G. 知的財産権の出願・登録状況

1. 特許取得

   無し

2. 実用新案登録

   無し

3. その他

   無し

H. 総括と展望

本研究費のサポートによりヒト多能性幹細胞をソースとする血小板製剤が作出可能である事の概念実証を呈示する事ができた。今後この概念実証を確実に臨床ベースにアプリケーションとして展開する為の次のステップの研究を展開していくことが重要であり，今後も厚生労働行政面からの研究支援を期待する。

II. 研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻	ページ	出版年
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III. 研究成果の刊行物・別刷

ELSEVIER

Experimental Hematology

Experimental Hematology 2008;36:897–906

Growth and maturation of megakaryocytes is regulated by Lnk/Sh2b3 adaptor protein through crosstalk between cytokine- and integrin-mediated signals

Hitoshi Takizawa[a,c,*], Koji Eto[b], Atsuko Yoshikawa[c], Hiromitsu Nakauchi[b], Kiyoshi Takatsu[a,d,e], and Satoshi Takaki[c]

[a]*Division of Immunology, Department of Microbiology and Immunology;* [b]*Laboratory of Stem Cell Therapy, Center for Experimental Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan;* [c]*Research Institute, International Medical Center of Japan, Tokyo, Japan;* [d]*Department of Immunobiology and Pharmacological Genetics, Graduate School of Medicine and Pharmaceutical Science for Research, University of Toyama, Toyama, Japan;* [e]*Prefectural Institute for Pharmaceutical Research, Toyama, Japan*

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Objective. **Various cytokines and growth factors control the differentiation and maturation of megakaryocytes (MKs). However, the mechanism regulating platelet release from MKs is not well understood. Here, we investigated a role of Lnk/Sh2b3, an intracellular adaptor protein, in megakaryopoiesis.**

Materials and Methods. **Number of MK progenitor in bone marrow (BM) of wild-type or *Lnk*$^{-/-}$ mice and their sensitivity to thrombopoietin (TPO) were determined in colony-forming unit assay. Using BM-derived wild-type or *Lnk*$^{-/-}$ MKs stimulated with TPO, activation of the signaling molecules was biochemically analyzed and effect of integrin stimulation on TPO signals was studied by addition of vascular cell adhesion molecule (VCAM-1). Platelet production from MKs in the presence of VCAM-1 was counted by flow cytometry and their morphological change was observed by time-lapse microscopy.**

Results. ***Lnk*$^{-/-}$ mice showed elevated platelets and mature MKs due to enhanced sensitivity of progenitors to TPO. Erk1/2 phosphorylation induced by TPO was augmented and prolonged in *Lnk*$^{-/-}$ MKs while activation of signal transducers and activators of transcription (Stat)3, Stat5, and Akt was normal. Wild-type MKs, but not in *Lnk*$^{-/-}$ MKs on VCAM-1 showed reduced Stat5 phosphorylation and mitogen-activated protein kinases activation upon stimulation with TPO. Additionally, the presence of VCAM in culture accelerated spontaneous platelet release from mature wild-type MKs, but not from *Lnk*$^{-/-}$ MKs.**

Conclusions. **Results suggest that contact of MKs with adhesion molecules via integrins might contribute to platelet release, which is under Lnk-mediated regulation of Stat-5 activation and show that Lnk functions in responses controlled by cell adhesion and in crosstalk between integrin- and cytokine-mediated signaling.** © **2008 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Megakaryocytes (MKs) and the platelets they produce are required for normal thrombosis and hemostasis [1]. Recent evidence indicates early roles of platelets in innate immune responses and tumor cell biology [2]. MK progenitors that reside in the bone marrow (BM) undergo endomitosis and differentiate into large, MKs with polyploidy in preparation for platelet production [3]. Thousands of platelets can be released from a single MK into the bloodstream. Thrombopoietin (TPO) is required for both MK development and maintenance of platelet production. Mutant mice lacking TPO or its receptor, c-Mpl, show severe thrombocytopenia [4,5]. However, the remaining platelets in those mice are morphologically and functionally normal [6]. Thus, the TPO/c-Mpl system plays a critical role in the survival and proliferation of MKs, but is not indispensable for either MK maturation or release of platelets.

*H. Takizawa's present address is Institute for Research in Biomedicine, 6500 Bellinzona, Switzerland.

Offprint requests to: Satoshi Takaki, M.D., Ph.D., Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-Ku, Tokyo 162-8655, Japan; E-mail: stakaki@ri.imcj.go.jp

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A critical step in thrombopoiesis is migration and adhesion of MKs to sinusoidal endothelial cells, where proplatelets elongate into the extravascular space [7–9]. The process is regulated by a variety of chemokines and cytokines as well as by adhesive interactions between adhesion molecules and extracellular matrix proteins. Mice lacking P-selectin, but not E-selectin, show increased numbers of interleukin (IL)-3–responsive MK progenitors and mature MKs with high ploidy [10]. A deficiency of platelet-endothelial cell adhesion molecule-1 leads to migration defects of MKs in response to stromal cell-derived factor-1 (SDF-1) and their increased adhesion to BM matrix proteins [11,12]. SDF-1 promotes localization of MK progenitors to the sinusoidal vascular zone in BM. This occurs through vascular cell adhesion molecule-1 (VCAM-1) and fibroblast growth factor-4 augmented very late antigen-4 (VLA-4)–mediated adhesion of MKs to endothelial cells, enhancing both MK survival and maturation [13]. These findings suggest pivotal roles of MK interaction with endothelial cells in BM via integrins and their ligands as well as cytokine-independent regulation in thrombopoiesis.

Lnk, recently designated as SH2B adaptor protein 3 (Sh2b3), belongs to an adaptor protein family composed of SH2-B (Sh2b1) and adaptor protein with PH and SH2 domains, APS (Sh2b2). Lnk negatively regulates lymphopoiesis and early hematopoiesis. *Lnk*-deficiency results in enhanced production of B cells partly due to enhanced signaling through c-Kit, and expansion as well as enhanced function of hematopoietic stem cells (HSCs) [14–16]. In addition, it appears that Lnk negatively controls erythropoietin- and TPO-dependent signals in erythropoiesis and thrombopoiesis [16–18]. Our previous study revealed that motility of hematopoietic progenitor cells on VCAM-1, an integrin ligand, was modulated by Lnk-mediated pathways [19]. Although the expansion of HSCs in $Lnk^{-/-}$ mice is largely TPO-dependent, it appears that the enhanced capability of $Lnk^{-/-}$ HSCs for repopulating irradiated host animals is not accomplished solely by a TPO-dependent pathway [20]. These observations suggest that Lnk might participate in control of integrin-mediated cell motility in addition to regulation of cytokine-dependent growth.

In this study, we investigated the possibility that Lnk might control thrombopoiesis by modulating integrin-mediated responses in addition to TPO-dependent growth signaling. Lnk specifically suppressed TPO-induced activation of Erk1/2, constraining the sensitivity of MKs to TPO. Costimulation of MKs with TPO and VCAM-1 resulted in downregulation of signal transducers and activators of transcription (Stat) 5 and upregulation of mitogen-activated protein kinase (MAPK) activation compared to stimulation by TPO alone. Those changes mediated by co-stimulation by TPO and VCAM-1 were not observed in MKs lacking Lnk. Our results revealed a formerly unrecognized regulatory mechanism in thrombopoiesis through integrin signaling and showed that Lnk modulates crosstalk between integrin-mediated signals and cytokine-induced signals.

Materials and methods

Mice and reagents

C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). $Lnk^{-/-}$ mice [14–16] and green fluorescent protein (GFP) transgenic mice [21], both are on C57BL/6 background, were bred and maintained under specific pathogen-free conditions at the animal facility of the Institute of Medical Science, the University of Tokyo. Recombinant human TPO, mouse IL-3, and mouse IL-6 were purchased from Peprotech (London, UK). Mouse VCAM-1/Fc chimera was provided by R&D Systems (Minneapolis, MN, USA). Purified human fibrinogen was from American Diagnostica Inc. (Stamford, CT, USA). Human collagen type I was from Nycomed Pharma GmbH (Munich, Germany). Anti–β-actin (clone 2.1) was obtained from Sigma (St. Louis, MO, USA), while anti–phospho-Stat5 (pTyr694), anti–phospho-Stat3 (pTyr705), anti-Stat3, anti–phospho-Akt (Ser473), anti-Akt, anti–phospho-Erk1/2 (pThr202/pTyr204), anti-Erk1/2, anti–phospho-p38 (pThr180/pTyr182), and anti-p38 were purchased from Cell Signaling Technology (Danvers, MA, USA). Additional antibodies included anti-Stat5a and anti-Stat5b (Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA), and secondary antibodies, horseradish peroxidase–coupled goat anti-rabbit IgG and sheep anti-mouse IgG (Amersham Pharmacia Biotechnology). Anti-Lnk antibodies were generated [14,15].

