MOLECULAR PHARMACOLOGY IN CHINA

Megakaryocytic Smad4 Regulates Platelet Function through Syk and ROCK2 Expression[§]

Yanhua Wang, Lirong Jiang, Xi Mo, Yu Lan, Xiao Yang, Xinyi Liu, Jian Zhang, Li Zhu, Junling Liu, and Xiaolin Wu

Department of Laboratory Medicine, The Fifth People's Hospital of Shanghai, Fudan University, Shanghai, People's Republic of China (Y.W.); Institute for Pediatric Translational Medicine, Shanghai Children's Medical Center, Shanghai, People's Republic of China (L.J., X.M.); State Key Laboratory of Proteomics, Genetic Laboratory of Development and Diseases, Institute of Biotechnology, Beijing, People's Republic of China (Y.L., X.Y.); Department of Pathophysiology, Key Laboratory of Cell Differentiation and Apoptosis of National Ministry of Education, Shanghai Jiao Tong University School of Medicine, Shanghai, People's Republic of China (X.L., J.Z.); Cyrus Tang Hematology Center, Soochow University, Suzhou, People's Republic of China (L.Z.); Department of Biochemistry and Molecular Cell Biology, Shanghai Key Laboratory of Tumor Microenvironment and Inflammation, Shanghai Jiao Tong University School of Medicine, Shanghai, People's Republic of China (J.L.); and The Central Laboratory of The Eighth People's Hospital of Shanghai, Shanghai, People's Republic of China (X.W.)

Received November 21, 2016; accepted June 21, 2017

ABSTRACT

Smad4, a key transcription factor in the transforming growth factor- β signaling pathway, is involved in a variety of cell physiologic and pathologic processes. Here, we characterized megakaryocyte/platelet-specific Smad4 deficiency in mice to elucidate its effect on platelet function. We found that megakaryocyte/platelet-specific loss of Smad4 caused mild thrombocytopenia and significantly extended first occlusion time and tail bleeding time in mice. Smad4-deficient platelets showed reduced agonist-induced platelet aggregation. Further studies showed that a severe defect was seen in integrin $\alpha_{IIb}\beta_3$ -mediated bidirectional (inside-out and outside-in) signaling in Smad4-deficient platelets, as evidenced by reduced fibrinogen binding and α -granule secretion, suppressed platelet spreading and clot

retraction. Microarray analysis showed that the expression levels of multiple genes were altered in Smad4-deficient platelets. Among these genes, spleen tyrosine kinase (Syk) and Rho-associated coiled-coil containing protein kinase 2 (ROCK2) were downregulated several times as confirmed by quantitative reverse-transcription polymerase chain reaction and immunoblotting. Further research showed that Smad4 directly regulates ROCK2 transcription but indirectly regulates Syk. Megakaryocyte/platelet-specific Smad4 deficiency caused decreased expression levels of Syk and ROCK2 in platelets. These results suggest potential links among Smad4 deficiency, attenuated Syk, and ROCK2 expression and defective platelet activation.

Introduction

Platelets play essential roles in hemostasis, angiogenesis, inflammation, and metastasis (George, 2000; Gay and Felding-Habermann, 2011; Semple et al., 2011). Platelets in

circulating blood contain high concentrations of transforming growth factor (TGF)- β 1 in their α -granules and release it during platelet activation (Labelle et al., 2011). Platelet-released TGF- β 1 substantially contributes to plasma levels of TGF- β 1 (Meyer et al., 2012), which can influence vascular function and hemostasis through the regulation of various cell functions (Redondo et al., 2012). A previous study demonstrated that TGF- β 1 could regulate platelet activity through a nontranscriptional signaling pathway. TGF- β 1-deficient mice exhibited a mild bleeding disorder and faulty platelet aggregation and fibrinogen (Fg) binding (Hoying et al., 1999).

This work was supported by the Program of National Natural Science Foundation of China [Grants 91439115, 81670129, and 81371922] and in part by the National Key Basic Research Program of China [Grant 2012CB518000].

J.L. and X.W. are co-corresponding authors.

<https://doi.org/10.1124/mol.116.107417>.

[§] This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: ANOVA, analysis of variance; CRP, collagen-related peptide; EP, epinephrine; Fg, fibrinogen; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPVI, glycoprotein VI; HEK293T, human embryonic kidney 293T; HHT, hereditary hemorrhagic telangiectasia; NIH, National Institutes of Health; PBS, phosphate-buffered saline; PE, phycoerythrin; PF4, platelet factor 4; PRP, platelet-rich plasma; ROCK, Rho-associated coiled-coil containing protein kinase; RP, reticulated platelet; RT, room temperature; RT-qPCR, real-time quantitative reverse transcription polymerase chain reaction; Syk, spleen tyrosine kinase; TEM, transmission electron microscopy; TGF, transforming growth factor.

Generally, TGF- β ligands convey signals intracellularly through the Smad signaling pathway. Among the Smad proteins, Smad4 is the common mediator Smad that forms a complex with the receptor-activated Smads (Smad1, 2, 3, 5, and 8). The activated complex accumulates in the nucleus and recruits transcriptional cofactors to control gene expression (Shi and Massague, 2003). However, the effects of Smad4 on platelet activation are still unknown. It has been reported that the specific inactivation of Smad4 in hematopoietic cells caused severe polyposis and anemia in mice (Pan et al., 2007), even though the peripheral platelet counts were not affected. Since Smad4 is necessary for TGF- β signaling, Smad4-deficient mice could be expected to show severe defects in platelet functions. However, Smad4-deficient mice die at approximately embryonic day 7.5 (Sirard et al., 1998; Yang et al., 1998). To investigate the role of Smad4 in platelets, we generated megakaryocyte/platelet-specific Smad4-deficient mice. Our study reveals a novel role for Smad4 as a positive regulator in platelet activation. Megakaryocyte/platelet-specific Smad4 deficiency indirectly interfered with integrin $\alpha_{IIb}\beta_3$ -mediated bidirectional signaling and caused severe platelet dysfunction.

Materials and Methods

Materials. Epinephrine (EP) (cat. no. E4250), apyrase (cat. no. A6535), prostaglandin E_1 (cat. no. P5515), Fg (cat. no. F3879), and the spleen tyrosine kinase (Syk)-specific inhibitor BAY61-3606 (cat. no. B9685) were purchased from Sigma-Aldrich (St. Louis, MO). ADP (cat. no. 0160) was purchased from Ameresco (Houston, TX). The CHRONO-LUME reagent was from CHRONO-LOG Corporation (Havertown, PA). The collagen-related peptide (CRP) was a gift from Professor Li Zhu (Soochow University, Suzhou, People's Republic of China). α -Thrombin (cat. no. HT4082A) was from Enzyme Research Laboratories (South Bend, IN). Fluorochrome-conjugated CD41 (cat. no. 558040), CD61 (cat. no. 553347), and P-selectin (CD62P; cat. no. 553744) antibodies as well as the Annexin V kit (cat. no. 556547) were from BD Biosciences (San Jose, CA). The Alexa Fluor 647-labeled anti-mouse Fg monoclonal antibody (cat. no. F35200) was from Molecular Probes (Life Technologies). The anti-Smad4 antibody (cat. no. D3M6U) was from Cell Signaling Technology (Danvers, MA). The anti-Syk antibody (cat. no. BS1344) was from Bioworld Technology (St. Louis Park, MN). Anti-Rho-associated coiled-coil containing protein kinase (ROCK) 1 (cat. no. 21850-1-AP) and anti-ROCK2 (cat. no. 21645-1-AP) antibodies were from Proteintech (Chicago, IL). The anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (cat. no. KC-5G4) was from Kangchen (Shanghai, People's Republic of China). The ROCK inhibitor Y-27632 (cat. no. IAD1011) was from Gene Operation (Ann Arbor, MI). Thiazole orange (CAS:107091-89-4) (cat. no. ES-SJ-S0265) was from EYSIN (Shanghai, People's Republic of China). The Sulfo-NHS-LC-Biotin (cat. no. 21335) was from Thermo Fisher Scientific (Waltham, MA). The Dual-Luciferase Reporter Assay kit (cat. no. E1910) was from Promega (Madison, WI), and the real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) kit (cat. no. 4367659) was from Applied Biosystems (Foster City, CA). The human embryonic kidney 293T (HEK293T) cell line was obtained from the cell bank of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Generation of Megakaryocyte/Platelet-Specific Smad4-Deficient Mice. Mice carrying the Smad4 gene flanked by loxP recognition sites (Smad4^{fl}) (Yang et al., 2002) were crossed with transgenic mice carrying the Cre recombinase under the control of the platelet factor 4 (PF4) promoter (PF4-Cre) (Tiedt et al., 2007) to generate Smad4^{fl/w} PF4-Cre⁺ mice (Smad4^{+/w}). Further mating gave rise to Smad4^{fl/fl} PF4-Cre⁺ (Smad4^{-/-}) mice that exhibit Smad4 deficiency in platelets. The mice were genotyped by PCR, and the Smad4 deficiency in the platelets was

confirmed by Western blotting. The animal research was approved by the Shanghai Jiao Tong University School of Medicine Animal Care and Use Committee.

