Materials and Methods

Materials

The SuperScript III One-Step RT-PCR System and Alexa Fluor 488-conjugated fibrinogen were purchased from Invitrogen (Carlsbad, CA). Anti-Dab2 (H-110) antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-Dab2 (p96) and fluorescein isothiocyanate (FITC)-conjugated anti-CD41 antibodies were purchased from BD Biosciences (San Diego, CA). Anti-serum albumin antibody was purchased from Abcam (Cambridge, MA). Anti-fibrinogen antibody was purchased from Dako (Glostrup, Denmark). Fibrinogen, thrombin and ADP were purchased from Calbiochem (Darmstadt, Germany). U46619 was purchased from Cayman (Ann Arbor, MI). The antibodies for PDK1, p-PDK1 (Ser241), Akt, p-Akt (Ser473), p-PKC (pan), p-PKCδ (T505), mTOR and p-mTOR (Ser2448) were purchased from Cell Signaling Technology (Danvers, MA). Anti-p-ROCKII (Ser1366) antibody was purchased from GeneTex (Pkwy Irvine, CA). Recombinant human thrombopoietin (TPO) was purchased from ProSpec (East Brunswick, NJ). The collagen was purchased from Chrono-Log (Havertown, PA). The phycoerythrin (PE)-labeled JON/A and FITC-labeled GPIbα antibodies were purchased from EMFRET Analytics (Wurzburg, Germany). The PE-labeled anti-CD62P antibody was purchased from eBioscience (San Diego, CA). The CellTiter-Glo reagent was purchased from Promega (Madison, WI). The β-actin antibody was purchased from Novus Biological (Mill Valley, CA). The $MnCl_2$, phospho(enol)pyruvic acid tri(cyclohexylammonium) salt, potassium acetate, ATP, Apyrase and FITC-conjugated phalloidin were purchased from Sigma (St Louis, MO). The pyruvate kinase was purchased from Roche (Indianapolis, IN). PAR4 peptide (AYPGKF-NH_2, purity > 95%) was synthesized from Kelowna International Scientific Inc. (Taiwan). The fluo 3-AM was purchased from AAT Bioquest (Sunnyvale, CA).

Generation of megakaryocyte lineage-restricted Dab2$^{-/-}$ mice

The Dab2$^{fl/fl}$ and PF4-Cre mice have been described previously.[1,2] Dab2$^{fl/fl}$ was cross-bred with PF4-Cre to generate PF4-Dab2$^{fl/+}$ mice which was then interbred to obtain Dab2$^{fl/fl}$ (hereafter named Dab2$^{fl/fl}$ or fl/fl) mice and PF4-Dab2$^{fl/fl}$ (hereafter named Dab2$^{-/-}$ or -/-) mice. The animal work has been reviewed and approved by the Institutional Animal Care and Use Committee (Approval ID: CGU09-113). Eight to twelve weeks old mice were used in this study.

Genotyping of Dab2$^{fl/fl}$, PF4-Cre and Dab2$^{-/-}$ mice

Genotyping of fl/fl, PF4-Cre, and -/- mice was performed as described

previously.[1,2] Genotyping of newborn pups for *DAB2* gene was performed by PCR using three primers: a forward primer (5'-GACCACGCTGTCCTTGAACTCAG-3') and two reverse primers (5'-CTGAAAAGAGAACACTGGAGGCTC-3' and 5'-GTAAATTCTCATGGCTGTGACTGG-3'). The resulting PCR products of 530, 460 and 250 bps were corresponded to the floxed, wild-type and deleted alleles, respectively. The PCR condition for *DAB2* was 1 cycle of 95 °C for 2 min, 40 cycles of 93 °C for 30 s, 59 °C for 30 s and 65 °C for 2 min, and 1 cycle of 65 °C for 5 min. For genotyping of *PF4*-promoter driven *Cre* gene, a forward primer located in the *PF4*-promotor (5'-CCCATACAGCACACCTTTTG-3') and a reverse primer in the *Cre* cDNA (5'-TGCACAGTCAGCAGGTT-3') were used to yield a 450-bp PCR product. The PCR condition for *PF4* was 1 cycle of 95 °C for 2 min, 40 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, and 1 cycle of 65 °C for 5 min.

