Ablation of collagen VI leads to the release of platelets with altered function

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Hemostatic abnormalities and impaired platelet function have been described in patients affected by collagen VI–related disorders and investigated the defects in platelet functionality, whose mechanisms are unknown. We demonstrated that megakaryocytes express collagen VI that is involved in the regulation of functional platelet production. By exploiting a collagen VI–null mouse model (Col6a1−/−), we found that collagen VI–null platelets display significantly increased susceptibility to activation and intracellular calcium signaling. Col6a1−/− megakaryocytes and platelets showed increased expression of stromal interaction molecule 1 (STIM1) and ORAI1, the components of store-operated calcium entry (SOCE), and activation of the mammalian target of rapamycin (mTOR) signaling pathway. In vivo mTOR inhibition by rapamycin reduced STIM1 and ORAI1 expression and calcium flows, resulting in a normalization of platelet susceptibility to activation. These defects were cell autonomous, because transplantation of lineage-negative bone marrow cells from Col6a1−/− mice into lethally irradiated wild-type animals showed the same alteration in SOCE and platelet activation seen in Col6a1−/− mice. Peripheral blood platelets of patients affected by collagen VI–related diseases, Bethlem myopathy and Ullrich congenital muscular dystrophy, displayed increased expression of STIM1 and ORAI1 and were more prone to activation. Altogether, these data demonstrate the importance of collagen VI in the production of functional platelets by megakaryocytes in mouse models and in collagen VI–related diseases.

Introduction

Collagen VI is an extracellular matrix protein that forms a distinct microfibrillar network and plays a remarkably broad range of key roles in different tissues. These include cytoprotection by counteracting apoptosis and oxidative damage, regulation of autophagy and cell differentiation, contribution to stem cell

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Data sharing requests should be sent to Alessandra Balduini (alessandra.balduini@unipv.it).

The full-text version of this article contains a data supplement.

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self-renewal and tissue regeneration, and promotion of tumor growth and progression.\textsuperscript{1} In humans, the major form of collagen VI is made up of 3 genetically distinct chains, \(\alpha 1(\text{VI}), \alpha 2(\text{VI})\), and \(\alpha 3(\text{VI})\), that are coded by the \textit{COL6A1}, \textit{COL6A2}, and \textit{COL6A3} genes, respectively.\textsuperscript{1} More recently, 3 additional genes (\textit{COL6A4}, \textit{COL6A5}, and \textit{COL6A6}) were identified that code for other collagen VI chains that can substitute for \(\alpha 3(\text{VI})\).\textsuperscript{2,3} Mutations in collagen VI genes in humans have been linked to a broad spectrum of muscular and neurological diseases, including Bethlem myopathy, Ullrich congenital muscular dystrophy (UCMD), congenital myosclerosis, and early-onset isolated dystonia.\textsuperscript{1,4} Different from other muscular dystrophies, collagen VI–related myopathies display remarkable alterations of the extracellular matrix of muscle and other connective tissues, including joints, tendons, and skin, and they can be considered hybrid disorders with clinical manifestations attributable to muscle and connective tissues.\textsuperscript{4}

The crucial roles exerted by collagen VI in vivo have been unveiled mainly by exploiting the collagen VI–null (Col6a1\textsuperscript{−/−}) mouse model, which was generated by targeted inactivation of the \textit{Col6a1} gene, resulting in the prevention of assembly and secretion of the entire platelet activation and aggregation, respectively.\textsuperscript{29-32} In addition to its role in Mk and platelet function, the PI3K/Akt/mTOR axis is pivotal in regulating Mk and platelets, STIM1 and ORAI1 have been demonstrated to drive activation of the intracellular Ca\textsuperscript{2+} channel ORAI1, which are activated upon depletion of the intracellular calcium (Ca\textsuperscript{2+}) homeostasis.\textsuperscript{6,7} The pathomolecular alterations characterizing the muscles of Col6a1\textsuperscript{−/−} mice have recently been found in other cells and tissues.\textsuperscript{1,8-10}

Some studies and case reports have described hemostatic abnormalities and platelet function defects in patients affected by heritable connective tissue disorders, such as collagenopathies.\textsuperscript{11-13} When assessing the blood parameters of patients affected by Bethlem myopathy and UCMD, we observed a mild bleeding tendency, suggesting a defect in platelet functionality. These findings led us to broaden the exploration of the defects caused by collagen VI mutations in the circulatory system.

Intracellular Ca\textsuperscript{2+} signaling is a fundamental regulator of megakaryocyte (Mk) and platelet function through modulation of several intracellular pathways.\textsuperscript{1,6-18} Store-operated calcium entry (SOCE) is a well-described mechanism regulating Ca\textsuperscript{2+} entry from the extracellular space following endoplasmic reticulum (ER) store depletion.\textsuperscript{18} We and other investigators have previously described the expression and function of SOCE in Mks and platelets.\textsuperscript{16,20-22} Mks express the 2 molecular components of SOCE, the ER Ca\textsuperscript{2+} sensor stromal interaction molecule 1 (STIM1) and the plasma membrane Ca\textsuperscript{2+} channel ORAI1, which are activated upon depletion of the intracellular Ca\textsuperscript{2+} pool. \textsuperscript{16} Ca\textsuperscript{2+} mobilization from the intracellular stores promotes Mk function and proplatelet extension.\textsuperscript{16} In platelets, STIM1 and ORAI1 have been demonstrated to drive activation processes in response to agonists.\textsuperscript{15,23-25} The Phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) axis is an intracellular signaling pathway enhanced by SOCE.\textsuperscript{26} In turn, mTOR regulates the expression of SOCE components.\textsuperscript{27,28} The PI3K/Akt/mTOR axis plays a pivotal role in regulating Mk and platelet function by controlling Mk proliferation and differentiation and platelet activation and aggregation, respectively.\textsuperscript{29-32} In addition to its role in Mk and platelet function, the PI3K/Akt/mTOR axis is known to be a crucial regulator of collagen I expression.\textsuperscript{33,34}