Peripheral Platelet Counting and Platelet Preparation. Peripheral platelet counting was performed using an HEMAVET 950 Veterinary Multi-species Hematology System. Washed platelets were prepared from mice as described previously (Weng et al., 2010). The platelet concentration was adjusted to 3×10^8 platelets/ml.

Platelet Aggregation. Aggregation experiments were performed with 300 μ l of washed platelets at 37°C with constant stirring in an optical aggregometer (CHRONO-LOG Corporation). An inhibitor was incubated with the platelets for 3 minutes prior to stimulation with different agonists, and the platelet aggregation was recorded.

Analysis of Annexin V Binding to Platelets. To detect platelet apoptosis, 5×10^6 washed platelets from Smad4^{fl/fl}, Smad4^{-/-}, and Smad4^{+/w} mice were resuspended in annexin V binding buffer and preincubated with fluorescein isothiocyanate (FITC)-conjugated annexin V for 15 minutes. The annexin V binding was analyzed using a FACSCalibur Flow Cytometer (BD Biosciences).

Bone Marrow Megakaryocyte Morphology and Counts. Bone marrow was collected from the bare femurs and tibiae of mice by flushing with a 10-ml empty syringe fitted with a 21-gauge needle rinsed with 10 mM EDTA. Drops of marrow were smeared on glass slides and stained with Wright and Giemsa stains. Images were captured with a Leica DM IRB Microscope (Leica Microsystems, Buffalo Grove, IL) in a 40 $\times$ objective field.

To measure the number of megakaryocytes in the bone marrow, red cells were lysed using erythrocyte-lysing reagent, and the bone marrow cells were washed twice in phosphate-buffered saline (PBS). The cells were stained with a CD41-phycoerythrin (PE) antibody, and the stained cells were counted using a FACSCalibur Flow Cytometer (BD Biosciences).

Flow Cytometric Measurement of Reticulated Platelets. To measure platelet reticulation, 5 μ l of whole blood was added to a tube containing sodium citrate. Platelet-rich plasma (PRP) was then prepared by centrifugation (180g for 5 minutes at room temperature (RT)). The PRP was diluted 20-fold with PBS and was stained with thiazole orange (100 ng/ml) and a CD41-PE antibody at saturating concentrations for 30 minutes at RT and analyzed with a FACSCalibur Flow Cytometer (BD Biosciences). Reticulated platelets (RPs) were identified as a discrete subpopulation of platelets exhibiting bright fluorescence in the FL-1 channel. The RPs were expressed as a percentage of the total number of counted platelets.

Platelet Life Span Analysis. In vivo platelet labeling was performed as described previously (Prislovsky et al., 2008). In brief, NHS-biotin was dissolved in 150 mM NaCl and injected into the tail vein of each mouse (35 μ g/g b.wt.). The injected mice were bled at the times indicated. Two hours after injection, 5 μ l of blood was taken from the tail vein. PRP was prepared as described above and stained with a CD41-PE antibody and streptavidin-allophycocyanin for 30 minutes at RT. The samples were analyzed with a FACSCalibur Flow Cytometer (BD Biosciences). The bleeds were repeated daily for a further 4 days to determine the percentage of labeled platelets.

Morphologic Analysis of Platelets by Transmission Electron Microscopy. Smad4^{fl/fl} and Smad4^{-/-} resting platelets were fixed with 2% glutaraldehyde in 0.1 M PBS (pH 7.2) and embedded in EPON. Thin sections were stained with lead citrate and examined under a CM120 Bio Twin Transmission Electron Microscope (Philips, Amsterdam, The Netherlands). Pictures were taken with a MegaView Camera (Olympus Soft Imaging Solutions GmbH, Muenster, Germany). One hundred platelets were measured using a 6000 $\times$ magnification random field with the National Institutes of Health (NIH) ImageJ software.

Ferric Chloride-Induced Carotid Artery Injury. A ferric chloride-induced carotid artery injury murine thrombosis model was processed as described previously (Chen et al., 2013). Monitoring of carotid artery blood flow was initiated at the time of FeCl₃ treatment. Carotid artery blood flow of <0.06 ml/min was scored as occlusion, allowing the time to first occlusion to be determined.

Bleeding Time Analysis. For tail-bleeding assays, the mice were anesthetized by peritoneal injection of 1% sodium pentobarbital. The tails were cut at 5 mm from the tip and bled onto a Whatman filter paper (GE Healthcare Bio-Sciences, Pittsburgh, PA). The filter paper was dabbed to the wound every 30 seconds without disrupting the forming clot. The experiment was continued until the bleeding stopped completely (Mahooti et al., 2000).

Flow Cytometry Analysis of the Expression of the Integrin $\alpha_{IIb}\beta_3$ and P-Selectin in Stimulated Platelets. To analyze the expression level of the surface integrin subunits α_{IIb} and β_3 on platelets, resting platelets were stained with PE-conjugated monoclonal antibodies (CD41 and CD61) at saturating concentrations for 30 minutes at RT and examined using a FACSCalibur Flow Cytometer (BD Biosciences). To monitor P-selectin expression levels and Fg binding when $\alpha_{IIb}\beta_3$ is activated, washed, unstirred platelets from Smad4^{fl/fl}, Smad4^{+/-}, and Smad4^{-/-} mice were incubated with saturating concentrations of FITC-conjugated P-selectin (CD62P) and Alexa Fluor 647-labeled anti-mouse Fg monoclonal antibodies, respectively. After stimulation with different agonists in a final volume of 100 μ l of modified Tyrode's buffer for 30 minutes at RT, the expression levels of activated $\alpha_{IIb}\beta_3$ and P-selectin were examined by a FACSCalibur Flow Cytometer (BD Biosciences).

Measurement of ATP Secretion. ATP secretion was evaluated using the CHRONO-LUME reagent (CHRONO-LOG Corporation) according to the manufacturer protocol. Stirred platelets at a concentration of 3×10^8 /ml were activated with the indicated agonists for 5 minutes at 37°C, and 10 μ l of the reagent was then added directly to each supernatant and monitored for 3 minutes to detect the ATP release (Niu et al., 2012).

Platelet Spreading on Immobilized Fg. The analysis of platelet spreading on immobilized Fg was performed as described previously (Chen et al., 2013). The resting state of washed platelets was evaluated using an optical aggregometer (CHRONO-LOG Corporation). Resting platelets (2×10^7 /ml) were then allowed to adhere on Fg (30 μ g/ml)-precoated glass coverslips for 120 minutes. Attached platelets were fixed with freshly prepared 4% paraformaldehyde then permeabilized with 0.2% Triton X-100 for 5 minutes, washed, and stained with rhodamine-conjugated phalloidin for 60 minutes at RT. Images of spread platelets stained by rhodamine-conjugated phalloidin were captured with a Leica DM IRB Microscope (Leica Microsystems) in a 100 $\times$ objective field, and the platelet size was quantified using NIH ImageJ software.

Clot Retraction. Clot retraction using mouse platelets was assayed as described previously (Flevaris et al., 2009). The retracting clots were photographed at the indicated times, the clot size was quantified from photographs using NIH ImageJ software, and the retraction was expressed as the retraction ratio [$1 - (\text{final clot size}/\text{initial clot size})$].

