Recent Advances in Platelet Production in Vitro and In Vivo

10/13/2018

Faculty Disclosures

The following faculty have no relevant financial relationships to disclose:

- Avital Mendelson

The following faculty have a relevant financial relationship:

- Joseph Italiano PhD
  - **Platelet BioGenesis:** Consultant
  - **VcanBio:** Scientific Advisory Board
- Wilbur Lam MD, PhD
  - **Sanguina, LLC:** Co-Founder

AABB
BOSTON

Learning Objectives

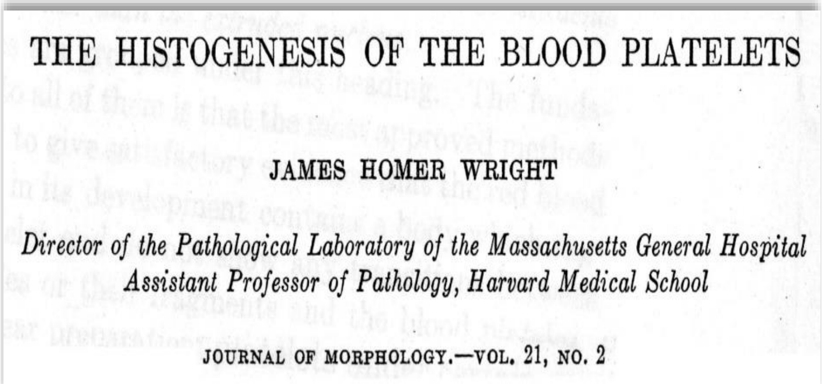
- Discuss the biology of platelet production *in vivo and* genetic engineering approaches to produce platelets
  - Joseph E. Italiano Ph.D., Associate Professor, Harvard Medical School
- Outline methods of *ex-vivo* platelet production with novel culture systems
  - Avital Mendelson Ph.D., Assistant Member, New York Blood Center
- Define the response of platelets to the mechanical microenvironment of blood vessels
  - Wilbur Lam M.D. Ph.D., Associate Professor, Emory University and Georgia Institute of Technology

AABB BOSTON 18

Molecular Drivers of Platelet Production

Joseph Italiano
Harvard Medical School
Brigham and Women’s Hospital
Boston Children’s Hospital
Platelet BioGenesis

History of Platelet Production



THE HISTOGENESIS OF THE BLOOD PLATELETS

JAMES HOMER WRIGHT

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JOURNAL OF MORPHOLOGY.—VOL. 21, NO. 2

NEWS AND VIEWS

BLOOD

Thrombopoietin — at last

Donald Metcalf

WHEN, for decades, a vital blood-cell growth factor has been believed to exist but has resisted all efforts to characterize it, resolution of the conundrum is a cause for celebration. So let celebrations begin — this issue contains four papers[1-4] indicating that the frustrations concerning the platelet-regulatory factor, thrombo-

Meanwhile, four other haemopoietic regulators with proliferative actions on megakaryocytes — interleukin-3, interleukin-6, leukaemia inhibitory factor and interleukin-11 — were characterized and proved to have some ability to increase platelet levels. From the clinical viewpoint, however, the action of each is

did this by using c-*mpl* antisense oligodeoxynucleotides to show that these inhibited megakaryocyte colony formation *in vitro*, but had no inhibitory effects on erythroid or granulocyte–macrophage colonies.

From this point on, the story demonstrates the power of the 'receptor first' approach for detecting growth factors. As an initial step, in all four of the present studies cell lines were engineered to express the c-Mpl receptor and these cell lines provided the essential tool for moni-

1906 Wright [PLATES]

1957 Yamada [DMS]

1976 Becker & Debruyn [PPLT]

1982 Radley

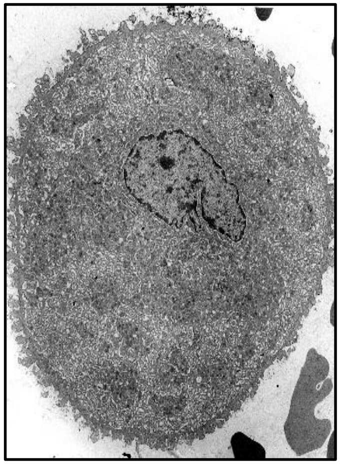
1984 Zucker-Franklin

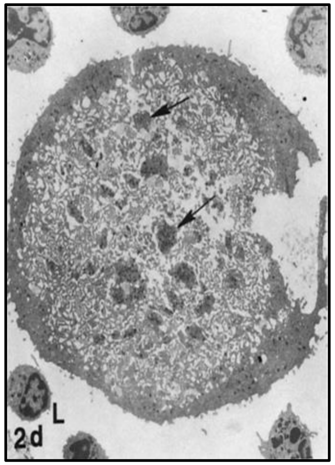
1986 Tablin & Leven

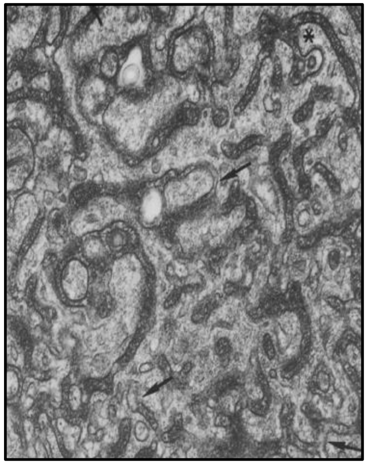
1994 [TPO]

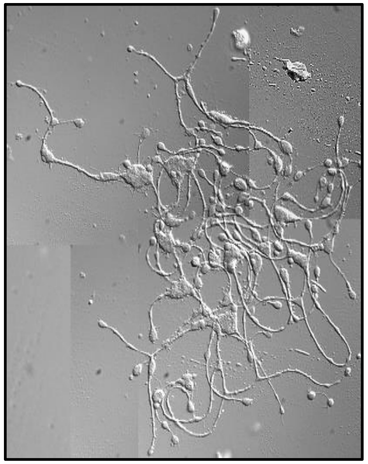
1996 Choi

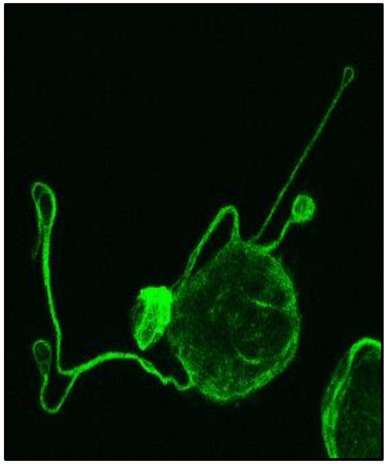
1998 Hartwig & Italiano



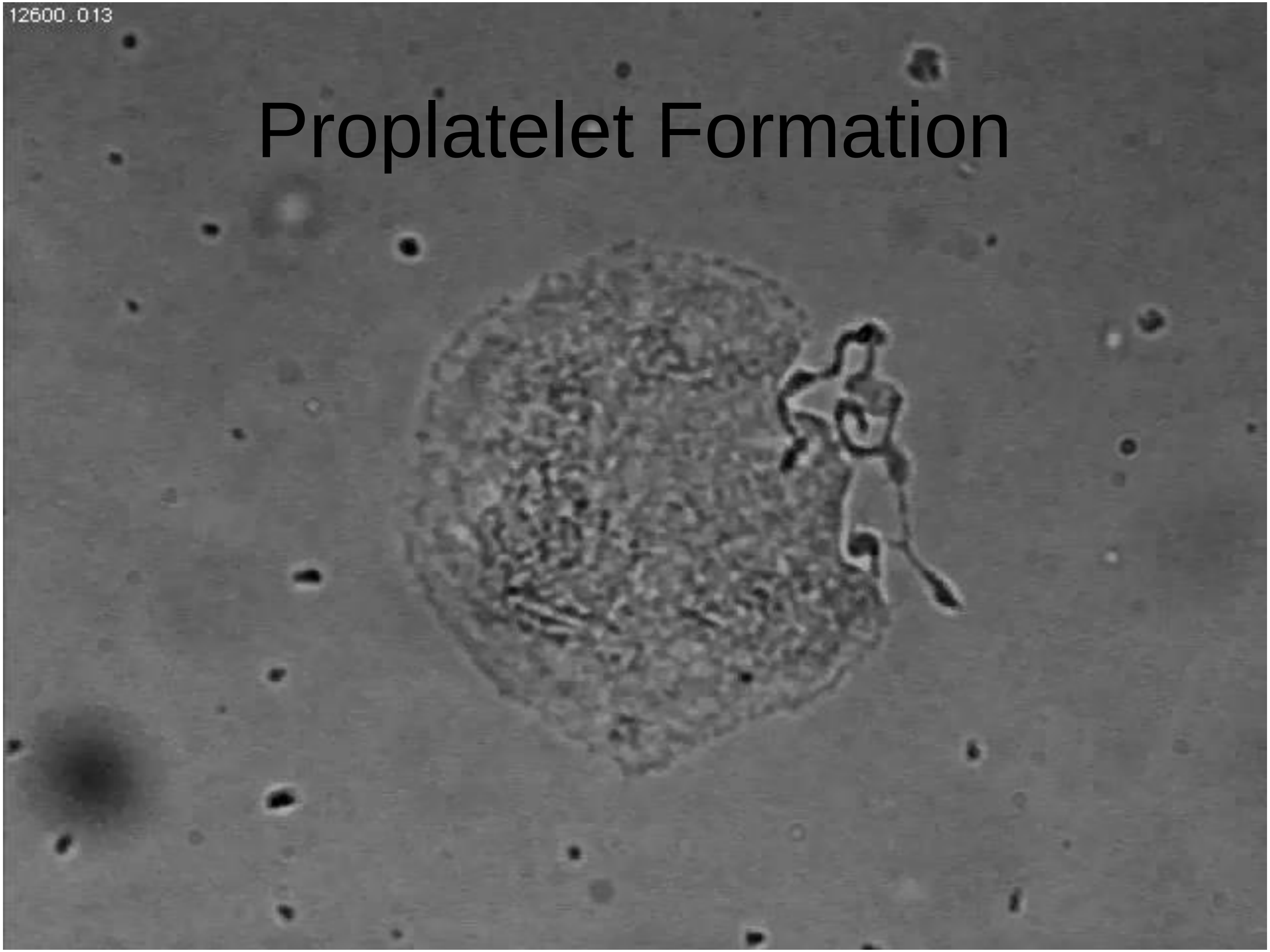






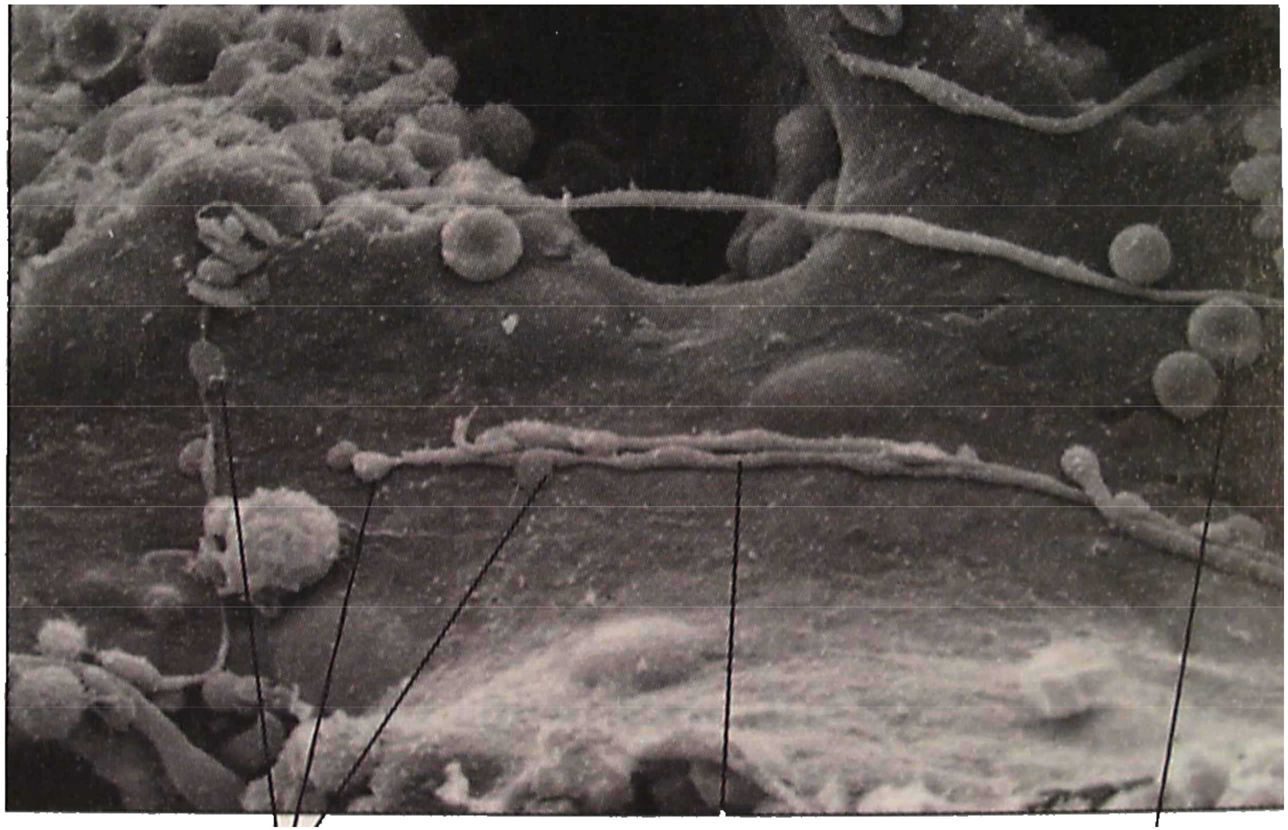


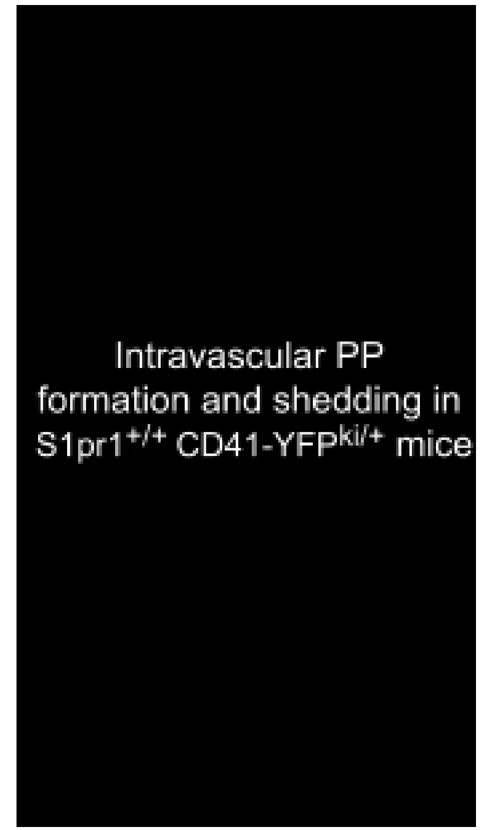
Proplatelet Formation



Italiano et al. JCB 1999, 147:1299-1312

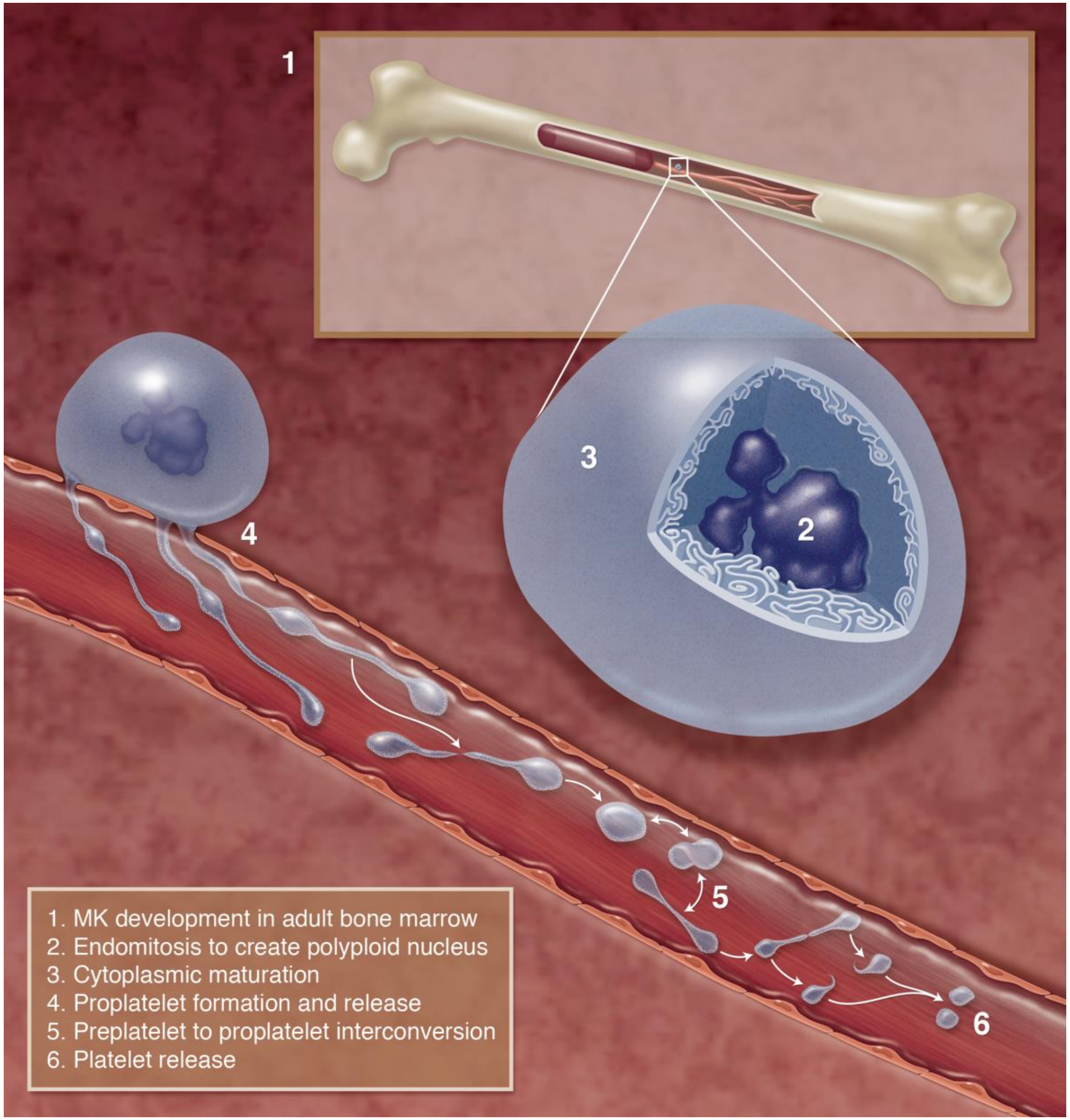
Proplatelets in vivo





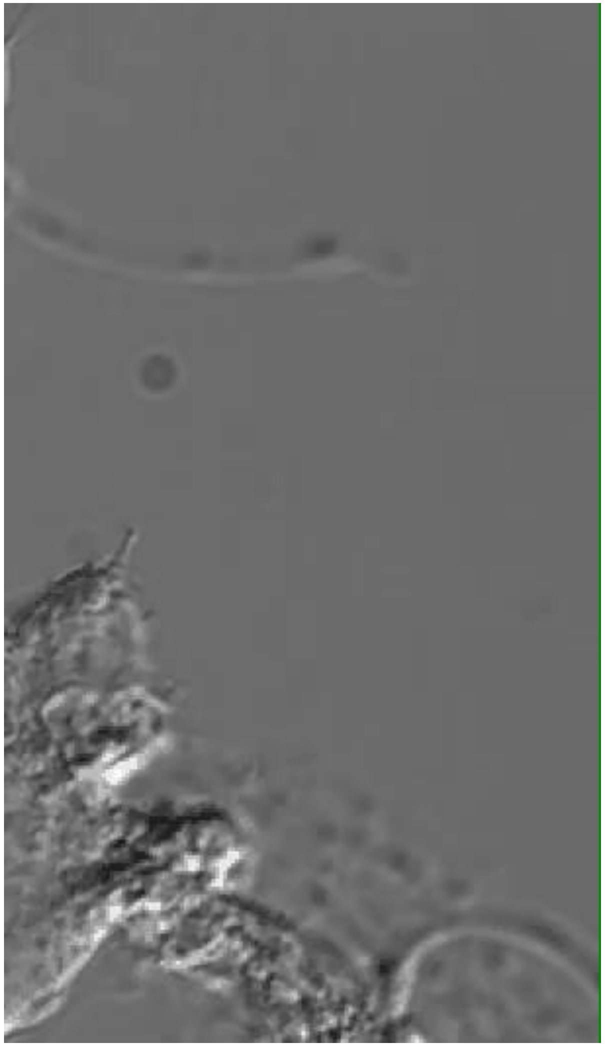
Massberg S. 2013 J Exp Med

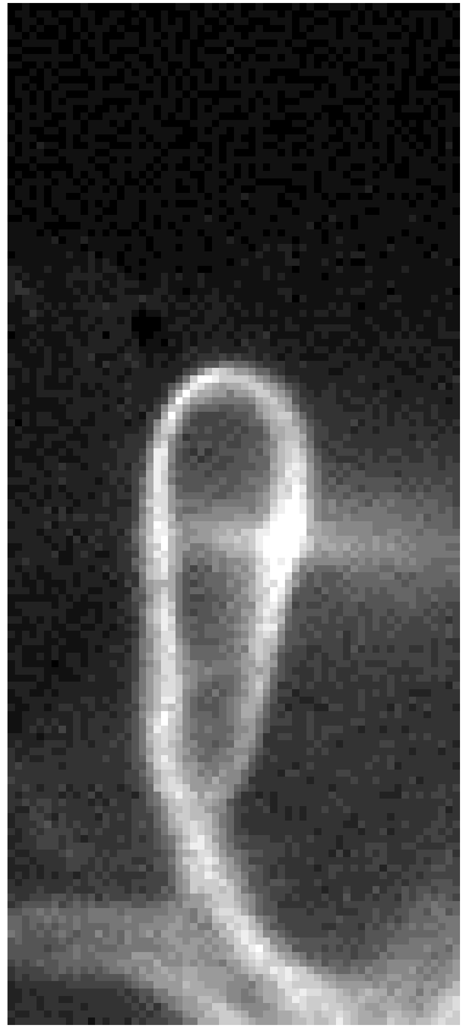
Platelet Production from Megakaryocytes



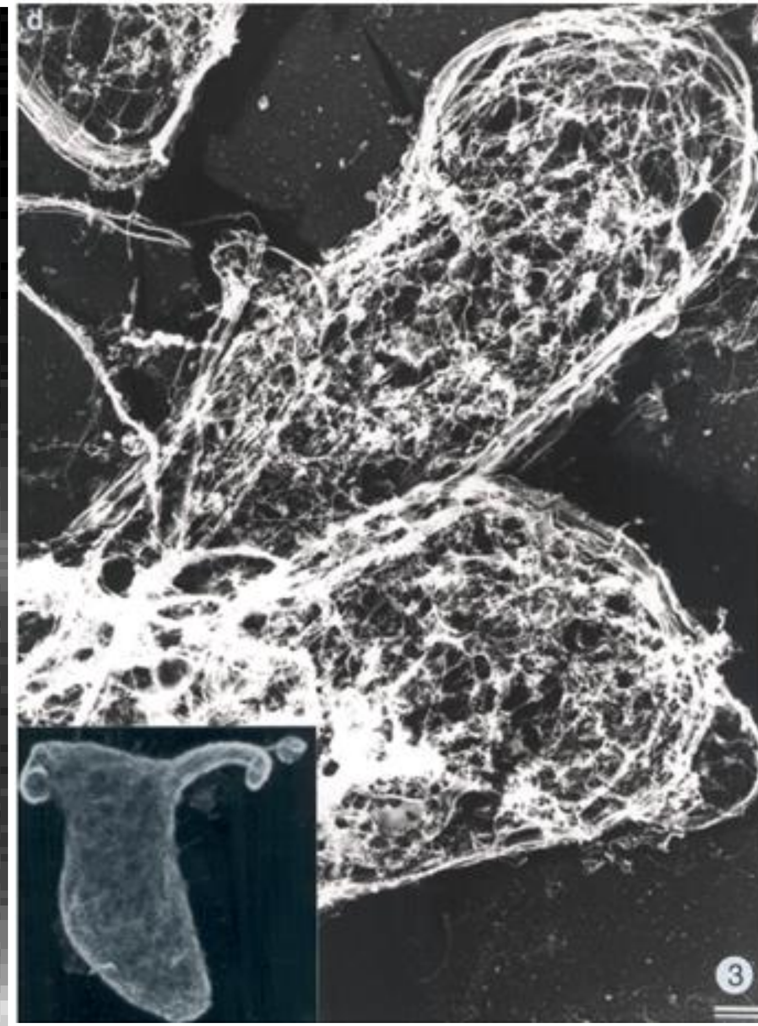
Machlus and Italiano. JCB 2013

Cytoskeleton powers platelet production



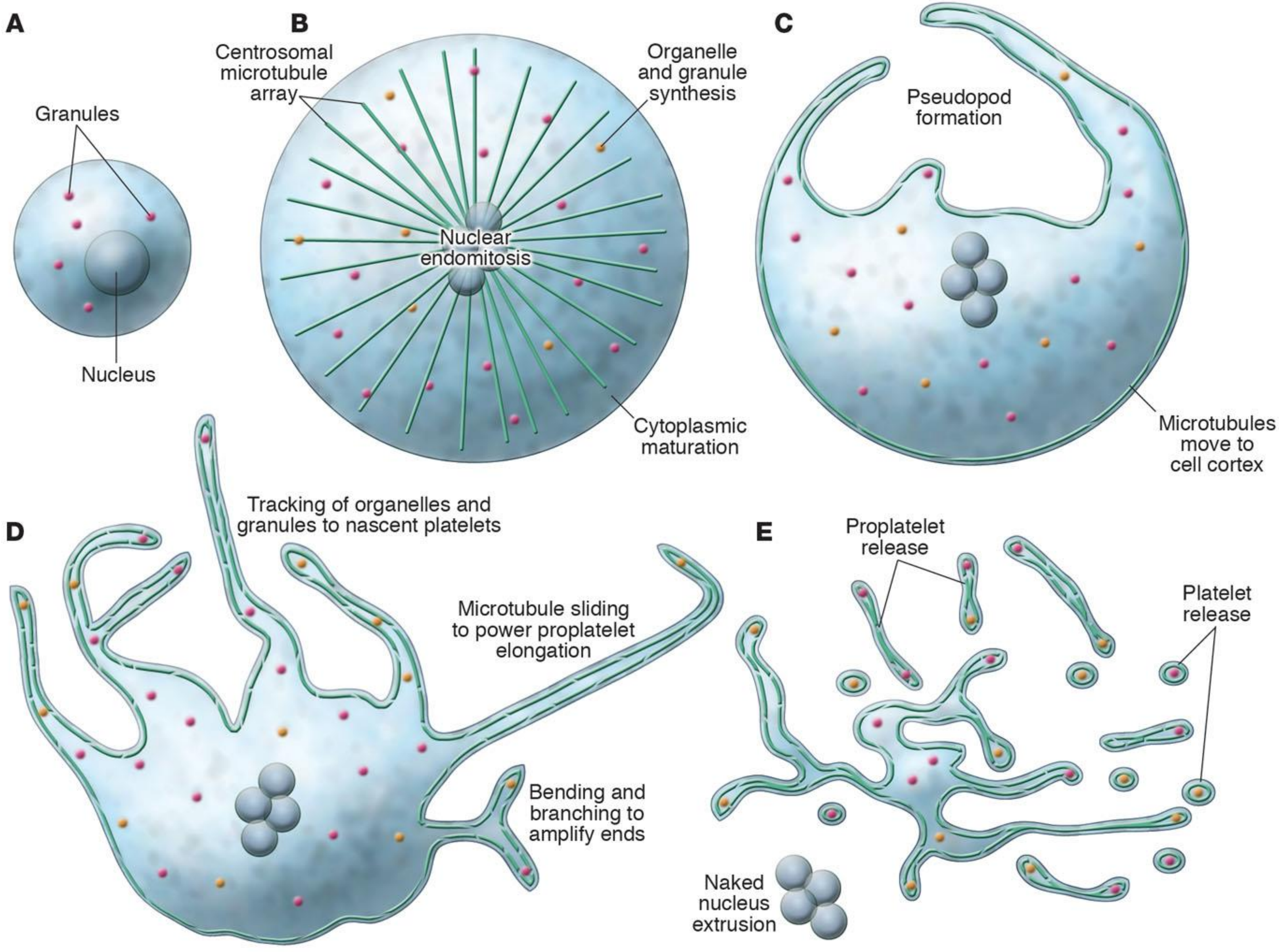


GFP-tubulin



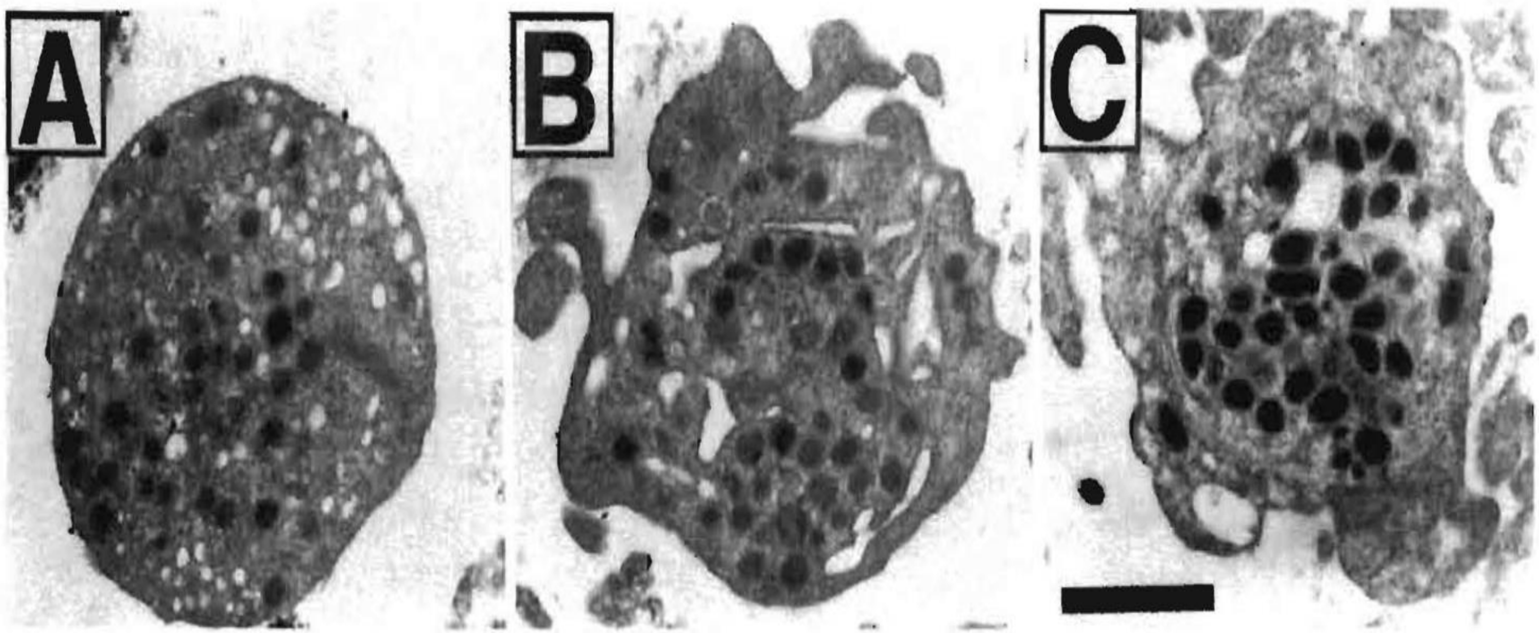
Italiano et al. JTH Supp 1:18-23

Model of Platelet Production



Megakaryocyte Cultures Provide a Source for In vitro Production of Platelets

Platelets Generated In Vitro From Proplatelet-Displaying Human Megakaryocytes Are Functional



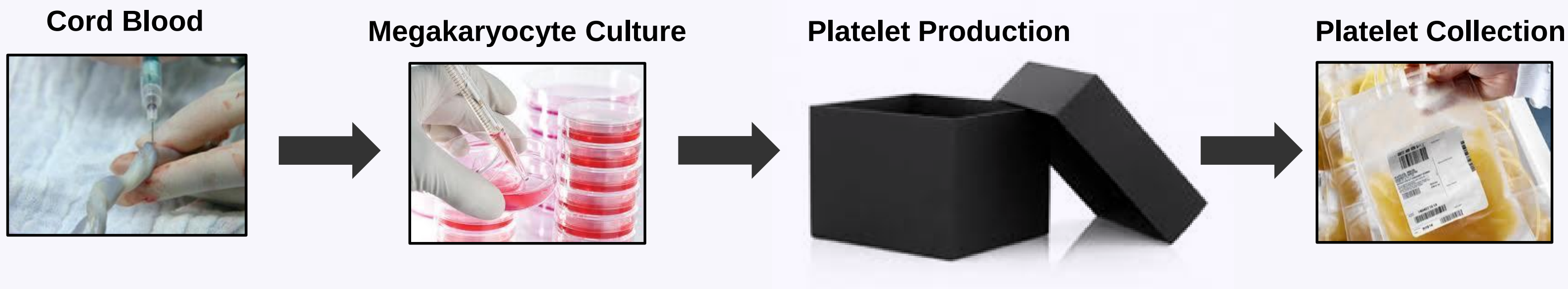
- **Culture Blood**

Culture

Choi et al. Blood. 1995

Strategy for Producing In Vitro Platelets

STEM CELL → MEGAKARYOCYTE DIFFERENTIATION → MEGAKARYOCYTE SOURCE → PLATELET PRODUCTION → PLATELET UNIT

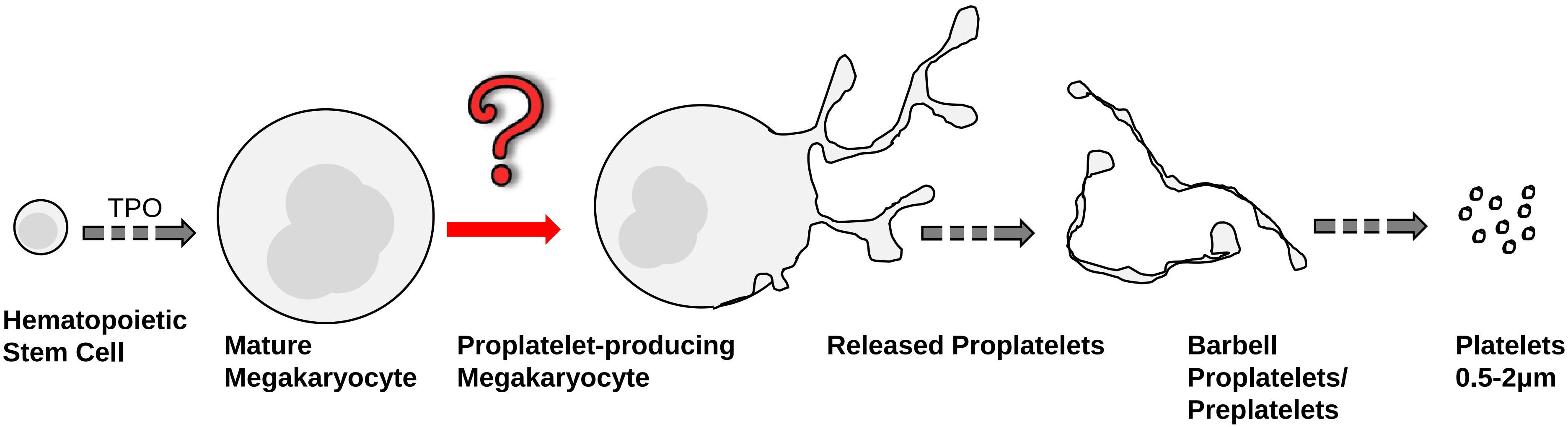


BARRIERS - While several groups have grown human megakaryocytes from a variety of sources, only a small percentage (10-15%) make platelets (Lambert et al., Blood 121:3319)

Bioreactor Design

Matsunaga et al. 2006 – 3 Phase co-culture system
Sullenbarger et al. 2009 – 3D modular perfusion system
Lasky et al. 2011- Continuous flow medium, oxygen
Pallotta et al. 2011 – Silk-based marrow system
DiBuduo et al. 2015 – Silk based, sponge, growth factors
Nakagawa et al. 2013 – Two differential flows
Thon et al. 2014 – Bioreactor on-a-chip
Blin et al. 2016 – Microfluidics, vWF coated micropillars
Avanzi et al. 2016 – Nanofiber mem, bidirectional flow
Guan et al. 2016 – 2 liter bottle turning device

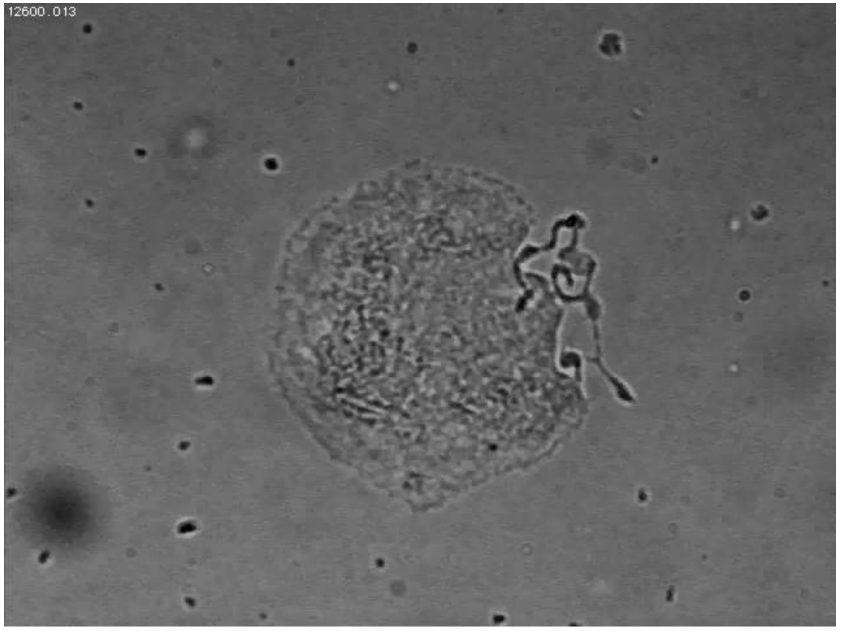
Little is Known About What Triggers Proplatelet Formation



Uncovering what stimulates proplatelet formation will lead to:

- Therapeutics that initiate auto-transfusion of platelets from existing bone marrow MKs
- Ability to control platelet formation in vitro

What Triggers Proplatelet Production?



1. Internal
2. External

1. Proplatelet-promoting factor (PPF) is an internal, cytosolic protein(s) that regulates proplatelet initiation.

2. Mimicking physiology (shear) triggers platelet production.

3. We have developed a scalable process to generate in vitro platelets from clinical grade iPS cells.

1. Are there internal, cytosolic factors that trigger platelet production?

Hypothesis: Mature megakaryocytes synthesize a cytosolic factor that triggers proplatelet initiation

Discovery of Mitosis Promoting Factor

Mitosis in tissue-cultured lung cell of a newt, *Traicha granulosa,* recorded with the new Pol-Scope.

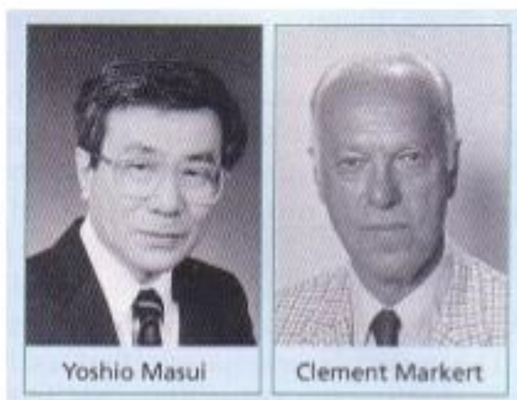
Cytoplasmic Control of Nuclear Behavior during Meiotic Maturation of Frog Oocytes

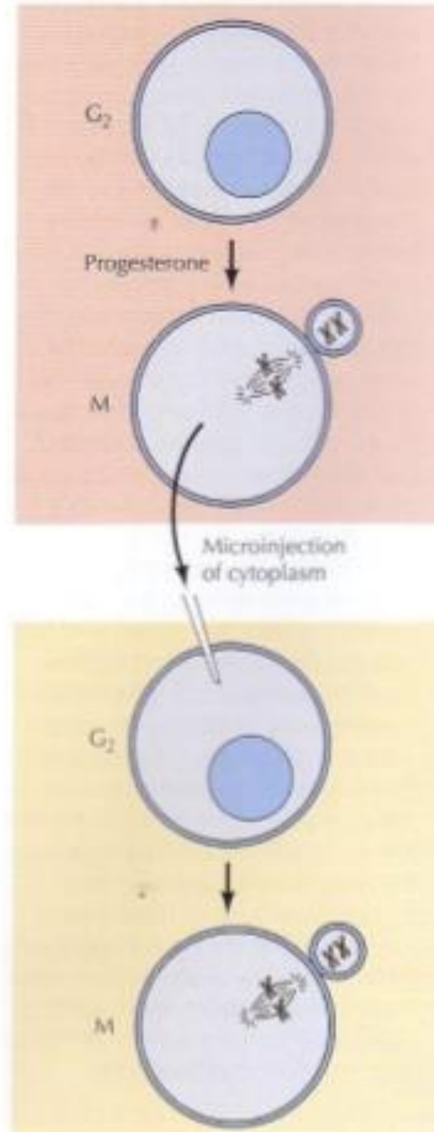
Yoshio Masui and Clement L. Markert

Yale University, New Haven, CT

Journal of Experimental Zoology, 1971, Volume 177, Pages 129–146

Further experiments showed that MPF is not restricted to oocytes and appeared to act as a general regulator of the transition from G2 to M .

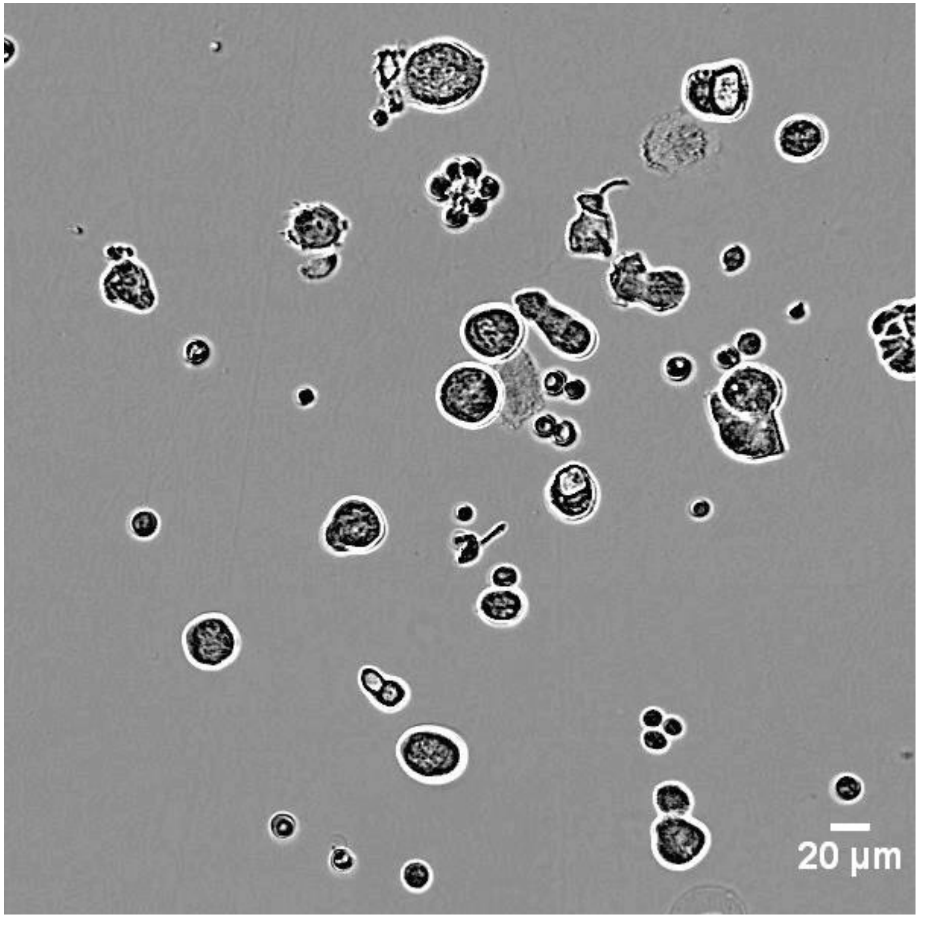




Do MKs contain a cytosolic factor that promotes proplatelet production?

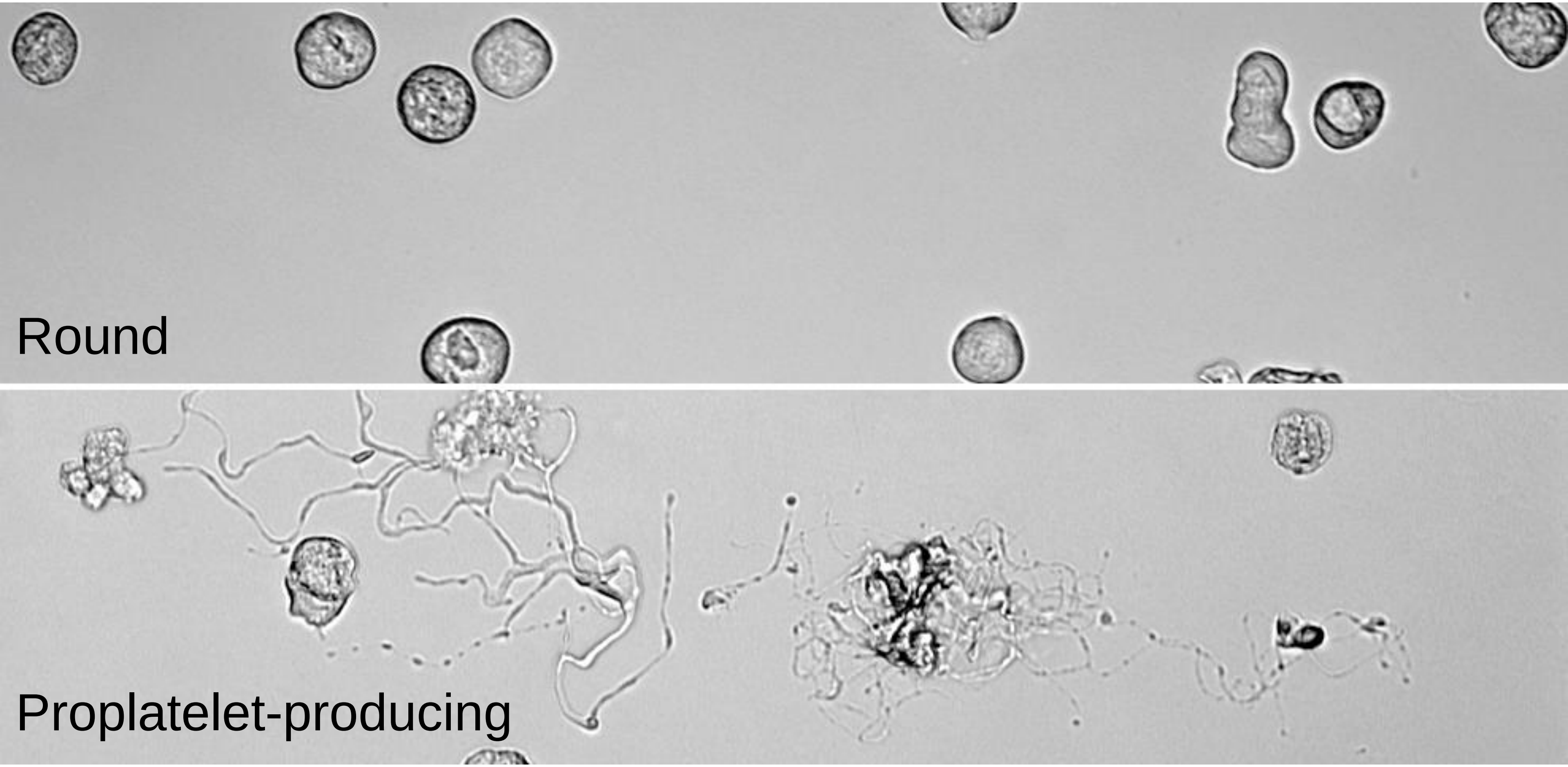
What would happen if we injected cytosol from a proplatelet-producing megakaryocyte into a round megakaryocyte?

