Roles of focal adhesion kinase (FAK) in megakaryopoiesis and platelet function: studies using a megakaryocyte lineage–specific FAK knockout

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Focal adhesion kinase (FAK) plays a key role in mediating signaling downstream of integrins and growth factor receptors. In this study, we determined the roles of FAK in vivo by generating a megakaryocyte lineage–specific FAK-null mouse (Pf4-Cre/FAK-floxed). Megakaryocyte and platelet FAK expression was ablated in Pf4-Cre/FAK-floxed mice without affecting expression of the FAK homologue PYK2, although PYK2 phosphorylation was increased in FAK−/− megakaryocytes in response to fibrinogen. Megakaryopoiesis is greatly enhanced in Pf4-Cre/FAK-floxed mice, with significant increases in megakaryocytic progenitors (CFU-MK), mature megakaryocytes, megakaryocyte ploidy, and moderate increases in resting platelet number and platelet recovery following a thrombocytopenic stress. Thrombopoietin (Tpo)–mediated activation of Lyn kinase, a negative regulator of megakaryopoiesis, is severely attenuated in FAK-null megakaryocytes compared with wild-type controls. In contrast, Tpo-mediated activation of positive megakaryopoiesis regulators such as ERK1/2 and AKT is increased in FAK-null megakaryocytes, providing a plausible explanation for the observed increases in megakaryopoiesis in these mice. In Pf4-Cre/FAK-floxed mice, rebleeding times are significantly increased, and FAK-null platelets exhibit diminished spreading on immobilized fibrinogen. These studies establish clear roles for FAK in megakaryocyte growth and platelet function, setting the stage for manipulation of this component of the Tpo signaling apparatus for therapeutic benefit. (Blood. 2008;111:596-604)

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Introduction

Focal adhesion kinase (FAK) is an essential nonreceptor protein tyrosine kinase that is expressed ubiquitously and is conserved in mammals and lower eukaryotic organisms.1-4 The principal FAK stimulus is integrin engagement (Guan and Shalloway5 and reviewed in Parsons6), although its direct interaction with, and activation via, platelet-derived and epidermal growth factor receptors suggests that it may also function downstream of other membrane-bound growth factor and cytokine receptors.7 Autophosphorylation of FAK Tyr397 occurs rapidly following integrin activation, and the resulting phosphorylated residue acts as a docking site for the SH2 domains of Src-family kinases.5,9 FAK catalytic activity is increased by subsequent Src-mediated phosphorylation of FAK residues Tyr576 and Tyr577,10 followed by Tyr861 and Tyr925, which act as binding sites for the SH3 domain of p130CAS and the SH2 domain of the adaptor protein GRB2, respectively.11,12 Tyr925, located in the focal adhesion targeting domain, also mediates interactions with integrin-associated proteins such as talin and paxillin,13 thereby recruiting FAK to focal adhesion sites.

Activated FAK modulates the activity of a broad range of downstream signaling proteins, including phosphoinositide 3-kinase (PI3-K)14 and phospholipase C (PLC)−γ15 as well as a number of small GTPases such as Ras, Rac, and Rho (reviewed in16). Extensive studies indicate that FAK is essential for normal cell migration. FAK-deficient cells migrate poorly in response to chemokines; form an increased number of prominent “immature” focal adhesions, apparently due to decreased focal adhesion turnover; and do not spread normally on extracellular matrices (ECMs).17,18

Megakaryopoiesis and platelet production are tightly regulated by a number of growth factors and cytokines to maintain a normal number of circulating platelets. The principal regulator of megakaryopoiesis is thrombopoietin (Tpo),21 although other factors such as interleukin-3 and stem-cell factor work in synergy with Tpo during the earlier stages of megakaryocytic progenitor cell expansion.22 It has become apparent that the microenvironments in which megakaryocytes function are also of critical importance to megakaryopoiesis.23-25 Indeed, direct cell-cell and cell-ECM interactions have been demonstrated to influence megakaryocyte differentiation and proplatelet formation.23-25 Although FAK is important in regulating cell spreading and migration in response to integrin-ECM interactions, the role of FAK in megakaryopoiesis remains unclear, partly because Fak deletion in mice is lethal at embryonic day 8.5, before the onset of significant definitive hematopoiesis. Although the role of proline-rich tyrosine kinase-2 (PYK2), which shares sequence homology and similar characteristics with FAK, has been characterized in megakaryocytes,8,26-28 its localization and dependence on intracellular calcium make the 2 proteins functionally different.29