Here, we exploited the collagen VI–null mouse model by generating Mk culture and radiation chimeras using bone marrow (BM) lineage-negative (Lin\textsuperscript{−}) cell transplantsations. We also investigated platelet function in Col6a1\textsuperscript{−/−} mice and in samples from patients affected by collagen VI–related disorders.

Methods

Commercial antibodies used are listed in supplemental Table 1. Primers for human and mouse collagen VI are listed in supplemental Table 2.

Information about reagents, platelet preparation, platelet aggregation, reverse transcription polymerase chain reaction (RT-PCR), western blotting, intracellular Ca\textsuperscript{2+} measurements in platelets, tissue collection and immunofluorescence, and BM transplant are provided in supplemental materials.

Cell cultures

Human Mks were obtained by differentiating cord blood–derived CD34\textsuperscript{+} or peripheral blood–derived CD45\textsuperscript{−} hematopoietic progenitors, as described previously.\textsuperscript{35,36} Mouse Mks were obtained by differentiating BM cells for 4 days in Dulbecco’s modified Eagle medium ( Gibco) supplemented with 1% penicillin/streptomycin, 1% l-glutamine, and 10% fetal bovine serum (Gibco), in the presence of 10 ng/mL recombinant murine thrombopoietin (PeproTech). On day 4, mature Mks were isolated by bovine serum albumin gradient sedimentation. For in vitro treatment with the propyl-4 hydroxylase inhibitor ethyl 3,4-dihydroxybenzoate (EDHB), Mks were cultured for 24 hours with 100 \(\mu\)M EDHB or with vehicle alone (0.1% ethanol). For in vitro treatment with ascorbic acid, Mks were cultured in the presence of 50 \(\mu\)g/mL ascorbic acid for 48 hours. For in vitro treatment with rapamycin, BM cells were cultured at day 0 for 4 days in the presence of 100 nM rapamycin or 0.1% dimethyl sulfoxide (DMSO) as vehicle control.

Flow cytometry

Stim1 and ORAI1 staining. To analyze STIM1 and ORAI1 in BM Mks, femurs were flushed, and red blood cells were lysed with 0.8% ammonium chloride solution. The remaining cells were washed by centrifugation with phosphate-buffered saline and stained with anti-ORAI1 antibody (1:100; Santa Cruz Biotechnologies) or anti-STIM1 antibody (1:100; Abcam). For STIM1 staining, cells were permeabilized with commercial buffers (BD Pharmingen) prior to staining with the antibody. To recognize BM Mks, we costained cells with an anti-CD41 antibody (0.1 mg/mL; BioLegend). All Mk samples were characterized as CD41/CD42b-positive and CD3/CD4/CD8/CD11b/CD19/CD33-negative cells, using appropriate antibodies (0.1 mg/mL; all from Beckman Coulter). Samples were acquired with a FACSDiva flow cytometer (Beckman Coulter). The analytical gating was set using unstained samples and relative isotype controls. Offline data were analyzed using the Beckman Coulter Kaluza software package (Beckman Coulter) and Flowing software 2.5.1 (University of Turku).

BM Mk sorting. Mks were sorted from BM cells as the CD11b\textsuperscript{−}/CD61\textsuperscript{−}/CD9\textsuperscript{+} population.\textsuperscript{37} The gating strategy is shown in supplemental Figure 9. Mk purity after sorting, by means of CD61\textsuperscript{+} /CD9\textsuperscript{−}/CD41\textsuperscript{+}, was routinely performed and assessed to be >95%. Cell viability was checked using Trypan Blue solution (Sigma Aldrich). Cell sorting experiments were performed using a
Platelet activation. For platelet activation, 2 x 10^8 platelets washed in modified Tyrode’s buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 29 mM KCl, 12 mM NaHCO_3, 20 mM HEPEs, 5 mM glucose) containing 1 mM CaCl_2 were incubated with 0.1 U/mL thrombin (Sigma-Aldrich), 25 μM adenosine diphosphate (ADP; Sigma-Aldrich), 20 ng/mL convulxin (Enzo Life Sciences), or vehicle alone in the presence of 2 μg/mL JON/A-PE (Emfret Analytics), 12 μg/mL PAC-1-FITC (BD Biosciences), or 10 μg/mL anti-CD62P-APC (BioLegend) and analyzed 10 minutes later.39 Platelet activation was expressed as the ratio between the mean fluorescence intensity measured after the stimulation with each agonist and the mean fluorescence intensity measured after incubation with vehicle alone.39