Ex vivo megakaryocytic differentiation of murine bone marrow cells

For *ex vivo* differentiation of murine bone marrow cells, CATCH buffer (1X PBS containing 0.38% sodium citrate, 1 mM adenosine and 2 mM theophylline) was used to flush out bone marrow cells from the femurs and tibias of $Dab2^{fl/fl}$ or $Dab2^{-/-}$ mice. After incubation with RBC lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$ and 0.1 mM EDTA) at 4°C for 10 min, cells were pelleted by centrifugation at 1500 rpm for 5 min and resuspended in the culture medium (OPTI-MEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) containing 10 ng/ml TPO for 5 days.

Purification of megakaryocytes by bovine serum albumin density gradient

The purification of megakaryocytes by bovine serum albumin (BSA) density gradient was performed as described previously.[3] The preparation of bovine serum albumin (BSA) density gradient (1.5/3/4.5%) in CATCH buffer was performed by adding 1.5 ml/gradient into 15 ml tube carefully without disturbing the demarcation. At day 5 after differentiation induction, bone marrow cells were collected by centrifugation at 1,500 rpm for 5 min and were resuspended in 1 ml OPTI-MEM. The bone marrow cell suspension was loaded on the BSA density gradient and the large size megakaryocytes were isolated by gravity at room temperature for 40 min. The large size megakaryocytes were collected from the 1 ml solution on the bottom of tube.

Cell sorting of megakaryocytes

Megakaryocytes obtained from BSA density gradient were incubated with FITC-conjugated anti-CD41 (2 μg) and PE-conjugated anti-Gr1 (1 μg) antibodies at room temperature for 20 min. After washing twice with CATCH buffer,[4] cell sorting

was performed by FACSAria using a 100 μm ceramic nozzle with the sheath pressure adjusted to 20 pounds per square inch and an acquisition rate of 10 events/sec.

Immunofluorescence staining

Megakaryocytes were fixed with 4% paraformaldehyde solution at room temperature for 20 min and permeabilized with 0.06% Triton X-100 at 4°C for 10 min. The fixed cells were blocked with 5% BSA and incubated with anti-Dab2 (H-110) antibody (1:200) at room temperature for 1.5 h. The Alexa Fluor 546 goat anti-rabbit antibody (1:1000) was then used for staining at room temperature for 1 h. Fluorescence images were observed using confocal microscopy (LSM 510 Meta, Zeiss). The fluorescent intensity was quantified by ImageJ software (National Institute of Health).

Single cell RT-PCR analysis

Single megakaryocyte from *ex vivo* differentiated bone marrow cells was collected by Pasteur pipette. Total RNA was converted to cDNA using the SuperScript III One-Step RT-PCR System. The RT-PCR condition was 1 cycle of 50°C for 15 min, 1 cycle of 95°C for 2 min and 18 cycles of 95°C for 15 sec and 60°C for 4 min. The secondary PCR condition was 1 cycle of 95°C for 2 min, 40 cycles of 95°C for 30 sec, 65°C for 30 sec and 72°C for 1 min, and 1 cycle of 72°C for 5 min in the presence of *DAB2* forward (5'-CCGAAAGCGCCATCAAAGAA-3') and reverse (5'-TCATTAGGGCCTCACTTCCA-3') primers. The PCR products of 535 and 395 bp correspond to the wild-type and truncated *DAB2* transcripts, respectively.