Flow cytometric analysis

BM cells or splenocytes from wild-type and Lnk-deficient mice were harvested with Iscove's modified Eagle's medium (IMDM; GIBCO, Invitrogen, Grand Island, NY, USA) containing 2% fetal bovine serum (FBS), incubated with anti-FcRγ2 antibody (2.4G2) to block nonspecific binding and stained with fluorescein isothiocyanate or phycoerythrin-conjugated anti-CD41 (MWReg30; BD Biosciences, San Jose, CA, USA). The percentage of $CD41^+$ cells in total BM was determined with a FACSCalibur instrument (BD Biosciences), and the absolute number of $CD41^+$ MKs in BM or spleen was calculated. The ratio of GFP^+ platelets in total $CD41^+$ platelets was also determined by flow cytometry. For polyploidy analysis, BM cells directly harvested from wild-type and $Lnk^{-/-}$ mice were dissociated in CATCH buffer (Hank's balanced salt solution [GIBCO, Invitrogen], including 0.38% sodium citrate, 1 mM adenosine, and 2 mM theophylline) and stained with fluorescein isothiocyanate-CD41 antibody. To stain nuclei, the labeled cells were washed and incubated overnight in 100 μg/mL propidium citrate and 50 μg/mL RNase in 1% sodium citrate solution at 4°C and subjected to flow cytometric analysis. For MKs used in the following biochemical analysis and in vitro platelet formation, bone marrow cells were cultured for 3 days and the ploidies of MKs purified from the culture were analyzed by the same method as described.

Platelet count

Peripheral blood was taken from wild-type and Lnk-deficient mice retro-orbitally and platelet count was analyzed on the flow cytometer programmed with mouse-specific parameter (Sysmex, Hyogo, Japan).

In vivo turnover of platelets

Platelet-rich plasma from GFP transgenic mice or GFP transgenic $Lnk^{-/-}$ mice was separated by centrifugation of whole blood at 150*g* for 15 minutes without braking. One micrometer prostaglandin E_1 and 5 U/mL apyrase (Sigma) were added followed by centrifugation at 750*g* for 10 minutes. The platelet pellet was resuspended in modified Tyrode-HEPES buffer pH 7.4 (10 mM HEPES, 12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, and 1 mM $MgCl_2$) and the concentration was determined on the flow cytometer (Sysmex). Resultant platelets (2.5×10^9) were intravenously injected into wild-type C57BL/6 mice or $Lnk^{-/-}$ mice. Peripheral blood was collected by plastic tip from the retro-orbital sinus of recipient mice at several time points and was incubated with biotinylated anti-CD41 and peridinin-chlorophyll-protein–conjugated streptavidin for 20 minutes at room temperature, and the percentage of GFP^+ platelets was determined on a FACSCalibur.

Immunohistochemistry

Freshly dissected nondecalcified femurs from 6- to 8-week-old wild-type C57BL/6 mice or $Lnk^{-/-}$ mice were embedded in 4% carboxymethyl cellulose (FINETEC, Fuchu, Japan) and snap-frozen in N-hexane chilled in a slurry of ethanol and dry ice. Sections were generated via Kawamoto's film method (Cryofilm transfer kit; FINETEC). The 150-μm–thick cryostat sections were first blocked with 5% FBS/phosphate-buffered saline and then stained with fluorescein isothiocyanate–conjugated anti-CD41 in blocking buffer. Cell nuclei were labeled with TO-PRO3 (Molecular Probes, Invitrogen). Sections were then mounted with PERMAFLUOR (Beckman Coulter, Fullerton, CO, USA), and confocal microscopic analysis was performed with an Olympus FV-500 confocal microscope.

In vitro colony assays

BM cells were flushed from femurs of wild-type or $Lnk^{-/-}$ mice with IMDM (GIBCO, Invitrogen) containing 2% FBS. Marrow cells (1×10^5) were cultured in double-chamber slides using MegaCult-C (Stem Cell Technologies, Vancouver, Canada) in the presence of different concentration (0, 0.1, 1, 10, and 100 ng/mL) of human TPO or 100 ng/mL TPO, 10 ng/mL mouse IL-3, and 20 ng/mL mouse IL-6. After 7 days of cultivation, slides were dehydrated in cold acetone for 5 minutes and colonies were stained for acetylcholinesterase activity with 5 mM sodium citrate solution, including 3 mM $CuSO_4$, 0.5 mM $K_3[Fe(CN)_6]$, and 75 mM Na_2HPO_4 (pH 6.0). Slides were scored microscopically, and megakaryocyte colonies were defined as colonies with at least three megakaryocytes.

Megakaryocyte culture and purification

Lineage-negative BM cells (depleted of cells expressing B220, CD3, Mac-1, Gr-1, and TER-119) were enriched using Hematopoietic Progenitor Cell Enrichment Set (BD Bioscience) following manufacturer's protocol. In brief, BM cells were labeled with a biotinylated cocktail of lineage-specific antibodies followed by Streptavidin-conjugated magnetic beads and lineage marker–negative (Lin^-) cells were collected using BD IMagnet. Lin^- cells (1×10^6) were incubated in IMDM containing 0.5% bovine serum albumin (BSA), 10 ng/mL human transferrin, human insulin, 1.6 μg/mL low density lipoprotein, 40 μM adenosine triphosphate, 40 μM uridine triphosphate, 40 μM cytidine triphosphate, 40 μM guanosine triphosphate (Sigma), 50 μM 2-mercaptoethanol, 29.2 μg/mL L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin (GIBCO, Invitrogen), in the presence of 20 ng/mL or 2 ng/mL TPO for wild-type or $Lnk^{-/-}$ cells, respectively. After culturing for 3 days, mature MKs were harvested by low-speed centrifugation (120*g* for 10 minutes) and purified by gravity sedimentation through discontinuous BSA (0%/1.5%/3%) for 45 minutes at room temperature.

Immunoblotting

Purified mature MKs after 3-day cultivation were washed with IMDM and further incubated in the absence of TPO for 16 hours for starvation. Subsequently, cells were stimulated with 10 ng/mL TPO for 0, 10, 30, and 60 minutes, and lysed in RIPA buffer (0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% sodium deoxycholate, 158 mM NaCl, 10 mM Tris-HCl, 1 mM ethylene glycol tetraacetic acid, 5 mM ethylene diamine tetraacetic acid, 100 U/mL aprotinin, 1 mM sodium vanadate, 10 mM sodium fluoride, 10 μg/mL leupeptin, and 2 mM phenylmethylsulfonyl fluoride). For integrin stimulation experiments, purified and starved MKs were stimulated with 10 ng/mL TPO and 50 μg/mL VCAM-1, fibrinogen or collagen type 1 in the presence of 2 mM manganese for 10 minutes and lysed. Thirty-five micrograms of total protein was subjected to immunoprecipitation and immunoblotting using anti-Lnk antibodies or other antibodies as described previously [14].

In vitro platelet formation

Twenty-four–well plates were precoated with 20 μg/mL mouse recombinant VCAM-1 overnight at 4°C and washed three times with MK-conditioned media. Purified mature MKs as described above were incubated on BSA- or VCAM-1–coated plates including 250 μL the conditioned media including 1 ng/mL TPO for 12 hours, and the culture supernatant was collected after addition of ethylene diamine tetraacetic acid at a final concentration of 10 mM. Flow-Count Fluorospheres (Beckman Coulter) were added and platelets gated on the same scatter properties as freshly prepared blood platelets were enumerated with a flow cytometer. For anti-integrin-α4 antibody treatment, purified cells were incubated with anti-CD49d, the α4 chain of the VLA-4 (PS/2; SouthernBiotech, Birmingham, AL, USA) at the concentration of 10 μg/mL for 30 minutes prior to the assay.

Proplatelet formation analysis

Harvested fetal liver cells (E13 to 15) were incubated in DMEM including 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 29.2 μg/mL L-glutamine, in the presence of 20 ng/mL or 2 ng/mL TPO for wild-type or $Lnk^{-/-}$ cells. After 3 to 5 days of cultivation, mature MKs were purified as described and washed with Leibovitz's L-15 medium (GIBCO, Invitrogen). MKs in suspension were mounted in semi-solid medium including 20% Leibovitz's L-15, 80% MegaCult M-3234 (Stem Cell Technologies) and 1 ng/mL TPO in 35-mm Petri dishes with a 10-mm glass hole (Matsunami Glass, Osaka, Japan) coated with 10 μg/mL BSA or VCAM-1. Images of proplatelet formation were obtained using confocal microscopy in a CO_2 chamber (FV500; Olympus, Tokyo, Japan).

Results

Mature platelets are increased in the absence of Lnk, but showed normal turnover in circulation

Platelets develop from HSCs in the BM. HSCs give rise to multipotent progenitors, differentiate into committed MK progenitors, and then further proliferate and differentiate into mature MKs. Platelets released from mature MKs circulate throughout the body for several days, and are eventually captured and degraded by macrophages in the spleen and liver. TPO plays key roles in the proliferation and maturation of MK progenitors and megakaryocytes, as well as in the expansion of HSCs. Previous studies by this and other groups have demonstrated that Lnk negatively regulates TPO-dependent signals in megakaryocytic cells as well as in HSCs [17,20,22]. Velazquez et al. reported deregulated hematopoiesis including thrombocytosis in *Lnk*-deficient mice [16]. Furthermore, Tong et al. showed that various signaling molecules downstream of c-Mpl (the TPO receptor), such as Jak2 tyrosine kinase, Stat-5, Erk1/2, and Akt are all hyperactivated in *Lnk*-deficient MK progenitors [17].