Microarray Assay and RT-qPCR. Total mRNA was extracted from washed platelets from Smad4^{fl/fl} or Smad4^{-/-} mice (for each group, $n = 6$). The total mRNA was labeled and hybridized to Mouse Genome 430 2.0 Chips (Affymetrix, Santa Clara, CA) according to the manufacturer instructions. The resultant transcriptomes were compared with generated differentially expressed gene lists. Validation assays were performed on the mRNA expression data set using RT-qPCR (7500 Real-Time PCR System, Applied Biosystems) with gene-specific primers. The relative fold change in mRNA expression level was normalized to GAPDH expression. The RT-qPCR primers used to analyze mouse platelet cDNA are detailed in Table 1.

Western Blotting. To detect target proteins by immunoblotting, the protein samples from platelets were subjected to SDS-PAGE and then transferred to polyvinylidene difluoride membrane. The membranes were blotted with the antibodies indicated in Fig. 4. The immunoreactive bands were detected using Super Signal Chemiluminescent Substrate (Pierce, Rockford, IL).

Luciferase Reporter Assay. Mouse Smad4 cDNA was generated from mouse livers by PCR using specific primers (Table 2). The PCR products were cloned into the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA). The promoter sequences of the mouse Syk, ROCK1,

TABLE 1

Primers used in RT-qPCR

Name	Sequence
GAPDH forward	5'CAGTATGACTCCACTCACGGC3'
GAPDH reverse	5'GAGGGGCCATCCACAGTCTTC3'
ROCK-1 forward	5'CTTGGAACGCTCCGAGAC3'
ROCK-1 reverse	5'TCTCACTGGCATTGCTGAAGG3'
ROCK-2 forward	5'GGATGGTTGTCATTGCCTGTG3'
ROCK-2 reverse	5'AAGGGTTGGACTGCTCTTTATC3'
Syk forward	5'TACGCCCC GAATGCATCAACTAC3'
Syk reverse	5'GCACCCCATCCGCTCTCCTTTCT3'
Integrin- β_1 forward	5'TGTGACCCATTGCAAGGAGAAGG3'
Integrin- β_1 reverse	5'GGATGATGTCGGGACCAGTAGG3'

and ROCK2 genes were generated from mouse genomic DNA by PCR using the primers shown in Table 2.

The PCR products were cloned into the pGL3-Basic vector (Promega), and all recombinant clones were sequenced. The pGL3-Basic or pGL3-promoters vectors and 1 ng of the Renilla Control Vector (Promega) together with or without the pcDNA3.1-Smad4 vector were transfected into HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer instructions. The cells were then harvested and analyzed using the Dual-Luciferase Reporter Assay Kit (Promega). The final amount of firefly luciferase activity was normalized to the Renilla luciferase activity. Each individual reaction was performed in triplicate.

Statistics. All data were analyzed using the GraphPad Prism version 6.01 statistical software. Results are presented as the mean $\pm$ S.E.M. All reported P values are two tailed, with statistical significance set at the $P < 0.05$. The statistical significance between two groups was determined by Student's t test. Comparisons among three or more groups were performed using analysis of variance (ANOVA), then all followed by post hoc comparisons (Tukey's multiple comparison test).

Results

Platelet-Specific Smad4 Deficiency Causes Mild Thrombocytopenia. To study the role of Smad4 in platelet activation and thrombus formation, the megakaryocyte/platelet-specific Smad4-deficient mice were examined. The Smad4^{fl/fl}, Smad4^{+/-}, (Smad4^{fl/fl}, PF4-Cre⁺) and Smad4^{-/-} (Smad4^{fl/fl}, PF4-Cre⁺) mice were genotyped by PCR (Fig. 1A), and the platelet-specific Smad4 deficiency was confirmed by Western blot analysis (Fig. 1B). The results showed that the Smad4 expression was significantly reduced in Smad4^{+/-} platelets and totally ablated in Smad4^{-/-} platelets. Because little is known about the function of Smad4 in platelets, the platelet number in these mouse was enumerated. The numbers of peripheral platelets were $996 \pm 29 \times 10^9$ /l, $980 \pm 37 \times 10^9$ /l, and $846 \pm 39 \times 10^9$ /l in Smad4^{fl/fl} mice ($n = 11$ mice/group), Smad4^{+/-} mice ($n = 8$ mice/group), and Smad4^{-/-} mice ($n = 10$ mice/group), respectively. Although there was no visible difference in platelet numbers between the Smad4^{fl/fl} and Smad4^{+/-} groups, the blood of Smad4^{-/-} mice contained $\sim 20\%$ fewer platelets than that of Smad4^{fl/fl} mice ($P < 0.05$) (Fig. 1C), indicating that the complete depletion of Smad4 in megakaryocytes/platelets causes mild thrombocytopenia. The proportion of RPs (%RP) in total platelets has been shown to reflect the production of platelets from megakaryocytes. Therefore, the %RP was evaluated in Smad4^{fl/fl} and Smad4^{-/-} mice. The average %RP of Smad4^{-/-} mice was 4.64% ($n = 12$ mice/group), compared with 5.65% ($n = 12$ mice/group) in Smad4^{fl/fl} mice. The complete depletion of Smad4 resulted in an $\sim 17.7\%$ reduction of the %RP ($P <$

TABLE 2
Primers used in Luciferase reporter assay

Name	Sequence
Smad4 forward	CGGGGTACCCCGTTACTCCAGAAATTGGAGAGTTGG (KpnI)
Smad4 reverse	GCTCTAGAGCGAGATCTCAGTCTAAAGGCTGTGGG (XbaI)
Syk forward	CGGGGTACCCCGTTAGCTCCACATCCGTGCAGAA (KpnI)
Syk reverse	GAAGATCTTC GCTTTGGCAGGGTTCAGAGTTT (Bgl II)
ROCK1 forward	GAAGATCTTC TACCTGTACTTTTTTGTGCGTTTCC (Bgl II)
ROCK1 reverse	CCCAAGCTTGGG ACTTGTGACGATACCTTCTTGCCC (HindIII)
ROCK2 forward	GGGGTACCCCTCAGGGGCATTACACTCTAGCTTCC (KpnI)
ROCK2 reverse	CCCAAGCTTGGGCTCCACACTGCGTGACACTTTC (Hind III)

0.01) (Fig. 1D). An analysis of the bone marrow revealed that mature megakaryocytes (CD41⁺) of Smad4^{-/-} mice were normal in number (Fig. 1E) and morphology (Supplemental Fig. 1A). To determine whether the thrombocytopenia in Smad4^{-/-} mice was caused by increased platelet turnover, the life span of Smad4^{-/-} platelets *in vivo* was determined. We found no significant difference in life span between Smad4^{+/+} and Smad4^{-/-} platelets (Fig. 1F). Annexin V binding to platelets was assayed to evaluate the role, if any, of apoptosis in the thrombocytopenia correlated with Smad4 deficiency. The results showed that Smad4 deficiency did not enhance annexin V binding to platelets (Supplemental Fig. 1B). This result suggested that it is impaired megakaryocyte differentiation, rather than enhanced platelet turnover, that caused the thrombocytopenia in megakaryocyte/platelet-specific Smad4-deficient mice. In addition, the platelet size in Smad4^{-/-} mice did not differ from that of Smad4^{+/+} mice, as revealed by transmission electron microscopy (TEM) (Fig. 1G). The average width of Smad4^{-/-} platelets was 0.82 ± 0.02 μm compared with 0.8 ± 0.02 μm for the Smad4^{+/+} controls (*n* = 100 platelets; *P* > 0.05) (Fig. 1H). Moreover, the TEM examination revealed that the intracellular structures of resting Smad4^{+/+} and Smad4^{-/-} platelets, including the α-granules and dense granules, were indistinguishable (Fig. 1G). Thus, instead of platelet turnover, Smad4 deficiency is more likely to affect the differentiation of megakaryocytes into platelets, causing thrombocytopenia.

Diminished Aggregation of Smad4-Deficient Platelets in Response to Agonists. The role of Smad4 in agonist-induced platelet aggregation was investigated by stimulating Smad4^{+/+}, Smad4^{+/-}, and Smad4^{-/-} platelets with thrombin, ADP, or CRP. As shown in Fig. 2A and Supplemental Fig. 2A, the aggregation of Smad4^{+/-} and Smad4^{-/-} platelets was diminished in response to different concentrations of thrombin (0.05 U/ml), ADP (40, 20, and 10 μM) or CRP (0.5, 0.35, and 0.2 μg/ml). Moreover, Smad4^{-/-} platelets showed more severely impaired aggregation in response to these agonists. Notably, markedly decreased aggregations of Smad4^{-/-} and Smad4^{+/-} platelets were seen upon activation with CRP. As CRP is a glycoprotein VI (GPVI)-specific agonist (Asselin et al., 1997; Kehrel et al., 1998), these results suggested that Smad4 deficiency mainly affects GPVI-associated downstream signal transduction in platelet activation.