FAK activation in platelets requires the costimulation of integrins and agonist receptors. Platelet adhesion to fibrinogen and collagen via integrins αIIbβ3 and α2β1, respectively, leads to rapid FAK phosphorylation on tyrosine residues in a manner dependent on a platelet agonist, such as adenosine diphosphate (ADP), and on


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596 BLOOD, 15 JANUARY 2008 • VOLUME 111, NUMBER 2
activation of protein kinase C.

FAK phosphorylation in response to costimulation with either fibrinogen or collagen and ADP accompanies changes in platelet spreading.

Using nonplatelet models, it has been inferred that FAK may mediate platelet spreading via phosphorylation of α-actinin, which colocalizes with a number of cytoskeletal proteins, including actin, vinculin, and zyxin, and is essential for the organization of the actin cytoskeleton.

However, any required role for FAK in platelet function remains unknown. To more fully evaluate the role of FAK in megakaryopoiesis and platelet function in vivo and in vitro, we have here successfully ablated FAK expression specifically in megakaryocytes and platelets by crossing conditional Fak-floxed mice with a recently described megakaryocyte lineage–specific platelet factor 4 (Pf4)-Cre mouse.

FAK ablation leads to a dramatic increase in the number of megakaryocyte progenitor cells and mature bone marrow megakaryocytes. In addition, megakaryocyte maturation was greatly enhanced, probably as a result of a dramatic increase in the number of megakaryocyte progenitor cells and mature bone marrow megakaryocytes. In addition, megakaryocyte maturation was greatly enhanced, probably as a result of altered Tpo signaling. Furthermore, Fak−/− mice exhibit increased bleeding times, and their platelets spread poorly on fibrinogen, establishing FAK as a requisite player in megakaryocyte and platelet biology.

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**Methods**

**Materials**

Tissue culture plastics were purchased from Corning (New York, NY) and standard tissue culture media from Invitrogen (Carlsbad, CA). Fibrinogen was purchased from Enzyme Research Laboratories (South Bend, IN). Human plasma fibronectin, mouse monoclonal anti-FAK, rat anti–mouse monoclonal anti-αIIb (MWReg30), and rat anti–mouse monoclonal CD41-FITC antibodies were purchased from BD Biosciences (La Jolla, CA). Rabbit anti-phosphoERK1/2, rabbit anti-phosphoAKT, rabbit anti-phosphoPYK2, rabbit anti-phosphoFAK, rabbit anti-PYK2, and rabbit anti–FAK antibodies were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti–mouse von Willebrand factor (VWF) antibody was purchased from Abcam (Cambridge, MA). Goat anti–rabbit horseradish peroxidase (HRP) and rabbit anti–mouse HRP were from Santa Cruz Biotechnology (Santa Cruz, CA). ECL-plus chemiluminescent reagent was purchased from GE Lifesciences (Piscataway, NJ). Goat anti–mouse FAK antibodies were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti–mouse von Willebrand factor (VWF) antibody was purchased from Abcam (Cambridge, MA). Goat anti–rabbit horseradish peroxidase (HRP) and rabbit anti–mouse HRP were from Santa Cruz Biotechnology (Santa Cruz, CA). ECL-plus chemiluminescent reagent was purchased from GE Lifesciences (Piscataway, NJ). Recombinant human Tpo was a gift from Don Foster, Zymogenetics (Seattle, WA).

**Animals**

Wild-type (WT), Pf4-Cre, and Fak-floxed mice were all previously bred and were previously bred onto a C57Bl/6 genetic background and were generated as previously described.36,37 Mice were routinely genotyped from tail DNA by polymerase chain reaction (PCR) using primers specific for the Pf4 promoter (CCCCATACGACACCTTGGT) and Cre-cDNA (TGGCACGTCA-CCAGGT), which amplified a 450–base pair (bp) product. Mice also were condition Fak-floxed (forward: GAAGTGACTAGGAAACCAATA and reverse: GAGAATCCAGGTTGGCGTGT), which amplified a 290-bp WT product and 400-bp Fak-floxed product. Mice were housed in a pathogen-free environment and the Animal Care Program of the University of California San Diego approved all protocols. For all experiments, 6- to 12-week-old mice were used.

