Evidence for a cytoplasmic proplatelet promoting factor that triggers platelet production

Megakaryocytes are specialized cells that enlarge and become polyploid through repeated cycles of DNA replication without cell division. During development, megakaryocytes become full of platelet-specific granules, expand their cytoplasmic content of cytoskeletal proteins, and develop a demarcation membrane system. Megakaryocytes then generate platelets by remodeling their cytoplasm into proplatelet extensions, which serve as assembly lines for platelet production.[1-3] Several laboratories have made fundamental discoveries in the mechanics of platelet biogenesis, including identifying the cytoskeletal forces that power proplatelet elongation, defining the mechanics of organelle transport and packaging, and establishing new mechanisms of the final stages of platelet production.[4-7] Despite this progress, our understanding of the cellular and molecular basis of the process by which megakaryocytes trigger proplatelet production has clearly lagged behind. Identification of a master regulator that triggers platelet production from megakaryocytes has the potential to reveal novel targets for treating thrombocytopenia. Here we report a rigorous set of microinjection experiments that reveal a cytoplasmic factor, designated proplatelet-promoting factor (PPF), that triggers platelet production in megakaryocytes. PPF was first detected when cytoplasm from proplatelet-producing megakaryocytes was isolated and microinjected back into round megakaryocytes lacking proplatelets, and this led to the immediate initiation of proplatelet production. Furthermore, the frequency with which proplatelet production was initiated was proportional to the volume of injected cytoplasm. Using various agents and treatments, we show that PPF was inactivated by protein modification procedures but resistant to inactivation by methods that modify nucleic acids. Specifically, PPF remained active after treatment with UV, nucleases, weak detergents, and dialysis, but was inactivated by treatment with proteases, sodium dodecylsulfate (SDS), phenol extraction, and heat. These observations provide compelling evidence that there is a protein, or multiple proteins, expressed during proplatelet formation that serve as an internal molecular trigger for proplatelet production.

We hypothesized that megakaryocytes undergoing proplatelet production may contain a cytoplasmic agent responsible for the initiation of proplatelet formation. This led us to ask if the microinjection of cytosol from proplatelet-producing megakaryocytes is sufficient to trigger round megakaryocytes to start proplatelet production. To test this, we adapted a microinjection approach that was initially used to identify mitosis promoting factor (Figure 1A).[8] We first separated proplatelet-producing megakaryocytes from round megakaryocytes (Figure 1B) and generated separate supernatant (S100) from both populations to use as the starting material for microinjection into round non-proplatelet-producing megakaryocytes (Figure 1C). Following injection of cytosol from proplatelet-producing megakaryocytes into round megakaryocytes, 83±6% of recipient megakaryocytes formed proplatelets within 1 hour (hr) (Figure 1D, E). Conversely, cytoplasm prepared from round, non-proplatelet-producing donor megakaryocytes had almost no effect when injected into recipient round megakaryocytes (3.8±2.1% of megakaryocytes made proplatelets). Proplatelet initiation was also minimally observed in control non-injected and sham saline-injected recipient round megakaryocytes at 1 hr (Figure 1E), demonstrating the mechanical force of microinjection did not cause proplatelet initiation. To ensure that round megakaryocytes injected with PPF did not just initiate proplatelet formation, but also continued to elongate and elaborate proplatelets, we examined these cells again after 12 hr. Round megakaryocytes microinjected with cytosol containing PPF continued to progress beyond proplatelet initiation and formed highly developed proplatelets with the hallmark beads-on-a-string appearance, emphasizing the physiological significance of this assay (Figure 2A, B). Of note, recipient cells injected with cytosol from round megakaryocytes had still not initiated proplatelet formation after 12 hr. Proplatelet initiation was also minimally observed in control non-injected and sham saline-injected recipient round megakaryocytes at the 12-hr time point (Figure 2B). However, cells injected with cytosol from proplatelet-producing megakaryocytes, round megakaryocytes, non-injected and saline-injected controls had all formed proplatelets at similar rates by 24 hr, suggesting our observations were not likely to be due to injection of an inhibitor in the cytosol from round donor megakaryocytes (Figure 2C). In order to confirm that the initiation of proplatelet formation was due to PPF, we performed a dose response, which revealed that the frequency of proplatelet initiation was directly proportional to the volume of injected cytosol from proplatelet-producing megakaryocytes (Figure 2D). To test the need for the cytosol being injected into cells *versus* megakaryocytes being incubated with the cytosol, we added S100 into the culture medium of round megakaryocytes. Cytosol at dilutions of 1:10 and 1:100 from both round and proplatelet-producing megakaryocytes was cultured with round (day 4) megakaryocytes (Figure 2E). Cytoplasm prepared from proplatelet-producing donor megakaryocytes had little effect when cultured with round megakaryocytes for 1 hr (1:10 dilution: 4.7±1.5% of megakaryocytes made

