

New insights into the expression and role of platelet FXIII-A

| | |
|-------------------------------|--|
| Journal: | <i>Journal of Thrombosis and Haemostasis</i> |
| Manuscript ID: | JTH-2009-00066.R2 |
| Manuscript Type: | Original Article - Platelets |
| Date Submitted by the Author: | |
| Complete List of Authors: | Jayo, Asier; Centro de Investigaciones Biológicas (C.S.I.C.), Cellular and Molecular Physiopathology Conde, Isabel; Centro de Investigaciones Biológicas (C.S.I.C.), Cellular and Molecular Physiopathology Lastres, Pedro; Centro de Investigaciones Biológicas (C.S.I.C.), Cellular and Molecular Physiopathology Jimenez-Yuste, Victor; Hospital Universitario La Paz, Laboratory of Hematology Gonzalez-Manchon, Consuelo; Centro de Investigaciones Biológicas (C.S.I.C.), Cellular and Molecular Physiopathology |
| Key Words: | Factor XIII-A, Platelet activation, Transglutaminase activity |
| | |

New insights into the expression and role of platelet FXIII-A

A. Jayo,^{*} I. Conde,^{*} P. Lastres,^{*} V. Jiménez-Yuste,[†] and C. González-Manchón^{*,‡}

^{*}Department of Cellular and Molecular Pathophysiology, Centro de Investigaciones Biológicas (CSIC), Madrid, Spain; [†]Laboratory of Hematology, Hospital Universitario La Paz, Madrid, Spain; and [‡]Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER, ISCIII)

Word count of abstract: 190

Word count of text: 1999

Running head: Role of FXIII-A in platelet function

Correspondence: Consuelo González-Manchón, Department of Cellular and Molecular Physiopathology, Centro de Investigaciones Biológicas, Ramiro de Maeztu 9, 28040 Madrid, Spain. Phone: 34-91-8373112; Fax: 34-91-5360432; E-mail: cgmanchon@cib.csic.es

Acknowledgments

This work was supported by grants from the Dirección General de Investigación del Ministerio de Educación y Ciencia (BFU2006-00914) and Fundación Rodríguez Pascual. A. Jayo was recipient of a fellowship from the Ministerio de Educación y Ciencia. I. Conde holds a research contract from the Consejo Superior de Investigaciones Científicas. We thank María Teresa Seisdedos for videomicroscopy technical assistance.

Summary

Background: The A subunit of factor XIII (FXIII-A) functions as an intracellular transglutaminase (TG) in the megakaryocyte/platelet lineage where, likely, it participates in the cytoskeletal remodeling associated with cell activation. However, so far, the precise role of cellular FXIII (cFXIII) and the functional consequences of its absence in FXIII-A-deficient patients are unknown. *Objectives and methods:* In this work, we have used platelets from four patients with congenital deficiency of FXIII-A to study the role of cFXIII in platelet functions. *Results:* We report that FXIII-A represents the only detectable source of TG activity in platelets and that the binding of fibrinogen in response to TRAP stimulation was significantly reduced in platelets from the patients. In agreement, in control platelets, monodansyl-cadaverine (MDC), a competitive amino-donor for transglutaminases, inhibited in a dose-dependent manner fibrinogen binding induced by TRAP. Moreover, upon adhesion to fibrinogen, normal platelets incubated with MDC as well as FXIII-A-deficient platelets showed a distinct extension pattern with reduced lamellipodia and increased filopodia formation, suggesting a delay in spreading. *Conclusions:* These findings provide evidence supporting that cFXIII-dependent TG activity may be directly involved in the regulation of platelet functions.

Key words: FXIII-A, platelet activation, transglutaminase activity

Introduction

Blood coagulation FXIII is a plasma transglutaminase (TG) that circulates as a heterotetramer composed of two catalytic A and two noncatalytic B subunits (A₂B₂) [1]. Much of plasma FXIII (pFXIII) is associated with fibrinogen, and it is activated by Ca²⁺ and thrombin in the final stage of the coagulation cascade, stabilizing the clot by cross-linking γ and α chains of fibrin and by attaching plasmin inhibitor to the fibrin α -chain [2,3]. The intracellular form of FXIII (cFXIII) is a dimer of A subunits that can be activated by low Ca²⁺ concentrations in the absence of thrombin [4,5] and is present in a variety of cells including platelets, megakaryocytes, and monocytes/macrophages. Platelets contain amounts of FXIII-A 150 fold more per volume than plasma, while FXIII-A concentration in monocytes is at least one magnitude less than in platelets [6].

FXIII is a member of the TG family, composed of Ca²⁺-regulated cross-linking enzymes structurally similar to the papain-like cysteine proteases. Mammalian TGs can be distinguished on the basis of their tissue distribution, localization, and substrate specificity [7,8]. TG2 or tissue TG, the first member to be discovered, has unique characteristics such as ubiquitous expression, widespread localization and capacity to bind to guanine nucleotides. Evidence on the presence of TG2 in platelets comes mainly from a study that attributed to cFXIII only the thrombin-dependent TG activity [9]. Later, it was shown that the intracellular activation of FXIII does not require proteolytic cleavage, and that increase in cytosolic Ca²⁺ concentration induced by platelet activation is sufficient to trigger the active configuration [4,5,10]. Therefore, whether TG2 is significantly expressed and is contributing as a source of platelet TG activity is so far unclear. Furthermore, the functional significance of the high expression of cFXIII in platelets remains also poorly understood.

No inherited disease has been identified associated to TG2 defect. However, a variety of mutations have been described in the FXIII subunit genes associated with bleeding tendency and pFXIII deficiency [11] (<http://www.hgmd.cf.ac.uk/ac/index.php>). The incidence of the disease, in the range of 1 in 2-3 million, is increasing as more laboratory support becomes available around the world. As a consequence of the absence of cross-linked fibrin, FXIII deficiency results in delayed bleeding after trauma and impaired wound healing, while primary hemostasis is apparently normal [12]. Umbilical bleeding in the first days after birth, repeated intracranial hemorrhages and recurrent early miscarriages are hallmarks of the disease. In patients carrying splice mutations,

1
2
3 small amounts of normally processed mRNA can produce FXIII to support partial
4 dimerization of fibrin, thus reducing the clinical consequences [13]. Although
5 heterozygous carriers are normally asymptomatic, some cases of bleeding have been
6 reported [14].
7
8
9

10 In this report we have characterized the molecular defect in three cases of bleeding
11 diathesis caused by FXIII-A deficiency. We used platelets from the patients as well as a
12 competitive amino-donor for transglutaminases, monodansyl-cadaverine (MDC), to
13 determine the source of platelet TG activity and to study the role of cFXIII in platelet
14 function.
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

Patients, materials and methods

Patients

Patient 1 is a 34-year old female from Morocco with a history of frequent mucocutaneous bleeding and spontaneous miscarriages. Her parents are relatives. One of her sisters died due to hemorrhage and other has severe bleeding. Spanish patients 2 and 3 are sisters with a lifelong bleeding tendency and abnormal wound healing. Their parents did not recognize consanguinity. Patient 4 is a Spanish 5-year old girl who has suffered two severe intracranial hemorrhages. Routine coagulation and platelet function tests were normal in all patients. Deficiency of pFXIII activity was determined by using the chromogenic Pefakit FXIII assay from Pentapharm (Basel, Switzerland). This study was approved by the hospital ethics Committee, and written informed consent for the use of blood sampling was obtained from the patients in accordance with the Declaration of Helsinki.