Decreased Secretion of α-Granules but Normal Dense Granule Release in Smad4-Deficient Platelets. P-selectin resides in the α-granule membrane of unstimulated platelets, which is translocated to the activated platelet surface via a secretory pathway (Polgar et al., 2005). The expression of P-selectin on the platelet surface as a readout for α-granule secretion was investigated by flow cytometry in

response to 0.1 U/ml thrombin, 2 μg/ml CRP, or 40 μM ADP with 0.4 μg/ml EP. The secretion of P-selectin was significantly decreased in Smad4^{-/-} platelets in response to CRP or ADP but not thrombin (Fig. 2B). These results indicated that the secretion of α-granules was impaired due to the homozygous deletion of Smad4 in platelets (Fig. 2B; Supplemental Fig. 2B). To test whether the release of dense granules was also affected, the amount of ATP released by stimulated platelets was measured. Surprisingly, no significant differences in ATP secretion were found between these three genotypes of platelets stimulated by either 0.35 μg/ml CRP, 20 μM ADP, or 0.05 U/ml thrombin (Fig. 2C). Therefore, the lack of Smad4 affects platelet α-granule secretion but has no impact on dense granule release.

Delayed Arterial Occlusive Thrombosis and Prolonged Bleeding Time in Smad4-Deficient Mice. An FeCl₃-induced arterial injury model and tail-bleeding time assays were used to evaluate the physiologic role of targeted molecules in thrombosis and hemostasis *in vivo* (Liu et al., 2006). Since lack of Smad4 results in defective agonist-induced platelet activation, thrombus formation in Smad4^{+/+}, Smad4^{+/-}, and Smad4^{-/-} mice was examined using the FeCl₃-induced carotid artery thrombosis model. The average time to first occlusion was 20.53 ± 2.56 minutes in Smad4^{-/-} mice (*n* = 6 mice/group), compared with 9.11 ± 0.29 minutes in Smad4^{+/+} mice (*n* = 6 mice/group; *P* < 0.01) and 17.13 ± 1.97 minutes in Smad4^{+/-} mice (*n* = 6 mice/group; *P* < 0.05) (Fig. 2D). These results indicated that Smad4 deficiency in platelets inhibited arterial thrombus formation *in vivo*. The effect of Smad4 deficiency on hemostasis was evaluated by measuring tail-bleeding time. The average bleeding time of Smad4^{-/-} mice was 16.62 ± 1.74 minutes (*n* = 7 mice/group), which is significantly longer than that of the Smad4^{+/+} mice, which was 3.93 ± 0.43 minutes (*n* = 8 mice/group; *P* < 0.001). In addition, the average bleeding time of the Smad4^{+/-} group was 8.4 ± 0.88 minutes (*n* = 6 mice/group; *P* < 0.05) (Fig. 2E). Thus, Smad4 deficiency in megakaryocytes/platelets caused a prolonged bleeding time, suggesting that Smad4 may regulate thrombus formation and hemostasis *in vivo*.

Lack of Smad4 Affects Platelet Integrin α_{IIb}β₃-Mediated Inside-Out Signaling. Integrin α_{IIb}β₃-mediated bidirectional signaling plays a critical role in thrombosis and hemostasis (Moser et al., 2008). Stimulation of platelets with various agonists will activate integrin α_{IIb}β₃ ("inside-out" signaling), which is then able to bind soluble Fg, resulting in stable platelet adhesion, platelet aggregation, and thrombus formation (Li et al., 2010). However, the function of Smad4 in the platelet integrin α_{IIb}β₃-related signaling is poorly understood. First, the expression levels of the α_{IIb} and β₃ subunits in platelets from Smad4-deficient mice were analyzed by flow cytometry using PE-conjugated anti-CD41

Fig. 1. Smad4 deficiency caused mild thrombocytopenia. (A) Genotyping results of Smad4^{+/ff} (Smad4^{wt/ff}, PF4-Cre⁺), Smad4^{ff/ff}, and Smad4^{-/-} (Smad4^{ff/ff}, PF4-Cre⁻) mice using PCR. (B) Western blot analysis demonstrated that Smad4 was totally deleted in Smad4^{-/-} platelets and that Smad4 expression was decreased by about half in the Smad4^{+/ff} platelets. GAPDH was used as a loading control. (C) Smad4 deficiency caused mild thrombocytopenia, which was demonstrated by an approximately 20% decrease of peripheral platelet counts in Smad4^{-/-} mice ($n = 11$ for Smad4^{ff/ff}; $n = 8$ for Smad4^{+/ff}; $n = 10$ for Smad4^{-/-}). * $P < 0.05$. (D) Smad4 deficiency resulted in a reduction of the average proportion of RP in total platelets ($n = 12$ for Smad4^{ff/ff}; $n = 12$ for Smad4^{-/-}). ** $P < 0.01$. (E) Mature megakaryocyte counts (CD41⁺) from the bone marrow were not affected by Smad4 deficiency. (F) Determination of the platelet life span in Smad4^{ff/ff} and Smad4^{-/-} mice. The results as determined by flow cytometry are the percentage of fluorescently labeled platelets at the indicated days after injection. The values are reported as the mean $\pm$ S.E.M. of three mice/group. (G) Representative TEM images from Smad4^{ff/ff} and Smad4^{-/-} platelets in the 6000 $\times$ and 29,500 $\times$ magnification fields. Scale bars: top (6000 $\times$), 5 μ m; bottom (29,500 $\times$), 1 μ m. (H) Platelet width was measured by NIH ImageJ software ($n = 100$ per group). Bars in the graph represent the mean $\pm$ S.E.M. N.s., not significant.

and anti-CD61 antibodies, respectively. The results showed that the expression levels of both subunits were similar among the Smad4^{ff/ff}, Smad4^{+/ff}, and Smad4^{-/-} mouse platelets (Fig. 3A). These results suggested that lack of Smad4 has no effect on the

expression of α_{IIb} and β_3 in platelets. We then examined the role of Smad4 in agonist-induced $\alpha_{IIb}\beta_3$ activation by detecting the binding efficiency of fluorescence-conjugated Fg to the stimulated Smad4^{ff/ff}, Smad4^{+/ff}, and Smad4^{-/-} platelets. We found that

Fig. 2. Smad4 deficiency caused the dysfunction of platelets. (A) The aggregation of $Smad4^{fl/fl}$, $Smad4^{+/-}$, and $Smad4^{-/-}$ platelets in response to agonists as indicated. The traces are representative of three experiments. (B) Washed, unstirred $Smad4^{fl/fl}$, $Smad4^{+/-}$, and $Smad4^{-/-}$ platelets at a concentration of 3×10^7 /ml were incubated with an FITC-conjugated P-selectin monoclonal antibody in the presence of 0.1 U/ml thrombin, 40 μ M ADP plus 0.4 μ g/ml EP, or 2 μ g/ml CRP. (C) Measurements of the ATP released in the supernatant of activated $Smad4^{fl/fl}$, $Smad4^{+/-}$, and $Smad4^{-/-}$ platelets induced by 0.35 μ g/ml CRP, 20 μ M ADP, or 0.05 U/ml thrombin. The bars in the graph represent the mean $\pm$ S.E.M. from three independent experiments. (D) Mouse carotid arteries were treated with 10% $FeCl_3$ as described. The times to occlusion were measured ($n = 6$ for each group; $*P < 0.05$; $**P < 0.01$). (E) Smad4 deficiency in platelets prolonged the mouse tail vein bleeding time ($n = 8$ for $Smad4^{fl/fl}$; $n = 6$ for $Smad4^{+/-}$; $n = 7$ for $Smad4^{-/-}$; $*P < 0.05$; $***P < 0.001$).

