

Metalloproteinase regulation improves *in vitro* generation of efficacious platelets from mouse embryonic stem cells

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Embryonic stem cells (ESCs) could potentially compensate for the lack of blood platelets available for use in transfusions. Here, we describe a new method for generating mouse ESC-derived platelets (ESPs) that can contribute to hemostasis *in vivo*. Flow cytometric sorting of cells from embryoid bodies on day 6 demonstrated that c-Kit⁺ integrin α IIb (α IIb)⁺ cells, but not CD31⁺ cells or vascular endothelial cadherin⁺ cells, are capable of megakaryopoiesis and the release of platelet-like structures by day 12. α IIb β 3-expressing ESPs exhibited ectodomain shedding of glycoprotein (GP)Ib α , GPV, and GPVI, but not α IIb β 3 or GPIIb β . ESPs showed impaired α IIb β 3 activation and integrin-mediated actin reorganization, critical events for normal platelet function. However, the administration of metalloproteinase inhibitors GM6001 or TAPI-1 during differentiation increased the expression of GPIb α , improving both thrombogenesis *in vitro* and posttransfusion recovery *in vivo*. Thus, the regulation of metalloproteinases in culture could be useful for obtaining high-quality, efficacious ESPs as an alternative platelet source for transfusions.

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Abbreviations used: ADAM, a disintegrin and metalloproteinase; EB, embryoid body; ESC, embryonic stem cell; ESP, ESC-derived platelet; Flk, fetal liver kinase; GC, glycolalicin; GP, glycoprotein; HSC, hematopoietic stem cell; MMP, matrix metalloproteinase; PC, platelet concentrate; TEM, transmission electron microscopy; TIMP, tissue inhibitors of metalloproteinase; TPO, thrombopoietin; VE, vascular endothelial; VWF, von Willebrand factor.

Platelet concentrates (PCs) from donated blood are required to treat severe thrombocytopenia in patients with various hematological diseases, such as those who have undergone cancer chemotherapy or are recovering from hematopoietic stem cell (HSC) transplantation (1, 2). Frequent transfusion of PCs is clinically necessary because the half-life of transfused human platelets is 4–5 d (3). Platelets cannot be stored frozen, thus the ability to generate platelets *in vitro* would provide significant advances for platelet replacement therapy in clinical settings. A novel culture method to generate human platelets from cord blood CD34⁺ cells was recently developed as an alternative source for PCs (4). However, technical difficulties in expanding *ex vivo*-cultured cord blood CD34⁺ cells on a large scale have limited this as a reasonable *in vitro* approach for generating platelets.

Human embryonic stem cells (ESCs) can be forced to differentiate along a megakaryocytic lineage and represent a promising *in vitro* source for platelets. Owing to their pluripotency, ESCs can potentially proliferate indefinitely in culture (5). Platelets, as anucleate fragments of cytoplasm, can be irradiated before transfusion to effectively eliminate any contaminating cell, such as an undifferentiated ESC. The possibility of irradiation is important, as ESCs can potentially form teratomas or, if present at high numbers, elicit an immune response (6, 7). Thus, although ESCs represent a potentially safe and unlimited source of platelets *in vitro*, there are technical obstacles that remain.

First, culture methods for efficient *in vitro* generation of platelets have not been established.

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Second, appropriate *in vivo* function of the *in vitro*-produced platelet must be achieved. In addition, contamination with nonhuman antigens resulting in immunological reactions must be prevented. We and other groups have developed a method to generate large numbers of megakaryocytes and platelets from mouse ESCs grown on OP9 stromal cells *in vitro* (8, 9). However, these methods have not consistently produced ESC-derived megakaryocytes or platelets in sufficient quantity or quality to be considered as an alternative platelet source. No pre-selection for megakaryocyte progenitors was included in these previous reports. Studies have shown that the *in vitro* generation of large numbers of mature megakaryocytes depends on increased numbers of ESC-obtained progenitors (7, 8). Therefore, the identification and selection of megakaryocyte progenitors might increase the efficiency of megakaryopoiesis.

The functional platelet paradigm in hemostasis and thrombosis is the initiation of platelet adhesion to the extracellular matrix (10). One key event in this process is the interaction between glycoprotein (GP)Ib α (the platelet receptor) and von Willebrand factor (VWF) present in the extracellular matrix (10). Simultaneously, platelets can interact with surface-bound collagen via platelet receptors GPVI and integrin α 2 β 1 (11). The net result is an activation of integrin α IIB β 3 to become a competent fibrinogen receptor leading to the formation of platelet aggregates (10). A recent report has also suggested that GPIb α contributes to arterial thrombosis *in vivo* independently of binding to VWF (12). Indeed, other studies have demonstrated that GPIb α associates with thrombin, kininogen, coagulation factors XI and XII, and thrombospondin-1 (13). In addition, the GPIb-V-IX complex, consisting of GPIb α , GPIb β , GPIX, and GPV (10, 14), can bind integrin α M β 2 on macrophages/monocytes or P-selectin on endothelial cells (13). Of note, aged human and mouse platelets shed GPV and an extracellular domain of GPIb α , which contains the binding sites for VWF and thrombin (13, 15). This process involves the action of a disintegrin and metalloproteinase (ADAM)17 (also referred to as tumor necrosis factor α converting enzyme) (16). This leads to decreased platelet function (16, 17).

In this study, we demonstrate that c-Kit⁺ integrin α IIB (α IIB)⁺ cells isolated from ESCs differentiate with high efficiency into megakaryocytes and ESC-derived platelets (ESPs) in the presence of thrombopoietin (TPO) and stromal cells. We also show that ESPs shed extracellular domains of GPIb α , GPV, and GPVI in culture, reducing α IIB β 3 activation and actin polymerization. Consequently, these ESPs are functionally impaired in thrombus growth (18, 19). However, the inhibition of metalloproteinases restores platelet function, making ESPs from induced pluripotent stem cells a source of platelets with therapeutic potential (20, 21).

RESULTS

Markers defining megakaryocyte differentiation from ESCs

The cell markers defining the megakaryocytic lineage in a culture system of ESCs or HSCs are not understood. It is known that megakaryocyte progenitors are highly enriched in the CD9⁺ α IIB subunit⁺ (α IIB⁺)Fc γ R¹⁰c-Kit⁺Sca-1⁻Lin⁻

fraction of bone marrow cells (22). The α IIB integrin subunit is an early primitive and definitive marker of hematopoiesis in the mouse embryo (17, 18) and a lineage-specific marker for postnatal megakaryocytes and platelets (23). We reasoned that CD31⁺, α IIB⁺, or c-Kit⁺ cells were candidates for megakaryocyte progenitors derived from ESCs because postnatal HSCs, mature megakaryocytes, and platelets all express CD31 and α IIB β 3, and postnatal megakaryocyte progenitors express both c-Kit and α IIB β 3 (22).

