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Dear Sir,

Shape change is one of the earliest visible responses after platelet activation with a soluble agonist or following contact with an adhesive substratum (1). Platelet shape change is a composite of several distinct morphological changes. Early observations reported that ADP-activated platelets undergo transformation from a smooth discoid shape, to discs extending a few short pseudopodia, and progressively to spheres with numerous long filopodial extensions (1). The same types of transformations have since been observed also in response to adhesive matrices such as von Willebrand factor (VWF) (2) and fibrinogen (3). On these matrices progressive growth of lamellipodia finally results in extensive platelet spreading. Whereas filopodia and lamellipodia are membrane protrusions driven by actin polymerisation, sphering of the platelet body has been linked to a distinct process powered by an actomyosin-driven contraction, tubulin depolymerisation, and cortical actin cytoskeleton severing.

Despite being one of the earliest manifestations of platelet stimulation, the mechanisms governing filopodia formation and their role in platelet physiology and haemostasis are still largely unknown. A physiological role has been postulated for the ability of filopodia to multiply $\alpha_{IIb}\beta_3$ fibrinogen contacts (4), thereby increasing platelet aggregation, as well as to slow-down platelet translocation under shear conditions, thus facilitating adhesion to the subendothelium (5). In motile and migrating cells, filopodia are proposed to participate in the migration process, in cell adhesion and exploring environmental cues (6). Filopodia are dynamic thin extensions made of parallel bundles of actin filaments. The mechanism of filopodia extension in platelets has been postulated to resemble that of other cell types, but in fact is still largely unknown. An important distinction between filopodia of adherent cells and those of platelets is that the latter does not require a pre-existing lamellipodial network and adherence to a substratum (7).

Among several filopodia regulatory proteins identified in other cell systems, vasodilator-stimulated phosphoprotein (VASP) has been proposed as an actin-binding positive modulator of filopodia extension (8). VASP belongs to the Ena/VASP family along with Mena and EVL (Ena-VASP-like), which is characterised by the presence of EVH1, EVH2 (Ena/VASP

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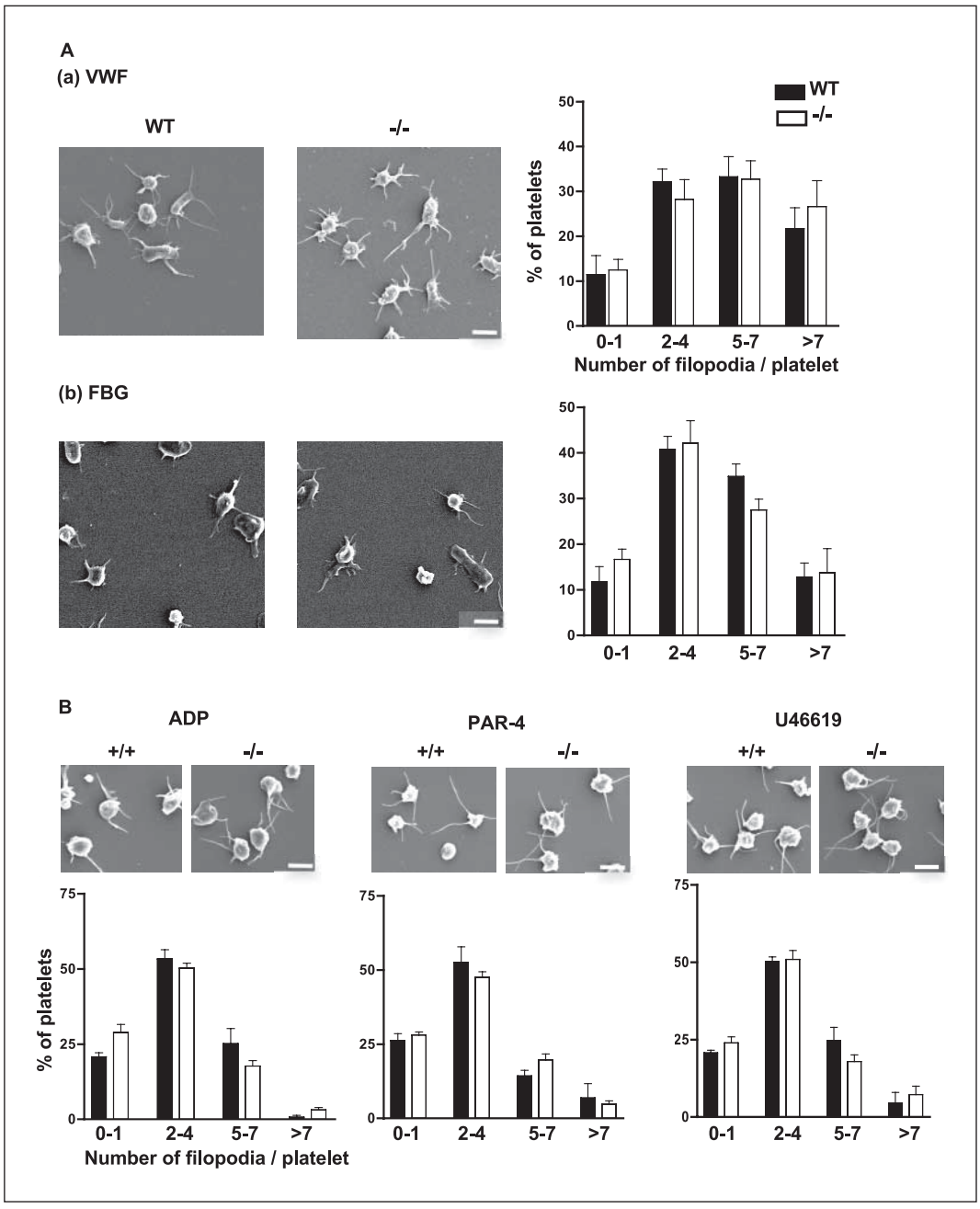