Would this trigger proplatelet initiation?



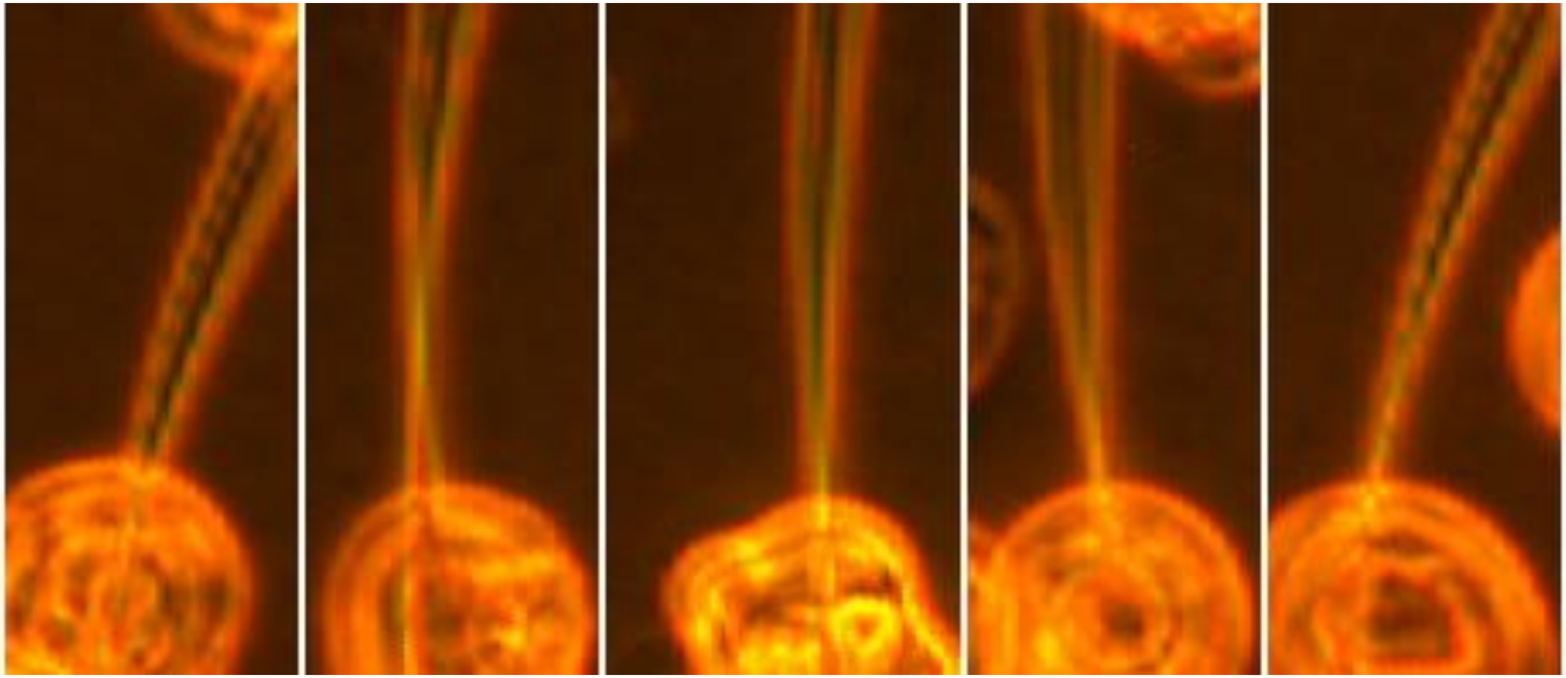
Microinjection of Megakaryocytes

Separation of Megakaryocyte fractions

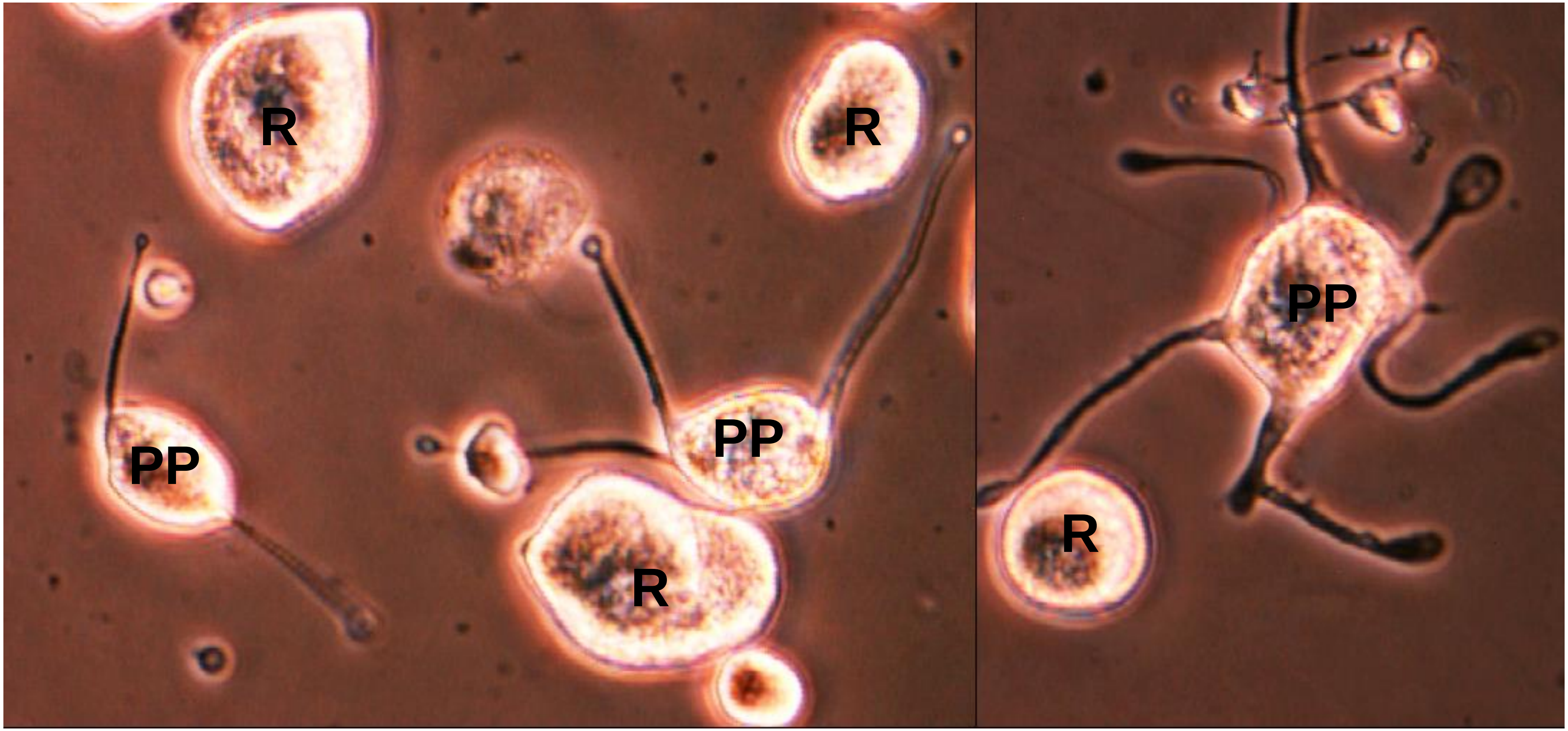


Thon et al. 2010 JCB 191:861

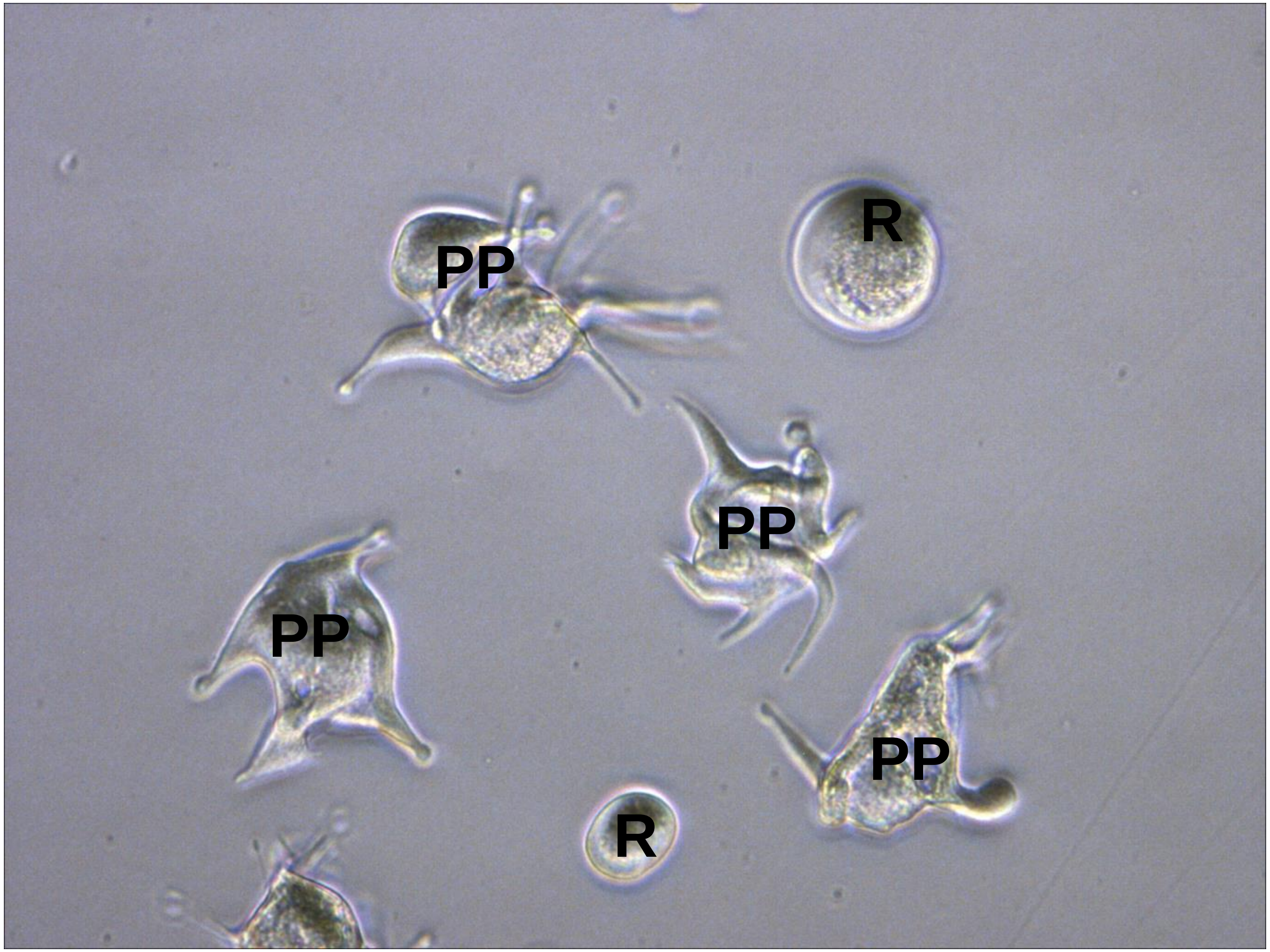
Megakaryocyte Microinjection



Injection of cytosol from proplatelet-producing MKs triggers platelet production



Injection of cytosol from PP-producing MKs triggers platelet production

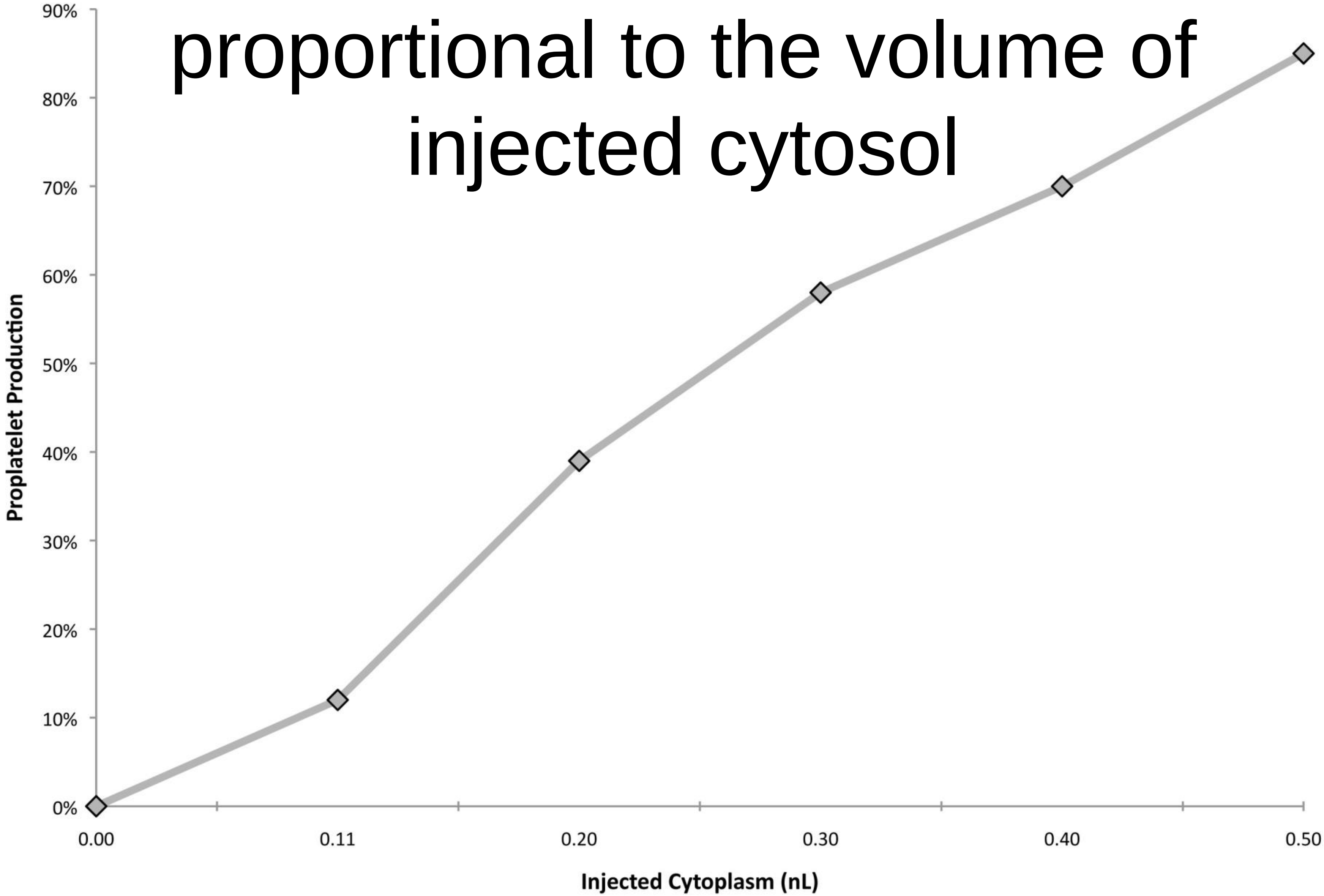


<u>Proplatelet initiation</u>

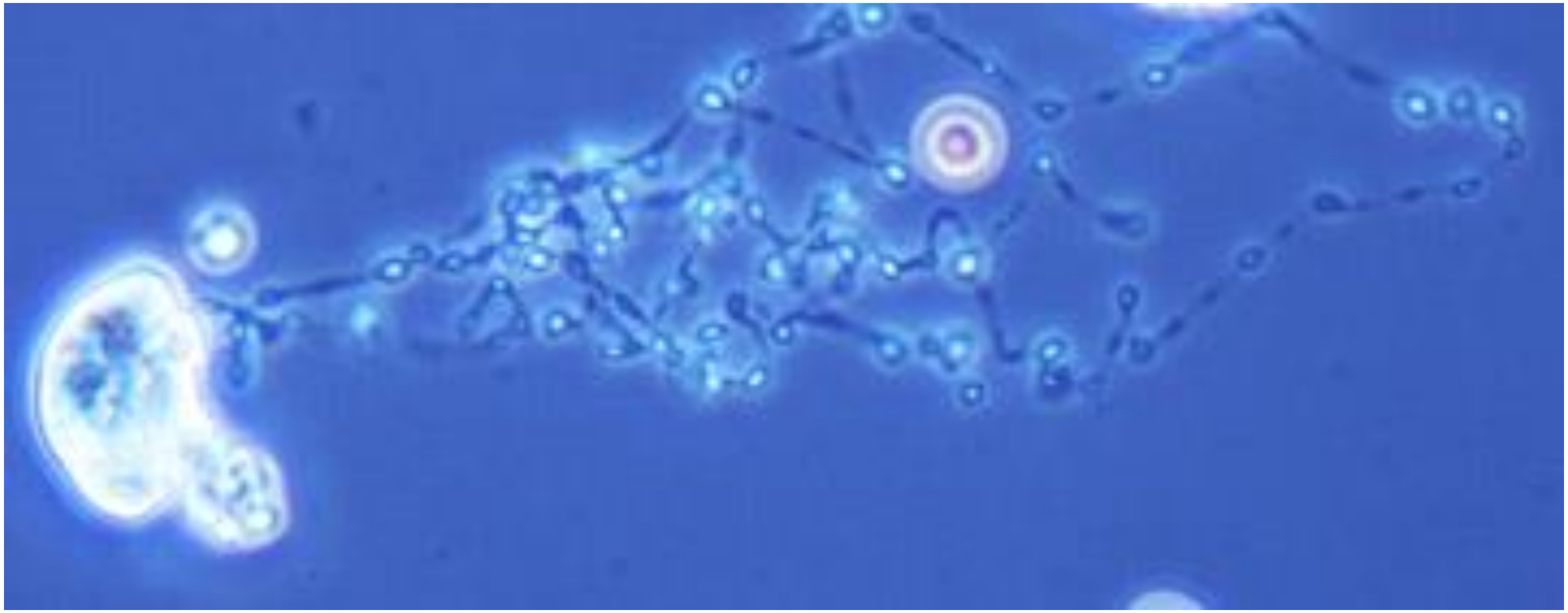
Rest cytosol- 7 %

PP cytosol - 83%

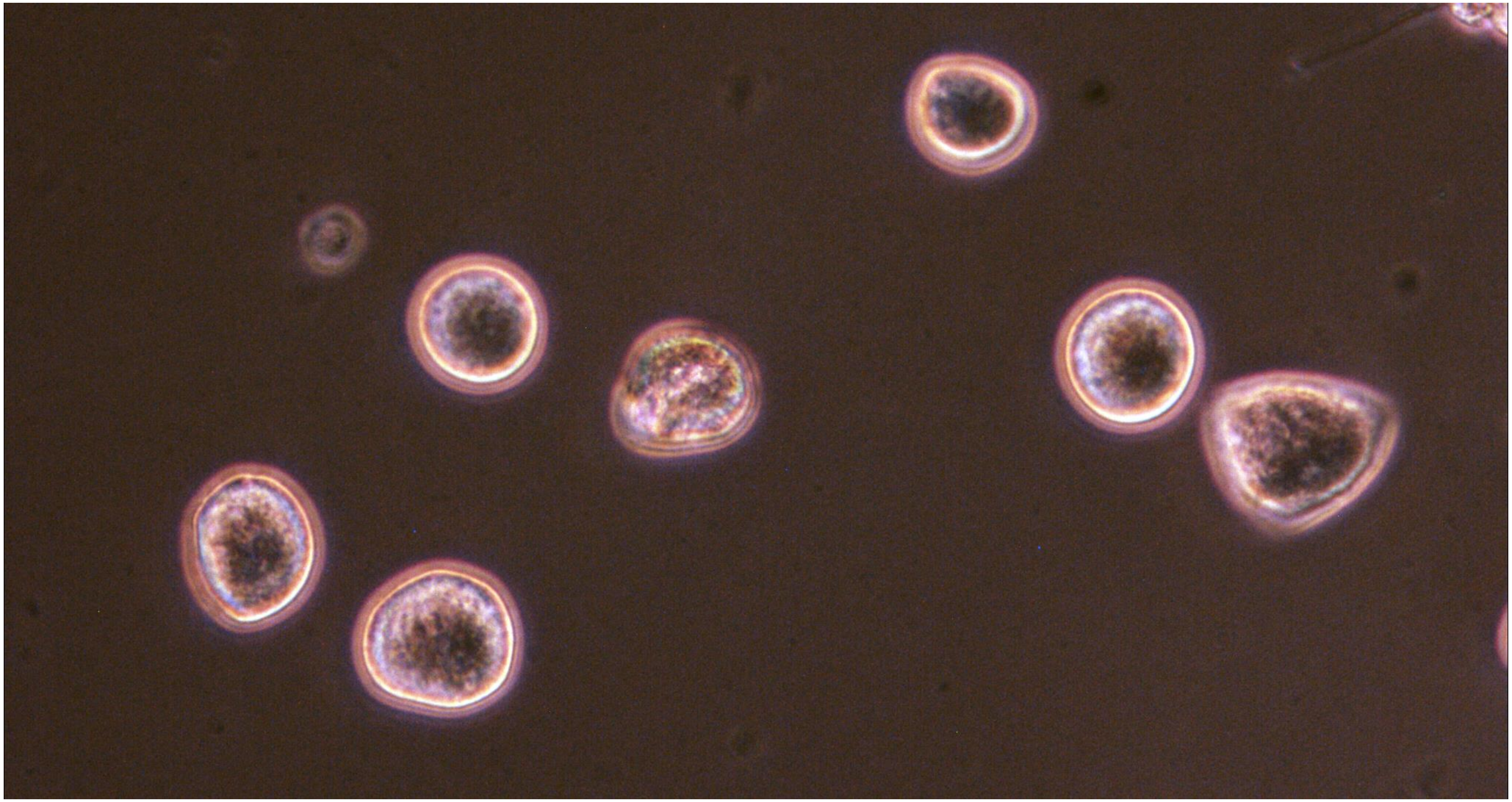
The frequency of PP production is proportional to the volume of injected cytosol



Microinjected megakaryocytes continue to form highly developed proplatelets



Sham-injected cells do not form proplatelets



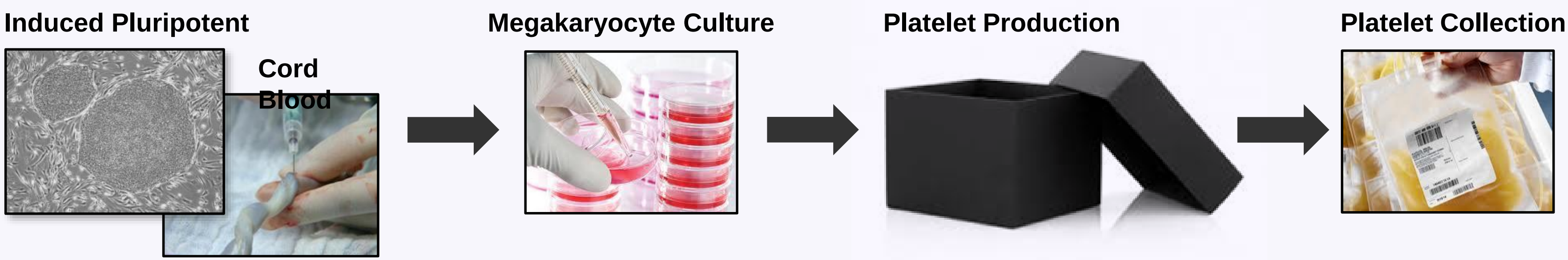
Proplatelet-promoting factor

Table 1. Characterization of proplatelet-promoting factor (PPF)

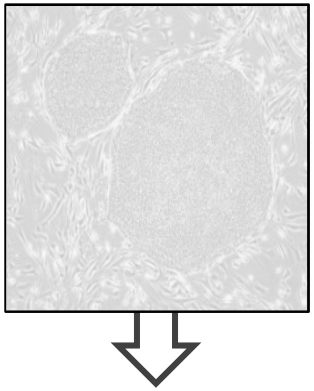
Treatment	**Stabile**	**Labile**
Nucleases	Ribonuclease, Deoxyribonuclease	
Temperature	4°C for 3 weeks	37°C, 25°C for 5 hrs
Protease Digestion		Proteinase K, trypsin
Detergents	Triton X-100, NP40	SDS
UV radiation	254 nm	
Centrifugation	High-speed to remove membranes	
Molecular Weight	> 100 KDa	< 100 KDa
Kinase inhibitors	staurosporine	
Phosphatase inhibitors		NaFl, NaOrthovanadate, Okadaic acid

2. How do we mimick physiology to trigger platelet production? and generate in vitro platelets?

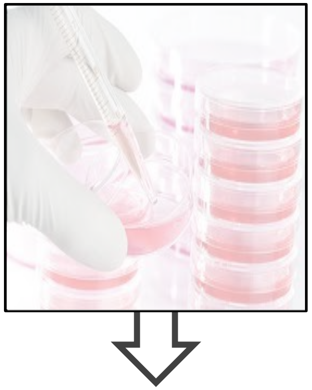
STEM CELL → MEGAKARYOCYTE DIFFERENTIATION → MEGAKARYOCYTE SOURCE → PLATELET PRODUCTION → PLATELET UNIT



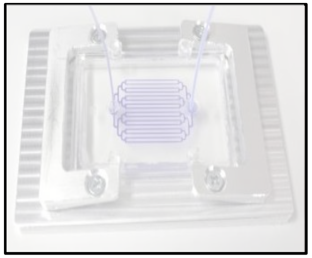
In Vitro Platelet Production



STEM CELL

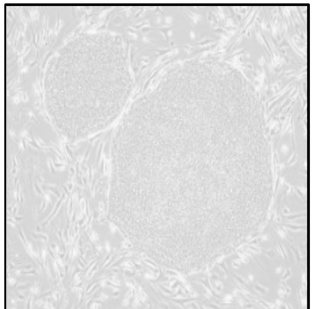


MEGAKARYOCYTE



PLATELET

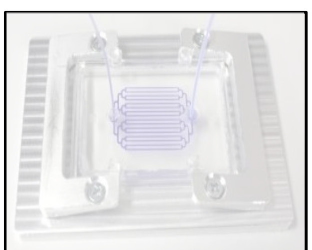
In Vitro Platelet Production



STEM CELL

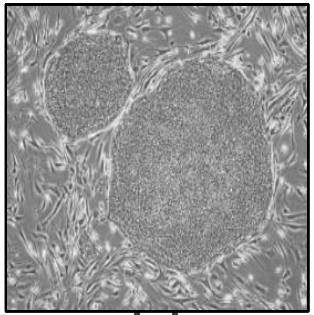
MEGAKARYOCYTE

1994 discovery of thrombopoietin (TPO) led to generation of 1st human platelets by Amgen (via cell culture).



PLATELET

In Vitro Platelet Production



STEM CELL

2006: Invention of human induced pluripotent stem cells (iPSC) allows for genetically consistent and scalable stem cells.

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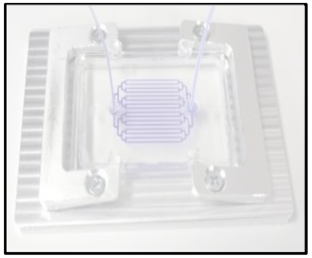
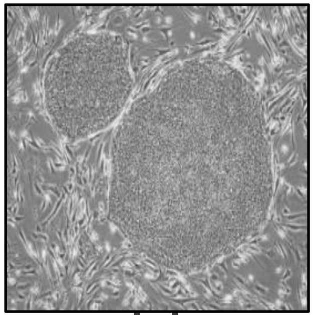


PLATELET

In Vitro Platelet Production



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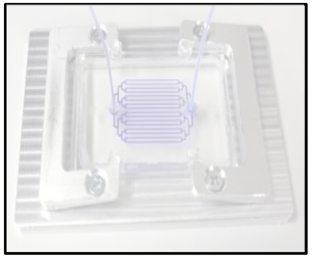
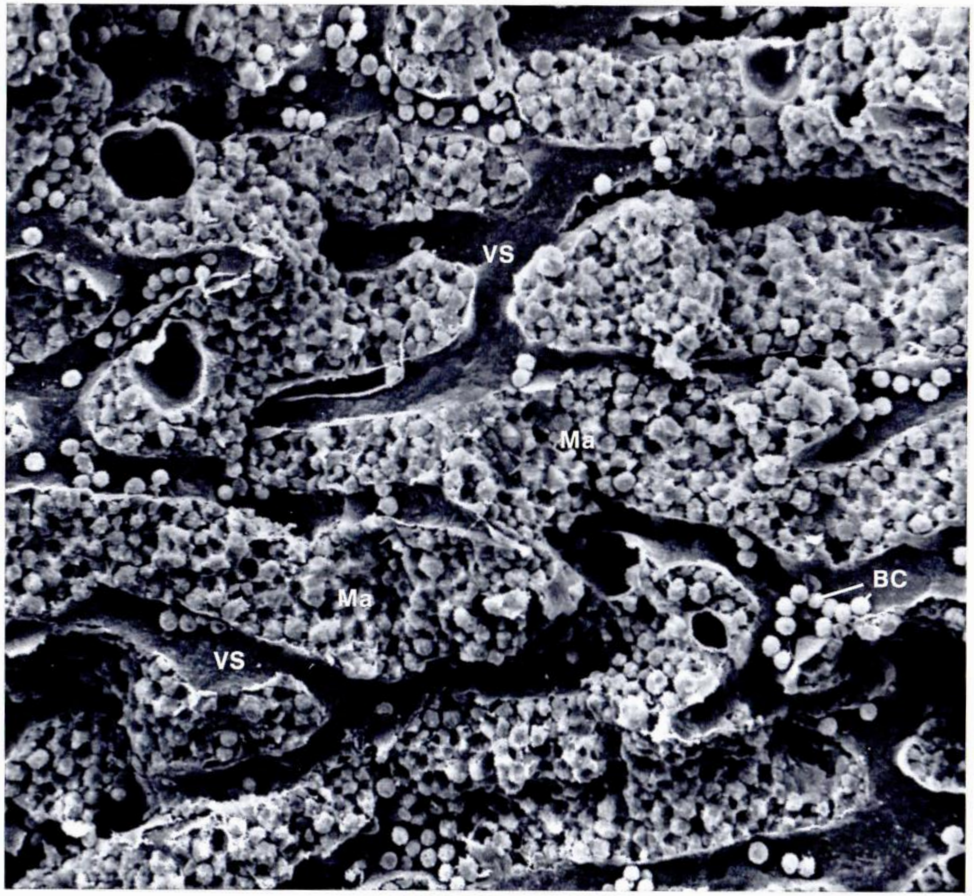


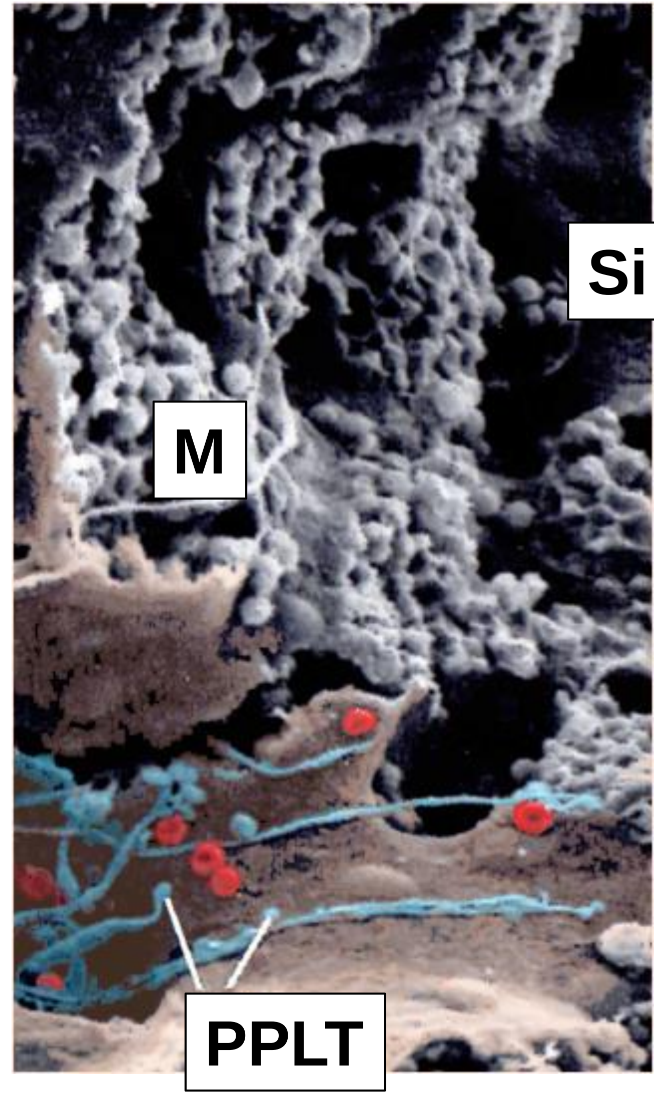
PLATELET

Hypothesis: Mimicking the native, physiological bone marrow vascular microenvironment will stimulate proplatelet production

Bone Marrow Microenvironment



Proplatelet production *in vivo*
Tissue and organs: a text atlas of scanning electron microscopy. 1975



Native Platelet Production

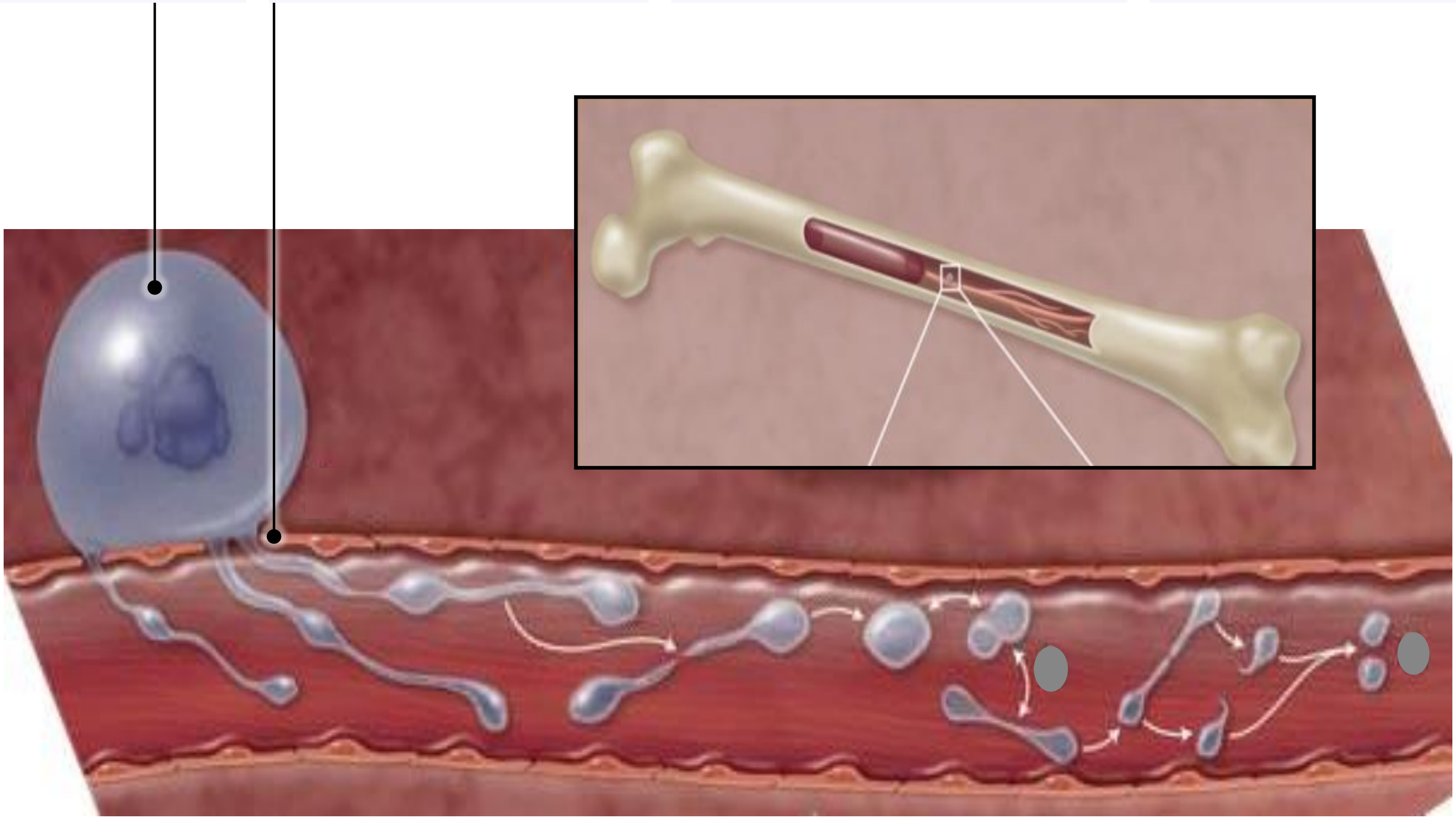
Human platelets are produced by megakaryocytes in the bone marrow

1. Megakaryocytes migrate to endothelial cells that line blood vessels

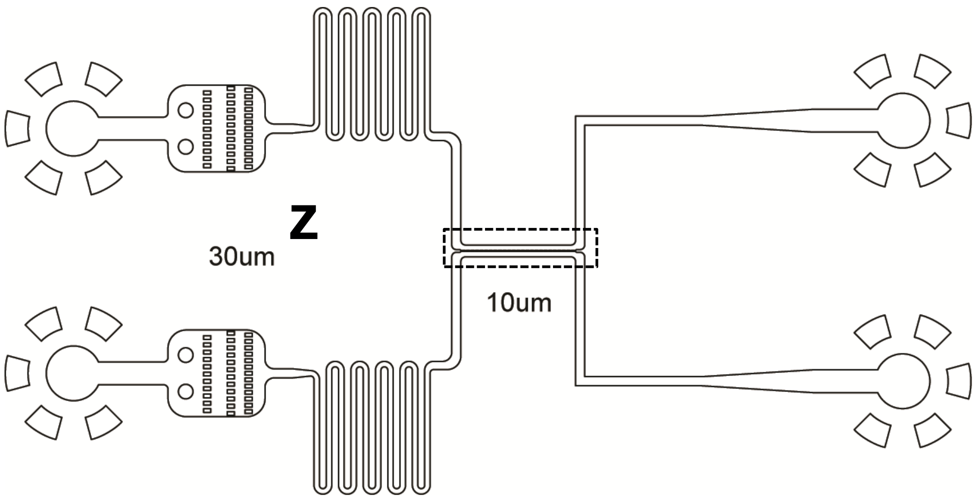
2. Megakaryocytes collect on a perforated barrier that mimics the endothelium

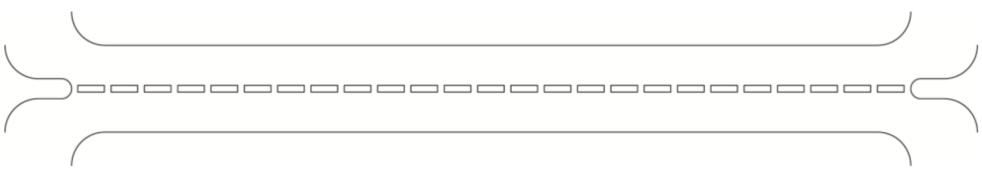
3. Proplatelets are extended as megakaryocytes “filter” through gaps/junctions in endothelium

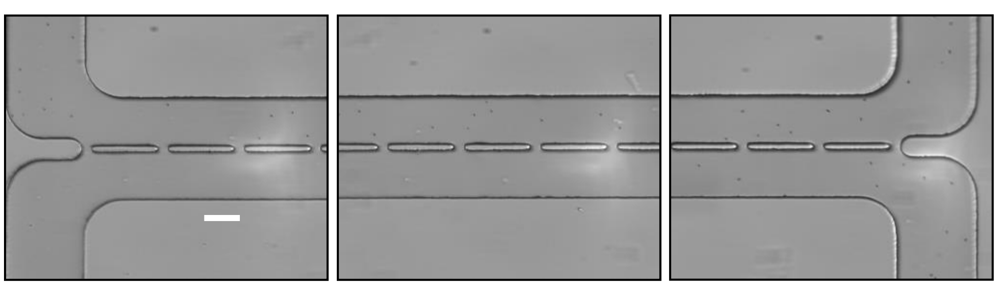
4. Proplatelets release platelets into blood vessels

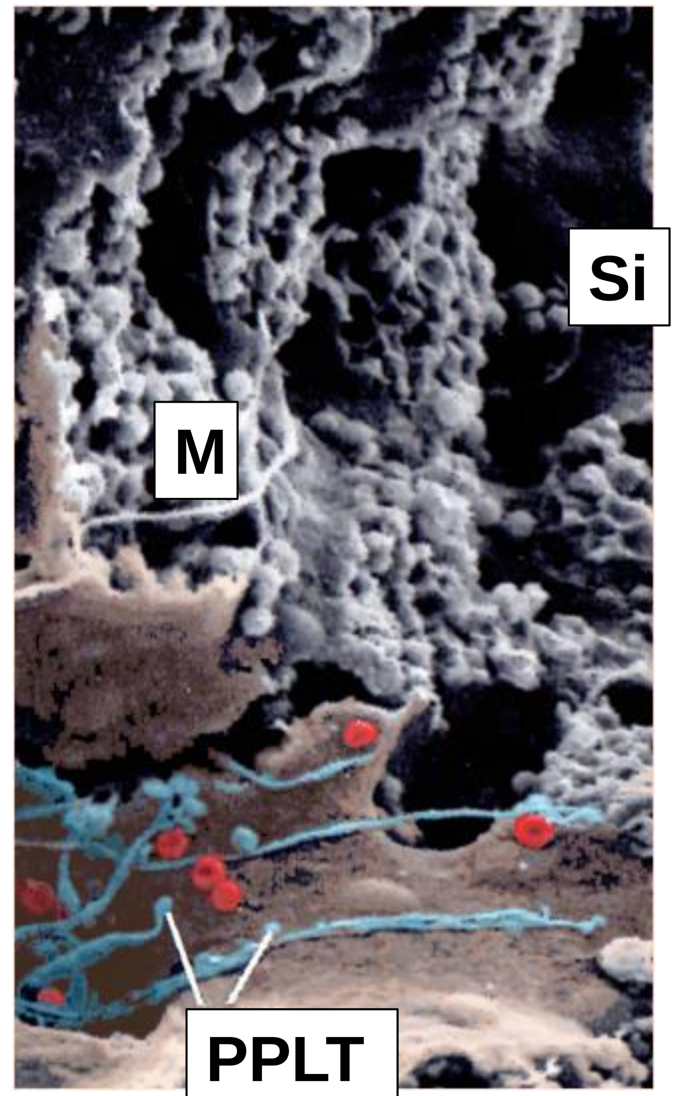


Concept and Design of Platelet Bioreactor On-A-Chip



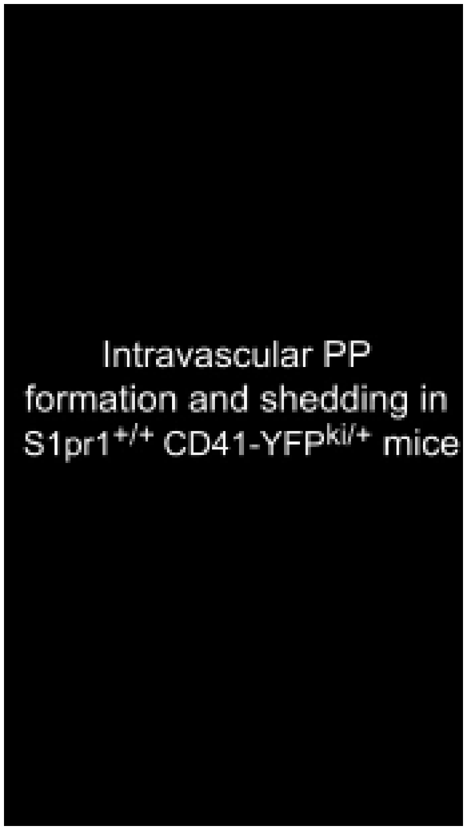




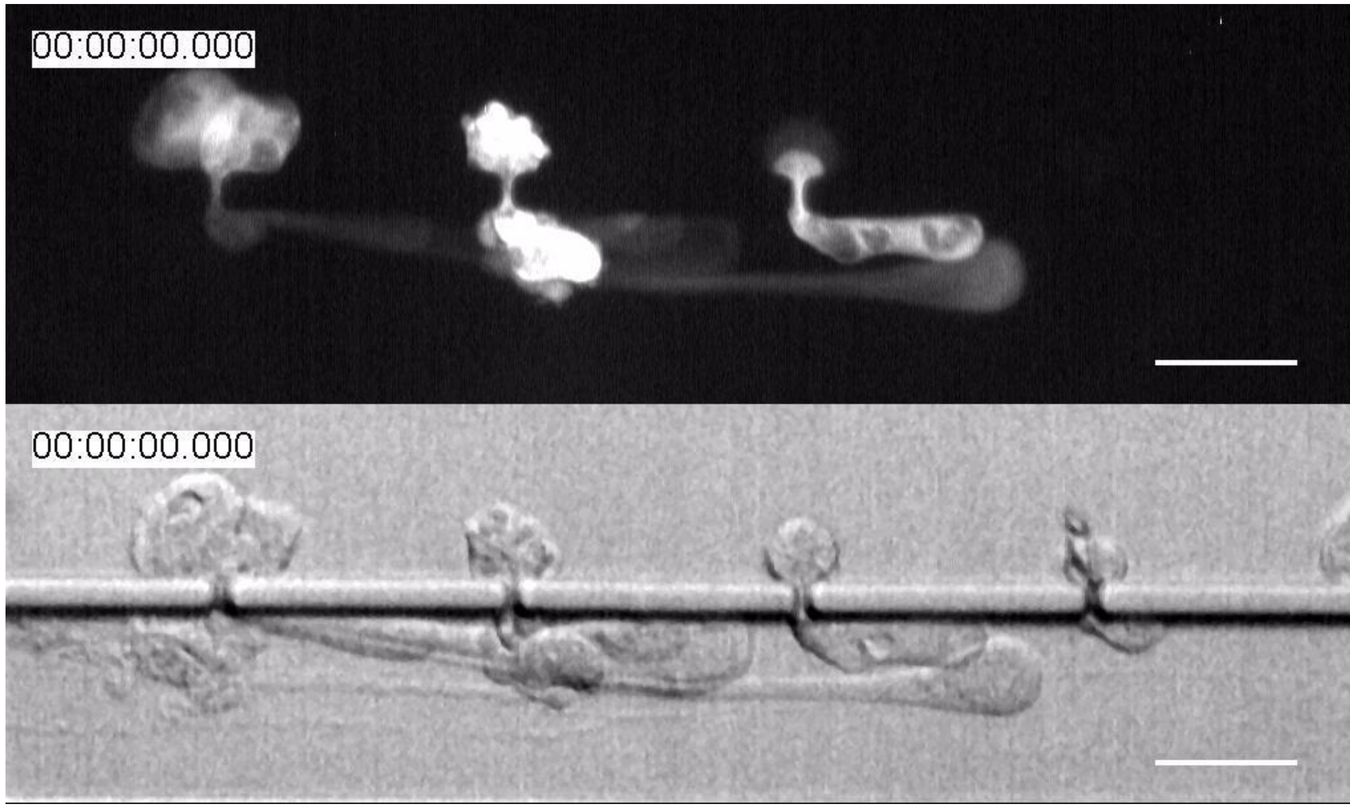


Platelet Production

In Vivo



Bioreactor



Massberg S. 2013 J Exp Med

Proplatelet Extension

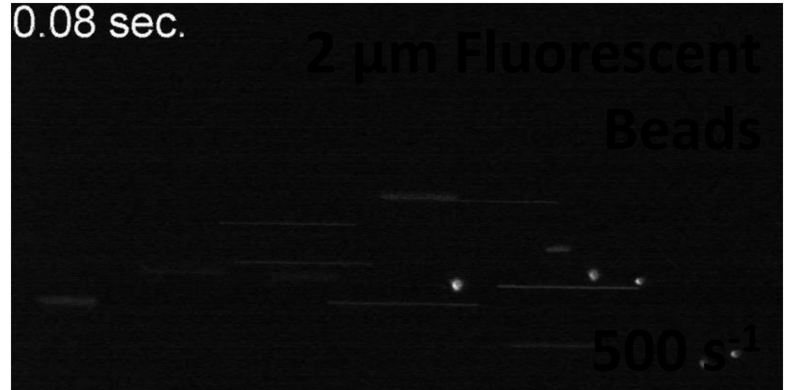
0.3-1.6 µm/min Static

6-24 µm/min (Intravital)