To determine which cells actually contribute to megakaryopoiesis, we chose to induce a liquid culture system of ESCs for embryoid body (EB) formation (24). Sorted cells were then applied onto OP9 stromal cells to differentiate along the megakaryocytic lineage (Fig. 1 A). This two-stage approach was used because EB formation has been reported to be more suitable than co-culture with OP9 cells in producing hematopoietic progenitors *in vitro* (25). Indeed, liquid culture for EB formation resulted in reproducibly higher expression of the α IIB integrin subunit than the use of the OP9 co-culture system (Fig. 1 B) (26). ESCs co-cultured with OP9 stromal cells preferentially expressed CD31 (Fig. 1 B, right) or vascular endothelial (VE)-cadherin (unpublished data) by days 4–5, as reported (27).

To trace newly developed megakaryocytes and platelets in culture, we generated a novel ESC line in which the platelet-specific GPIb α promoter supports expression of a human GPIb α -EGFP fusion protein (Fig. 1 C, GPIb-ESCs) (28–30). Earlier reports showed selective expression in megakaryocytes and platelets using this system (29–31). The GFP-tagged cells were detectable by day 9 in the presence of TPO (Fig. 1 C). In addition, GFP expression was detectable only in α IIB⁺GPIb α ⁺ megakaryocytes derived from cultures treated with TPO but not in Ter119⁺ erythroblasts isolated from erythropoietin-containing cultures (not depicted). We further separated EB cells on day 6 to identify which fraction could differentiate into GFP-expressing megakaryocytes (Fig. 1 D). Murine ESCs (2 \times 10⁵ per 100-mm dish) typically produced 10⁶ c-Kit⁺ α IIB⁺ cells by day 6 and 2.5 \times 10⁶ α IIB⁺ megakaryocytes by day 12. Cells derived from the c-Kit⁺ α IIB⁺ fraction at day 6 expressed GFP on day 12 at 10-fold higher levels compared with cells in other fractions or in unfractionated whole EB-derived cells (Fig. 1 E). Fetal liver kinase (Flk)-1⁺CD31⁺ or VE-cadherin⁺c-Kit⁺ cells from day 6 EB did not contribute to megakaryopoiesis in this system (Fig. 1, D and E). ESC-derived VE-cadherin⁺c-Kit⁺CD45⁻ cells in OP9 co-culture had been reported to contribute to definitive hematopoiesis (32).

Serial RT-PCR studies to detect GPIb α or GPV, both megakaryocytic-specific markers (33), along with the essential transcription factors for megakaryopoiesis, GATA-1, GATA-2, FOG-1, Fli-1, or NF-E2 (34, 35), indicated that day 6 EB c-Kit⁺ α IIB⁺ cells were positive for these markers in the presence of TPO on OP9 stromal cells (Fig. 1 F) (34). Indeed, day 6 EB c-Kit⁺ α IIB⁺ cells corresponded to CD9⁺Sca-1⁻ cells in bone marrow-derived megakaryocyte progenitors (Fig. 1 D) (22), and their morphological features resembled immature hematopoietic progenitor cells derived from adult bone marrow (Fig. 1 G) (22). We concluded from these results that