We attempted to confirm those observations, and also to define the steps in thrombopoiesis, which were regulated by Lnk-dependent pathway(s). First, we examined expression of Lnk protein in MK-lineage cells, and found that substantial amounts of Lnk protein were expressed in MKs and maintained by mature platelets (Fig. 1A, and data not shown). The number of platelets in peripheral blood was increased nearly fivefold in *Lnk*-deficient mice compared with normal mice (Fig. 1B), consistent with previous observations by Velazquez et al. The absence of Lnk in mature platelets might change their survival and turnover rate in circulation. We assessed the half-life of platelets and the phagocytic function of macrophages by adoptive transfer. Platelet-rich fractions were prepared from the blood of GFP-transgenic mice or GFP-transgenic $Lnk^{-/-}$ mice, and transferred into *Lnk*-sufficient normal mice (Fig. 1C) or *Lnk*-deficient mice (Fig. 1D). In both conditions, transferred *Lnk*-deficient platelets disappeared from peripheral blood at almost the same rate as normal mature platelets. The survival of mature platelets in circulation as well as removal and degradation of old platelets by peripheral macrophages were not affected by the absence of Lnk.

Increased MK precursors in $Lnk^{-/-}$ mice and their distribution in BM

Next, we examined thrombopoiesis in the BM. $CD41^+$ MKs were significantly increased threefold in BM and nearly tenfold in the spleen (Fig. 2A). To determine whether MKs accumulated within specific regions of the BM of $Lnk^{-/-}$ mice, we undertook an immunohistochemical analysis. As shown in Figure 2B, numerous $CD41^+$ MKs were diffusely present throughout the BM in the femur in $Lnk^{-/-}$ mice, and no particular accumulating spot of $CD41^+$ cells was observed.

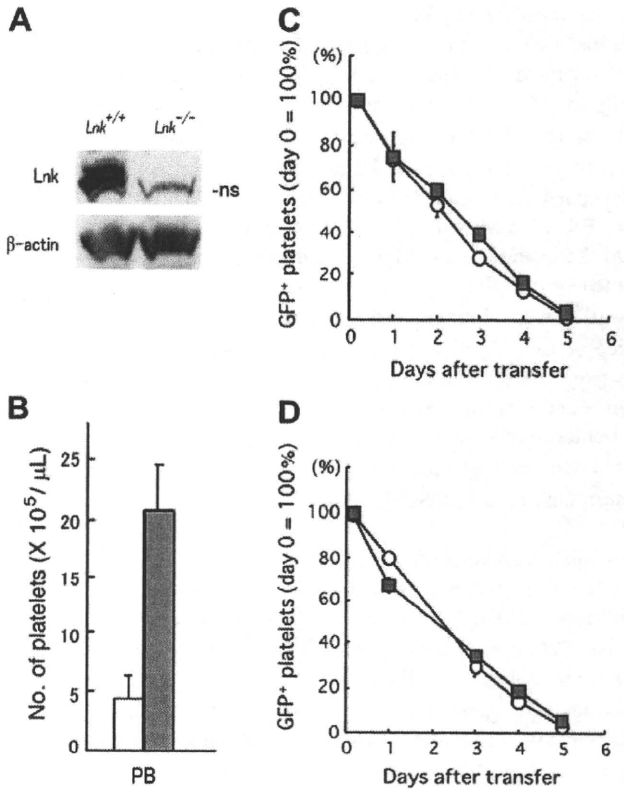

Figure 1. Lnk expression in megakaryocytes (MKs) and thrombocytosis in Lnk-deficient mice. (**A**) Substantial expression of Lnk in MKs. Lin^- cells obtained from bone marrow (BM) of $Lnk^{+/+}$ or $Lnk^{-/-}$ mice were cultured in the presence of 20 ng/mL or 2 ng/mL thrombopoietin (TPO), respectively. After a 3-day incubation, mature MKs were purified on density gradients and lysed. Total proteins were separated and immunoblotted with an anti-Lnk antibody (upper panel) or with an anti-actin antibody, as loading control (lower panel). NS = nonspecific band. (**B**) Fivefold increase in number of platelets in $Lnk^{-/-}$ mice. Peripheral blood was obtained retro-orbitally from $Lnk^{+/+}$ (open column) or $Lnk^{-/-}$ (closed column) mice and platelet counts were measured by flow cytometry. (**C, D**) Platelet turnover in $Lnk^{-/-}$ mice. Platelets obtained from green fluorescent protein-positive (GFP^+) wild-type (open circle) or GFP^+ $Lnk^{-/-}$ (closed square) mice were transferred to $Lnk^{+/+}$ (**C**) or $Lnk^{-/-}$ mice (**D**). Peripheral blood was taken from the recipient mice and the percentage of GFP^+ platelets was determined by flow cytometry. The relative percentage of GFP^+ platelets was calculated by dividing the percentage of GFP^+ platelets at each indicated time point by that at the day of transfer (Day 0).

$Lnk^{-/-}$ MK precursors are hypersensitive to TPO and hypermature

The increase in MKs may be due to increased proliferation of progenitors and/or prolonged survival and maturation of MKs. Growth of MK progenitors and their sensitivity to TPO were evaluated by colony-forming assays. $Lnk^{-/-}$ progenitors formed colonies even at very low concentrations of TPO whereas normal progenitors hardly responded. Furthermore, the number of colony-forming cells upon full stimulation with TPO, IL-6, and IL-3 was twofold higher in $Lnk^{-/-}$ BM than in wild-type (Fig. 3A). Maturation of

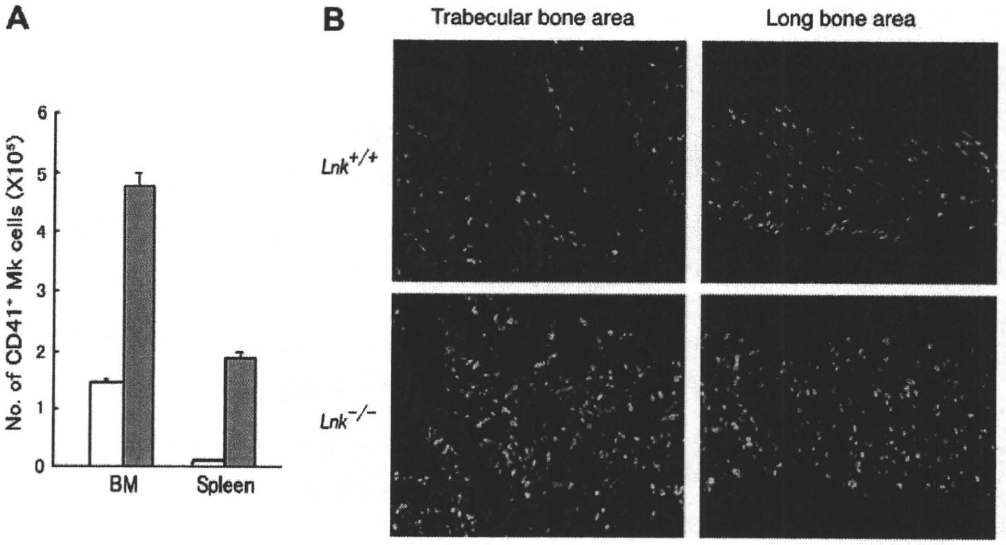

Figure 2. Megakaryocytes (MKs) numbers are increased in bone marrow (BM) and spleen of $Lnk^{-/-}$ mice. (**A**) Significant increase in MKs in BM and spleen. BM cells (left panel) or splenocytes (right panel) were harvested from $Lnk^{+/+}$ (open column) or $Lnk^{-/-}$ (closed column) mice and the percentage of $CD41^+$ MKs was determined by flow cytometry. The absolute number of $CD41^+$ MKs in each tissue was calculated by multiplying the percentage by the absolute number of total mononuclear cells. Shown are mean ± SD of results obtained from two experiments. (**B**) Immunofluorescence microscopic analysis of $Lnk^{-/-}$ BM. Frozen sections of femurs from $Lnk^{+/+}$ (upper) or $Lnk^{-/-}$ (lower) mice were stained with anti-CD41 (green) and TO-PRO3 (blue) and observed in confocal microscopy. Representative images of trabecular bone (left column) and long bone regions (right column) are shown.

MKs in BM also was quantified by measuring their ploidies. Polyploidy analysis of MKs in BM using flow cytometry showed that mature MKs with high polyploidy (32N and 64N) were more frequent in $Lnk^{-/-}$ BM. In contrast, a majority of MKs in normal BM had 16 nuclei (Fig. 3B). These data indicate that Lnk regulates the proliferation of MK progenitors by limiting their sensitivity to TPO, and *Lnk*-deficiency promoted expansion of MK-lineage cells. It seemed that Lnk also played a role in controlling maturation of MKs.

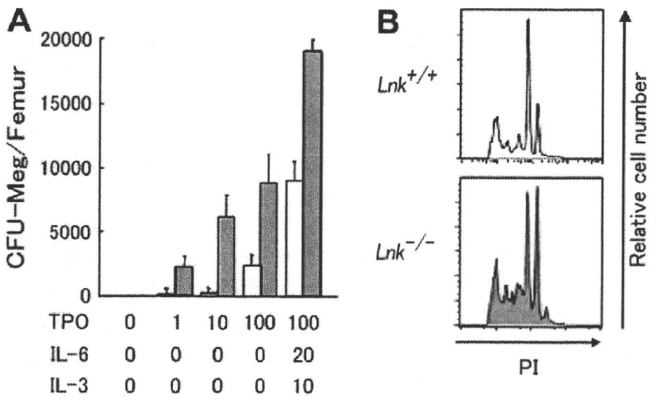

Figure 3. Megakaryocytosis in $Lnk^{-/-}$ mice. (**A**) Increased number of megakaryocytes (MK) progenitors and their hypersensitivity to thrombopoietin (TPO). Total bone marrow (BM) cells obtained from the BM of $Lnk^{+/+}$ (open column) or $Lnk^{-/-}$ (closed column) mice were cultured at the indicated concentration of TPO, interleukin (IL)-3, and IL-6 for 7 days. The mean ± SD of the number of colonies per femur are shown from three independent experiments. (**B**) BM cells of $Lnk^{+/+}$ (upper column) or $Lnk^{-/-}$ (lower column) mice were analyzed for DNA content. Representative data of multiple experiments are shown.