Recapitulating Physiology

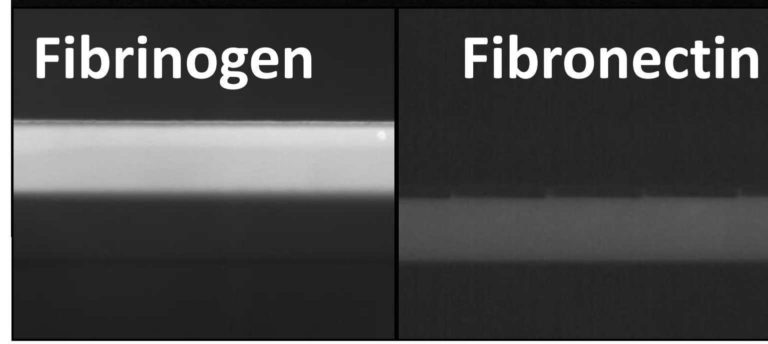
Physiological Shear Rates

500-2500 s^{-1}



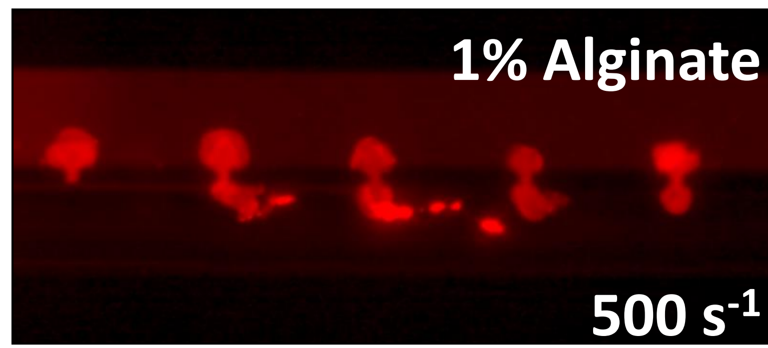
Extracellular Matrix Composition

Fibrinogen, Fibronectin,
Laminin,
Type IV Collagen



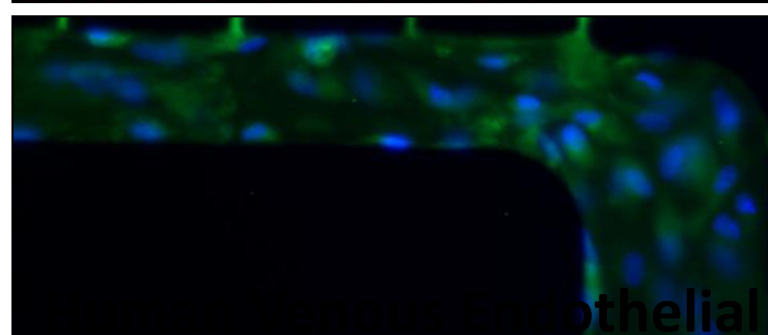
Extracellular Matrix Stiffness

200-1000 Pa

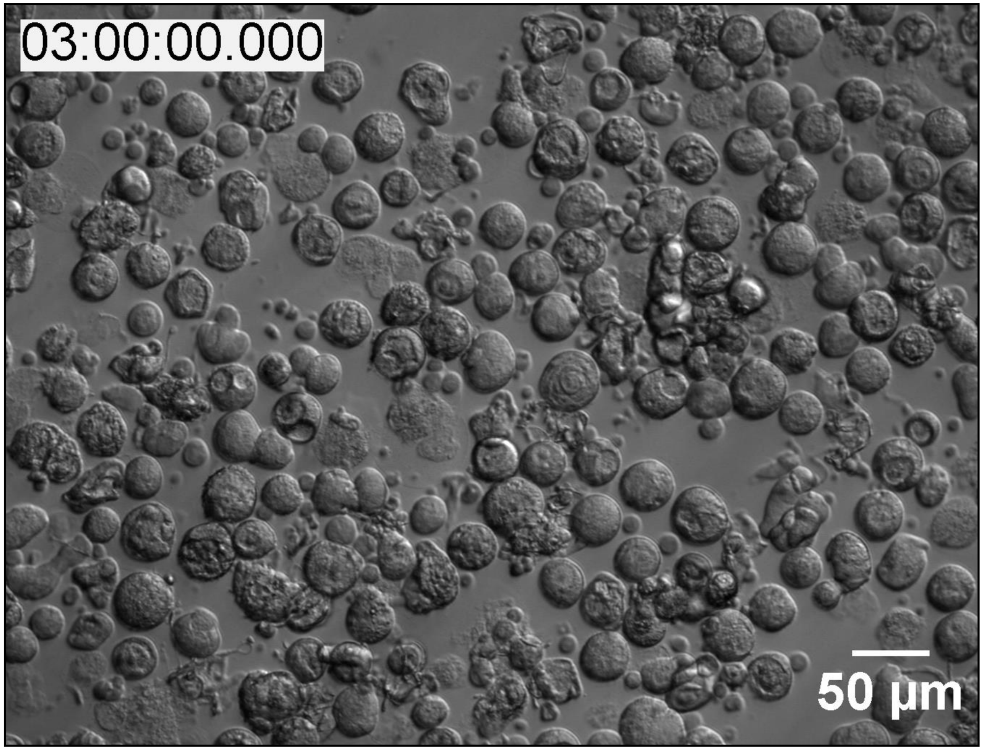


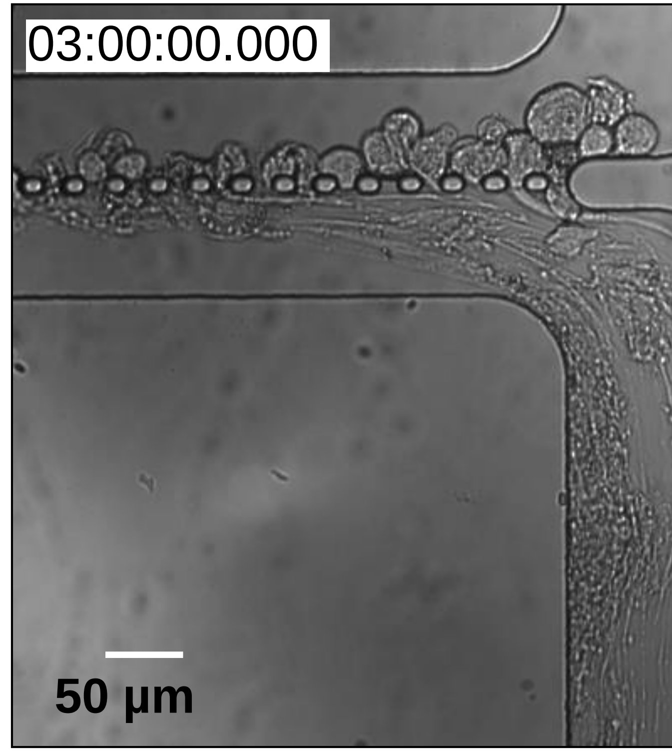
Blood Vessel Physiology

Endothelial Cell Contact



Shear triggers platelet production



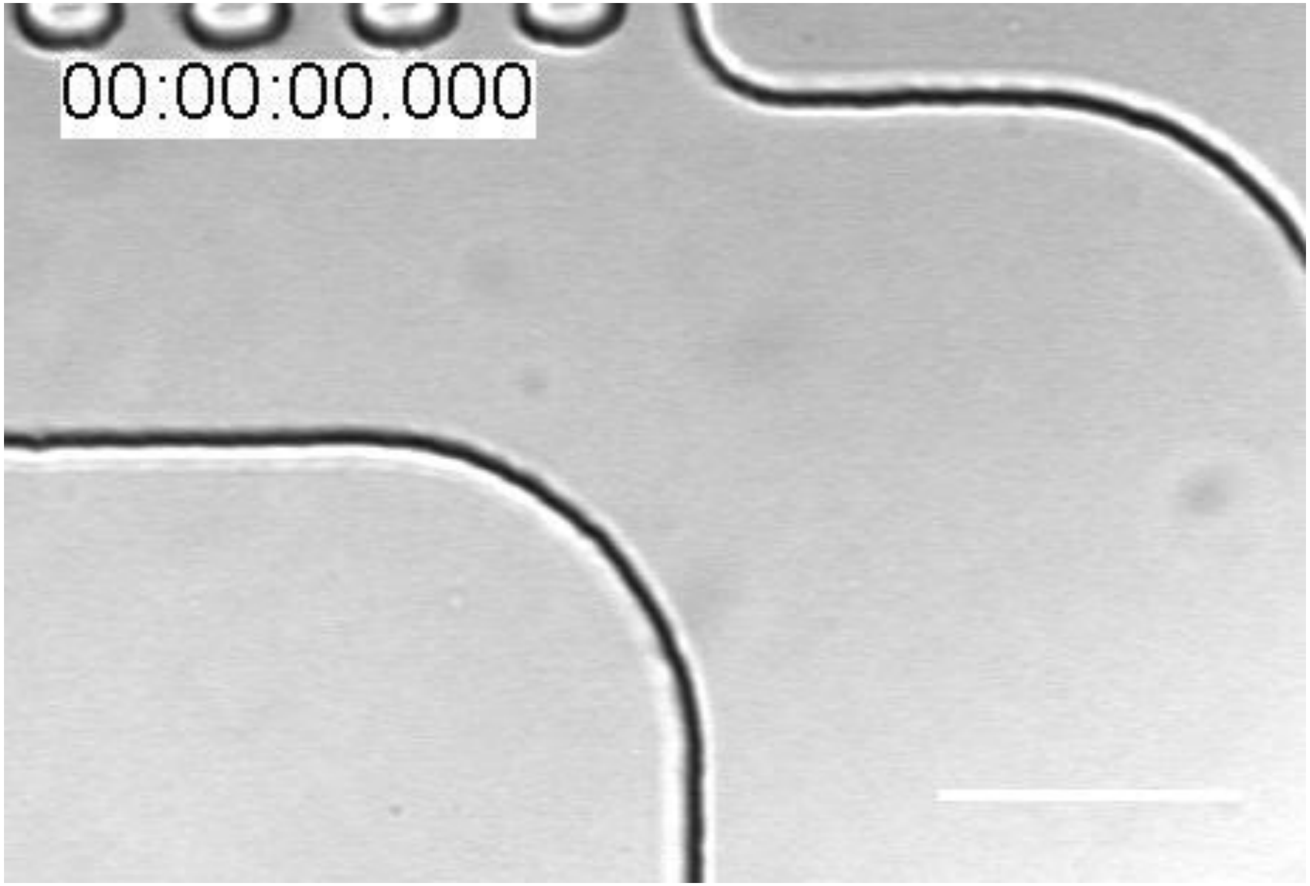


Static

Shear: 500 s^{-1}

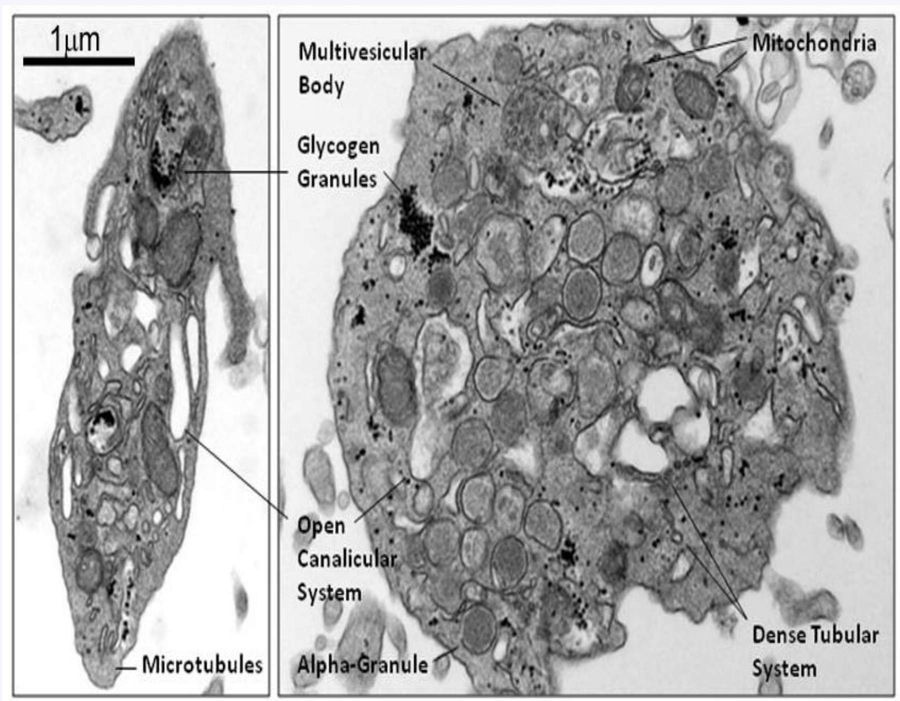
Thon et al. Blood 2014, 124:1857

Shear triggers platelet production

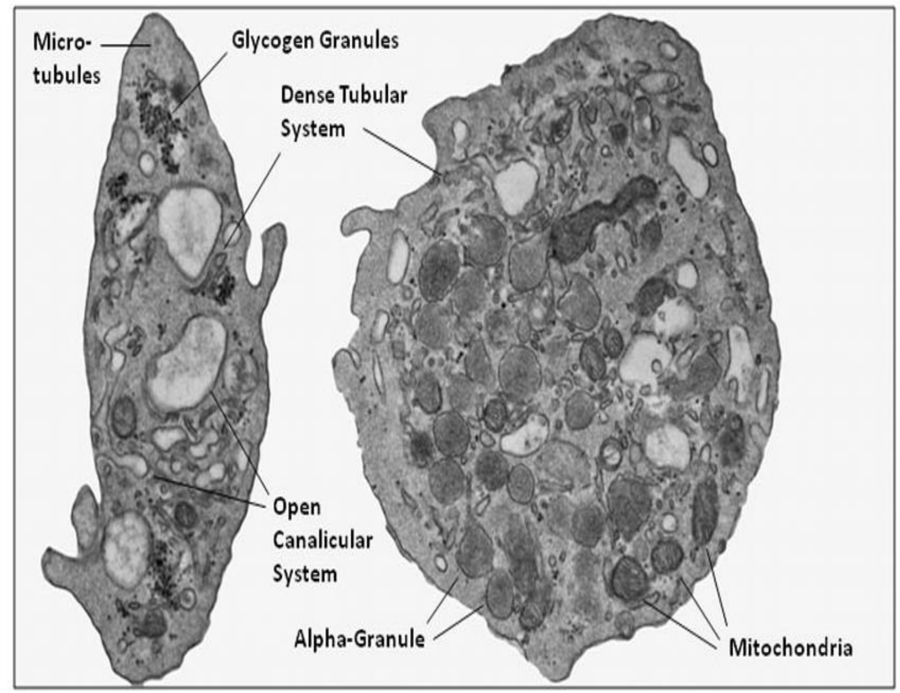


Biochip-derived platelets manifest structural properties of blood platelets

Bioreactor Platelet



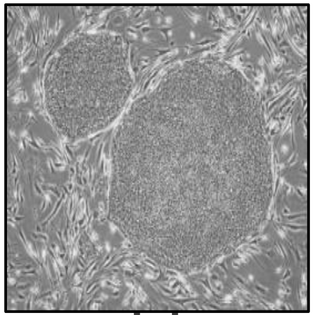
Blood Platelet



- Lu, S.J., et al. **Platelets generated from human embryonic stem cells are functional in vitro and in the microcirculation of living mice. Cell Res.** 2011;21:530-45
- Thon, J.N., et al. **Platelet bioreactor-on-a-chip. Blood.** 2014;124:1857-67
- Feng, Q., et al. **Scalable generation of universal platelets from human induced pluripotent stem cells. Stem Cell Reports.** 2014;11:817-31
- Bender, M.*, Thon, J.N.*, et al. **Microtubule sliding drives proplatelet elongation and is dependent on cytoplasmic dynein. Blood.** 2015;125:860-8

3. How do we use biologically-inspired engineering to scale and commercialize in vitro platelet production?

In Vitro Platelet Production

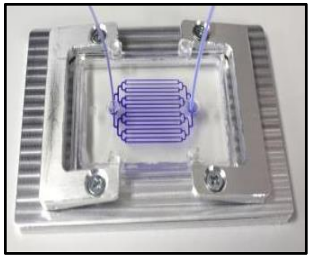


STEM CELL

2006: Invention of human induced pluripotent stem cells (iPSC) allows for genetically consistent and scalable stem cells.

MEGAKARYOCYTE

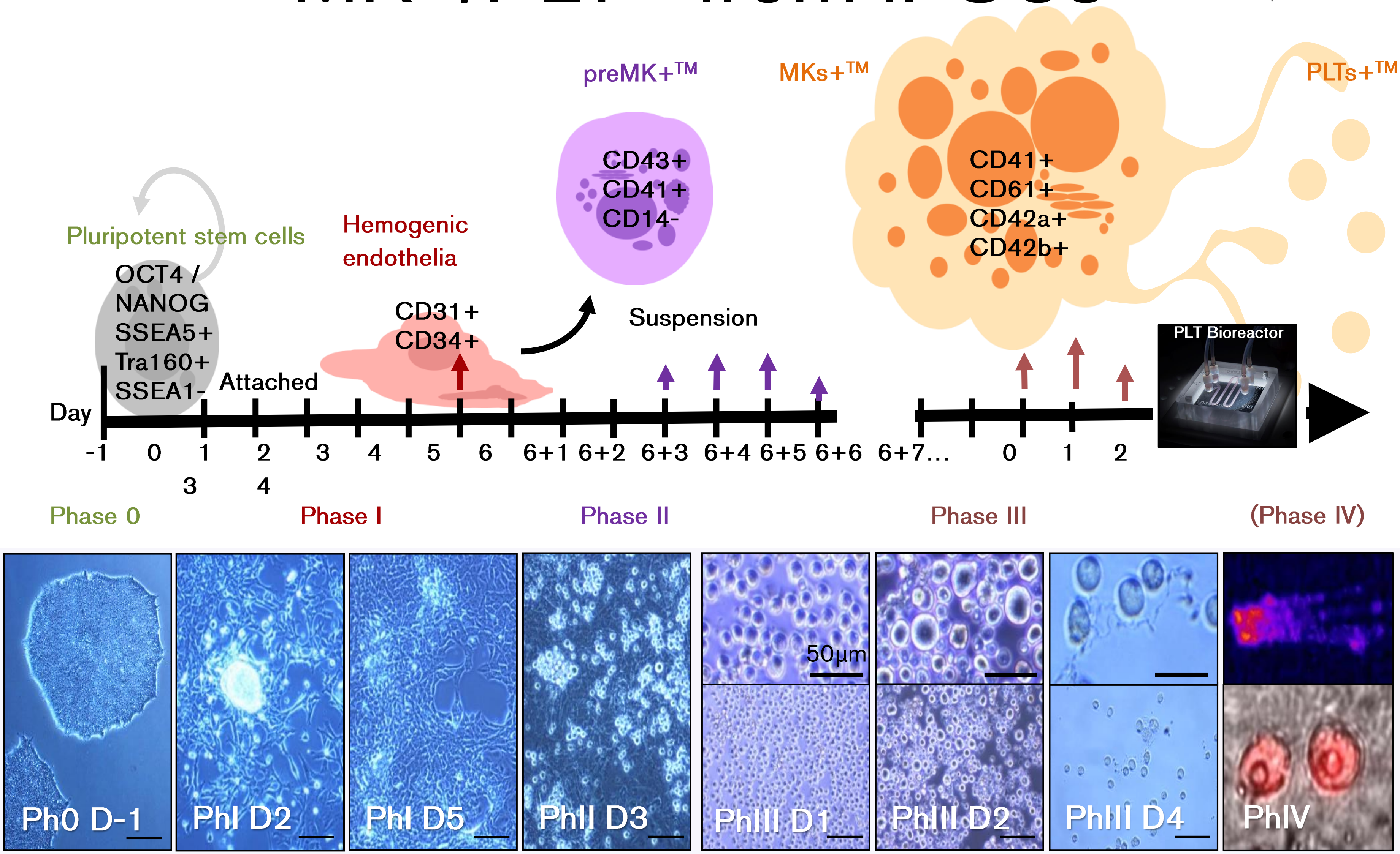
1994 discovery of thrombopoietin (TPO) led to generation of 1st human platelets by Amgen (via cell culture).



PLATELET

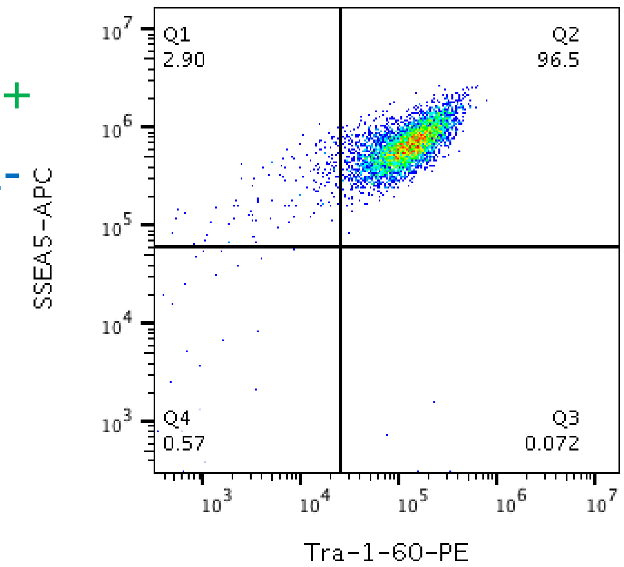
2014 development of platelet bioreactor that models bone marrow *and* allows scalable generation of platelets

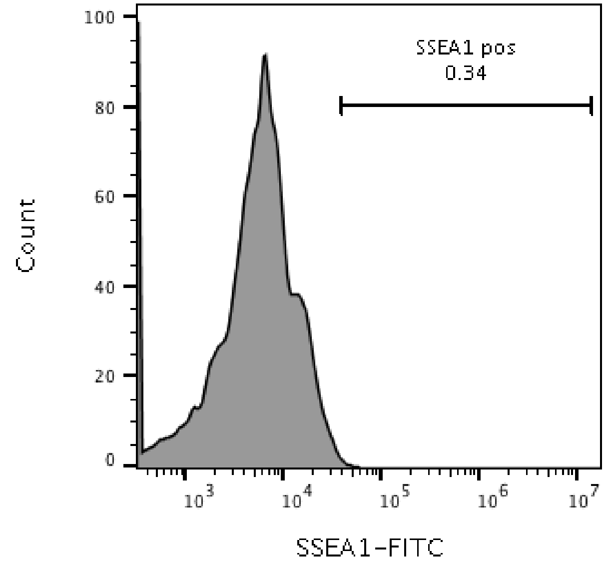
Directed Differentiation of MK+/PLT+ from iPSCs



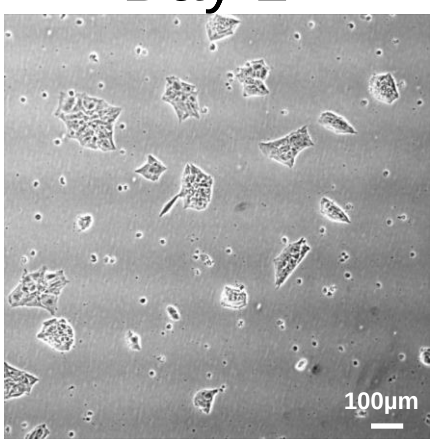
Phase 0. Undifferentiated iPSC

hiPSCs are Oct4+ Nanog+ SSEA5+ Tra160+ SSEA1-

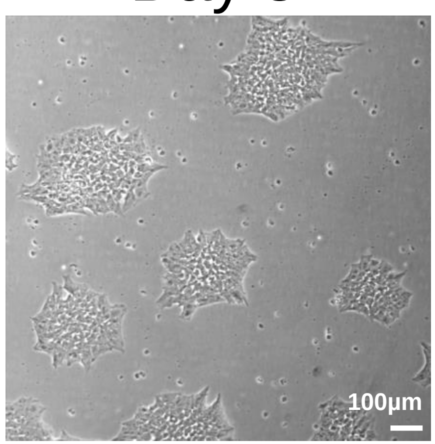




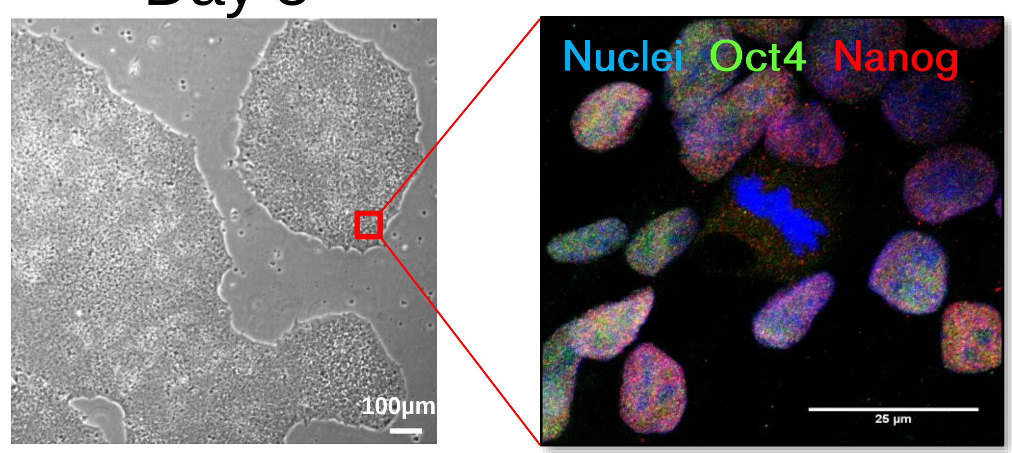
Day 1



Day 3

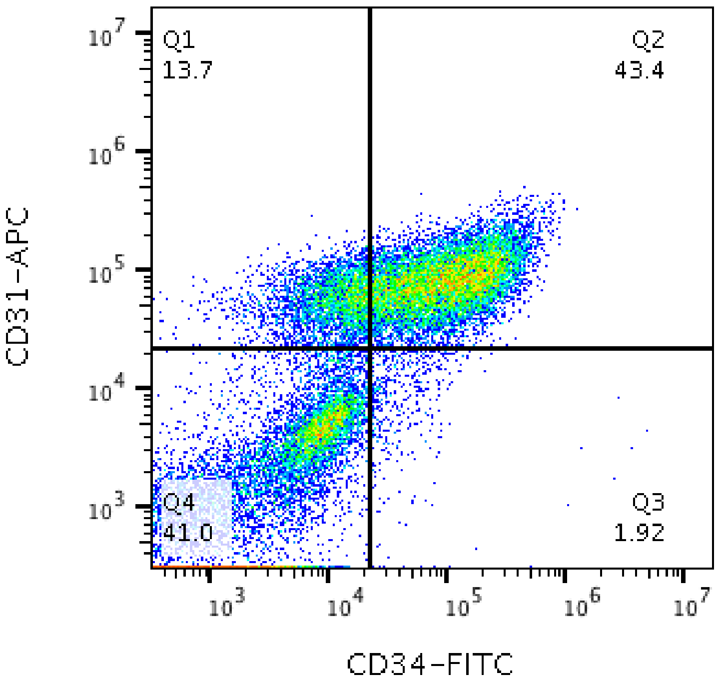


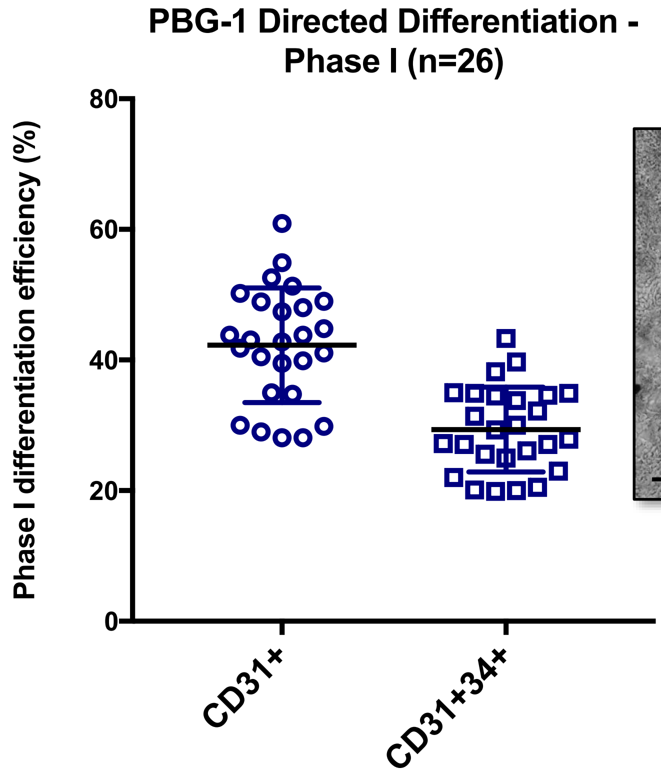
Day 5

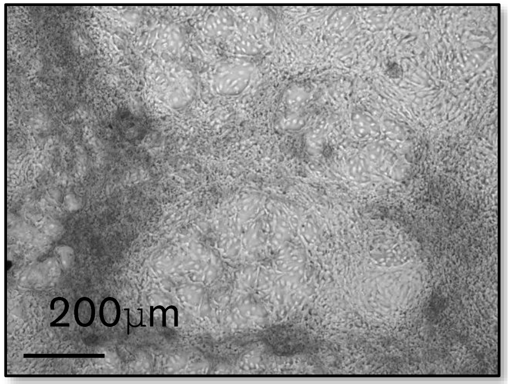


Phase I. iPSC → Hemogenic Endo

- Hemogenic endothelium are CD31+ CD34+
- Propidium Iodide-



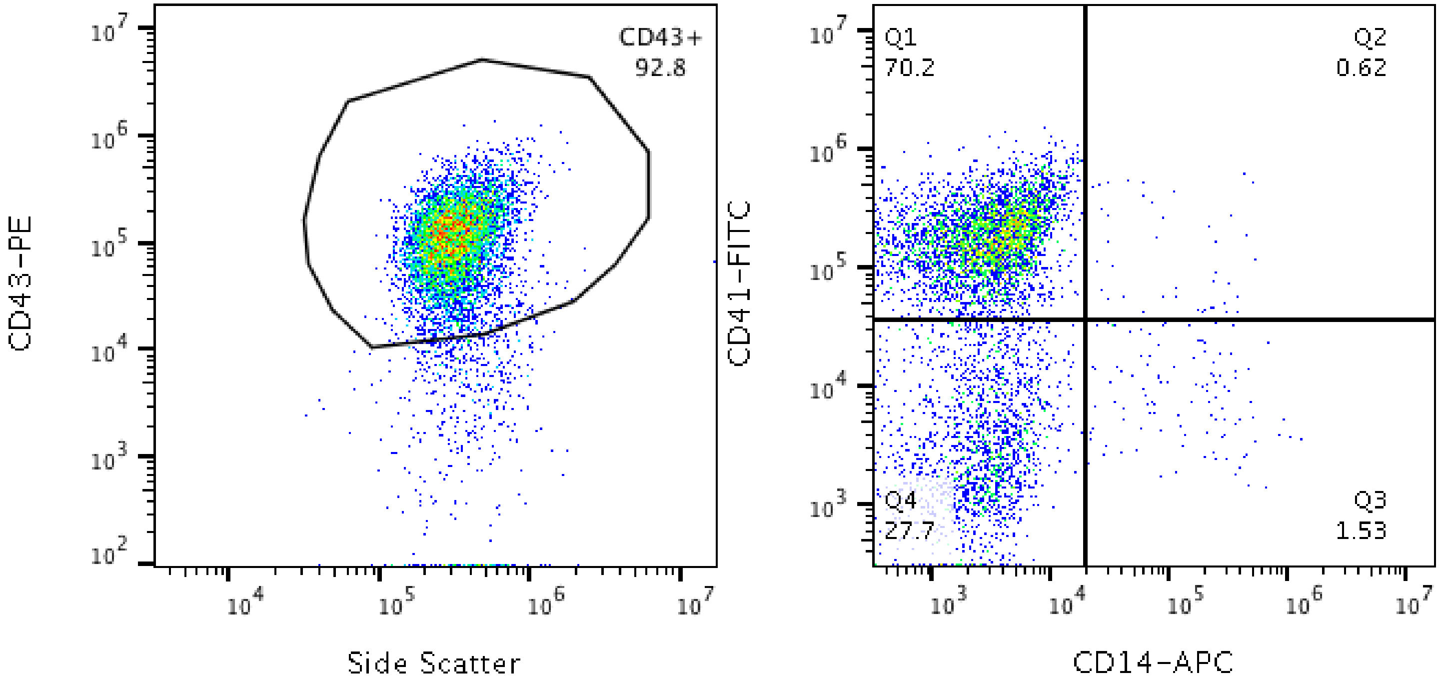




Phase II.Hemogenic Endo→preMKs+™

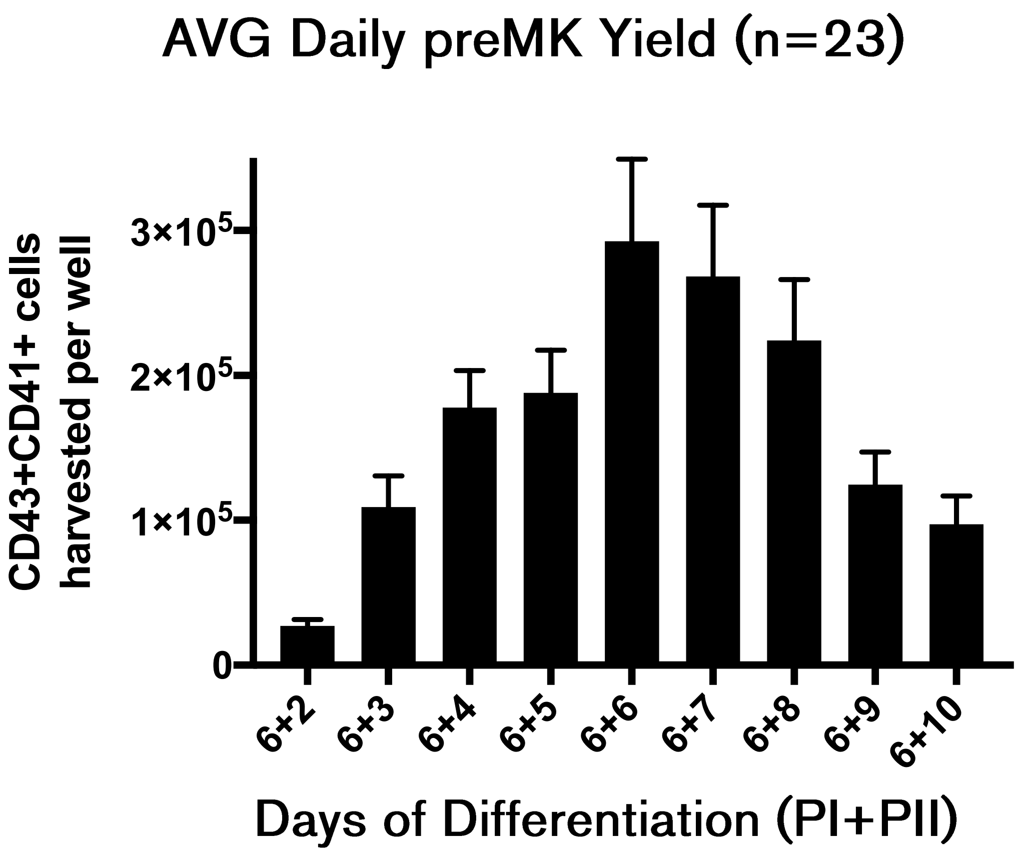
preMKs+™ are CD43+ CD41+ CD14-

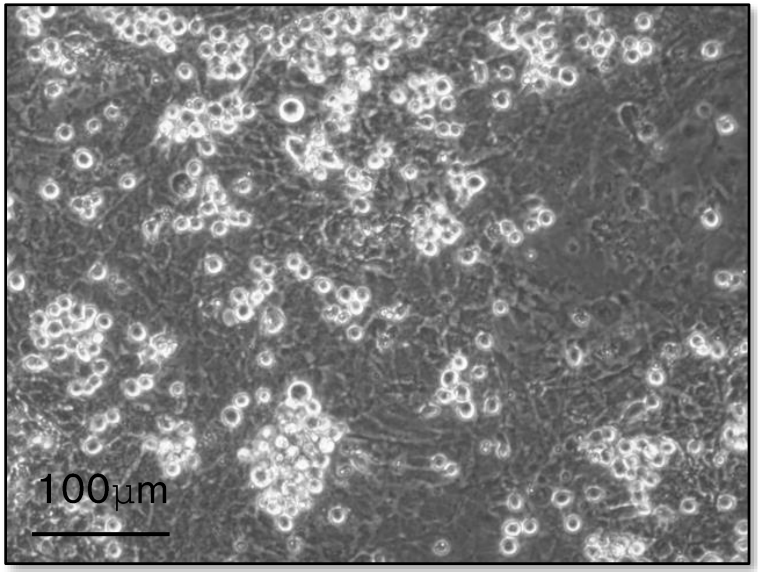
Propidium Iodide-



Phase II. HE → preMKs+™

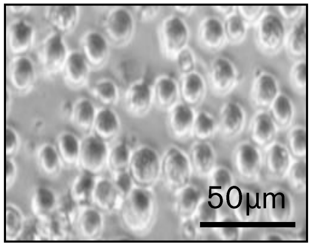
- preMKs+™ are CD43+ CD41+ CD14-
- Propidium Iodide-



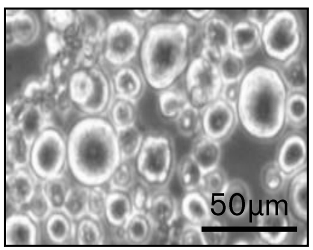


Phase III: Maturation of MKs+™ from preMKs+™

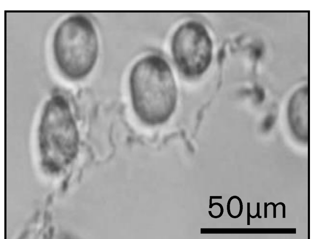
PhIII D1

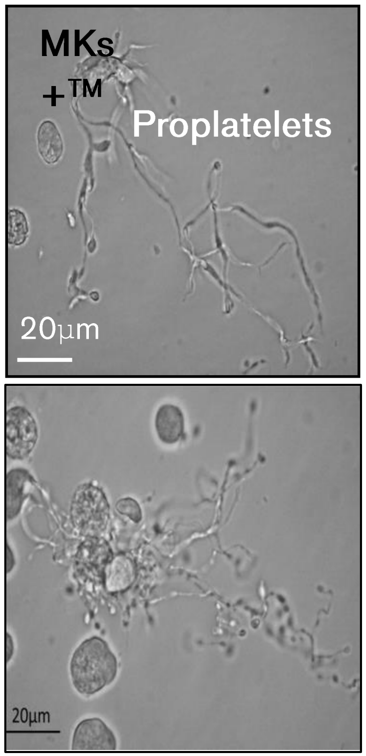


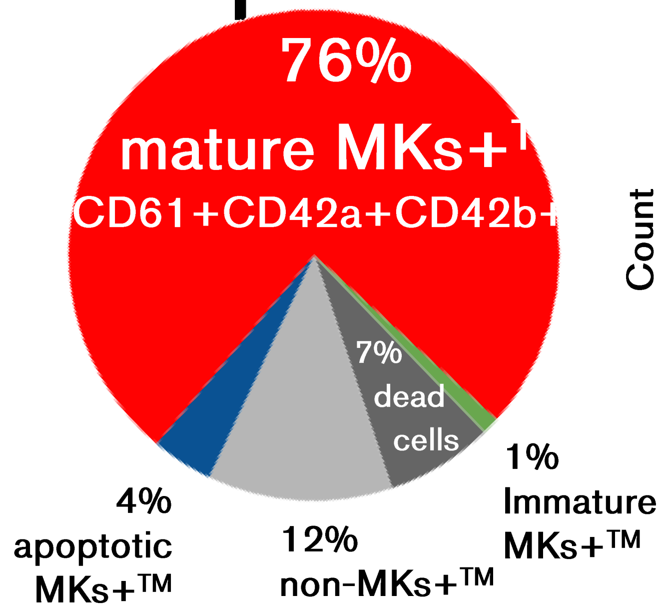
PhIII D2

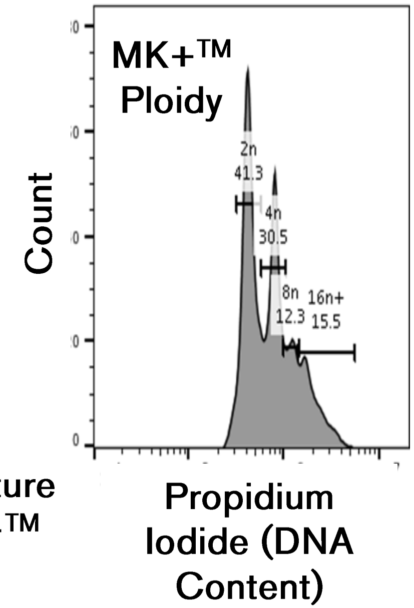


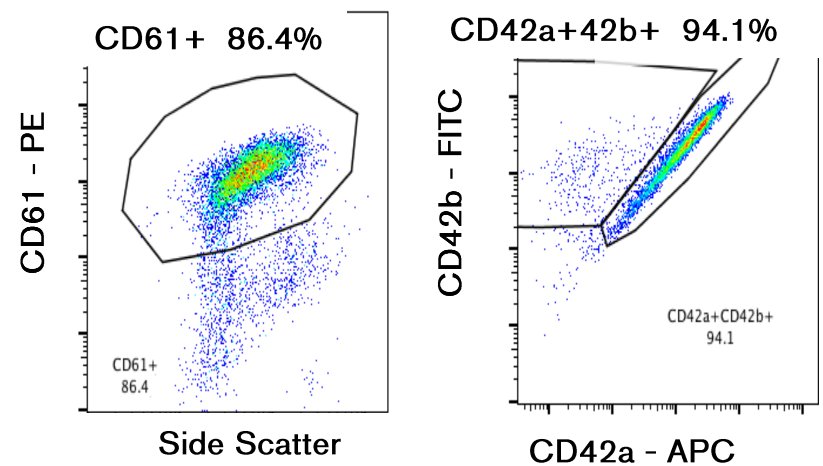
PhIII D4



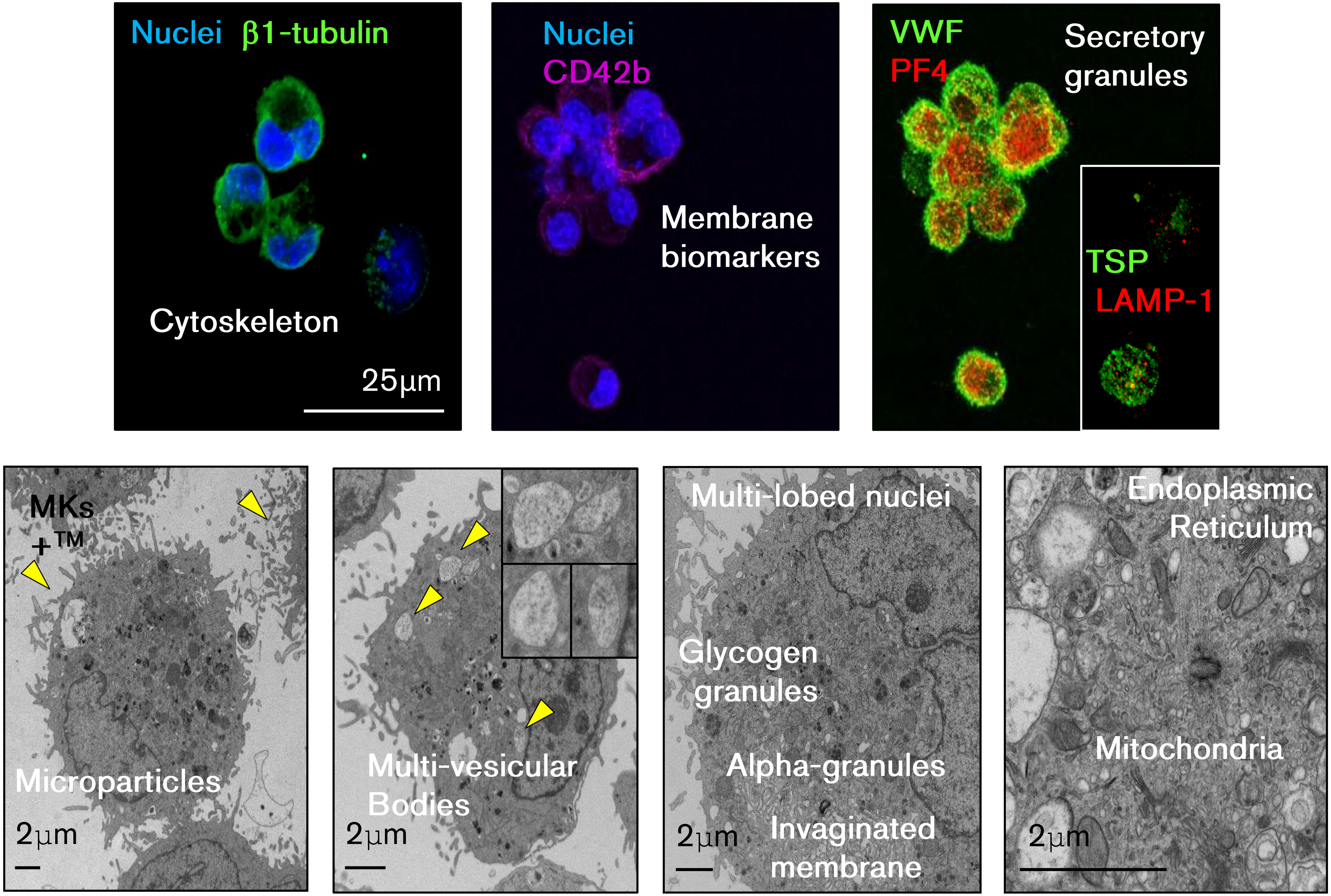








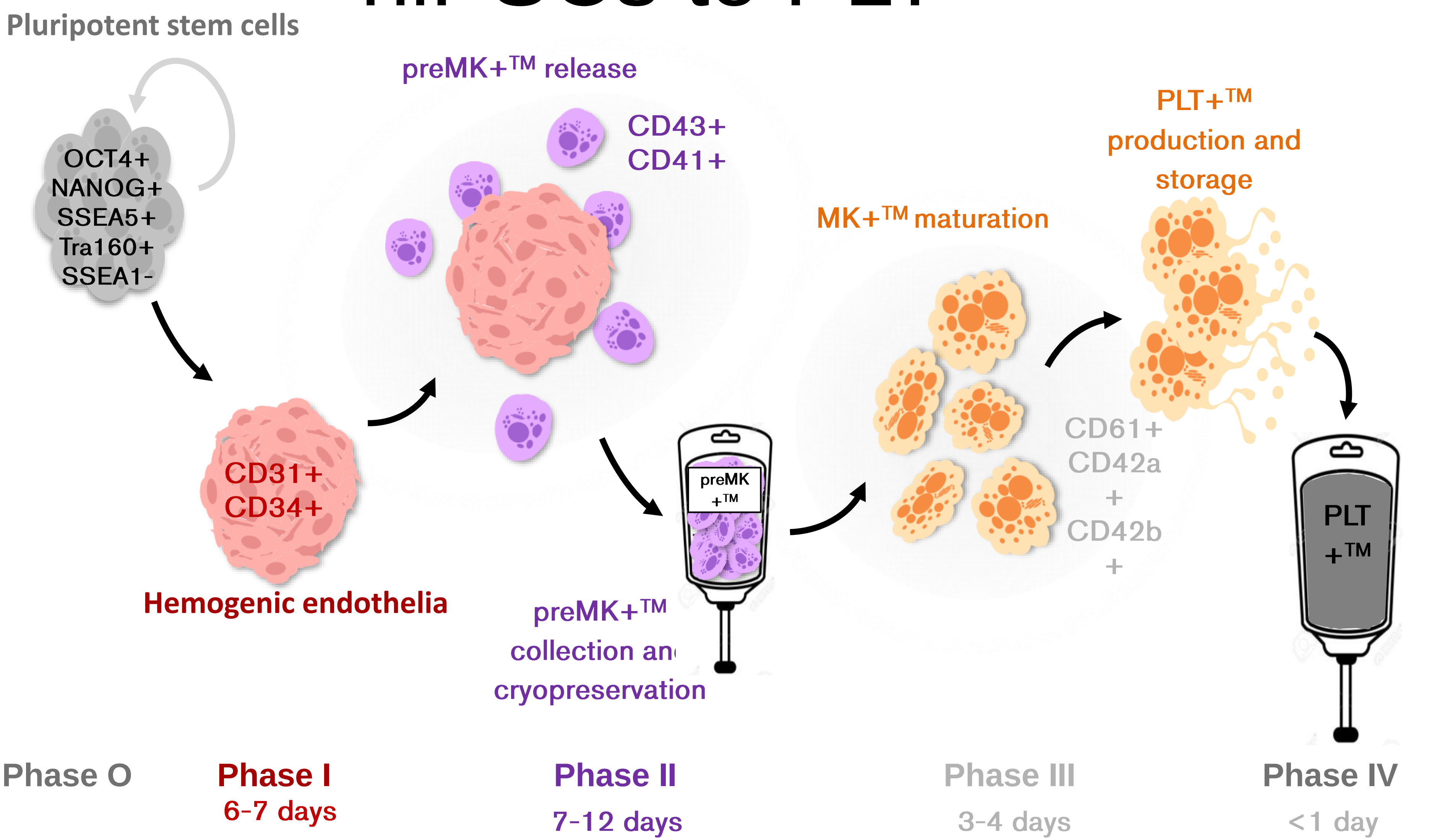
MK+™ Biomarker Expression and EM characterization