Mutation identification

Total platelet RNA was obtained using the guanidinium isothiocyanate procedure. Screening for mutations was performed by direct sequencing of PCR-amplified overlapping fragments of reversed transcribed FXIII-A mRNA.

Genomic DNA was extracted from peripheral blood cells and the sequence of PCR products containing exons and flanking regions of introns of the *F13A1* gene were analyzed following standard procedures.

PCR-based relative quantification of FXIII-A and TG2 mRNA

To compare the relative expression of FXIII-A and TG2 in platelets and human umbilical vein endothelial cells (HUVECS), a series of 30 μ L-reverse transcription reactions were performed using a common antisense oligonucleotide, 5'-AGGATGCCATCTTCAAAGT-3', targeting sequences shared by both mRNAs. After cDNA synthesis, 5 μ L-aliquots were used as template in 25, 30, 35 and 40-cycle PCR reactions, using specific sense oligonucleotides, 5'-GTCTGTGCGGCTGTCCATC-3' and 5'-GCAAGACTGCACCTCG-3', to amplify 285-bp cDNA fragments of FXIII-A and TG2, respectively. Amplification of a 163-bp fragment of β -actin cDNA was

1
2
3 carried out in parallel to verify that similar amounts of total RNA were used. The
4 amplification products were resolved on agarose gels for densitometry analysis.
5
6
7

8 9 **Western analysis of platelet lysates**

10 Platelet proteins were solubilized in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl,
11 1% Triton X-100, 0.05% Tween 20) supplemented with 1 mM phenylmethyl sulfonyl
12 fluoride (PMS) and 5 $\mu\text{L}/100 \mu\text{L}$ protease inhibitor cocktail (SIGMA). After 20 min on
13 ice, lysates were cleared by centrifugation, quantified and resolved on SDS-10% PAGE
14 under reduced conditions, transferred to PVDF membranes and incubated with a
15 polyclonal antibody against FXIII-A (USBiological, Swampscott, MA, USA). The
16 specific bound antibody was detected with a peroxidase-conjugated goat anti-rabbit IgG
17 (BioRad) and visualized using the ECL chemiluminescent system.
18
19
20
21
22
23
24
25

26 27 **Binding of fibrinogen to activated platelets**

28 Purified human fibrinogen (Calbiochem) was labeled with FITC as previously
29 described [15].
30

31 Washed platelets were resuspended in HEPES buffer, pH 7.4 (10 mM HEPES, 136
32 mM NaCl, 2.7 mM KCl, 1 mM CaCl, 1 mM MgCl, 0.1% glucose) at a final
33 concentration of approximately $5 \times 10^7/\text{mL}$. After 30 min of incubation at 37°C with
34 different concentrations of monodansyl-cadaverine (MDC), 100 μL aliquots were
35 treated for 5 min at room temperature with 50 μM ADP plus 1 mM epinephrine, 25 μM
36 TRAP-6, or 20 nM PMA. Then, FITC-Fg was added at 50 $\mu\text{g}/\text{mL}$ and, after 20 min,
37 platelets were washed and analyzed in a Coulter flow cytometer model EPICS XL.
38
39
40
41
42
43
44
45

46 47 **Platelet adhesion, spreading and immunofluorescence analysis**

48 Washed platelets were resuspended at approximately $4 \times 10^8/\text{mL}$ in HEPES buffer and
49 left at room temperature for 1 hour. For adhesion assays, 100 μL -aliquots were added to
50 96 well plates coated with 3 $\mu\text{g}/\text{mL}$ of purified human fibrinogen and incubated at 37°C
51 for 15 min. After washing twice, the number of adhering platelets was quantified in a
52 colorimetric assay by measuring alkaline phosphatase activity [16].
53
54
55
56

57 For spreading and immunofluorescence assays, 500 μL -aliquots of platelet
58 suspension were plated on glass coverslips coated with 3 $\mu\text{g}/\text{mL}$ fibrinogen in 24-well
59 culture dishes. After 15 min, they were fixed for 20 min in PBS containing 4%
60

1
2
3 paraformaldehyde, and blocked and permeabilized with 0.5% Triton X-100 in PBS-
4 0.5% BSA for 20 min at room temperature. Then, platelets were incubated with anti- β 3
5 (H1AG11) monoclonal antibody, and/or with an anti-FXIII A polyclonal antibody in
6 PBS containing 0.1% Triton X-100 and 10% normal goat serum for 1 hour. After
7 washing, platelets were incubated with Alexa Fluor 488-conjugated anti-mouse and/or
8 Alexa Fluor 546-conjugated anti-rabbit antibodies. After washing, the preparations were
9 mounted on FluorSave reagent (Calbiochem) and visualized with an x63 objective with
10 a Zeiss Axioplan epifluorescence microscope (Göttingen, Germany) equipped with a
11 cooled CCD camera (Leica DFC 350 FX). Platelets were counted and classified
12 according its spreading pattern in three categories: (i) platelets with sustained calcium
13 induced phenotype (SCIP), (ii) platelets forming filopodia, and (iii) spread platelets
14 forming lamellipodia.
15
16
17
18
19
20
21
22
23
24
25

26 **Multidimensional microscopy**

27
28 Platelets were prepared as described for spreading experiments and plated in
29 microslides for live-cell-imaging (IBIDI, München, Germany) previously coated with 3
30 μ g/mL fibrinogen. Adhesion and spreading processes were monitored during 30 min in
31 a multidimensional Leica AF6000 LX microscope, equipped with a CCD Hamamatsu
32 C9100-02 camera in a CO₂ and temperature controlled incubator.
33
34
35
36
37
38

