Evidence for a cytoplasmic proplatelet promoting factor that triggers platelet production

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Evidence for a cytoplasmic proplatelet promoting factor that triggers platelet production

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Data Sharing Statement: Please contact the corresponding author at joseph.italiano@childrens.harvard.edu for access to the original data related to this manuscript.

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Authorship Contributions
Contribution: J.E.I. conceptualized and designed the research, conducted the experiments, analyzed and interpreted the data, and wrote the manuscript; K.R.M. designed and interpreted the research and edited and reviewed the manuscript.

Disclosure of Conflicts of Interest
Conflict-of-interest disclosure: J.E.I. has a financial interest in and is a founder of StellularBio, a biotechnology company focused on making donor-independent platelet-like cells at scale. J.E.I. has a financial interest in and is a founder of SpryBio, a biotechnology company focused on using shelf-stable platelets to treat osteoarthritis. Boston Children's Hospital manages the interests of J.E.I. KRM declares no competing financial interests.
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.\textsuperscript{1-3} Several laboratories have made fundamental discoveries into 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.\textsuperscript{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. 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 caused immediate initiation of proplatelet production. Further, 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 retained activity after treatment with UV, nucleases, weak detergents, and dialysis, but was inactivated by treatment with proteases, 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.

Cytoplasmic microinjections reveal a proplatelet-promoting factor (PPF) that triggers proplatelet production. To understand mechanisms that activate platelet 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 triggers round megakaryocytes to start proplatelet production. To test this, we adapted a microinjection approach that was initially used to identify mitosis promoting factor (Fig 1A).\textsuperscript{8} We first separated proplatelet-producing megakaryocytes from round megakaryocytes (Fig 1B) and generated separate supernatant (S100) from both populations to use as the starting material for microinjection into round, non-proplatelet-producing megakaryocytes (Fig 1C). Following injection of cytosol from proplatelet-producing megakaryocytes into round megakaryocytes, 83+/−6% of recipient megakaryocytes formed proplatelets within 1 hr (Fig 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 1hr (Fig 1E), suggesting 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 hours. Round megakaryocytes microinjected with cytosol containing PPF continued to progress beyond proplatelet initiation and form highly developed proplatelets with the hallmark beads-on-a-string appearance, emphasizing the physiological significance of this assay (Fig 2A, B). Of note, recipient cells injected with cytosol from round megakaryocytes still did not initiate 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 (Fig 2B). However, cells injected with cytosol from proplatelet-producing megakaryocytes, round megakaryocytes, non-injected and saline-injected controls all formed proplatelets at similar rates by 24 hrs, suggesting our observations were not likely due to injection of an inhibitor in the cytosol from round donor megakaryocytes (Fig 2C).

To substantiate 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 (Fig 2D). To test the necessity of the cytosol being injected into cells versus megakaryocytes being incubated with the cytosol, we added S100 to 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 (Fig 2E). Cytoplasm prepared from proplatelet-producing donor megakaryocytes had almost no effect when cultured with round megakaryocytes at 1 hr (1:10 dilution, 4.7 +/- 1.5% of megakaryocytes made proplatelets and 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 and 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 and 1:100 dilution, 3.2 +/- 6.1% of megakaryocytes made proplatelets) or 12 hr (1:10 dilution, 4.0 +/- 2.6% of megakaryocytes made proplatelets and 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 and systematically added chemical agents and detergents to PPF to determine whether they altered its ability to induce proplatelet formation (Fig 3A). We found that the proplatelet-triggering activity of the crude S100 extract was stable for months at -80°C, and for at least 3 weeks at 4°C. However, it was unstable at higher temperatures; at 25°C and 37°C, the activity was lost at 5 hrs. 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. Nondenaturing, 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 cut off 5,000 kDa), suggesting PPF is not a small molecule.
Given that our previous studies demonstrated that inhibitors of protein synthesis blocked proplatelet initiation\(^\text{10}\), 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 (Fig. 3B, C, 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. We also recognize that external factors in the bone marrow microenvironment may signal to regulate this internal factor during proplatelet production.
References