Fig. 3. Smad4 deficiency impaired integrin $\alpha_{IIb}\beta_3$ -mediated inside-out and outside-in bidirectional signaling. (A) Washed, resting Smad4^{fl/fl}, Smad4^{+/-}, and Smad4^{-/-} platelets at a concentration of 3×10^7 /ml were incubated with PE-conjugated CD41 and CD61 monoclonal antibodies. The expression levels of the integrin subunits α_{IIb} and β_3 were detected using a flow cytometer. (B) Binding of Alexa Fluor 647-Fg to washed, unstirred Smad4^{fl/fl}, Smad4^{+/-}, and Smad4^{-/-} platelets stimulated with 0.1 U/ml thrombin, 40 μ M ADP plus 0.4 μ g/ml EP, or 2 μ g/ml CRP. (C) Spreading of Smad4^{fl/fl}, Smad4^{+/-}, and Smad4^{-/-} platelets on immobilized Fg for 2 hours. (D) Quantification of the area (pixel number) of three random fields in a $100\times$ objective field. Statistical analysis of the spreading size of platelets was performed using Tukey's test after ANOVA ($n = 3$; mean $\pm$ S.E.M., *** $P < 0.001$). (E) The clot retraction of PRP containing Smad4^{fl/fl}, Smad4^{+/-}, or Smad4^{-/-} platelets. (F) The clot retraction was measured using NIH ImageJ software, and the data were expressed as retraction ratios (mean $\pm$ S.E.M. from three separate experiments; ** $P < 0.01$, *** $P < 0.001$).

Smad4 deficiency significantly lowered the Fg binding to platelets induced by 2 μ g/ml CRP under nonstirring conditions. However, no obvious differences in Fg binding were found among the three genotypes when platelets were stimulated by either 0.1 U/ml thrombin or 40 μ M ADP plus 0.4 μ g/ml EP (Fig. 3B; Supplemental Fig. 2C). These results suggest that Smad4 plays a considerable role in the CRP-stimulated integrin $\alpha_{IIb}\beta_3$ -mediated inside-out signaling.

Smad4 Deficiency Affects Integrin $\alpha_{IIb}\beta_3$ -Mediated Outside-In Signaling. Platelet spreading on immobilized Fg is dependent on cytoskeletal reorganization driven by integrin $\alpha_{IIb}\beta_3$ -mediated outside-in signaling (Calderwood et al., 2002). To examine the role of Smad4 in $\alpha_{IIb}\beta_3$ -mediated outside-in signaling, the spreading of Smad4^{+/+}, Smad4^{+/-}, and Smad4^{-/-} platelets on immobilized Fg was assessed under the same conditions for 2 hours. Smad4^{-/-} platelets were barely able to spread on immobilized Fg and only formed filopodia, in contrast to Smad4^{+/+} platelets. Similarly, the spreading of Smad4^{+/-} platelets was also significantly affected, but Smad4^{+/-} platelets displayed more obvious filopodia extensions than did Smad4^{-/-} platelets (Fig. 3C). The statistical analysis showed that the average size of the platelets that spread on the Fg was 1169 ± 60.47 pixels for Smad4^{-/-} platelets compared with 2439 ± 110.3 pixels for Smad4^{+/+} platelets and 1350 ± 70.37 pixels for Smad4^{+/-} platelets ($P < 0.001$) (Fig. 3D). These findings demonstrated that Smad4 deficiency in platelets severely interferes with platelet spreading on immobilized Fg. Since platelet clot retraction is driven by integrin $\alpha_{IIb}\beta_3$ -mediated outside-in signaling, the effect of Smad4 deficiency on clot retraction was also assessed. The results presented in Fig. 3, E and F, demonstrated that the average ratio of clot retraction of PRP containing Smad4^{+/+} platelets was 0.75 ± 0.018 in 2 hours versus 0.52 ± 0.034 in PRP containing Smad4^{+/-} platelets ($P < 0.01$) and 0.35 ± 0.04 in PRP containing Smad4^{-/-} platelets ($P < 0.001$). Similar results were also observed at 4 and 12 hours (Supplemental Fig. 3, A and B). Therefore, Smad4 deficiency in platelets severely delayed clot retraction in PRP.

Smad4 Regulates Syk and ROCK Kinase Transcription.

Since Smad4 is an important transcription factor, its deficiency may affect the expression of genes in megakaryocytes. Since platelets do not have nuclei, all of their mRNA originates from megakaryocytes, we therefore evaluated the mRNA levels in platelets. Total mRNA was extracted from Smad4^{+/+} and Smad4^{-/-} platelets for microarray analysis. The results demonstrated that the mRNA levels of more than 1700 genes were upregulated or downregulated based on the cutoff of a 2-fold change [Y. Wang and J. Liu, manuscript in preparation (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43814>)]. Among these genes, we focused on those that are closely associated with platelet function, such as Syk, ROCK1, ROCK2, and integrin- β_1 (Fujita et al., 1997; Poole et al., 1997; Inoue et al., 2006). The mRNA levels of Syk and ROCK2 in Smad4^{-/-} platelets, as shown by RT-qPCR, were downregulated more than 4-fold compared with that of the Smad4^{+/+} controls. The levels of integrin β_1 and ROCK1 in Smad4^{-/-} platelets dropped to about half of the levels observed in the Smad4^{+/+} controls (Fig. 4A). Immunoblotting results further confirmed that the expression levels of Syk and ROCK2 dropped significantly in Smad4^{-/-} platelets. However, no obvious change was observed in the expression of integrin β_1 and ROCK1 in Smad4^{-/-} platelets (Fig. 4B). Therefore, these results suggested that the expression of Syk and ROCK2 genes are probably under the control of Smad4 in megakaryocytes.

Syk-Specific Inhibitor BAY61-3606 and the ROCK Inhibitor Y-27632 Both Suppress Platelet Aggregation Induced by Agonists.

The participation of Syk in platelet integrin-mediated outside-in signaling has been reported previously (Oberfell et al., 2002). ROCKs, as predominant downstream factors of Rho GTPases, generally regulate cytoskeletal reorganization and cell behaviors in a variety of cell lines (Riento and Ridley, 2003). We then assessed the roles of the kinases Syk and ROCK in Smad4-regulated platelet functions. The Syk-specific inhibitor BAY61-3606 (Yamamoto et al., 2003) and the ROCK inhibitor Y-27632 (Narumiya et al.,

Fig. 4. Smad4 deficiency downregulated the expression of Syk and ROCK2 in platelets. (A) Validation assays of integrin β_1 , Syk, ROCK1, and ROCK2 expression in Smad4^{-/-} platelets were performed using RT-qPCR. The data presented are from three separate experiments. The sequences of PCR primers are listed in Table 1. (B) The target proteins were detected in Smad4^{+/+} and Smad4^{-/-} platelets using Western blot analysis.

2000) were used for analyzing their effect on platelet aggregation. Smad4^{fl} platelets were preincubated with different concentrations of BAY61-3606 or 10 μ M Y-27632 prior to being stimulated with different agonists. The results showed that the Smad4^{fl} platelet aggregations induced by 0.05 U/ml thrombin or 20 μ M ADP were hardly affected by treatment with 2.5 μ M BAY61-3606. However, an obvious inhibition was observed when Smad4^{fl} platelets were treated with 5 μ M BAY61-3606, which was similar to the suppressive effect of 10 μ M Y-27632 treatment (Fig. 5A). In addition, a more significant inhibitory effect was achieved when the platelets were stimulated with 0.5 μ g/ml CRP after treatment with these inhibitors (Fig. 5A). The effect of BAY61-3606 and Y-27632 on the platelet aggregation induced by these agonists was consistent with that of Smad4 deficiency. The inhibitory effects of BAY61-3606 and Y-27632 were more obvious on CRP-induced platelet aggregation (Fig. 5A; Supplemental Fig. 4). These results suggested that the kinases Syk and ROCK are significantly involved in CRP-associated GPVI signal transduction in platelets.