**Preparation of megakaryocytes and platelets**

Megakaryocytes were expanded and separated as previously described.38 Briefly, flushed bone marrow was cultured in serum-free medium containing 3% Tpo supernatant (previously prepared and quantified from a mouse Tpo-secreting baby hamster kidney [BHK] cell line) for 72 hours. Mature megakaryocytes were isolated by passing the culture through a discontinuous bovine serum albumin (BSA) density gradient. For hematopoietic colony assays, fresh bone marrow was cultured according to the manufactur-
12 hours. DNA was stained by adding 10 μg/mL propidium iodide (Sigma) and incubating for 1 hour. Analyses were performed using a FACsCaliber flow cytometer (BD Biosciences). DNA plots were taken from large CD41 high-expressing cells only. On average, large CD41 high-expressing cells represented approximately 0.5% of the total bone marrow population.

Cell-spreading assays
Sterile coverslips were coated with 100 μg/mL fibrinogen in phosphate-buffered saline (PBS; pH 8.0) overnight and blocked for 2 hours in PBS containing 1% BSA. Platelets were resuspended in Tyrode buffer (150 mM NaCl, 2.5 mM KCl, 12 mM NaHCO3, 2 mM MgCl2, 2 mM CaCl2, 1 mg/mL BSA, 1 mg/mL dextrose, pH 7.4) at 107 platelets/mL. Cells were incubated on fibrinogen for 1 hour at 37°C in the presence or absence of specific agonists. Nonadhered cells were removed by washing with PBS before adhered cells were fixed with 4% PFA for 10 minutes at room temperature and permeabilized with PBS, 0.1% Triton X-100. Following antibody staining, platelet samples were mounted in Citifluor mounting medium (Ted Pella, Redding, CA) and visualized using a Nikon Eclipse E800 microscope (Nikon, Melville, NY) with platelet surface area measured in pixels. An overall average of 800 platelets per data point were enumerated from 6 mice of each genotype.

For cytofospin preparations, megakaryocytes were resuspended at 104 cells per milliliter and cytospun onto a Superfrost/Plus microscope slide (Fisher Scientific, Pittsburgh, PA) using a Cytospin 4 cytocentrifuge (Fisher Scientific, Pittsburgh, PA) with platelet surface area measured in pixels. An overall average of 800 platelets per data point were enumerated from 6 mice of each genotype.

For cytofospin preparations, megakaryocytes were resuspended at 104 cells per milliliter and cytospun onto a Superfrost/Plus microscope slide (Fisher Scientific, Pittsburgh, PA) using a Cytospin 4 cytocentrifuge (Fisher Scientific, Pittsburgh, PA). Platelet fibrinogen–binding assays and clot-retraction assays were performed as previously described.40

Immunofluorescence microscopy
Platelets were allowed to adhere to fibrinogen-coated coverslips as described in “Cell-spreading assays.” Nonspecific antibody binding was blocked using 10% rabbit or goat serum in PBS before primary antibody incubations. Primary antibodies were used at 1:400 dilutions (vinculin and β3 integrin) in 10% serum PBS and incubated for 45 minutes at 37°C. Secondary antibodies (goat polyclonal anti-β3 integrin-Alexa-Fluor488 and mouse monoclonal anti-vinculin Alexa-Fluor594 antibodies; Invitrogen) were incubated at 1:400 dilutions for 30 minutes at room temperature. Following antibody staining, platelet samples were mounted in Citifluor mounting medium and visualized using a Nikon Eclipse E800 microscope with a 60×/1.4 oil objective (0.17 WD 0.21). Images were captured using an Applied Photometrics Cool Snap HQ camera (Photometrics, Tucson, AZ) and QED In Vivo version 2.0 imaging software (Media Cybernetics, Silver Spring, MD). Images were processed using Photoshop 5.5 software (Adobe, San Jose, CA). Quantification of spreading was performed blindly using ImageJ software (http://rsb.info.nih.gov/ij/) with platelet surface area measured in pixels. An overall average of 800 platelets per data point were enumerated from 6 mice of each genotype.