proplatelets; 1:100 dilution: 4.3±2.5% of megakaryocytes made proplatelets) or 12 hr (1:10 dilution: 4.3±1.5% of megakaryocytes made proplatelets; 1:100 dilution: 5.3±2.5% of megakaryocytes made proplatelets) suggesting PPF does not have an effect from an extracellular environment. Proplatelet initiation was also minimally observed in controls, in which cytoplasm prepared from round non-proplatelet-producing megakaryocytes was cultured with round megakaryocytes at 1 hr (1:10 dilution: 6.0±2.6% of megakaryocytes made proplatelets; 1:100 dilution: 3.2±6.1% of megakaryocytes

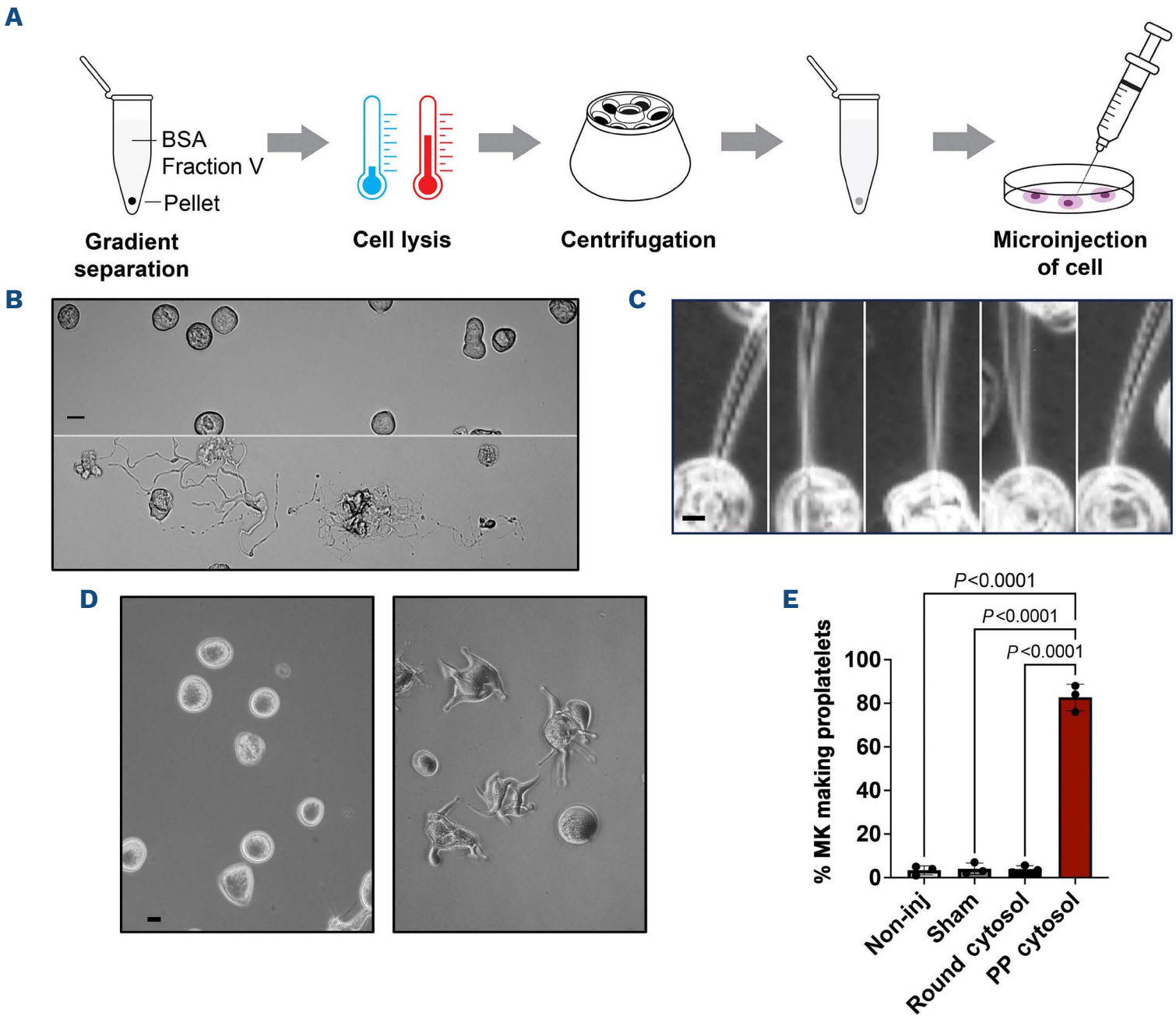


Figure 1. Evidence supporting a proplatelet-promoting factor. (A) Overview of approach to identify proplatelet-promoting factor starting with separation of proplatelet-producing megakaryocytes (MK) from round MK. Murine fetal liver cells were recovered from wild-type CD1 mice (Charles River Laboratories) on embryonic day 13.5 and cultured as previously described.[11] Fetal liver cell cultures (15,000 MK per fetal liver) were layered on a single-step gradient (1.5-3.0% BSA) on day 4, and MK were allowed to sediment for 1 hour (hr). The MK pellet was resuspended in media and cultured for an additional 24 hr, during which proplatelet production was readily observed. MK cultures were layered on a second single-step gradient (1.5-3.0%) on day 5 and intermediate stages in platelet production were resolved within different fractions of the gradient. Proplatelet-producing MK localized to the BSA fraction, whereas round, non-proplatelet-producing MK localized to the pellet. Isolated MK fractions were washed with PBS and then lysed by 3 freeze-thaw cycles and then centrifuged at 100,000 g