Lnk-deficiency causes augmented MAPK activation in MKs upon stimulation with TPO

To understand the molecular mechanism by which Lnk regulates TPO-induced MK proliferation, we examined overall protein phosphorylation following TPO stimulation using BM-derived MKs. We generated MKs with the same ploidy from wild-type and $Lnk^{-/-}$ mice by adjusting TPO concentration to levels that induced relatively comparable proliferative responses, and used them for biochemical analyses (Fig. 4A). Purified MKs were starved for 16 hours in TPO-free medium, stimulated with 10 ng/mL TPO and then lysed at several time points. The cell lysates were subjected to immunoblotting analyses for signaling molecules activated and phosphorylated downstream of c-Mpl (Fig. 4B). Stat3 and Akt was phosphorylated ten minutes after TPO stimulation and lasted >60 minutes in wild-type cells. Activation of Stat5, p38, and Erk1/2 was transiently observed, with peak phosphorylation 10 minutes after TPO stimulation. In $Lnk^{-/-}$ MKs, Erk1/2 activation was prolonged while phosphorylation levels of the other signaling molecules were essentially comparable to those induced in wild-type cells. Thus, our results indicated that Lnk specifically inhibited activation or regulates inactivation of Erk1/2 in TPO-dependent (c-Mpl–mediated) signaling pathways in MKs.

Megakaryocytic responses to combined exposure to TPO and integrins are modulated by expression of Lnk

Our previous study showed that the motility of hematopoietic progenitor cells on VCAM-1 was regulated by Lnk-mediated pathways [19]. We, therefore, asked whether

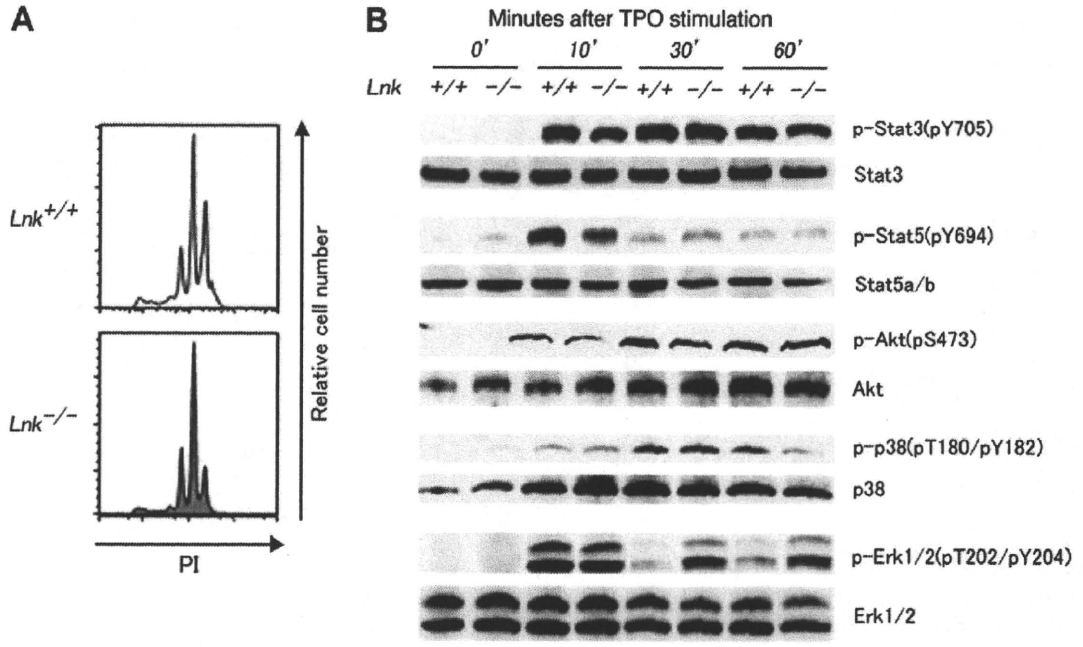

Figure 4. Thrombopoietin (TPO)-mediated hyperactivation of Erk1/2 in $Lnk^{-/-}$ megakaryocytes (MKs). (**A**) Equivalent ploidy between $Lnk^{+/+}$ and $Lnk^{-/-}$ MKs. Lin^- populations harvested from $Lnk^{+/+}$ (upper column) or $Lnk^{-/-}$ (lower column) mice were cultured in the presence of 20 ng/mL or 2 ng/mL TPO, respectively. (**B**) Augmented phosphorylation of Erk1/2 in Lnk-deficiency. Purified mature MKs were starved for 16 hours in the absence of TPO and re-stimulated with 10 ng/mL TPO for the indicated period. Total cell lysates were subjected to immunoblotting using indicated antibodies. Typical results of three independent experiments are shown.

additional stimuli through integrins might affect TPO-mediated megakaryopoiesis. It was also important to determine whether Lnk might be involved in integrin-mediated signaling pathways in MKs. Phosphorylation of cellular proteins was examined in both wild-type and $Lnk^{-/-}$ MKs, which were exposed to VCAM-1 in the presence of TPO and manganese to activate integrins. In wild-type cells, costimulation with TPO and VCAM-1 significantly inhibited Stat5 phosphorylation, and enhanced activation of p38 and Erk1/2 (Fig. 5A). On the other hand, in $Lnk^{-/-}$ MKs, TPO-dependent Stat5 activation was clearly induced even in the presence of VCAM-1 costimulation, while p38 activation was not enhanced (Fig. 5A). These observations suggest that costimulation by integrin engagement changed the nature of signals downstream of c-Mpl in MKs. Downregulated activation of Stat5 by VCAM-1 as well as augmented activation of p38 and Erk1/2 by VCAM-1 seemed to require Lnk-mediated pathways.

Costimulation by VCAM-1 and TPO reduces the release of platelets by $Lnk^{-/-}$ MKs

VCAM-1 is a ligand for $\alpha4\beta1$ and $\alpha4\beta7$ integrins and is expressed mainly on endothelial cells [23,24]. Adhesive interaction between endothelial cells and MKs through the VLA-4/VCAM-1 axis promotes maturation of megakaryocytes [25]. To examine how VCAM-1–mediated changes in TPO-induced cellular responses affected certain MK functions, we evaluated platelet release from mature MKs in vitro. Purified MKs were incubated on BSA- or VCAM-1–coated plates for 12 hours, after which the number of released platelets was counted by flow cytometry. In the absence of TPO, wild-type and $Lnk^{-/-}$ MKs produced similar numbers of platelets on BSA- and VCAM-1–coated plates. In the presence of TPO, wild-type MKs on VCAM-1 released greater numbers of platelets than those on BSA. In contrast, the number of platelets released by TPO-stimulated $Lnk^{-/-}$ cells on VCAM-1 was comparable to that observed with BSA. The number of released platelets from $Lnk^{-/-}$ MKs was significantly lower than that from normal cells on VCAM-1 (Fig. 5B and Table 1). Pretreatment by anti-integrin-$\alpha4$ antibody completely abolished the difference in platelet production between $Lnk^{-/-}$ and wild-type cells on VCAM-1 (Fig. 5B). Apoptosis of MKs leads to generation of functional platelets [26]. There was, however, no difference in the percentage of apoptotic cells or their ploidy between wild type and $Lnk^{-/-}$ cells either on BSA- or VCAM-1–coated plate (data not shown).

We examined the possibility that reduced platelet formation by $Lnk^{-/-}$ MKs on VCAM was due to morphological changes in MKs. We generated proplatelet-forming MKs from wild-type and $Lnk^{-/-}$ fetal liver progenitors and monitored their morphological changes on BSA and VCAM-1 by time-lapse confocal microscopic analysis (Fig. 6). While the wild-type MKs elongated proplatelets with pseudopodia shape on BSA, they produced more branched proplatelets from firmly attached and well-expanded cell body on VCAM-1. $Lnk^{-/-}$ MKs on BSA showed proplatelet formation comparable to that of wild-type cells on BSA.