Immunohistochemistry
Femurs from P44-Cre/FAK-floxed and age-matched control mice were fixed in 4% PFA for 24 hours and decalcified in 5% EDTA for 48 hours. Paraffin sections (5-μm thickness) were deparaffinized and antigen retrieval was performed in EDTA buffer (pH8) at 95°C for 30 minutes. All solutions were made in Tris-buffered saline (TBS) plus or minus 0.1% Tween-20 (TBS-T) and slides washed in 3 changes of TBS-T for 3 minutes each time, unless otherwise stated. Endogenous peroxidase activity was inhibited with 0.6% H2O2, and nonspecific binding was blocked using 10% goat serum. Sections were incubated with rabbit anti-VWF primary antibody (1:300 dilution) for 2 hours at 37°C followed by a goat anti-rabbit biotinylated secondary antibody (1:300 dilution; Vector Laboratories) for 30 minutes at room temperature. Positive staining was detected using streptavidin reagent (avidin-biotin complex [ABC]; Vector Laboratories) and diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories) before samples were counterstained using Harris hematoxylin (Fisher Scientific) mounted in Clarion mounting medium (Sigma) and visualized using a Leica DMLS upright microscope with a 40× N-plan ×0.17/D objective. Images were captured using a Diapadic SPOT camera and SPOT-advanced software and imported into Corel Photo-Paint 11. For quantitative analysis of positively stained megakaryocytes in the bone marrow, 25 images (×200 magnification) were taken of either P44-Cre/FAK-floxed or WT marrow. Megakaryocyte enumeration was performed blindly.

Results
Specific ablation of Fak expression from the megakaryocyte lineage in P44-Cre/FAK-floxed mice
To determine the roles of FAK in megakaryocytes and platelets, we generated a megakaryocyte lineage–specific FAK knockout mouse. FAK protein expression, determined by Western blot, was completely ablated in mature bone marrow–derived megakaryocytes and in washed platelets from P44-Cre/FAK-floxed mice, in contrast to WT controls (Figure 1A). Expression levels of the FAK homologue PYK2 in megakaryocytes and in platelets from P44-Cre/ FAK-floxed mice were comparable to those in WT controls. Immunofluorescence microscopy on cytofospin preparations confirmed the absence of FAK expression in mature megakaryocytes derived from P44-Cre/FAK-floxed bone marrow (Figure 1B). Although expression levels of PYK2 were comparable, we tested whether PYK2 activation in megakaryocytes and in platelets derived from P44-Cre/FAK-floxed was different from that of WT mice. Megakaryocytes were exposed to either BSA or fibrinogen-coated tissue culture plastics and stimulated with 1 mM MnCl2 for 30 minutes. All solutions were made in Tris-buffered saline (TBS) plus or minus 0.1% Tween-20 (TBS-T) and slides washed in 3 changes of TBS-T for 3 minutes each time, unless otherwise stated. Endogenous peroxidase activity was inhibited with 0.6% H2O2, and nonspecific binding was blocked using 10% goat serum. Sections were incubated with rabbit anti-VWF primary antibody (1:300 dilution) for 2 hours at 37°C followed by a goat anti-rabbit biotinylated secondary antibody (1:300 dilution; Vector Laboratories) for 30 minutes at room temperature. Positive staining was detected using streptavidin reagent (avidin-biotin complex [ABC]; Vector Laboratories) and diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories) before samples were counterstained using Harris hematoxylin (Fisher Scientific) mounted in Clarion mounting medium (Sigma) and visualized using a Leica DMLS upright microscope with a 40× N-plan ×0.17/D objective. Images were captured using a Diapadic SPOT camera and SPOT-advanced software and imported into Corel Photo-Paint 11. For quantitative analysis of positively stained megakaryocytes in the bone marrow, 25 images (×200 magnification) were taken of either P44-Cre/FAK-floxed or WT marrow. Megakaryocyte enumeration was performed blindly.

Tail rebleeding
Tail bleeding assays were performed as previously described.41 Briefly, the distal 3 mm of the tail tip from age- and sex-matched P44-Cre/FAK-floxed and WT mice were dissected with a sharp scalpel. After cutting, the mice were placed back in cages and clotting was allowed to progress for approximately 10 minutes. Tails were then immersed in a saline solution kept at 37°C to restart bleeding. Rebleeding time was measured as the time taken for the stream of blood from the tail tip to stop. If rebleeding time for any mouse had exceeded 120 seconds, that mouse would have been removed from the experiment.

