow cytometry before (dashed line) and after (solid line) culture. (D) Semiquantitative RT-PCR analysis on the expression of AIM-1 and STK15 mRNA. Total cellular RNA was isolated at the times indicated and cDNA was synthesized. The amounts of cDNA products were normalized according to the amounts of PCR products of GAPDH. The adjusted amounts of cDNA products from each sample were subjected to the PCR reaction for AIM-1, STK15, GPIIb, and GAPDH. The PCR products were size fractionated on 3.5% polyacrylamide gels, dried, and autoradiographed. NTC, no template control.

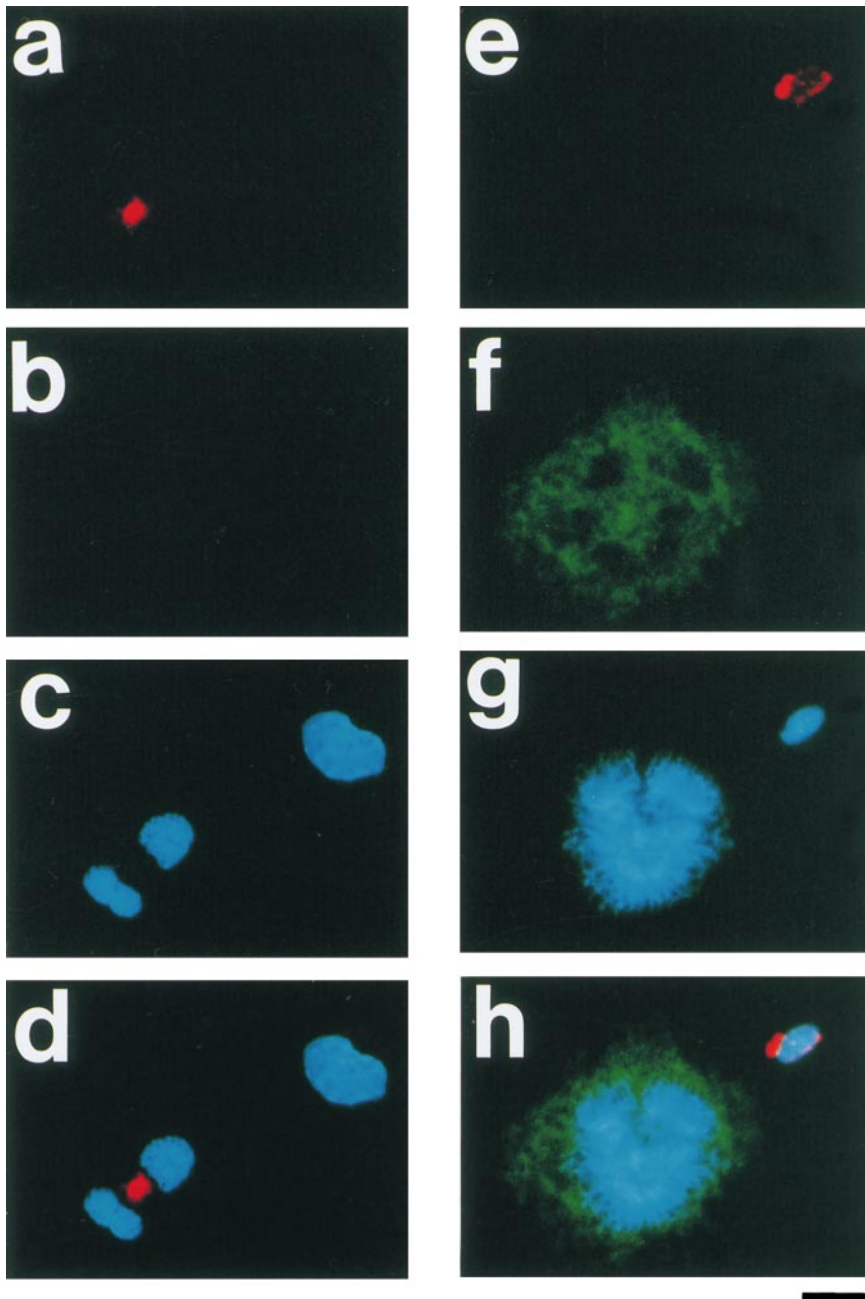


Figure 5. Immunocytochemical analysis of murine proliferating cells and polyploid megakaryocytes. Murine bone marrow mononuclear cells were cultured with rmIL-3 (10 ng/ml) and rmSCF (100 ng/ml) (a–d) or with rhTPO (100 ng/ml) (e–h) for 3–5 d. Cyto centrifugation preparations were stained with murine anti-AIM-1 antibody and Alexa-conjugated goat anti-mouse IgG antibody (a and e), FITC-conjugated anti-CD41 mAb (b and f), and DAPI (c and g). The triple staining of the same cell is shown in d and h. Bar, 10 μ m. a–d show a CD41-negative (nonmegakaryocytic) proliferating cell, and e–h show a CD41-positive megakaryocyte and a CD41-negative cell also stained for AIM-1.

lines, which possess higher levels of cyclin B and cdc2 activity than normal megakaryocytes.