Intracellular Ca^{2+} measurements in Mks

To study SOCE, Mks were treated with 10 mM cyclopiazonic acid (CPA) in Ca^{2+}-free (Ca^{2+} 0) solution to deplete the stores. Similar to thapsigargin treatment, CPA treatment causes a transient increase in the intracellular Ca^{2+} concentration ([Ca^{2+}]), due to the passive emptying of Ca^{2+} stores. After Ca^{2+} levels return to baseline, external Ca^{2+} is restored to 1.5 mM, causing a second increase in intracellular Ca^{2+} levels due to activated SOCE. As an internal control, Mks were treated with vehicle alone (0.03% volume-to-volume ratio of DMSO), which did not elicit any significant increase in [Ca^{2+}]. (supplemental Figure 10). Intracellular Ca^{2+} measurements in Mks were performed as previously described.16,40 For a detailed description, please refer to supplemental Methods.

Mice and in vivo treatments

We performed experiments in wild-type (WT) mice of the inbred C57BL/6NCr strain and in Col6a1^{−/−} mice that we previously backcrossed on the C57BL/6NCr strain for 8 generations, as previously described.7 We obtained data for 3-month-old mice by crossing sex-matched WT and COL6A1^{−/−} animals. Mouse procedures were approved by the Italian Ministry of Health (approvals #282-2017 and #877-2018). In situ hybridization and the Declaration of Helsinki. Every patient provided written informed consent. Mouse procedures were approved by the Italian Ministry of Health (approvals #282-2017 and #877-2018).

Statistics

Data are expressed as mean ± standard deviation (SD) or standard error of the mean (SEM). A 2-tailed Student t test was used for comparisons between 2 groups. For comparisons of 1 factor across multiple groups, 1-way ANOVA was performed followed by the post hoc Tukey test. GraphPad Prism 8 (GraphPad Software) was used for statistical analyses and graphing. P values < .05 were considered statistically significant. All experiments were independently replicated 3 times.

Results

Human and mouse Mks express collagen VI

To investigate collagen VI expression, human Mks were differentiated from cord blood, whereas mouse Mks were derived from BM progenitors. As shown in Figure 1, Mks of human and murine origin expressed the 3 major collagen VI chains (α1, α2, α3) at RNA (Figure 1A) and protein (Figure 1B) levels. To confirm the specificity of the protein bands, Mks were treated for 24 hours with the prolyl-4 hydroxylase inhibitor EDH3.48 This treatment reduced the synthesis of collagen VI chains in human and murine Mks cultures with respect to treatment with vehicle alone (Figure 1B). Mature murine Mks cultured in the presence of ascorbic acid were able to secrete collagen VI, which localized around the cell membrane and was also detectable in the culture medium by immunoblotting (Figure 1C-D).

Platelets from collagen VI-null mice are more susceptible to activation

We studied the ex vivo functionality of platelets from WT and Col6a1^{−/−} mice. The absence of the collagen VI α1 chain in Col6a1^{−/−} Mks was confirmed by western blot analysis (supplemental Figure 1). Collagen VI-null platelets showed an increased tendency toward activation and aggregation. Platelets from Col6a1^{−/−} mice exhibited increased levels of activated integrin αIIbβ3 and CD62P (P-selectin) exposure on the cell membrane after stimulation with thrombin, ADP, or the GPVI collagen receptor agonist convulxin (CVX) (Figure 2A). On the other hand, the expression levels of integrin αIIbβ3, PAR-4, P2Y12, and GPVI were not different between WT and Col6a1^{−/−} platelets (supplemental
Table 1. Summary of the clinical features, genetic characterization, and International Society on Thrombosis and Hemostasis-Bleeding Assessment Tool for patients with Bethlem myopathy or UCMD included in this study

| Onset | Patient 1/F | Patient 2/M | Patient 3/M | Patient 4/F | Patient 5/F |
|-------|-------------|-------------|-------------|-------------|-------------|
| Age   | Adolescence | Neonatal    | Neonatal    | Adolescence | Childhood   |
| Symptoms | Difficulties in sport | Hypotonia, respiratory distress | Hypotonia, finger contractures | Shoulder and knee dislocation | Slower than peers |
| Last evaluation | | | | | |
| Age | 20 | 20 | 18 | 47 | 54 |
| Best motor function | Run | Run | Walk | Walk | |
| COL6A mutations | COL6A3: c.5035G>T; p.Gly1679Trp | COL6A1: c.930 + 1890C>T, intron 11 (pseudohomozygosity) | COL6A3: c.4859C>T; p.Pro1620Leu | COL6A3: c.5035G>T; p.Gly1679Trp | COL6A3: c.5035G>T; p.Gly1679Trp |
| Platelet count (×10^9/L)* | 251 | 269 | 212 | 267 | 237 |
| Bleeding phenotype | Menorrhagia, mild trauma-related bruises | Not applicable | Not applicable | Menorrhagia, mild trauma-related bruises, mild oral cavity bleeding, postpartum hemorrhage | Menorrhagia, mild trauma-related bruises, mild oral cavity bleeding |
| ISTH-BAT† | 2 | 0 | 0 | 4 | 7 |

AD, autosomal dominant; BetMy, Bethlem myopathy; ISTH-BAT, International Society on Thrombosis and Hemostasis-Bleeding Assessment Tool; WCB, wheelchair bound.