Figure 1: Role of vasodilator-stimulated phosphoprotein (VASP) in platelet filopodia extension. Blood from wild-type and VASP knock-out (-/-) mice was drawn from the abdominal aorta into acid citrate dextrose (ACD) anticoagulant and a washed platelet suspension (300000 platelet/µl) was prepared in Tyrode's albumin buffer as described previously (20). (A) Platelets (a) were treated with 40 µg/ml Integrilin and 1 µg/ml botrocetin and allowed to adhere for 20 min to cover slips coated with 10 µg/ml of mouse von Willebrand factor (VWF), (b) were left untreated and allowed to adhere for 60 min on cover slips coated with 100 µg/ml of human fibrinogen (FBG). (B) Platelets treated with 40 µg/ml Integrilin were activated during 2 min in suspension without stirring by 1 µM ADP, 200 µM protease activated receptor 4 peptide (PAR-4) or 1 µM thromboxane A2 analog U46619. All experiments were performed at 37°C. Samples were fixed with 2,5% glutharaldehyde, processed for Scanning Electron Microscopy and analysed. Filopodia formation was scored according to the number of extensions per platelet (0 to 1, 2 to 4, 5 to 7, >7) in five different fields corresponding to a total surface of 9215 µm^2. Bar represents 2,5 µm. The results are mean values (±SEM) from three separate experiments with 2 animals/experiment. The results are not statistically different using a Student's t test.

homology 1 and 2), and proline-rich central domains. The EVH2 domain contains a G- and F-actin-binding region (9) which contributes to the ability of VASP to regulate actin polymerisation with anti-capping, nucleation and bundling activities (10). The anti-capping activity is reinforced by the capacity of the VASP proline-rich domain to recruit profilin. Moreover, VASP interacts with proteins involved in filopodia extension and cytoskeletal dynamics such as src and Abl kinases (Abelson protein tyrosine kinase) (11).

VASP is phosphorylated by cAMP and cGMP-dependent protein kinases PKA and PKG resulting in decreased anti-capping and bundling activities (12, 13). Clinically, phosphorylated VASP is used as a marker to monitor the efficiency of treatments with the P2Y12 ADP receptor targeting drug clopidogrel (14, 15).

VASP$^{-/-}$ mice have shown slight increases in *in vitro* platelet aggregation, secretion and fibrinogen-binding responses (16, 17). A more pronounced difference was noted under conditions in which cAMP and especially cGMP levels where increased. With VASP-deficient platelets, especially the cGMP-mediated inhibition of agonist-evoked platelet aggregation and FBG binding was impaired indicating a role of VASP as a negative modulator of integrin $\alpha_{IIb}\beta_3$ activation (16, 17). Also, the NO donor SNP (sodium-nitroprusside) induced inhibition of thrombus-formation in flow-chambers with collagen-coated cover slips was strongly impaired with VASP KO platelets (18). *In vivo*,

VASP-deficiency resulted in increased platelet adhesion to the injured vascular wall as well as impairment of the NO-induced inhibition using a model of arterial thrombosis (19).