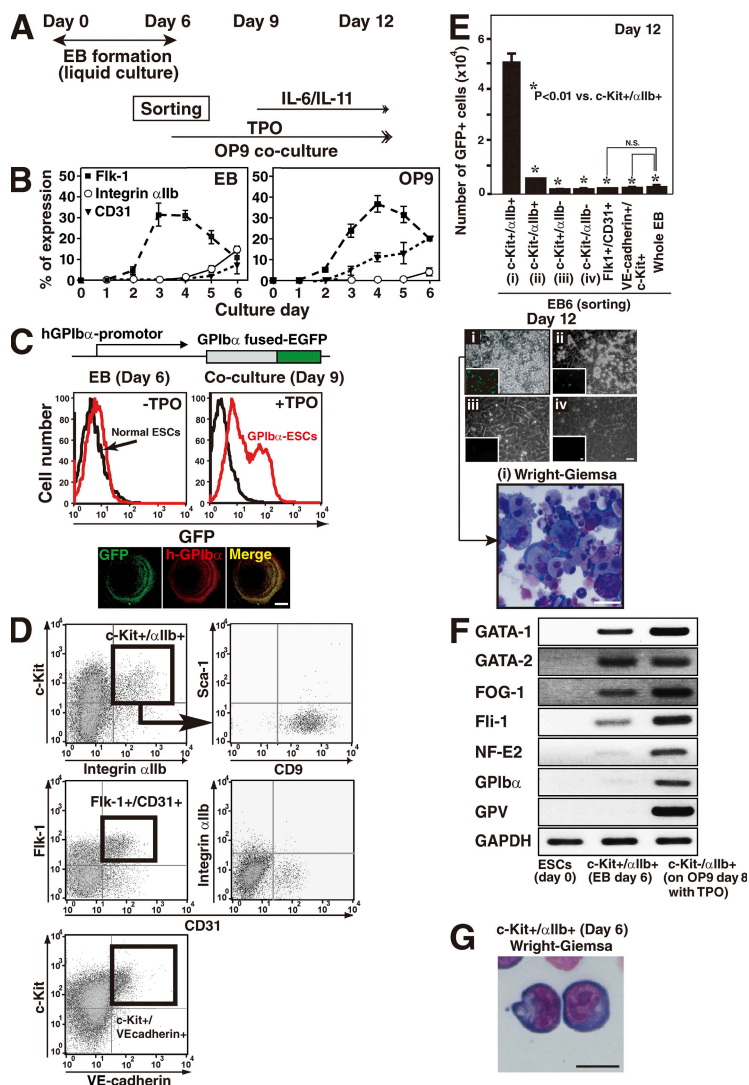


Figure 1. c-Kit $^+$ α IIb $^+$ cell selection from day 6 EB determines the megakaryocytic lineage. (A) Protocol used in this study. (B) EB formation or OP9 co-culture was used for ESC differentiation from day 0 through day 6 in vitro. On the indicated days of culture, expression of Flk-1, integrin α IIb subunit (CD41), or CD31 was examined in both groups of cultured cells. The graph shows mean \pm SEM from three independent experiments. (C) GPIb α and GFP expression in GPIb α -ESCs is regulated by the human GPIb α promoter. GFP is detectable in cultured cells that exhibit megakaryopoiesis (day 9) but not in progenitors (day 6). In the bottom box, cells with surface-expressed GFP also express human GPIb α . The cells on day 9 were fixed and stained to mark human GPIb α to confirm the colocalization with GFP expression at the membrane of the cells. Bars, 10 μ m. (D) Representative dot plots for the expression of individual antigens on 10,000 living cells from day 6 EB. c-Kit $^+$ α IIb $^+$ cells corresponded with Flk-1 $^+$ cells (not depicted) but not with VE-cadherin $^+$ cells. And >96% of c-Kit $^+$ α IIb $^+$ cells are found in the CD9 $^+$ Sca-1 $^-$ fraction. (E) EB cells were sorted as shown in D, and 20,000 cells per well of a 6-well plate were seeded onto OP9 stroma cells in the presence of TPO. The left graph shows the number of generated GFP $^+$ cells (mean \pm SEM) per well of a 6-well plate on day 12 of culture. Representative images from day 12 culture dishes (phase or fluorescence microscopy [insets; bar, 50 μ m]) of four different fraction-derived cells at day 6; i, c-Kit $^+$ / α IIb $^+$; ii, c-Kit $^-$ / α IIb $^+$; iii, c-Kit $^+$ / α IIb $^-$; iv, c-Kit $^-$ / α IIb $^-$ [phase; bar, 50 μ m]) and of Wright-Giemsa-stained cytopsin preparation on day 12 derived from c-Kit $^+$ / α IIb $^+$ fraction of day 6 were also shown. Bar, 50 μ m. Results indicate that c-Kit $^+$ / α IIb $^+$ fraction of day 6 (i) selectively yields megakaryocytes on day 12. Similar results have been obtained from three independent experiments. (F) Expression of genes related to the megakaryocytic lineage as determined by RT-PCR of cells from day 0, 6, or 8 cultures as indicated. (G) Wright-Giemsa-stained cytopsin preparation of sorted c-Kit $^+$ α IIb $^+$ cells from EB at day 6 is shown. Bar, 10 μ m.

c-Kit $^+$ α IIb $^+$ cells derived from ESCs can differentiate displaying several markers unique to the megakaryocytic lineage.

GPIb α expression on ESPs is reduced during in vitro culture
EB generation from ESC in liquid culture (6 d) followed by sorting for c-Kit $^+$ α IIb $^+$ cells (Fig. 1 A) resulted in the constant

generation of megakaryocytes and platelet-like particles, an improvement over our previously reported culture conditions (9). After 8–14 d in culture (2–8 d on co-culture with OP9 stromal cells), culture supernatants contained proplatelets (Fig. 2 A, i) and platelet-sized particles consistent with ESPs (Fig. 2 B). The granularity and size of these ESPs varied from those of

circulating murine platelets as determined by transmission electron microscopy (TEM) (Fig. 2 A) and the forward scatter profiles produced by flow cytometry (Fig. 2 B). Thus, the culture conditions seem to yield various developmental stages of a platelet (36). ESPs displayed an extensive surface-connected open canalicular system and multiple α or dense granules (Fig. 2 A, ii and iii). As compared with a normal mouse platelet, ESPs had a reduced number of granules per ESP (unpublished data).

We examined the relative expression of receptors by flow cytometry comparing ESPs and freshly isolated murine platelets. Expression of the integrin α IIb, GPIb β , and GPIX was similar in platelets from the two sources (Fig. 2 B). However, expression of GPIb α , GPV, and GPVI was significantly less for ESPs as compared with mouse platelets (Fig. 2 B). Cells with an α IIb $^+$ GPIb α^- GPIb β^+ phenotype composed 35–65% of the cellular population after 12–14 d in culture. Reduced GPIb α expression of ESPs was not detected at day 8 (Figs. 2 C, i and ii), consistent with a longer time in culture leading to a loss of GPIb α^+ cells or shedding of GPIb α from the cell. Unlike ESPs, ESC-derived megakaryocytes did not exhibit reduced GPIb α expression, even when cultured for >8 d (Fig. 2 C, i).

ADAM17 can cleave the extracellular domain of GPIb α on human and mouse platelets abrogating platelet function by its metalloproteinase activity (16, 17). Thus, we investigated whether a metalloproteinase supported the shedding of GPIb α in culture. Two potent metalloproteinase inhibitors, GM6001 (Ilomastat) or TAPI-1, were added to the co-culture system to evaluate the potential involvement of a matrix metalloproteinase (MMP). Because both inhibitors also inhibit MMP9, which has been reported to facilitate megakaryopoiesis (37), the drugs were administered only after day 10 (day 4 of co-culture) when megakaryocyte polyploidy is observed (9). Indeed, we found that the addition of GM6001 at the co-culture start impaired megakaryopoiesis (not depicted). GM6001 and TAPI-1 (Fig. 3 B, 1 and 10 μ M) increased GPIb α expression on released ESPs at day 12 (Fig. 3, A and B). This increased expression did not affect the total number of α IIb $^+$ ESPs (α IIb $^+$ GPIb α^+ plus α IIb $^+$ GPIb α^-) at days 12 (Fig. 3 C) or 14 (not depicted). But the reduction of GPIb α was not observed on megakaryocytes during the culture (Figs. 2 C, i, and 3 A). Similarly, GM6001 addition to the culture restored expression of GPV and GPVI in α IIb $^+$ ESPs (Fig. 3 D) as reported