Increased megakaryocyte progenitor number in P44-Cre/FAK-floxed mice
Complete blood counts demonstrated no differences in measured blood lineages, apart from a moderate but significant increase in platelet numbers in P44-Cre/FAK-floxed mice (Table 1;
Based on the increase in platelets, several assays were performed to determine whether the absence of FAK has a direct effect on megakaryopoiesis or thrombopoiesis. The number of megakaryocyte colony-forming units (CFU-MK) in Pf4-Cre/FAK-floxed mice was determined using standard semisolid collagen-based colony assays with increasing concentrations of Tpo to support megakaryocyte lineage growth. CFU-MK number was increased nearly 4-fold in Pf4-Cre/FAK-floxed mice compared with WT at 0.5 ng/mL Tpo (Figure 2A; 2.67 ± 0.22 colonies per 2 × 10⁴ bone marrow cells in WT mice vs 10.67 ± 1.04 colonies per 2 × 10⁴ bone marrow cells in Pf4-Cre/FAK-floxed mice). At higher Tpo concentrations (5 and 50 ng/mL), CFU-MK number was approximately 2-fold greater in Pf4-Cre/FAK-floxed mice than in WT mice. Fifty ng/mL of Tpo resulted in growth of WT CFU-MK colonies, and no colonies were observed in the absence of Tpo (data not shown). In contrast to the results for CFU-MK, the numbers of other hematopoietic colonies (CFU-G/M, BFU-E and CFU-E) were comparable in WT and Pf4-Cre/FAK-floxed mice (Figure 2B).

Increased mature megakaryocyte number and ploidy in Pf4-Cre/FAK-floxed mice

Next we determined whether the increase in CFU-MK number in Pf4-Cre/FAK-floxed mice leads to an increase in the number of mature bone marrow megakaryocytes. Sections of tibiae and
femora from WT and P4f-Cre/FAK-floxed mice were counterstained with hematoxylin and eosin and evaluated by light microscopy. The number of morphologically recognizable megakaryocytes was significantly increased in P4f-Cre/FAK-floxed mice compared with WT (Figure 3A). To quantify megakaryocyte number, we counted the VWF-positive megakaryocytes per field of view (FOV; 200X magnification) in 3 mice from each group. The number of megakaryocytes was significantly increased in P4f-Cre/FAK-floxed mice compared with WT (15.2 ± 0.83 per FOV in WT vs 28.9 ± 1.1 per FOV in P4f-Cre/FAK-floxed; Figure 3B). In addition to megakaryocyte number, we also determined the effect of FAK ablation on megakaryocyte ploidy in vivo. We found a marked shift to higher ploidy in P4f-Cre/FAK-floxed megakaryocytes compared with WT (Figure 3C). By quantifying the number of CD41+ megakaryocytes in each ploidy group, we determined that the geometric mean increased from 63.37 in WT to 10.03 in P4f-Cre/FAK-floxed, and the percentage of megakaryocytes greater than 4N increased from 33% in WT to 52% in P4f-Cre/FAK-floxed mice (Figure 3D). These results suggest that enhanced megakaryopoiesis might account for the increased platelet counts observed in P4f-Cre/FAK-floxed mice.

To determine whether the enhanced megakaryopoiesis might affect platelet recovery after induction of thrombocytopenia in P4f-Cre/FAK-floxed mice, we generated thrombocytopenia using an antiplatelet CD41 antibody (Figure 4A). No difference was observed in the time for recovery to physiologic platelet counts (90 hours), but the extent of rebound thrombocytosis after recovery was significantly increased in the P4f-Cre/FAK-floxed mice (170 hours; 1254 ± 10^3/µL (± 36) in WT, compared with 1570 ± 10^3/µL (± 63) in Fak−/− mice). Considering the greater megakaryocyte and platelet numbers in P4f-Cre/FAK-floxed mice, we determined levels of Tpo in the plasma by ELISA. However, Tpo levels were not significantly different from those in WT mice (Figure 4B).