Hypothesis: To enable commercialization we need to

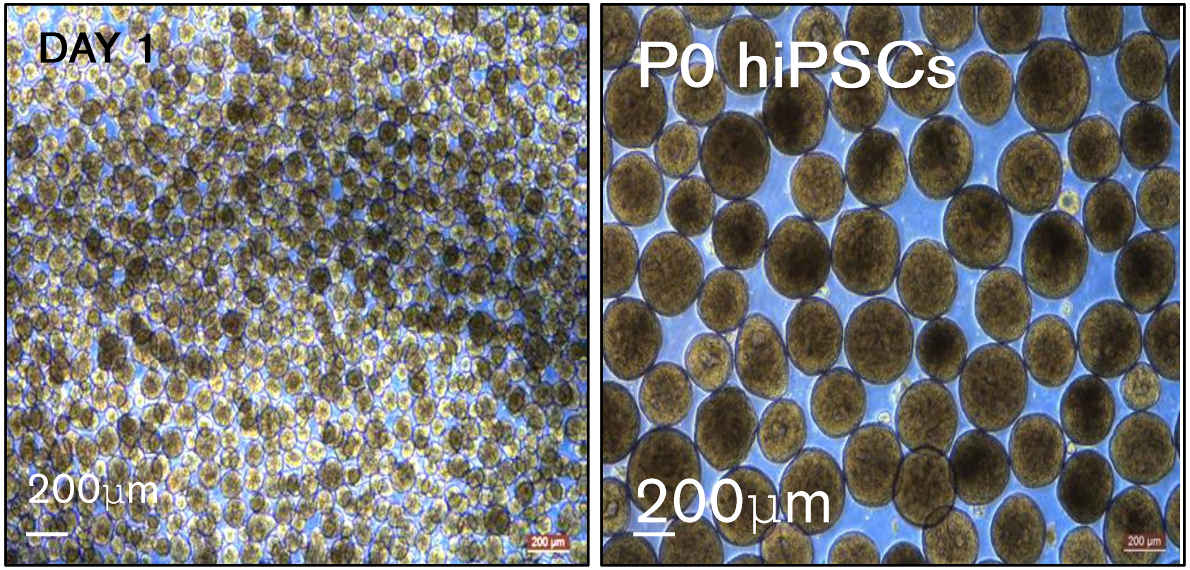
1. Source a clinical grade iPSC line as an inexhaustible source of human MKs and PLT
2. Establish a feeder-free, serum-free, animal component-free differentiation protocol
3. Develop a scalable production process for industrial manufacture

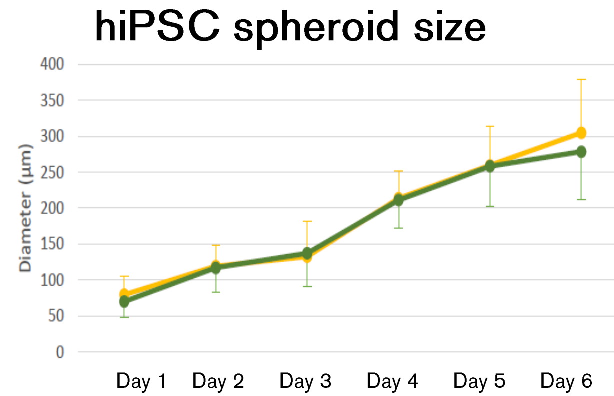
Directed Differentiation of hiPSCs to PLT+™

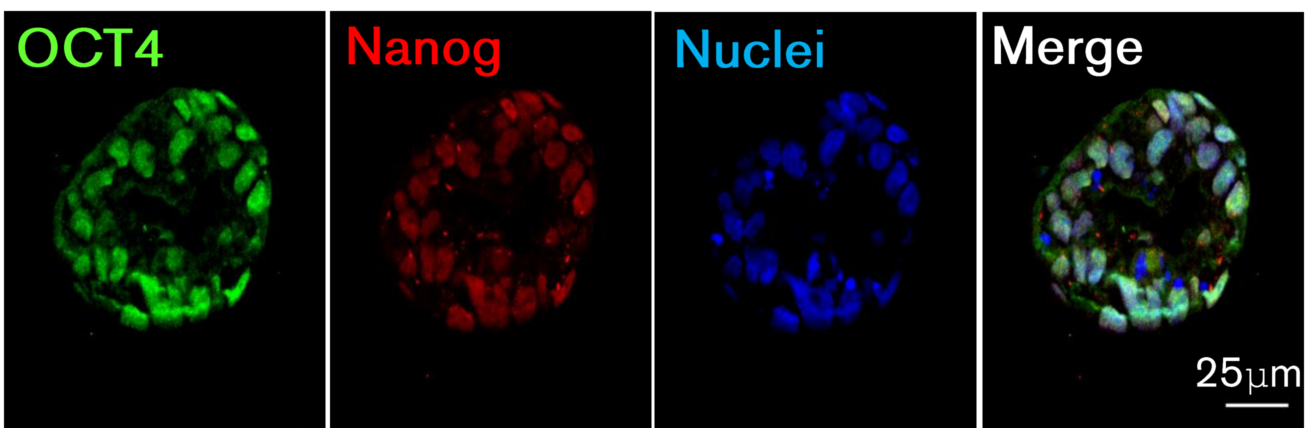


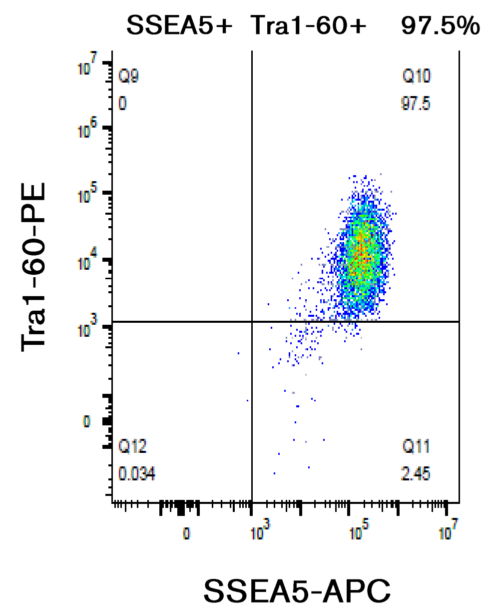
Phase 0: hiPSC Seed Train

3D Expansion of induced pluripotent stem cells as spheroids





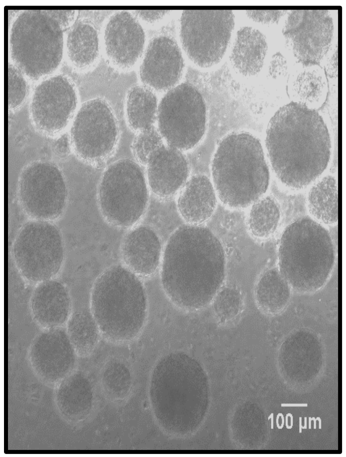




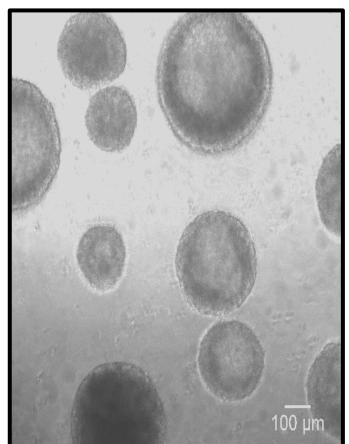
Phase I: iPSC Differentiation to Hemogenic Endothelium

Day -1

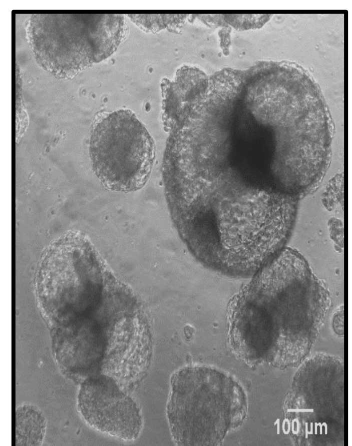
Day 0



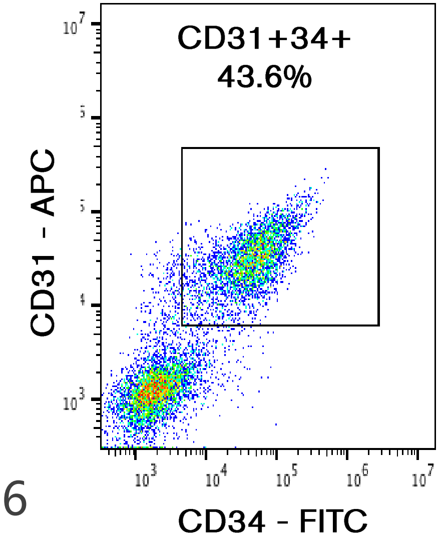
Day 3



Day 6

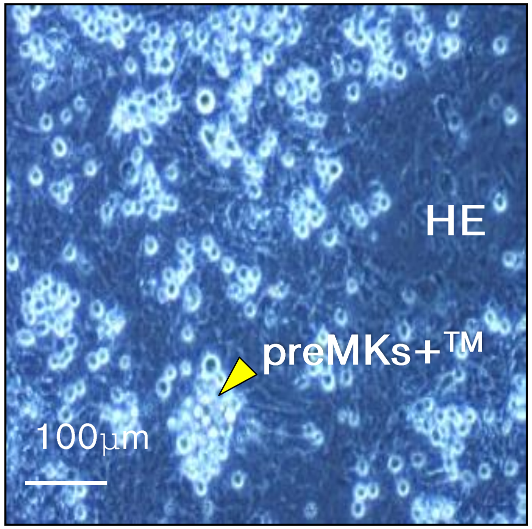


HE differentiation efficiency day 6

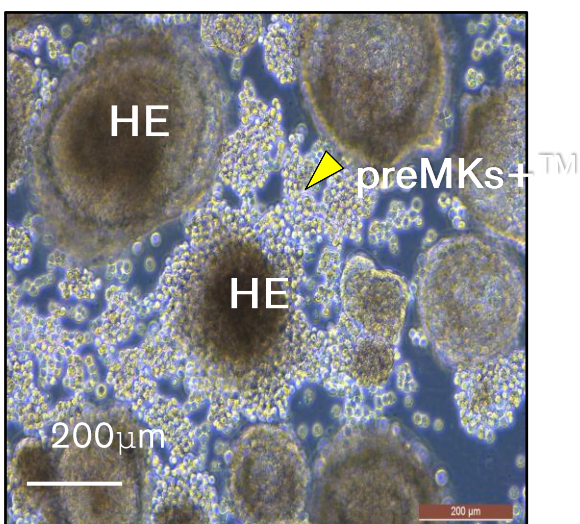


Phase II: Production and release of preMKs+™

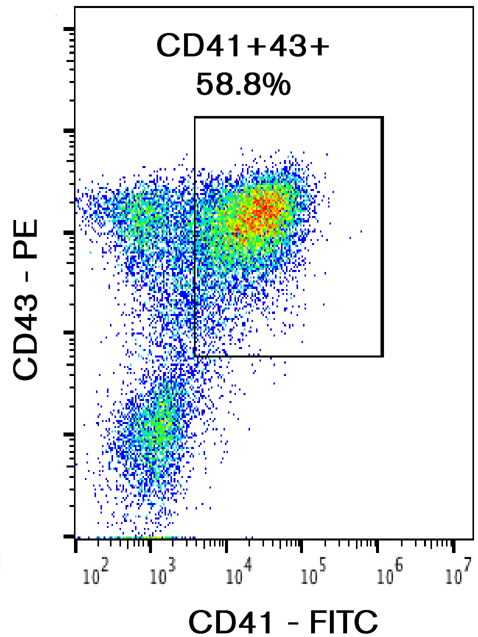
2D differentiation culture 6+6



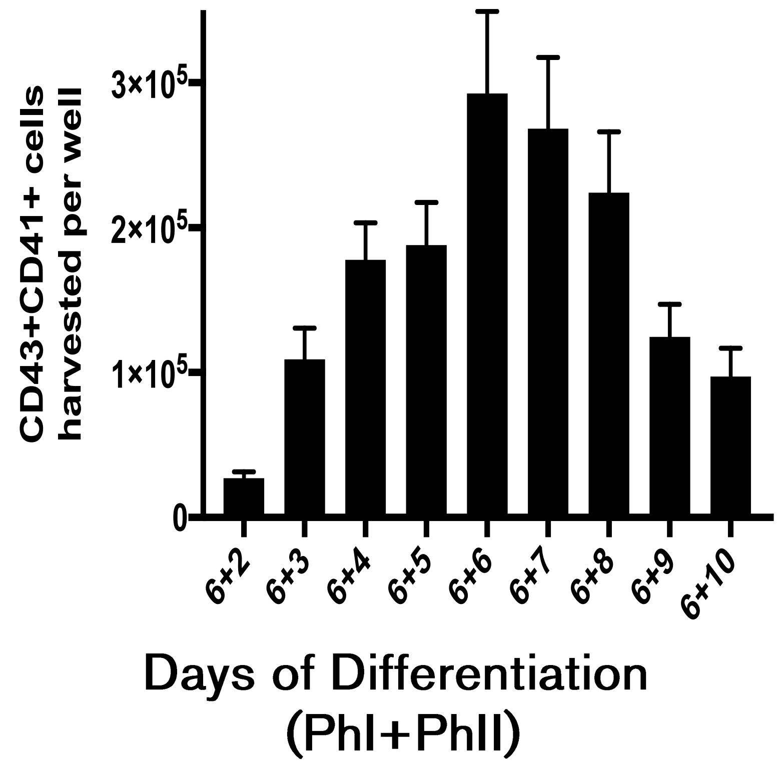
3D differentiation culture 6+4



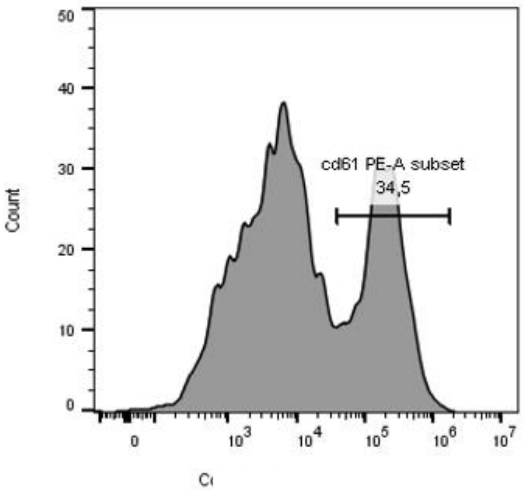
preMK+™ purity
(3D: day 6+4)



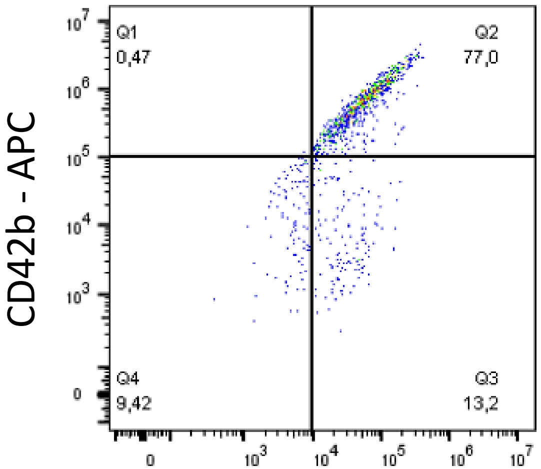
Daily preMK+™ Yield
(2D: n=23)



Phase III Cultures of 3D Directed Differentiated hiPSC

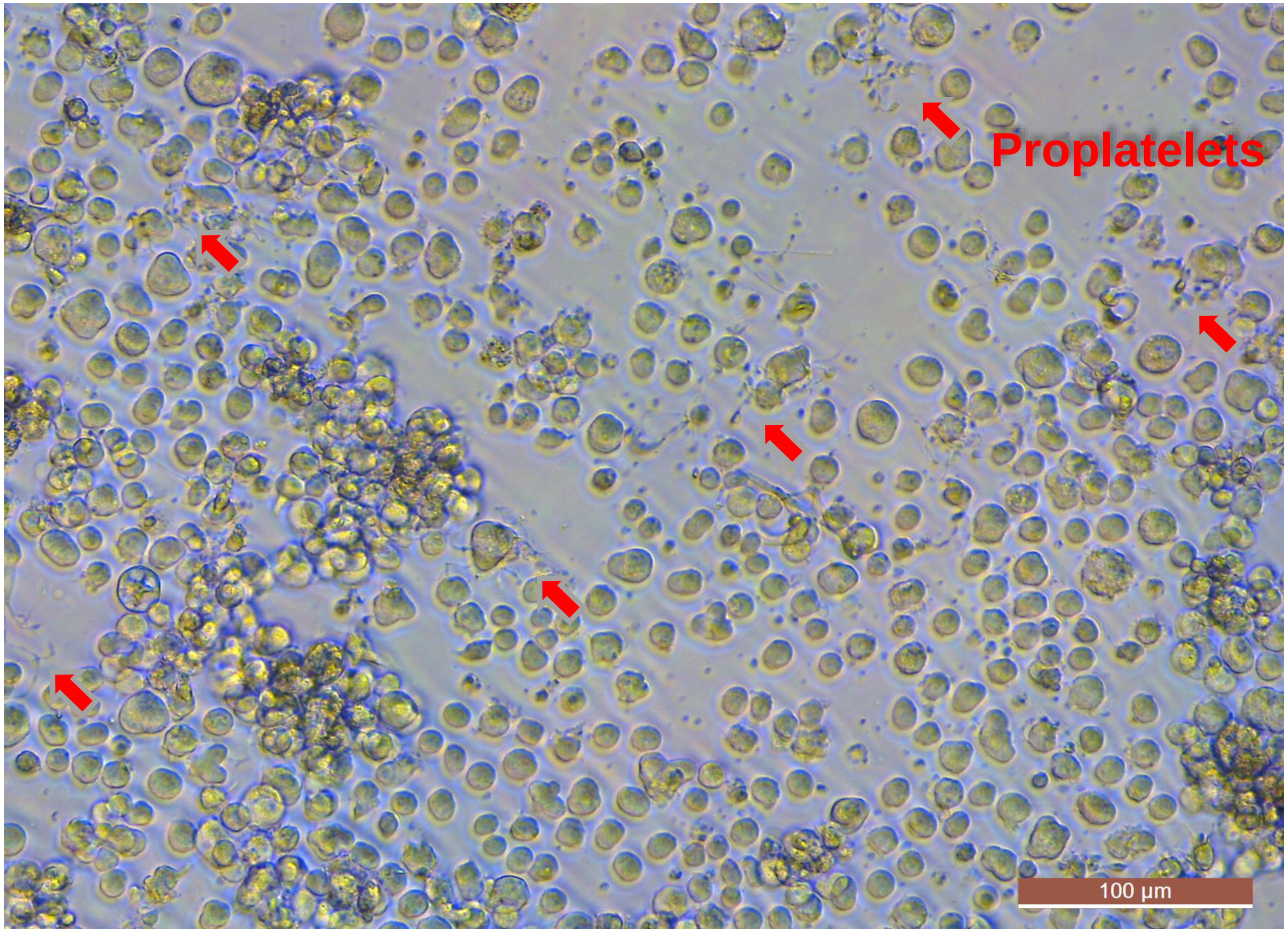


CD61 - PE

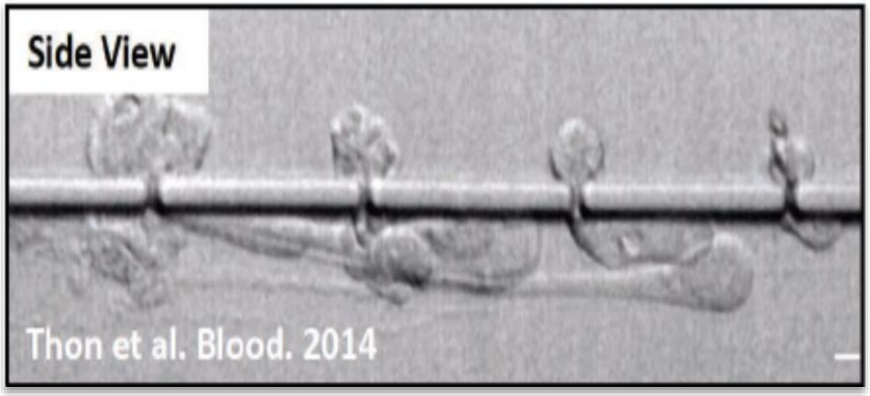


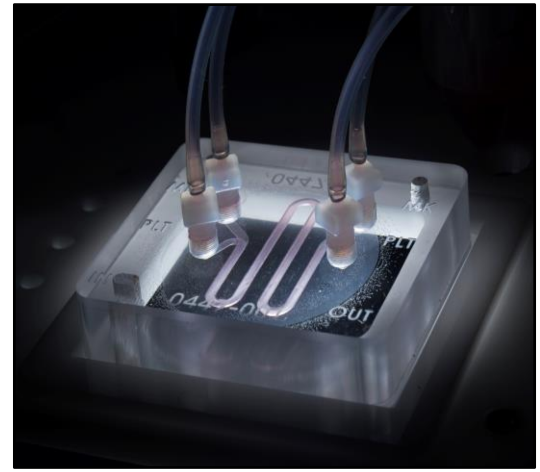
CD42a-FITC

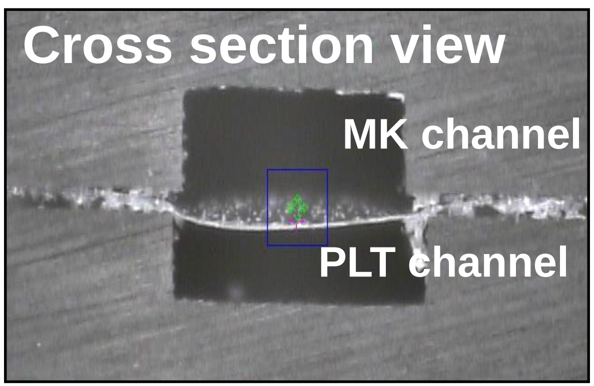
Phase III – Day 6+4+3



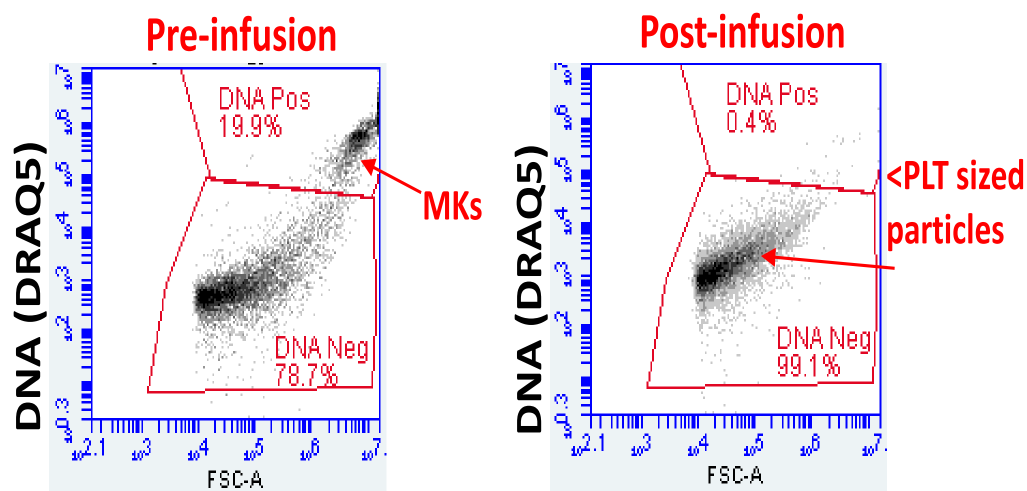
Phase IV: PLT+^{TM} bioreactor



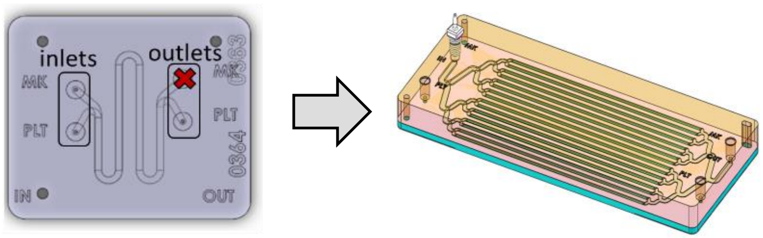




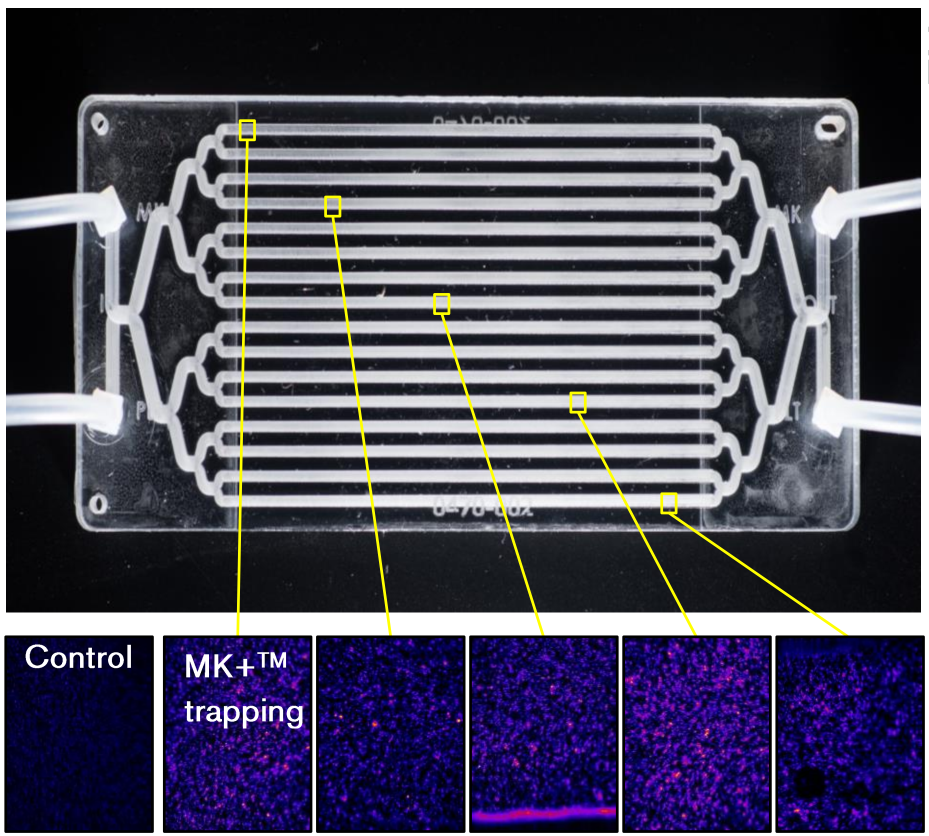
Nucleated cells retained in PLT+^{TM} bioreactor



PLT+™ Bioreactor Scale Up

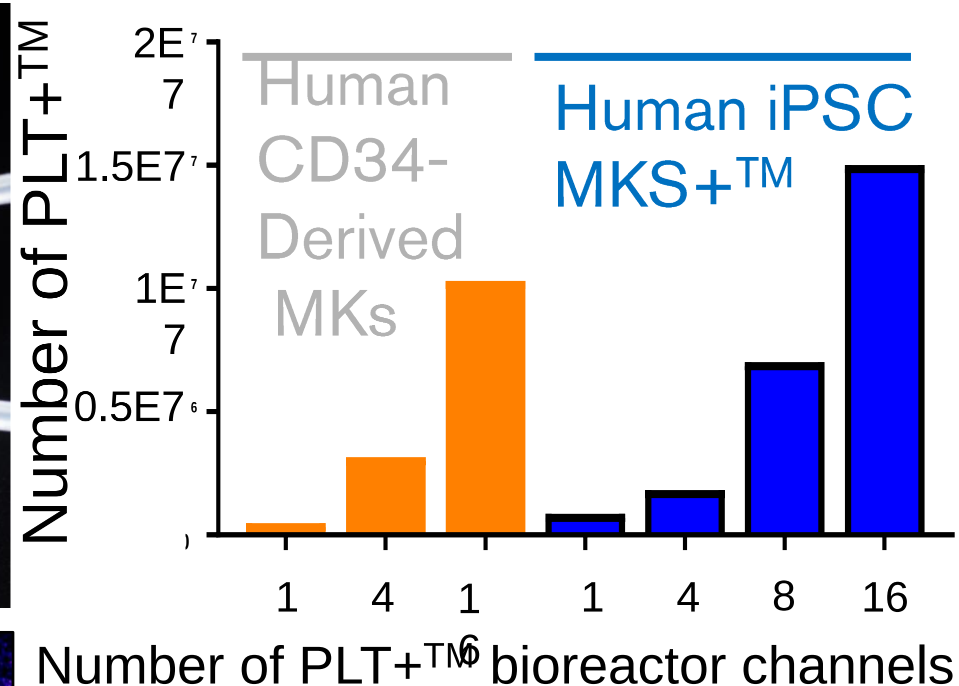


Even seeding across channels



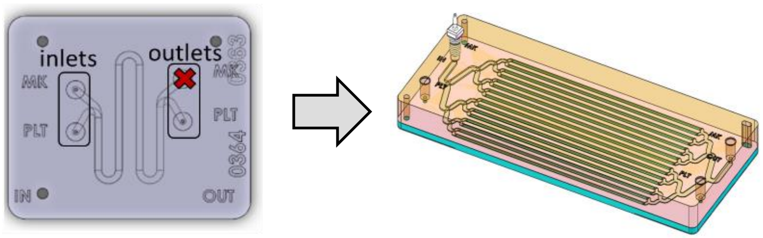
Signal = nuclei (Hoescht)

Linear increase in production

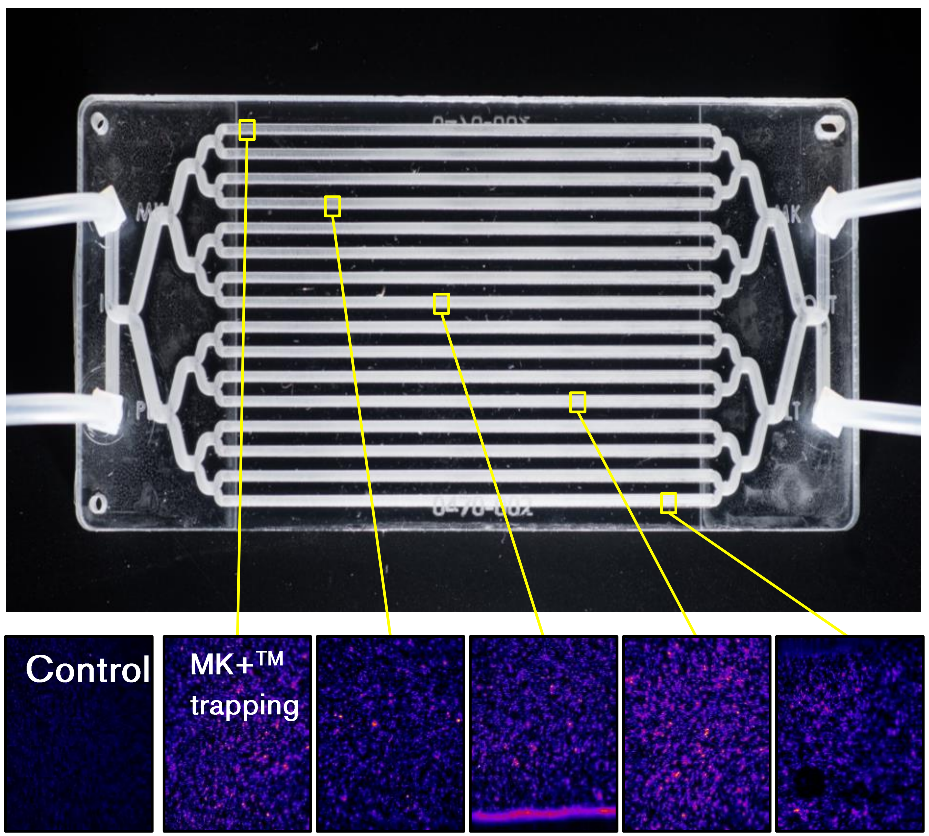


(t=3hrs)

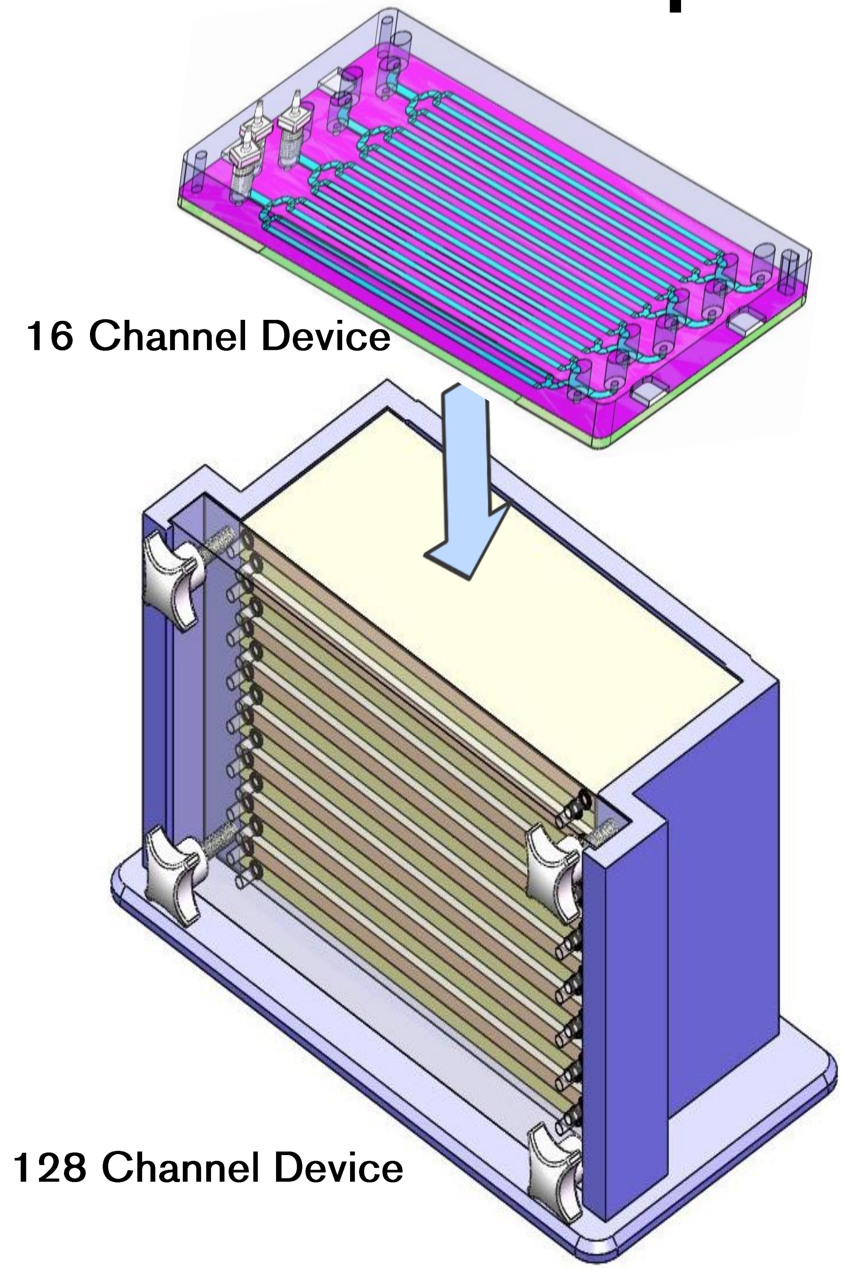
PLT+™ Bioreactor Scale Up



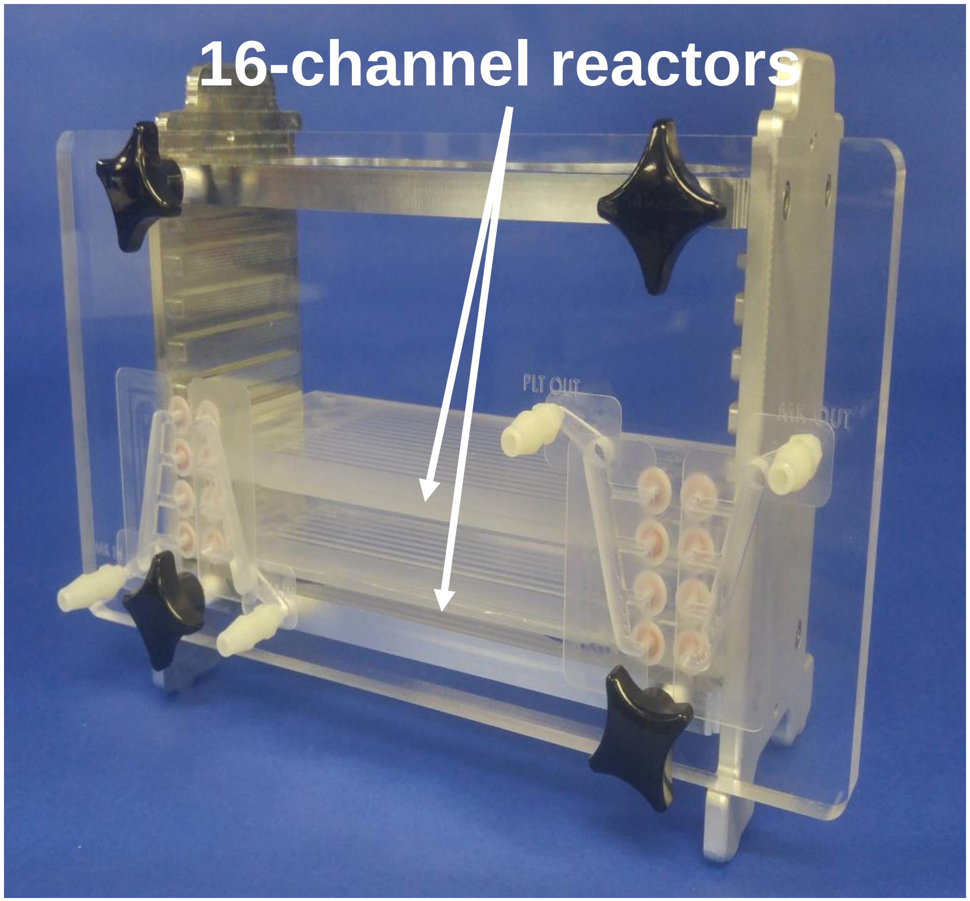
Even seeding across channels



Signal = nuclei

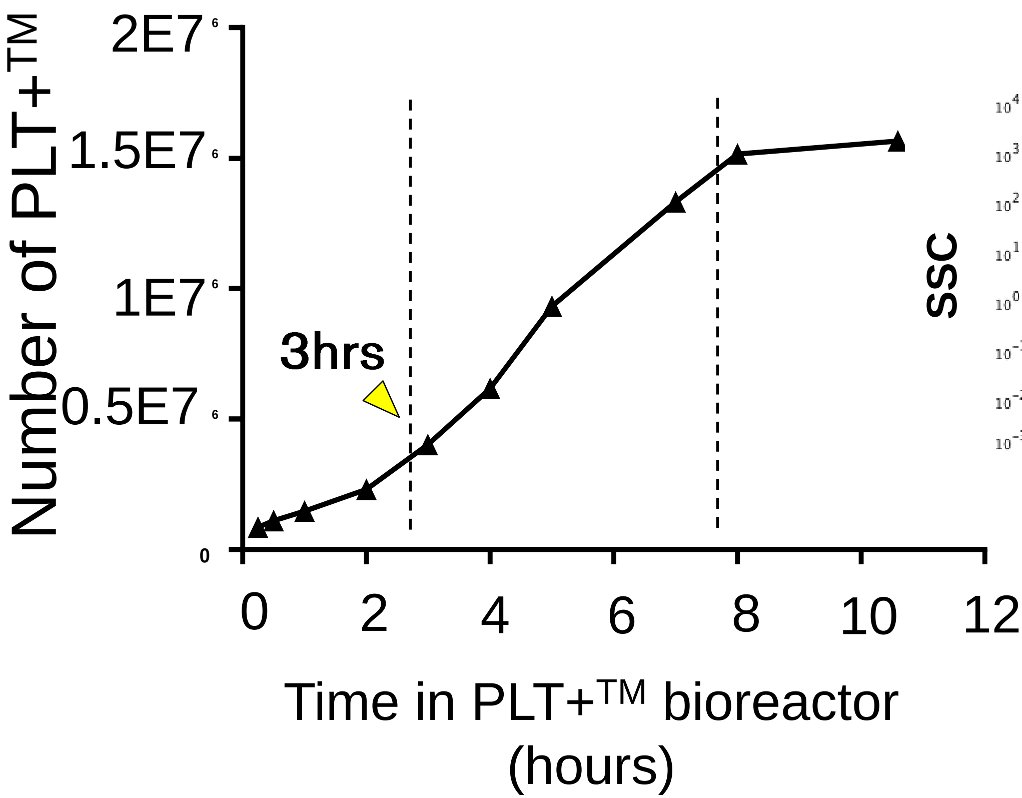


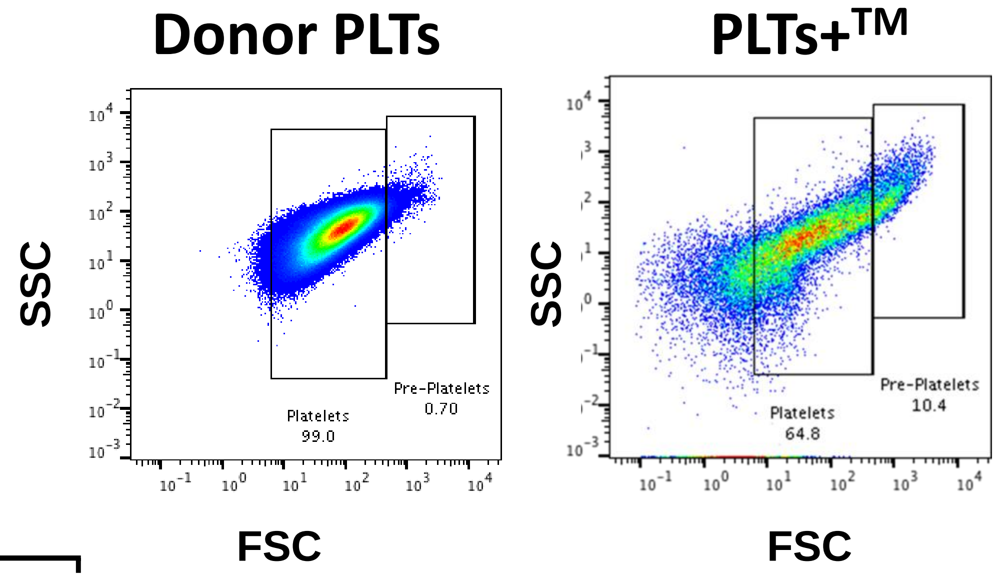
PLT+™ Bioreactor Prototype in operation



PLT+^{TM} Bioreactor Makes PLTs+^{TM}

PLTs+^{TM} Production Kinetics



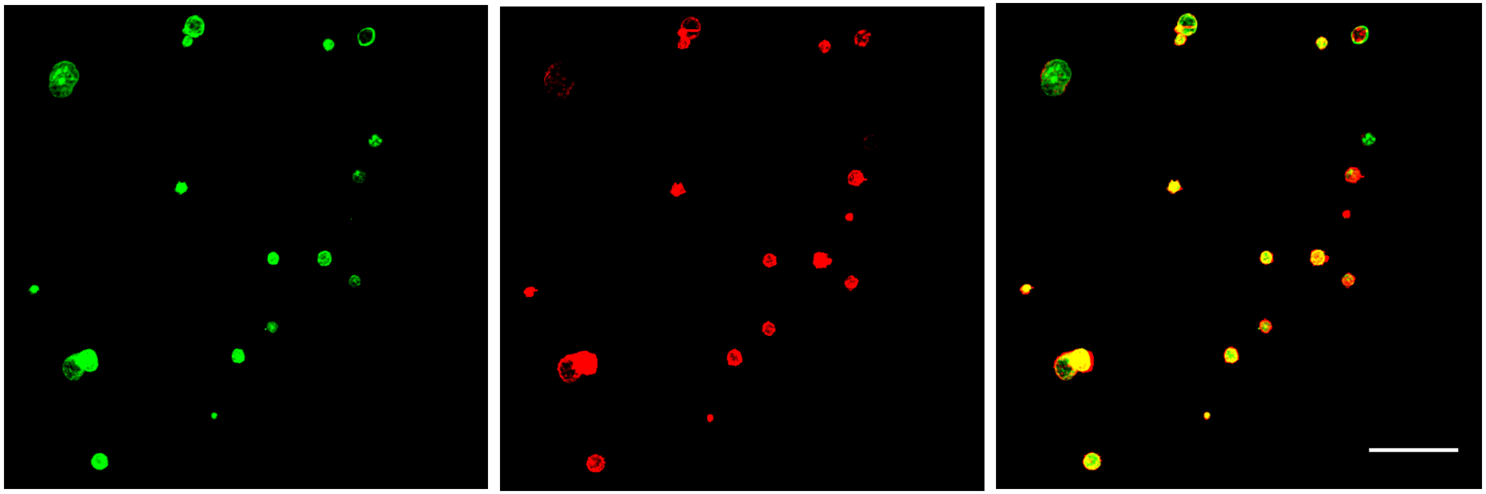


<u>Pre gating on:</u>

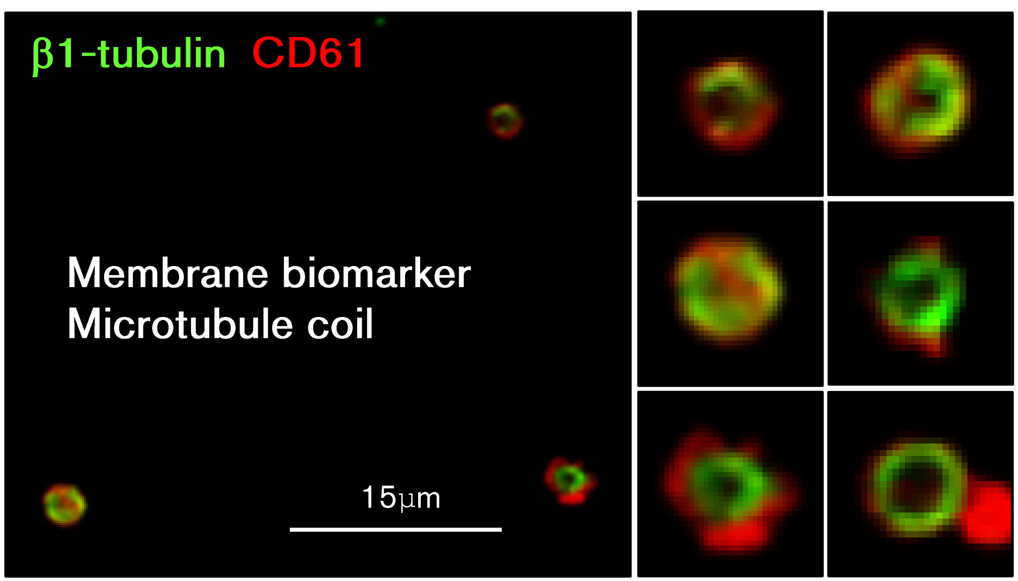
CD61+
DRAQ (DNA)-
Calcein+
CD42a+

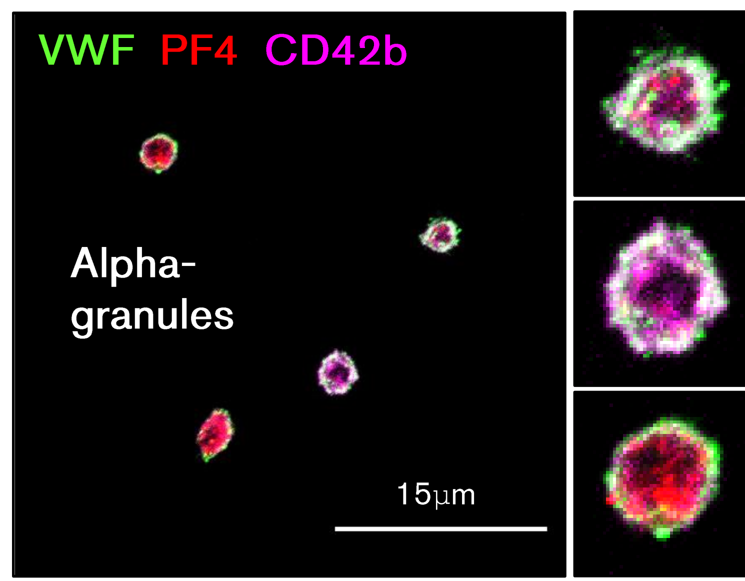
Platelet+^{TM} Imaging

B-tubulin | CD61 | Merge

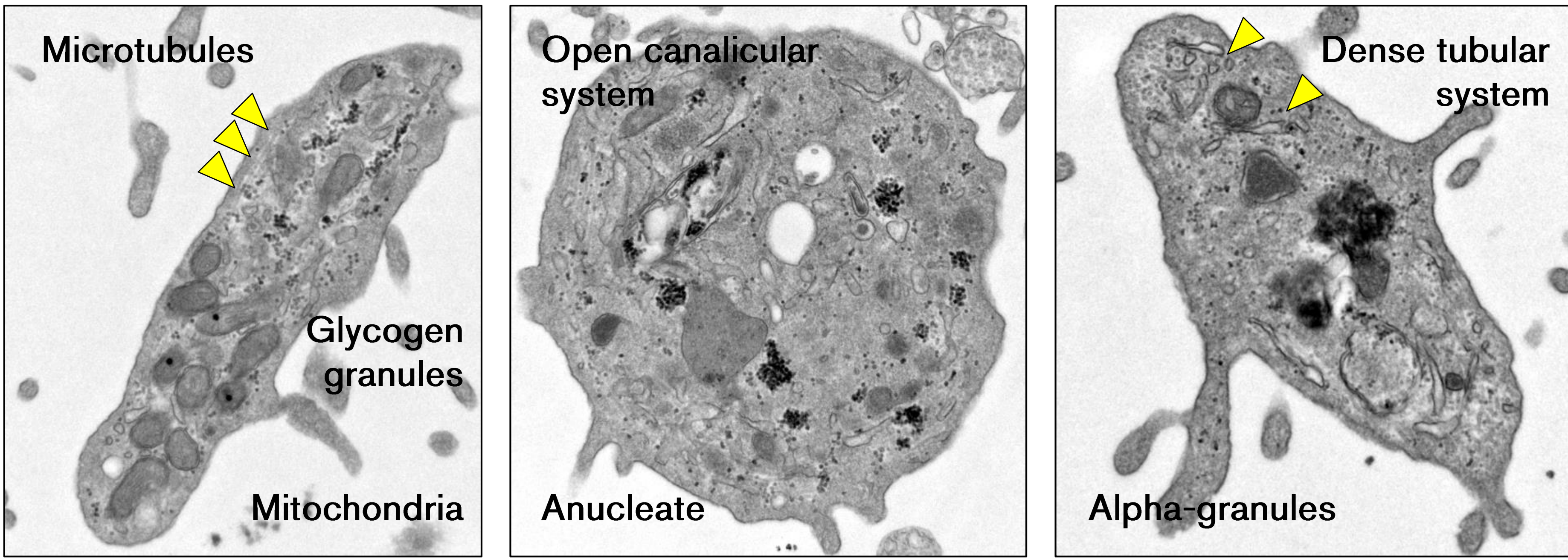


Scale bar = 25 µm
% Platelets = 79.8%
Average Diameter = 4.41 um
Std Dev = 1.43 um