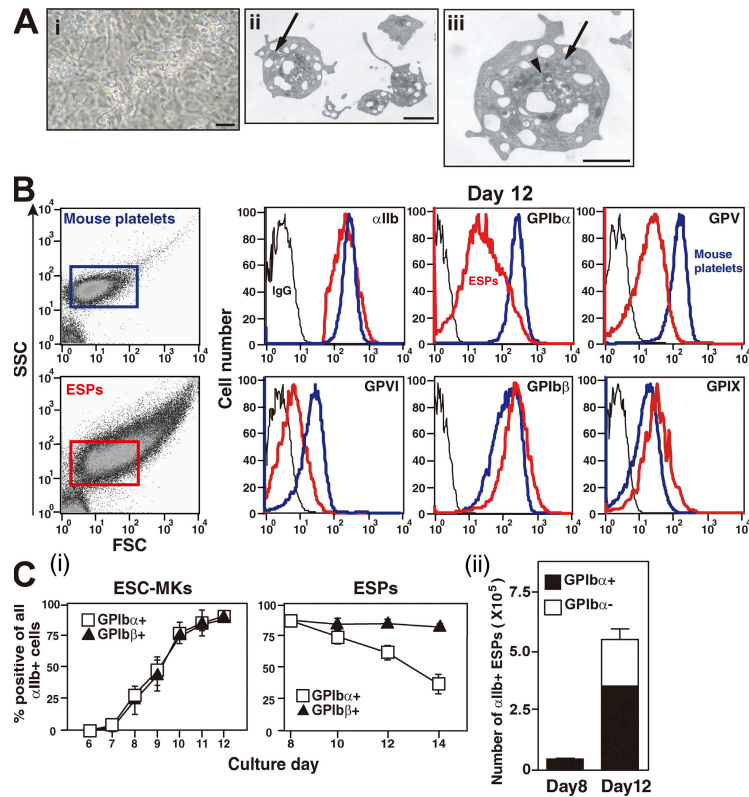


Figure 2. GPIb α expression is reduced on cultured ESPs but not megakaryocytes. (A) On day 12 of the culture, ESC-derived megakaryocytes bearing proplatelets are represented in panel (i) (phase contrast image in culture dish). Bar, 100 μ m. Panels (ii) and (iii) show TEM images of ESPs. The subcellular structure of ESPs showed an open canalicular system, dense granules (arrowhead), and α granules (arrow), which were similar to those of peripheral blood platelets. Bars, 1 μ m. (B) Mouse platelets (12 wk old) or ESPs (day 12) were subjected to flow cytometry. Graphs show representative forward scatter (FSC) or side scatter (SSC) dot plots. ESPs vary in size compared with mouse platelets. The remaining six graphs show surface expression of α IIb integrin subunit, GPIb α , GPIb β , GPV, GPVI, and GPIX on mouse platelets (red lines) or ESPs (blue lines) with control IgG (black lines). (C) (i) On the indicated days of culture (x axis), expression of GPIb α and GPIb β in α IIb $^+$ megakaryocytes derived from ESCs and ESPs is shown. Panel (ii) shows the number of α IIb $^+$ ESPs on days 8 and 12 that are either GPIb α^+ (black bar) or GPIb α^- (white bar). All results are mean \pm SEM from four independent experiments.

for aged blood platelets (15, 38). These results suggest that metalloproteinase(s) impairs the retention of GPIIb α , GPV, and GPVI on ESPs. Biochemical analysis confirmed that ESPs in culture shed the extracellular domain of GPIIb α , referred to as “glycocalicin” (GC), in the absence of GM6001 but not in the presence of GM6001 (Fig. 3 E). As ESC-derived megakaryocytes do not show receptor shedding (Fig. 3, A and E, i), we removed them from the day 12 cell culture population. The remaining specimens were centrifuged, and pellets along with the corresponding supernatant were analyzed. Western blot analysis revealed GPIIb α and GC antigen in the absence of GM6001, but a single band of GPIIb α alone in the presence of GM6001 (Fig. 3 E, ii). A single band of GC was detectable only in the absence of GM6001 in ESP-derived supernatant (Fig. 3 E, iii).

To study whether pre-activation by plasma membrane injury or the activation state of ESPs is associated with metalloproteinase activity during culture, we examined the an-

nexin V binding and P-selectin expression in the absence or presence of GM6001. Annexin V is a marker of platelet activation that detects the translocation of phosphatidylserine to the outer membrane, while P-selectin expression is a hallmark marker of activation (13, 39). Annexin V binding, but not P-selectin expression, was inhibited in the presence of GM6001 (Fig. 3 F). These data indicate that metalloproteinase-dependent cellular changes occurring during ESP generation leads to a reduced viability of these cells. Comparable results were obtained using a variety of different murine ESC lines (R1, EB3, TT2, or E14.1; unpublished data).

Inhibition of metalloproteinase activity restores integrin α IIb β 3 bidirectional signaling in ESPs

To explore whether metalloproteinases induce extracellular shedding of GPIIb α and affect α IIb β 3 inside-out signaling, we used flow cytometry to assess specific fibrinogen binding upon agonist stimulation (9). GM6001 restored specific fibrinogen

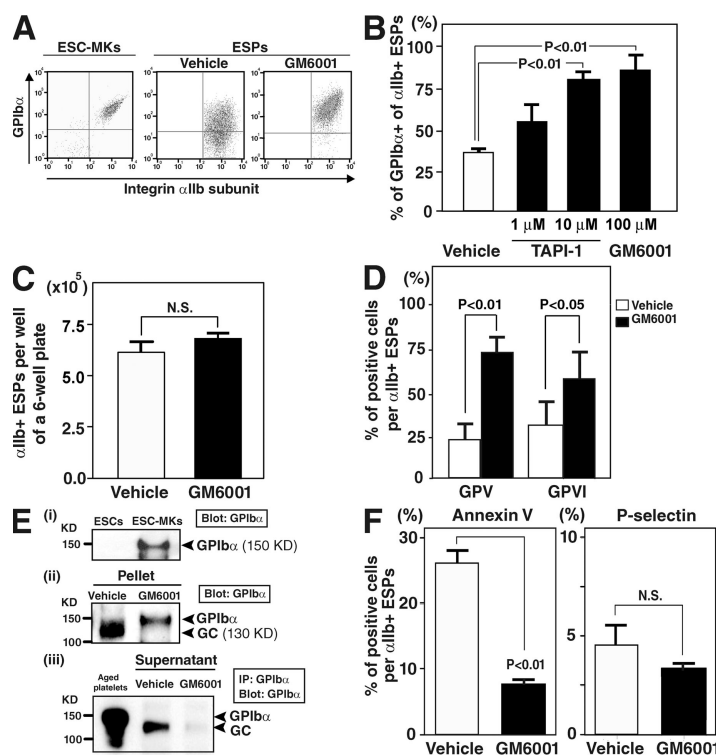


Figure 3. Inhibition of metalloproteinases in culture restores GPIIb α expression in ESPs. (A) On day 10 of ESC culture, 1% DMSO alone (vehicle) or 100 μ M GM6001 dissolved with 1% DMSO was added to the culture medium. On day 12 of culture, representative flow cytometry dot plots of mature megakaryocytes derived from ESCs (ESC-MKs) in the absence of GM6001 (left) and ESPs in the absence or presence of GM6001 are shown. (B) The graph shows the percentage of GPIIb α ⁺ of all α IIb⁺ ESPs on day 12 in the absence or presence of 1–10 μ M TAPI-1 or 100 μ M GM6001. Results are shown as mean \pm SEM from three independent experiments. (C) The graph summarizes total numbers of α IIb⁺ (α IIb⁺GPIIb α ⁺ plus α IIb⁺GPIIb α ⁻) ESPs per well of a 6-well plate on day 12. Results are the mean \pm SEM from four independent experiments. (D) The graph shows the effects of the metalloproteinase inhibitor GM6001 on the surface expression of GPV or GPVI in α IIb⁺ ESPs on day 12. Results are mean \pm SEM from four independent experiments. (E) Expression of GPIIb α or GC in lysates from ESC-MKs (i), in lysates from pellets containing ESPs depleted of MKs (ii), or in supernatant (iii). In panel (i) or (ii), lysates were analyzed by Western blot (7.5% SDS-PAGE) with an anti-GPIIb α antibody. In panel (iii), supernatant derived from culture media of ESPs pretreated with or without GM6001 were subjected to immunoprecipitation followed by immunoblotting with anti-GPIIb α . In vitro-aged platelets from an adult mouse were used as a positive control in panel (iii). Similar results were obtained from three independent experiments. (F) The graphs show the percentage of positive cells of annexin V or P-selectin from the total α IIb⁺ ESPs on day 12 in the absence or presence of GM6001. Results are the mean \pm SEM from three independent experiments.