Next, we examined changes in expression levels of AIM-1 and STK15 during the developmental processes of normal megakaryocytes. CD34⁺ cells were purified from human bone marrow cells and cultured with rhTPO and PPP for the indicated times. After the 6-d culture, we found that most of the surviving cells became large in size by phase-contrast microscopic analysis (Fig. 4 A). Also, cytospin preparations of the cultured cells revealed that ~95% of the cells surviving and developing after the 6-d culture of CD34⁺ cells with rhTPO and PPP were megakaryocytes (a typical cell is shown in Fig. 4 B). In agreement with morphologic changes, DNA content analysis revealed that most of the cultured cells possessed polyploid nuclei (solid line), whereas almost all of the cells were diploid before the culture (dashed line) (Fig. 4 C). Consistent with these findings, the expression level of megakaryocyte-specific

surface antigen GPIIb was upregulated gradually until day 6 by semiquantitative RT-PCR analysis (Fig. 4 D). The expressions of AIM-1 and STK15 were induced at day 2, and then decreased to an undetectable level from days 4 to 6 (Fig. 4 D). In this culture condition, the purified CD34⁺ cells temporarily proliferated in response to TPO around day 2 (data not shown), and then underwent polyploidization until day 6. Thus, it was speculated that the expressions of AIM-1 and STK15 were transiently induced at the proliferative process (day 2) and were continuously suppressed during the subsequent polyploidizing process (days 4–6).

To examine the expression of AIM-1 in polyploidizing megakaryocytes by immunocytochemical analysis, we prepared murine bone marrow mononuclear cells and cultured them with rhTPO. Also, to obtain proliferating cells as a positive control for the AIM-1 staining, the murine bone marrow mononuclear cells were cultured with rmSCF

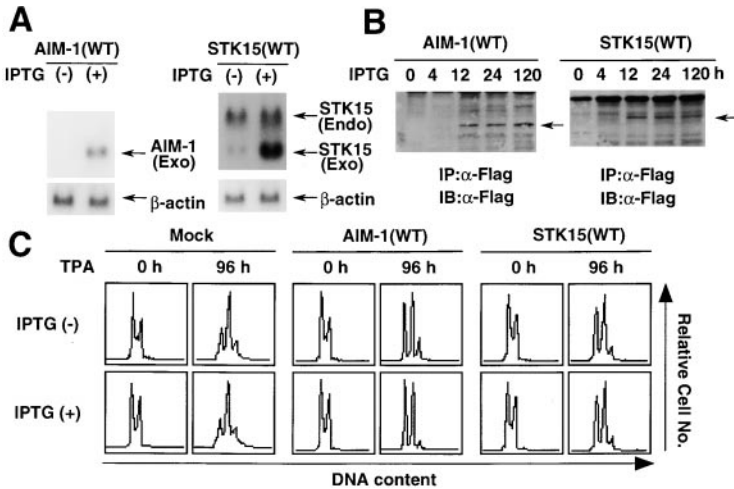


Figure 6. The effects of the induced expression of WT AIM-1 and STK15 on TPA-induced polyploidization of K562 cells. (A) Northern blot analysis on the induction of Flag-AIM-1(WT) and Flag-STK15(WT) before and after IPTG treatment. Total cellular RNA was isolated before and after the 4-h IPTG treatment and subjected to Northern blot analysis. The filters were hybridized with 32 P-labeled probes for AIM-1 or STK15. Exo, exogenous mRNA; Endo, endogenous mRNA. (B) Western blot analysis on the induction of Flag-AIM-1(WT) and Flag-STK15(WT) before and after IPTG treatment. The cells were cultured with 1 mM IPTG for the times indicated. Total cell lysates were immunoprecipitated (IP) and immunoblotted (IB) with the anti-Flag antibody M2. (C) Flow cytometric analysis of DNA content for each clone before and after TPA treatment in the culture, with or without IPTG. Cells were cultured with or without 1 mM of IPTG for 24 h, and then TPA was added to the culture medium (1 nM, final concentration). 96 h after adding TPA, the cultured cells were subjected to DNA content analysis.

and rmIL-3. The cells obtained from both culture conditions were subjected to the double staining with an anti-CD41 antibody and an anti-AIM-1 antibody in batched and parallel reactions. As a positive control, we could find several CD41-negative dividing cells after the 5-d culture with rmIL-3 and rmSCF, in which AIM-1 expression was condensed at their midbody (Fig. 5, a–d). In contrast, all CD41-positive polyploid megakaryocytes that developed after the culture with TPO were essentially negative for anti-AIM-1 staining, even in metaphase or anaphase, whereas some CD41-negative small cells were stained with

an anti-AIM-1 antibody on the same glass slide (Fig. 5, e–h). These results again suggested that AIM-1 expression was continuously downregulated at the protein level in normal megakaryocytes during polyploidization.