39 **Cross-linking activity of platelet FXIII-A**

40 We measured TG activity in lysates of washed platelets using biotinylated
41 cadaverine. Briefly, washed platelets were resuspended in 10 mM Tris-HCl, pH 7.4,
42 containing 0.1% Triton X-100, 140 mM NaCl, 0.4 mM PMSF and 1 mM DTT, and
43 lysed on ice for 20 min followed by three freezing-thawing cycles. After clearing by
44 centrifugation, protein concentration was determined using the BIO-RAD Bradford dye
45 method, and 30 μ g of protein were incubated with 400 μ M biotinylated cadaverine with
46 or without 5 mM EDTA or 5 mM CaCl₂, in a final volume of 15 μ l of lysis buffer
47 containing 3 mM DTT. After 1 hour at 37°C the reactions were stopped by adding
48 loading buffer and, then, resolved in 10% SDS-PAGE under reducing conditions, and
49 transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk,
50 incubated with 1:4500 dilution of peroxidase-conjugated avidin, and visualized using
51 the ECL chemiluminescent system.
52
53
54
55
56
57
58
59
60

Results

Transglutaminase (TG) activity in platelets from FXIII-deficient patients

Diagnosis of FXIII deficiency was based on the clinical history of hemorrhagic tendency and the absence of plasma FXIII activity revealed by an enzymatic assay. To determine whether the disorder was caused by a defect in the FXIII-A subunit, TG activity was determined in platelets lysates using cadaverine as competitive substrate. We observed that control platelets contained detectable basal TG activity that was blocked by EDTA and stimulated in the presence of calcium (Fig. 1A). This activity was absent in the patients suggesting that the disease is due to FXIII-A subunit deficiency.

Identification of mutations in the *F13A1* gene

cDNA sequence analysis identified the Gly562Arg mutation in patient 1 (Fig. 2). Its homozygous status was verified by analyzing the gDNA. Platelets were found to contain normal mRNA levels of mutant FXIII-A, but they showed absence of protein in western and fluorescence microscopy analysis (Figs 1 and 3). Sequencing analysis revealed the presence of two main transcripts in patients 2 and 3. In one, the first 7 bases of exon 6 were deleted; the other carried additional deletion of exon 5 (Fig. 2B). Analysis of gDNA identified the same mutation, IVS5-1G>A (Fig. 2A), in two different alleles for FXIII-A, as assessed by detection of Pro564Leu and Gln651Glu polymorphisms in heterozygosis. The results suggest that the IVS5-1G>A mutation activated a cryptic splice acceptor site in the 5' sequence of exon 6 that is also preferentially used during splicing of intron 4 (Fig. 2B). The 7 bp-deleted transcript was also detected in a previously reported patient carrying the IVS5-1G>A mutation and low FXIII-A-mRNA levels [17]. In patient 4, cDNA sequencing revealed heterozygosis of Val34Leu polymorphism and similar amounts of two transcripts, one of them carrying deletion of exon 14 and, as consequence, frame-shift and premature stop codon. Sequence of the normal size transcript identified the G1985A substitution in exon 14 that would change Arg661 to Gln. Analysis of gDNA revealed heterozygous status for G1985A and identified, in the last nucleotide of exon 14, a heterozygous G>A transition (G2045A) which disrupts the donor splice sequence of intron 14. This mutation was not present in the PCR product corresponding to the normal size transcript, indicating that it always induces skipping of exon 14.

Platelet TG activity relies on the presence of cFXIII

TG activity in platelet from FXIII^{-/-} mice was recently shown to be higher than in normal mice, suggesting a compensatory up-regulation of another TG in FXIII-A-deficient platelets [18]. The absence of TG activity in platelets from the patients (Fig. 1) suggests that cFXIII is, practically, the only source of platelet TG activity and that the murine compensatory TG activation is not operating in human platelets. Consistent with these findings, TG2 mRNA expression was undetectable relative to FXIII-A-mRNA levels in control platelets. In contrast, TG2, but not FXIII-A, was easily amplified in endothelial cells (Fig. 3). mRNA-TG2 was also barely expressed in FXIII-A-deficient platelets, independently of whether platelets contain normal (Fig. 3) or decreased (not shown) mRNA levels.

Role of cFXIII in platelet functions

Since cFXIII represents the main platelet TG activity we considered the possibility that it could modulate platelet activation response. We first determined the effect of different stimulators on fibrinogen binding to control and FXIII-A-deficient platelets. Platelets from the patients and, at least, two control individuals were analyzed together in two or more independent assays and, then, the values were pooled together for comparative analysis. The broad dispersion of the values does not imply variability in the response to activators; it is due, at least in part, to inter-experimental variability in the basal activation state of platelets during manipulation, as reflected in fibrinogen binding values in non-stimulated platelets. As shown in Fig. 4A, significant differences were only found in platelets stimulated with the thrombin receptor agonist hexapeptide TRAP. Consistent with this observation, monodansyl-cadaverine (MDC), a competitive amino-donor for transglutaminases [19], blocked in a dose-dependent manner the effect of TRAP on fibrinogen binding of control platelets (Fig 4B).

We next compared adhesion of control and FXIII-A-deficient platelets to 3 µg/mL fibrinogen-coated surfaces. As shown in Fig. 5A, an altered spreading phenotype characterized by higher percent of platelets emitting filopodial versus lamellipodial extensions was observed in platelets from the patients. Consistent with this, incubation with MDC induced a dose-dependent increase of the proportion of control platelets showing filopodial processes. The effect of MDC was similar in control platelets seeded

1
2
3 on surfaces coated with 50 $\mu\text{g}/\text{mL}$ fibrinogen or 33 $\mu\text{g}/\text{mL}$ type I collagen (results not
4 shown), but it was not observed in FXIII-A-deficient platelets (Fig. 5B).
5
6

7 Complementary video microscopy analysis suggests that the higher number of FXIII-
8 defective platelets emitting filopodia reflects a delay in the rate of spreading (Fig. 6).
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

Discussion

In the present work we failed to detect TG activity in four patients with diagnosis of plasma FXIII deficiency, which indicated that the defect was located in the A subunit of FXIII. Absence of TG activity in FXIII-deficient monocytes was previously reported [20]. Further immunofluorescence microscopy and western analysis of platelets revealed the absence of FXIII-A antigen, indicating that the molecular lesions affect mRNA expression and/or protein stability.