*Normal value: 200 to 450 ×10^9/L.

Figure 2. Col6a1−/− platelets displayed more avidity for adhesion to collagen I and fibrinogen than did WT platelets (supplemental Figure 3). In vitro Col6a1−/− platelet stimulation with the platelet agonists collagen, thrombin, or ADP plus adrenaline led to a robust amplification of the aggregation, thus confirming their hyperreactive behavior (Figure 2B).

Collagen VI-null platelets and Mks have increased levels of SOCE effectors

Platelet activation is directly dependent on the increase in [Ca^{2+}]_{i} due to SOCE activation.21 To assess whether the alterations observed in Col6a1−/− platelets may depend on changes in the molecular components of SOCE, we analyzed the expression of STIM1, the ER Ca^{2+} sensor, and ORAI1, a plasma membrane Ca^{2+} channel, which detect any significant decrease in ER Ca^{2+} levels and mediate the ensuing extracellular Ca^{2+} entry, respectively.19 The protein levels of both SOCE effectors were significantly increased in circulating platelets from Col6a1−/− mice compared with WT mice (Figure 3A). We exposed cells to thapsigargin to evaluate the impact of increased STIM1 and ORAI1 expression on platelets, we sorted BM Mks and analyzed the expression of STIM1 and ORAI1 transcripts. We found that the levels of both messenger RNAs (mRNAs) were significantly increased in BM Mks from Col6a1−/− mice compared with WT mice (Figure 3C). These data were further supported by the analysis of STIM1 and ORAI1 protein levels in BM Mks by flow cytometry (Figure 3Cii). The increase in STIM1 and ORAI1 expression in Col6a1−/− Mks resulted in an elevation in SOCE-dependent Ca^{2+} signaling. In the absence of extraacellular Ca^{2+}, treatment of Mks with CPA, an inhibitor of SERCA that is structurally unrelated to thapsigargin, induced mobilization of Ca^{2+} from the intracellular stores that was comparable between WT and Col6a1−/− Mks. Conversely, the addition of extracellular Ca^{2+} in the presence of CPA elicited a significant increase in Ca^{2+} entry from the extracellular space in Col6a1−/− Mks (Figure 3D), thus confirming the increase in SOCE.

Altered mTORC1 signaling in collagen VI-null Mks and platelets

It was demonstrated previously that the mTOR signaling axis regulates Mk growth and platelet activation,29–31 as well as STIM1 and ORAI1 expression.27,28 Based on this information, we investigated the activation of Akt and the downstream effectors of the mTORC1 signaling complex, S6 and 4E-BP1, in mouse BM Mks and circulating platelets. All of these proteins were significantly more phosphorylated in BM Mks (Figure 4A; supplemental Figure 4) and resting platelets (Figure 4B) derived from Col6a1−/− mice compared with WT mice.

Collagen VI-null Mks and platelets maintain SOCE alterations after BM transplantation in WT recipient mice

We investigated whether the alterations in SOCE expression and function in Col6a1−/− Mks and platelets were due to the lack of collagen VI in the BM environment or to the intrinsic absence of self-produced collagen VI in Mks. Lin− BM cells isolated from WT and Col6a1−/− donor mice were transplanted into lethally irradiated WT recipient mice. All mice except 1 survived the transplant and recovered their body weight with no signs of clinical suffering. All analyzed mice restored their white blood cell count, indicating a full engraftment of transplanted cells. Mks were then sorted from BM
and analyzed. Col6a1−/− Mks maintained increased expression of STIM1 and ORAI1, as demonstrated by quantitative RT-PCR (qRT-PCR) and flow cytometry (Figure 5A). These alterations were transferred to platelets, as indicated by western blot for STIM1 and ORAI1 (Figure 5B). Also, platelets isolated from WT mice that were transplanted with Col6a1−/− Lin− BM cells showed increased levels of phosphorylated (p)S6 and p4E-BP1 (supplemental Figure 5). Platelets produced by Col6a1−/− Mks in a WT BM environment maintained the increased susceptibility to activation, relative to WT platelets, as demonstrated by αIibβ3 activation and CD62P (P-selectin) exposure on the cell membrane after stimulation with major agonists (thrombin, ADP, and CVX) (Figure 5C).

Rapamycin treatment normalizes STIM1 and ORAI1 expression and SOCE

To test whether the increased expression of SOCE effectors was directly dependent on mTORC1 signaling, we cultured flushed BM cells from WT and Col6a1−/− mice in the presence of thrombopoietin to induce Mk differentiation and treated them
with the mTOR inhibitor rapamycin or with vehicle alone. Mks were purified by gradient sedimentation and lysed. Western blot analysis confirmed higher protein levels of STIM1 and ORAI1 in Col6a1<sup>-/-</sup> Mks (Figure 6A). Rapamycin treatment induced a significant reduction in STIM1 and ORAI1 levels (Figure 6A), with a consequent reduction in CPA-induced SOCE activation (Figure 6B) that was similar in WT and Col6a1<sup>-/-</sup> Mks following rapamycin treatment. mTOR signaling was assessed in vivo by injecting mice with rapamycin or vehicle. In vivo, rapamycin treatment resulted in a significant reduction in pS6 and p4E-BP1 in Col6a1<sup>-/-</sup> platelets to a level comparable to WT platelets under resting conditions and after stimulation with thrombin (supplemental Figure 6). In vivo, rapamycin administration also resulted in a normalization of STIM1 and ORAI1 protein expression in Col6a1<sup>-/-</sup> BM Mks (Figure 6C; supplemental Figure 7) and platelets (Figure 6D). The reduction in the expression of SOCE effectors resulted in a significant decrease in platelet activation upon thrombin stimulation, detected by active integrin αIIbβ3, to a level comparable to WT platelets (Figure 6E). Rapamycin treatment also normalized the peripheral blood platelet count of Col6a1<sup>-/-</sup> mice, which showed a mild thrombocytopenia (Figure 6F).