Supplement of AIM-1, but Not STK15, Activities Inhibits TPA-induced Polyploidization of K562 Cells

Because the sustained reduction of AIM-1 and STK15 expression was coupled with polyploidization of megakaryocytes, it was speculated that downregulation of AIM-1

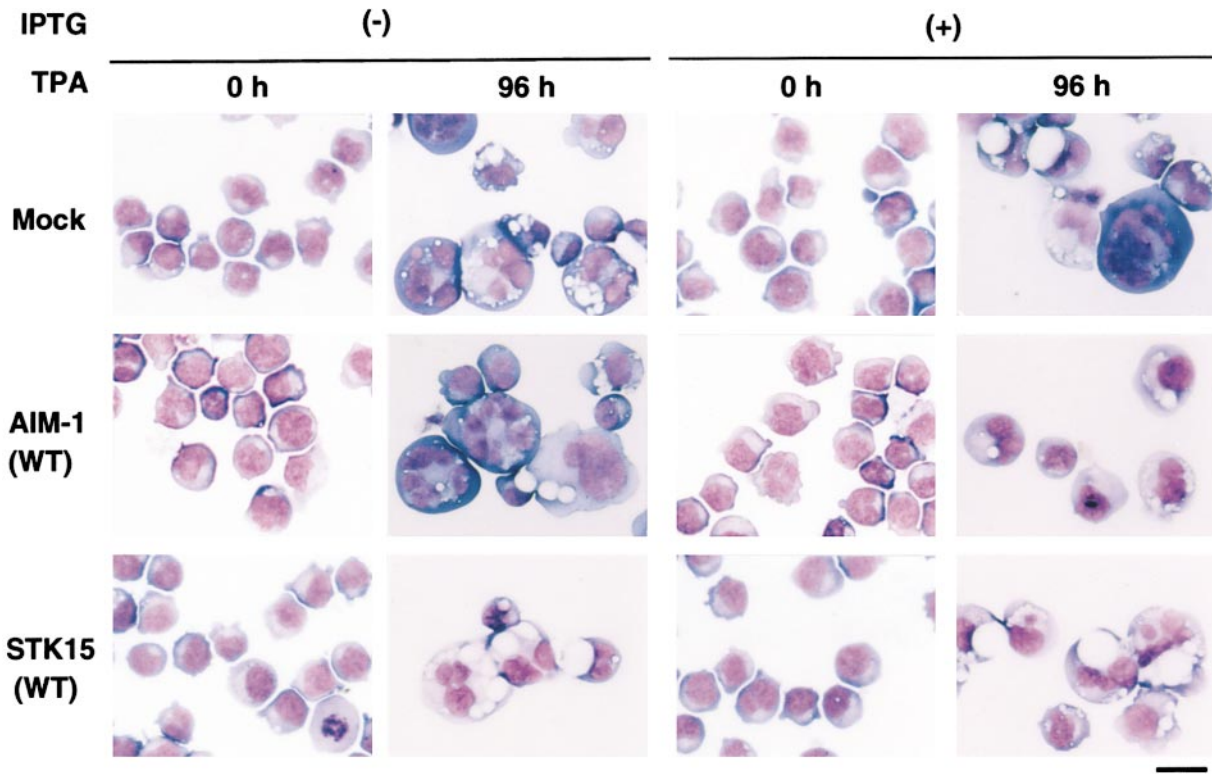


Figure 7. The effects of induced Flag-AIM-1(WT) and Flag-STK15(WT) on TPA produced morphologic changes in K562 cells. The cells were cultured as described in the legend to Fig. 6. A cytocentrifugation preparation from each culture was stained with May-Grunwald-Giemsa. Bar, 10 μ m.

Table I. DNA Ploidy Analysis of K562 Clones after TPA Treatment with or without IPTG Treatment

	IPTG	Percent of cells in each DNA content		
		2N	4N	8N
Mock	-	25.4 ± 2.3	57.6 ± 4.6	17.0 ± 1.1
	+	24.6 ± 1.9	57.9 ± 4.1	17.5 ± 1.4
AIM-1 (WT)	-	37.8 ± 2.5	45.9 ± 3.8	16.3 ± 1.5
	+	48.6 ± 3.1	45.7 ± 2.9	5.7 ± 0.6*
STK15 (WT)	-	38.8 ± 2.9	49.3 ± 3.9	11.9 ± 1.1
	+	39.1 ± 3.3	49.2 ± 3.7	11.7 ± 1.3

The results are shown as the mean ± SD of 10 independent experiments.

*Denotes significant difference ($P < 0.01$) compared to the mock clone by two-sample rank test.

and/or STK15 might be a prerequisite for abortive mitosis and consequent polyploidization of megakaryocytes. To examine this possibility, we inducibly expressed Flag-tagged AIM-1(WT) and STK15(WT) in K562 cells with a Lac-inducible system in which expression of the target protein was induced by IPTG treatment, and investigated their effects on TPA-induced polyploidization of K562 cells; the clones were designated K562/AIM-1(WT) and K562/STK15(WT), respectively. As shown in Fig. 6 A, the expression of AIM-1 and STK15 mRNA was induced after the 4-h IPTG treatment at levels almost similar to or more than that of respective endogenous mRNA in unsynchronized cells. Also, immunoblotting with anti-Flag antibody demonstrated that AIM-1 and STK15 proteins were effectively induced by IPTG treatment after 12–120 h (Fig. 6 B). With or without the 24-h IPTG pretreatment, K562/AIM-1(WT), K562/STK15(WT), and a control clone K562/mock transfected with an empty vector were cultured with TPA in the presence or absence of IPTG for 4 d. Without IPTG treatment, DNA content analysis showed that TPA treatment induced similar levels of poly-