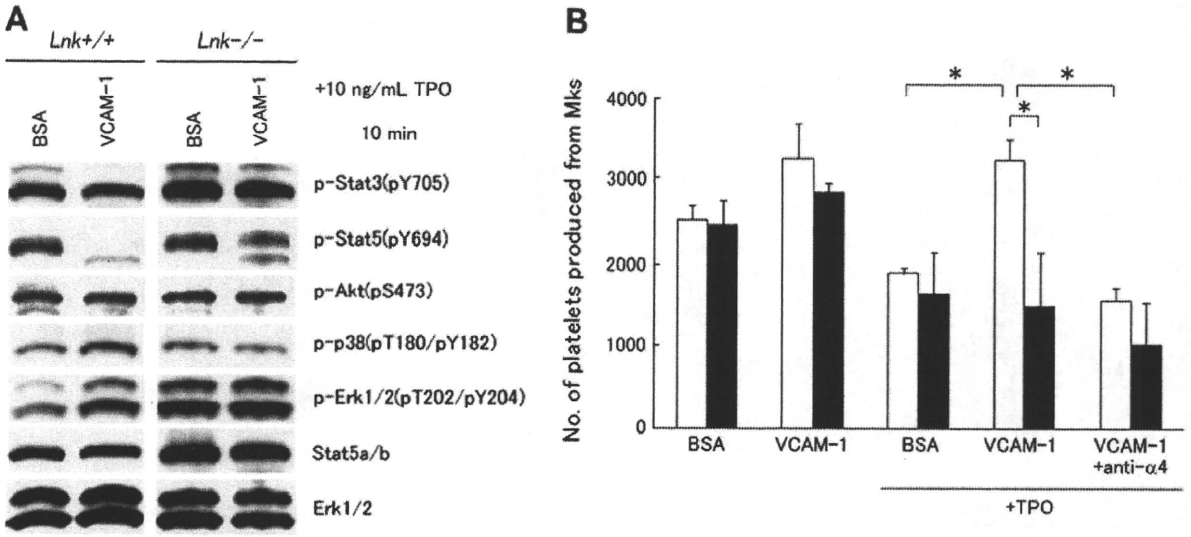

Figure 5. Changes of thrombopoietin (TPO)-induced cellular response by integrin ligands. **(A)** Modulation of TPO signaling pathways by integrin ligands. Mature megakaryocytes (MKs) obtained from $Lnk^{+/+}$ or $Lnk^{-/-}$ mice were starved for 16 hours without TPO and restimulated with both 10 ng/mL TPO and 50 μg/mL of vascular cell adhesion molecule-1 (VCAM-1). After 10 minutes incubation, cells were lysed and the lysates subjected to immunoblotting using the indicated antibodies. Results of several experiments are shown. **(B)** Augmented platelet production in the presence of VCAM-1. Purified $Lnk^{+/+}$ (open column) or $Lnk^{-/-}$ (closed column) MKs were incubated on either 20 μg/mL bovine serum albumin (BSA)- or VCAM-1–coated plates with or without addition of 1 ng/mL TPO for 12 hours. For anti-integrin-α4 antibody treatment, cells were preincubated with 10 μg/mL anti-CD49d for 30 minutes prior. The number of platelets was measured by flow cytometry. Shown are mean ± SD of results obtained from three independent experiments. $*p < 0.01$ comparison of $Lnk^{+/+}$ cells on VCAM-1–coated plates with that of $Lnk^{+/+}$ cells on BSA-coated plates, of $Lnk^{-/-}$ cells on VCAM-1–coated plates and of antibody-treated $Lnk^{+/+}$ cells on VCAM-1–coated plates.

However, $Lnk^{-/-}$ MKs on VCAM-1 developed proplatelets from not only the edge but also the center of the cell body with enlarged pseudopodial ends. This result suggests that a stimulation of integrin bound to VCAM-1 promoted platelet production by forming more proplatelets in wild-type MKs, and Lnk plays a role in regulating morphological changes for proplatelet formation.

Discussion

In this study, we demonstrated that $Lnk^{-/-}$ MK progenitors were hypersensitive to TPO, causing increased MK numbers, hypermaturation, and delayed platelet production on VCAM-1, which in turn led to thrombocytosis. It has been previously reported that *Lnk*-deficiency resulted in hyperactivation of TPO-dependent signaling molecules including Stat3, Stat5, Akt and Erk1/2 in MKs [17]. We also observed a significant enhancement in TPO-induced activation of Erk1/2 in $Lnk^{-/-}$ MKs, but not Stat3, Stat5 nor Akt in our experimental settings. The reasons for the discrepancy are currently unknown, but might be ascribed to the differences in the preparation of MKs used for biochemical analysis. Because the sensitivity of MKs to TPO declines during maturation and increase in ploidy [27], we prepared wild-type and $Lnk^{-/-}$ MKs with equalized ploidy and high purity from cultivated BM cells. MKs isolated by flow cytometric sorting from normal and *Lnk*-deficient mice might have different ploidy and purity because of low frequency and fragility of MKs in BM. Enhanced responses against other cytokines that activate Jak/Stat pathway, such as IL-3 or IL-7, have been suggested in the absence of Lnk. However, we did not observe any enhancements of cellular responses by IL-3, IL-7, and IL-5 in $Lnk^{-/-}$ precursors ([14,22] and unpublished observations). We believe that the inhibition of TPO-induced signals by

Table 1. VCAM-1 increases platelet release by megakaryocytes in the presence of Lnk

	Platelet production ratios in the presence of VCAM-1[a]	
	Genotype	
Cytokine addition	$Lnk^{+/+}$	$Lnk^{-/-}$
–TPO	1.19 ± 0.07	1.05 ± 0.18
+TPO	1.86 ± 0.15*	1.25 ± 0.27**

[a]Lin^- bone marrow (BM) precursor cells from wild-type or $Lnk^{-/-}$ mice were incubated for 3 days in the presence of 20 ng/mL or 2 ng/mL thrombopoietin (TPO), respectively. Purified megakaryocytes were incubated with or without 1 ng/mL TPO for an additional 12 hours. The number of platelets released in culture on vascular cell adhesion molecule (VCAM-1)–coated plates was counted by flow cytometry and compared with that on bovine serum albumin (BSA)–coated plates (see also Fig. 5B). Platelet production ratios were calculated by dividing the number of platelets on VCAM-1–coated membranes by that on BSA-coated membranes in the absence or presence of TPO. Data are presented as mean ± SD of results from three independent experiments.

$*p < 0.003$ compared with that of $Lnk^{-/-}$ in the presence of TPO or that of $Lnk^{+/+}$ in the absence of TPO.

$**p > 0.2$ compared with that of $Lnk^{-/-}$ in the absence of TPO.

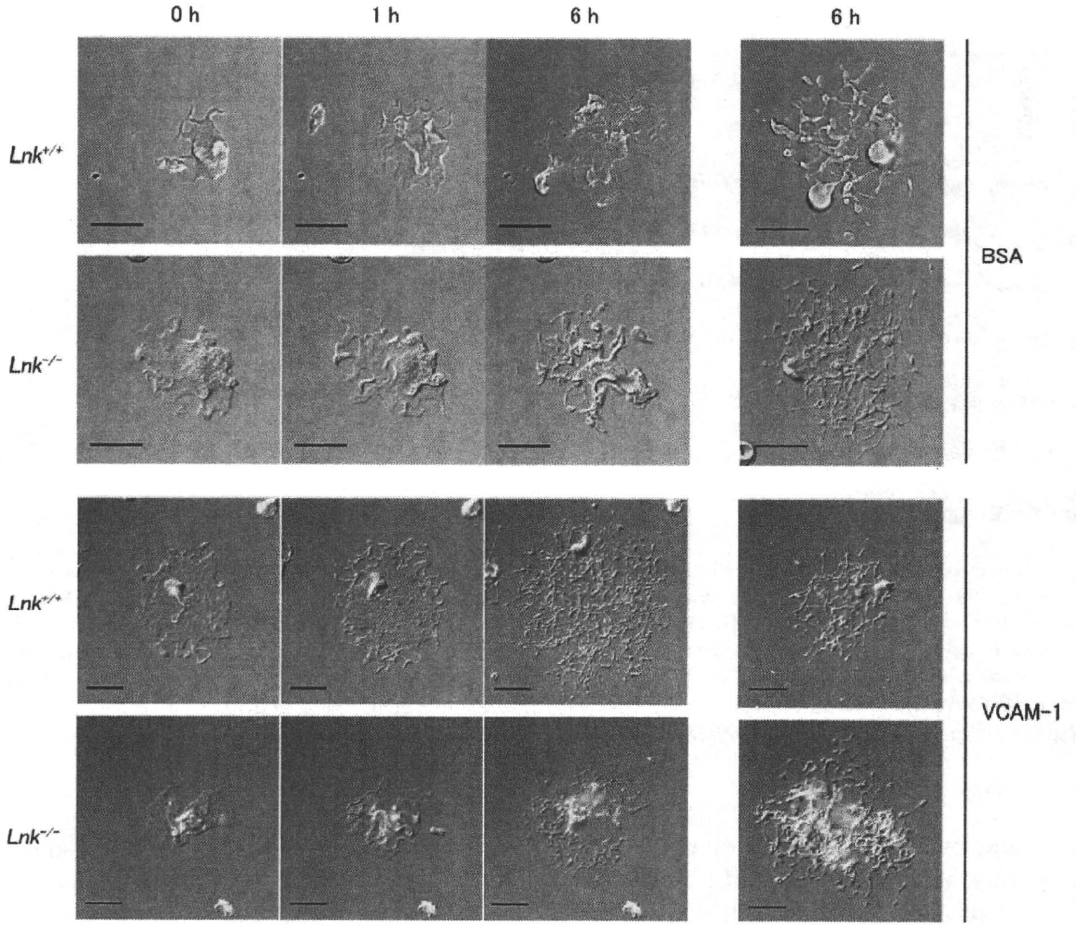

Figure 6. Morphological changes of $Lnk^{-/-}$ megakaryoctyes (MKs) producing proplatelets on vascular cell adhesion molecule-1 (VCAM-1). Fetal liver–derived $Lnk^{+/+}$ or $Lnk^{-/-}$ MKs were cultured on bovine serum albumin (BSA)– (upper panels) or VCAM-1–coated plates (lower panels) in microscope chambers and images were taken every hour up to 24 hours. Shown are representative images taken at the beginning of proplatelet elongation (0 hours), and following 3- and 6-hour observation. Images of another independent MKs at 6 hours were also shown. Scale bars: 50 μm.

Lnk involves cellular machinery specific to c-Mpl and does not act via Jak2 activation.