The Gly562Arg mutation identified in patient 1 had been previously described [21]. Although normally synthesized, FXIII-A carrying Gly562Arg was reported to rapidly disappear in transfected cells, suggesting an altered conformation leading to rapid degradation [21]. According to these data, platelets from patient 1 contained normal levels of mutant mRNA but absence of FXIII-A antigen. Patients 2 and 3 inherited two different alleles of the *FXIII A1* gene carrying the same mutation, IVS5-1G>A, identified also in a previously reported case [17], suggesting that this recurrent mutation has occurred more than once on a different genetic background. The mutation disrupts the AG splice acceptor site of intron 5 and activates a cryptic AG dinucleotide in the 5' sequence of exon 6, leading to the practical absence of normally spliced transcripts and the generation of reduced levels of a frame-shifted transcript due to deletion of the 7 first bases of exon 6. Moreover, the RT-PCR analysis detected similar levels of another mutant transcript carrying an additional deletion of the upstream exon 5. Mutations in splice-donor sequences result in skipping of the entire upstream neighboring exon, while alterations at a splice acceptor site cause aberrant splicing of the downstream exon [22]. As far as we know, this is the first report of an acceptor sequence mutation resulting in skipping of the upstream exon. Since the outcome of splice site mutations may reflect the order of intron removal [23,24], the abnormal outcome of the IVS5-1G>A mutation, together with the absence of mutant transcripts carrying only exon-5 deletion, suggests that utilization of the new acceptor site in exon 6 speeds removal of intron 5 with respect to intron 4. Although most of the mRNA is probably degraded due to premature termination of the frame-shifted transcript, the GGTCAG/ATGGCA sequence generated in the new 7-bp deleted RNA intermediate may be preferred as an alternative to the normally used atgcag/ATGATG at intron 4-exon 5 boundary, resulting in skipping of exon 5. Even though deletion of exon 5 restores the correct reading frame, the scarce amount of mRNA and/or the likely aberrant folding of the mutant

1
2
3 protein may unable its detection. In patient 4 we found compound heterozygosis for two
4 non-previously described mutations in exon 14 that result in normal amounts of two
5 different transcripts. Thus, the absence of immunodetectable FXIII-A in platelets is
6 probably due to the synthesis of misfolded polypeptides that are either unstable or
7 rapidly degraded.
8
9

10
11 Our results showing the practical absence of TG activity in FXIII-A deficient
12 platelets indicate that platelet TG activity depends, basically, on the presence of cFXIII.
13 Supporting this, TG2 mRNA levels were markedly reduced compared to those of FXIII-
14 A. Moreover, our data may also reveal a new difference between mouse and human
15 platelet. As established for the species differences in PAR isoforms used to mediate
16 thrombin signaling [25], human and mouse platelets may show a distinct relative
17 expression and/or regulation of the TG family members.
18
19

20
21 The functional implications of the high expression level of FXIII-A in platelets
22 remain poorly understood [8]. Our results suggest a role for cFXIII in regulating platelet
23 functions. A significant reduction of soluble fibrinogen binding in response to TRAP
24 was observed in FXIII-A-deficient platelets, suggesting a role of cFXIII in TRAP-
25 induced platelet activation. Supporting these data, the competitive amino-donor of
26 transglutaminases MDC was shown to block fibrinogen binding to control platelets
27 stimulated by TRAP. It should be noted that in these assays the effect of MDC might
28 also be due to competitive inhibition of plasma FXIII contained in the commercial
29 preparation of fibrinogen.
30
31

32
33 Platelet activation by most agonists results in a rise of free Ca^{2+} , due to both influx
34 across plasma membrane and release from internal stores. On the other hand, TG
35 activity has been reported to increase in cytoskeletal fractions of activated versus non-
36 activated platelets [26]. Therefore, calcium concentration in TRAP-stimulated platelets
37 could reach levels required for FXIII-A subunit activation. However, the apparent
38 normal platelet response mediated by PMA or ADP plus epinephrine suggests that other
39 mechanism could operate in platelets activated by TRAP. Increasing evidences suggest
40 that dimerization of G-coupled protein receptors (GPCRs) regulate many receptor-
41 specific functions [27]. TG activity of cFXIII has been shown to be required for AT_1
42 receptor dimerization, which correlated with enhanced monocyte adhesion to
43 endothelial cells at the onset of atherosclerosis [28]. Whether this mechanism could be
44 applied to other seven transmembrane domain receptors is unknown, but is unlikely that
45 it would be specific to AT_1 . Thus, the possibility exists that basal and/or stimulated
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 cFXIII transglutaminase activity plays a role in maintaining a level of protease-activated
4 receptor (PAR) dimerization that, in turn, modulated platelet response upon TRAP
5 stimulation.
6
7

8
9 Platelet FXIII has been proposed to negatively regulate α IIB β 3 integrin function in
10 collagen-adherent platelets [29]. The authors suggested that activation of platelet FXIII
11 is involved in a mechanism by which sustained increases in cytosolic calcium flux,
12 promoted by platelet adhesion to collagen, induce a switch from a pro-adhesive to a
13 procoagulant phenotype, named as sustained calcium-induced platelet (SCIP)
14 morphology, characterized by marked contraction of lamellipodial membranes,
15 microvesiculation and platelet fragmentation. In our experimental conditions we
16 observed a dose-dependent inhibitory effect of MDC on SCIP morphology in control as
17 well in FXIII-A-deficient platelets adhered to fibrinogen (results not shown), suggesting
18 that protein activities other than cFXIII may be involved in this process. In contrast, our
19 experiments revealed that normal platelet spreading on fibrinogen-coated surfaces
20 requires platelet expression and activity of cFXIII, suggesting a role for cFXIII in up-
21 regulating α IIB β 3 adhesive functions. These findings are consistent with the reported
22 interaction of cFXIII with specific cytoskeletal proteins upon activation of intact
23 platelets [26], and suggest an important role of cFXIII in the cytoskeletal remodeling
24 associated with the adhesive function and/or contractility of platelets. In line with this
25 observation, impaired phagocytosis has been described in monocytes from FXIII-
26 deficient patients [30].
27
28
29
30
31
32
33
34
35
36
37
38
39

40 The severity of bleeding symptoms due to plasma FXIII deficiency has made
41 difficult to determine whether patients with mutations in FXIII-A subunit have a
42 distinctive phenotype due to the concurrent lack of platelet cFXIII. The impaired
43 platelet response observed in FXIII-A-deficient patients in the present report suggests
44 that platelet dysfunction may represent a precipitating factor of bleeding in high-risk
45 situations. Additional work is required to determine the precise function of platelet
46 cFXIII. Moreover, it would be also of interest to define whether the apparently different
47 TG activity in human and mouse platelets may have some haemostatic impact.
48
49
50
51
52
53
54
55
56
57
58
59
60