Figure Legends

**Figure 1. Evidence supporting a proplatelet-promoting factor.** (A) Overview of approach to identify proplatelet-promoting factor starting with separation of proplatelet-producing megakaryocytes from round megakaryocytes. Murine fetal liver cells were recovered from wild-type CD1 mice (Charles River Laboratories) on embryonic day 13.5 and cultured as previously described. Fetal liver cell cultures (15,000 megakaryocytes per fetal liver) were layered on a single-step gradient (1.5-3.0% BSA) on day 4, and megakaryocytes were allowed to sediment for 1 hr. The megakaryocyte pellet was resuspended in media and cultured for an additional 24hr, during which proplatelet production was readily observed. Megakaryocyte 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 megakaryocytes localized to the BSA fraction, whereas round, non-proplatelet-producing megakaryocytes localized to the pellet. Isolated megakaryocyte fractions were washed with PBS and then lysed by three freeze-thaw cycles and then centrifuged at 100,000g 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 megakaryocytes were microinjected with untreated S100 or S100 that was treated as indicated in each experiment. Experiments were repeated in biological triplicates. To determine the percent of megakaryocytes making proplatelets, megakaryocytes 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 megakaryocytes. Cells were injected with 0.075 nL unless noted otherwise. All data are presented as mean +/- SD and N = 3 biological replicates. (B) Representative images showing isolation of round (top) and proplatelet-producing (bottom) megakaryocytes. Scale bar = 20 μm. (C) Representative images showing microinjection of round megakaryocytes. Scale bar = 10 μm. (D) Representative images of 1-hour time-point showing microinjection of round megakaryocytes with cytosol from round megakaryocytes (left panel) and cytosol from proplatelet-producing megakaryocytes (right panel). Scale bar = 20 μm. (E) Percentage of megakaryocytes making proplatelets 1 hour post microinjection. N = 3 biological replicates. Data are presented as mean +/- SD.

**Figure 2. Data substantiating a proplatelet-promoting factor.** (A) Representative images of megakaryocytes 12-hours post injection showing that round megakaryocytes injected with S100 from proplatelet-producing megakaryocytes continue to develop classic beads-on-string proplatelets. Scale bar = 10 μm, original magnification = 40x. (B) Percentage of megakaryocytes making proplatelets 12-hours post microinjection. N = 3 biological replicates. Data are presented as mean +/- SD. (C) Percentage of megakaryocytes making proplatelets 24-hours 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 proplatelet-producing megakaryocytes. Volume is in nL and cells assayed 1
hour after injection. N = 3 biological replicates. Data are presented as mean +/- SD. Round megakaryocytes were injected with indicated volume of S100 from proplatelet-producing megakaryocytes. (E) Representative images showing the effect of the addition of cytosol on round megakaryocytes. Cytosol (S100 supernatant) was placed in the culture medium at dilutions of 1:10 and 1:100. The top panel shows representative images of round megakaryocytes cultured with cytosol from proplatelet-producing megakaryocytes and the lower panel shows representative images of round megakaryocytes cultured with cytosol from round megakaryocytes. N = 3 biological replicates, Scale bar = 80 μm, original magnification = 10x.

Figure 3. Molecular qualities of proplatelet-promoting factor (PPF). (A) Table showing molecular treatments done to S100 derived from proplatelet-producing megakaryocytes, and corresponding percentage of megakaryocytes making proplatelets after 1 hour when treated S100 was microinjected into round megakaryocytes (indicated in red). UV irradiation was carried out at 254 nm. S100 was irradiated on ice and placed beneath GE germicidal lamps (emax = 254nm) at approximately 5 cm. A Spectroline UV dosimeter was used to measure the delivered doses, calculated at 7.2 +/- 1.2 J/m2/sec. The calculated dose on the S100 was 10,000 J/m2. For high-speed centrifugation, S100 was centrifuged at 18,000 g for 90 min in a Thermo Scientific Sorvall Lynx 4000 centrifuge. For size separation, 100 μL of S100 was placed in a Millipore filter molecular weight cutoff at 5 kDa and centrifuged for 2 hours. The lower layer was collected, and upper layer reconstituted to the original volume in 10mM 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 hour 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 4,000g 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 formation⁴ (4.6 +/- 1.7% proplatelet production). Representative images of 8-hour time-point after puromycin treatment (original magnification 40x). (C) S100 from proplatelet-producing megakaryocytes was microinjected into puromycin-treated megakaryocytes, which introduced new proteins and triggered proplatelet production. Representative images of 1-hour time-point (original magnification 40x) after injection of S100 obtained from proplatelet-producing megakaryocytes (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. N = 3 biological replicates. Data are presented as mean +/- SD.
Figure 1

A

Gradient separation → Cell lysis → Centrifugation → Microinjection of cell

B

C

D

E

% MKs Making Proplatelets

Non-Inj  Sham  Round Cytosol  PP Cytosol

<0.0001  <0.0001  <0.0001
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<th>Inactive – No PPs</th>
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**Figure 3**