ploidization in K562/mock, K562/AIM-1(WT), and K562/STK15(WT) cells (Fig. 6 C). In contrast, the induced expression of AIM-1(WT) reduced the proportion of 8N fraction from $16.3 \pm 1.5\%$ to $5.7 \pm 0.6\%$, with a significant difference ($P < 0.01$, two-sample rank test) (Fig. 6 C and Table I). In addition, IPTG-induced AIM-1(WT) was required to inhibit TPA-induced polyploidization as observed in several K562/AIM-1(WT) clones (data not shown). In contrast, STK15(WT) did not show an apparent effect on TPA-induced polyploidization in K562/STK15(WT) cells (the proportion of 8N fraction: IPTG[-] $11.9 \pm 1.1\%$ versus IPTG[+] $11.7 \pm 1.3\%$) (Fig. 6 C and Table I). Consistent with these data, in morphologic analysis, IPTG-induced AIM-1(WT) significantly suppressed TPA-induced polyploidization, whereas the induced STK15 showed little or no effect (several typical cells are shown in Fig. 7). These results suggested that downregulation of AIM-1, but not STK15, might be required for TPA-induced polyploidization of K562 cells.

To further clarify the mechanisms by which the induced AIM-1(WT) abrogated TPA-induced polyploidization of K562 cells, TPA- and IPTG-treated K562/AIM-1(WT) cells were subjected to immunocytochemical analyses with anti-Flag (Fig. 8 a) and anti- α -tubulin antibodies (Fig. 8 b). Also, the nuclei were visualized with DAPI staining (Fig. 8 c). During interphase, Flag-tagged AIM-1(WT) was dispersed in the cytoplasm, and then visualized by staining with anti-Flag mAb (data not shown). After 36–48 h, some of the cultured cells were supposed to enter mitosis, even in the presence of TPA, because separation of sister chromatids was observed in these cells (a representative cell is shown in Fig. 8). In addition, it was noted that Flag-tagged AIM-1(WT) was localized at the midzone of the central spindle in the cell around anaphase B (Fig. 8), telophase, and cytokinesis (data not shown). These results mimic the activities of endogenous AIM-1.

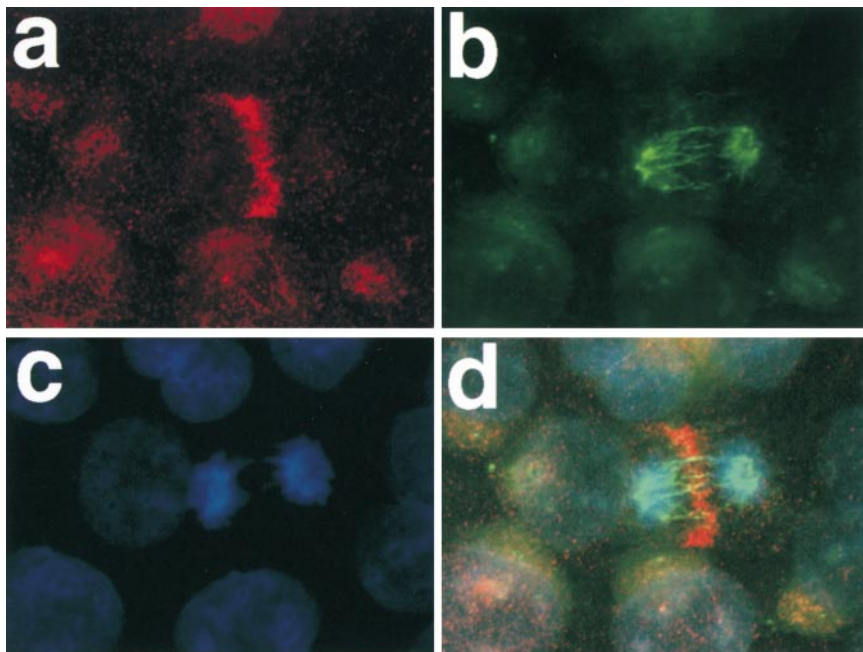


Figure 8. Immunocytochemical analysis of K562/AIM-1(WT) cells during IPTG and TPA treatment. The cells were pre-treated with 1 mM of IPTG for 24 h, and then TPA was added to the culture medium (1 nM, final concentration). 48 h after adding TPA, the cultured cells were stained with murine anti-Flag antibody, M2, and Alexa-conjugated goat anti-mouse IgG antibody (a), FITC-conjugated murine anti- α -tubulin antibody (b), and DAPI (c). The triple staining of the same cell is shown in d. Bar, 5 μ m.