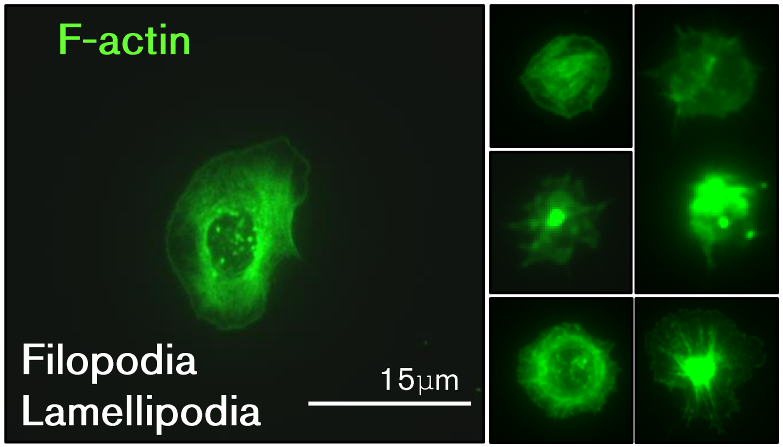


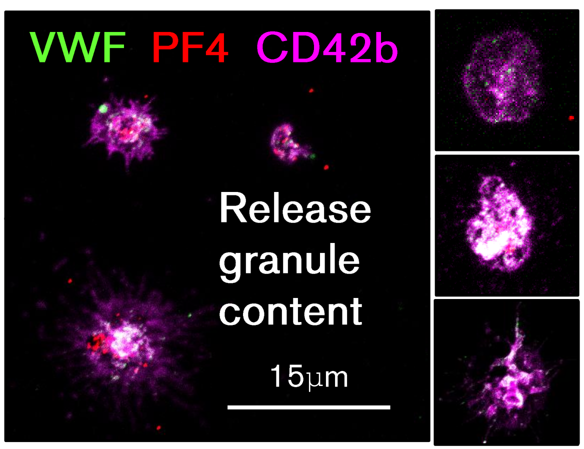


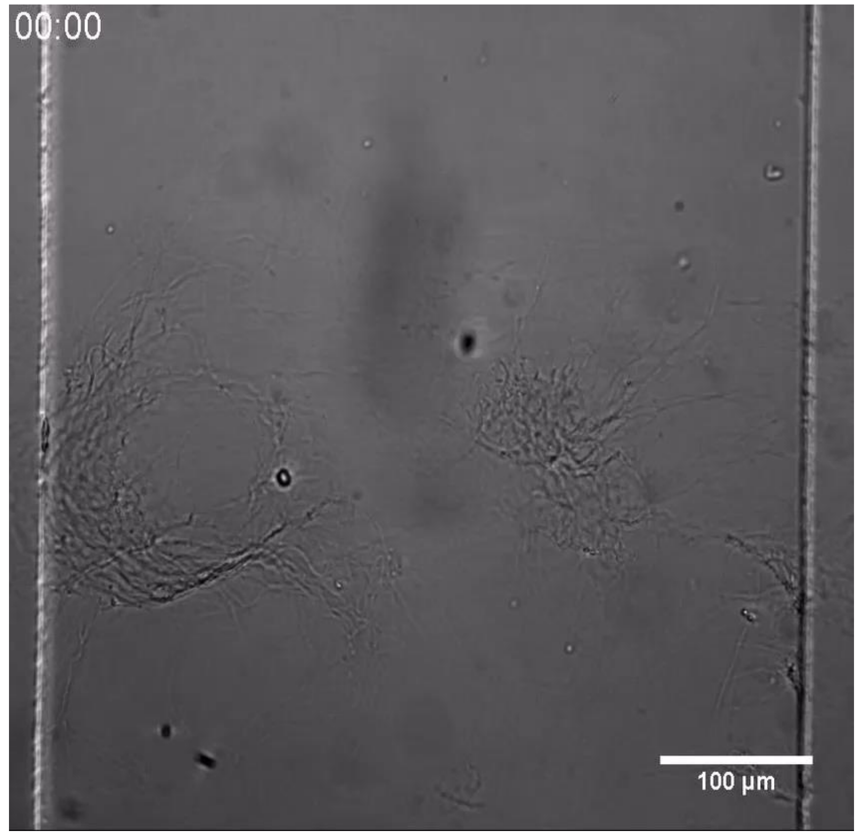
Electron microscopy characterization of Platelet+™



Functional characterization of PLT+^{TM}







PLTs+^{TM} in Culture Media (no plasma)

Collagen I

CONCLUSIONS

1. Proplatelet-promoting factor (PPF) is an internal, cytosolic protein(s) that regulates proplatelet initiation.

2. Mimicking physiology triggers platelet production.

3. We have developed a scalable process to generate in vitro platelets from clinical grade iPS cells.

ACKNOWLEDGEMENTS

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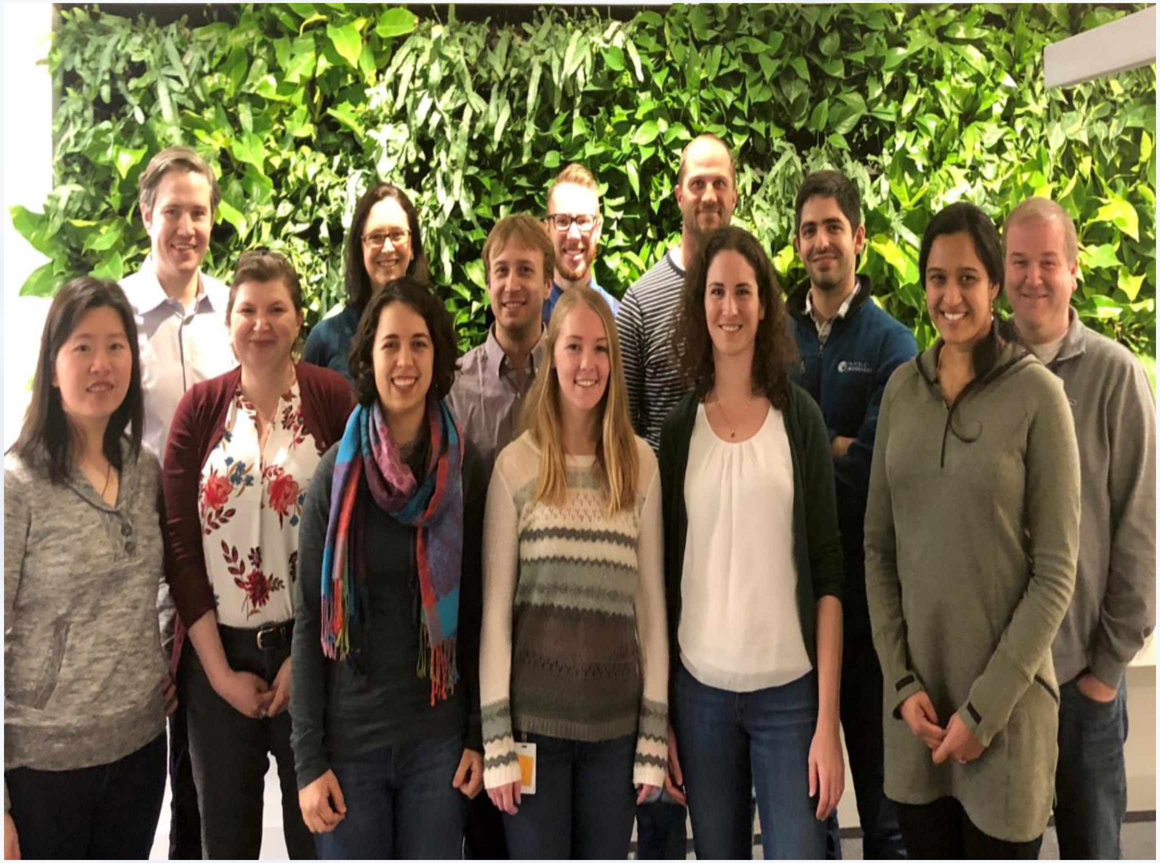
NATIONAL INSTITUTES OF HEALTH

AMERICAN SOCIETY OF HEMATOLOGY

BWH BRIGHAM AND WOMEN'S HOSPITAL
A Teaching Affiliate of Harvard Medical School

Platelet BioGenesis Team



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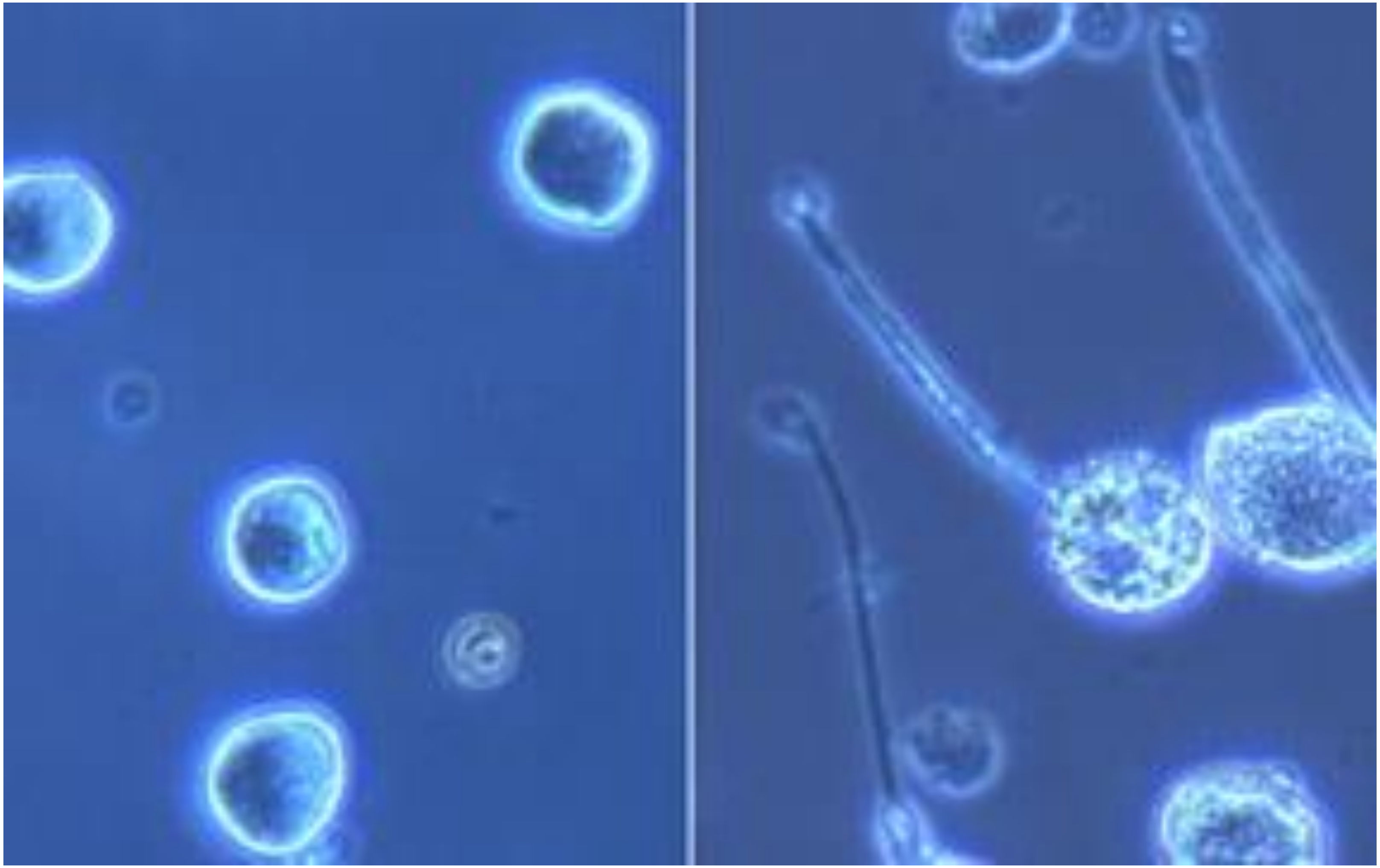
Jorge Valdez, PhD Bioreactor

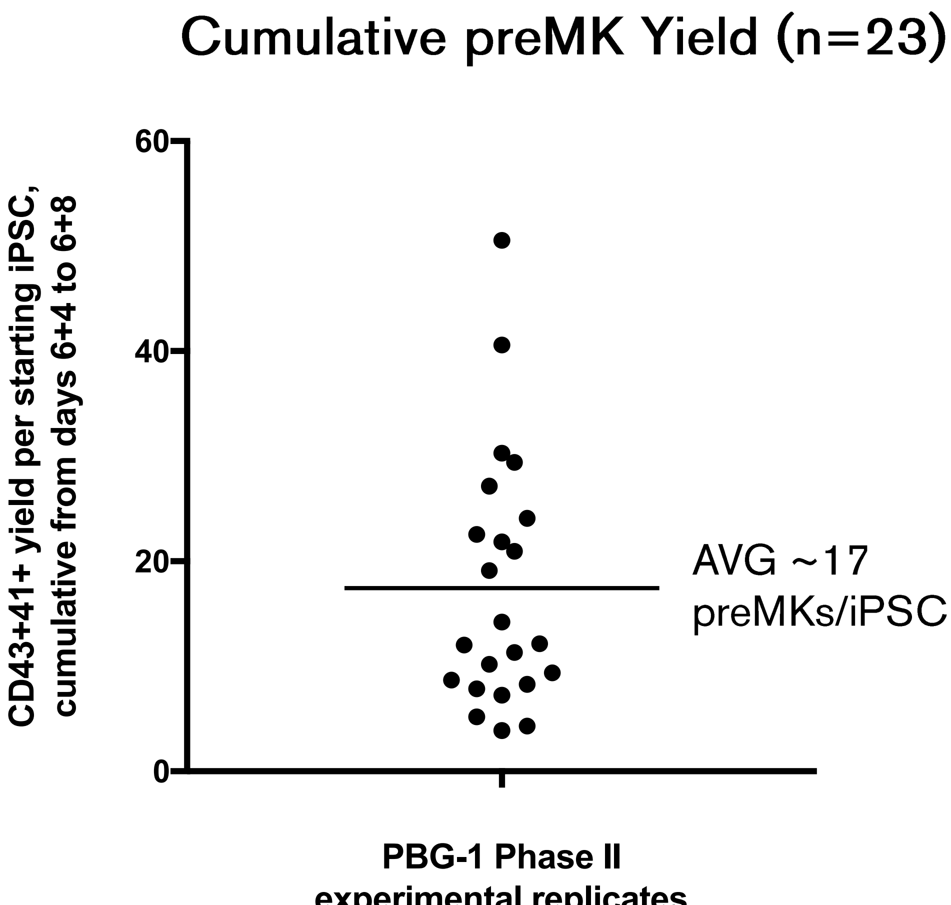
Christ Peters, PhD Characteriazation

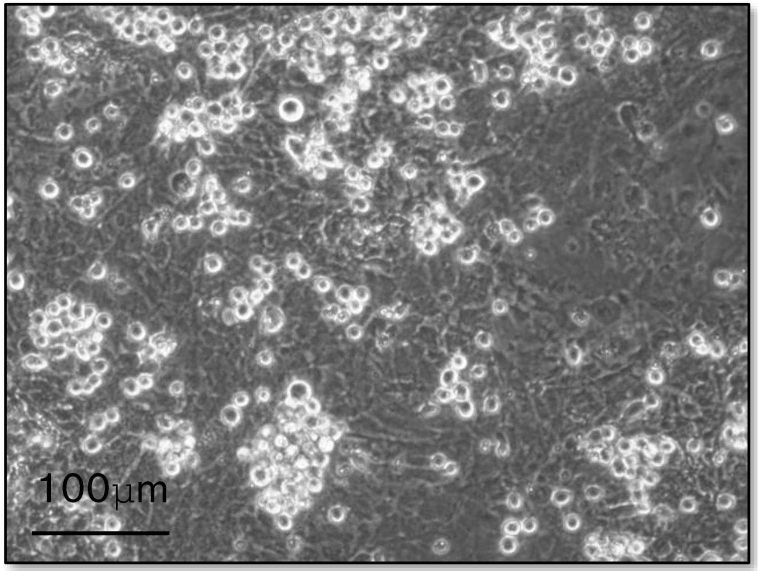
Microinjection of platelet cytosol triggers proplatelet initiation



Phase II. HE → preMKs+™

- preMKs+™ are CD43+ CD41+ CD14-
- Propidium Iodide-





Defined Regulatory Pathway

Plan developed from discussions with the CBER and FDA

1. Quality Assessment	▪ Assess platelet: circulation time, structure, function, and storage profile
2. Purity Assessment	▪ Assess nucleated cell (non-platelet) contamination, and platelet unit sterility
3. Safety Assessment	▪ Assess immunogenicity risk, septic risk, teratoma risk, & inter-batch variability

4. Pre-IND Consultation 5. File IND	Application was reviewed in the **Office of Tissues and Advanced Therapies** by the **Center for Biologics Evaluation and Research** (pre-IND completed April 2018) ▪ Human iPSC-derived megakaryocytes will be reviewed by CBER/OTAT and qualified for clinical grade manufacture *Code of Federal Regulations (Title 21, Sections 1271).* ▪ Clinical indication for bioreactor-derived platelets will be reviewed by the **Office of Blood Research and Review** *Code of Federal Regulations (Title 21, Sections 221 and 640).*

2018 → **2020**

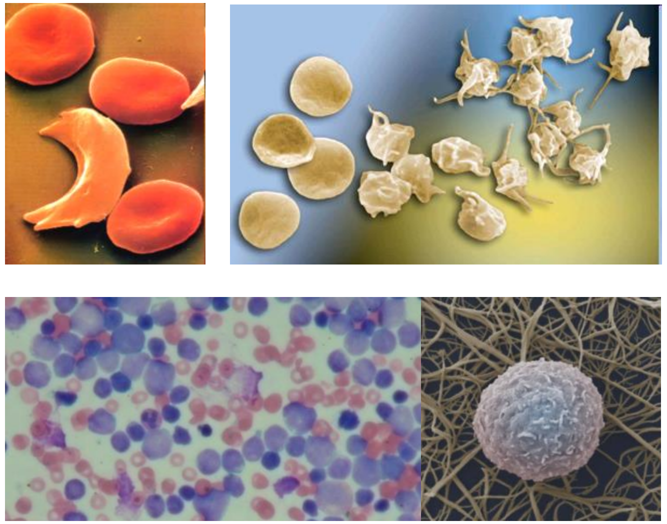
how platelets "feel" and respond to their mechanical microenvironment during clot formation

Wilbur A. Lam, MD, PhD

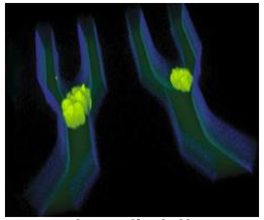
Associate Professor
Department of Pediatrics
Aflac Cancer and Blood Disorders Center
Division of Pediatric Hematology/Oncology
Children’s Healthcare of Atlanta/Emory University School of Medicine
Wallace H. Coulter Department of Biomedical Engineering
Georgia Institute of Technology and Emory University

Aflac Cancer & Blood Disorders Center CHILDREN'S HEALTHCARE OF ATLANTA · Georgia Tech · Wallace H. Coulter Department of Biomedical Engineering at Georgia Tech and Emory University · EMORY UNIVERSITY

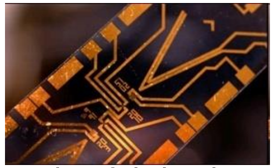
overview of our laboratory approach



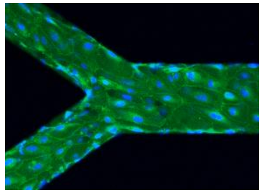
cell biophysics of hematology in health and disease



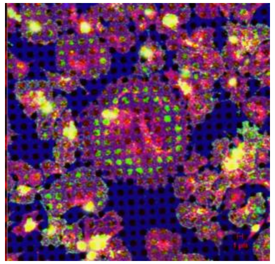
microfluidics



microfabrication

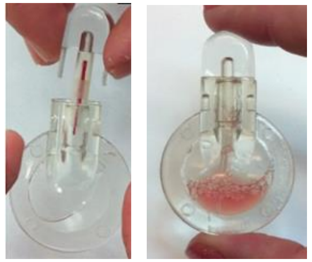


microvasculature-on-a-chip



protein nano/microcontact printing

development of research-enabling nano/microsystems



point-of-care anemia diagnostic (FDA cleared)

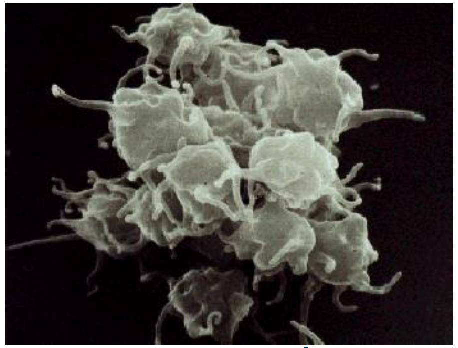
smartphone-based diagnostics

diagnostic and medical device development

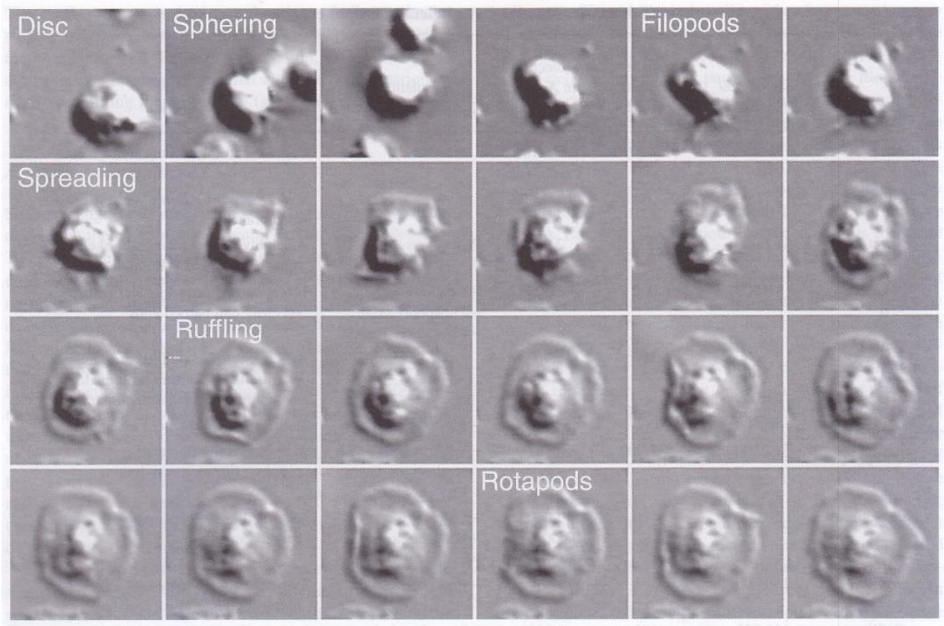
translation to our patients

how “smart” are platelets?

resting



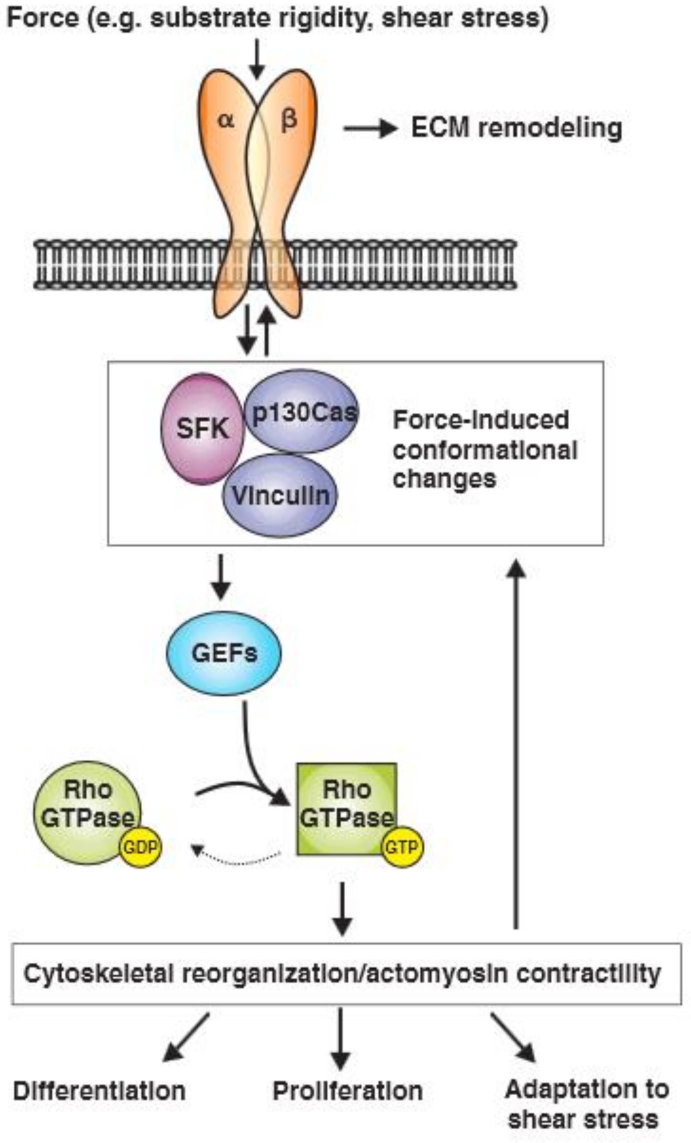
activated



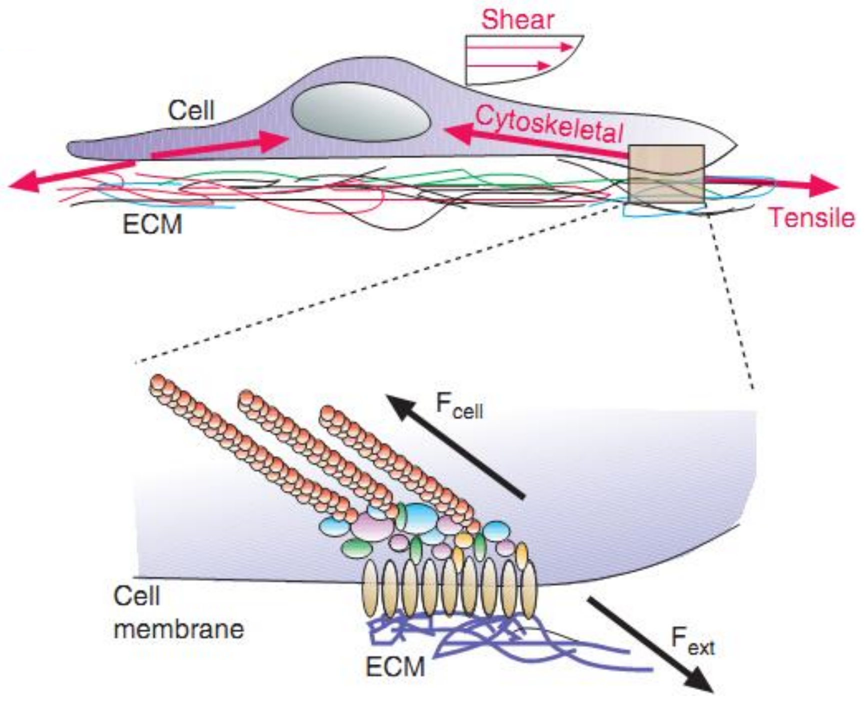
Hartwig JH, *Platelets* 2002

how do platelets physiologically react to the biophysical microenvironment?

do platelets sense and physiologically respond to their mechanical microenvironment?

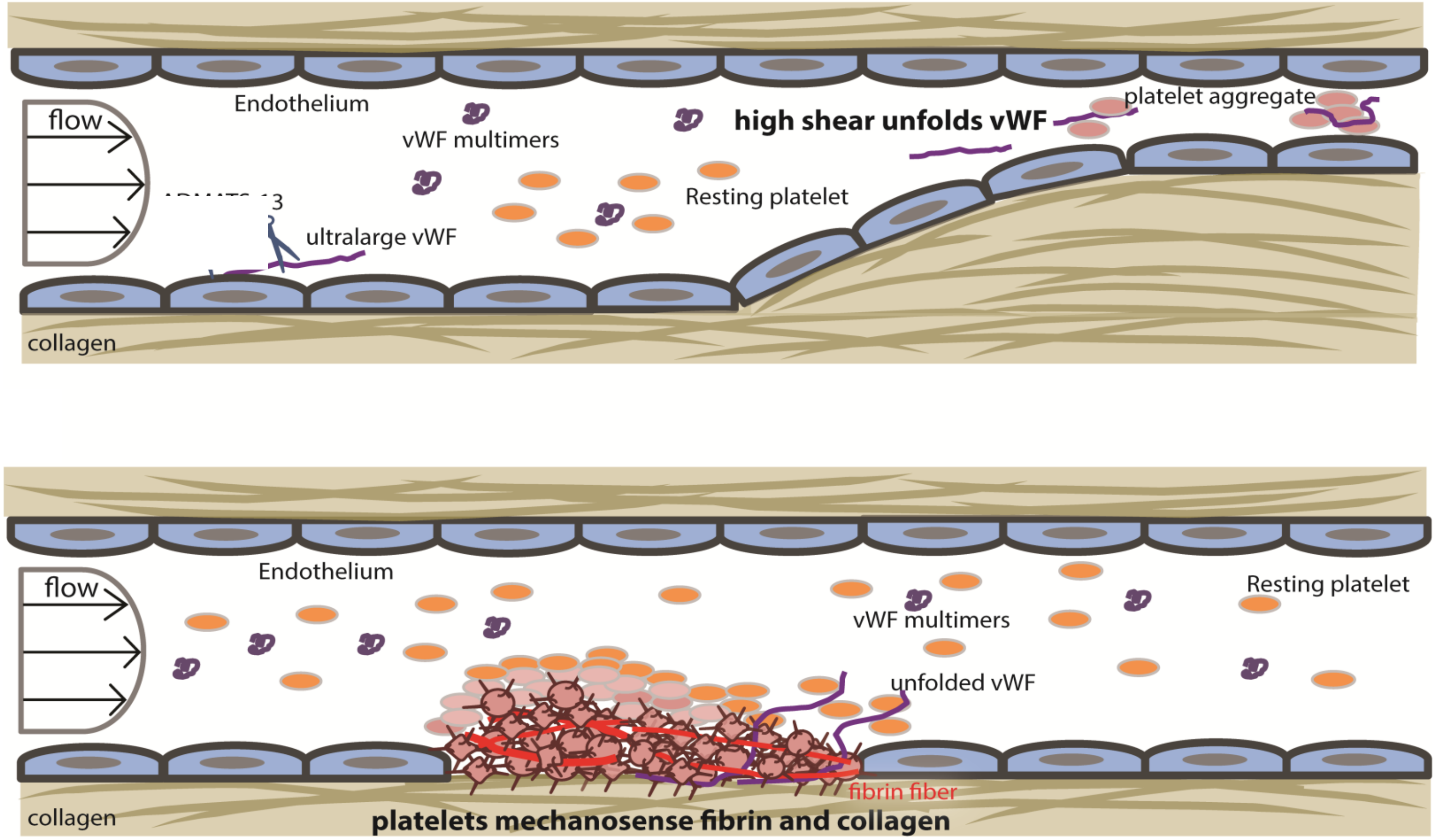


Huveneers et al. *J Cell Sci* 2009



Chen, *J Cell Sci* 2008

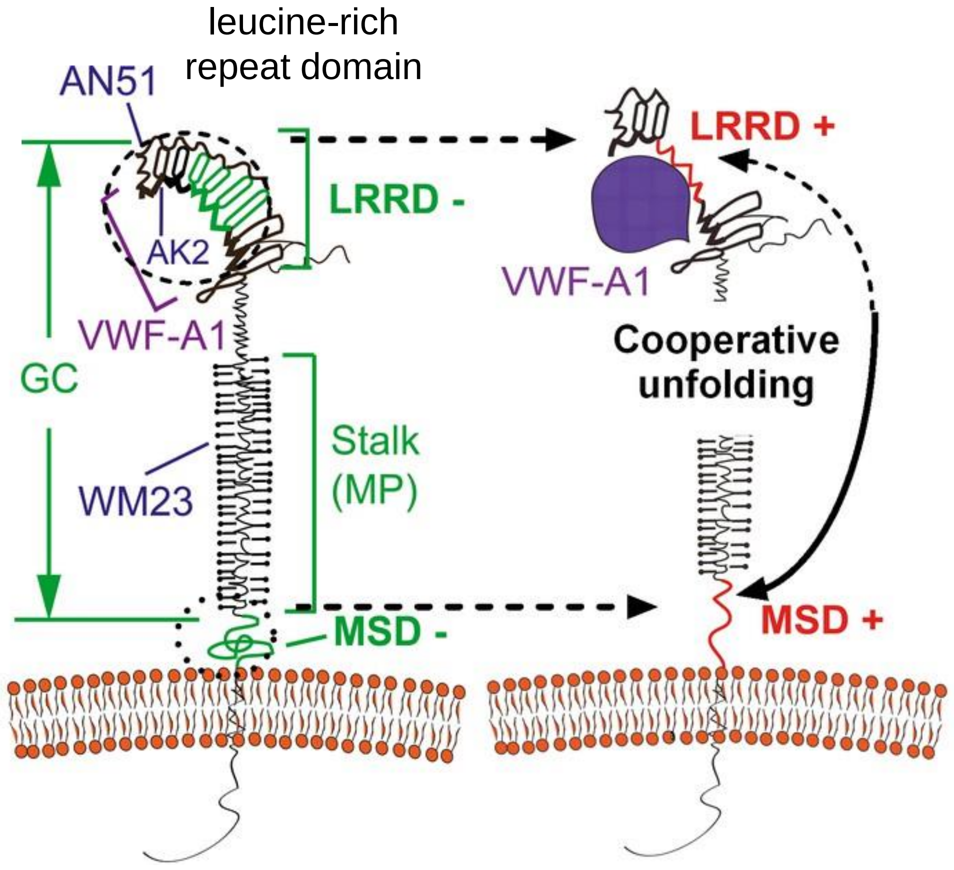
platelets function in a dynamic mechanical microenvironment



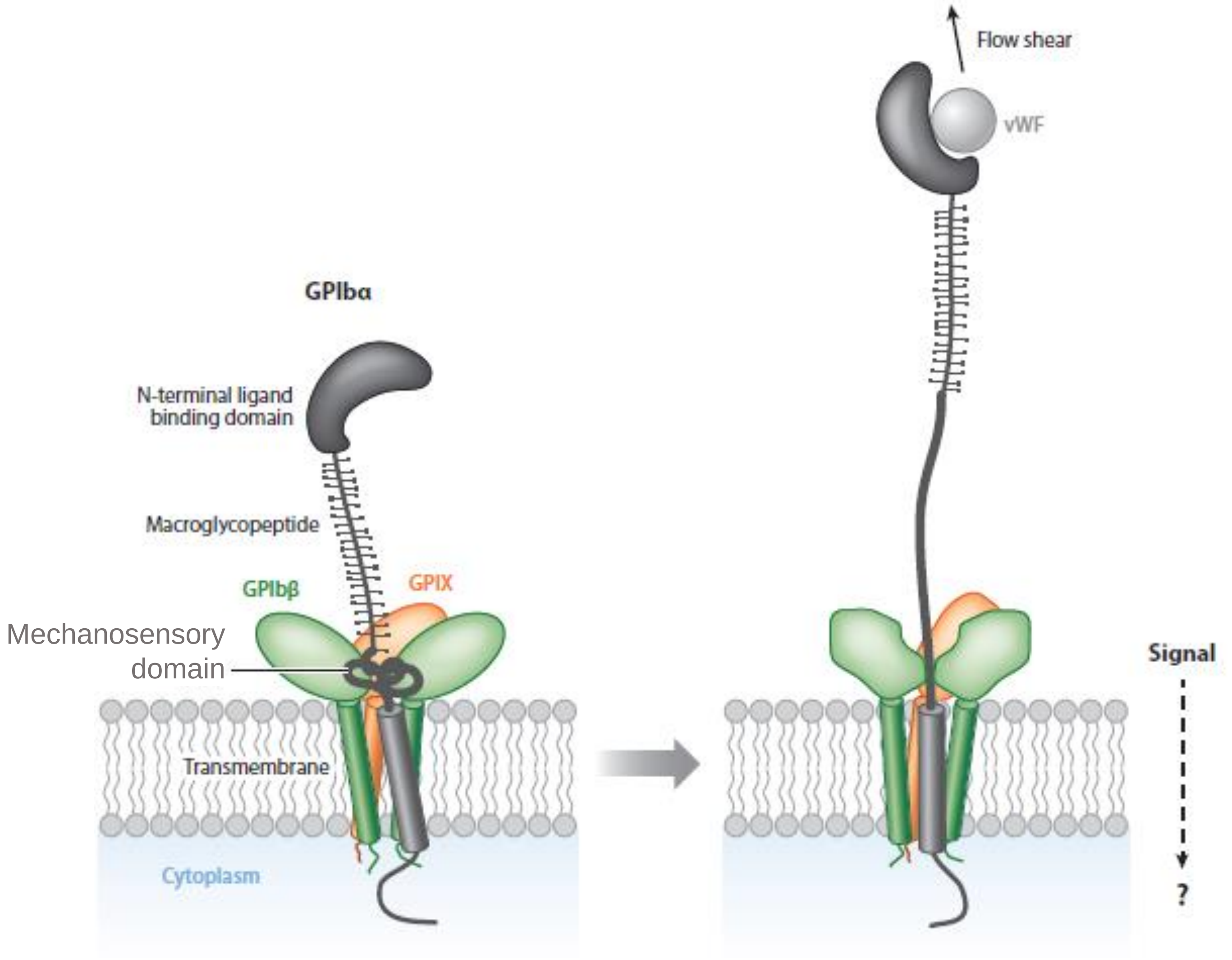
Qiu Y et al, *Blood Rev* 2015

how do fluid mechanics affect platelet physiology?
how do clot mechanics affect platelet physiology?

platelet mechanosensing under flow conditions focus on von willebrand factor-platelet interactions

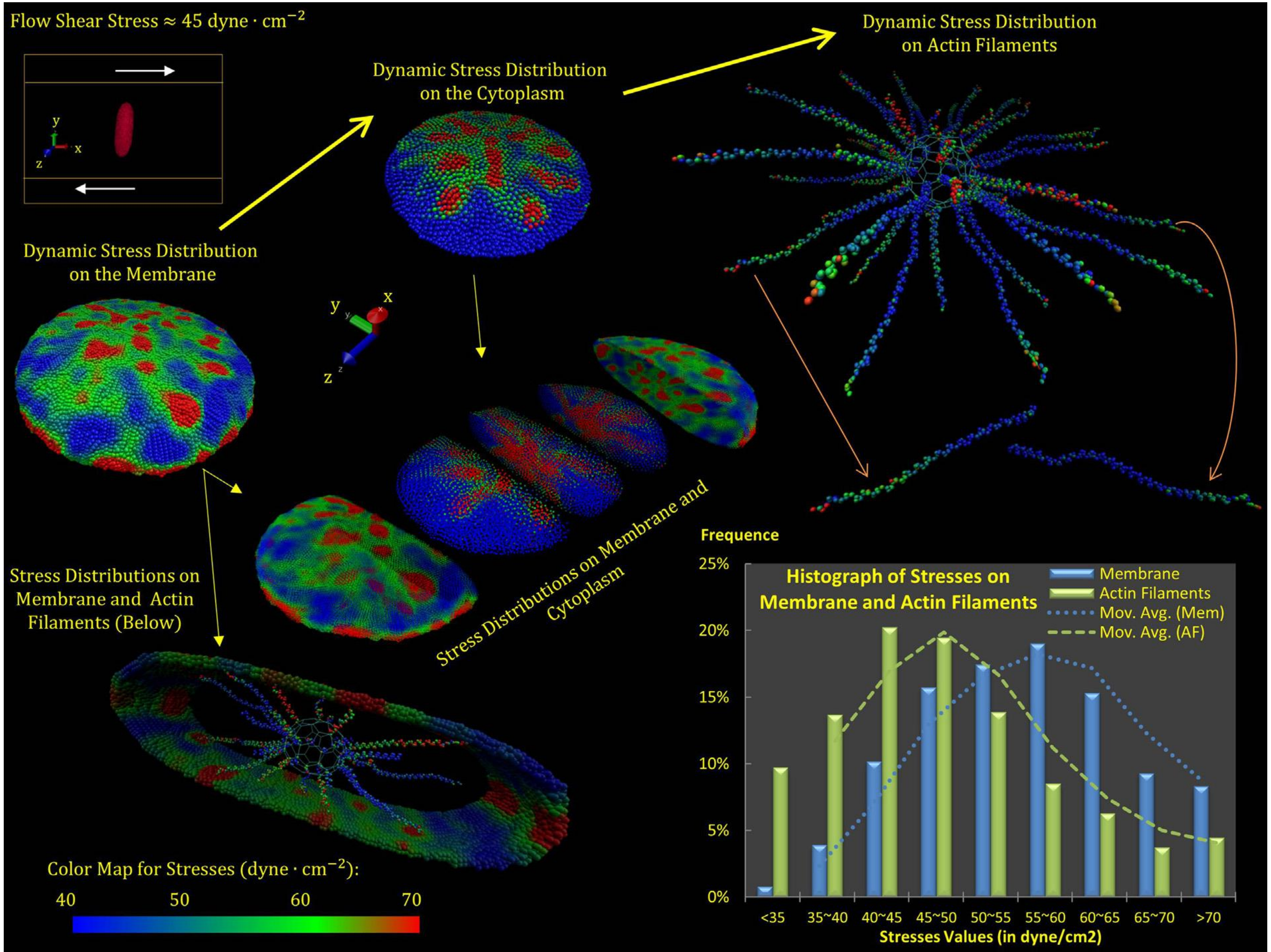


Ju L et al, *Elife* 2016



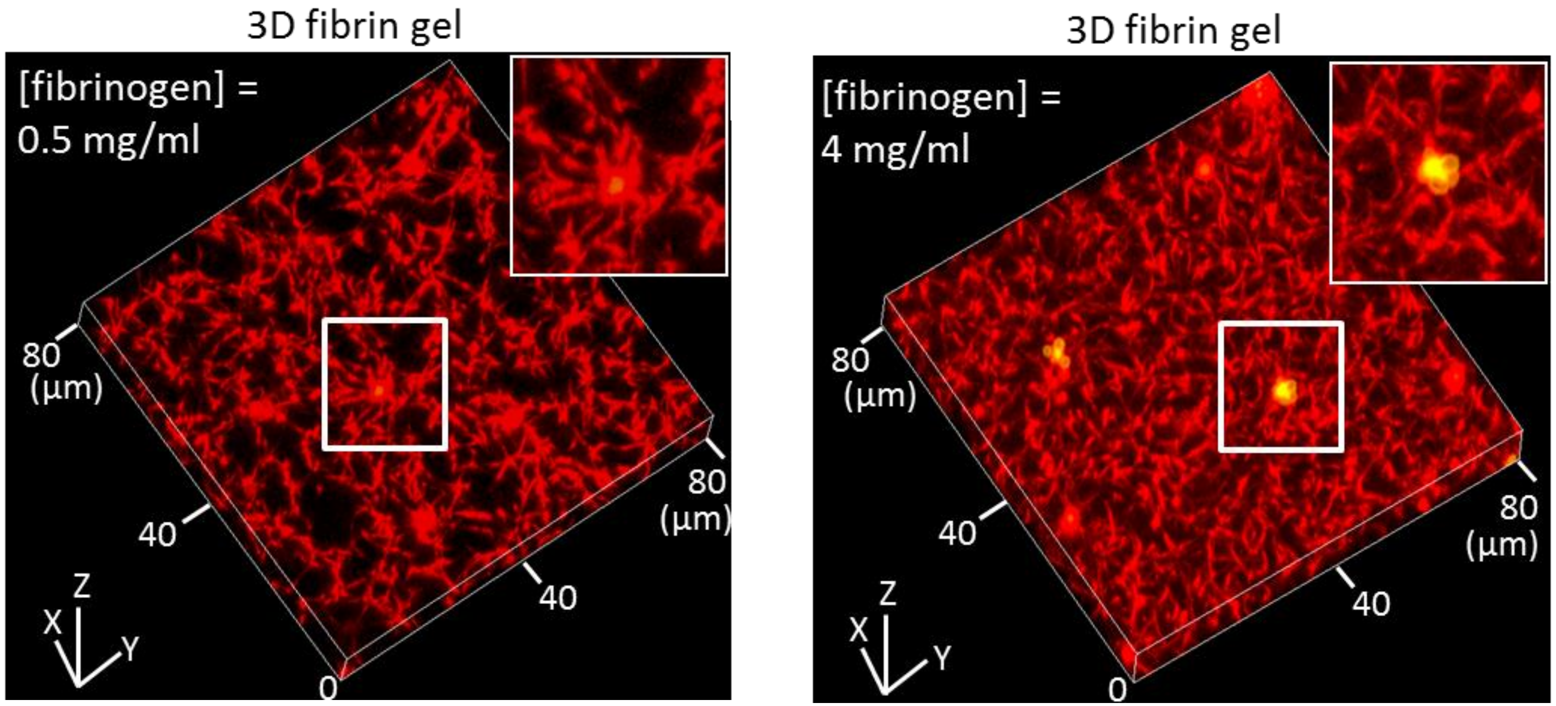
Zhang W et al, *Blood* 2015

in silico approaches have revealed stress profiles of individual platelets under flow conditions



Zhang P et al, *J Biomech* 2017

can clot and vascular mechanics affect platelet physiology?