binding when washed ESPs were stimulated with thrombin or ADP (Fig. 4 A). To rule out the possibility that metalloproteinases directly impair integrin structure in ESPs, $MnCl_2$ was also used (40). Binding of Alexa 488–conjugated fibrinogen to ESPs was comparable in the presence or absence of GM6001 (Fig. 4 A) and similar to that in blood platelets (not depicted). Moreover, $\alpha IIb\beta 3$ -based actin cytoskeletal changes (outside-in signaling) of ESPs in culture were unexpectedly enhanced in the presence of GM6001, apparent when lamellipodia formation was facilitated by the addition of thrombin (Fig. 4 B, arrow). To address whether our observation of defects in ESPs also applied to blood platelets, we generated *in vitro*-injured human platelets by incubation at 37°C for 24 h. Reduced GPIIb α expression was determined by flow cytometry and Western blot analysis (unpublished data), and a defect in lamellipodia formation in these aged platelets was observed. Impaired lamellipodia formation, apparent even upon thrombin stimulation, in aged human platelets was partially restored by the administration of GM6001 in culture for 24 h (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20071482/DC1>). These data demonstrate that deregulated $\alpha IIb\beta 3$ -mediated bidirectional signaling (both inside-out and outside-in) may be associated with an increase in metalloproteinase activity in both ESPs and human platelets.

Metalloproteinases may regulate thrombus formation under flow conditions and posttransfusion recovery *in vivo* by ESPs

Thrombus formation is a dynamic process, and we sought to examine platelet function under physiologically relevant conditions found in the arterial circulation. An experimental system frequently used to study platelet thrombus formation is perfusion of whole blood over a monolayer of collagen at high shear rates, features mimicking those that occur *in vivo* (41). In this model, the initial event is tethering of platelets via binding of GPIIb α to VWF, the latter being immobilized by collagen (10, 11, 13, 14). Direct interaction between integrin $\alpha 2\beta 1$ /GPVI on platelets and collagen follows (10). We prepared reconstituted whole blood consisting of mouse blood labeled with mepacrine, as a marker for platelets (green fluorescence), mixed (1,000:1) with Ds-red–labeled ESC-derived ESPs (red fluorescence). As a control, washed mouse platelets labeled with PE–conjugated anti- αIIb antibody were used (Fig. 5 A) (42). ESPs pretreated with 1% DMSO failed to adhere to collagen–VWF matrices, but pretreatment with GM6001 improved adhesion (Fig. 5, B and C). Most importantly, treating ESPs with GM6001-treated ESPs increased their ability to participate in thrombogenesis. However, their thrombus-forming potential was less than that of uninjured fresh platelets derived from adult mice (Fig. 5 C).

Aged or injured platelets are cleared out of the body after being trapped in the liver (unpublished data) and spleen (43). To confirm the effect of GM6001 on *in vivo* function of ESPs, we examined the time course kinetics of residual ESPs after transfusion. Because murine platelets do not express Ly5 antigen, we chose GFP-expressing ESCs for *in vivo* assays after transfusion (Fig. 6 A). More than 80% of ESPs on day 12

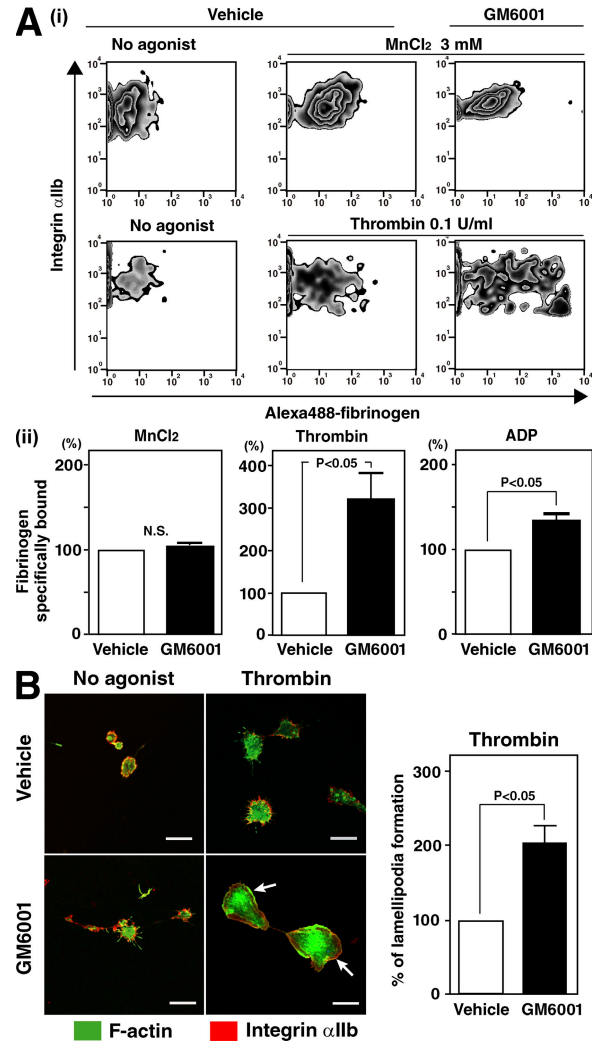


Figure 4. Inhibition of metalloproteinases is required for platelet function mediated through integrin $\alpha IIb\beta 3$ in ESPs. (A) (i) Representative flow cytometry dot plots showing three mM $MnCl_2$ -stimulated or 0.1 U/ml thrombin-stimulated fibrinogen binding to integrin $\alpha IIb\beta 3$ in ESPs after pretreatment with 1% DMSO as a vehicle or 100 μM GM6001. (ii) The graphs show specific fibrinogen binding to $\alpha IIb\beta 3$ stimulated with 3 mM $MnCl_2$ (reference 40), 0.1 U/ml thrombin, or 500 μM adenosine diphosphate. The value of control (vehicle) is defined as 100%. Results are the mean \pm SEM from three independent experiments. (B) On day 14 of culture, washed ESPs pretreated with 1% DMSO or 100 μM GM6001 were plated on fibrinogen-coated coverslips for 45 min. An aliquot of each preparation was assayed in the presence of 1 U/ml thrombin. Cells were fixed, permeabilized, and stained with Alexa 488–phalloidin to stain F-actin (green) and with an anti- αIIb antibody followed by Alexa 567 (red). Bar, 10 μm . The value of control (vehicle) is defined as 100%. The right graph summarizes three independent experiments (mean \pm SEM).

expressed GFP in culture. GM6001-treated ESPs were transfused into the tail veins of mice with significant thrombocytopenia ($\sim 10^4/\mu l$) as a result of irradiation 10 d earlier (Fig. 6 A). 2, 24, 48, or 72 h after transfusion, whole blood was obtained from recipient mice, and the percentage of GFP-expressing platelets among all αIIb^+ platelets was determined.