Our results revealed novel regulatory mechanisms in thrombopoiesis mediated by Lnk in addition to its role in constraining TPO signals. Additional stimulation by integrin ligand modulated TPO-induced signal responses of MKs. Engagement of integrin ligand induced strong mitogenic responses in MKs as well as in fibroblasts [28,29]. Interestingly, costimulation by TPO and VCAM-1 led to suppression of Stat5 phosphorylation and upregulation of p38 and Erk1/2 activation, which might change the nature of TPO-induced signals from ones supporting cell growth to those inducing cell-cycle arrest and differentiation. Consistently with this, we showed that normal MKs released greater numbers of platelets on VCAM-1 than on BSA. Administration of SDF-1 and fibroblast growth factor-4 to mice facilitates both MK maturation and platelet production by enhancing migration and adhesion of MK progenitors to BM endothelial cells through adhesion molecules including VCAM-1/VLA-4 [13]. Cellular interaction through VCAM-1 might trigger MKs to release platelets into the blood stream by terminating expression of Stat5-inducible genes after attachment of MKs to sinusoidal endothelial cells (Fig. 7A).

Inactivation of Stat5 was not observed in $Lnk^{-/-}$ MKs treated with TPO and VCAM-1. Additionally, platelet production from $Lnk^{-/-}$ MKs was not increased in the presence of VCAM-1. $Lnk^{-/-}$ MKs on VCAM-1 showed some morphological changes in proplatelet formation, which might be due to insufficient activation of demarcation membrane system of MKs [30], leading to inefficient platelet release in vitro upon integrin ligation by VCAM-1. In the absence of Lnk, MKs might continue undergoing endomitosis without producing platelets due to impaired Stat5 inactivation even if they contacted endothelial cells. This may lead to their hypermaturation in BM and result in more platelets production (Fig. 7B), because MKs with high ploidy are able to produce more platelets than those with low ploidy [31]. Thrombocytosis in $Lnk^{-/-}$ mice might be a consequence of increased numbers of MKs due to hypersensitivity of MK progenitors (TPO-dependent), in combination with accumulation of hypermature MKs attached

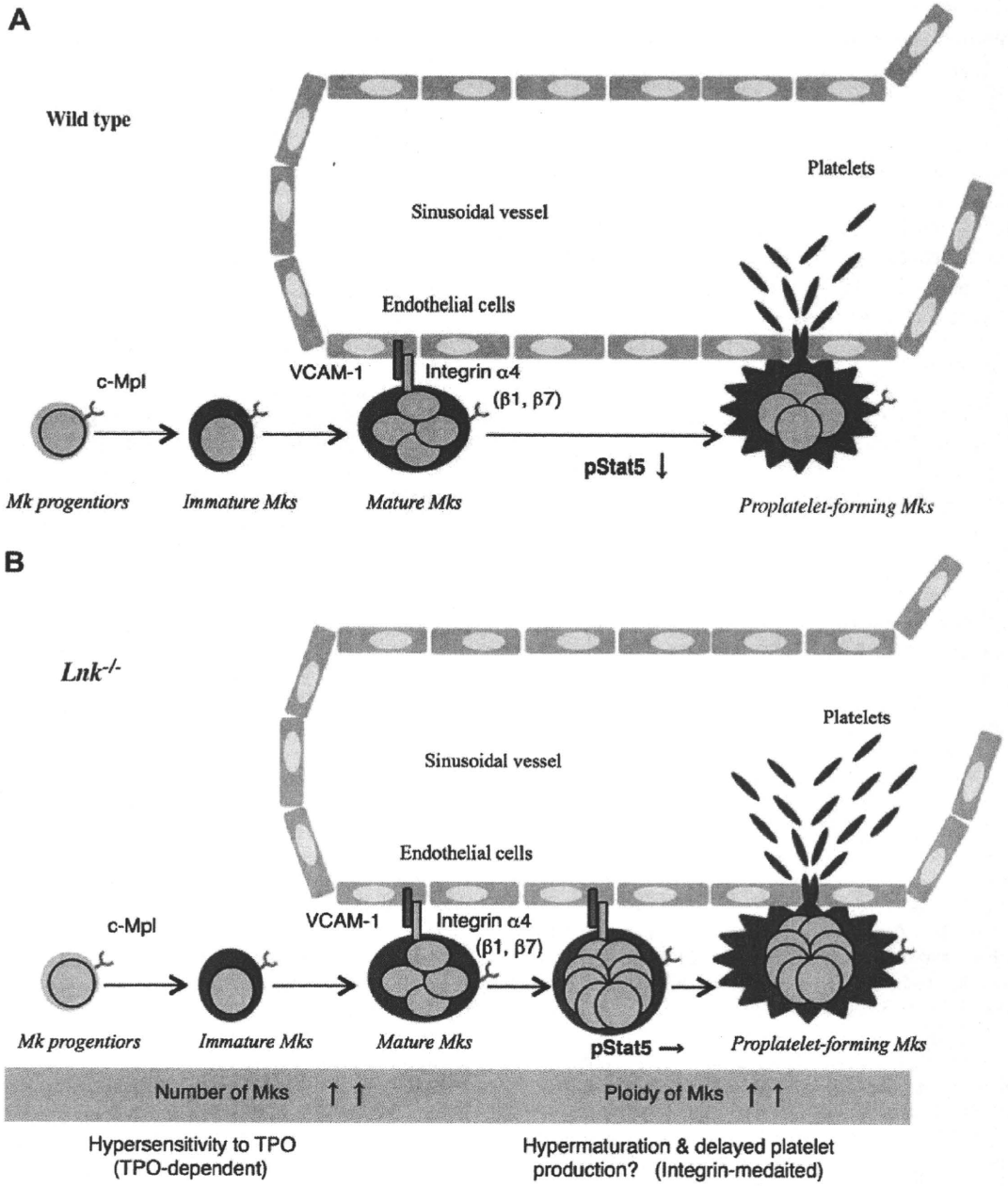

Figure 7. (**A**) Wild-type megakaryocytes (MK) progenitors proliferate and differentiate into mature MKs in response to thrombopoietin (TPO). When they contact the endothelial cells forming the sinusoidal vessels in bone marrow (BM) though integrinα4–vascular cell adhesion molecule-1 (VCAM-1) axis, platelets are released into bloodstream through proplatelet formation, which is possibly triggered by reduction of TPO-induced signal transducers and activators of transcription 5 (Stat5) activity upon VCAM-1 stimulation. (**B**) In the case of Lnk-deficiency, the number of mature MKs is increased due to enhanced sensitivity of $Lnk^{-/-}$ MK progenitor to TPO (TPO-dependent). After attaching the endothelial cell, MKs might keep undergoing endomitosis without platelet release, possibly due to impaired Stat5 inactivation, leading to their hypermaturation with an increase of ploidy.

to endothelial cells due to delayed platelet release (integrin-mediated) in Figure 7B. Future experiments will determine whether regulation of Stat5 is directly involved in initiation of platelet release, and how activation of Stat5 and MAPK in TPO-stimulated MKs is regulated by integrin- and Lnk-mediated events.

In summary, our results shed light on formerly unrecognized regulatory mechanisms in thrombopoiesis mediated by Lnk, and crosstalk between cytokines and integrins in MKs. TPO-induced Erk1/2 phosphorylation, but not activation of Stats and Akt, was constrained by Lnk, and was augmented in the absence of Lnk. Reduction of TPO-induced Stat5 and MAPKs activation in the presence of VCAM-1 co-ligation was mediated by a Lnk-dependent manner. Adhesion molecules play important roles in the biological properties of hematopoietic cells as environmental factors that determine cell fate. Understanding how integrin-mediated signals affect gene regulation or cytokine- or growth

factor–induced signaling pathways will be important to efforts to fully understand thrombopoiesis, hematopoiesis, and cellular responses.

Acknowledgments

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Generation of functional platelets from human embryonic stem cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors

Naoya Takayama,[1] Hidekazu Nishikii,[1] Joichi Usui,[1] Hiroko Tsukui,[1] Akira Sawaguchi,[2] Takashi Hiroyama,[3] Koji Eto,[1] and Hiromitsu Nakauchi[1]

[1]Laboratory of Stem Cell Therapy, Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo; [2]Department of Anatomy, University of Miyazaki Faculty of Medicine, Miyazaki; and [3]Cell Engineering Division, Rikagaku Kenkyusho (RIKEN) BioResource Center, Tsukuba, Japan

Human embryonic stem cells (hESCs) could potentially represent an alternative source for blood transfusion therapies and a promising tool for studying the ontogeny of hematopoiesis. When we cultured hESCs on either C3H10T1/2 or OP-9 cells to facilitate hematopoiesis, we found that exogenous administration of vascular endothelial growth factor promoted the emergence of sac-like structures, which we named embryonic stem cell–derived sacs (ES-sacs). These ES-sacs consisted of multiple cysts demarcated by cellular monolayers that retained some of the properties of endothelial cells. The spherical cells inside ES-sacs expressed primarily CD34, along with VE-cadherin, CD31, CD41a, and CD45, and were able to form hematopoietic colonies in semisolid culture and to differentiate into mature megakaryocytes by day 24 in the presence of thrombopoietin. Apparently, ES-sacs provide a suitable environment for hematopoietic progenitors. Relatively large numbers of mature megakaryocytes could be induced from the hematopoietic progenitors within ES-sacs, which were then able to release platelets that displayed integrin αIIbβ3 activation and spreading in response to ADP or thrombin. This novel protocol thus provides a means of generating platelets from hESCs, which could serve as the basis for efficient production of platelets for clinical transfusion and studies of thrombopoiesis. (Blood. 2008;111:5298-5306)