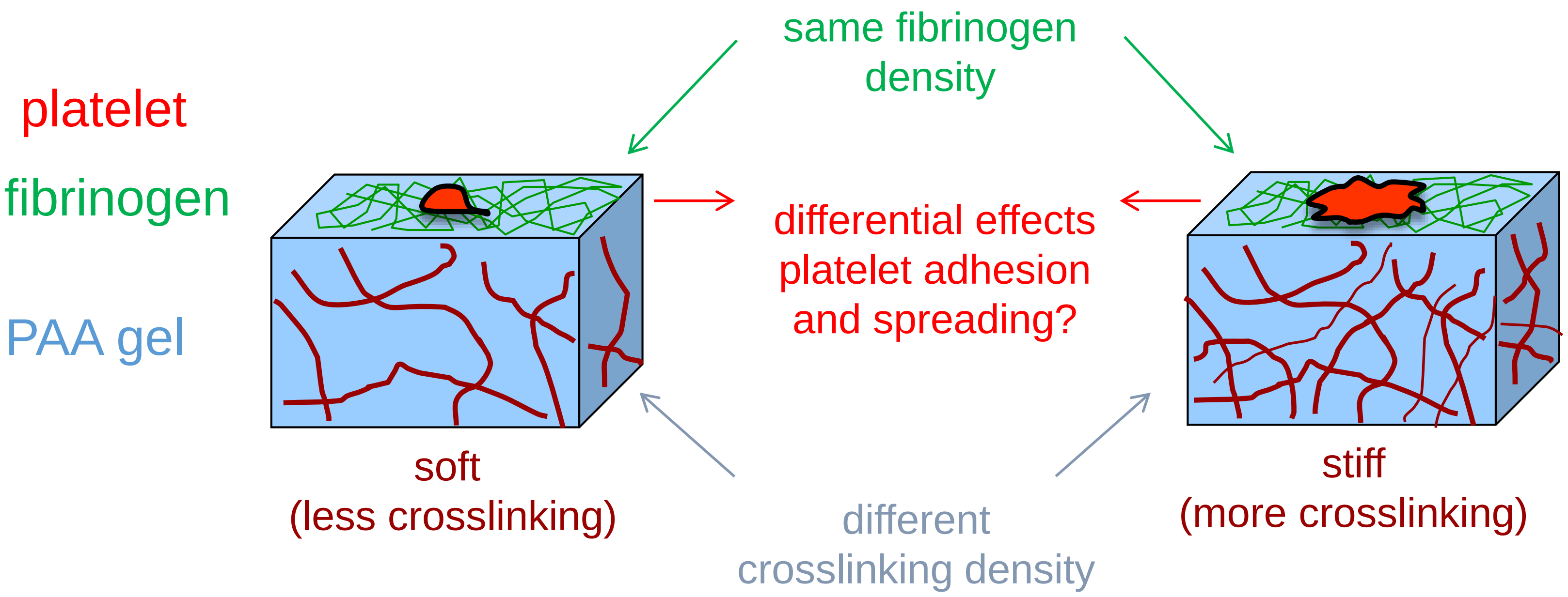


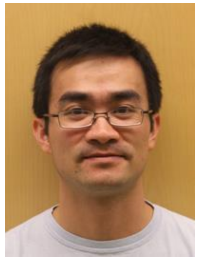
yellow-green = annexin V staining for phosphatidylserine

confounding factors associated with 3D fibrin gels: architecture, branching, ligand density all change with varying fibrinogen concentration

Qiu Y, et al, *PNAS* 2014

using fibrinogen-conjugated poly-acrylamide (PAA) gels to investigate how substrate stiffness affects platelet function

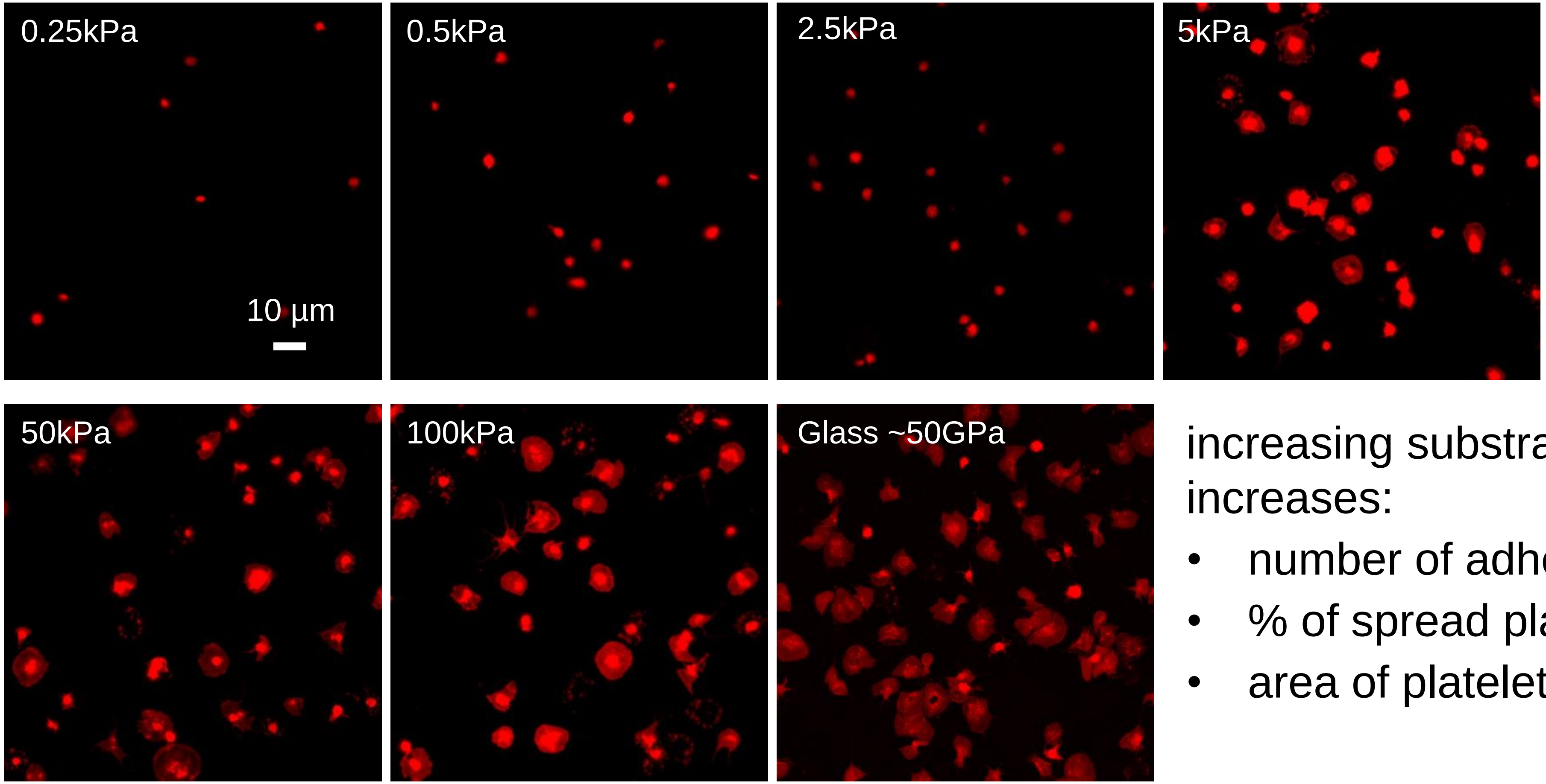




Yongzhi Qiu, PhD

Qiu Y, et al, *PNAS* 2014

substrate stiffness mediates platelet adhesion and spreading on fibrinogen

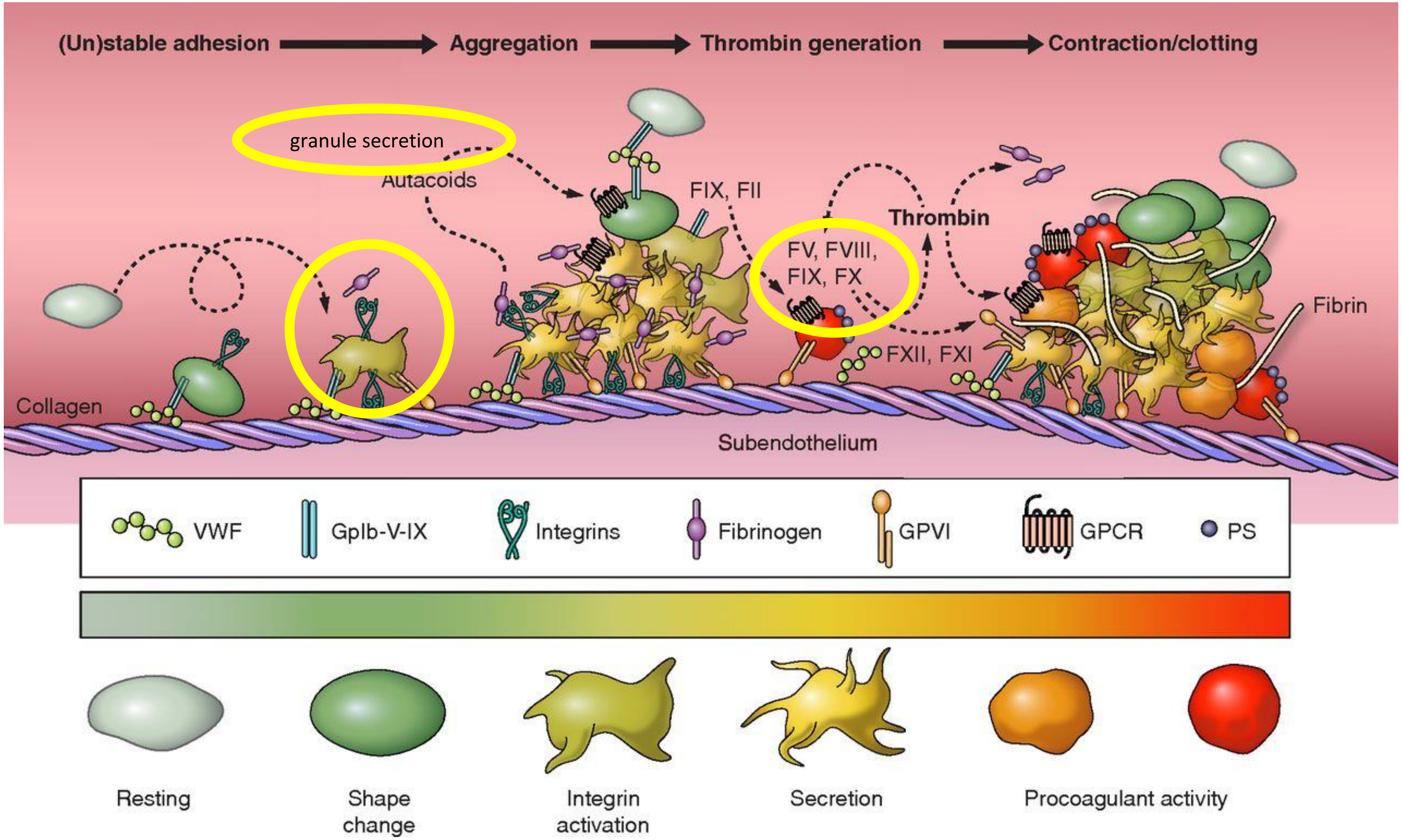


increasing substrate stiffness increases:

- number of adherent platelets
- % of spread platelets
- area of platelet spreading

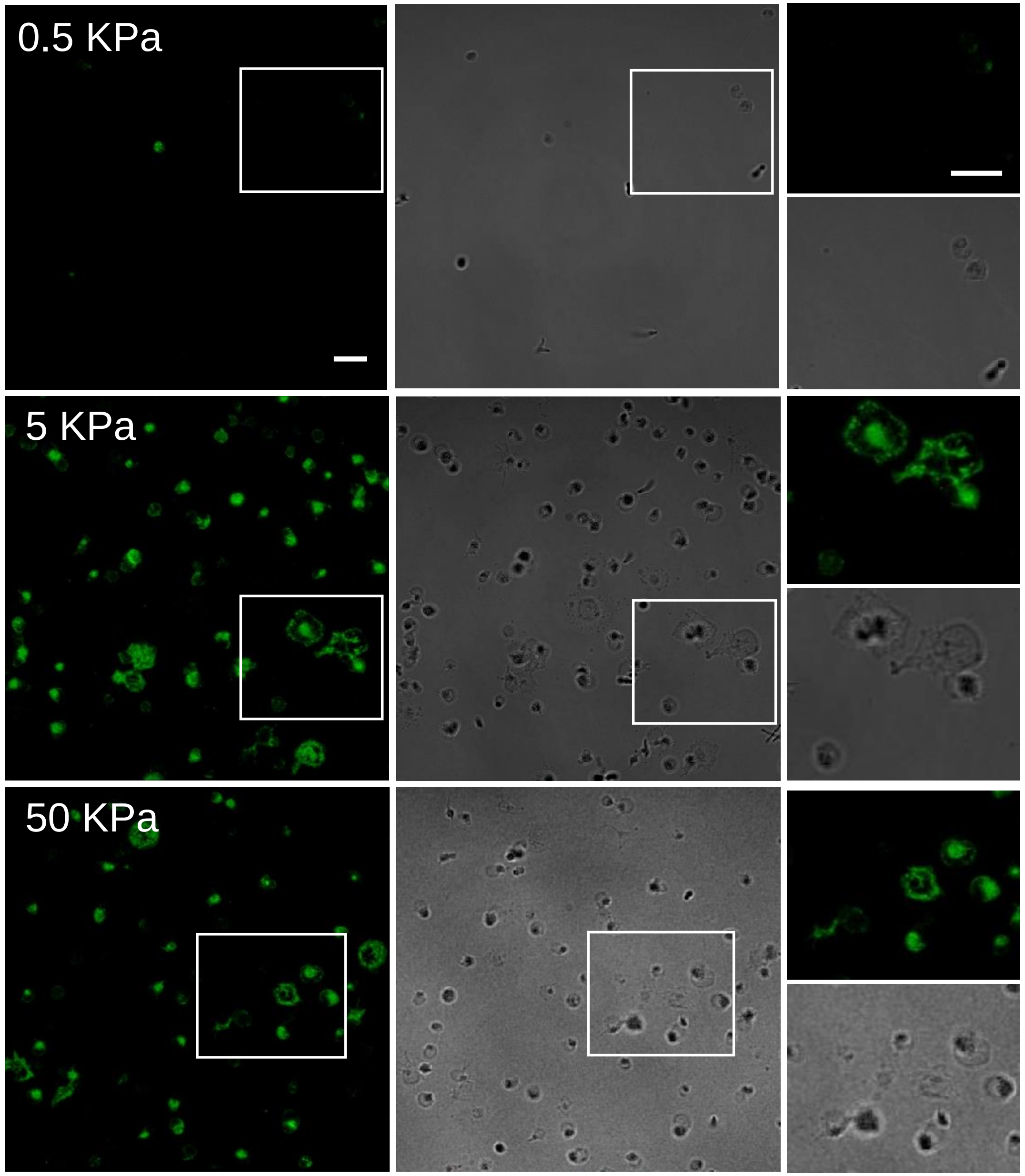
Qiu Y, et al, *PNAS* 2014

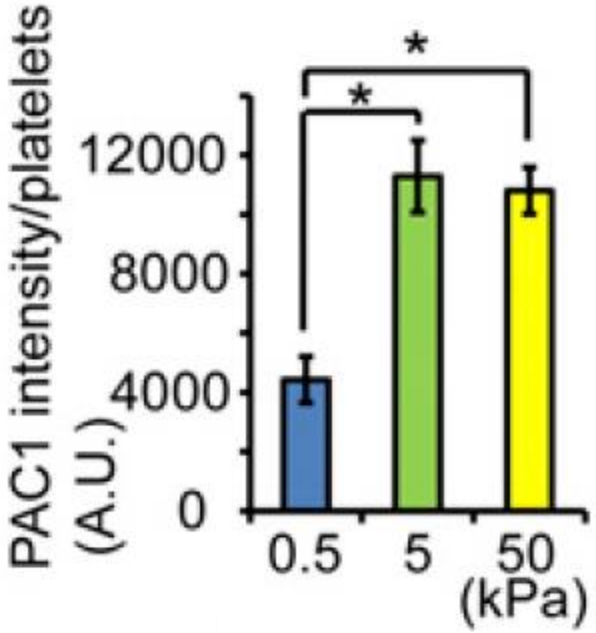
how else does microenvironmental mechanotransduction affect platelet function?



Versteeg et al. *Physiol Rev* 2013

substrate stiffness affects platelet integrin $\alpha_{IIb}\beta_3$ activation



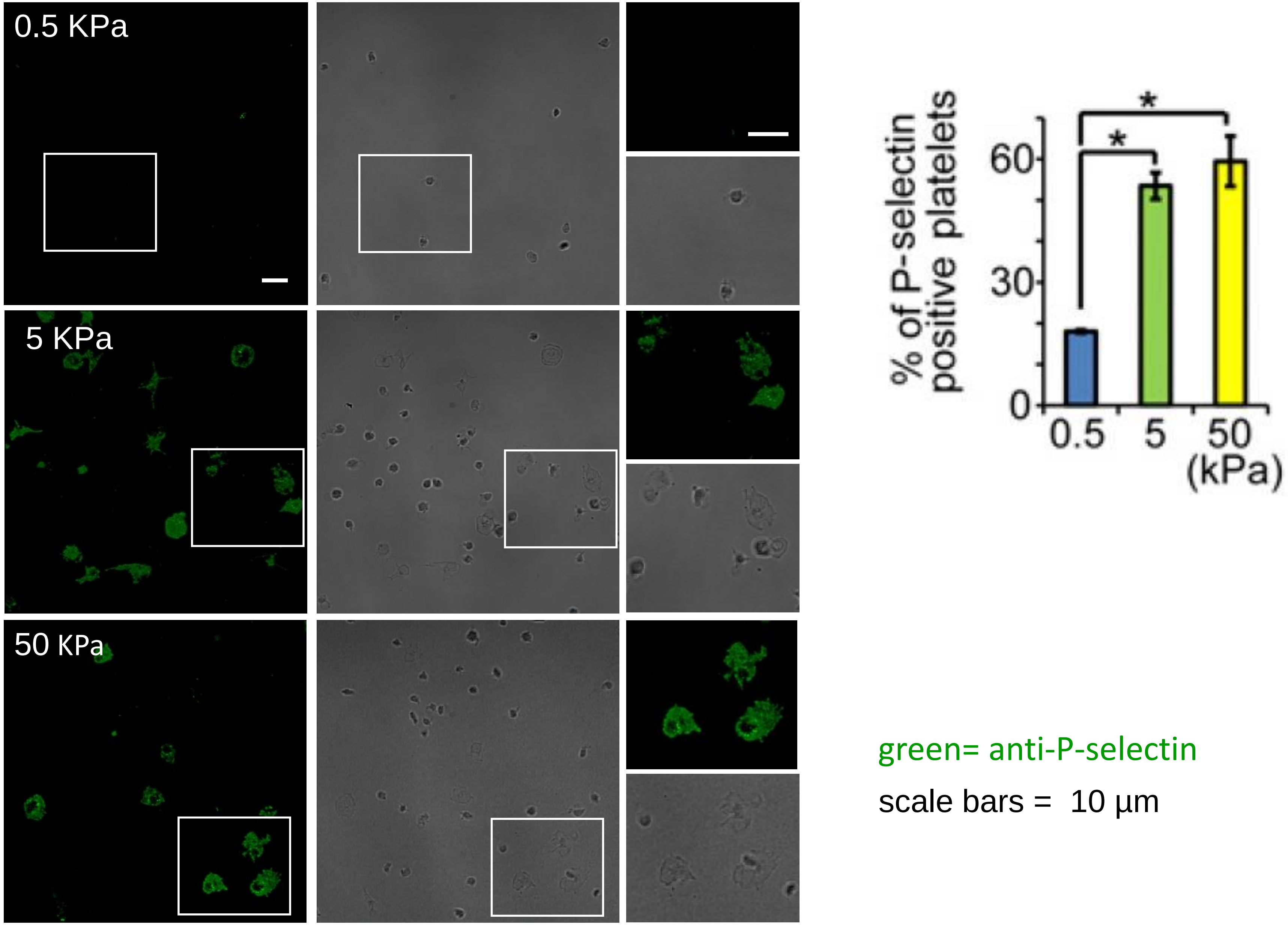


green = PAC-1 antibody → labels only activated $\alpha_{IIb}\beta_3$ integrins

scale bars = 10 µm

Qiu Y, et al, *PNAS* 2014

substrate stiffness affects platelet α-granule secretion

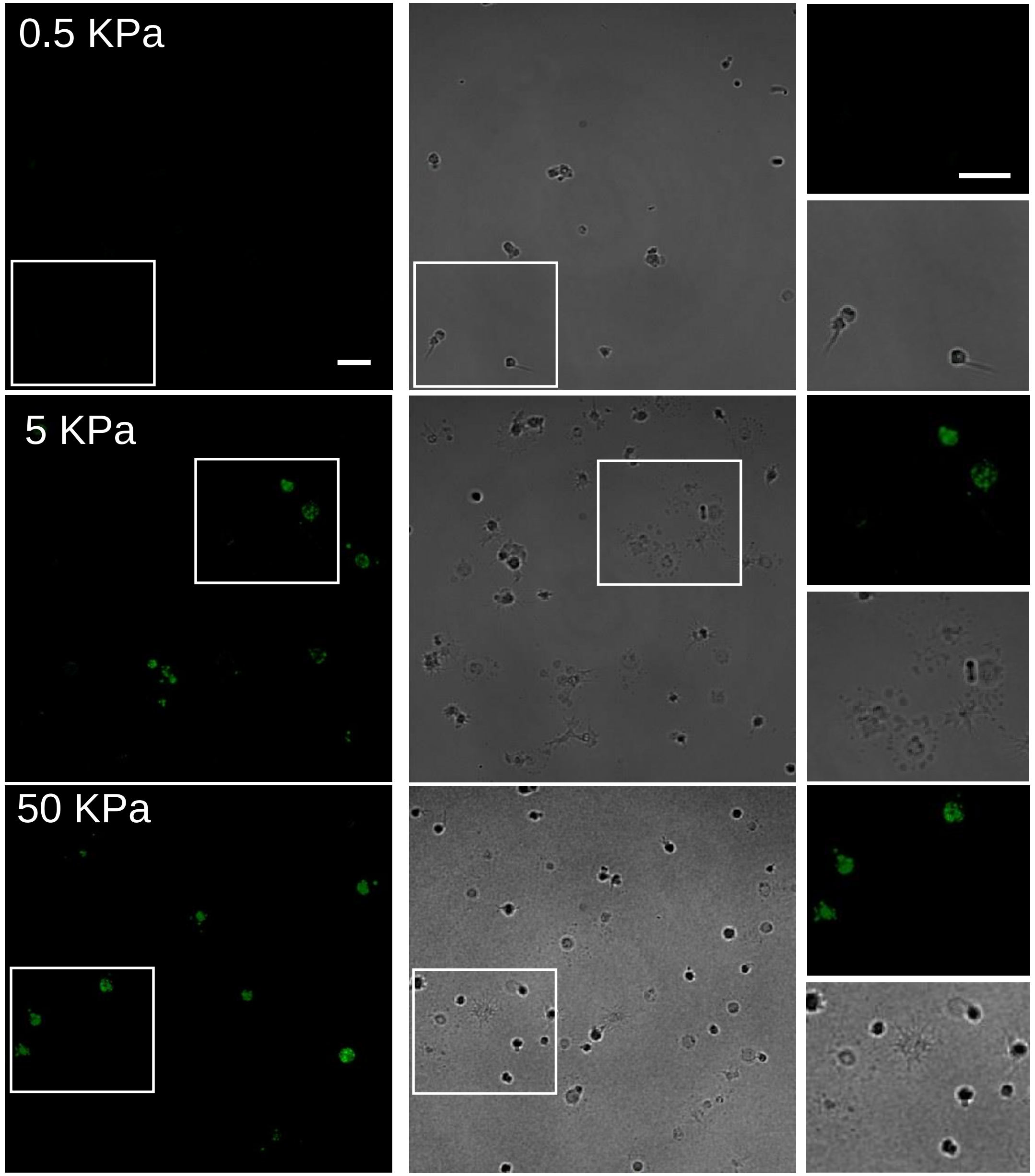


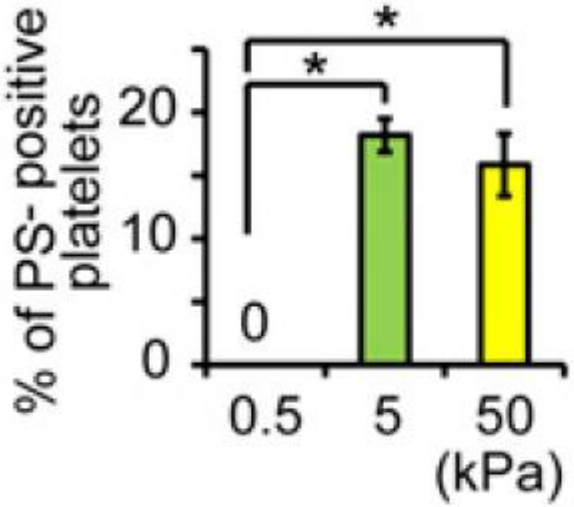
green= anti-P-selectin

scale bars = 10 µm

Qiu Y, et al, *PNAS* 2014

substrate stiffness affects platelet phosphatidylserine (PS) exposure



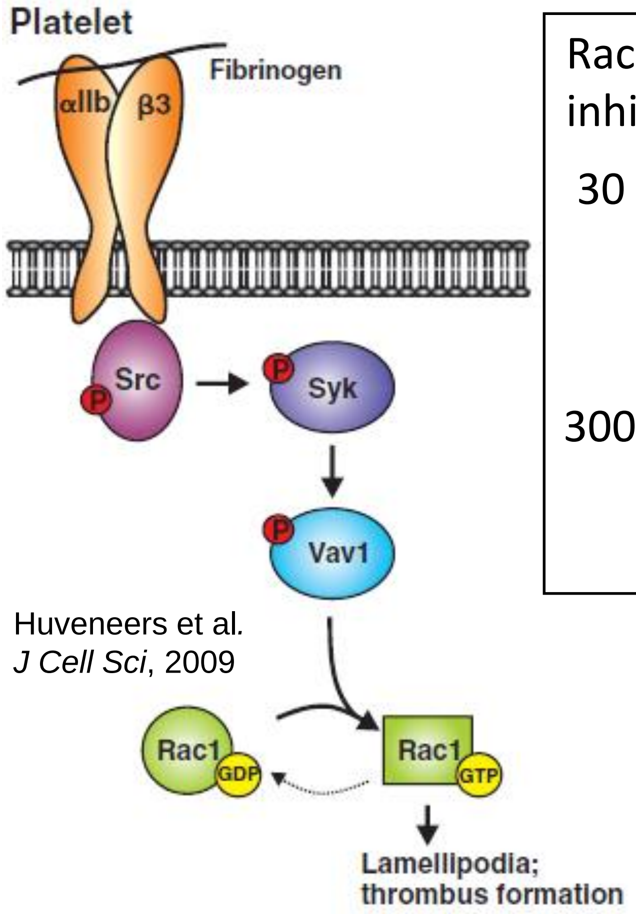


green = Annexin V → labels PS

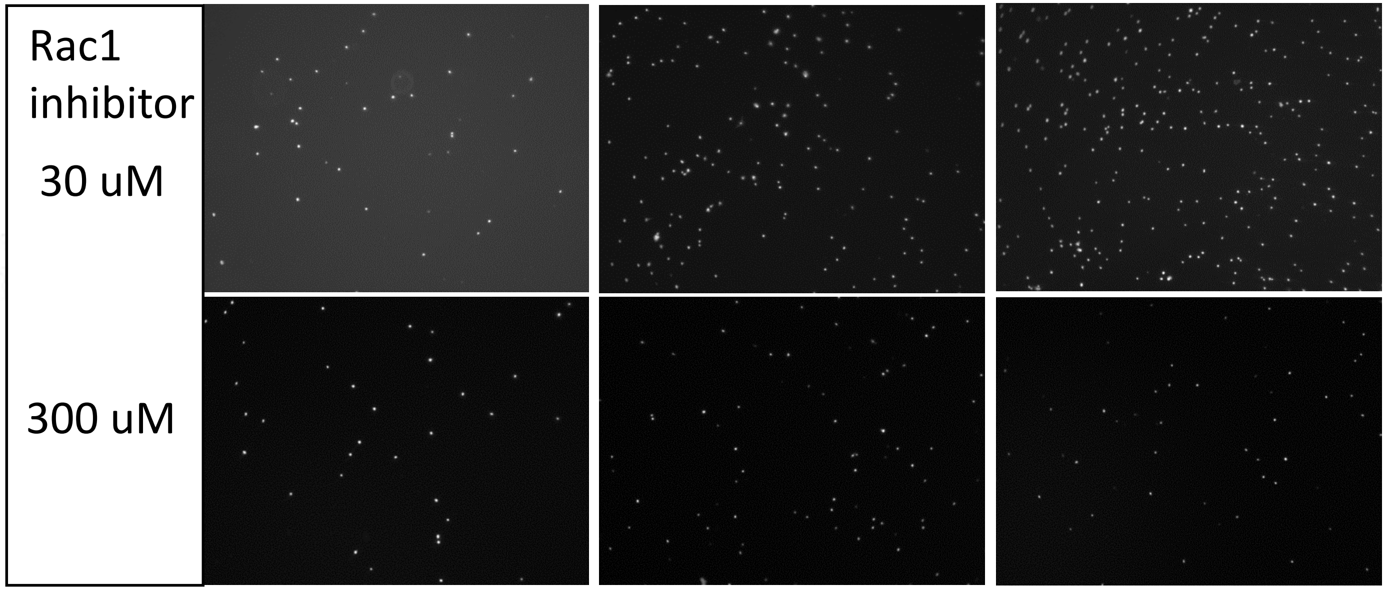
scale bars = 10 µm

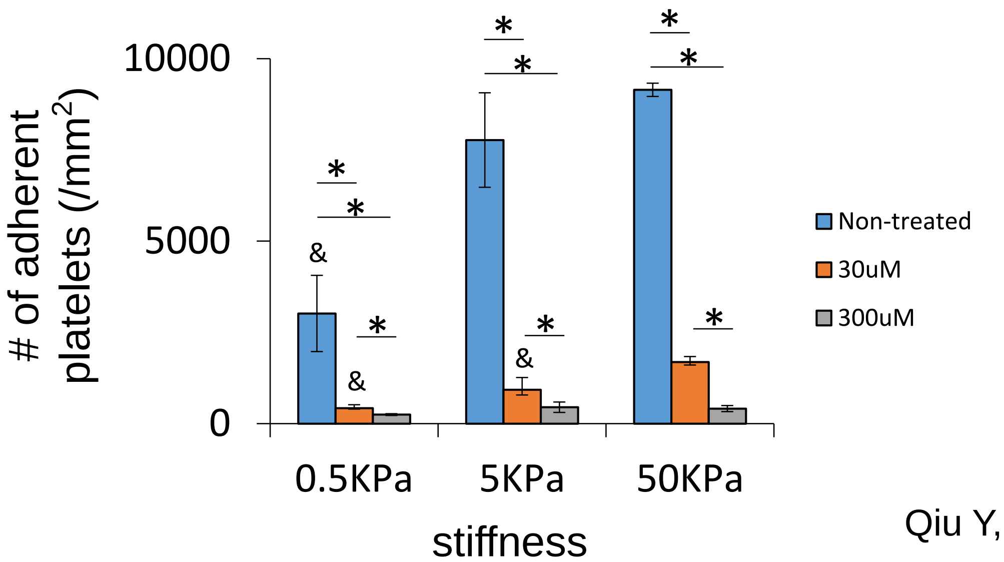
Qiu Y, et al, *PNAS* 2014

Rac1 mediates substrate stiffness dependence on platelet adhesion on fibrinogen



Huveneers et al.
J Cell Sci, 2009

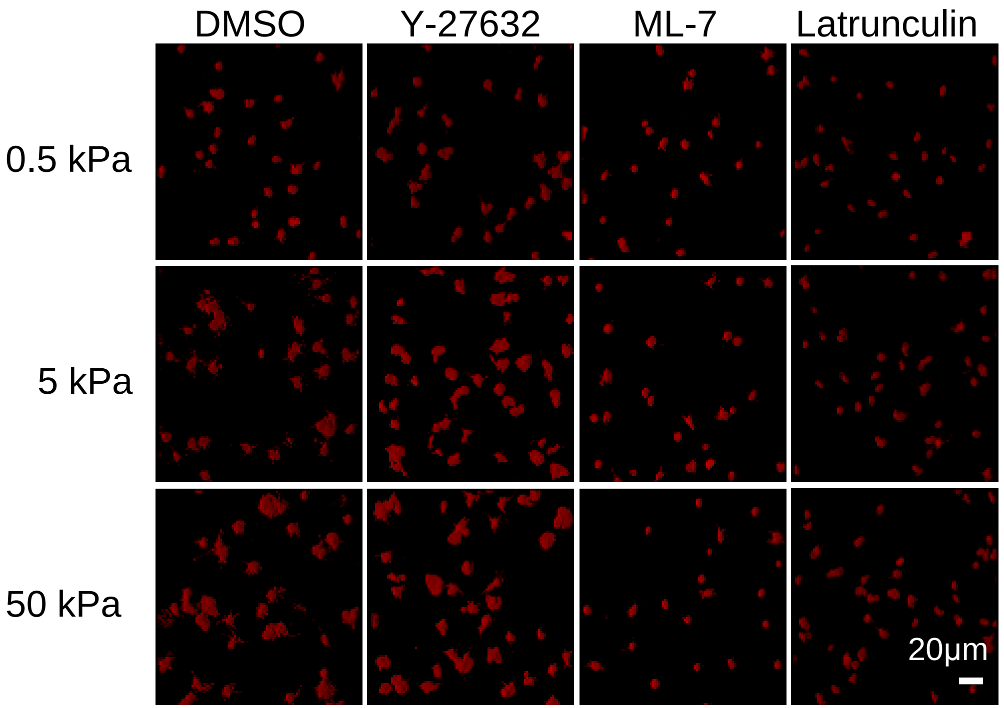


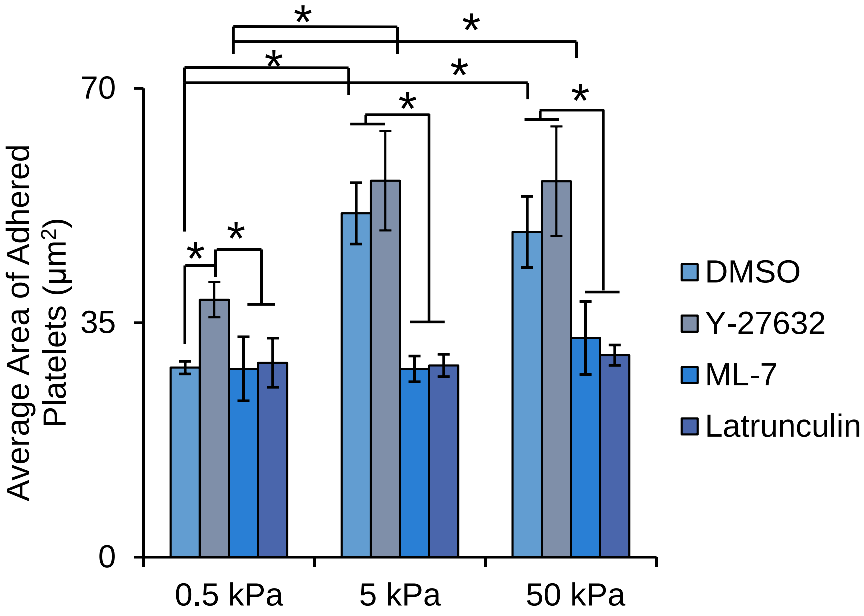


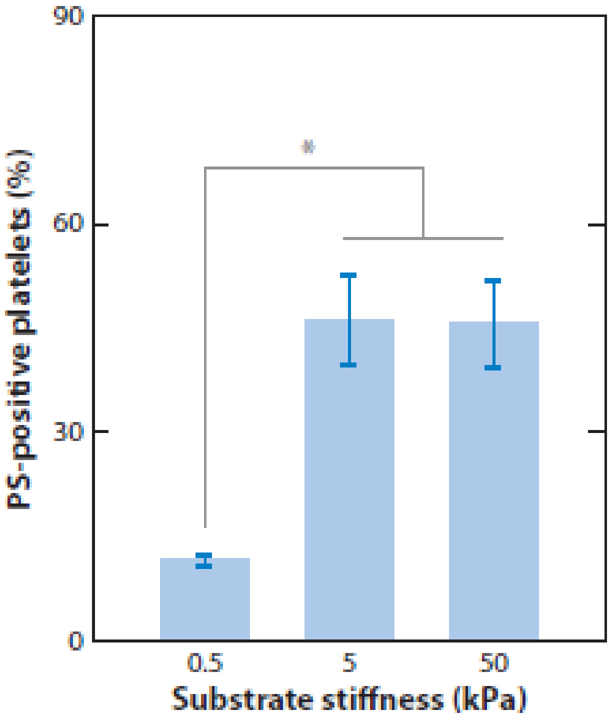
ROCK, MLCK, and actin polymerization affect substrate stiffness-dependent platelet spreading but not adhesion

Qiu Y, et al, *PNAS* 2014

substrate stiffness mediates platelet adhesion and spreading on collagen via actomyosin

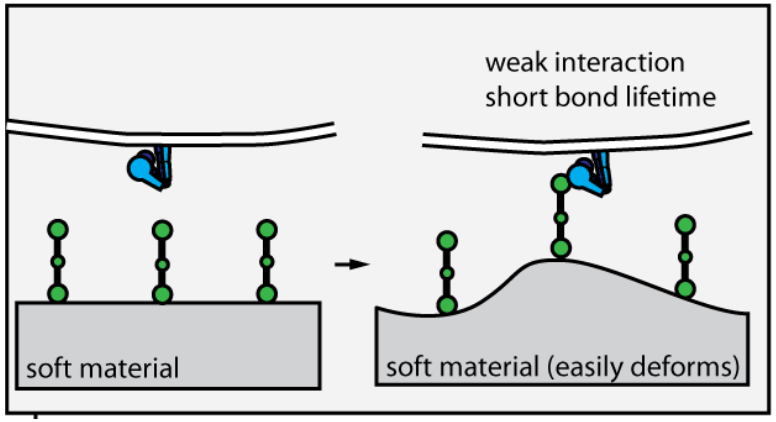






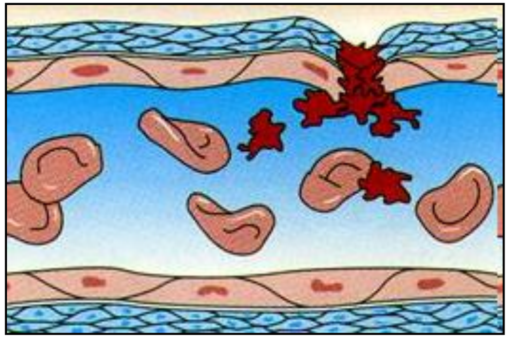
Kee M, et al, *PLoS ONE* 2015

potential role of platelet mechanosensing of substrate stiffness during clot formation

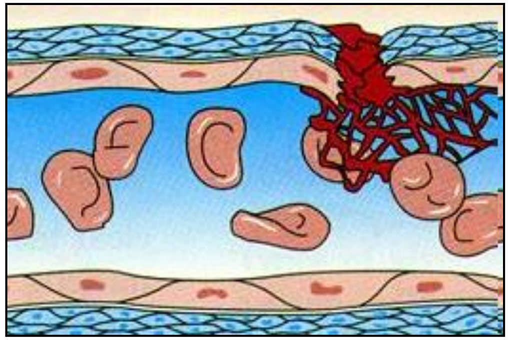


Qiu Y, et al, *PNAS* 2014

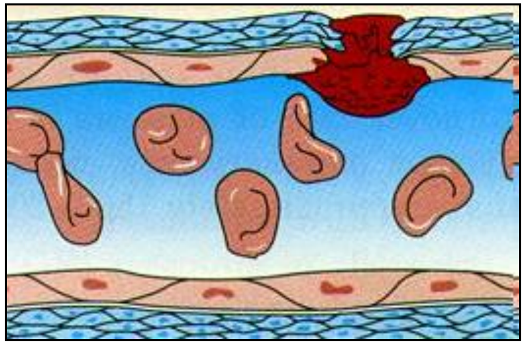
how does platelet mechanotransduction affect clot mechanics and stability?



platelet plug at injury site



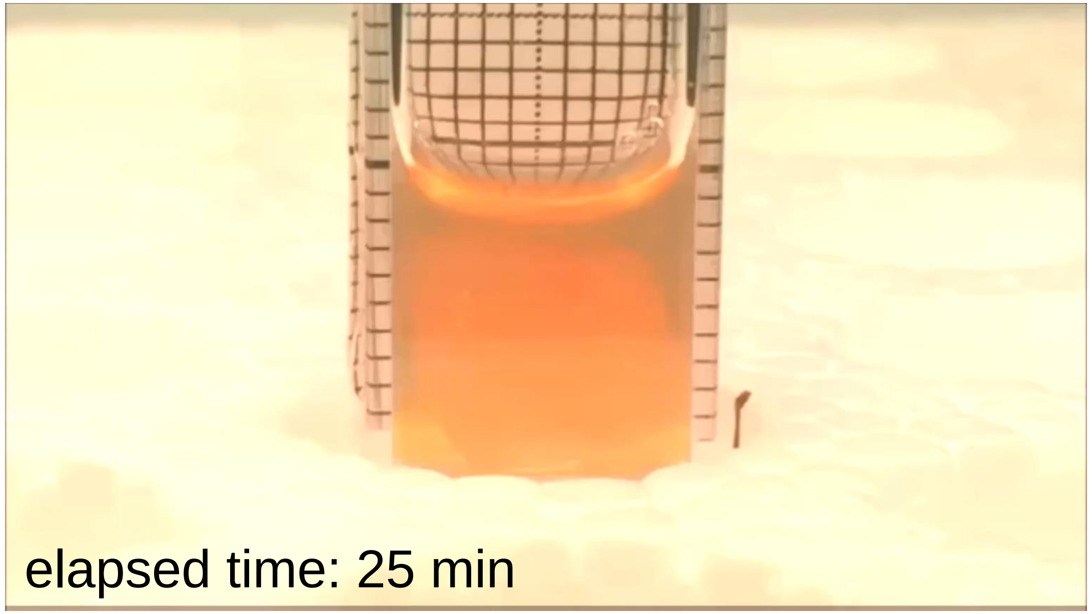
fibrin formation

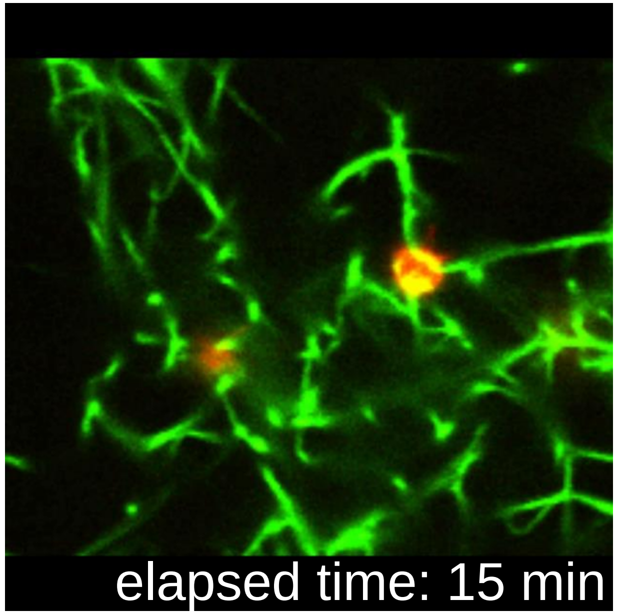


clot contraction

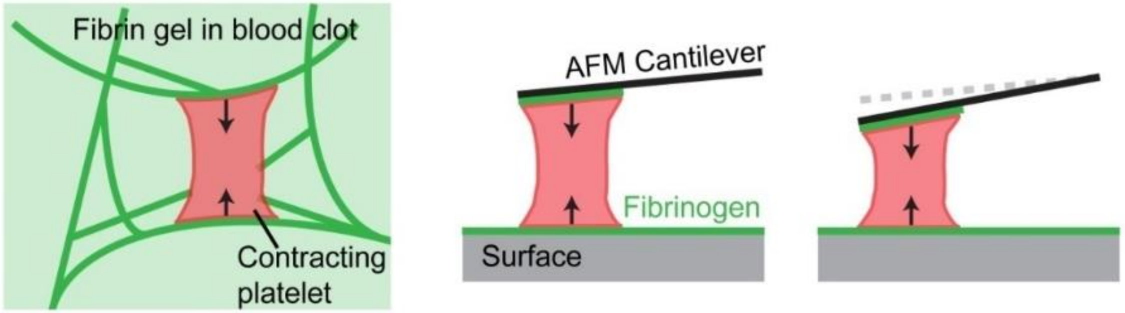
platelet-mediated contraction mechanically reduces clot volume via actomyosin signaling

platelets + fibrinogen + thrombin

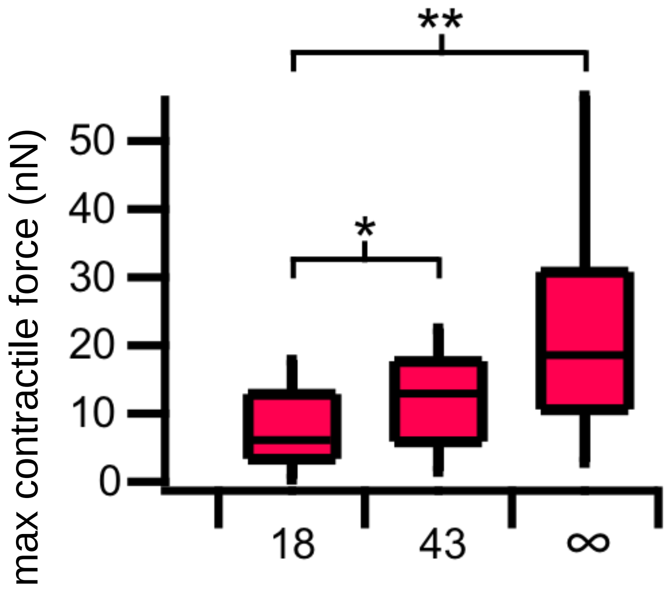




platelet contraction occurs at high forces and is mediated by the mechanical microenvironment



atomic force microscopy (AFM) measurements of single platelet contraction



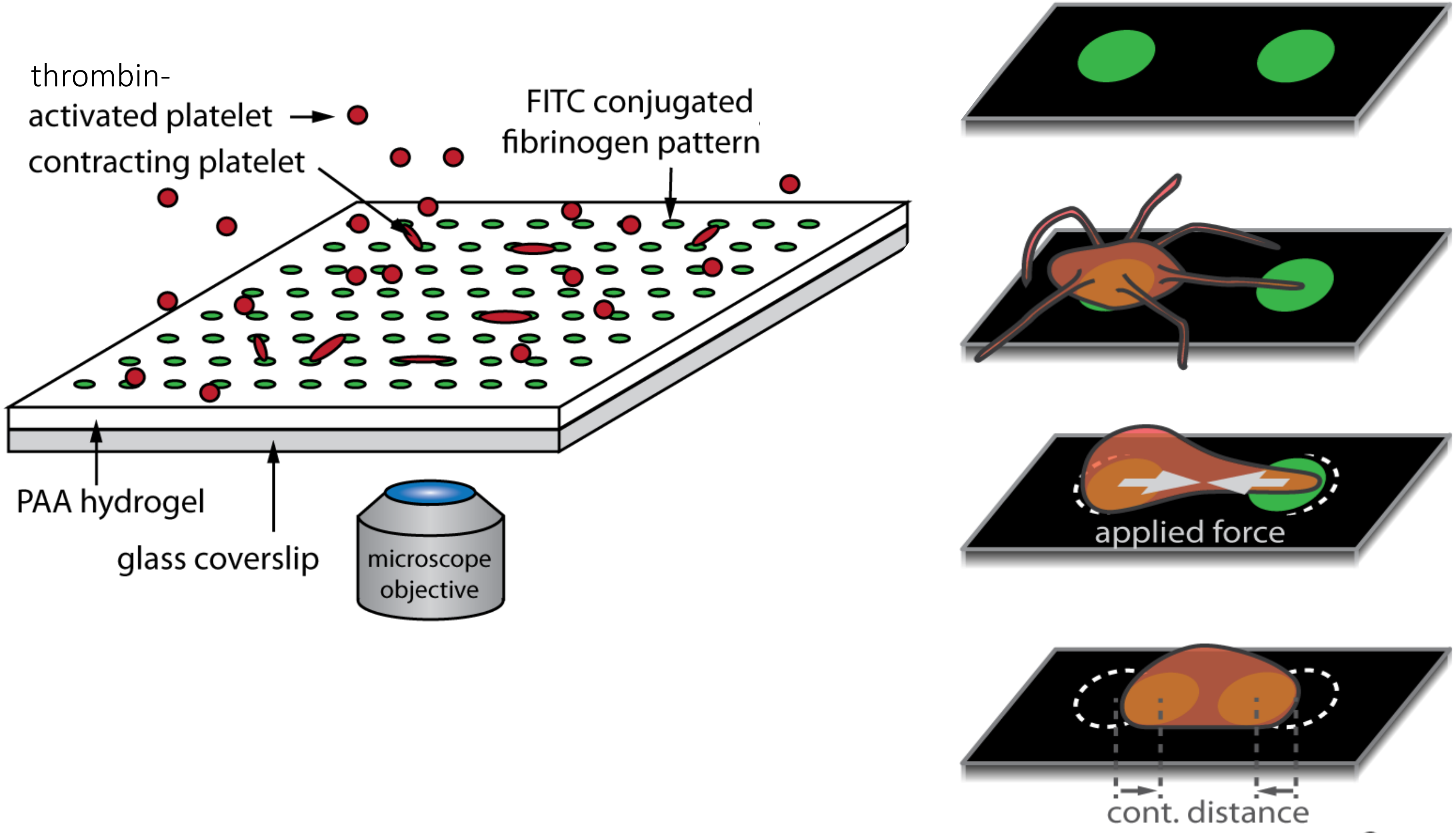
cantilever stiffness (pN/nm)