Both BAY61-3606 and Y-27632 Inhibit Platelet Spreading on Immobilized Fg. Smad4^{fl} platelets were preincubated with either 5 μ M BAY61-3606 or 10 μ M Y-27632, and then the spreading of Smad4^{fl} platelets on immobilized Fg was assessed under the same conditions for 120 minutes. As shown in Fig. 5B, pretreatment of the platelets with 5 μ M BAY61-3606 or 10 μ M Y-27632 obviously decreased the spreading of Smad4^{fl} platelets. The statistical analysis showed that the average size of the platelets that spread on Fg was 1151 ± 73.2 pixels in response to 5 μ M BAY61-3606 treatment ($P < 0.001$) and 1705 ± 87.46 pixels in response to 10 μ M Y-27632 treatment ($P < 0.001$) compared with 2317 ± 101.1 pixels for Smad4^{fl} platelets (Fig. 5C). Thus, the two inhibitors had an effect similar to that of Smad4 deficiency. These results suggest that decreased Syk or ROCK expression in Smad4-deficient platelets could be responsible for defective platelet spreading.

Both BAY61-3606 and Y-27632 Inhibit Platelet Clot Retraction. The role of Syk and ROCK in platelet clot retraction was also examined. Smad4^{fl} platelets were pretreated with either 5 μ M BAY61-3606 or 10 μ M Y-27632, and the average ratios of clot retraction were analyzed after 120 minutes. The results indicated, similar to the effect of Smad4 deficiency, that both BAY61-3606 and Y-27632 significantly delayed platelet clot retraction in PRP (Fig. 5D). The average ratio of clot retraction of the PRP containing Smad4^{fl} platelets was 0.13 ± 0.014 in response to 5 μ M BAY61-3606 treatment and 0.38 ± 0.043 to 10 μ M Y-27632 treatment, whereas the average ratio was 0.82 ± 0.037 in the PRP containing Smad4^{fl} control platelets under the same conditions ($P < 0.001$) (Fig. 5E). The platelets treated with BAY61-3606 and Y-27632 displayed similar defects in platelet clot retraction to that of Smad4 deficiency. These results demonstrated that functional blockade of Syk or ROCK has the same suppressive effect on platelet clot retraction as that of Smad4 deficiency.

Smad4 Directly Regulates ROCK2 Transcription but Indirectly Regulates Syk. Having shown that the defective platelet function in Smad4^{-/-} platelets is due to the reduced transcription of Syk and ROCK2 genes in megakaryocytes, we speculated that the promoter of these genes may be under the control of Smad4. A dual-luciferase reporter assay was used to evaluate the mechanism of the Smad4-dependent

transcriptional regulation of Syk and ROCK genes. A Smad4 expression plasmid was transfected into HEK293T cells with plasmids containing the Syk, ROCK1, or ROCK2 promoters. The results showed that the relative luciferase levels increased more than five times compared with cells transfected with the pGL3-basic control vector, suggesting that each promoter is transcriptionally activated in this system. The relative luciferase activity of the ROCK2 promoter when cotransfected with the Smad4 expression plasmid was two times higher than that observed upon cotransfection with the pcDNA 3.1(+) vector (Fig. 6C). However, Smad4 had no such effect on the promoters of Syk and ROCK1. The mean relative luciferase activity of the Syk promoter-containing and ROCK1 promoter-containing plasmids exhibited no visible difference between cells cotransfected with the Smad4 expression plasmid or with the pcDNA 3.1(+) vector (Fig. 6, A and B). The results indicated that Smad4 regulates the promoter activation of ROCK2 in a direct manner but indirectly regulates Syk gene.

Discussion

It has been reported that TGF- β 1 plays an active role in platelet aggregation and the maintenance of integrin function (Hoying et al., 1999). Smad4 is a key molecule in the TGF- β family signaling pathway (Shi and Massague, 2003), and the effects of Smad4 on platelet function are unclear. Here, we observed that megakaryocyte/platelet-specific inactivation of Smad4 impaired the platelet aggregation induced by thrombin, ADP, and CRP as well as thrombus formation and hemostasis. The results from Fg binding (Fig. 3B), platelet aggregation (Fig. 2A), platelet spreading, and clot retraction studies (Fig. 3, C–F) revealed that lack of Smad4 results in defective platelet activation by affecting integrin $\alpha_{IIb}\beta_3$ -mediated bidirectional signaling. The data shown in Figs. 4 and 5 indicated that the platelet functions regulated by Smad4 are mediated through the transcriptional regulation of key platelets signaling proteins, Syk, and ROCK2 in megakaryocytes.

The quantity of peripheral platelets is a balance between platelet formation by megakaryocytes and platelet clearance. Previous studies have reported that TGF- β 1 negatively regulated murine megakaryocytopoiesis (Ishibashi et al., 1987; Jeanpierre et al., 2008) and that the numbers of peripheral blood platelets are elevated in TGF- β 1-deficient mice (Hoying et al., 1999). Recent studies showed that the hematopoietic (Vav-Cre) deficiency of Smad4 has no effect on platelet count (Pan et al., 2007) but that Smad4 is critical for the self-renewal of hematopoietic stem cells (Karlsson et al., 2007; Rörby et al., 2012). In this study, the platelet number was slightly reduced in megakaryocyte/platelet-specific Smad4-deficient mice. The results from a platelet turnover study suggested that instead of enhanced platelet clearance, it may be impaired megakaryocyte differentiation that led to thrombocytopenia (Fig. 1D). These findings suggested that Smad4 may be involved in regulating megakaryocyte differentiation, thus affecting the number of platelets. This effect of Smad4 may be achieved by transcriptional regulation of molecules in other signaling pathways. One example is ROCK2, a downstream effector of RhoA that was demonstrated to be regulated by Smad4 in our study (Fig. 6) and may also be involved in platelet formation by megakaryocytes (Pleines et al., 2012).

Fig. 5. The kinases Syk and ROCK were both involved in platelet function. (A) The inhibitory effects of 2.5 and 5 μ M BAY 61-3606 and 10 μ M Y-27632 on Smad4^{fl/fl} platelet aggregation in response to either 0.05 U/ml thrombin, 20 μ M ADP, or 0.5 μ g/ml CRP. (B) Spreading of Smad4^{fl/fl} platelets on immobilized Fg in the presence of 5 μ M BAY 61-3606 or 10 μ M Y-27632. (C) Quantification of the area (pixel number) of spreading platelets in three random fields. The statistical analysis was performed using Tukey's test after ANOVA ($n = 3$; mean $\pm$ S.E. M., *** $P < 0.001$). (D) The clot retraction of PRP containing Smad4^{fl/fl} in the presence of 5 μ M BAY61-3606 or 10 μ M Y-27632. Both inhibitors significantly delayed the platelet-mediated clot retraction in PRP. (E) NIH ImageJ software was used for the statistical analysis of clot retraction area and the data were expressed as retraction ratios. *** $P < 0.001$. BAY, BAY 61-3606.

TGF- β 1 had no direct effect on thrombin- or collagen-induced aggregation of Smad4^{fl/fl} and Smad4^{-/-} platelets (data not shown), indicating that Smad4 may not regulate platelet activation directly. As the most abundant platelet surface receptor, integrin $\alpha_{IIb}\beta_3$ -mediated bidirectional signaling is required for various aspects of platelet functions (Li et al., 2010). Some of the functional deficits in the Smad4-deficient

platelets could arise from defects in the expression of the integrin $\alpha_{IIb}\beta_3$. However, we failed to find any effect of Smad4 deficiency on the expression of either α_{IIb} or β_3 in platelets (Fig. 3A). On the other hand, Smad4 deficiency significantly lowered Fg binding to platelets after CRP-induced $\alpha_{IIb}\beta_3$ activation and inhibited platelet spreading on immobilized Fg as well as clot retraction in PRP (Fig. 3, B–F). These results revealed that the

Fig. 6. Smad4 directly regulates ROCK2 transcription but indirectly regulates Syk. Statistical analysis of the relative fold change of luciferase values when plasmids containing the Syk (A), ROCK1 (B), or ROCK2 (C) promoter were transfected into HEK293T cells with a Smad4 expression vector. N.s., not significant. *** $P < 0.001$. The primers for the promoter sequences of the mouse Syk, ROCK1, and ROCK2 genes, and Smad4 cDNA are listed in Table 2.

dysfunction of Smad4^{-/-} platelets arose from reduced expression of ROCK2 and Syk, which translates into defects in integrin $\alpha_{IIb}\beta_3$ -mediated bidirectional signaling.