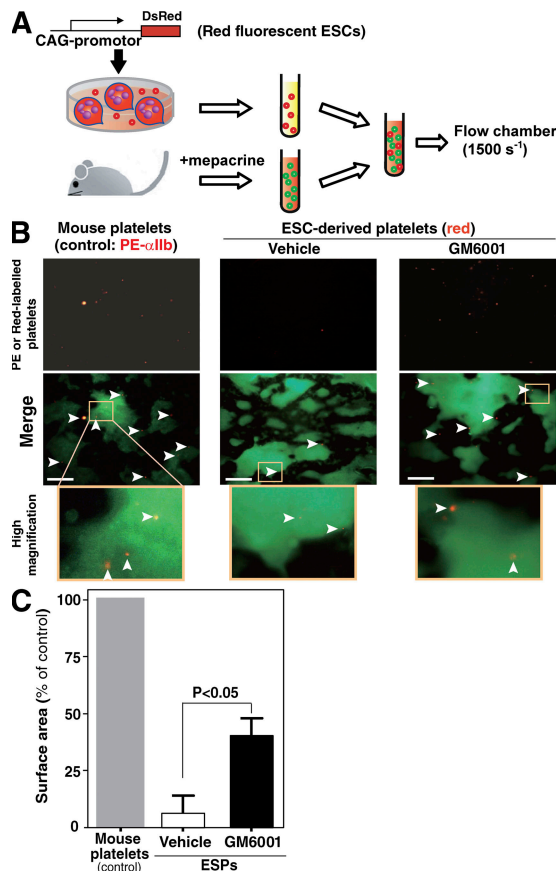


Figure 5. ESPs treated with GM6001 but not without GM6001 contribute to thrombus formation under flow conditions. (A) Schema of the experimental design. (B) Representative two-dimensional fluorescence images at 6 min. Glass slides coated with type I collagen were perfused at a wall shear rate of $1,500 \text{ s}^{-1}$ for 6 min. Samples included reconstituted whole blood composed of ESPs (red, arrowheads) in blood obtained from 10–12 mice (per experiment), or a mixture of PE-labeled mouse platelets (red, arrowheads) in whole blood pooled from 10–12 mice. All murine platelets or ESPs were stained with mepacrine (green). ESPs capture fluorescent mepacrine as indicated by their dual expression of green and red by flow cytometer. Bars, $100 \mu\text{m}$. (C) After 6 min, platelet thrombi formed on the collagen surface were quantified using NIH Images software from a recorded video. The results summarize four independent experiments (mean \pm SEM).

No difference in the size of endogenous platelets and ESPs transfused into recipient mice was observed (not depicted), suggesting that the *in vivo* circulation that may induce shear stress is an important determinant of platelet size (44). Preincubation with GM6001 consistently increased the percentage of GFP⁺ ESPs after transfusion compared with controls (Fig. 6 B).

DISCUSSION

This study has shown that (a) GPIIb α , GPV, and GPVI, but not integrin α IIb β 3 or GPIIb β , are shed from ESPs during culture; (b) the process is specific for ESPs and not relevant for megakaryocyte differentiation from ESCs; (c) the use of a metalloproteinase inhibitor retains the complete GPIIb–V–IX

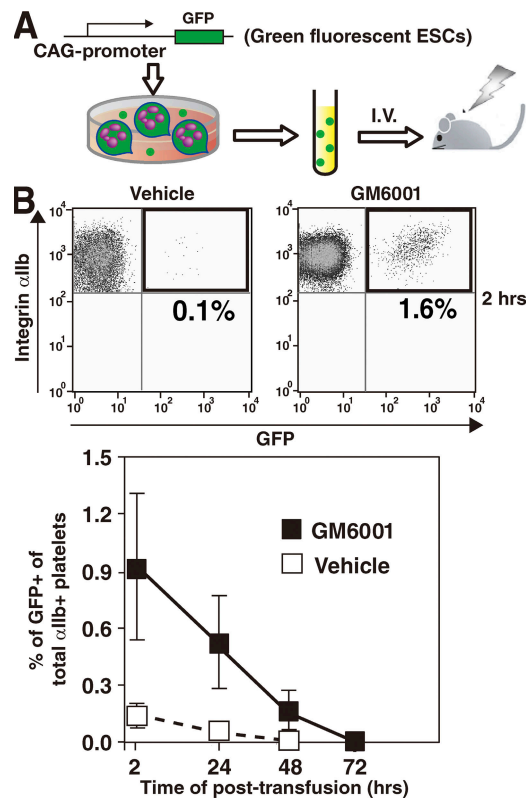


Figure 6. The effect of metalloproteinase inhibition on posttransfusion recovery by ESPs in mice. (A) Schema of the experimental design. (B) 2 h after transfusion of 3×10^6 ESPs, using a 1290la strain mouse model in which platelet numbers are severely reduced, blood obtained from the retroorbital venous plexus was treated with 3.8% sodium citrate and subjected to flow cytometry to detect GFP⁺ platelets (ESPs) among all α IIb⁺ platelets. Additional serial evaluations were performed 24, 48, and 72 h after transfusion. Pretreatment with GM6001 increased the ratio of GFP⁺ ESPs per a recipient at all points examined. Results are the mean \pm SEM from three independent experiments.

complex on ESPs; and (d) both inside–out and outside–in signaling of α IIb β 3 might be associated with metalloproteinase activity. A link between metalloproteinase regulation and α IIb β 3–mediated signaling has not been reported previously. Preventing shedding of the α -subunit of the GPIIb–V–IX complex retains key binding sites for VWF, thrombin, coagulation factors, P-selectin, and Mac-1, all potentially important for normal platelet function (13). The N-terminal motif of GPIIb α is essential for arterial thrombosis independent of VWF (12), and the signaling cascade from the GPIIb–V–IX complex to α IIb β 3 is well known to regulate integrin activation (45). However, the mechanisms whereby metalloproteinase activity regulates integrin signaling remain to be identified.

Metalloproteinases are functionally regulated by endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (46). When we considered the reason why megakaryocytes maintained GPIIb α in culture, as opposed to ESPs, we hypothesized that TIMP-3, also known to inhibit ADAM17 (47), might be highly expressed in megakaryocytes but not in

platelets (48). We therefore determined TIMP-3 expression in ESC-derived megakaryocytes and ESPs, but found no significant difference by RT-PCR (unpublished data). Alternatively, receptor shedding may be occurring with megakaryocytes but is not detectable owing to their gene and protein expression potential, which differs from the anucleate platelet (49).