*p<0.05, ** p<0.001

- platelet contraction occurs near instantaneously upon contact
- platelet contractile force nears myocyte contractile force
- platelets "sense" their mechanical microenvironment and contract with higher forces on stiffer substrates

Lam et al, *Nature Materials* 2011

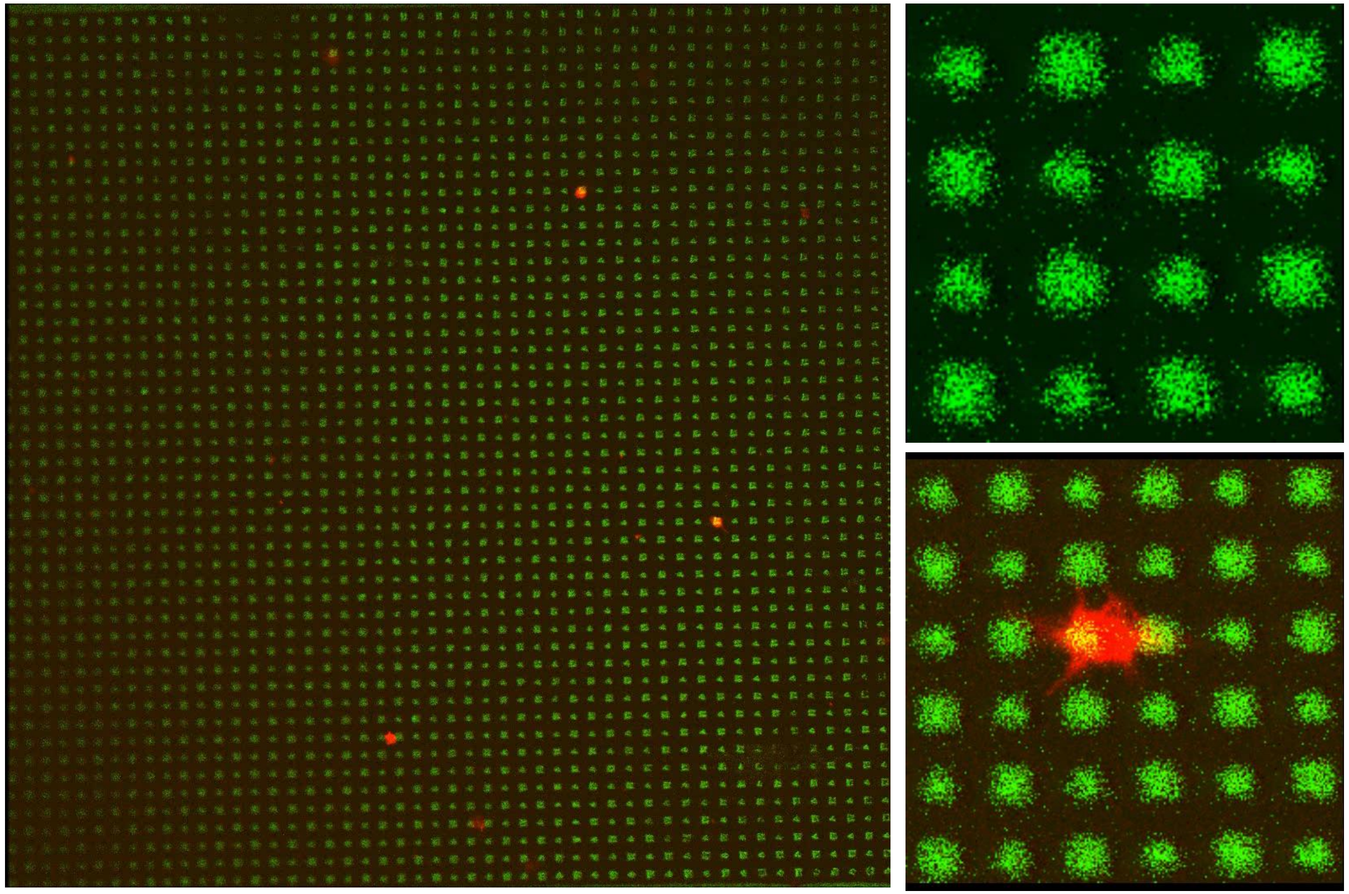
higher throughput "platelet contraction cytometry" via a hydrogel-based approach



David R. Myers, PhD

Myers et al, *Nature Materials* 2017

live imaging videomicroscopy enables tracking of single platelet contraction

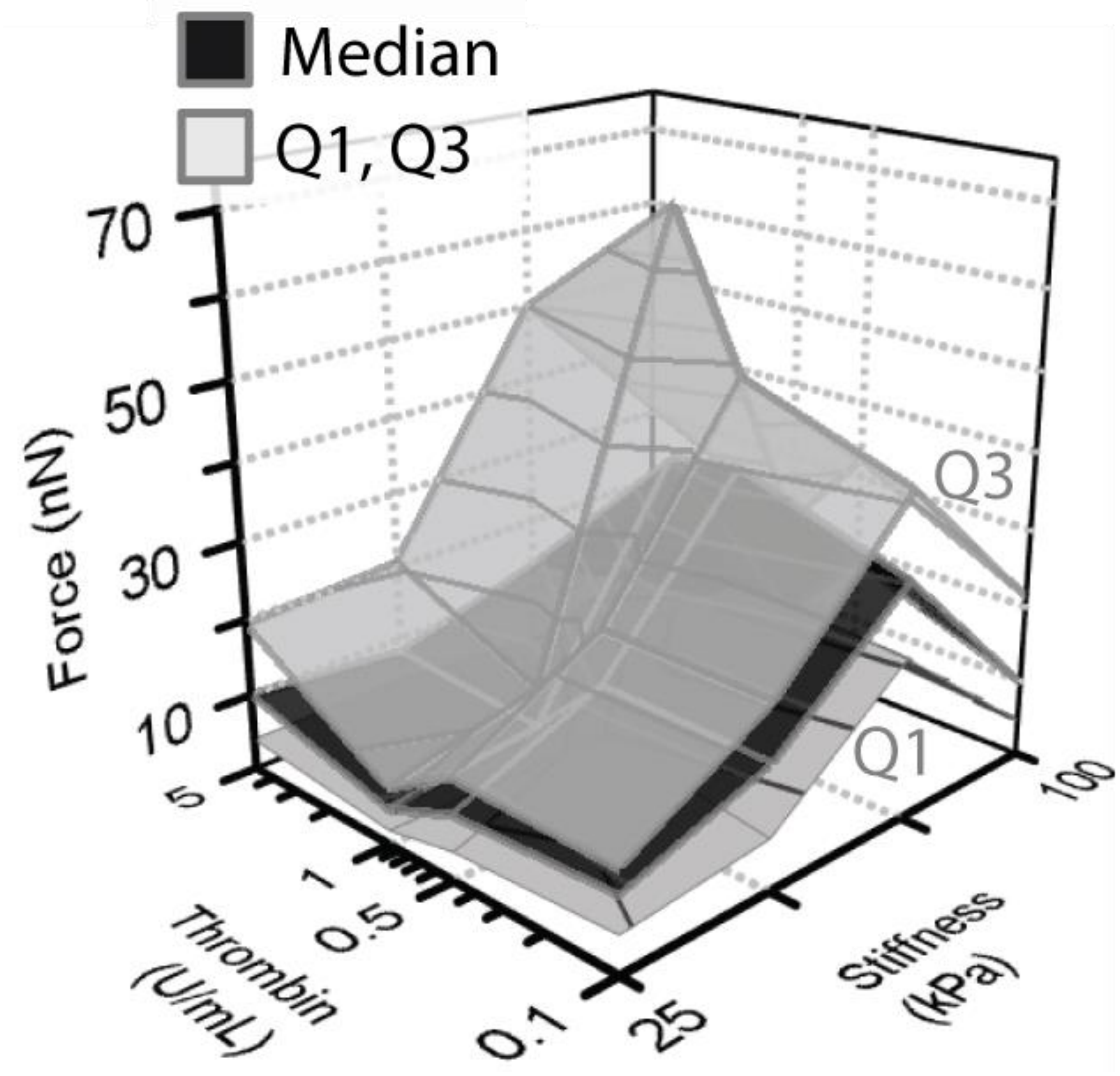


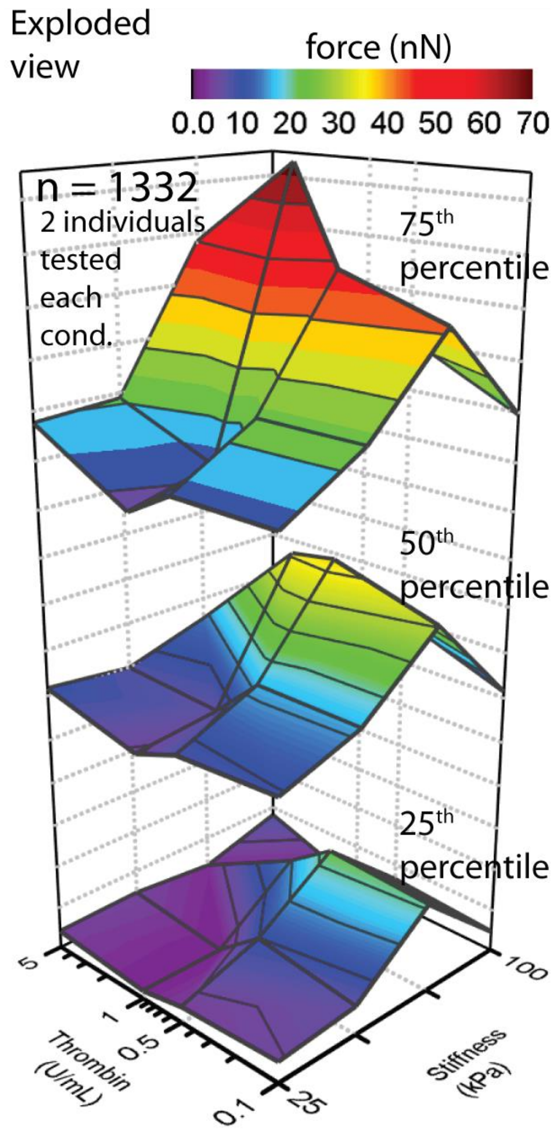
20 min

platelets = red   fibrinogen = green

Myers et al, *Nature Materials* 2017

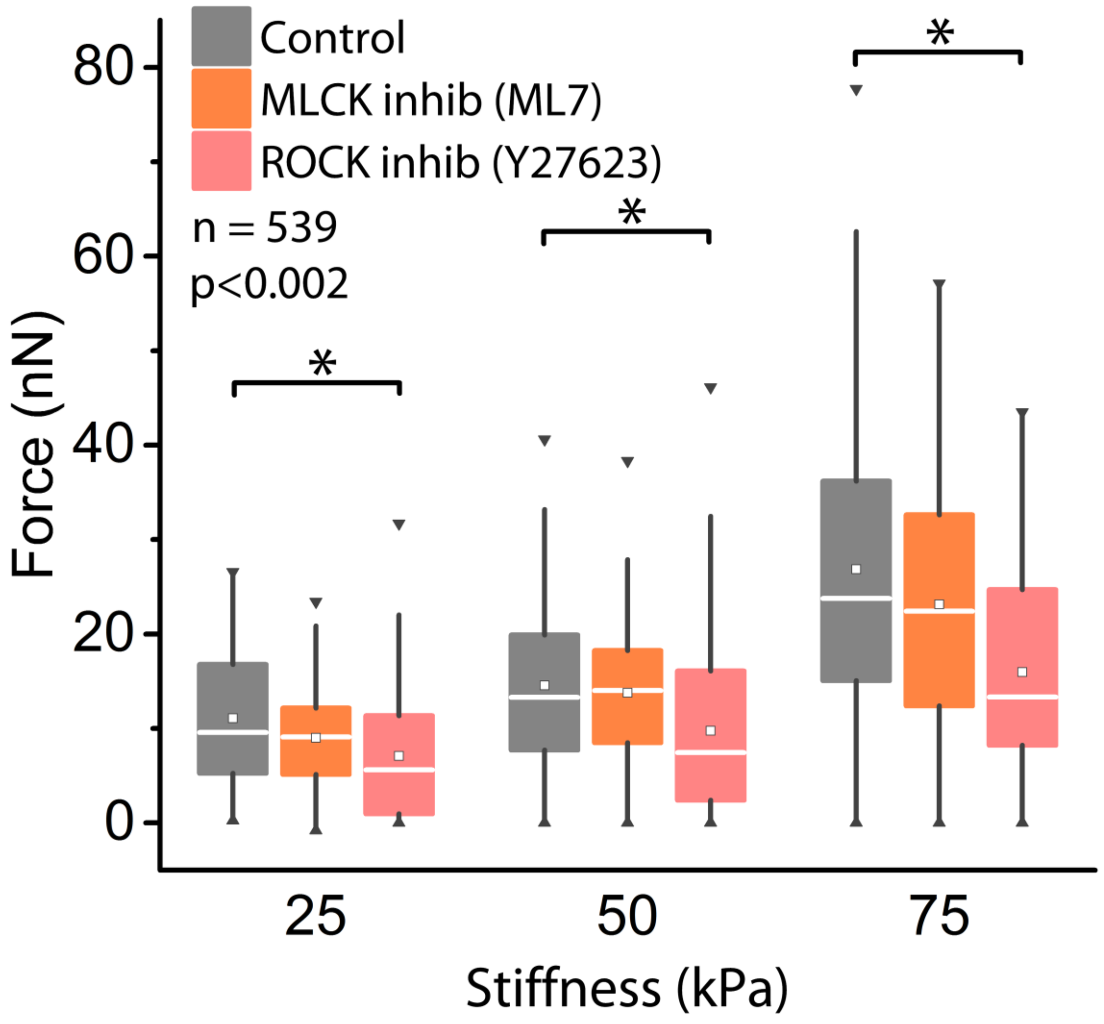
biochemical and mechanical cues synergistically mediate platelet contractile force





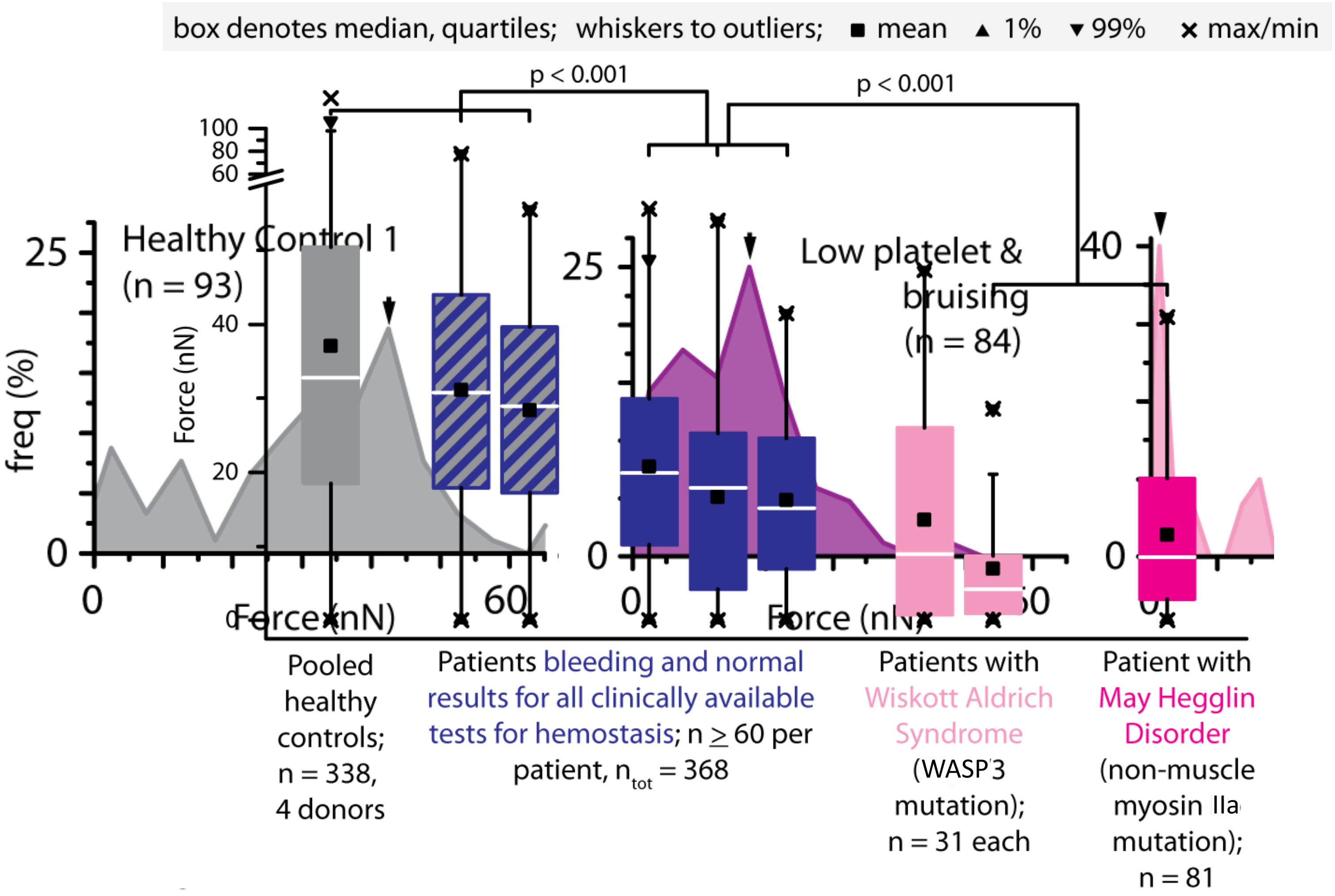
Myers et al, *Nature Materials* 2017

mechanotransductive platelet contraction is mediated by the Rho-associated protein kinase (ROCK) pathway



Myers D, et al…Lam WA, *Nature Materials* 2017

can platelet contraction cytometry be used as a potential clinical biophysical biomarker of platelet function?



contraction force does not correlate with P-selectin expression, PS exposure, $\alpha_{IIb}\beta_3$ activation, or shear stress

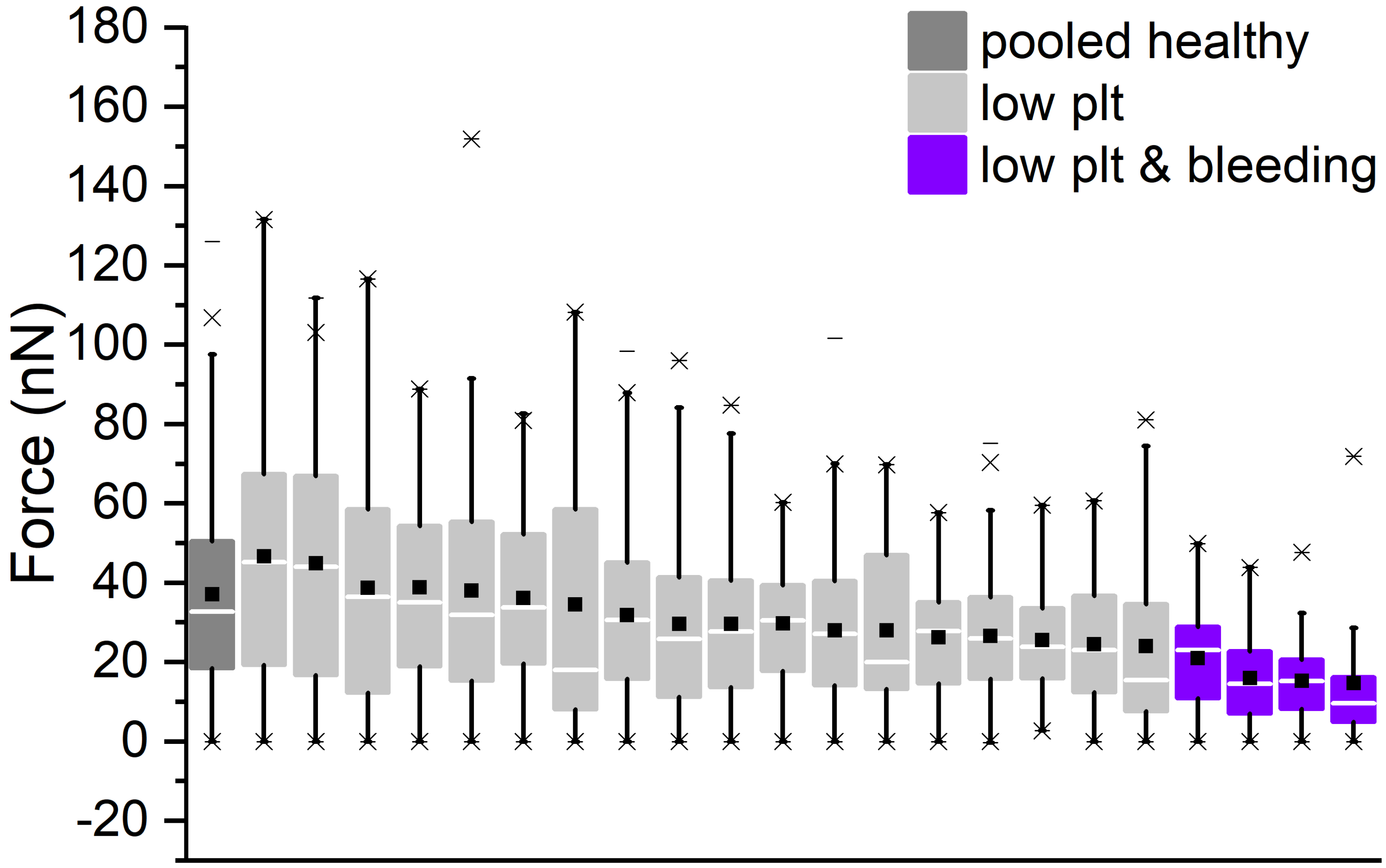
75 kPa gels

Myers et al, *Nature Materials* 2017

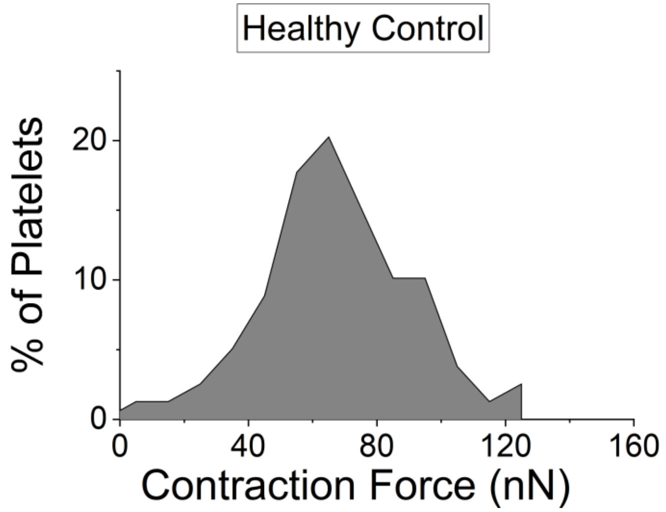
can platelet contraction cytometry be used as a clinical <u>biophysical</u> biomarker for immune thrombocytopenia (ITP)?

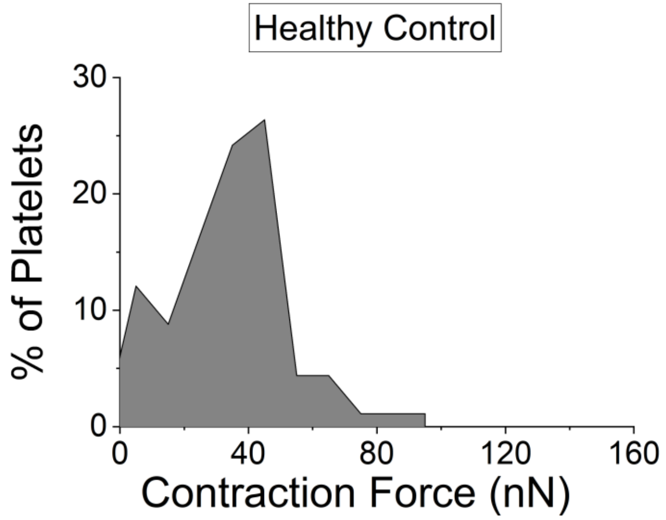
- low platelet count due to autoimmune destruction
- bleeding is a significant risk
    - 10% of patients have major bleeding
    - 0.5% have life-threatening intracranial hemorrhage
- most patients self-resolve, but a minority develop chronic ITP
- typical clinical tests of platelet function are confounded by the low platelet count
- no existing biomarker reliably predicts
    - who needs treatment
    - who will become chronic
    - which patients are prothrombotic

preliminary platelet contraction cytometry data reveal lower average platelet contractile forces in symptomatic ITP patients



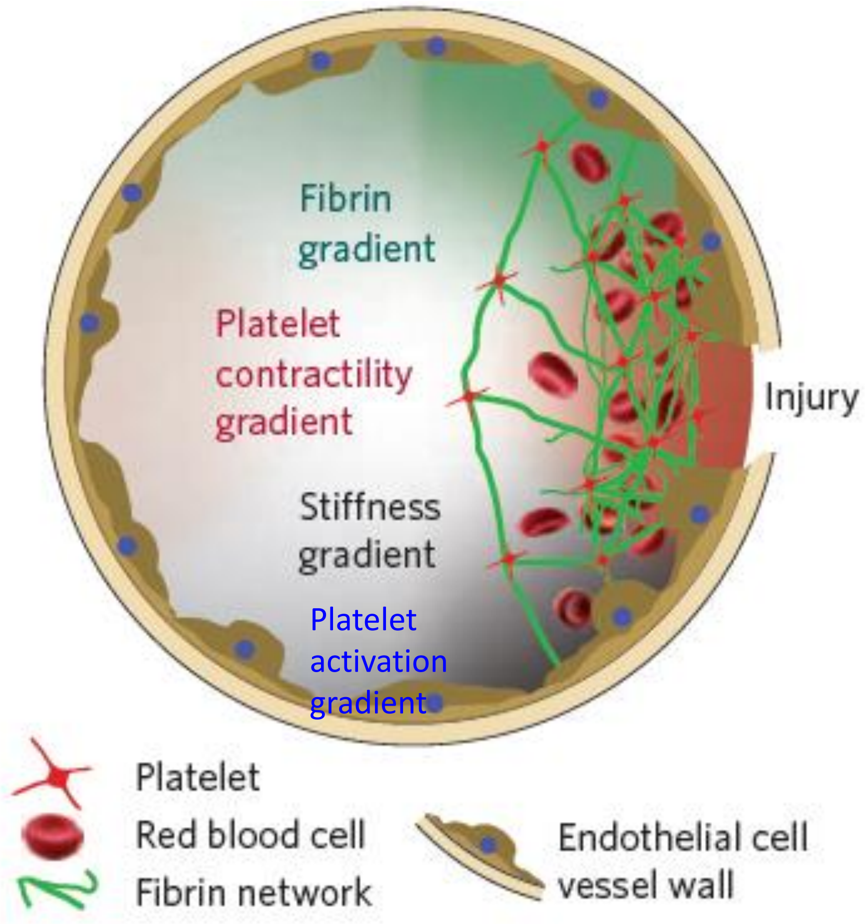
platelet contraction cytometry detects distinct platelet contractile subpopulations in ITP patients





conclusions

- mechanical microenvironment of the nascent clot and vessel wall directly mediates, and is mediated by, platelet physiology and function
- platelet mechanotransduction:
  - drives platelet contraction, which further alters the mechanics of the nascent clot
  - may be leveraged for diagnostic applications as “biophysical biomarkers”
- provides insight into platelet aggregation and help explain the heterogeneity of platelet activation in a growing thrombus
- important implications for implications for improving the biocompatibility of engineered biomaterials and medical implants



Commentary by Erlicher and Hartwig, *Nature Materials* , 2011

acknowledgements

Christina Caruso, Meredith Fay, Caroline Hansen, Elaissa Hardy, Rob Mannino, David Myers, Yumiko Sakurai, Yongzhi Qiu, Reggie Tran, Erika Tyburski, Kendall Williams

collaborators

Emory/Georgia Tech
- Carolyn Bennett, MD, MSc
- Michael Briones, DO

Boston University
- Michael Smith, PhD

Blood Center of Wisconsin
- Shawn Jobe, MD, PhD

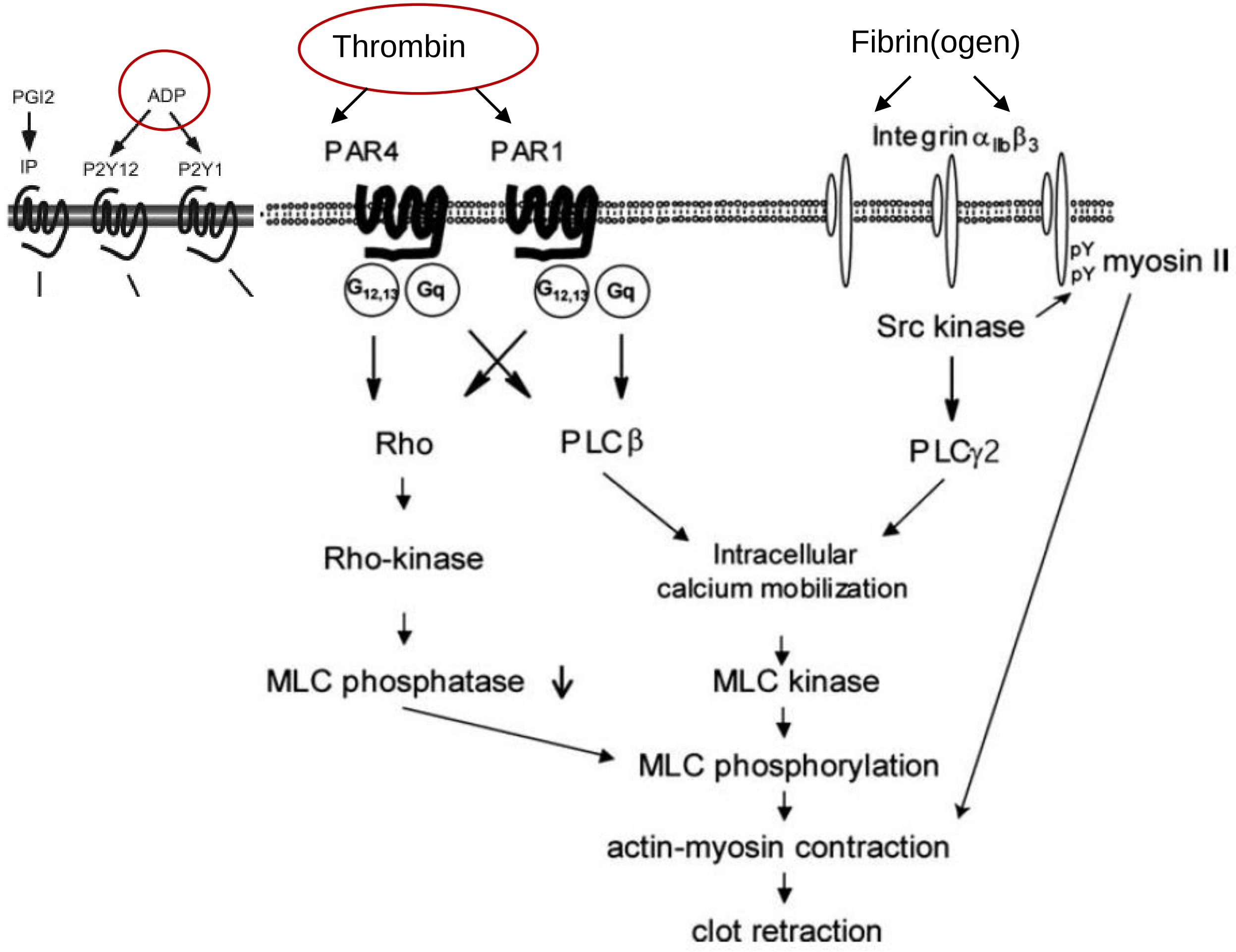
UNC-Chapel Hill/NC State
- Ashley Brown, PhD

University of Virginia
- Tom Barker, PhD

funding

National Institutes of Health
National Heart Lung and Blood Institute
National Eye Institute
NSF
American Heart Association
Department of Defense United States of America
FDA

the biological signaling of platelet contraction is well characterized, but the associated biophysics is poorly understood



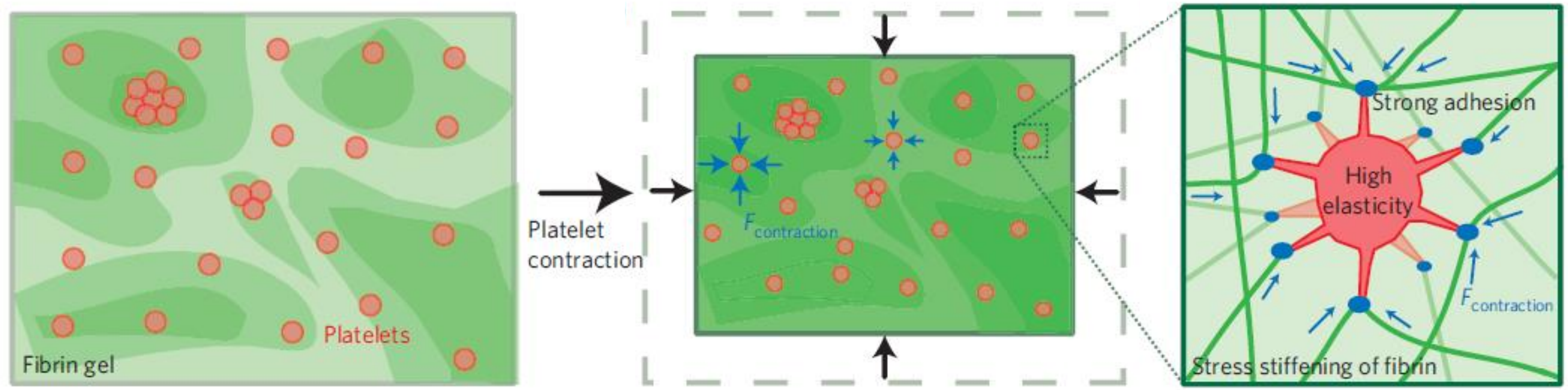
Li, Z. et al. *Arterioscler Thromb Vasc Biol* (2010)

Suzuki-Inoue et al, *Thrombosis Res* (2007)

proposed cumulative effects of platelets on clot mechanics

platelets may directly increase elasticity through multiple mechanisms

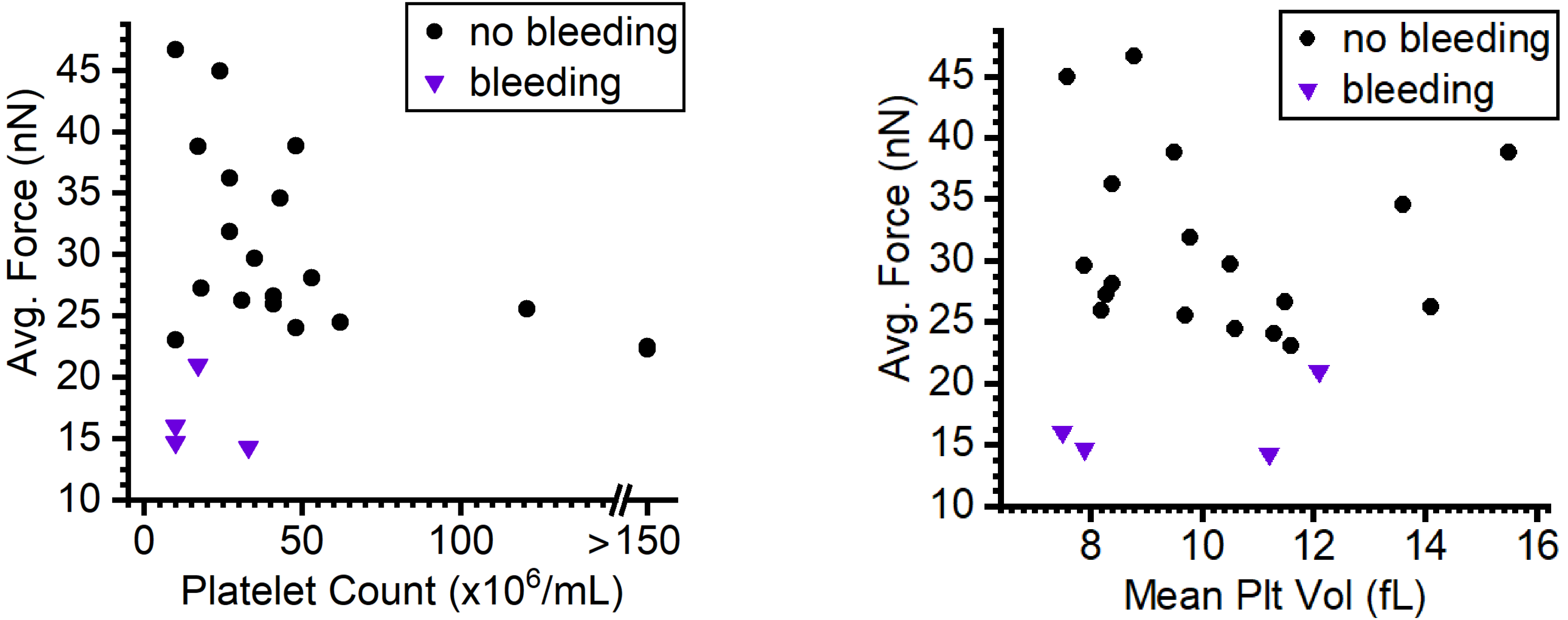
light shading = less dense, less stiff
dark shading = more dense, more stiff



uniform contraction

Lam et al, *Nature Materials* (2011)

how do platelet count and platelet size relate to contractile force in ITP?



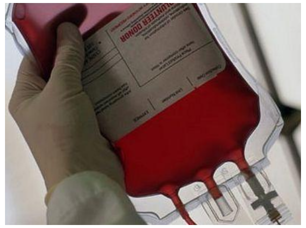
Biomimetic systems for *ex-vivo* platelet production

Avital Mendelson Ph.D.
Assistant Member, Lindsley F. Kimball Research Institute
Head, Stem Cell Biology and Engineering Research Program
New York Blood Center

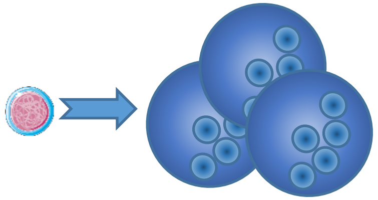
It's A Numbers Game

Goal:

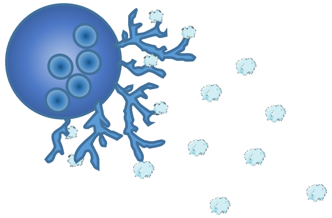
Platelet unit: 300 billion platelets



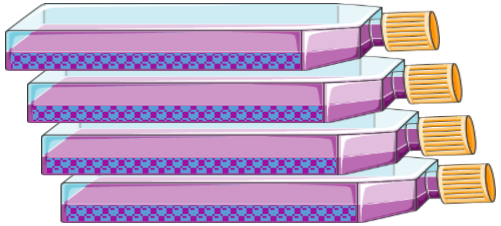
UCB unit: 5 x 10^6 CD34+ Cells



1 CD34+ cell can yield 1000 MKs *in vitro*



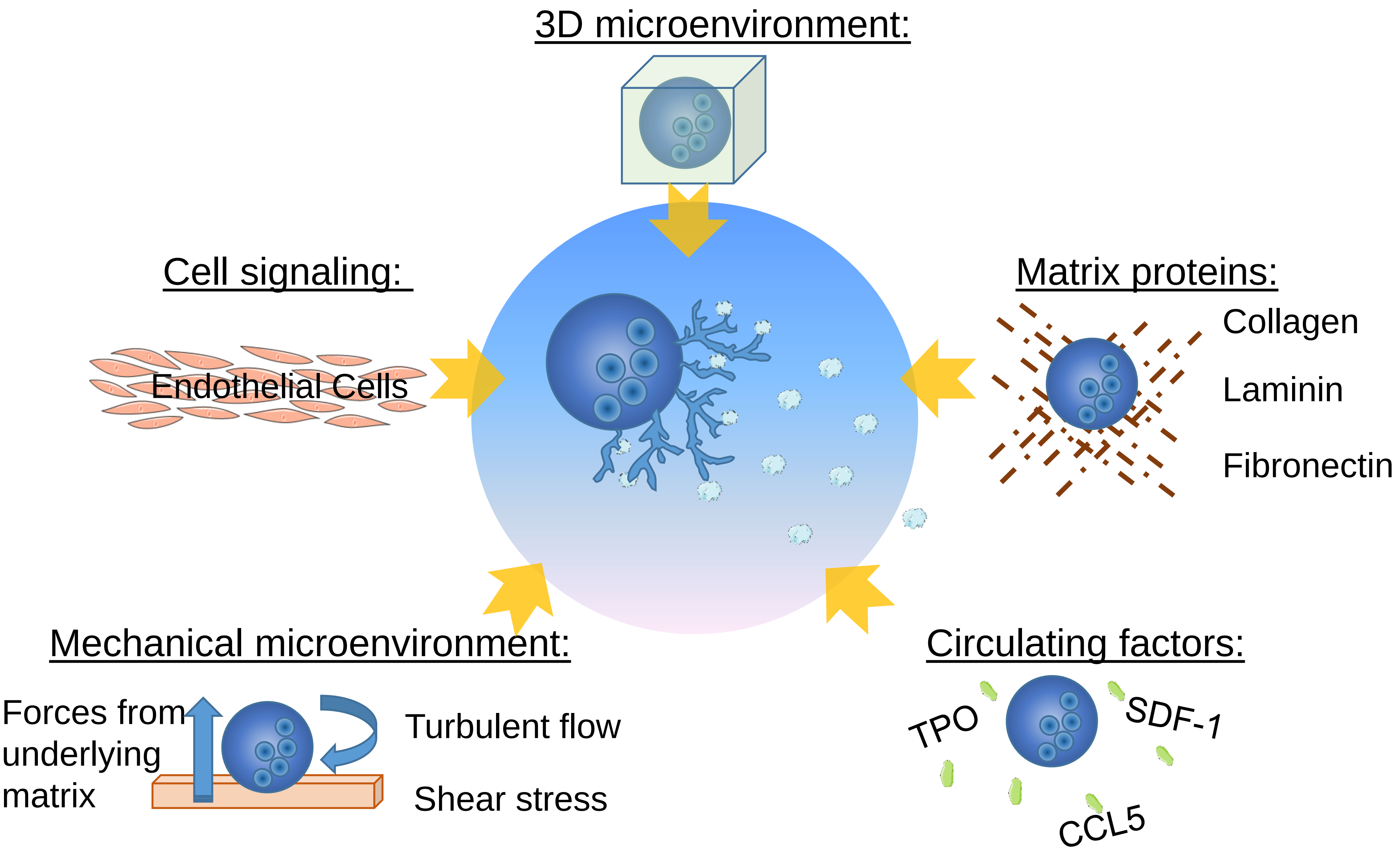
1 mK *in vivo* produces 2000-5000 platelets.



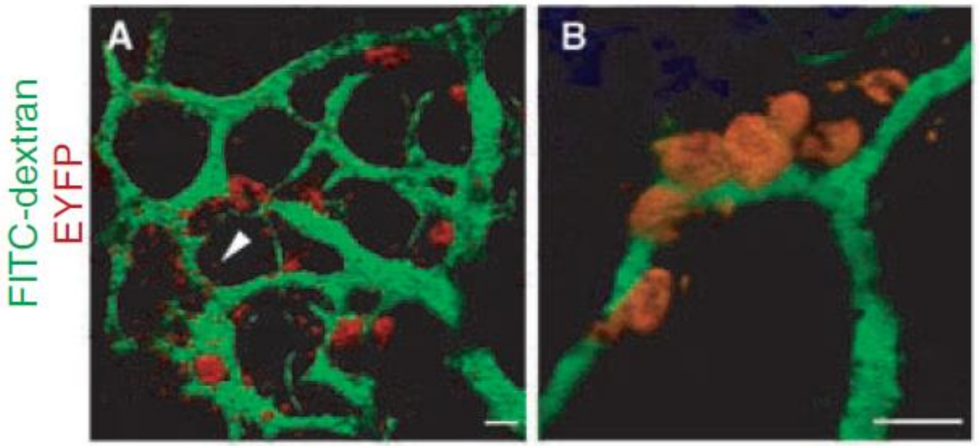
5 x 10^6 UCB CD34+ cells should produce 5 billion MKs, which should make 10,000 billion platelets.