In an attempt to better understand the mechanisms and role of filopodia formation in platelets, we investigated the consequences of VASP-deficiency on filopodia formation of platelets exposed to adhesive matrices (VWF and FBG) and soluble agonists (ADP, PAR-4 and the thromboxane A2 analogue U46619). Filopodia formation has been identified as a GPIb-dependent response of platelets adhering to a VWF matrix (2, 20). A majority of the mouse platelets adhering for 20 minutes on mouse VWF in the presence of botrocetin loose their discoid shape and extend filopodia independently of an RGD-integrin $\alpha_{IIb}\beta_3$ mechanism. This response was not prevented in VASP$^{-/-}$ mice in which, similar to wild type, 90% of adherent platelets extended one or more filopodia and exhibited a similar distribution in number of filopodia per platelet (Fig 1. A. a). Platelet adhesion to immobilised FBG also results in filopodia extension via an $\alpha_{IIb}\beta_3$ dependent process. As observed for the VWF matrix, VASP deficiency did not modify the capacity of platelets to extend filopodia on FBG (Fig 1. A. b).

Filopodia formation was also analysed in platelets kept in suspension and activated by agonists acting on separate receptors (ADP, a PAR-4 agonist peptide and U46619). Under conditions in which aggregation was prevented (no stirring, no added FBG and presence of an $\alpha_{IIb}\beta_3$ antagonist), a similar proportion of wild type and VASP-deficient platelets (75% of total) exhibited filopodia with these different stimuli. Here also, a normal distribution was observed in VASP$^{-/-}$ after scoring for the number of filopodia per platelet (Fig 1. B).

Altogether, the above results indicate that lack of VASP has little impact on filopodia formation after exposure to adhesive matrices or soluble agonists. In contrast, a previous study suggested a role of VASP downstream of PKCdelta mediating its negative effect on filopodia formation (21). The discrepancy could originate from differences in the type of matrix, the chronology and the nature of filopodia being analysed. Pula et al, had examined platelets adhering on a collagen matrix and at times where full spreading had occurred. Under these conditions, platelets with PKCdelta deficiency or blockade harboured a mixed morphology qualified as *'filopodia formation...superimposed upon a persistent lamellipodia formation in the fully spread platelet'* which was absent in VASP deficient platelets. One hypothesis is that a separate event was evaluated here, whereby PKCdelta blockade engaged filopodia extension from existing lamellipodia, whereas the present work focused on filopodia formation prior to spreading. The response in the former study could resemble that documented in nucleated cells migrating on an adhesive matrix, which extend filopodia from preformed lamellipodia at their leading edge, and where a positive role of VASP has been postulated (22). Although this has not been formally documented in their study, the fact that VASP-deficient platelets normally spread on collagen in the absence of PKC delta blockade (21), suggests that the early filopodia transformation might also be normal on this matrix.

The normal filopodia response reported here in VASP deficiency contrasts with the increased FBG-binding, aggregation and secretion responses noted under conditions in which cAMP and cGMP levels where increased (16, 17). However, attempts to reveal a putative increase in filopodia formation in VASP$^{-/-}$ under similar conditions of increased cAMP and cGMP levels were obviated by the normal capacity of WT platelets to extend filopodia on VWF or FBG after pretreating with Sp-5,6-DCI-cBIMPS or 8-pCPT-cGMP, thus making it unlikely that VASP plays any role here (Supplementary Figure, available online at http://www.thrombosis-online.com). Normal filopodia extension under conditions of increased cAMP has been previously reported by Savage et al. in PGE1-treated platelets adhering to fibrinogen, supporting the present observation (23).

In conclusion, the present evaluation of VASP$^{-/-}$ mice suggests that VASP is not essential in the early process of filopodia formation in platelets. Additional work is needed to clarify the mechanisms of filopodia formation and to test the *in vivo* importance of this platelet response in haemostasis. Despite the recognised value of the analysis of VASP phosphorylation in the surveillance of patients treated with the antiplatelet drug clopidogrel (15), the exact function(s) and importance of VASP and VASP phosphorylation in platelet function remain to be fully elucidated. Since VASP is an established substrate of cGMP and cAMP dependent kinases, and is involved in cyclic-nucleotide-(and especially cGMP-) mediated inhibition of platelet adhesion (19) its role may be more significant under situations where these nucleotides are increased in response to prostacyclin and/or nitric oxide.

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