Platelets derived from patients harboring collagen VI mutations show increased levels of SOCE effectors

We analyzed peripheral blood platelets derived from healthy subjects and patients affected by Bethlem myopathy and UCMD of intermediate severity (Table 1). The phosphorylation of S6 ribosomal protein and of 4E-BP1 appeared significantly increased in platelets isolated from patients, relative to those isolated from healthy subjects (Figure 7A). Further, the amounts of STIM1 and ORAI1 proteins were increased in patient platelets compared with healthy subjects (Figure 7B). Additionally, when we tested platelet activation in response to thrombin stimulation, we observed increased activation of integrin αIIbβ3 in patients compared with healthy subjects (Figure 7C).

Discussion

The release of functional platelets from Mks is regulated by environmental and autocrine factors. In previous studies, we demonstrated that Mks express different extracellular matrix components that regulate platelet production. Here, we demonstrated that
Figure 3. *Col6a1**/** platelets and Mks have increased STIM1 and ORAI1 expression and increased SOCE.* (Ai) Western blot for STIM1 and ORAI1 in WT and *Col6a1**/** platelets. β-Actin was used as a loading control. (Aii) Band densities were quantified and expressed relative to WT. Data are mean ± SD (n = 6). Student t test. (Bi) Representative FLUO3 fluorescence–based [Ca**2⁺**]i traces of WT (red line) and *Col6a1**/** (blue line) platelets incubated in Ca**2⁺**0 conditions. Where indicated, successive additions of 5 μM thapsigargin (TG) and 1 mM Ca**2⁺** were carried out. (Bii) Quantification of basal and peak [Ca**2⁺**]i following the addition of thapsigargin (ER release) and Ca**2⁺** (SOCE) in WT and *Col6a1**/** platelets. Data are mean ± SEM (n = 5). Student t test. (Ci) Quantitative RT-PCR analysis of STIM1 and ORAI1 mRNA expression in sorted BM Mks from WT and *Col6a1**/** mice. Data are mean ± SD (n = 3). Student t test. (Cii) Flow cytometry analysis of STIM1 and ORAI1 protein levels in BM Mks from WT and *Col6a1**/** mice. Data are mean ± SD (n = 4). Student t test. (Di) Representative Fura-2 fluorescence ratio reflecting [Ca**2⁺**]i in WT and *Col6a1**/** differentiated Mks. [Ca**2⁺**]i variations were monitored in the presence of 10 μM CPA in Ca**2⁺**0 and after addition of 1.5 mM extracellular Ca**2⁺**. (Dii) Analysis of Ca**2⁺** flows (ER release and SOCE) in WT and *Col6a1**/** Mks. Data are mean ± SEM (n = 4). Student t test. *P < .05, **P < .01.
human and mouse Mks express collagen VI, as confirmed by 3 recent studies. In this study, we took advantage of a collagen VI-null mouse model and found that Col6a1−/− platelets display a hyperactivation tendency, as revealed by different in vitro tests that we conducted. Based on our results, this phenotype is due to an intrinsic platelet defect because the platelet function tests were performed on washed platelets that were devoid of extrinsic factors involved in platelet activation and aggregation (eg, plasma, interaction with other cells, blood flow rate). Platelet activation and aggregation are directly dependent on an increase in [Ca^{2+}]. The SOCE mechanism has a major role in regulating intracellular Ca^{2+} content in platelets through the coordinated action of its main effectors STIM1 and ORAI1. Contrary to platelets derived from mice with a constitutive activation of Stim1 in Col6a1−/− platelets we observed a normal basal level of cytosolic calcium. Also, the level of Ca^{2+} released by the ER, upon SERCA inhibition, in Col6a1−/− platelets was normal. In collagen VI-null platelets, the increased expression of STIM1 and ORAI1 resulted in a higher level of cytosolic Ca^{2+} influx from the extracellular space upon SOCE activation. The consequent increase in [Ca^{2+}], leads to increased platelet reactivity.

We previously proved that Mks express STIM1 and ORAI1 and that an increase in [Ca^{2+}], prompted by SOCE activation is crucial to promote proplatelet extension and platelet release. In this study, we found overexpression of STIM1 and ORAI1 and increased SOCE in Col6a1−/− Mks, indicating that the defects displayed in collagen VI-null platelets may originate from Mks.

It was demonstrated previously that STIM1 and ORAI1 expression is under the control of the mTOR pathway. A distinctive signaling defect found in Col6a1−/− tissues is the hyperactivation of the Akt/mTOR pathway, which, in turn, results in autophagy deregulation. mTOR includes 2 complexes: mTORC1 and mTORC2. The mTOR pathway was proven to positively regulate platelet activation and aggregation, as well as thrombus formation and platelet aggregate stability, by regulating protein synthesis.