Table II. Effects of AIM-1(K/R) and STK15(K/R) on the DNA Content of K562 Cells

	IPTG	Percent of cells in each DNA content			
		2N	4N	8N	16N
Mock	0 h	62.2 ± 6.0	37.8 ± 3.5	0.0 ± 0.0	0.0 ± 0.0
	120 h	57.9 ± 5.7	42.1 ± 4.0	0.0 ± 0.0	0.0 ± 0.0
AIM-1 (K/R)	0 h	63.6 ± 6.3	36.4 ± 3.5	0.0 ± 0.0	0.0 ± 0.0
	120 h	33.3 ± 2.9	42.1 ± 3.9	21.9 ± 1.8*	2.7 ± 0.1
STK15 (K/R)	0 h	64.8 ± 6.6	35.2 ± 3.6	0.0 ± 0.0	0.0 ± 0.0
	120 h	59.6 ± 6.1	40.4 ± 4.2	0.0 ± 0.0	0.0 ± 0.0

The results are shown as the mean ± SD of 10 independent experiments. *Denotes significant difference ($P < 0.001$) compared to the mock clone by two-sample rank test.

The Induced Expression of dn-*AIM-1* but Not dn-*STK15* Leads to Polyploidization of K562 Cells

Next, we prepared stable clones that can inducibly express the Flag-tagged dn form (K/R type) of AIM-1 and STK15 from K562-LacR cells; these clones were named K562/AIM-1(K/R) and K562/STK15(K/R), respectively. The in-

duction of AIM-1(K/R) and STK15(K/R) proteins by IPTG treatment was confirmed by immunoblot analysis using the anti-Flag antibody (data not shown). Because the data presented here suggested that AIM-1 was involved in megakaryocytic polyploidization, we initially performed immunocytochemical analyses of K562/AIM-1(K/R) cells during IPTG treatment. After the 24–36-h IPTG treatment, Flag-tagged AIM-1(K/R) was localized at the midbody of cells, with two nuclei at late mitosis in a significant proportion of the cultured cells (data not shown). After the 72-h IPTG treatment, K562/AIM-1(K/R) cells with two nuclei (4N cells) entered into mitosis and progressed to metaphase (a representative cell is shown in Fig. 9), at which point Flag-tagged AIM-1(K/R) was dispersed in the cytoplasm (Fig. 9 a). After the 96-h IPTG treatment, several cells possessed four nuclei, and expression of Flag-tagged AIM-1(K/R) was again found at the midbody of the cells at late mitosis (Fig. 9 e). These results mimic the activities of endogenous AIM-1. After the 120-h IPTG treatment, ~30% of K562/AIM-1(K/R) cells revealed polyploid features in morphologic analysis (Fig. 10 A). In contrast, an apparent morphologic change was not

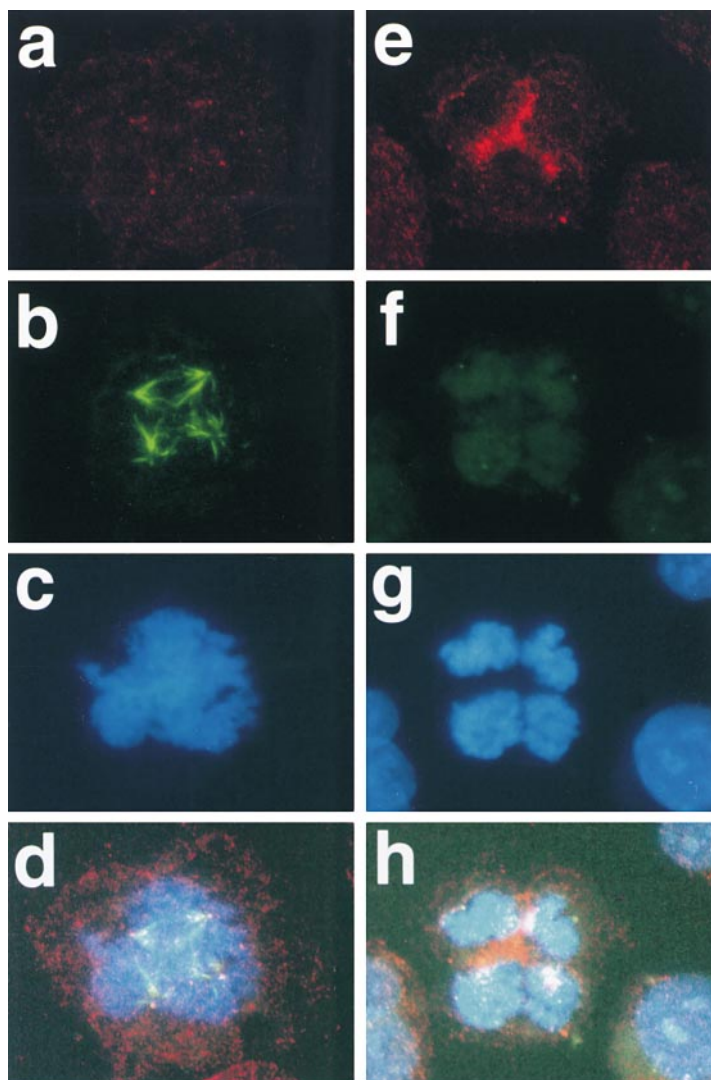


Figure 9. Immunocytochemical analysis of K562/AIM-1(K/R) cells during IPTG treatment. Cells were cultured with 1 mM of IPTG, and then subjected to cytospin preparation at 72 (a–d) and 96 h (e–h). The cytospin preparations were stained with murine anti-Flag antibody M2, and Alexa-conjugated goat anti-mouse IgG antibody (a and e), FITC-conjugated murine anti- α -tubulin antibody (b and f), and DAPI (c and g). The triple staining of the same cell is shown in d and h. Bar, 5 μ m.