for 1 hr at 4°C. The supernatant (S100) was recovered and used immediately as the starting material (at 45 mg/mL protein concentration) for microinjection or stored at -80°C until use. A Harvard apparatus PII-100A injector was used for microinjection. Needles were purchased from World Precision Instruments. For each experiment, at least 40 round MK were microinjected with untreated S100 or S100 that was treated as indicated in each experiment. Experiments were repeated in biological triplicates. To determine the percentage of MK making proplatelets, MK were observed on a Nikon Eclipse TS2 microscope with either a 20x/0.40 Ph1 Adl or 40x/0.55 Ph2 Adl objective using an Olympus O-color 3 camera using QCapture Pro 7 software and categorized as round or proplatelet-producing (with cellular extensions) by eye. Data are expressed as percentage of proplatelet-producing MK. Cells were injected with 0.075 nL unless noted otherwise. All data are presented as mean ± Standard Deviation (SD). N=3 biological replicates. (B) Representative images showing isolation of round (top) and proplatelet-producing (bottom) MK. Scale bar = 20 μm. (C) Representative images showing microinjection of round MK. Scale bar = 10 μm. (D) Representative images of 1-hr time-point showing microinjection of round MK with cytosol from round MK (left) and cytosol from proplatelet-producing MK (right). Scale bar = 20 μm. (E) Percentage of MK making proplatelets 1 hr post microinjection. N=3 biological replicates. Data are presented as mean ± SD. Non-inj: not injected. *P*<0.0001.

made proplatelets) or 12 hr (1:10 dilution: 4.0±2.6% of megakaryocytes made proplatelets; 1:100 dilution: 3.5±2.0% of megakaryocytes made proplatelets).