Introduction

Human embryonic stem cells (hESCs) are pluripotent cells that can proliferate almost infinitely in vitro,[1] and thus could represent a potent source for cell transplantation therapies. Indeed, studies are currently under way to obtain a variety of differentiated cell types from hESCs, including hematopoietic cells, endothelial cells, cardiomyocytes, hepatocytes, and neuronal cells.[2-7] Although, at present, derivation of hESCs from an individual patient is difficult, technologies to establish patient-derived multipotent stem cells that should, in theory, overcome the issue of immunologic rejection are being developed. These include nuclear-transfer ESCs,[8] induced pluripotent stem (iPS) cells through gene manipulation,[9,10] germ stem cell–derived pluripotent stem cells,[11] and establishment of hESC banks for various and distinct types of human leukocyte antigen (HLA).[12] Another issue that must be overcome is the potential for teratoma formation that can occur if undifferentiated or immature hESCs are contaminated with transplanted mature cells.[13]

The aforementioned problems must be resolved before hESC-derived cells can be applied in clinical settings. Platelets, which are terminally differentiated and anucleate cells, can be irradiated to eliminate residual white blood cells involved in immediate immune reactions before transfusion, particularly in immunocompromised patients.[14] Irradiation of hESC-derived platelets would also kill passenger hESCs and their derivatives that could form teratomas or induce immunologic rejection through expression of polymorphic major histocompatibility complex molecules. Therefore, when an hESC bank containing various types of HLA,[12] or the clinical use of iPS cells,[9,10] becomes applicable, it might be feasible to use hESC/human iPS cell–derived platelets for transfusion. Such generation of platelets from hESCs could potentially lead to a constant and safe source of platelets that would eliminate the need to obtain platelets through blood donation.

With this in mind, we developed a system in which large numbers of mature, proplatelet-producing megakaryocytes can be derived from murine ESCs cultured on OP-9 stromal cells.[15] Using this coculture system, Gaur et al recently succeeded in producing megakaryocytes from H9 hESCs; however, their yield of megakaryocytes was low, and they failed to obtain functional platelets.[16] The aim of the present study was to modify this in vitro culture system such that hESCs would be able to differentiate into platelet-forming megakaryocytes. Here we describe a novel protocol that enables hESCs to efficiently develop into hematopoietic progenitors and megakaryocytes capable of releasing functional platelets via the formation of unique sac-like structures.

Methods

Reagents and cell lines

All reagents were from Sigma-Aldrich (St Louis, MO) unless indicated otherwise. Three hESC lines, Kyoto hESC-1 (KhES), KhES-2 and KhES-3, were obtained from the Institute for Frontier Medical Science, Kyoto University (Kyoto, Japan), with approval for hESC use granted by the Minister of Education, Culture, Sports, Science, and Technology

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of Japan. The Review Board of the Institute of Medical Science, University of Tokyo approved this research. The entire study was conducted in accordance with the Declaration of Helsinki. KhESCs were maintained as described previously[17]; they were cultured on irradiated mouse embryonic fibroblasts in a 1:1 mixture of Dulbecco modified Eagle medium and Ham F-12 medium supplemented with 0.1 mM nonessential amino acids (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Invitrogen), 20% knockout serum replacement (Invitrogen), 0.1 mM 2-mercaptoethanol, and 5 ng/mL basic fibroblast growth factor (bFGF; Upstate, Lake Placid, NY). The cells were passaged every 3 days to maintain them in an undifferentiated state. The mouse C3H10T1/2 cell line was purchased from the RIKEN BioResource Center (Tsukuba, Ibaraki, Japan) and was cultured in Eagle basal medium (Invitrogen) containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine. The OP-9 cell line was a gift from Dr T. Nakano (Osaka University, Osaka, Japan) and was cultured in α-minimum essential medium (MEM; Invitrogen) containing 20% FBS and 2 mM L-glutamine. ESC differentiation medium was Iscove modified Dulbecco medium supplemented with a cocktail of 10 μg/mL human insulin, 5.5 μg/mL human transferrin, 5 ng/mL sodium selenite, 2 mM L-glutamine, 0.45 mM α-monothioglycerol, 50 μg/mL ascorbic acid, and 15% highly filtered FBS (Cellect Gold; ICN Biomedicals, Aurora, OH) in the absence or presence of the cytokines/mediators listed in Figures 3 and 5. Human vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and human bone morphogenetic protein-4 (BMP-4) were from R&D Systems (Minneapolis, MN). Human thrombopoietin (TPO), human interleukin-6 (IL-6), IL-11, insulin-like growth factor II (IGF-II), and human stem cell factor (SCF) were from Peprotech (Rocky Hill, NJ). The following antibodies were used: phycoerythrin (PE)–conjugated anti-CD31, PE- or fluorescein isothiocyanate (FITC)–conjugated anti-CD34, unconjugated CD41a (integrin αIIb subunit), PE-conjugated CD41a or allophycocyanin (APC)–conjugated anti-CD41a, FITC-conjugated anti-CD42a (GPIX), PE-conjugated anti-CD42b (GPIbα), Alexa 405–conjugated anti-CD45, unconjugated anti–vascular endothelial (VE)–cadherin, and APC-conjugated anti–VEGF-receptor 2 (VEGF-R2). Antihuman VEGF neutralizing antibody (bevacizumab)[18] was from Roche (Basel, Switzerland). Antihuman c-Mpl antibody was a kind gift from Kirin (Tokyo, Japan). FITC-conjugated PAC-1 antibody (BD Biosciences, San Jose, CA) was used for integrin activation studies.[19] Tirofiban,[20] a specific antagonist to human integrin αIIbβ3, was from Merck (Whitehouse Station, NJ).

Cell culture

C3H10T1/2 cells or OP-9 cells were irradiated (50 Gy) in 100-mm dishes before use. We compared 2 different protocols, as depicted in Figure 1Ai-ii. Protocol 1: Small clumps (< 100 cells) of hESCs were transferred onto untreated OP9 cells and cultivated in the presence of 100 ng/mL TPO throughout the culture. On days 7 and 11, the cells were passed onto fresh OP-9 cells, leading to the generation of mature megakaryocytes on days 15 to 17, as previously demonstrated.[16] Protocol 2: Small clumps of hESCs (suspended in PBS containing 0.25% trypsin, 1 mM $CaCl_2$, and 20% KSR) were transferred onto C3H10T1/2 or OP-9 cells and cultured in hESC differentiation medium, which was refreshed every 3 days. On days 14 to 15 of culture, embryonic stem cell–derived sacs (ES-sacs) were collected into a 50-mL tube, gently crushed with a pipette, and passed through a 70-μm cell strainer to obtain hematopoietic progenitors. These cells were transferred onto fresh, irradiated feeder cells at a density of 2 to 3 × 10^4 cells per a well in 6-well plates and maintained in differentiation medium supplemented with human TPO or other combinations of cytokines/mediators (human IL-6, IL-11, human SCF, and heparin; Pharmacia & Upjohn, Bridgewater, NJ). The medium was replaced every 3 days; nonadherent cells were collected and analyzed after 20 to 32 days.

Immunohistochemical studies and flow cytometric analyses

Immunohistochemical staining of ES-sacs was carried out on days 14 to 15. Intact ES-sacs were fixed with either 10% methanol or 4% formaldehyde in PBS and then stained with antibodies against CD31, CD34, VEGF-R2, and/or FITC-conjugated *Ulex europaeus* agglutinin-1 (UEA-1) lectin, an endothelial cell marker (Vector Laboratories, Burlingame, CA), after which they were labeled with secondary antibodies and observed under a fluorescence microscope (Leica DM IRBE; Leica Microsystems, Wetzlar, Germany).

To investigate the internal structures of ES-sacs, immunohistochemistry was carried out with serial 2-μm paraffin sections. Samples were fixed with 4% formaldehyde in PBS and stained with biotinylated UEA-I lectin, after which they were incubated with peroxidase-conjugated streptavidin (Nichirei, Tokyo, Japan). Peroxidase activity was visualized using 3′3-diaminobenzidine in PBS with 0.01% H_2O_2. Parallel sections were also stained with hematoxylin and eosin. An ECLIPSE50i light microscope (Nikon, Tokyo, Japan) was used for evaluation.

Expression of cell surface molecules was analyzed by flow cytometry (FACS Aria; Becton Dickinson Japan, Tokyo, Japan). To determine precise numbers of megakaryocytes, the cells were stained with antihuman CD41a, antihuman CD42a, and antihuman CD42b, and were accompanied with True Count Beads (BD Biosciences) when analyzed by flow cytometry.

Semiquantitative RT-PCR

On day 24 of culture, hematopoietic cells were sorted into $CD34^+/CD41a^-$ and $CD34^+/CD41a^+$ fractions by flow cytometry and lysed with Trizol (Invitrogen). Total RNA was extracted as recommended by the manufacturer, after which cDNAs were obtained using a Thermo Script reverse-transcription–polymerase chain reaction (RT-PCR) system and oligo-dT primer (Invitrogen). Samples were normalized to intrinsic *GAPDH*. The following primer sequences (5′ to 3′) were used: for *GAPDH*, AAC AGC CTC AAG ATC ATC AGC (forward) and TTG GCA GGT TTT TCT AGA CGG (reverse); for *GATA-1*, TCA ATT CAG CAG CCT ATT CC (forward) and TTC GAG TCT GAA TAC CAT CC (reverse); for *GATA-2*, TGT TGT GCA AAT TGT CAG ACG (forward) and ACT TTG ACA GCT CCT CGA AGC (reverse); for *FOG-1*, GCC ACC GCA GTG ATC AAC AAA (forward) and AAG TGG CTG TAG AGG ATG TCC (reverse); for *Fli-1*, TAA GAA TAC AGA GCA ACG GCC (forward) and GGC ATG TAG GAG ATG TCA GAA (reverse); for *NF-E2*, ATG AGC TAT TGG CAA GGT ACC (forward) and TAC TCT TCA GGA GAG TAG CTG (reverse); for *GPIbα*, AAT CCA CTA CTG AAC CAA CCC (forward) and GGG TGG AGA AAA GGG TCA TTT (reverse).