Another interesting observation was that an administration of GM6001 or TAPI-1 from the beginning of culture impaired the production of $c\text{-Kit}^+\alpha\text{IIb}^+$ cells by day 6 of the liquid culture (unpublished data). We observed that the use of inhibitors to metalloproteinases, which cover both MMPs and ADAMs, is advantageous for the production of efficacious platelets only after day 10 of co-culture. Nonspecific inhibition to metalloproteinases may affect the early phase of hematopoiesis from ESCs as demonstrated in postnatal hematopoiesis through MMP9-mediated mechanisms (50). In addition, it has been reported that MMP2 or MMP9 may have an effect on platelet function. MMP2 activates platelet aggregation through an increase in phospholipase C, protein kinase C, Ca^{2+} mobilization, and phosphatidylinositol 3 kinase (46, 51, 52). MMP9 blocks phospholipase C, protein kinase C, Ca^{2+} mobilization, and thromboxane A2 production leading to the inhibition of the effects of MMP2 (46, 52, 53). Integrins share some of these pathways and therefore might explain how both metalloproteinases and integrin activation might influence each other. In a search for specific MMPs regulating platelet function, we analyzed mice deficient in MMP9 and found comparable platelet numbers, integrin activation, and platelet spreading on fibrinogen comparing wild-type and MMP9-deficient mice (Fig. S2, A, C, and D, available at <http://www.jem.org/cgi/content/full/jem.20071482/DC1>). In addition, extracellular shedding of GPIIb α was observed using in vitro-injured platelets from *MMP9*^{-/-} mice as well as platelets from *MMP9*^{+/+} mice (Fig. S2 B). These results indicate that MMP9 per se is not implicated in thrombopoiesis and platelet function.

Our results suggest that the administration of metalloproteinase inhibitors prolongs the half-life of circulating ESPs in vivo (Fig. 6), presumably by maintaining the repertoire of membrane receptors, such as GPIIb-V-IX and GPVI. In patients with thrombocytopenia, platelet destruction is proportional to plasma concentrations of GC, a shedding product that includes the N-terminal domain of GPIIb α (54). The ectodomain of GPIIb α , GPV, and GPVI is easily shed in stocked human PCs possibly due to increased activities of ADAM17 and ADAM10 (12, 13, 55). Consistent with our study, GM6001 has been recently shown to prevent inactivation of refrigerated platelets by inhibiting ADAM17 activity (56). Specific inhibition to ADAM17 (and ADAM10) with spatial-temporal regulation not affecting hematopoiesis in ESCs may increase generation and storage of efficacious ESPs.

In conclusion, the inhibition of metalloproteinases in murine cultures of ESC-derived $c\text{-Kit}^+\alpha\text{IIb}^+$ primitive cells represents an improvement in the production of efficacious ESPs. Confirming these observations starting with human cells is a future direction potentially defining an experimental framework to produce ESPs in sufficient quantity for clinical application.

MATERIALS AND METHODS

Plasmid preparation, reagents, and mice. All reagents were purchased from Sigma-Aldrich unless otherwise indicated. All animal and recombinant DNA experiments were approved by the Institutional Animal Care and Use Committee of the Institute of Medical Science, University of Tokyo. C57BL/6 mice were purchased from SLC. 129Ola mice (transfusion recipients) were purchased from The Jackson Laboratory. *MMP9*^{-/-} mice were provided by Z. Werb (University of California, San Francisco, San Francisco, CA) (45). Rhodamine- and Alexa 488-conjugated phalloidin, Alexa 488-conjugated fibrinogen, Alexa 568-conjugated bovine IgG, and Alexa 647-conjugated mouse IgG antibodies were from Invitrogen. Purified human fibrinogen was from American Diagnostica Inc. FITC- and allophycocyanin (APC)-conjugated, PE-conjugated, and unconjugated anti-mouse integrin αIIb subunit and APC-conjugated anti- $c\text{-Kit}$, PE-conjugated anti-CD31, PE-conjugated anti-Sca-1, biotin-conjugated anti-CD9, FITC-conjugated anti-P-selectin, unconjugated human anti-GPIIb α , and streptavidin-APC-cyanine 7 (APC-Cy7) antibodies were from BD Biosciences. An annexin V-FITC apoptosis detection kit was purchased from BD Biosciences. PE-conjugated or unconjugated anti-mouse GPIIb α , FITC-conjugated anti-mouse GPIIb β , GPV, GPVI, and GPVI antibodies were from Emfret. Biotin-conjugated anti-VE-cadherin antibody was provided by M. Ogawa (Kumamoto University, Kumamoto, Japan). Human TPO and erythropoietin were provided by H. Miyazaki (Kirin, Takasaki, Japan). Mouse leukemia inhibitory factor (^hESGRO) was from Millipore. Human TPO, IL-6, and IL-11 were purchased from PeproTech. GM6001 and TAPI-1 (57) were from EMD.

Growth and differentiation of ES cells. The murine ESC lines E14tg2A (58), E14 (5), R1 (8), EB3 (37), and TT2 (7) were maintained as described previously (9). For EB formation, 2×10^5 ESCs were placed in 100-mm bacterial Petri dishes containing Iscove's modified Dulbecco's medium supplemented with a cocktail of 300 $\mu\text{g}/\text{ml}$ human transferrin, 0.45 mM monothioglycerol, 50 $\mu\text{g}/\text{ml}$ ascorbic acid, and penicillin-streptomycin/L-glutamine solution (Invitrogen). On day 5 or 6 of culture, cells were dissociated with 0.25% trypsin/EDTA and subjected to sorting by FACS MoFlo (Dako). Sorted cells (2×10^4 per well) were seeded onto OP9 stroma cells in 6-well plates with 20 ng/ml TPO as described previously (9). After 3 d of culture, a cocktail of 10 ng/ml TPO, 5 ng/ml IL-6, and 10 ng/ml IL-11 was added. Cell surface antigen expression was examined by flow cytometry (FACS Aria; BD Biosciences).

Establishment of GPIIb-ESC line. An expression construct containing a human genomic DNA fragment containing the *GPIIb α* promoter (28), followed by coding sequence for a human *GPIIb α -EGFP* fusion, was inserted into pcDNA 3.1 (+)/zeo vector (Invitrogen). All sequences were subsequently confirmed by nucleotide sequencing. Completed pcDNA3.1 zeo vector was linearized with NheI site and transfected into E14tg2A ESCs by electroporation. After drug selection with 65 $\mu\text{g}/\text{ml}$ zeocin for 7 d, resistant colonies were collected. Viable ESCs were confirmed by PCR to detect *zeocin* and human *GPIIb α -EGFP* as a positive clone. To define useful ESC clones further, the intensity of GFP expression was assessed after differentiation into megakaryocytes (Fig. 1 C).