To begin to define PPF, we carried out a detailed characterization of the proplatelet-promoting activity contained in the S100 cytosol. We adapted a strategy used to characterize prions[9] and systematically added chemical agents and detergents to PPF to determine whether they altered its ability to induce proplatelet formation (Figure 3A). We found that the proplatelet-triggering activity of the crude S100 extract was stable for months at -80°C, and for at least three weeks at 4°C. However, it was unstable at higher temperatures; at 25°C and 37°C, the activity was lost after 5 hr. Further, PPF lost its ability to stimulate proplatelet production when exposed to protein-destroying treatments such as phenol, proteinase K, or trypsin for 1 hr at 25°C. However, pre-incubation of proteinase K with PMSF restored PPF activity. Notably, there was no loss in PPF activity after nucleic acid-destroying treatments including UV radiation, ribonuclease, or deoxyribonuclease. Non-denaturing, ionic detergents (at 2.5%) such as Triton X-100, Nonidet P-40, octyl glucoside, and Tween-20 also did not inactivate the agent. In contrast, the denaturing detergent SDS inactivated PPF at a concentration of 1%. High-speed centrifugation to remove membranes from the S100 also failed to inactivate PPF. There was also no loss in PPF activity when S100 was dialyzed (molecular weight cutoff 5,000 kDa), suggesting PPF is not a small molecule.

Given that our previous studies demonstrated that inhibitors of protein synthesis blocked proplatelet initiation,[10] we wondered whether injection of PPF could rescue this phenotype and overcome the loss of proplatelet formation with protein synthesis inhibition. Indeed, injection of PPF caused megakaryocytes treated with puromycin to avidly begin proplatelet production (Figure 3B-D). Together, these data provide evidence for a megakaryocyte-intrinsic protein or multiple proteins, PPF, that is a master trigger for platelet production. Further, they suggest that PPF is present in megakaryocytes as they undergo proplatelet formation and justify future studies examining its potential existence in platelets. While PPF is sufficient to trigger proplatelet formation, we recognize that external factors in the bone