### Differences in Tpo-mediated activation of Lyn kinase, ERK, and AKT in Fak−/− megakaryocytes

Having demonstrated the involvement of FAK in megakaryocyte maturation, to elucidate the molecular mechanism responsible, we determined the effects of Fak ablation on signaling pathways previously found to be important in megakaryocyte differentiation. Mature bone marrow–derived megakaryocytes were Tpo starved for 16 hours before Tpo stimulation (50 ng/mL) for 5 minutes. In WT megakaryocytes, Tpo stimulation resulted in a 10-fold increase in Lyn kinase activity. However, Tpo did not stimulate Lyn kinase activity in Fak−/− megakaryocytes (Figure 5A). Western blot analyses clearly demonstrated a significant enhancement in the Tpo-mediated phosphorylation of both ERK1/2 and AKT in Fak−/− megakaryocytes compared with WT (Figure 5B,C). There was no difference in Tpo-mediated phosphorylation of JAK2, STAT3, or STAT5 (data not shown). These findings strongly suggest that FAK has a role in the negative regulation of Tpo-mediated signaling events important for megakaryocyte maturation downstream of JAK2 activation. Studies using the Tpo-dependent hematopoietic progenitor cell line UT7/Tpo indicate that Tpo is able to rapidly phosphorylate FAK in a dose-dependent manner in the absence of integrin engagement, suggesting a potential mechanism to alter the Tpo response (Figure 5D).
Platelet adhesion and aggregation are the 2 main mechanisms through which blood platelets ensure the preservation of cardiovascular-system integrity in the event of an injury. To demonstrate a role for FAK in the signaling events involved in the activation of platelets and general thrombus stability, the hemostatic abilities of WT and Pf4-Cre/FAK-floxed mice were tested in a tail rebleeding assay. Rebleeding times for Pf4-Cre/FAK-floxed mice were almost double those for WT (19 seconds for the WT vs 37.8.3 seconds for the Pf4-Cre/FAK-floxed mice; Figure 6A). Because FAK is known primarily for its involvement in cell adhesion and migration, we then investigated the ability of Fak-/- platelets to spread on immobilized fibrinogen. WT and Fak-/- platelets were incubated on fibrinogen-coated slides in the presence or absence of high concentrations of either ADP, collagen-related peptide (CRP), or the PAR4 agonist peptide (Figure 6B). Fak-/- platelets displayed significantly impaired spreading compared with WT platelets, both in the absence of added agonist and in the presence of ADP, collagen-related peptide (CRP), or PAR4 receptor–activating peptide. To determine whether impaired spreading in Fak-/- platelets was due to a loss of vinculin-rich focal adhesion structures, 42 fibrinogen-bound platelets were stained for vinculin (Figure 6C) and analyzed by fluorescence microscopy. While Fak-/- platelets clearly displayed reduced spreading, their ability to form vinculin patches was unaltered, suggesting that the absence of FAK does not affect the formation of these particular adhesion structures. Fibrinogen-binding and clot-retraction assays were also performed on WT and Fak-/- platelets, and no significant difference was observed (data not shown).

Discussion

FAK regulates the activity of a number of diverse signaling molecules, including Rho- and Arf-family GTPases, PI3-K, ERK, and PLC-γ, both independently and through the formation of signaling crosstalk networks with growth factor receptors.14,15,43-45 Although we have a good understanding of the roles and signaling properties of FAK in vitro, our knowledge of its role in vivo is restricted by the fact that Fak knockout is lethal to mice at embryonic day 8.5.46 To overcome this problem, lineage-conditional Fak-null mice are used to study the roles of FAK in vivo.36 In this study we have explored the roles of FAK in megakaryocyte maturation and platelet function.

Figure 4. Platelet recovery following immune-induced thrombocytopenia in Pf4-Cre/FAK-floxed mice. (A) The data represent blood platelet levels measured at various time points following CD41 antibody administration. *P < .05. **P < .01. The numbers represent the mean platelet counts (± SEM) taken from 3 mice for each time point in each group. The data are representative of 2 independent experiments. (B) Levels of Tpo in plasma as determined by ELISA. Data represent mean (± SEM) from 5 mice in each group.