In our experimental conditions, Y27632 affected platelet spreading on immobilized Fg (Fig. 5B). This result suggested that Rho/ROCK signaling is involved in integrin $\alpha_{IIb}\beta_3$ -dependent platelet spreading. Although, the reports about the role of RhoA in platelet spreading on immobilized Fg are debatable. Pleines et al. (2012) reported that RhoA gene deletion did not negatively affect platelet spreading, indicating that RhoA is not required for platelet spreading. Gong et al. (2010) reported that $G_{\alpha_{13}}$ -mediated integrin $\alpha_{IIb}\beta_3$ -dependent inhibition of RhoA is important in stimulating platelet spreading. Some groups reported (Leng et al., 1998; Akbar et al., 2016) that pharmacological inhibition or genetic ablation of RhoA inhibits platelet spreading on immobilized Fg. The reasons for these opposing results about the role of RhoA in platelet spreading is not very clearly understood at this time. It is known that RhoA inhibitors dramatically inhibited platelet granule secretion (Suzuki et al., 1999; Pandey et al., 2006; Getz et al., 2010). We thus cannot exclude the possibility that the effect of Rho kinase inhibitors on platelet spreading may be secondary to their effect on platelet granule secretion. The role of the RhoA pathway in platelet granule secretion may be responsible for the discrepancies observed in these studies.

Our molecular mechanism studies indicated that the Smad4-dependent regulation of platelet functions may be mediated by the downstream effectors Syk and ROCK2 (Figs. 4–6). Syk is a nonreceptor tyrosine kinase that is activated by proteins containing immunoreceptor tyrosine-based activation motifs (Mócsai et al., 2010). It has been reported that GPVI-mediated platelet function relies on Syk (Poole et al., 1997). In this study, Smad4-deficient platelets had severe defects in response to CRP-induced platelet activation, suggesting that Smad4 dependent Syk expression may predominantly dictate GPVI-related downstream signal transduction. Consistent with our findings, previous data from chimeric Syk-deficient mice demonstrated that Syk is essential for collagen-induced platelet activation (Poole et al., 1997) and integrin $\alpha_{IIb}\beta_3$ -mediated signal transduction in platelets (Law et al., 1999; Obergfell

et al., 2002). However, chimeric Syk-deficient mice have a normal bleeding time (Poole et al., 1997). This is different from megakaryocyte/platelet-specific Smad4-deficient mice, which showed an increasing bleeding time (Pleines et al., 2012). The prolonged bleeding time may be the consequence of decreased expression of ROCK2 or other undetermined molecules in Smad4-deficient platelets. Moreover, RhoA deficiency in megakaryocytes/platelets also caused defective platelet activation and prolonged bleeding time. In this study, Smad4 could directly regulate the transcription of ROCK2 (Fig. 6) in megakaryocytes, suggesting that the defects in the hemostasis of Smad4-deficient platelets may be mediated through the Rho/ROCK signaling pathway.

Smad4 mutation in human beings is involved in the third subtype of hereditary hemorrhagic telangiectasia (HHT), which is caused by mutations in a number of genes of the TGF- β signaling pathway (Sharathkumar and Shapiro, 2008). HHT is an autosomal-dominant disorder (Fuchizaki et al., 2003). It has been reported that HHT develops in patients with platelet dysfunction (Endo et al., 1984). In our study, the mice with heterozygous Smad4 deficiency displayed a series of impaired platelet functions, such as prolonged artery thrombus formation time and bleeding time, suggesting that Smad4 may contribute to bleeding disorders in patients with HHT.

In summary, Smad4 deficiency in megakaryocytes/platelets leads to mild thrombocytopenia. The functional defects in Smad4-deficient platelets are due to impaired integrin $\alpha_{IIb}\beta_3$ -mediated bidirectional signaling. The effect of Smad4 was at least partially mediated by the downstream effectors Syk and ROCK2 transcription in megakaryocytes, suggesting that GPVI-mediated signaling and the Rho GTPase/ROCK signaling pathways are involved. These findings identified some important aspects of the TGF- β /Smad4 signaling pathway in megakaryocytes that affect platelet activation, thrombus formation, and hemostatic process, but further work is required for a comprehensive understanding of the role of Smad4 in this process. The data in this study also implied that in addition to regulating platelet quantity, the TGF- β signaling pathway in megakaryocytes plays important roles in normal platelet formation and function.

Acknowledgments

The authors thank Li Zhu (Soochow University) for providing the CRP and Professor Xiao Yang (Institute of Biotechnology, Beijing) for providing mice carrying the Smad4 gene flanked by loxP recognition sites (Smad4^{fl}). The authors also thank Professor Ulhas P. Naik for assistance in polishing the final manuscript.

Authorship Contributions

Participated in research design: J. Liu and Wu.

Conducted experiments: Wang, Jiang, Mo, Lan, X. Liu, and Wu.

Performed data analysis: Yang, Zhang, Zhu, J. Liu, and Wu.

Wrote or contributed to the writing of the manuscript: Wang, J. Liu, and Wu.