Constitutive mTOR activation stimulates mitochondrial biogenesis and function and, consequently, reactive oxygen species (ROS) production. Higher levels of platelet ROS are associated with increased platelet activation, and ROS scavenger administration reduces platelet activation in aged mice, which are characterized by increased mTOR signaling activity. In addition, a reciprocal interplay between SOCE and mitochondria has been reported because cytosolic calcium oscillations are decoded in the mitochondria. Thus, the increase in cytosolic calcium upon SOCE activation, as...
observed in Col6a1<sup>2/2</sup> Mks and platelets, might result in an imbalance in intracellular Ca<sup>2+</sup> dynamics between organelles deputed to Ca<sup>2+</sup> handling. Future studies will unravel whether STIM1 and ORAI1 overexpression may interfere with the mitochondrial function in Col6a1<sup>2/2</sup> Mks.

To understand whether the endogenous Mk self-produced collagen VI was the principal regulator of functional platelet production, we produced radiation chimeras by transplanting BM Lin<sup>2</sup> cells from Col6a1<sup>2/2</sup> mice into WT recipients. At 7 to 8 weeks posttransplant, when peripheral blood count was recovered, BM sorted Col6a1<sup>2/2</sup> Mks showed increased expression of STIM1 and ORAI1, despite having undergone differentiation in the presence of collagen VI in the BM environment. As in Col6a1<sup>2/2</sup> mice, STIM1 and ORAI1 overexpression in Mks resulted in the production of platelets with increased susceptibility to activation and overexpressing pS6 and...
Figure 6. In vitro and in vivo rapamycin treatment normalizes STIM1 and ORAI1 expression and SOCE in Col6a1−/− Mks. (A) Western blot for STIM1 and ORAI1 in WT and Col6a1−/− differentiated Mks, in the presence of 100 nM rapamycin (Rapa) or vehicle alone as control (0.1% DMSO). β-Actin was used as a loading control. (Ai) Band intensities were quantified and expressed relative to control. Data are mean ± SD (n = 5). 1-way ANOVA. (B) Representative Fura-2 fluorescence ratios reflecting [Ca^{2+}]i variations in WT and Col6a1−/− differentiated Mks, in the presence of 100 nM Rapa or vehicle alone. Ca^{2+} flows were monitored in the presence of 0.1 nM CPA in Ca^{2+}0 conditions and after the addition of 1.5 mM extracellular Ca^{2+}. (Bi) Analysis of Ca^{2+} flows (ER release and SOCE) in WT and Col6a1−/− Mks. A minimum of 40 Mks were analyzed per experiment. Data are mean ± SEM (n = 4). 1-way ANOVA. (C) Flow cytometry analysis of STIM1 and ORAI1 protein expression in BM Mks from WT and Col6a1−/− mice treated with Rapa (2 mg/kg body weight) or vehicle as control (5% PEG-400/5% Tween-80 in saline). Data are mean ± SD (n = 5). 1-way ANOVA. (D) Western blot for STIM1 and ORAI1 in platelets from WT and Col6a1−/− mice treated with Rapa or vehicle as control. (Di) Band densities were quantified and expressed relative to control. Data are mean ± SD (n = 5). 1-way ANOVA. (E) Flow cytometry analysis of integrin αIIbβ3 activation (JON/A antibody binding) in WT and Col6a1−/− platelets, after stimulation with thrombin (0.1 U/mL), from mice treated with Rapa or vehicle as control. Data are mean ± SD (n = 5). 1-way ANOVA. (F) Peripheral blood platelet (Plt) count in WT and Col6a1−/− mice following administration of Rapa or vehicle as control (n = 3). Data are mean ± SD (n = 3). 1-way ANOVA. *P < .05, **P < .01. CTRL, control; MFI, mean fluorescence intensity.
These results proved that the Mk self-produced collagen VI plays a significant role in the production of functional platelets, despite the composition of the BM extracellular matrix environment.

mTOR may have an immediate effect on thrombus formation and stability through a mechanism independent from mRNA translation. Our data show that in vitro and in vivo inhibition of the mTOR signaling by rapamycin treatment restores STIM1 and ORAI1 expression and SOCE in Col6a1/2 Mks, leading to a normalization of the platelet activation response. Platelet function abnormalities have been described in patients affected by connective tissue disorders, such as collagenopathies; however, the mechanisms underlying these defects remain unknown. We revealed that peripheral blood platelets of patients affected by collagen VI-related diseases express higher levels of STIM1 and ORAI1, resulting in a hyperactivation state. Interestingly, platelet count was normal in all of the analyzed patients.