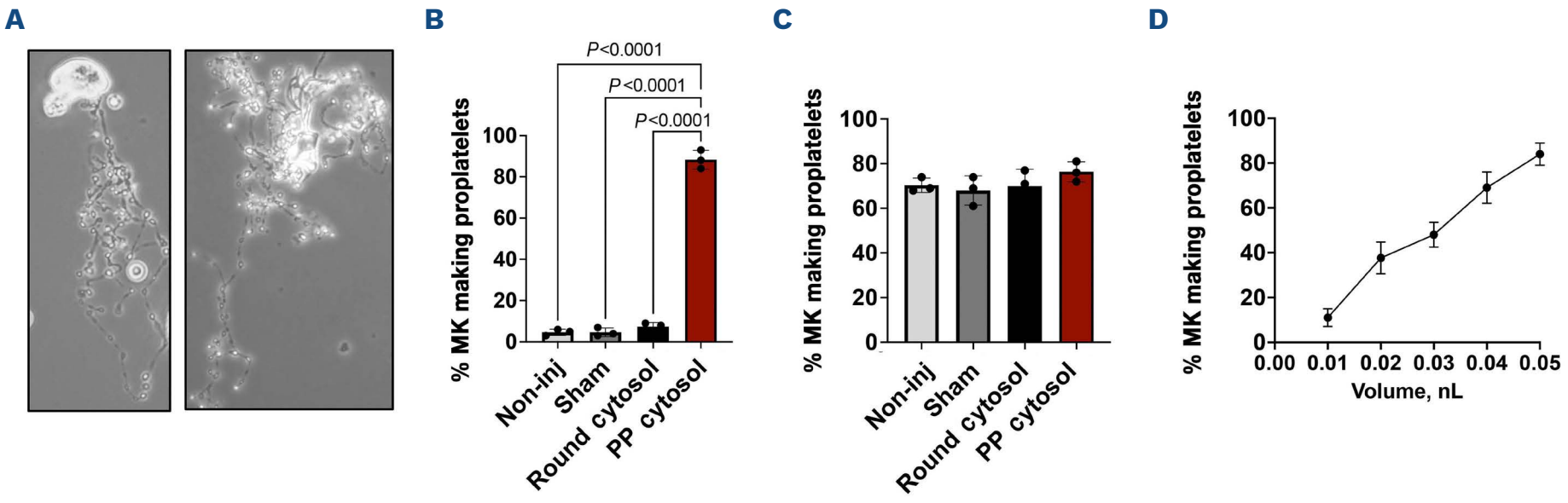


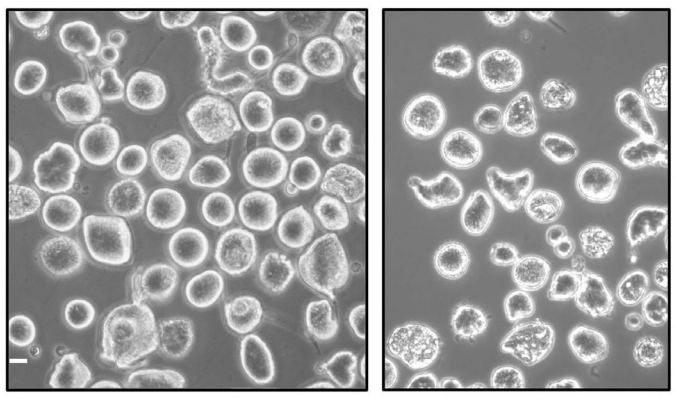
Figure 2. Data substantiating a proplatelet-promoting factor. (A) Representative images of megakaryocytes (MK) 12-hours (hr) post microinjection showing that round MK injected with S100 from proplatelet-producing (PP) MK continue to develop classic beads-on-string proplatelets. Scale bar = 10 μm. Original magnification = 40x. (B) Percentage of MK making proplatelets 12-hr post microinjection. N=3 biological replicates. Data are presented as mean ± Standard Deviation (SD). (C) Percentage of MK making proplatelets 24-hr post microinjection. N=3 biological replicates. Data are presented as mean ± SD. (D) Graph showing that the frequency of proplatelet initiation is proportional to the volume of injected S100 from PP MK. Volume is in nL and cells assayed 1 hr after injection. N=3 biological replicates. Data are presented as mean ± SD. Round MK were injected with indicated volume of S100 from PP MK. (E) Representative images showing the effect of the addition of cytosol on round MK. Cytosol (S100 supernatant) was placed in the culture medium at dilutions of 1:10 and 1:100. (Top) Representative images of round MK cultured with cytosol from PP MK. (Bottom) Representative images of round MK cultured with cytosol from round MK. N=3 biological replicates. Scale bar = 80 μm. Original magnification = 10x. *P*<0.0001.

A

Treatment	Active – Stimulates PP	Inactive – No PP
Ultra-violet radiation	254nm *82.3±9*	
Protease	Proteinase K + PMSF *77.3±6.4*	Proteinase K *1.5±1.2* Trypsin *1.3±1.2*
Nucleases	Ribonuclease *83.6±8%* Deoxyribonuclease *81.3±8.6*	
Detergents	Triton X-100 *81±8.9* NP40 *85±4.3* Octyl glucoside *80.3±10.5* Tween-20 *83.6±3.6*	SDS *8.0±13.6*
Temperature	4 °C for 3 weeks *87.6±7.7* -80 for months *86.3±8*	37 °C *4.3±2.5* 25°C *6.3±4* for 5 hr
Phenol		Phenol *1.5±1.1*
Centrifugation	High-speed to remove membranes *81.0±2.5*	
Molecular weight	>5 kDa	< 5 kDa