References

- Akbar H, Duan X, Saleem S, Davis AK, and Zheng Y (2016) RhoA and Rac1 GTPases differentially regulate agonist-receptor mediated reactive oxygen species generation in platelets. *PLoS One* **11**:e0163227.
- Asselin J, Gibbins JM, Achison M, Lee YH, Morton LF, Farndale RW, Barnes MJ, and Watson SP (1997) A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase C $\gamma 2$ in platelets independent of the integrin $\alpha 2$ - $\beta 1$. *Blood* **89**:1235–1242.
- Calderwood DA, Yan B, de Pereda JM, Alvarez BG, Fujioka Y, Liddington RC, and Ginsberg MH (2002) The phosphotyrosine binding-like domain of talin activates integrins. *J Biol Chem* **277**:21749–21758.
- Chen X, Zhang Y, Wang Y, Li D, Zhang L, Wang K, Luo X, Yang Z, Wu Y, and Liu J (2013) PDK1 regulates platelet activation and arterial thrombosis. *Blood* **121**:3718–3726.
- Endo Y, Mamiya S, Niitsu H, Miura A, and Nishimura S (1984) Reduced platelet aggregation induced by epinephrine and idiopathic thrombocytopenic purpura in hereditary haemorrhagic telangiectasia. *Thromb Haemost* **52**:369.
- Flevaris P, Li Z, Zhang G, Zheng Y, Liu J, and Du X (2009) Two distinct roles of mitogen-activated protein kinases in platelets and a novel Rac1-MAPK-dependent integrin outside-in retractile signaling pathway. *Blood* **113**:893–901.
- Fuchizaki U, Miyamori H, Kitagawa S, Kaneko S, and Kobayashi K (2003) Hereditary haemorrhagic telangiectasia (Rendu-Osler-Weber disease). *Lancet* **362**:1490–1494.
- Fujita A, Saito Y, Ishizaki T, Maekawa M, Fujisawa K, Ushikubi F, and Narumiya S (1997) Integrin-dependent translocation of p160ROCK to cytoskeletal complex in thrombin-stimulated human platelets. *Biochem J* **328**:769–775.
- Gay LJ and Felding-Habermann B (2011) Contribution of platelets to tumour metastasis. *Nat Rev Cancer* **11**:123–134.
- George JN (2000) Platelets. *Lancet* **355**:1531–1539.
- Getz TM, Dangelmaier CA, Jin J, Daniel JL, and Kunapuli SP (2010) Differential phosphorylation of myosin light chain (Thr18 and (Ser)19 and functional implications in platelets. *J Thromb Haemost* **8**:2283–2293.
- Gong H, Shen B, Flevaris P, Chow C, Lam SC, Voyno-Yasenetskaya TA, Kozasa T, and Du X (2010) G protein subunit $\alpha 12$ binds to integrin $\alpha 11\beta$ and mediates integrin “outside-in” signaling. *Science* **327**:340–343.
- Hoying JB, Yin M, Diebold R, Ormsby I, Becker A, and Doetschman T (1999) Transforming growth factor $\beta 1$ enhances platelet aggregation through a non-transcriptional effect on the fibrinogen receptor. *J Biol Chem* **274**:31008–31013.
- Inoue O, Suzuki-Inoue K, McCarty OJ, Moroi M, Ruggeri ZM, Kunicki TJ, Ozaki Y, and Watson SP (2006) Laminin stimulates spreading of platelets through integrin $\alpha 6\beta 1$ -dependent activation of GPVI. *Blood* **107**:1405–1412.
- Ishibashi T, Miller SL, and Burstein SA (1987) Type beta transforming growth factor is a potent inhibitor of murine megakaryocytopoiesis in vitro. *Blood* **69**:1737–1741.
- Jeanpierre S, Nicolini FE, Kaniewski B, Dumontet C, Rimokh R, Puisieux A, and Maguer-Satta V (2008) BMP4 regulation of human megakaryocytic differentiation is involved in thrombopoietin signaling. *Blood* **112**:3154–3163.
- Karlsson G, Blank U, Moody JL, Ehinger M, Singbrant S, Deng CX, and Karlsson S (2007) Smad4 is critical for self-renewal of hematopoietic stem cells. *J Exp Med* **204**:467–474.
- Kehrel B, Wierwille S, Clemetson KJ, Anders O, Steiner M, Knight CG, Farndale RW, Okuma M, and Barnes MJ (1998) Glycoprotein VI is a major collagen receptor for platelet activation: it recognizes the platelet-activating quaternary structure of collagen, whereas CD36, glycoprotein IIb/IIIa, and von Willebrand factor do not. *Blood* **91**:491–499.
- Labelle M, Begum S, and Hynes RO (2011) Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. *Cancer Cell* **20**:576–590.
- Law DA, Nannizzi-Alaimo L, Ministri K, Hughes PE, Forsyth J, Turner M, Shattil SJ, Ginsberg MH, Tybulewicz VL, and Phillips DR (1999) Genetic and pharmacological analyses of Syk function in $\alpha 11\beta$ signaling in platelets. *Blood* **93**:2645–2652.
- Leng L, Kashiwagi H, Ren XD, and Shattil SJ (1998) RhoA and the function of platelet integrin $\alpha 11\beta$. *Blood* **91**:4206–4215.
- Li Z, Delaney MK, O'Brien KA, and Du X (2010) Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol* **30**:2341–2349.
- Liu J, Fitzgerald ME, Berndt MC, Jackson CW, and Gartner TK (2006) Bruton tyrosine kinase is essential for botrocetin/VWF-induced signaling and GPIIb-dependent thrombus formation in vivo. *Blood* **108**:2596–2603.
- Mahooti S, Graesser D, Patil S, Newman P, Duncan G, Mak T, and Madri JA (2000) PECAM-1 (CD31) expression modulates bleeding time in vivo. *Am J Pathol* **157**:75–81.
- Meyer A, Wang W, Qu J, Croft L, Degen JL, Coller BS, and Ahamed J (2012) Platelet TGF- $\beta 1$ contributions to plasma TGF- $\beta 1$, cardiac fibrosis, and systolic dysfunction in a mouse model of pressure overload. *Blood* **119**:1064–1074.
- Mócsai A, Ruland J, and Tybulewicz VL (2010) The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat Rev Immunol* **10**:387–402.
- Moser M, Nieswandt B, Ussar S, Pozgajova M, and Fässler R (2008) Kindlin-3 is essential for integrin activation and platelet aggregation. *Nat Med* **14**:325–330.
- Narumiya S, Ishizaki T, and Uehata M (2000) Use and properties of ROCK-specific inhibitor Y-27632. *Methods Enzymol* **325**:273–284.
- Niu H, Chen X, Gruppo RA, Li D, Wang Y, Zhang L, Wang K, Chai W, Sun Y, Ding Z, et al. (2012) Integrin $\alpha 11\beta$ -mediated PI3K/Akt activation in platelets. *PLoS One* **7**:e47356.
- Obergfell A, Eto K, Mócsai A, Buensuceso C, Moores SL, Brugge JS, Lowell CA, and Shattil SJ (2002) Coordinate interactions of Csk, Src, and Syk kinases with $[\alpha 11\beta]$ initiate integrin signaling to the cytoskeleton. *J Cell Biol* **157**:265–275.
- Pan D, Schomber T, Kalberer CP, Terracciano LM, Hafen K, Krenger W, Hao-Shen H, Deng C, and Skoda RC (2007) Normal erythropoiesis but severe polyposis and bleeding anemia in Smad4-deficient mice. *Blood* **110**:3049–3055.
- Pandey D, Goyal P, Bamburg JR, and Siess W (2006) Regulation of LIM-kinase 1 and cofilin in thrombin-stimulated platelets. *Blood* **107**:575–583.
- Pleines I, Hagedorn I, Gupta S, May F, Chakarova L, van Hengel J, Offermanns S, Krohne G, Kleinschnitz C, Brakebusch C, et al. (2012) Megakaryocyte-specific RhoA deficiency causes macrothrombocytopenia and defective platelet activation in hemostasis and thrombosis. *Blood* **119**:1054–1063.
- Polgar J, Matuskova J, and Wagner DD (2005) The P-selectin, tissue factor, coagulation triad. *J Thromb Haemost* **3**:1590–1596.
- Poole A, Gibbins JM, Turner M, van Vugt MJ, van de Winkel JG, Saito T, Tybulewicz VL, and Watson SP (1997) The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J* **16**:2333–2341.
- Prislovsky A, Marathe B, Hosni A, Bolen AL, Nimmerjahn F, Jackson CW, Weiman D, and Strom TS (2008) Rapid platelet turnover in WASP(-) mice correlates with increased ex vivo phagocytosis of opsonized WASP(-) platelets. *Exp Hematol* **36**:609–623.
- Redondo S, Navarro-Dorado J, Ramajo M, Medina U, and Tejerina T (2012) The complex regulation of TGF- β in cardiovascular disease. *Vasc Health Risk Manag* **8**:533–539.
- Riento K and Ridley AJ (2003) Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol* **4**:446–456.
- Rörby E, Hägerström MN, Blank U, Karlsson G, and Karlsson S (2012) Human hematopoietic stem/progenitor cells overexpressing Smad4 exhibit impaired reconstitution potential in vivo. *Blood* **120**:4343–4351.
- Semple JW, Italiano, Jr JE, and Freedman J (2011) Platelets and the immune continuum. *Nat Rev Immunol* **11**:264–274.
- Sharathkumar AA and Shapiro A (2008) Hereditary haemorrhagic telangiectasia. *Haemophilia* **14**:1269–1280.
- Shi Y and Massagué J (2003) Mechanisms of TGF- β signaling from cell membrane to the nucleus. *Cell* **113**:685–700.
- Sirard C, de la Pompa JL, Elia A, Itie A, Mirtsos C, Cheung A, Hahn S, Wakeham A, Schwartz L, Kern SE, et al. (1998) The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev* **12**:107–119.
- Suzuki Y, Yamamoto M, Wada H, Ito M, Nakano T, Sasaki Y, Narumiya S, Shiku H, and Nishikawa M (1999) Agonist-induced regulation of myosin phosphatase activity in human platelets through activation of Rho-kinase. *Blood* **93**:3408–3417.
- Tiedt R, Schomber T, Hao-Shen H, and Skoda RC (2007) Pf4-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo. *Blood* **109**:1503–1506.
- Weng Z, Li D, Zhang L, Chen J, Ruan C, Chen G, Gartner TK, and Liu J (2010) PTEN regulates collagen-induced platelet activation. *Blood* **116**:2579–2581.
- Yamamoto N, Takeshita K, Shichijo M, Kokubo T, Sato M, Nakashima K, Ishimori M, Nagai H, Li YF, Yura T, et al. (2003) The orally available spleen tyrosine kinase inhibitor 2-[7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]nicotinamide dihydrochloride (BAY 61-3606) blocks antigen-induced airway inflammation in rodents. *J Pharmacol Exp Ther* **306**:1174–1181.
- Yang X, Li C, Herrera PL, and Deng CX (2002) Generation of Smad4/Dpc4 conditional knockout mice. *Genesis* **32**:80–81.
- Yang X, Li C, Xu X, and Deng C (1998) The tumor suppressor SMAD4/DPC4 is essential for epiblast proliferation and mesoderm induction in mice. *Proc Natl Acad Sci USA* **95**:3667–3672.

Address correspondence to: Xiaolin Wu, The Eighth People's hospital of Shanghai, Shanghai 200235, People's Republic of China. E-mail: wuxiaolin999@hotmail.com or Junling Liu, Department of Biochemistry and Molecular Cell Biology, Shanghai Key Laboratory of Tumor Microenvironment and Inflammation, Institute of Medical Science, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, People's Republic of China. E-mail: liujl@shsmu.edu.cn