Functional analysis of platelet activation

Platelet preparation. Platelets in culture medium were gently collected, and a one-ninth volume of acid citrate dextrose solution (85 mM sodium citrate, 104 mM glucose, and 65 mM citric acid) was added. The modified medium containing the cells was then centrifuged at 150*g* for 10 minutes to eliminate any large cells. The supernatant was transferred to a new tube, 1 μM prostaglandin E_1 and 1 U/mL apyrase were added to prevent platelet activation, and the mixture was centrifuged at 400*g* for 10 minutes to sediment a platelet pellet. The pellet was then resuspended in an appropriate volume of modified Tyrode-HEPES buffer at pH 7.4 (10 mM HEPES, 12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, and 1 mM $MgCl_2$) and finally used after addition of 1 mM $CaCl_2$.

Studies of agonist-mediated integrin αIIbβ3 activation. To investigate integrin αIIbβ3 activation, 50-μL aliquots of hESC-derived platelets in buffer were incubated for 20 minutes at room temperature with PE-conjugated anti-CD42b and FITC-conjugated PAC-1 in the absence or presence of human thrombin or ADP. The binding of PAC-1 to platelets was quantified using flow cytometry. Nonspecific binding was determined in the presence of 10 μM tirofiban, a specific antagonist to human integrin αIIbβ3.[20] Specific binding was defined as total minus nonspecific binding.

Confocal studies. All observations of cytoskeletal changes in platelets were made using a confocal microscopic system (Leica TCS SP2; Leica Microsystems) equipped with a 63×/1.40 numeric aperture oil-immersion objective (Leica Microsystems). Images were assembled using Adobe Photoshop (Adobe Systems, San Jose, CA). Human washed platelets[19] or

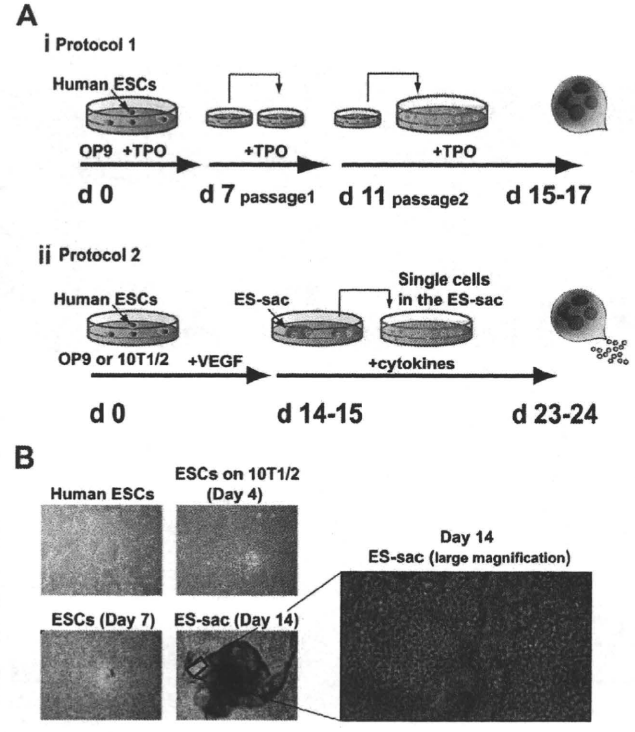

Figure 1. Human ESC-derived sac-like structure (ES-sac). (A) Schematic diagrams of 2 different in vitro differentiation protocols for human embryonic stem cell (hESC)–derived megakaryocytes and platelets. (i) Megakaryocytes are generated on OP-9 stromal cells, as described previously.[16] (ii) Megakaryocytes are generated from the cells within ES-sacs on days 14 to 15. (B) Photomicrographs showing undifferentiated hESCs (day 0) and differentiated stages on C3H10T1/2 cells (days 4, 7, and 14). On day 14, ES-sacs appeared. Original magnification, 40×. A high-magnification view (day 14) shows an ES-sac containing numerous bright, spherical cells (200×). VEGF indicates vascular endothelial growth factor; TPO, thrombopoietin.

hESC-derived platelets were spread on fibrinogen-coated cover glass in the absence or presence of an agonist (ADP or thrombin) that accelerated actin cytoskeletal changes. The cells were fixed, permeabilized, and stained with rhodamine-phalloidin to label F-actin (1:50 dilution) or an anti-CD41a antibody (10 μg/mL) followed by Alexa 488–conjugated secondary antibody (1:500 dilution).

Results and discussion

Multipotent hematopoietic progenitors that efficiently generate mature megakaryocytes are enriched inside ES-sacs

Using the protocol depicted in Figure 1Ai,[15] Gaur et al successfully generated megakaryocytes with high ploidy from H9 hESCs, but no release of platelets was observed.[16] We also failed to obtain large numbers of platelet-like particles from hESCs[17] when following this basic protocol, or even after application of additional hematopoietic cytokines, including SCF, IL-6, and IL-11 (data not shown). During the course of these studies, however, we noticed the appearance of inflated sac-like structures in cultures maintained for 2 weeks without additional reseeding procedures (Video S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). What is more, some of these sac-like structures contained round, hematopoietic-like cells inside; those, we termed embryonic stem cell–derived sacs (ES-sacs).

The protocol used to obtain ES-sacs required stromal cells, such as OP-9 or C3H101/2 cells. While OP-9 and C3H10T1/2 cells generated equal numbers of ES-sacs (data not shown), we primarily used C3H10T1/2 cells in the subsequent studies summarized in the results.

Each ES-sac consisted of a morphologically distinct external layer that enveloped several thousand spherical cells (Figure 1B). Immunohistochemical studies of ES-sacs revealed that the cells in the external layer preferentially expressed VEGF-R2, CD31, CD34, and UEA-1 lectin-binding activity (UEA-1$^+$), indicating differentiation along an endothelial cell lineage (Figure 2A arrows).[21] Hematoxylin-eosin staining revealed that ES-sacs contain multiple cystic structures demarcated by UEA-1$^+$ septa (arrowheads in the higher-magnification view of Figure 2B). To characterize the spherical cells within ES-sacs, they were stained with anti-CD31, anti-CD34, anti–VEGF-R2, anti–VE-cadherin (a hematoendothelial cell marker),[2] anti-CD41a (an early hematopoietic progenitor and megakaryocyte marker),[22] and anti-CD45 (a panhematopoietic cell marker) and then analyzed by flow cytometry. Most spherical cells within ES-sacs expressed CD31 (Figure 2C), whereas only approximately one-third of the CD31$^+$ cells expressed CD34. VEGF-R2, VE-cadherin, CD41a, and CD45 were present on 8%, 70%, 50%, and 41% of CD34$^+$ cells, respectively (Figure 2C). Moreover, these cells effectively formed multilineage colonies under semisolid liquid conditions (Figure S1).

Collectively, the results suggest the cells inside ES-sacs include multipotent hematopoietic progenitors. By contrast, hESC-derived clumps within cultures that did not form ES-sacs (they appeared in the absence of round cells) failed to differentiate into hematopoietic cells. Thus, our new protocol (protocol 2, summarized in Figure 1Aii) enabled hESCs to develop into hematopoietic progenitors, once they formed an ES-sac structure (Figure 2C). Indeed, when the cells inside ES-sacs were harvested, reseeded onto feeder cells in the presence of TPO, and cultured according to protocol 2, megakaryocytes were generated with much greater efficiency than has been seen with other in vitro methods, including protocol 1 and the floating cell method with protocol 2 (Figure 2D). This is noteworthy in that we recently succeeded in using the floating cells but not adherent cells to efficiently generate megakaryocytes and platelets from monkey ES cells (CMK6).[23] There is thus a clear difference in the optimal methodologies between CMK6 ESCs and hESCs.

In addition, we found that exogenous administration of VEGF significantly increased the number of ES-sacs (Figure 3A). While the synergistic action of IGF-II plus VEGF is required for more efficient production of hematopoietic progenitors from CMK6 ESCs,[23] VEGF (up to 20 ng/mL), by itself, induced ES-sacs from hESCs as efficiently as VEGF plus IGF-II (Figure 3A). On the other hand, administration of BMP-4, which is known to be a potent accelerator for hESC-derived hematopoiesis through embryoid bodies,[2] inhibited the positive action of VEGF on production of ES-sacs and hematopoietic progenitors (Figure 3A). To test whether VEGF specifically acts on the formation of ES-sacs, we next examined the effects of antihuman VEGF neutralizing antibody (bevacizumab).[18] For these experiments, 20 ng/mL VEGF was preincubated with 500 ng/mL bevacizumab for 2 hours at 37°C. As shown in Figure 3B, bevacizumab completely reversed the VEGF-induced increase in ES-sac formation seen on day 15, as well as the production of platelets seen on day 24 (Figure 3Bi,ii).