Quantification by RT-PCR. Sorted cells from day 6 EB or day 8 $\alpha\text{IIb}^+c\text{-Kit}^-$ megakaryocytes cultured on OP9 were lysed with Trizol-LS (Invitrogen) for total RNA extraction. cDNAs were obtained by using Thermo Script RT-PCR System and oligo-dT primer (Invitrogen). Final results were normalized with intrinsic GAPDH and PCR Taqman PCR probe in quantity (Applied Biosystems). RT-PCR was performed to determine expression levels of genes of interest. Amplification proceeded for 30–39 cycles. PCR products were separated on agarose gels and visualized by ethidium bromide staining. The primer sets used are shown in Table S1, which is available at <http://www.jem.org/cgi/content/full/jem.20071482/DC1>.

ESP preparation and TEM study. Platelets in culture supernatant were gently collected. Acid citrate dextrose solution was added to yield final

concentrations of 8.5 mM sodium citrate, 6.5 mM citric acid, and 10.4 mM glucose. The collected fluid was centrifuged at 150 *g* for 10 min to eliminate large cells (e.g., megakaryocytes). The supernatant was transferred into a new tube, 1 μ M prostaglandin E₁ and 1 U/ml apyrase were added to prevent platelet activation, and the mixture was centrifuged at 400 *g* for 10 min to sediment a platelet pellet. The pellet was resuspended in an appropriate volume of modified Tyrode-Hepes buffer, pH 7.4 (10 mM Hepes, 12 mM NaHCO₃, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, and 1 mM MgCl₂ without Ca²⁺), or in 2% fetal bovine serum in PBS. To determine the number of platelets in culture, cells were mixed with True Count Beads (BD Biosciences). To detect ESPs in flow cytometry dot plots, the side scatter and forward scatter gates for murine plasma platelets (from mice aged 8–12 wk) were used. TEM studies were performed by using a JEOL 1200EX transmission electron microscope operating at 80 kV (JOEL). Specimens were treated with a mixture of 0.5% glutaraldehyde and 2% paraformaldehyde, followed by 1% osmium tetroxide for observation.

Biochemical studies. For immunoprecipitation, lysis buffer (2% Triton X-100 or 1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, 0.5 mM EDTA, 1 mM Na₃VO₄, 0.5 mM NaF, 0.5 mM PMSE, and 50 μ g/ml leupeptin) was used. Separated supernatant in culture at day 12 of culture was immunoprecipitated with anti-GPIIb α antibody and immunoblotted with anti-GPIIb α antibody (clone Xia G7).

Integrin activation and actin cytoskeletal changes. To investigate integrin α IIb β 3 activation, 50- μ l aliquots of ESPs were incubated with APC-conjugated anti- α IIb and 200 μ g/ml Alexa 488-fibrinogen in the absence or presence of thrombin or ADP for 30 min at room temperature. Binding of Alexa 488-fibrinogen to ESPs was quantified using flow cytometry. Nonspecific binding was determined in the presence of 10 mM EDTA or 20 μ g/ml 1B5, a specific inhibitor of mouse α IIb β 3 (provided by B. Coller, The Rockefeller University, New York, NY). Specific binding was defined as total minus nonspecific binding. To investigate outside-in signaling via α IIb β 3, all observations of cytoskeletal changes (morphology of spreading) in ESPs were performed using a confocal microscopic system (TCS SP2; Leica) as described previously (9).

Flow chamber study. To study the effect of inhibition on metalloproteinases, reconstituted whole blood was prepared by combining ESPs and mouse blood. ESPs were generated from ESCs in which the CAG promoter consistently controls Ds-red expression (provided by H. Niwa, RIKEN, Kobe, Japan) (Fig. 5 A). ESPs in which red fluorescent protein was expressed were combined (1:1,000 ratio) with mouse whole blood that had been labeled with mepacrine to mark mouse platelets; the total volume per experiment was 7–8 ml. Washed platelets obtained from mice aged 10–12 wk were stained with 4 μ g/ml of PE-labeled anti- α IIb antibody (to avoid blocking effect) (42). This whole blood was also obtained from 11–12 C57/BL6 mice aged 10–12 wk per single experiment; argatrovan, an anti-thrombin drug (Mitsubishi Pharma), was added at a final concentration of 100 μ M. Samples of the treated blood, containing ESPs, were injected into the chamber with a syringe pump (Harvard Apparatus) at a constant flow rate to achieve high wall shear rates (i.e., 1,500 s⁻¹) on collagen surfaces. Platelet thrombi forming on the collagen surfaces were visualized with an inverted-stage epifluorescence videomicroscope system (DM IRB; Leica) as described previously (59). The microscopic images were digitized online with a photosensitive color CCD camera (L-600; Leica). Image-J software (National Institutes of Health [NIH] Image) was used to quantify the percentages of surfaces covered by platelets.

Kinetics of residual ESPs in vivo after transfusion. ESPs were generated from ESCs in which the CAG promoter consistently controls GFP expression (provided by H. Niwa, RIKEN, Kobe, Japan) (Fig. 6 A). ESPs were generated in culture in the absence or presence of metalloproteinase inhibitor. On day 12 of culture, 3 \times 10⁶ ESPs were transfused into mice (129Ola strain) in which platelet numbers had been reduced by irradiation (650 cGy)

10 d beforehand. To detect GFP-expressing ESPs at the indicated time points, blood samples were collected with micro-capillaries from the retro-orbital venous plexus and stained with APC-conjugated anti- α IIb antibody. The percentage of GFP⁺ ESPs was determined by flow cytometry for each specimen.

Statistical analysis. Differences between experimental and control results (mean \pm SE median) were analyzed by Student's *t* test. Probability values of *P* < 0.05 were considered significant.

Online supplemental material. Table S1 depicts the primers for Fig. 1 F. Fig. S1 shows the effects of GM6001 on the spreading of aged human platelets on fibrinogen. Fig. S2 shows the platelet number and functions via an integrin bidirectional signaling in *MMP9*^{-/-} mice and their control *MMP9*^{+/+} mice. The online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20071482/DC1>.

The authors thank Drs. H. Miyazaki, M. Ogawa, H. Niwa, B. Coller, and Z. Werb for providing reagents and mice. We are also very grateful to H. Tsukui for her excellent help and Drs. A.S. Knisely and M. Mahaut-Smith for manuscript review.

H. Nishikii, K. Eto, N. Tamura, T. Kanaji, A. Sawaguchi, and S. Goto performed the study. K. Hattori, B. Heissig, and J. Ware provided critical reagents. H. Nishikii and K. Eto designed the study. K. Eto and H. Nakauchi wrote the manuscript.

This work was supported by grants from the Ministry of Education, Culture, Sport, Science and Technology of Japan (to K. Eto and H. Nakauchi) and by a grant from Vehicle Locomotion Foundation (Tokyo, Japan, to K. Eto and S. Goto).

The authors have no conflicting financial interests.

Submitted: 18 July 2007

Accepted: 30 June 2008

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