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Off-targets effects underlie the inhibitory effect of FAK inhibitors on platelet activation: studies using *Fak*-deficient mice

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Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase critical in mediating adhesion and migration in many cell types via direct association with integrins. Considering its role in controlling events downstream of integrin activation, the function of FAK in platelet signaling has been well studied. Platelet adhesion to fibrinogen via integrin $\alpha_{IIb}\beta_3$ leads to rapid FAK phosphorylation in an agonist-dependent manner and on activation of protein kinase C [1,2]. FAK phosphorylation in response to co-stimulation with either fibrinogen or collagen and adenosine 5′-diphosphate (ADP) accompanies changes in platelet spreading [3]. However, as *Fak* deletion in mice is embryonic lethal at day 8.5, before the onset of significant hematopoiesis, the exact roles that FAK plays in platelet function *in vivo* remain elusive.

To fully evaluate the role of FAK in platelet function, we successfully ablated *Fak* expression specifically in megakaryocytes and platelets by crossing conditional *Fak-floxed* mice [4] with megakaryocyte lineage–specific *platelet factor 4* (*Pf4*)-*Cre* mice [5]. We found that *Pf4-Cre*/*Fak-floxed* ($Fak^{-/-}$) mice exhibit increased bleeding times by tail bleed assays and attenuated platelet spreading. Recently, it was demonstrated an FAK inhibitor, PF-573,228, significantly attenuated platelet aggregation, spreading, and calcium release [6], suggesting that targeting FAK with specific pharmacological inhibitors may prevent thrombosis in high-risk patients.

In this study, we further investigate the role of FAK in platelet function using platelet-specific *Fak* knockout mice and determine the effectiveness of FAK inhibitors, PF-573,228 (PF-228) and PF-573,271 (PF-271), in mediating platelet activity, in the presence and absence of FAK.

We found that platelet aggregation was not significantly different in *WT* and $Fak^{-/-}$ mice in response to thrombin, ADP, or collagen (Fig. 1A). Platelet integrin expression was also not significantly different between the two groups (data not shown). Next, we determined the effects of FAK inhibitors PF-228 and PF-271 on platelet function *in vitro*. PF-228 and PF-271 both completely inhibited thrombin-mediated FAK phosphorylation in isolated *WT*

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Addendum
M. E. Roh designed and performed research, analyzed data, and wrote the manuscript. M. Cosgrove performed research and analyzed data. K. Gorski performed research and analyzed data. I. S. Hitchcock designed and performed research, analyzed data, and wrote the manuscript.

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The authors state that they have no conflict of interests.

platelets, while FAK was absent in *Fak*$^{-/-}$ platelets (data not shown). Despite the absence of FAK, however, PF-228 and PF-271 significantly inhibited platelet aggregation in response to thrombin, ADP, and collagen in *Fak*$^{-/-}$ platelets as well as *WT* (Fig. 1B).

Arterial occlusion times were measured using age-matched *WT* and *Fak*$^{-/-}$ mice (8–12 weeks) after injury with 7.5% $FeCl_3$ to the carotid artery as described previously [7]. At 30 min before artery occlusion assays, mice received 50 mg/kg^{-1} PF-228 or PF-271 solubilized in cremephore DL/DMSO/ethanol (3:2:3) via intraperitoneal injection. Control mice received vehicle alone. Arterial occlusion time was not different between vehicle-treated *WT* and *Fak*$^{-/-}$ mice (*WT*, 545 ± 87 s; *Fak*$^{-/-}$, 552 ± 71 s; Fig. 1C). Significantly, PF-271–treated *WT* and *Fak*$^{-/-}$ mice failed to occlude following injury throughout the 30-min test period. However, PF-228 had no effect on arterial thrombosis *in vivo* (Fig.1C).

We have shown that the absence of FAK has no significant effects on arterial thrombosis following injury or platelet aggregation in response to ADP, collagen, or thrombin. One potential explanation for the apparent lack of platelet phenotype in *Fak*$^{-/-}$ mice is the compensatory role of the FAK homologue protein Pyk2. A number of reports describe increased expression and phosphorylation of Pyk2 when *Fak* is ablated and the increase in Pyk2 function is able to compensate for the absence of FAK [8,9]. Similarly, we observed that Pyk2 phosphorylation and expression are significantly increased in *Fak*$^{-/-}$ platelets (data not shown). Importantly, a recent publication determined the importance of Pyk2 in regulating integrin $\alpha_{IIb}\beta_3$ outside-in signaling in platelets, showing that *Pyk2* ablation inhibited platelet adhesion and spreading on fibrinogen [10], further supporting the significance of Pyk2 in platelet function.

Given the roles of FAK in cellular motility, adhesion, invasion, metastasis, and angiogenesis, the potential of FAK inhibitors as antioncogenic drugs has received considerable attention [11]. Both of the FAK inhibitors we have used in our studies, which directly affect the ATP binding site and thereby lower FAK kinase activity, have been shown to inhibit tumor growth in murine models [12,13]. However, the development of these drugs for clinical trials has been complicated by the structural similarities of the ATP-binding domain of many kinases, resulting in off-target effects of the inhibitors. We have shown that the FAK inhibitors have a significant effect on platelet aggregation in response to thrombin, collagen, and ADP, similar to the conclusions made previously [6]. However, we have shown that these effects are observed in both the presence and the absence of FAK.