Most current *in vitro* platelet generating protocols only produce 10-150 platelets/MK

Biomimetic culture systems to improve platelet formation



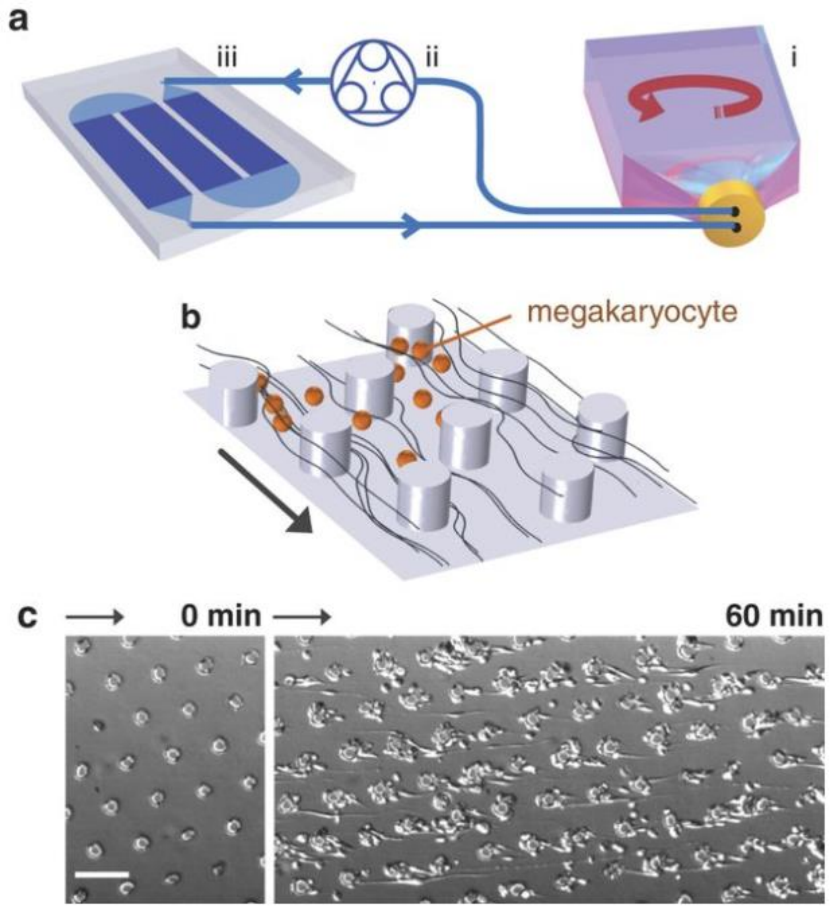
Shear stress enhances thrombopoiesis



- Using multiphoton intravital microscopy, observed MK extend proplatelets into BM sinusoids.
- Proplatelets are sheared by flowing blood and enter peripheral blood
- *In vitro*, proplatelet production is enhanced with shear stress.

(Junt T. et al. *Science*, 2007)

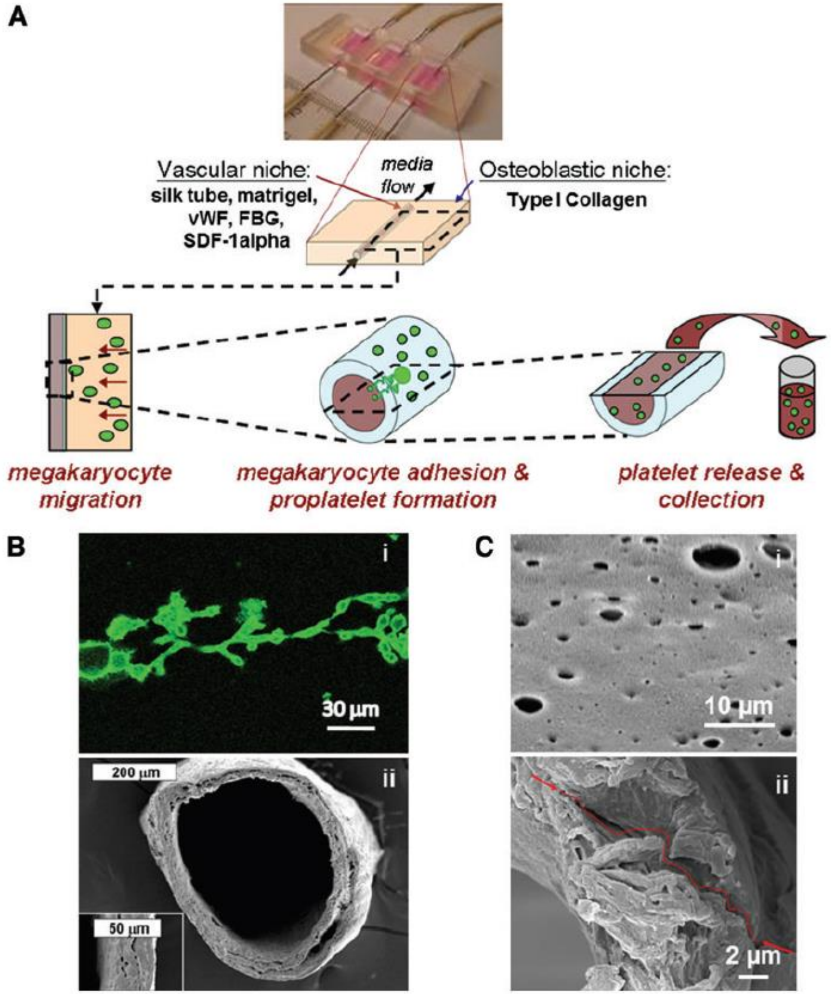
Microfluidic device mimicking megakaryocyte capture, proplatelet elongation and fragmentation



- Allowed for the visualization of the platelet formation process.
- Could lead to platelet production within 2hrs of perfusion.
- Bioreactor fabrication was fast and cheap.
- Fairly low numbers of MKs could be processed at once (1 million).

(Blin, A. et al. *Scientific Reports*, 2016)

3D bone marrow niche for platelet formation *ex-vivo*

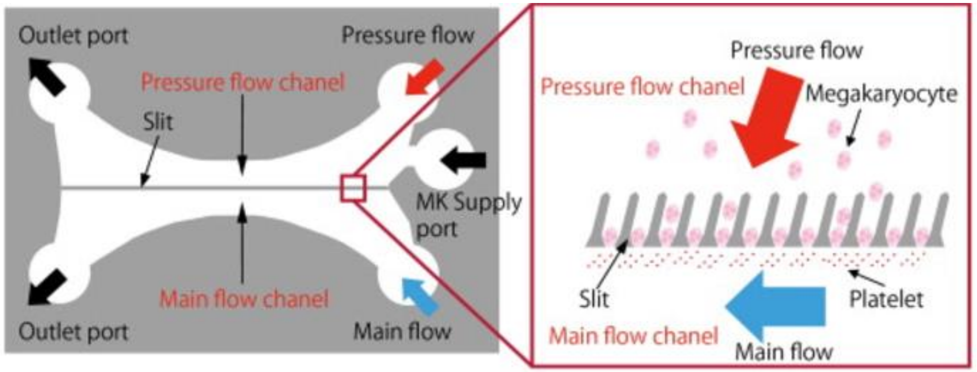


- Modeled silk to control for topography and stiffness, and seeded with endothelial cells, to enhance MK adhesion and proplatelet formation.
- Platelet yields are low compared to *in vivo* levels.
- Slow platelet fragmentation time of 12-16hrs.

(Di Buduo, C.A. et al. *Blood*, 2015)

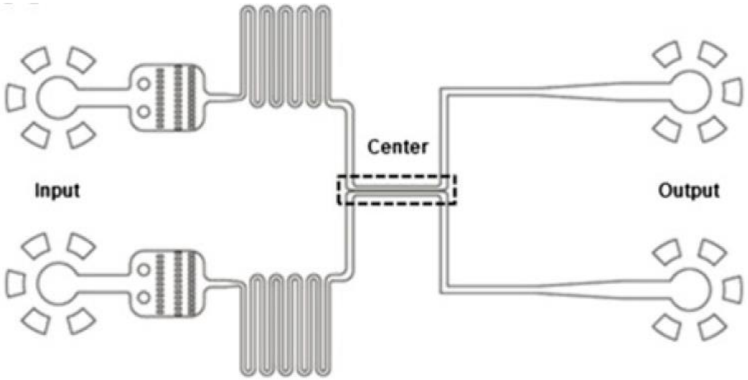
Application of shear stress to *in vitro* systems

1



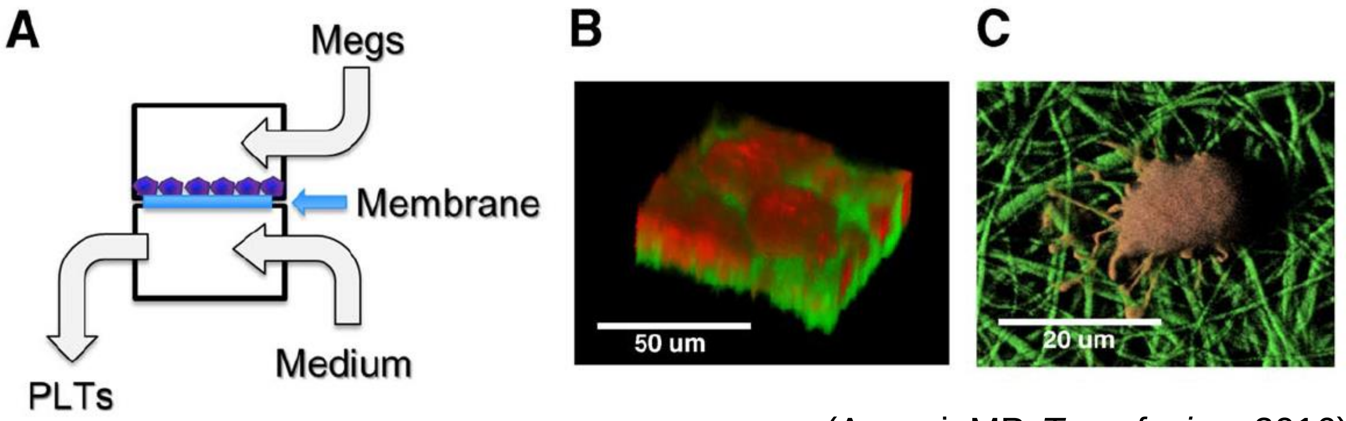
(Nakagawa, Y., *Experimental Hematology*, 2013)

2



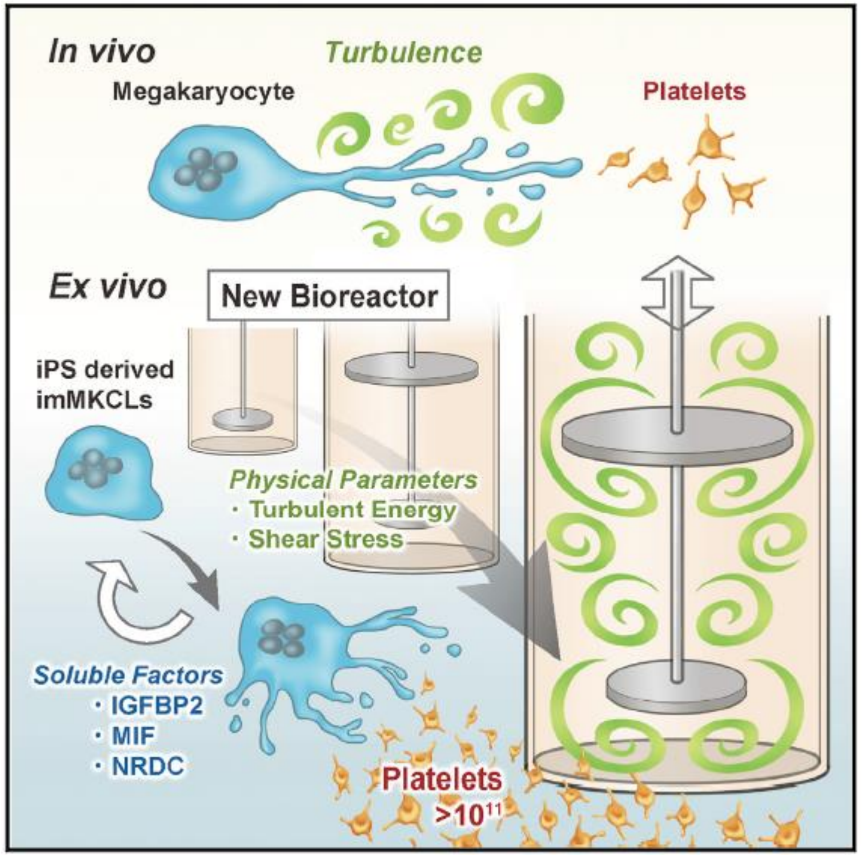
(Thon, J.N., *Blood*, 2014)

3



(Avanzi, MP, *Transfusion*, 2016)

Turbulent flow for increasing thrombopoiesis

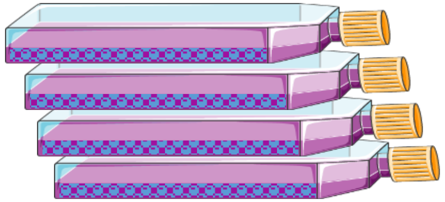


- Achieved clinical scale platelet numbers: 1-1.3 x 10^{11} platelets with *in vitro* and *in vivo* functional properties
- Platelets derived from immortalized MK cell lines varied in size, level of baseline activation and survival *in vivo*.
- Takes 26 days to produce
- High production cost under GMP.

(Ito, Y. et al. *Cell*, 2018)

Remaining hurdles to be addressed before bringing *ex-vivo* produced platelets to the clinic

Platelet production time

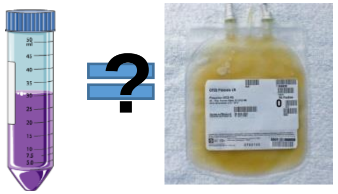
Safety regulation hurdles

- Platelet quality/functionality
- Purity
- Safety

Production cost

- Cost of manufacturing
- Price of safety testing
- Must have comparable pricing to donor units

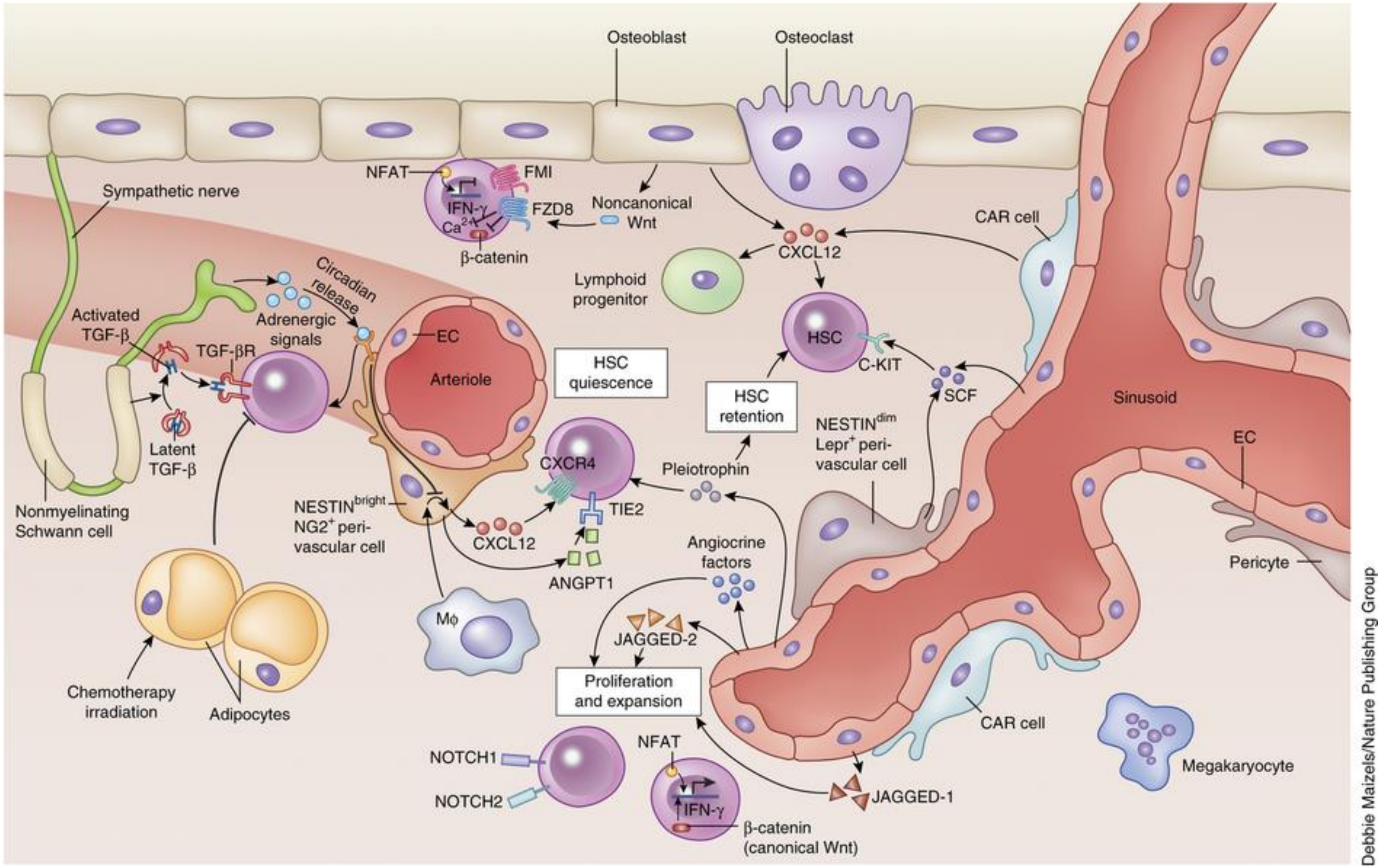
Platelet yield and shelf-life



- 2 days for safety testing
- 1 day for transportation
- 1.5 day shelf life

Investigation of an alternative biomimetic approach to improve platelet formation *in vitro*.

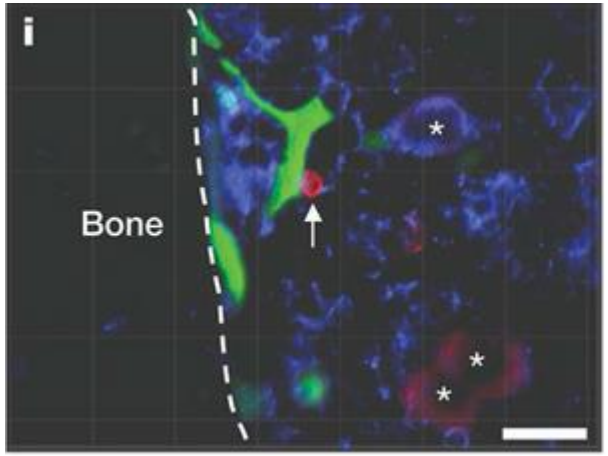
Bone marrow HSC niche



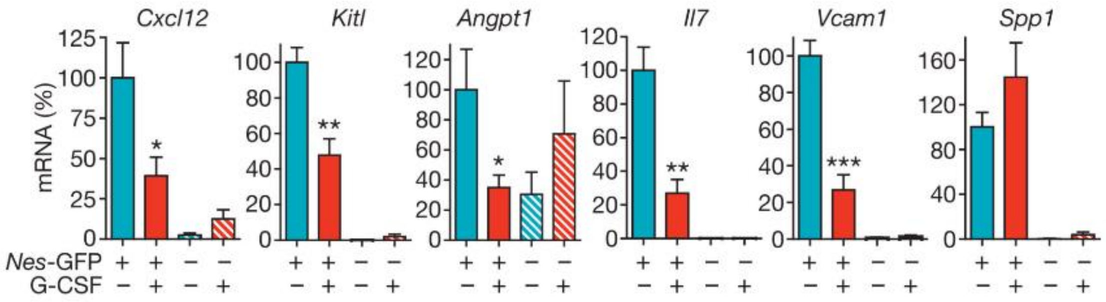
(Mendelson, A, *Nature Medicine*, 2014)

MSCs in the BM

- Represent the CFU-F fraction of stromal cells in the BM
- Are distinct from vascular endothelial cells (do not express CD31) and have a perivascular distribution
- Form an essential HSC niche component:
  - Highly express HSC maintenance genes
  - Share a close physical association with HSCs in the bone marrow.



CD150 CD48/Lin

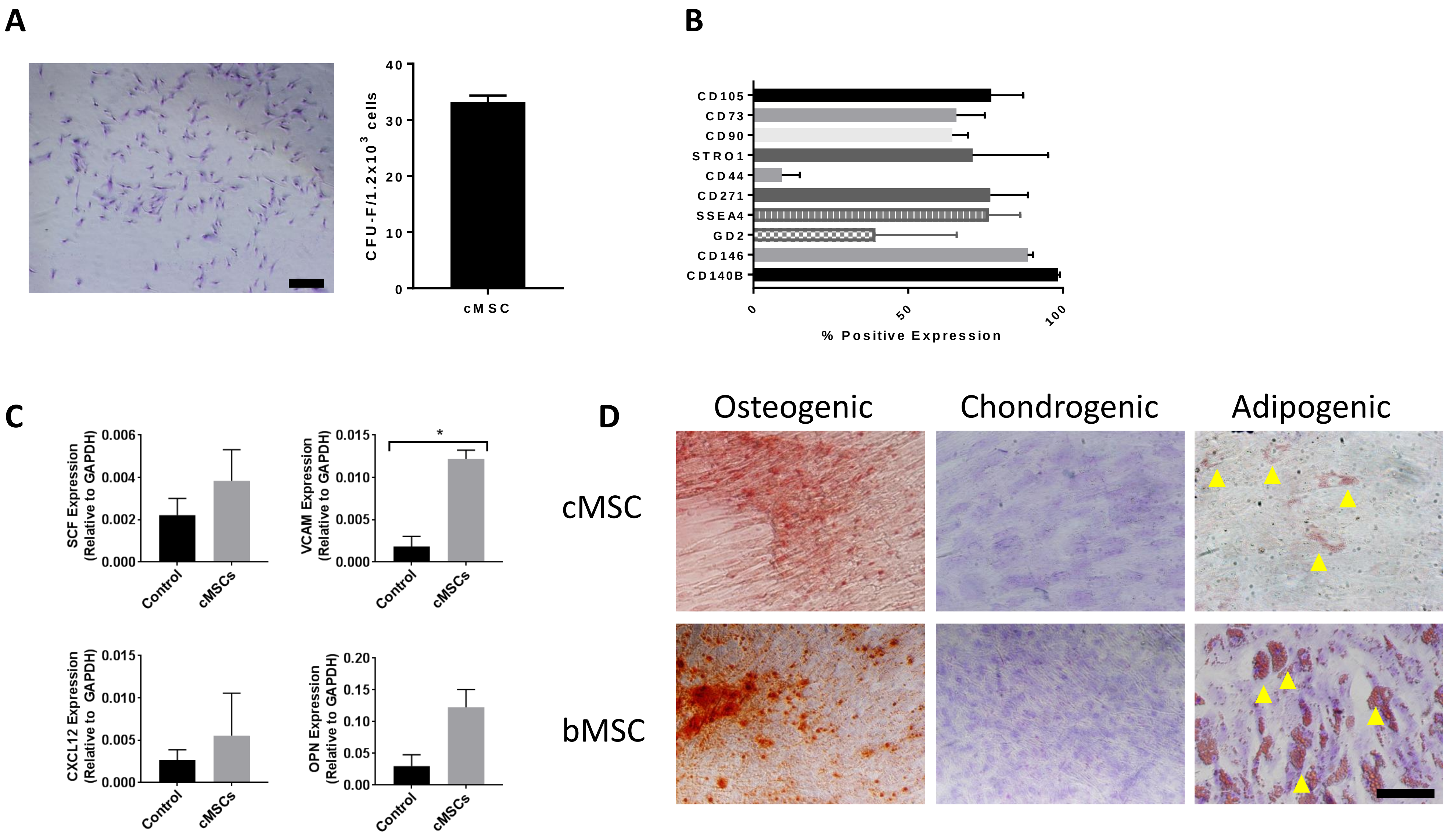


HSC Maintenance Genes

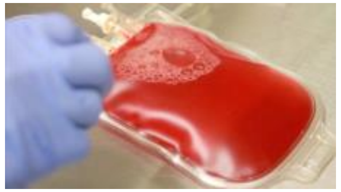
(Mendez-Ferrer, S., *Nature*, 2010)

(Pinho, S., *JEM*, 2013)

cMSC stromal cell characterization



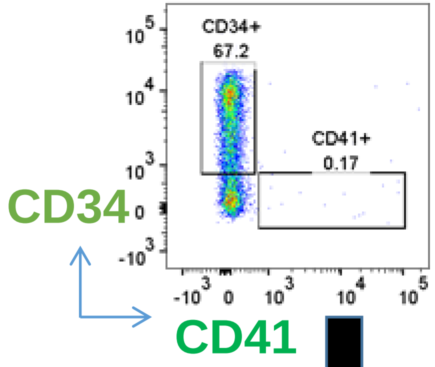
Platelet formation from UCB stem cells



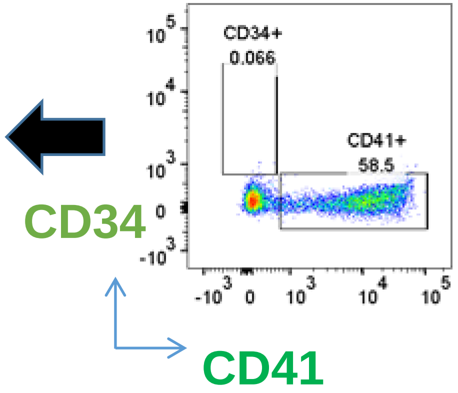
Unit of cord blood

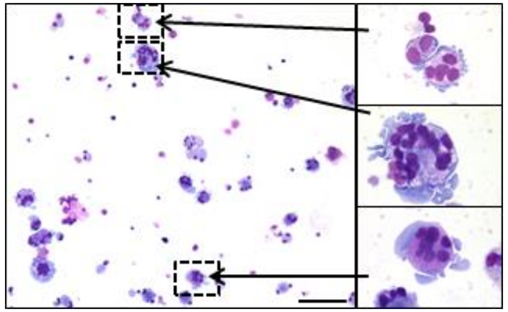
Day 0: Enrichment of CD34$^+$ stem cells

Day 4: Cultured Cells Express Stem Cell

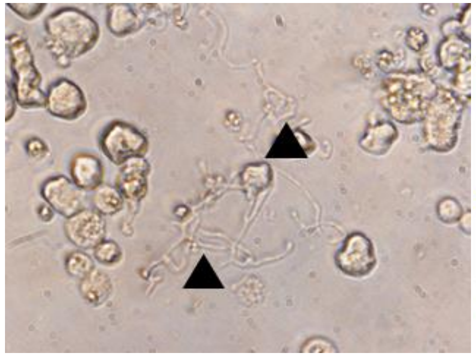


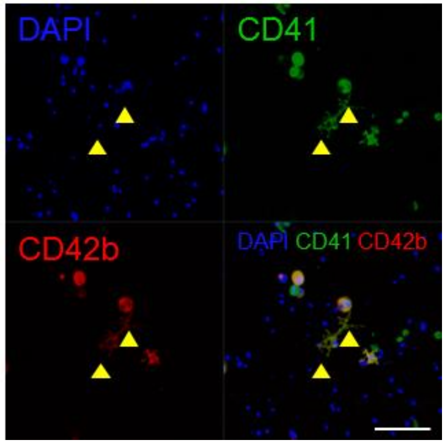
Day 8: Differentiation of CD34$^+$ cells into polypoid CD41$^+$ CD42b$^+$ megakaryocytes



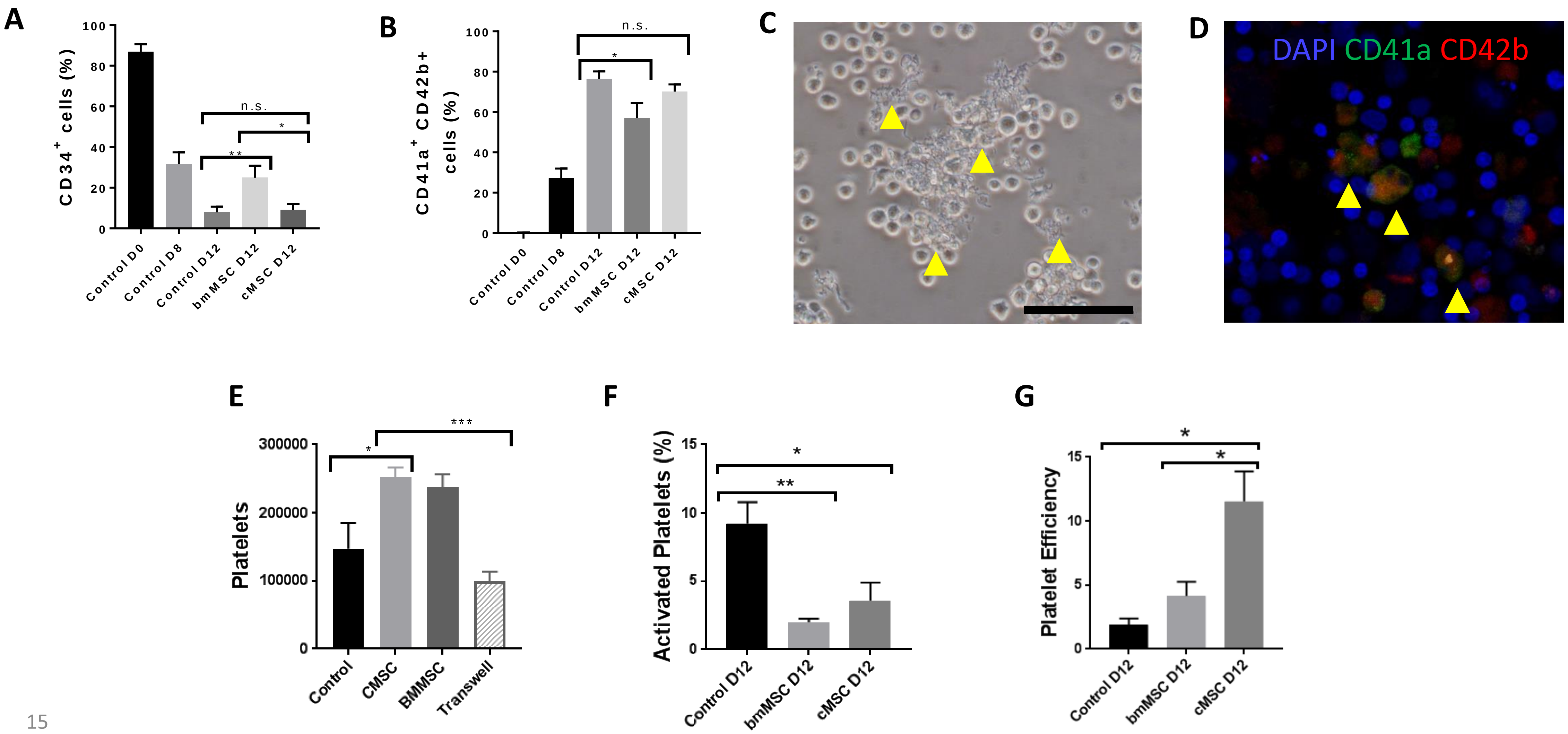


Day 11: Formation of Proplatelets

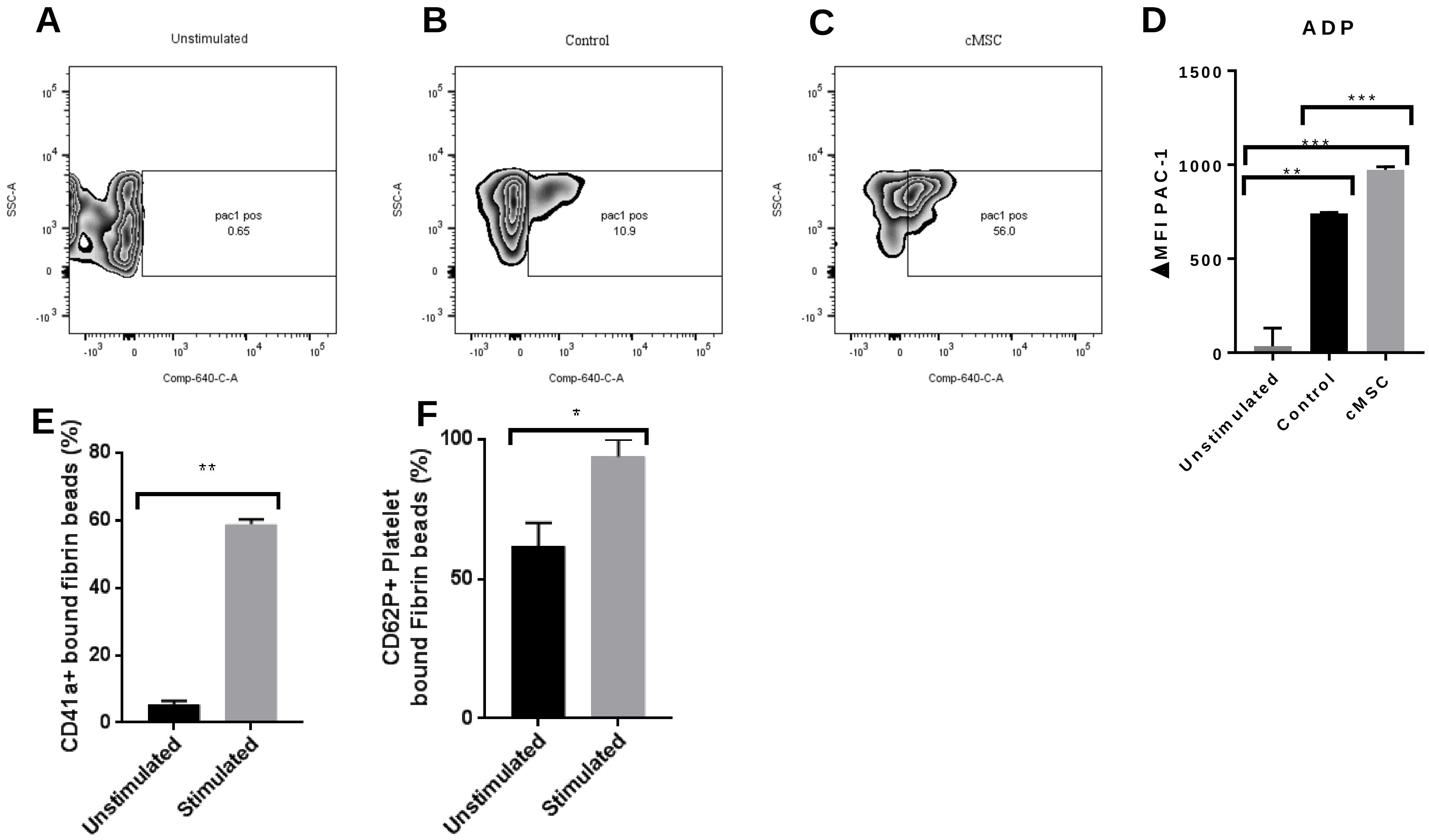




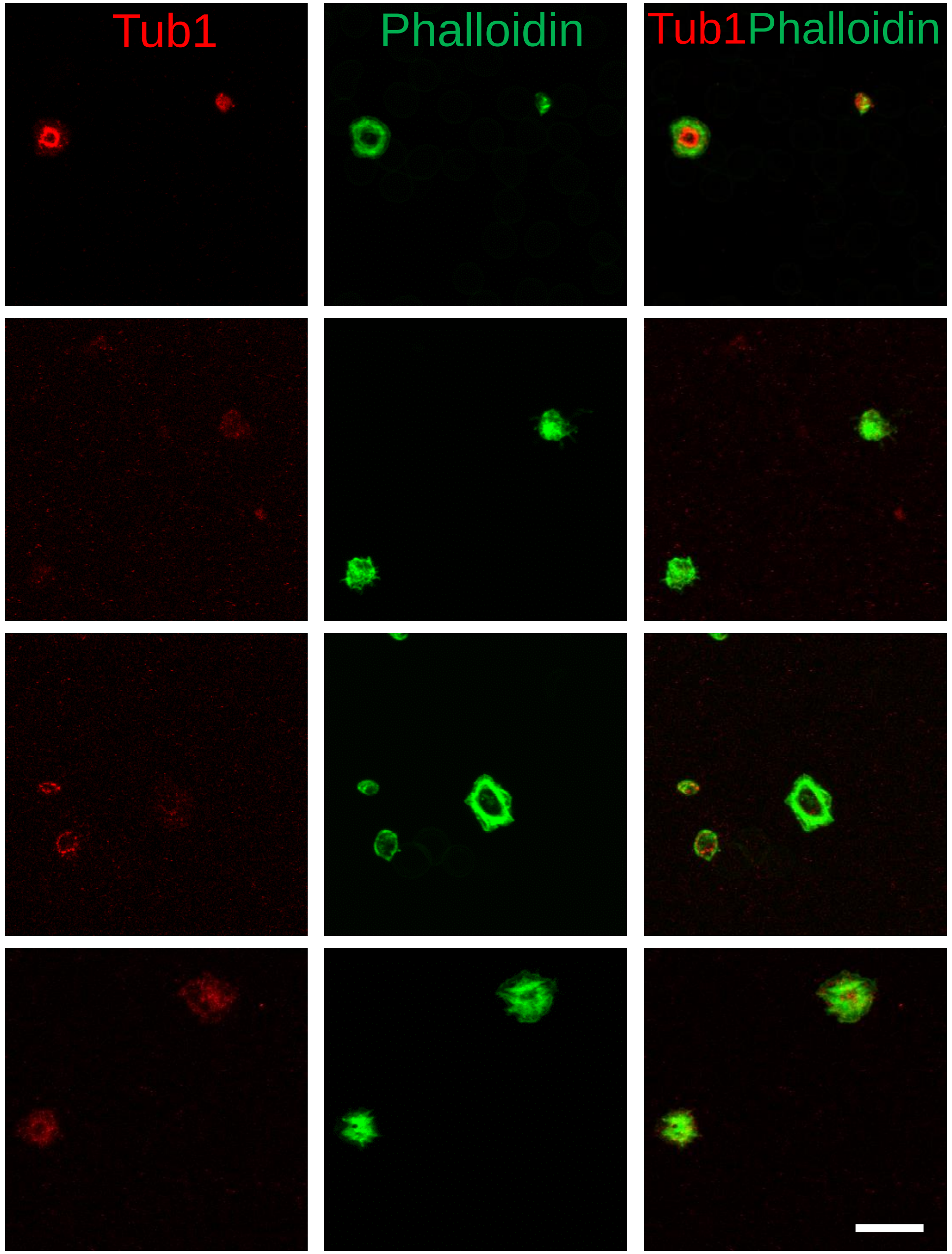
cMSCs enhance platelet formation *in vitro*



Functional platelet characterization: activation in response to ADP stimulation



Generated platelets can spread on glass and matrix proteins



Platelets from blood uncoated

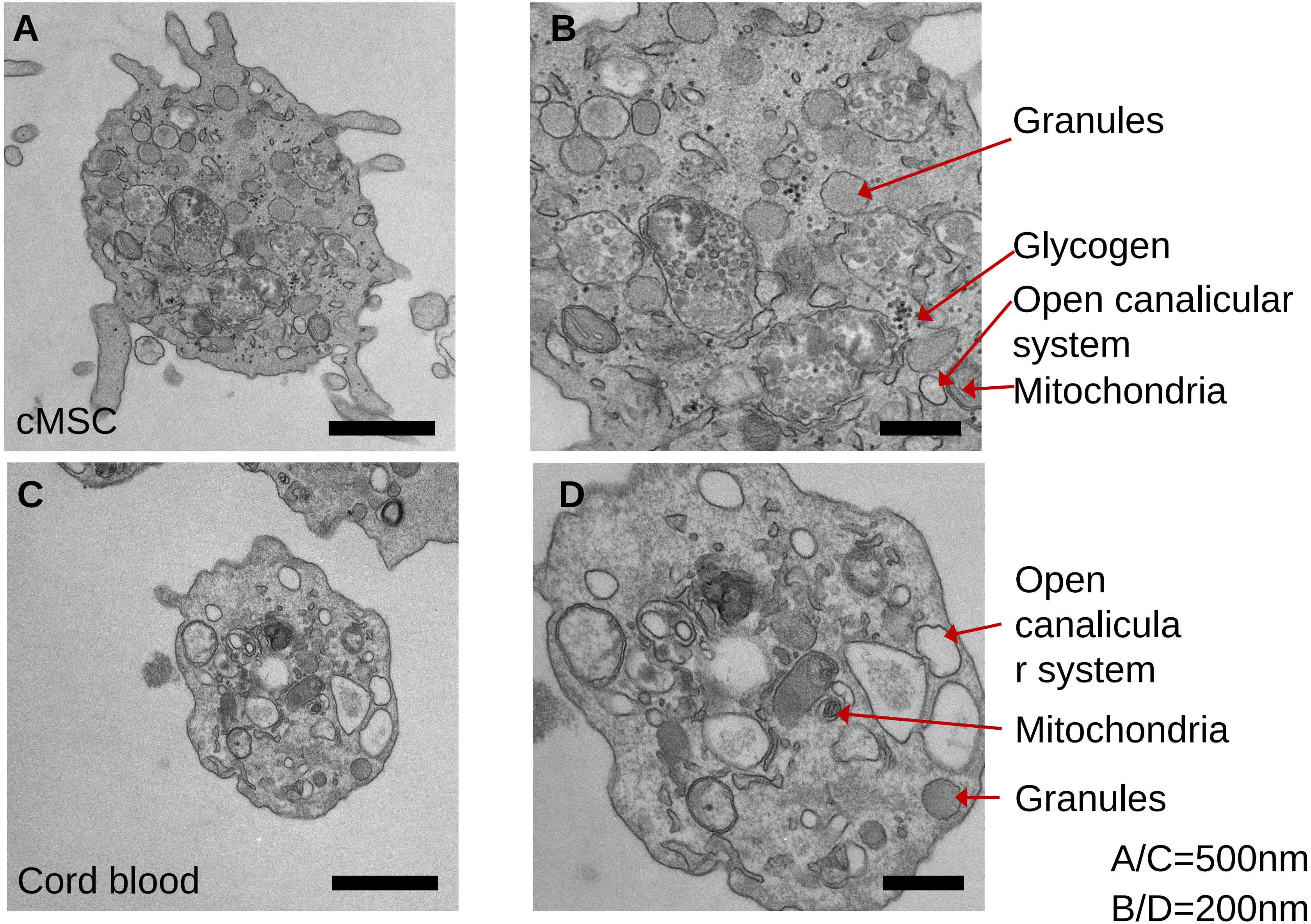
Platelets from cMSC co-culture uncoated

Platelets from blood collagen

Platelets from cMSC co-culture collagen

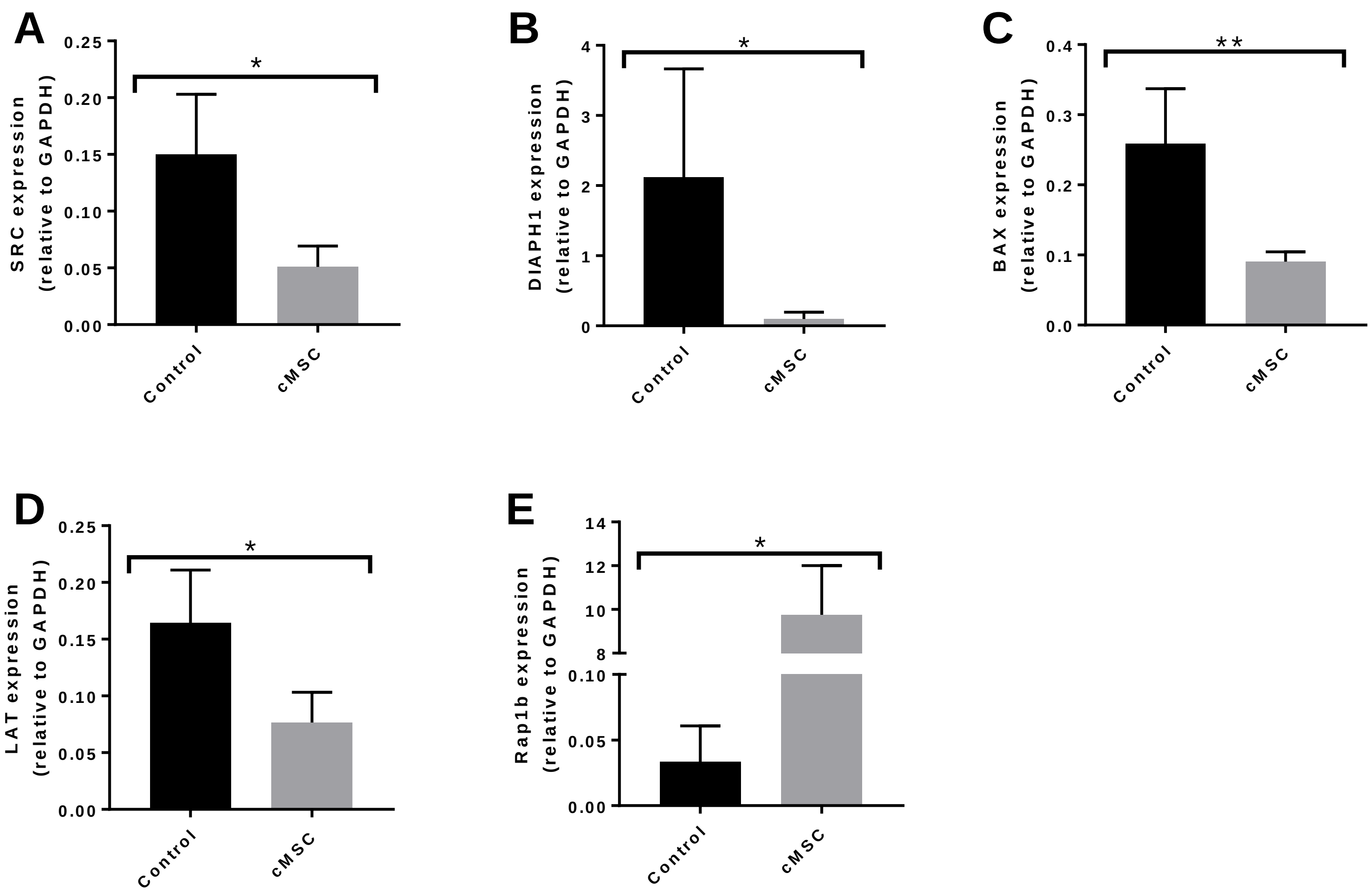
Scale = 10µm

Generated platelets have a similar ultrastructure to primary platelets

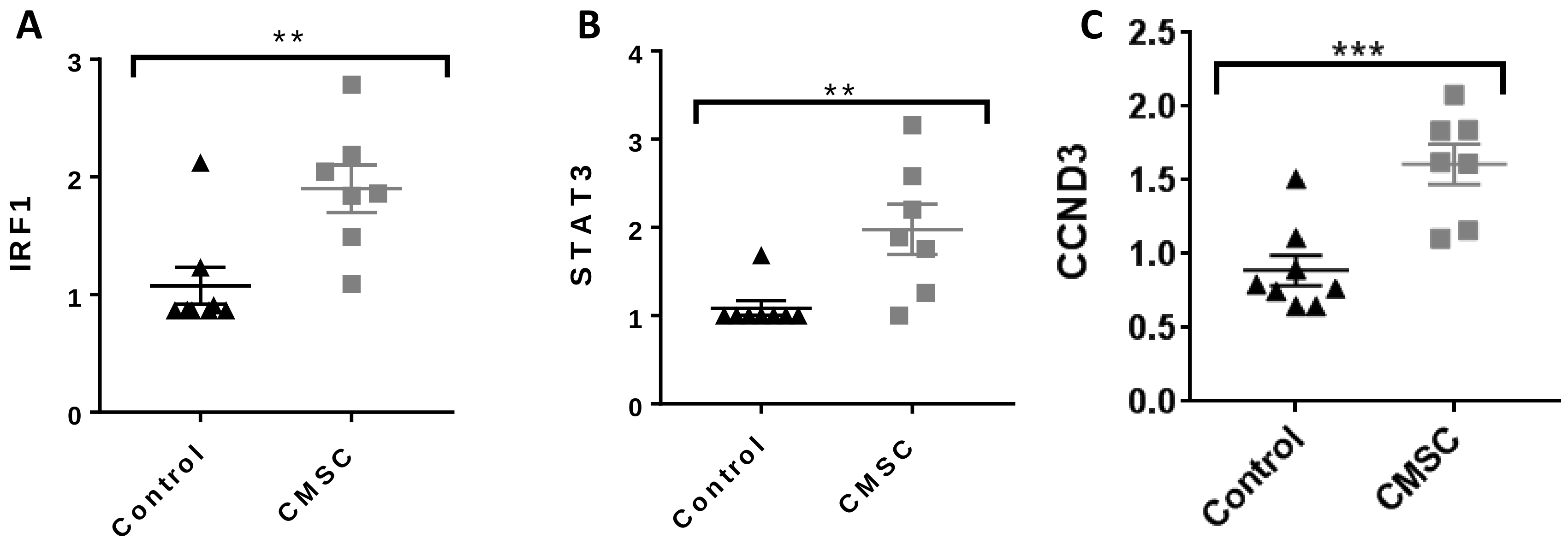


A/C=500nm
B/D=200nm

cMSCs alter gene expression in megakaryocytes leading to pro-platelet formation and decreased platelet activation.



cMSCs assist in promoting megakaryocyte maturation at the protein level



Discussion

- A novel population of stromal cells can be isolated from cord tissue, which assist in promoting platelet formation in megakaryocyte progenitor cells, with low basal activation levels.

- Generated platelets contain ultrastructure similar to native platelets, can adhere and spread to matrix proteins, and activate in response to agonist stimulation.

- Ideal tissue source for cMSC isolation: umbilical cord tissue is normally discarded after birth.

- Proof-of-concept study: scale-up is necessary to approach therapeutic platelet levels.

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