References

- 1 Lorand L. Factor XIII and the clotting of fibrinogen: from basic research to medicine. *J Thromb Haemost* 2005; **3**: 1337-48.
- 2 Greenberg CS, Shuman MA. The zymogen forms of blood coagulation factor XIII bind specifically to fibrinogen. *J Biol Chem* 1982; **257**: 6096-101.
- 3 Siebenlist KR, Meh DA, Mosesson MW. Plasma factor XIII binds specifically to fibrinogen molecules containing gamma chains. *Biochemistry* 1996; **35**: 10448-53.
- 4 Polgar J, Hidasi V, Muszbek L. Non-proteolytic activation of cellular protransglutaminase (placenta macrophage factor XIII). *Biochem J* 1990; **267**: 557-60.
- 5 Muszbek L, Polgar J, Boda Z. Platelet factor XIII becomes active without the release of activation peptide during platelet activation. *Thromb Haemost* 1993; **69**: 282-5.
- 6 Muszbek L, Yee VC, Hevessy Z. Blood coagulation factor XIII: structure and function. *Thromb Res* 1999; **94**: 271-305.
- 7 Lorand L, Graham RM. Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nat Rev Mol Cell Biol* 2003; **4**: 140-56.
- 8 Adany R, Bardos H. Factor XIII subunit A as an intracellular transglutaminase. *Cell Mol Life Sci* 2003; **60**: 1049-60.
- 9 Puszkin EG, Raghuraman V. Catalytic properties of a calmodulin-regulated transglutaminase from human platelet and chicken gizzard. *J Biol Chem* 1985; **260**: 16012-20.
- 10 Muszbek L, Haramura G, Polgar J. Transformation of cellular factor XIII into an active zymogen transglutaminase in thrombin-stimulated platelets. *Thromb Haemost* 1995; **73**: 702-5.
- 11 Ichinose A. Physiopathology and regulation of factor XIII. *Thromb Haemost* 2001; **86**: 57-65.
- 12 Ozsoylu S, Hicsonmez G. Platelet aggregation in congenital factor XIII deficiency. *Acta Haematol* 1976; **56**: 314-7.
- 13 Mikkola H, Muszbek L, Laiho E, Syrjala M, Hamalainen E, Haramura G, Salmi T, Peltonen L, Palotie A. Molecular mechanism of a mild phenotype in coagulation factor XIII (FXIII) deficiency: a splicing mutation permitting partial correct splicing of FXIII A-subunit mRNA. *Blood* 1997; **89**: 1279-87.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
- 14 Egbring R, Seitz R, Gürten M. Bleeding complications in heterozygotes with congenital factor XIII deficiency. In: Mosesson M, Amrani D, Siebenlist K, Diorio J, eds. Fibrinogen: biochemistry, biological functions, gene regulation and expression: Amsterdam Elsevier Science, 1988, 341-6.
 - 15 Pabón D, Jayo A, Xie J, Lastres P, González-Manchón C. Thrombin induces GPIb-IX-mediated fibrin binding to α IIb β 3 in a reconstituted Chinese hamster ovary cell model. *J Thromb Haemost* 2006; **4**: 2238-47.
 - 16 Bellavite P, Andrioli G, Guzzo P, Arigliano P, Chirumbolo S, Manzato F, Santonastaso C. A colorimetric method for the measurement of platelet adhesion in microtiter plates. *Anal Biochem* 1994; **216**: 444-50.
 - 17 Vreken P, Niessen RW, Peters M, Schaap MC, Zuithoff-Rijntjes JG, Sturk A. A point mutation in an invariant splice acceptor site results in a decreased mRNA level in a patient with severe coagulation factor XIII subunit A deficiency. *Thromb Haemost* 1995; **74**: 584-9.
 - 18 Jobe SM, Leo L, Eastvold JS, Dickneite G, Ratliff TL, Lentz SR, Di Paola J. Role of FcR{gamma} and factor XIIIa in coated platelet formation. *Blood* 2005; **106**: 4146-51.
 - 19 Parameswaran KN, Velasco PT, Wilson J, Lorand L. Labeling of epsilon-lysine crosslinking sites in proteins with peptide substrates of factor XIIIa and transglutaminase. *Proc Natl Acad Sci U S A* 1990; **87**: 8472-5.
 - 20 Muszbek L, Adany R, Kawai M, Boda Z, Lopaciuk S. Monocytes of patients congenitally deficient in plasma factor XIII lack factor XIII subunit A antigen and transglutaminase activity. *Thromb Haemost* 1988; **59**: 231-5.
 - 21 Takahashi N, Tsukamoto H, Umeyama H, Castaman G, Rodeghiero F, Ichinose A. Molecular mechanisms of type II factor XIII deficiency: novel Gly562-Arg mutation and C-terminal truncation of the A subunit cause factor XIII deficiency as characterized in a mammalian expression system. *Blood* 1998; **91**: 2830-8.
 - 22 Krawczak M, Thomas NS, Hundrieser B, Mort M, Wittig M, Hampe J, Cooper DN. Single base-pair substitutions in exon-intron junctions of human genes: nature, distribution, and consequences for mRNA splicing. *Hum Mutat* 2007; **28**: 150-8.
 - 23 Takahara K, Schwarze U, Imamura Y, Hoffman GG, Toriello H, Smith LT, Byers PH, Greenspan DS. Order of intron removal influences multiple splice outcomes, including a two-exon skip, in a COL5A1 acceptor-site mutation that results in

- 1
2
3 abnormal pro-alpha1(V) N-propeptides and Ehlers-Danlos syndrome type I. *Am J*
4 *Hum Genet* 2002; **71**: 451-65.
5
6
7 24 Attanasio C, David A, Neerman-Arbez M. Outcome of donor splice site mutations
8 accounting for congenital afibrinogenemia reflects order of intron removal in the
9 fibrinogen alpha gene (FGA). *Blood* 2003; **101**: 1851-6.
10
11
12 25 Kahn ML, Zheng YW, Huang W, Bigornia V, Zeng D, Moff S, Farese RV, Jr.,
13 Tam C, Coughlin SR. A dual thrombin receptor system for platelet activation.
14 *Nature* 1998; **394**: 690-4.
15
16
17 26 Serrano K, Devine DV. Intracellular factor XIII crosslinks platelet cytoskeletal
18 elements upon platelet activation. *Thromb Haemost* 2002; **88**: 315-20.
19
20
21 27 Terrillon S, Bouvier M. Roles of G-protein-coupled receptor dimerization. *EMBO*
22 *Rep* 2004; **5**: 30-4.
23
24
25 28 AbdAlla S, Lothar H, Langer A, el Faramawy Y, Quitterer U. Factor XIIIa
26 transglutaminase crosslinks AT1 receptor dimers of monocytes at the onset of
27 atherosclerosis. *Cell* 2004; **119**: 343-54.
28
29
30 29 Kulkarni S, Jackson SP. Platelet factor XIII and calpain negatively regulate integrin
31 alphaIIb beta3 adhesive function and thrombus growth. *J Biol Chem* 2004; **279**:
32 30697-706.
33
34
35 30 Sarvary A, Szucs S, Balogh I, Becsky A, Bardos H, Kawai M, Seligsohn U,
36 Egbring R, Lopaciuk S, Muszbek L, Adany R. Possible role of factor XIII subunit
37 A in Fc gamma and complement receptor-mediated phagocytosis. *Cell Immunol*
38 2004; **228**: 81-90.
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Legends to the figures

Fig. 1. Intracellular FXIII-A expression and transglutaminase activity in platelets.