No platelet alterations were previously reported for Bethlem myopathy or UCMD. On the other hand, platelet abnormalities are well known in patients harboring gain-of-function mutations in ORAI1 or STIM1 and displaying a multisystemic clinical phenotype that is characterized by muscle weakness, with a clinical spectrum ranging from isolated tubular aggregate myopathy to the more complex Stormorken syndrome (STRMK) or York syndrome. A characteristic feature of STRMK is a mild to pronounced thrombocytopenia and thrombocytopathy. Platelets from patients with STRMK were found to be in an activated state, with increased levels of CD63 and CD62P, whereas other aspects of platelet function are impaired. Despite platelet activation and increased SOCE, no thrombotic episodes were observed in patients with STRMK, with only 1 patient presenting with pulmonary embolism and thrombosis. While, it has been observed a rather common bleeding tendency, possibly related to thrombocytopenia and abnormal platelet function. Patients with STRMK or with collagen VI-related diseases showed increased platelet activation, which may suggest an increased thrombotic predisposition. A mild bleeding tendency, which is well established in STRMK, may also be present in patients affected by collagen VI-related myopathies (Table 1). Consistent with this hypothesis, we observed a significantly prolonged

**Figure 7.** Increased STIM1 and ORAI1 expression in platelets derived from patients affected by collagen VI-related disorders. (A) Representative western blot of phosphorylated (p) S6 protein and 4E-BP1 protein in peripheral blood platelets of healthy subjects (HS; n = 3) and patients (P) with Bethlem myopathy/UCMD (n = 5). (Ai) Band densities were quantified and expressed relative to HS. Data are mean ± SD. Student t test. (B) Representative western blot of STIM1 and ORAI1 protein expression in peripheral blood platelets from HS (n = 3) and P with Bethlem myopathy/UCMD (n = 5). (Bi) Band densities were quantified and expressed relative to HS. Data are mean ± SD. Student t test. (C) Flow cytometry analysis of integrin αIIbβ3 activation (PAC-1 antibody binding) in peripheral blood platelets from HS (n = 3) and P with Bethlem myopathy/UCMD (n = 5) after stimulation with thrombin (0.5 U/mL). Data are mean ± SD. Student t test. ~, patient 1/F; ▲, patient 2/M; ●, patient 3/M; ▼, patient 4/F; ▲, patient 5/F. Student t test. *P < .05. MFI, mean fluorescence intensity.
bleeding time in Col6a1−/− mice with respect to WT mice in a tail bleeding assay (supplemental Figure 8). This apparent paradox could be explained, in part, by considering the documented key role of collagen VI on platelet adhesion and aggregation under low and high shear conditions. By linking the subendothelial basement membrane to the interstitial collagen network, collagen VI microfilaments play a pivotal role in the hemostatic process that is triggered upon the damage of blood vessels.72,73 Additional and further studies in larger patient populations are needed to establish whether and to what extent patients affected by collagen VI–related disorders have a risk for bleeding.15,74 Nevertheless, these data suggest that mutations in different genes resulting in deregulation of Ca2+ entry may underlie a shared pathophysiological mechanism involving muscle and platelets.

In conclusion, our data provide a fundamental step toward understanding the interaction between self-produced collagen VI and Ca2+ signaling in the regulation of Mk function and production of functional platelets. These data represent clinically relevant evidence to be taken into account for the long-term management of patients affected by collagen VI–related diseases, including Bethlem myopathy and UCMD.

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Authorship

Contribution: V.A. conceived the study, designed and performed experiments, analyzed data and wrote the manuscript; C.G. designed and performed the experiments, analyzed data, and edited the manuscript; M.B., A.Z., C.A.D.B., M.C., P.-A.L., and E.L. performed experiments, analyzed data, and edited the manuscript; M.M., F.M., V.P., P. Braghetta, and P. Bernardi analyzed data and edited the manuscript; C.S., L.B., and E.P. provided patient samples and analyzed data; L.S. and A.V. assisted with radiation chimeras, analyzed data, and edited the manuscript; L.D.M. and P. Bonaldo designed experiments, analyzed data, and edited the manuscript; and A.B. conceived the study, designed experiments, analyzed data, supervised the project, and wrote the manuscript.

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References

1. Cescon M, Gattazzo F, Chen P, Bonaldo P. Collagen VI at a glance. J Cell Sci. 2015;128(19):3525-3531.
2. Fitzgerald J, Rich C, Zhou FH, Hansen U. Three novel collagen VI chains, alpha4(VI), alpha5(VI), and alpha6(VI). J Biol Chem. 2008;283(29): 20170-20180.
3. Gara SK, Grumati P, Urciuolo A, et al. Three novel collagen VI chains with high homology to the alpha3 chain. J Biol Chem. 2008;283(16):10658-10670.
4. Bönemann CG. The collagen VI-related myopathies: muscle meets its matrix. Nat Rev Neurol. 2011;7(7):379-390.
5. Bonaldo P, Braghetta P, Zanetti M, Piccolo S, Volpin D, Bressan GM. Collagen VI deficiency induces early onset myopathy in the mouse: an animal model for Bethlem myopathy. Hum Mol Genet. 1998;7(13):2135-2140.
6. Bernardi P, Bonaldo P. Dysfunction of mitochondria and sarcoplasmic reticulum in the pathogenesis of collagen VI muscular dystrophies. Ann N Y Acad Sci. 2008;1147(1):303-311.
7. Irwin WA, Bergamin N, Sabatelli P, et al. Mitochondrial dysfunction and apoptosis in myopathic mice with collagen VI deficiency. Nat Genet. 2003; 35(4):367-371.
8. Cescon M, Chen P, Castagnaro S, Gregorio I, Bonaldo P. Lack of collagen VI promotes neurodegeneration by impairing autophagy and inducing apoptosis during aging. Aging (Albany NY). 2016;8(8):1083-1101.
9. Chen P, Cescon M, Zuccolotto G, et al. Collagen VI regulates peripheral nerve regeneration by modulating macrophage recruitment and polarization. Acta Neuropathol. 2015;129(1):97-113.
10. Castagnaro S, Chrisam M, Cescon M, Braghetta P, Grumati P, Bonaldo P. Extracellular collagen VI has prosurvival and autophagy instructive properties in mouse fibroblasts. Front Physiol. 2018;9:1129.
11. Antoni A, Bassotti A, Abbattista M, et al. Hemostatic abnormalities in patients with Ehlers-Danlos syndrome. J Thromb Haemost. 2018;16(12): 2425-2431.
51. Malara A, Mollah S, Grabell J, et al; Zimmerman Program Investigators. Normal range of bleeding scores for the ISTH-BAT: adult and pediatric data from the merging project. *Haemophilia*. 2014;20(6):831-835.