Bleeding time and rebleeding rate analysis

The mice were anesthetized by inhalation of isoflurane. The extremity of mouse tail was cut in 0.5 cm length and was immediately immersed in 1X PBS prewarmed to 37°C. Bleeding time was defined as the time required from the excision to cessation of bleeding. The test was terminated when bleeding lasted for more than 10 min. The mice that were rebled within 2 min after blood flow stop were considered as having the tendency of rebleeding.

$FeCl_3$-induced mesenteric arteriole/venule thrombosis

Mouse was anesthetized with 2.5% avertin (20 μl/g body weight) by intraperitoneal injection and the mesentery was exteriorized via an abdominal incision. Platelets (2 X 10^8) were labeled with Calcein red-orange AM (1 μg/μl) for 30 min at room temperature and injected into recipient mouse with the same genotype through orbital venous plexus. Mesenteric arteriole and venule injury were induced by $FeCl_3$

for 5 min (Whatman paper drenched with 10% $FeCl_3$). Mesentery was placed under a fluorescence microscope for video recording of thrombus formation until the blood flow stops or for a maximal time interval of 40 min.

Washed platelets preparation

Whole blood was mixed with sodium citrate (3.15%) and was centrifuged at 500 g for 20 min. Platelet-rich-plasma (PRP) was collected by centrifugation at 200 g for 8 min. Platelets were then obtained by centrifugation of PRP at 2000 g for 6 min in the presence of 0.5 μM prostaglandin I_2. After washing twice with Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.42 mM $NaH_2PO_4 \cdot H_2O$, 5.5 mM glucose and 5 mM HEPES, pH 7.4),[5] the washed platelets were resuspended in Tyrode's buffer containing Ca^{2+} and Mg^{2+} (1 mM $MgCl_2 \cdot 6H_2O$ and 2 mM $CaCl_2 \cdot 2H_2O$).

Platelet aggregation assay

Platelet aggregation was performed as described in the supplementary methods.[6] 400 μl of a washed platelet suspension (3 X 10^8/ml) and 95 μl Tyrode's buffer containing Ca^{2+} and Mg^{2+} was added into a cuvette with continuous stirring at 37°C for 1 min. The indicated agonists (5 μl) were then added into the cuvette with continuous stirring at 37 °C for an additional 10 min. The aggregation status of platelets was monitored with a platelet aggregometer (Chrono-Log) by measuring changes in light transmission.

Clot retraction assay

Citrated platelet-rich-plasma (cPRP; adjusted to 6 X 10^8 platelets/ml) obtained by centrifugation of whole blood at 250 g for 10 min was stimulated with thrombin (0.5 U/ml) in the presence of 3 μl $CaCl_2$ (100 mM) and incubated at 37°C for up to 2 h. Clot retraction was photographed and the size of the retracted clot was determined by ImageJ.

Platelet spreading assay

Coverslips were coated with 100 μg/ml fibrinogen for 2 h at 37°C and were blocked for 30 min by 1% denatured BSA. The washed platelets (100 μl, 3 X 10^7 platelets/ml) were subjected to the platelet spreading assay in the presence or absence of thrombin (0.05 U/ml), $MnCl_2$ (0.5 mM) or ADP (100 μM).[7] The adhered platelets were fixed with 3.7% formaldehyde for 10 min at room temperature for microscopy analysis. The number of adhered and spread platelets was quantified by ImageJ. The increase in surface area of the spread platelets after thrombin stimulation was determined following subtraction of the surface area of resting

platelets.

Complete blood count

Whole bloods from fl/fl or -/- mice were collected into 0.5 ml EDTA.K3 tube (BD Vacutainer). The complete blood count was determined using SYSMEX XT-1800i.

Flow cytometry analysis

Platelets were stimulated with indicated agonists and incubated with either PE-conjugated JON/A antibody (5 μl), FITC-conjugated anti-CD41 antibody (4 μl), PE-conjugated anti-CD62P antibody (5 μl), FITC-conjugated anti-GPIbα antibody (4 μl) or Alexa Fluor 488-conjugated fibrinogen for 15 min at room temperature in the dark. The reactions were stop by adding 400 μl 1X PBS and the samples were analyzed within 30 min using the Accuri C6 Flow Cytometer with CFlow® Software (BD Biosciences).