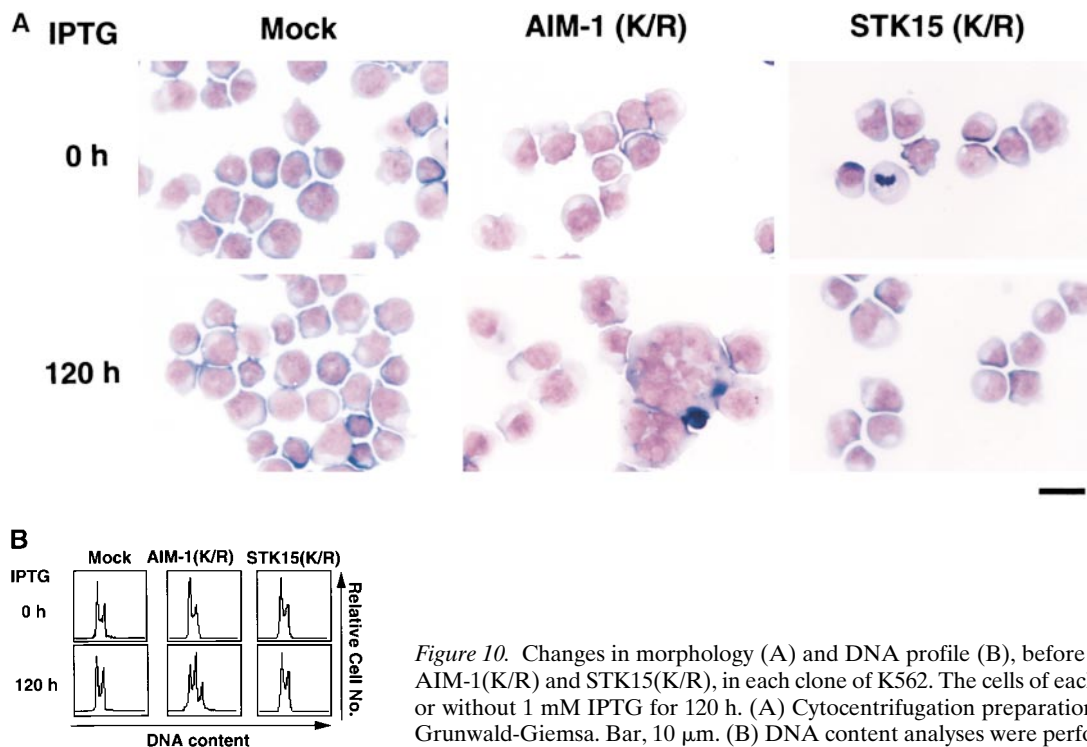


Figure 10. Changes in morphology (A) and DNA profile (B), before and after the induction of AIM-1(K/R) and STK15(K/R), in each clone of K562. The cells of each clone were cultured with or without 1 mM IPTG for 120 h. (A) Cytocentrifugation preparations were stained with May-Grünwald-Giemsa. Bar, 10 μ m. (B) DNA content analyses were performed by flow cytometry.

induced in K562/mock or K562/STK15(K/R) cells after IPTG treatment (Fig. 10 A). Consistent with the changes in morphology, the induced expression of AIM-1(K/R) yielded 8N fraction in $21.9 \pm 1.8\%$ of the cultured cells in DNA content analysis, which was a significant difference ($P < 0.001$) with two-sample rank test. In contrast, STK15(K/R) hardly affected the DNA content profile of K562 cells (Fig. 10 B and Table II). Similar results were observed in several other clones of K562/AIM-1(K/R).

In addition to K562 cells, we also prepared several stable clones from CMK in which AIM-1(K/R) was inducibly expressed; these clone were named CMK/AIM-1(K/R). As shown in Fig. 11 A, induction of AIM-1(K/R) protein (IPTG treatment) was confirmed by immunoblot analysis using an anti-Flag antibody (Fig. 11 A). In a clone of CMK/AIM-1(K/R), DNA content analysis revealed that the induced AIM-1(K/R) provoked prominent polyploidization up to 32N (2N, 20.9%; 4N, 24.4%; 8N, 27.9%; 16N, 22.1%; and 32N, 4.7%) after the 5-d IPTG treatment (Fig. 11 B). Similar levels of polyploid formation were also observed in other clones of CMK/AIM-1(K/R) after IPTG treatment (data not shown). However, the AIM-1(K/R)-induced polyploidization lacked discrete twofold multiples of DNA content in the FACS[®] profile compared with the TPA-induced polyploidization (Fig. 11 B). Thus, in morphologic analyses, we found that the AIM-1(K/R)-induced mature megakaryocytes with multiple nuclei (typical cells are shown in Fig. 11 C) were more liable to result in apoptosis than the TPA-induced megakaryocytes. Because TPA has been reported to activate anti-apoptotic Ras/mitogen-activated protein kinase pathways, it was speculated that the AIM-1(K/R)-induced polyploid megakaryocytes were more sensitive to apoptosis than the TPA-induced polyploid megakaryocytes due to the lack of anti-apoptotic signals. In addition to K562 and CMK cells, the induced expression of AIM-1(K/R) was able to provoke

polyploidization of F-36P-*mpl* cells, as in the case of TPO (data not shown). Taken together, these results implied that the suppression of AIM-1 activities by the induced AIM-1(K/R) was, at least in part, sufficient for inducing polyploidization of K562, CMK, and F-36P-*mpl* cells.