(A) Platelet lysates from controls and plasma FXIII-deficient patients were incubated with biotinylated cadaverine in the absence or presence of EDTA or calcium, labelled in the figured as B, E and C, respectively, resolved by SDS-PAGE and blotted with peroxidase-conjugated avidin, as described in “Material and methods”. C: Control; P: Patient. (B) Control and FXIII-deficient platelets were seeded on fibrinogen coated coverslips, fixed and labelled with anti- β 3 and FXIII-A antibodies, and analyzed by epifluorescence microscopy. Bar: 20 μ m. (C) Western analysis of platelet FXIII-A in control and FXIII-A deficient platelets.

Fig. 2. Identification of mutations in the *F13A1* gene. (A) Direct sequencing of cDNA or genomic DNA (gDNA) amplification products of FXIII-A, showing homozygous Gly562Arg and IVS-1G>A in patients 1 and 2, respectively, and heterozygous Arg661Gln and G2045A in patient 4. (B) Dotted lines in the diagrams indicate the alternative splicing pattern induced by IVS6-2G>A and G2045A mutations in patients 2 and 4, respectively.

Fig. 3. Relative expression of FXIII-A and TG2 mRNA in control and FXIII-A deficient platelets. PCR-based relative quantification of FXIII-A and TG2 mRNA was carried out as described in “Materials and methods”. (A) The amplification products from control and patient-1 platelets and from HUVEC cells were resolved on agarose gels y visualized by ethidium bromide staining. The figure shows a representative determination. (B) The analysis was repeated three times using different amounts of template mRNA and amplification products in a linear range were quantified by densitometry.

Fig. 4. Agonist-induced fibrinogen binding to control and FXIII-A-deficient platelets. (A) Flow cytometry analysis of agonist-induced FITC-Fg binding to platelets from 10 control individuals and 4 FXIII-A-deficient patients. Washed platelets were stimulated with agonists for 5 min and, then, incubated with FITC-Fg as described in “Materials and methods”. In parentheses, the number of independent determinations. (B) Dose-dependent effect of MDC on TRAP-induced fibrinogen binding. Control

1
2
3 platelets were incubated with MDC at 37°C for 30 min and, then processed, as before.
4
5 The fluorescence values were calculated as products of the percent of gated positive
6
7 platelets and the value of the mean channel, and expressed as percent of TRAP effect in
8
9 the absence of MDC. Results are means \pm SD of determinations in, at least, platelets
10
11 from five individuals.
12

13
14 **Fig. 5. Effect of MDC on spreading of control and FXIII-A-deficient platelets.**

15
16 Washed platelets were seeded on glass coverslips coated with 3 μ g/mL fibrinogen and,
17
18 after 15 min, fixed and labelled with anti- β 3 moAb, and visualized as described in
19
20 “Materials and methods”. (A) Representative spreading pattern of control and FXIII-A-
21
22 deficient platelets. F: platelets forming filopodia; L: spread platelets forming
23
24 lamellipodia. Bar: 20 μ m. (B) Dose-dependent effect of MDC on the relative number of
25
26 filopodia- and lamellipodia-forming platelets from four controls and four FXIII-A-
27
28 deficient patients.
29

30
31 **Fig. 6. Real-time imaging of spreading of control and FXIII-A-deficient platelets**

32 **on fibrinogen.** Platelets from control and patient-1 were plated on 3 μ g/mL-coated
33
34 chambers and the adhesion-spreading process was monitored during 30 min using a
35
36 multidimensional microscope as described in “Materials and methods”. Observation of
37
38 a significant number of platelets revealed a delay in the rate of spreading of FXIII-A-
39
40 deficient platelets. The figure shows the behaviour of representative control and FXIII-
41
42 deficient platelets.
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

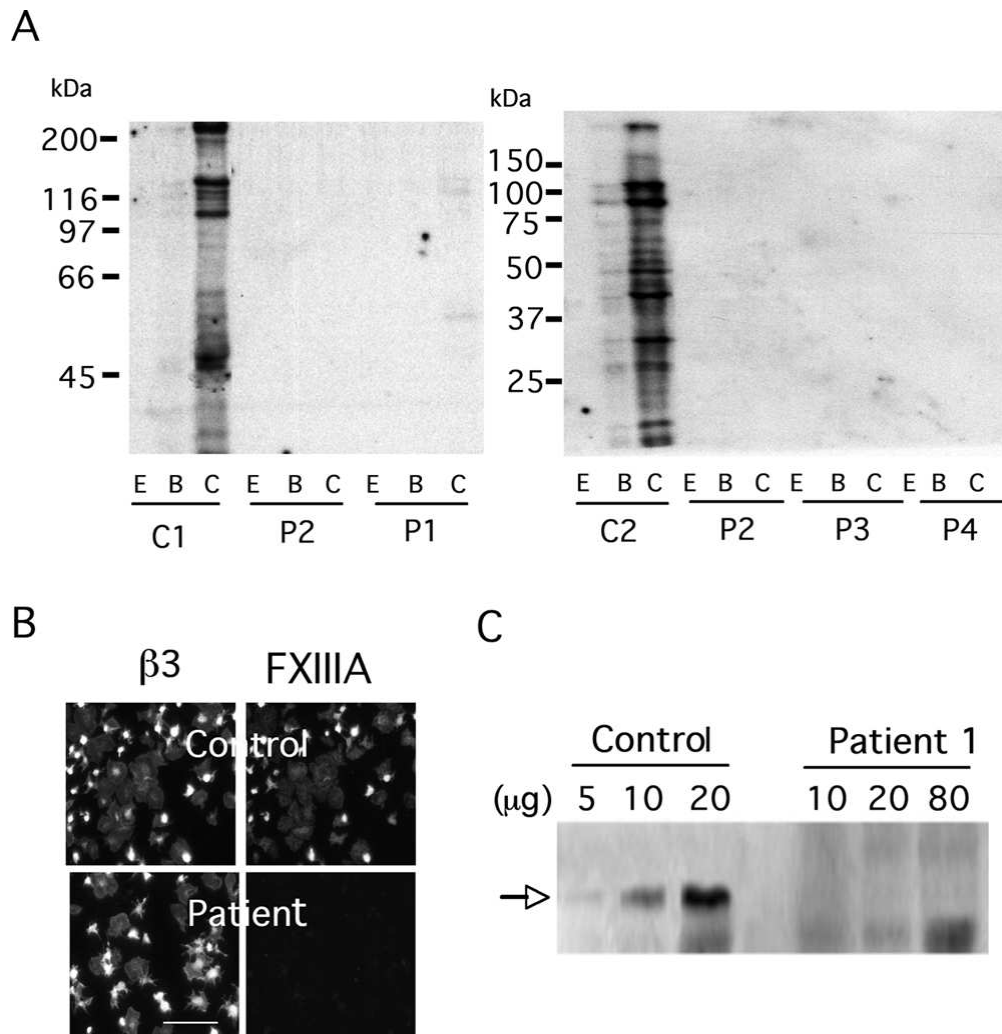


Fig. 1
84x86mm (300 x 300 DPI)