52. Chiu SK, Orive SL, Moon MJ, et al. Shared roles for Gpvi and Itga10 in megakaryocyte production and platelet function. *Blood*. 2019;134(12):911-923.

53. Semeniak D, Kulawig R, Stegner D, et al. Proplatelet formation is selectively inhibited by collagen type I through Syk-independent Gpvi signaling. *J Cell Sci*. 2019;132(18):3473-3484.

54. Wang H, He J, Xu C, et al. Decoding Human Megakaryocyte Development. *Cell Stem Cell*. 2021;28(3):535-549.e8.

55. Chiu SK, Orive SL, Moon MJ, et al. Shared roles for *Scl* and *Lyt1* in murine platelet production and function. *Blood*. 2019;134(10):826-835.

56. Stritt S, Beck S, Becker IC, et al. Twinfilin 2α regulates platelet reactivity and turnover in mice. *Blood*. 2017;130(15):1746-1756.

57. Pietra D, Rumi E, Ferretti VV, et al. Differential clinical effects of different mutation subtypes in CALR-mutant myeloproliferative neoplasms. *Leukemia*. 2016;30(2):431-438.

58. Chrisam M, Pirozzi M, Castagnaro S, et al. Reactivation of autophagy by spermidine ameliorates the myopathic defects of collagen VI-null mice. *Autophagy*. 2015;11(12):2142-2152.

59. Grumati P, Coletto L, Sabatelli P, et al. Autophagy is defective in collagen VI muscular dystrophies, and its reactivation rescues myofiber degeneration. *Nat Med*. 2010;16(11):1313-1320.

60. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012;149(2):274-293.

61. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease [published correction appears in *Cell*. 2017;168(6):960-976]. *Cell*. 2017;168(6):960-976.

62. Pabla R, Weyrich AS, Dixon DA, et al. Integrin-dependent control of translation: engagement of integrin αIIbbβ3 regulates synthesis of proteins in activated human platelets. *J Cell Biol*. 1999;144(1):175-184.

63. Weyrich AS, Dixon DA, Pabla R, et al. Signal-dependent translation of a regulatory protein, Bcl-3, in activated human platelets. *Cell Metab*. 2013;18(5):698-711.

64. Yang J, Zhou X, Fan X, et al. mTORC1 promotes aging-related venous thrombosis in mice via elevation of platelet volume and activation. *Blood*. 2016;128(5):615-624.

65. Hajnóczky G, Robb-Gaspers LD, Seitz MB, Thomas AP. Decoding of cytosolic calcium oscillations in the mitochondria. *Cell*. 1995;82(3):415-424.

66. Jiménez-Escarniación E, Vilá LM. Recurrent venous thrombosis in Ehlers-Danlos syndrome type III: an atypical manifestation. *BMJ Case Rep*. 2013;2013:bcr2013008922.

67. Misceo D, Holmgren A, Louch WE, et al. A dominant STIM1 mutation causes Stormorken syndrome. *Hum Mutat*. 2014;35(5):556-564.

68. Malfait F, De Paepe A. Bleeding in the heritable connective tissue disorders: mechanisms, diagnosis and treatment. *Blood Rev*. 2009;23(5):191-197.

69. Lacruz RS, Facskó S. Diseases caused by mutations in ORAI1 and STIM1. *Blood*. 2014;123(18):3385-3396.

70. Manon-Jensen T, Kjeld NG, Karsdal MA. Collagen-mediated hemostasis. *J Thromb Haemost*. 2016;14(3):438-448.

71. Mazzuccato M, Spessotto P, Masotti A, et al. Identification of domains responsible for von Willebrand factor type VI collagen interaction mediating platelet adhesion under high flow. *J Biol Chem*. 1999;274(5):3033-3041.

72. Jarre A, Gowert NS, Donner L, et al. Pre-activated blood platelets and a pro-thrombotic phenotype in APP23 mice modeling Alzheimer’s disease. *Cell Signal*. 2014;26(9):2040-2050.

73. Mazzuccato M, Spessotto P, Masotti A, et al. Identification of domains responsible for von Willebrand factor type VI collagen interaction mediating platelet adhesion under high flow. *J Biol Chem*. 1999;274(5):3033-3041.

74. Jarre A, Gowert NS, Donner L, et al. Pre-activated blood platelets and a pro-thrombotic phenotype in APP23 mice modeling Alzheimer’s disease. *Cell Signal*. 2014;26(9):2040-2050.