Discussion

Cell growth and differentiation are tightly regulated by a series of cell cycle regulatory molecules, such as cyclins, cyclin-dependent kinases, and CDK inhibitors (for review see Sherr and Roberts, 1995). After the completion of DNA replication, M phase is triggered by M phase promoting factor (MPF or *cdc2*-cyclin B complex) in proliferating cells. In contrast, polyploidizing megakaryocytes are supposed to skip mitosis due to the lack of *cdc2* activities. However, recent studies demonstrated that *cdc2* activities and cyclin B were retained at a level sufficient for entry into M phase in normal human and murine megakaryocytes (Vitrat et al., 1998; Bassini et al., 1999). In addition, immunocytochemical analysis clearly demonstrated that megakaryocytes actually entered mitosis during TPO-induced polyploidization (Nagata et al., 1997; Vitrat et al., 1998; Bassini et al., 1999).

Previously, Nagata et al. (1997) observed by immunocytochemical analysis that during TPO-induced polyploidization, normal murine megakaryocytes entered mitosis and progressed through normal prophase, prometaphase, metaphase, and up to anaphase A, but not to anaphase B, telophase, or cytokinesis. They found that the spindle pole pairs did not show outward movement during anaphase B and were located in closer proximity to each other compared with those in proliferating cells. They speculated that dysregulation of microtubule motor proteins might be involved in this process. Also, a similar functional defect of microtubules in anaphase B was re-

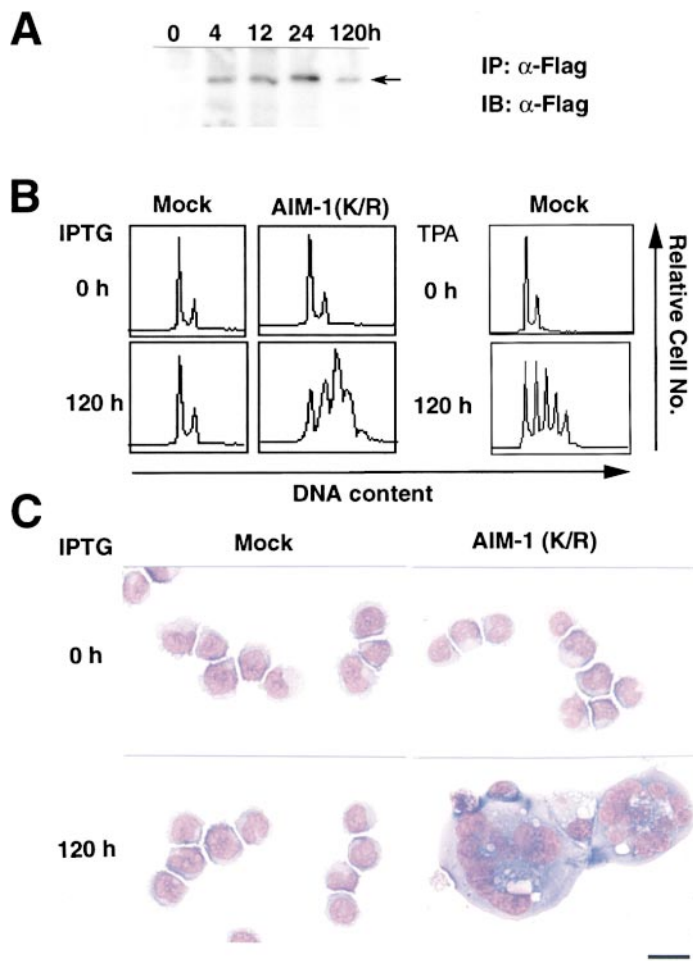


Figure 11. Effects of the induced expression of Flag-AIM-1(K/R) on CMK cells. (A) Western blot analysis on the induction of Flag-AIM-1(K/R) before and after IPTG treatment. CMK/AIM-1(K/R) cells were cultured with 1 mM of IPTG for the times indicated. Total cell lysates were immunoprecipitated (IP) and immunoblotted (IB) with the anti-Flag antibody M2. (B) DNA content analyses on mock-transfected CMK and CMK/AIM-1(K/R). Mock-transfected CMK and CMK/AIM-1(K/R) were culture with IPTG for 120 h. Also, mock-transfected CMK was treated with 1 nM TPA for 120 h. DNA content analyses were performed before and after the cultures. (C) Light micrographs of cells. Cyto-centrifugation preparations of each clone, before and after the 120-h IPTG treatment, were stained with May-Grunwald-Giemsa. Bar, 10 μ m.

ported by Vitrat et al. (1998). However, Bassini et al. (1999) recently reported that normal human polyploidizing megakaryocytes could go through anaphase and complete telophase, because they detected nuclear envelope reconstitution in these cells by transmission electron microscopic analysis. This result suggested that only cytokinesis is neglected in polyploidizing megakaryocytes (even if anaphase B may be disrupted partially), and that the failure of cytokinesis is a major cause of megakaryocytic polyploidization.