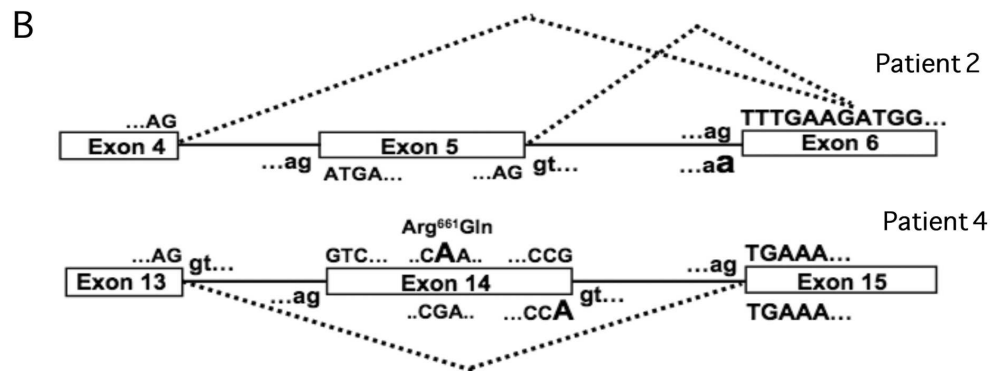
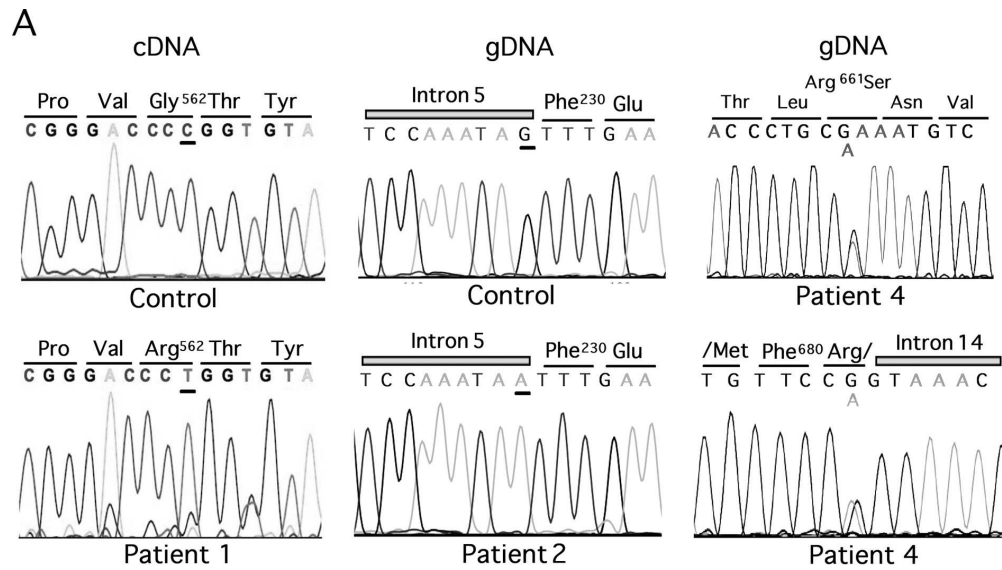
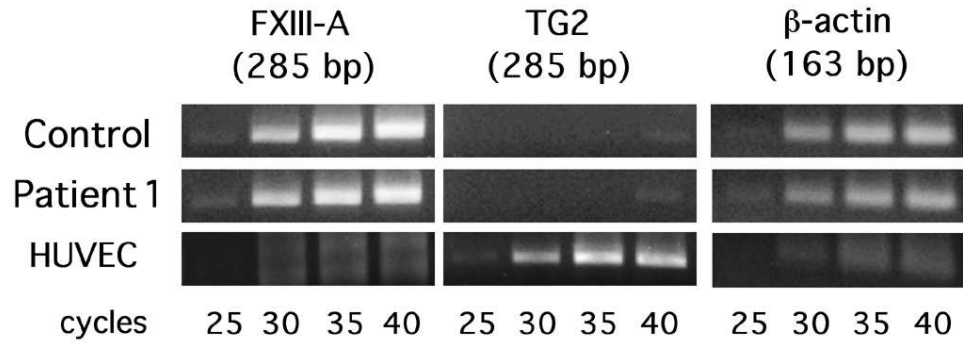


Fig. 2
120x117mm (400 x 400 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

A



B

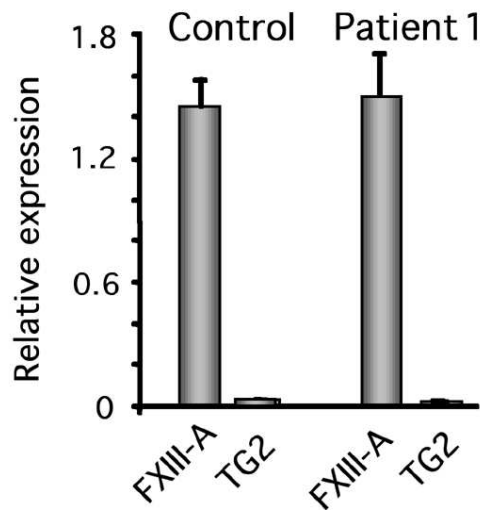
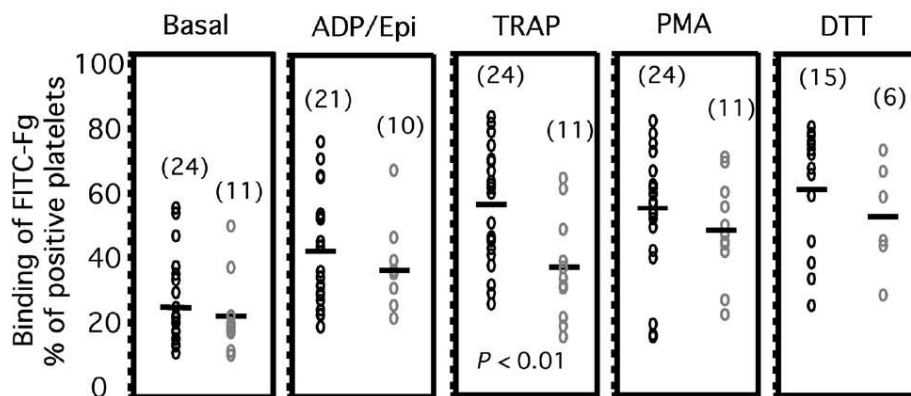