Figure 5. Increased Tpo-mediated signaling in FAK-/- megakaryocytes. (A) Lyn kinase assay of megakaryocytes derived from WT and Pf4-Cre/FAK-floxed mice in response to Tpo. Data represent autoradiography (top) and scintillation counts (bottom) of samples in triplicate. Data are representative of the mean (± SEM) of 2 independent experiments. (B,C) Western blot analyses of Tpo-mediated phosphorylation of ERK1/2 and AKT in WT and Fak-/- megakaryocytes. (D) Tpo-stimulated phosphorylation of FAK (Tyr925) in UT7/Tpo cells. The blotting results are representative of 3 independent experiments.
Our measurements of megakaryocyte progenitors, megakaryocyte number in the bone marrow, and platelet counts indicate that FAK plays a significant role as a negative regulator of megakaryopoiesis. Controlling megakaryocyte progenitor proliferation and megakaryocyte differentiation requires an intricate network of growth factor–mediated signaling events, hematopoietic and nonhematopoietic cell interactions, and gene transcription. Our finding that FAK negatively regulates megakaryopoiesis, potentially via direct effects on the signals that emanate from the Tpo-stimulated c-Mpl receptor, adds an important and novel signaling mechanism to the several mechanisms already determined. It has been well documented that FAK contains a novel signaling mechanism to the several mechanisms already discussed, SFKs are required for FAK activation following integrin engagement.28 We have shown no change in expression of Fak in Fak+/null models these cell behaviors represent the major phenotype. One possible explanation for the lack of a spreading and migration phenotype involves the FAK homologue PYK2. Previous work using Fak−/− fibroblasts demonstrated an increase in PYK2 expression,54 and targeting PYK2 to β1 integrin–containing focal adhesion sites using a chimeric PYK2-FAK-C-terminal protein rescues the Fak−/− phenotype.55 Using our model, we demonstrated no change in expression of PYK2 in Pf4-Cre/FAK-floxed mice. However, PYK2 phosphorylation in response to fibrinogen and MnCl2 is increased in Fak−/− megakaryocytes compared to WT controls. It is possible that increased PYK2 activity is able to compensate in the absence of FAK to maintain normal megakaryocyte adhesion and spreading. This theory is supported by previous findings that suggest PYK2 is able to localize to focal adhesion sites in megakaryocytic cells and is activated following integrin engagement.28 We have shown no increase in PYK2 activity in response to various agonists in FAK-null platelets. The more pronounced difference in FAK-null
platelet spreading compared with megakaryocyte spreading may be due to the lack of PYK2 compensation in platelets.

The potential roles of FAK in platelet function have been previously described. Those earlier studies and our research described here together demonstrate that FAK is rapidly phosphorylated following a combination of integrin and agonist signals. However, before this study, the lack of a viable FAK-null mouse has prevented confirmation of these findings in vivo. Although our studies using Fak-/- platelets are just beginning, it appears from the observed defects in platelet spreading and tail rebleeding times that FAK may be of considerable importance for platelet αIIbβ3 integrin outside-in signaling and thrombus stability. Our results suggest that one of the major roles of FAK in platelets is the reorganization of the actin cytoskeleton to initiate spreading rather than to recruit proteins such as vinculin to nascent adhesion structures. Additional studies will be required, and are now possible, to more fully understand the role of platelet FAK in hemostasis.

In this study, we further demonstrated the great potential of the Pf4-Cre mouse in determining the roles of specific proteins in megakaryocytes and platelets. The ability to ablate a specific floxed gene from these cells now allows in vivo characterization of genes of interest with functions that had been previously undescribed because of their vital role or roles in development of other cell lineages. Using these mice, we have been able to demonstrate novel roles for FAK in mediating megakaryocyte differentiation in vivo, and Tpo-mediated intracellular signaling in vitro. In addition, we have provided the first insights into the phenotype of Fak-/- platelets. Further studies with these mice may reveal potential future therapeutic targets for the control of platelet functions.

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Authorship

Contribution: All authors substantially contributed to the content of the paper and have agreed to the submission in its current format. I.S.H. designed and performed research, analyzed data, and wrote the manuscript; N.E.F. and K.S. designed and performed experiments and analyzed data; N.P. designed and performed experiments, analyzed data, and wrote the manuscript; S.J.S. designed experiments and wrote the manuscript; and K.K. designed experiments, interpreted data, and wrote the manuscript.

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