These data confirm that attenuation of platelet activity by treating with FAK inhibitors PF-228 and PF-271 is due to off-target effects rather than FAK inhibition. Considering PF-271 is now in phase I clinical trials, the significant inhibitory effects on platelet function should be considered as a potential side effect, although currently there are no reports of bleeding diatheses in treated patients.

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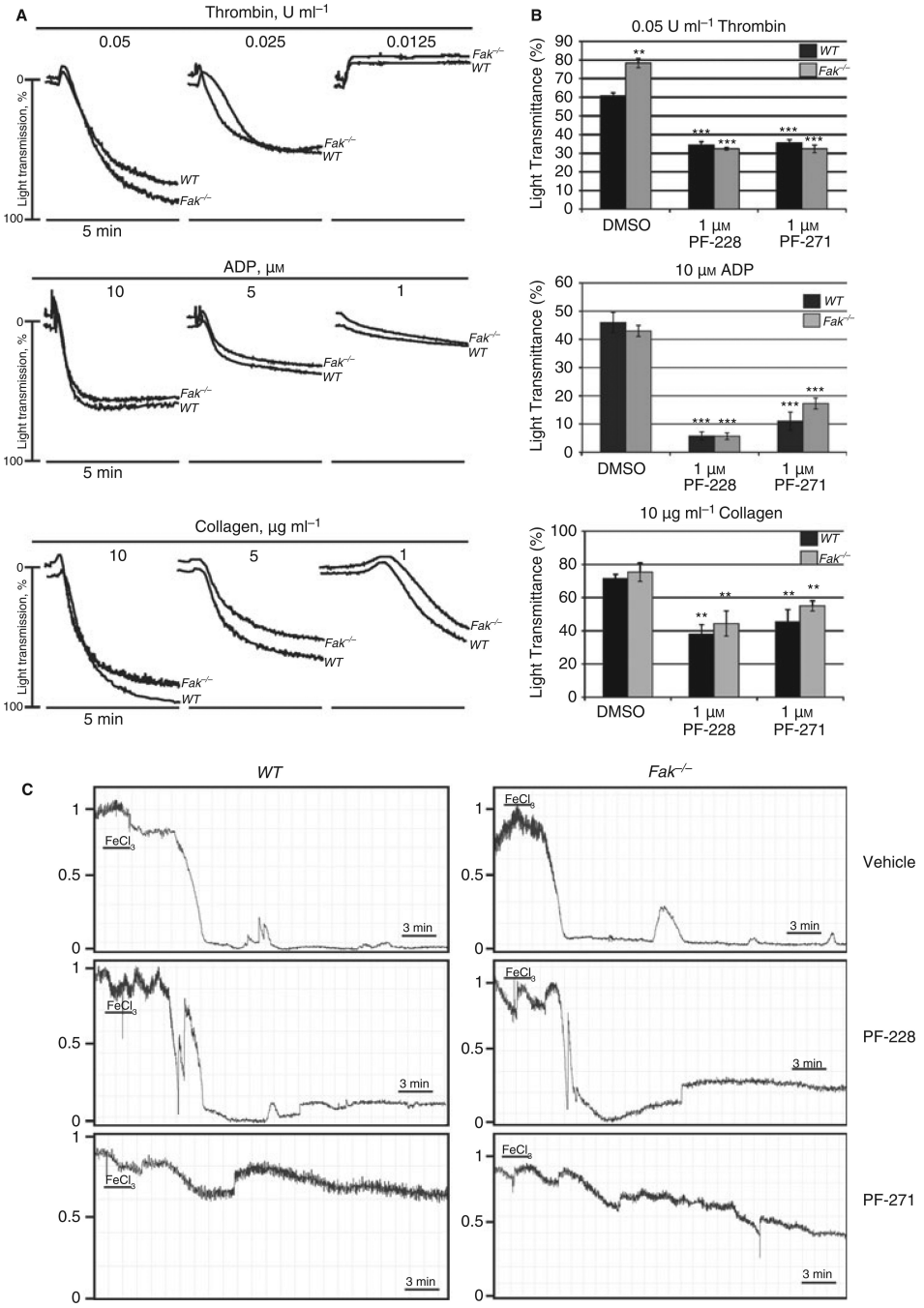

Fig. 1.

Effects of *Fak* ablation and FAK inhibitors on platelet function and thrombosis. Animal procedures were performed in accordance to protocols approved by the Institutional Animal Care and Use Committee, Stony Brook University. (A) Platelet aggregation was determined using washed platelets stimulated with decreasing concentrations of thrombin, adenosine 5′diphosphate (ADP), and collagen. Data are representative of at least three separate experiments. (B) Platelet aggregation was determined in the absence and presence of FAK inhibitors in *WT* and $Fak^{-/-}$ platelets following stimulation with maximal concentrations of thrombin, ADP, and collagen. Data is representative of the SEM of three independent experiments (**$P < 0.01$; ***$P < 0.005$). (C) Carotid artery occlusion assays were used to

determine the effects of FAK inhibitors on *in vivo* thrombosis. Mice were treated with vehicle or PF-228 or PF-271 (50 mg/kg^{-1}) for 30 min before occlusion assay. Data are representative of four mice per group.