Fig. 3
90x91mm (300 x 300 DPI)

A



B

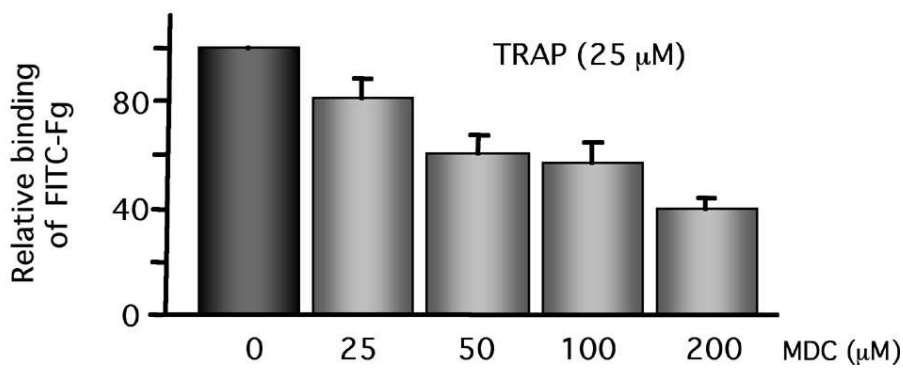


Fig. 4
90x82mm (300 x 300 DPI)



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

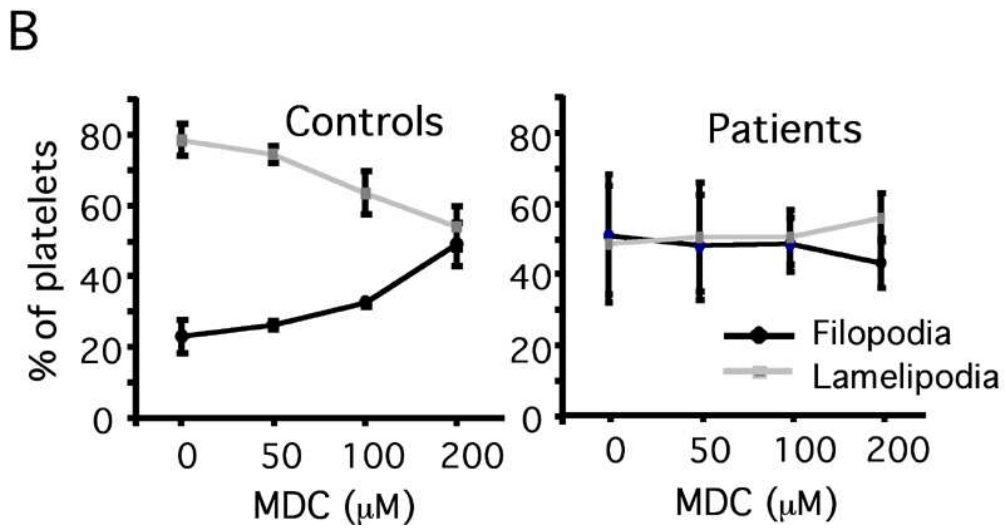
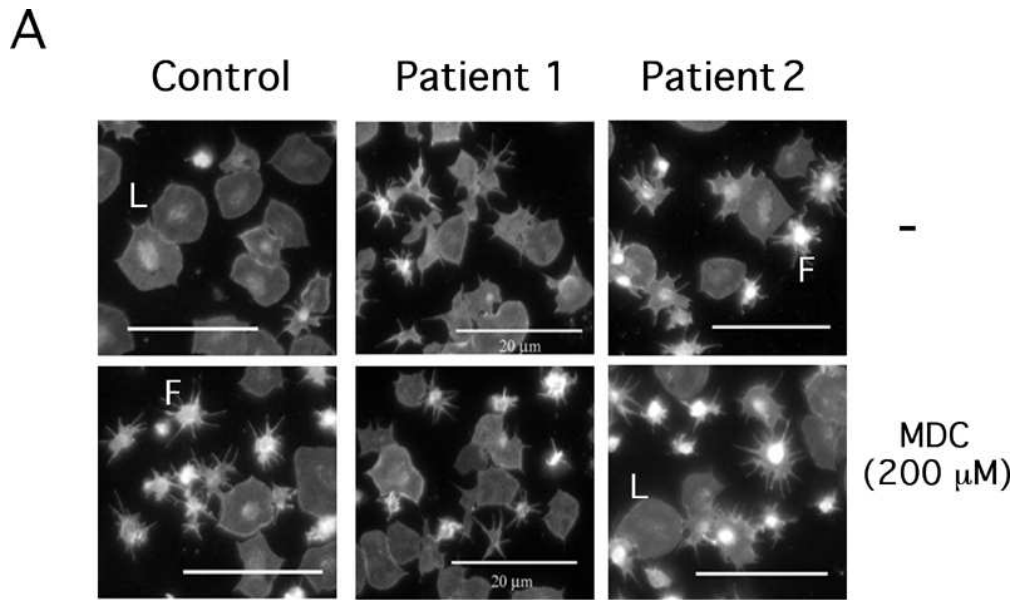


Fig. 5
89x105mm (225 x 225 DPI)

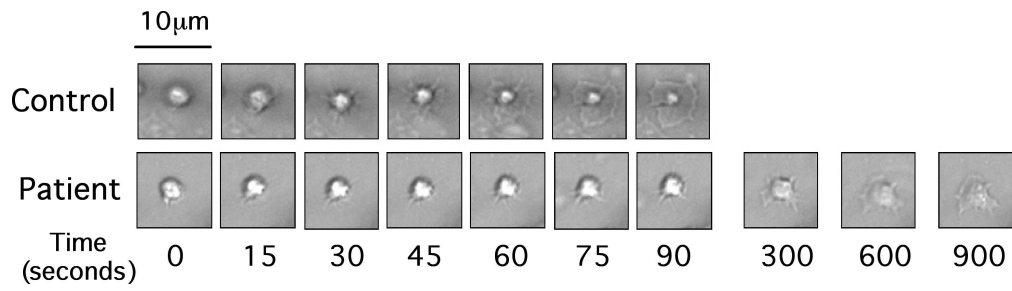


Fig. 6

For Peer Review