Aurora/Ipl1 family proteins associate with spindles and regulate the interaction between kinetochores and microtubules by controlling the phosphorylation status of the kinetochore component (Golsteyn et al., 1995; Biggins et al., 1999). Among this family, STK15 is considered to be involved in centrosome separation, whereas AIM-1 is supposed to regulate cytokinesis. Here, the expressions of AIM-1 and STK15 were both downregulated during polyploidization of megakaryocytes, however, TPA-induced polyploidization of K562 cells was significantly canceled by AIM-1(WT), but not by STK15(WT). In addition, the induced expression of dn-AIM-1, but not of dn-STK15, was effective in inducing polyploidization of K562 cells. These results suggested that the inhibition of cytokinesis by downregulation of AIM-1 activities was both required and sufficient for inducing polyploidization of K562 cells, and that the lack of AIM-1 activities may be a major cause of the abortive mitosis and the consequent polyploidiza-

tion of megakaryocytes. However, we can not still deny the possibility that STK15 might play some roles in megakaryocytic polyploidization, because its roles might be replaced by other members of the STK15 subfamily. Thus, though dn-STK15 scarcely induced polyploidization of K562 cells, further studies are needed to define the roles of STK15 in megakaryocytic polyploidization.

Here, the induced expression of AIM-1(WT) significantly inhibited TPA-induced polyploidization of K562 cells. Because AIM-1(WT) was assumed to exert its effects through microtubules, TPA-treated K562 cells must be in mitosis to receive the effects of AIM-1(WT) efficiently. This result seems to conflict with the previous findings that megakaryocytic cell lines HEL and Meg01 do not enter mitosis during TPA-induced polyploidization due to the lack of cdc2 activities. However, in our preliminary experiments, the immunocytochemical analysis showed that a significant proportion of TPA-treated K562 cells certainly entered mitosis (data not shown). Therefore, we speculated that the induced AIM-1 may inhibit TPA-induced polyploidization of K562 cells, possibly through the completion of their cell division.

We previously reported that overexpression of AIM-1(WT) induced polyploidization in a small fraction (~3%) of MV1Lu cells (Terada et al., 1998). This result also seems to be somewhat inconsistent with the present finding that the induced AIM-1(WT) inhibited TPA-induced polyploidization. However, since AIM-1(WT) was in-

duced in a nonsynchronized condition in our previous report, AIM-1(WT) was supposed to be overexpressed, even at inappropriate phases of cell cycle, such as G1, S, and early G2 phases. Therefore, it was possible that this inappropriate expression of AIM-1(WT) might perturb normal cell cycle progression and lead to inadequate DNA duplication and/or segregation, resulting in polyploid formation in a part of MV1Lu cells.

As in other cell cycle regulatory molecules, the expression levels of AIM-1 protein are supposed to be regulated at both transcriptional and posttranscriptional levels. Here, the expression of AIM-1 mRNA was specifically induced at G2/M phase during IL-3-induced proliferation, whereas its expression was continuously downregulated during polyploidization. To further elucidate the molecular mechanisms of megakaryocytic polyploidization, it may be useful to examine the transcriptional regulation of the AIM-1 gene in endomitotic megakaryocytes, as well as in proliferating cells. Particularly, the analysis on ras/mitogen-activated protein kinase (MAPK) pathway-mediated regulation might provide some clue to identify the mechanistic basis of megakaryocytopoiesis, since this pathway is supposed to regulate megakaryocytic maturation (Matsumura et al., 1998; Fichelson et al., 1999; Rojnuckarin et al., 1999).

Because of the requirement for DNA synthesis in polyploidization, D-type cyclins, which are crucial regulators of G1/S progression (for review see Sherr, 1995), are thought to participate in polyploid formation of megakaryocytes. Recent studies showed that cyclin D3 was intensely expressed in mature megakaryocytes and was necessary for megakaryocytic differentiation, because antisense oligonucleotides to cyclin D3 inhibited megakaryocytic polyploidization in a murine bone marrow culture system (Wang et al., 1995, 1999). In addition, bone marrow megakaryocytes obtained from the transgenic mice, in which cyclin D3 was overexpressed exclusively in a megakaryocytic lineage, showed a significant increase in polyploidization (Zimmer et al., 1997). Also, we recently reported that the enforced expression of each D-type cyclin (1, 2, and 3), together with dn-cdc2, effectively induced megakaryocytic polyploidization of F-36P cells (Matsumura et al., 2000a). These lines of evidence suggested that the increased expression of D-type cyclin is required for polyploidization of megakaryocytes. Together with the data presented here, it was speculated that inhibition of cytokinesis and increased expression of D-type cyclins were both required for efficient polyploidization.

In summary, our data provide unique evidence that downregulation of AIM-1 is involved in megakaryocytic polyploidization. Further studies to elucidate the regulation and function of AIM-1 would lead to a better understanding of megakaryocytopoiesis.

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