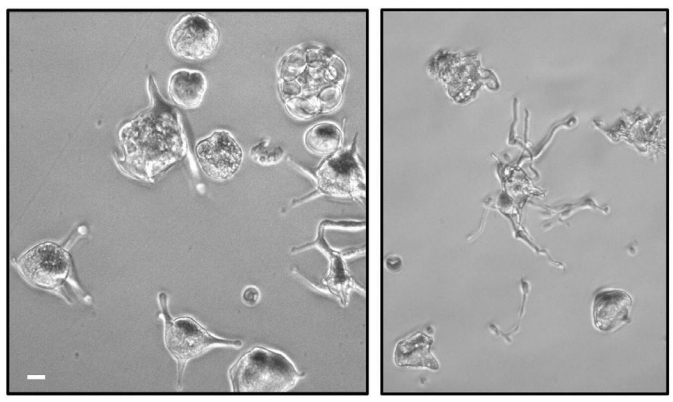
B

Puromycin



C

Puromycin + PP cytosol



D

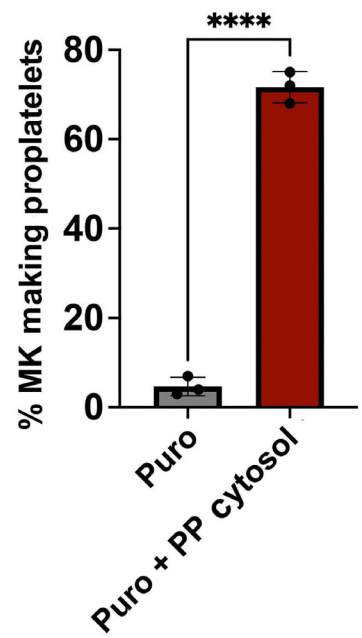


Figure 3. Molecular qualities of proplatelet-promoting factor. (A) Table showing molecular treatments done to S100 derived from proplatelet-producing megakaryocytes (MK), and corresponding percentage of MK making proplatelets after 1 hour (hr) when treated S100 was microinjected into round MK (indicated in red). UV irradiation was carried out at 254 nm. S100 was irradiated on ice and placed beneath GE germicidal lamps (emax = 254 nm) at approximately 5 cm. A Spectroline UV dosimeter was used to measure the delivered doses, calculated at 7.2 ± 1.2 J/m^2/sec. The calculated dose on the S100 was 10,000 J/m^2. For high-speed centrifugation, S100 was centrifuged at 18,000 g for 90 minutes (min) in a Thermo Scientific Sorvall Lynx 4000 centrifuge. For size separation, 100 μL of S100 was placed in a Millipore filter with a molecular weight cut off at 5 kDa and centrifuged for 2 hr. The lower layer was collected, and upper layer reconstituted to the original volume in 10 mM KH2HPO4 (pH 6.8) buffer. Proteinase K, trypsin, PMSF, sodium dodecyl sulfate, puromycin, Triton X-100, Nonidet P-40, octyl glucoside, and Tween-20 were all obtained from Sigma. Proteinase K digestion was carried out at 100 μg/mL for 1 hr at 37°C. PhMeSO2F (PMSF) was used at 0.1 mM to inhibit proteinase K. Digestion with RNase A or DNase I was carried out at 50 μg/mL for 1 hr. Trypsin was used at 100 μg/mL for 1 hr. For phenol extraction, prior to extraction with phenol, cytosol was suspended in 30 mM Tris-acetate, pH 8.3. Equal volumes of cold phenol were used to extract the samples at 4°C. Phase separation was accomplished by centrifugation at 4,000 g for 10 min at 4°C. The aqueous phase was recovered. N=3 biological replicates. Data are presented as mean ± SD. (B) Puromycin treatment (250 μg/mL) caused a block in proplatelet formation[10] (4.6±1.7% proplatelet production). Representative images of 8-hr time-point after puromycin treatment (original magnification 40x). (C) S100 from proplatelet-producing MK was microinjected into puromycin-treated MK, which triggered proplatelet production. Representative images of 1-hr time-point (original magnification 40x) after injection of S100 obtained from proplatelet-producing MK (71.6±8.2% proplatelet initiation). Scale bars = 20 μm. (D) Proplatelet production in the presence of puromycin and after microinjection with S100 from proplatelet-producing megakaryocytes. N=40 cells per condition per replicate. N=3 biological replicates. Data are presented as mean ± SD. ****P=0.0001. PPF: proplatelet-promoting factor.

marrow microenvironment may provide signaling to regulate this internal factor during proplatelet production.

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Disclosures

JEI has a financial interest in and is a founder of StellularBio, a biotechnology company focused on making donor-independent platelet-like cells at scale, and has a financial interest in and is a founder of SpryBio, a biotechnology company focused on using shelf-stable platelets to treat osteoarthritis. His interests are managed by the Boston Children's Hospital. KRM has no conflicts of interest to disclose.

Contributions

JEI conceptualized and designed the research, conducted the experiments, analyzed and interpreted the data, and wrote the manuscript. KRM designed and interpreted the research, and edited and reviewed the manuscript.

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Data-sharing statement

Please contact the corresponding author for access to the original data related to this manuscript.

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