Platelet lysates preparation and Western blot analysis

Platelet suspensions were lysed in 5X lysis buffer (50 mM Tris-HCl (pH 7.4), 500 mM NaCl, 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 5% Triton X-100, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and 5 mM EGTA). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition, transferred to PVDF membrane and blocked with 5% non-fat milk for 1 h. The membrane was incubated with primary antibody at room temperature for 1.5 h followed by secondary antibody for 1 h. Protein expression was detected by ECL-Plus reagents.

Transmission electron microscopy (TEM)

Subcellular morphology of fl/fl and -/- platelets was analyzed by TEM. Platelet-rich plasma was collected and spun at 590 g for 5 min. Supernatant was removed from the platelet pellet, and the pellet was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). The pellet was washed in PB and incubated in 1% osmium tetroxide for 30 min at room temperature. After washing in PB and deionized water, the pellet was incubated in 3% uranyl acetate and incubated for 30 min at room temperature. After washing with deionized water, the pellet was dehydrated in a graded series of increasing amounts of ethanol (70%, 80%, 90%, 96%, 100%, and 100%, with each step lasting for 10 min). After removal of the 100% ethanol, the pellet was incubated in pure Epon for 2 h at room temperature. Thereafter, the Epon was replaced with fresh Epon, and the platelet block was

hardened overnight in a 60°C oven. Ultrathin counterstained sections (~75 nm-thick) were cut on the ultramicrotome (Leica EMUC7) and imaged on a transmission electron microsope (model Hitachi H-7500 TEM, Tokyo, Japan). To determine the dense granule and α-granule content, total numbers of granules in equivalent-sized fields of view were counted.

ADP release measurement

Platelets were stimulated with thrombin at the indicated concentrations. After 10 min, the reactions were stopped by adding 50 μl EDTA (100 mM) and transferred to an eppendorf tube. The supernatants collected by centrifugation at 2000 g for 5 min were treated with equal volumes of 100% EtOH followed by centrifugation at 2000 g for 5 min. The supernatants were then incubated with CellTiter-Glo reagent (A), potassium acetate/pyruvate kinase/PEP mixture (B), ATP (0.001 pmol, C) and ADP (0.001 pmol, D) at room temperature for 10 min, respectively. Luminescent signal was measured by a GloMax 20/20 luminometer (Promega). The calculation of ADP content for each sample was: ADP (pmol) = 0.001 × (B-A)/(C-B). The value of 100 × (D-C)/(B-C) was considered as quality control with the acceptable range of 100 ± 10.

Intracellular calcium measurement

Washed platelets (3 X 10^8/ml) were suspended in Tyrode's buffer and loaded with fluo 3-AM (5 μM, Kd = 390) for 30 min at 37°C in the dark. After labeling, platelets were washed once and resuspended in Tyrode's buffer containing 2 mM calcium. Aliquots (1.5 ml) of fluo 3-AM-loaded platelets (1 X 10^8/ml) were transferred to a glass cuvette and pre-warmed for 2 min at 37°C. Platelets were stimulated with the indicated agonists and the intracellular calcium concentration was measured by fluorescence spectrophotometer (model Hitachi F7000, Tokyo, Japan) using the excitation wavelength of 505 nm and emission wavelength of 525 nm. F_{max} was the fluorescence obtained after addition of Triton X-100 (0.1%) and F_{min} was the fluorescence obtained after addition of EGTA (0.2 mM). The changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were calculated by $[Ca^{2+}]_i = Kd * (F - F_{min})/(F_{max} - F)$.

Statistical analysis

Statistical analysis of bleeding time was determined by log-rank test.[8] Unpaired Student's *t* test was used for all the other analyses. The data were presented as mean ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant.

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