Scientific Category: Platelets and Thrombopoiesis

Article Title/Short Title: Platelet Bioreactor-on-a-Chip

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Key Points:

- We have developed a biomimetic microfluidic platelet bioreactor that recapitulates bone marrow and blood vessel microenvironments.
- Application of shear stress in this bioreactor triggers physiological proplatelet production, and platelet release.

Abstract:

Platelet transfusions total >2.17 million apheresis-equivalent units/year in the United States and are derived entirely from human donors despite clinically significant immunogenicity, associated risk of sepsis, and inventory shortages due to high demand and 5-day shelf life. To take advantage of known physiological drivers of thrombopoiesis we have developed a microfluidic human platelet bioreactor that recapitulates bone marrow stiffness, extracellular matrix composition, micro-channel size, hemodynamic vascular shear stress, and endothelial cell contacts, and supports high-resolution live-cell microscopy and quantification of platelet production. Physiological shear stresses triggered proplatelet initiation, reproduced ex vivo bone marrow proplatelet production, and generated functional platelets. Modeling human bone marrow composition and hemodynamics in vitro obviates risks associated with platelet procurement and storage to help meet growing transfusion needs.
Introduction:

Although platelets (PLTs) play critical roles in hemostasis\(^1\), angiogenesis\(^2\) and innate immunity\(^3\), PLT production remains poorly understood. Consequently, PLT units are derived entirely from human donors, despite serious clinical concerns owing to their immunogenicity and associated risk of sepsis\(^4\). More than 2.17 million apheresis-equivalent PLT units are transfused yearly in the United States\(^5,6\) at a cost of >$1 billion per year. While demand for PLT transfusions has increased markedly in the past decade, a near-static pool of donors and a 5-day PLT unit shelf life resulting from bacterial contamination\(^7\) and storage-related PLT deterioration\(^8\), have resulted in significant PLT shortages\(^9\). Furthermore artificial platelet substitutes have failed to replace physiological platelet products\(^10\). An efficient, donor-independent PLT bioreactor capable of generating clinically significant numbers of functional human PLTs is necessary to obviate risks associated with PLT procurement and storage, and help meet growing transfusion needs.

In vivo, megakaryocytes (MKs, PLT progenitors) sit outside blood vessels in the bone marrow (BM) and extend long, branching cellular structures designated proPLTs into the circulation from which PLTs are released\(^11-15\). Nearly 100% of human adult MKs must produce ~10\(^3\) PLTs each to account for circulating PLT counts\(^16\). While functional human PLTs were first grown in vitro in 1995\(^17\), to date only ~10% of human MKs initiate proPLT production in culture. This results in yields of 10\(^1-2\) PLTs per CD34\(^+\) cord blood- or embryonic stem cell-derived MK\(^18\), which are themselves of limited availability, constituting a significant bottleneck in the ex vivo production of a PLT transfusion unit. Although second generation cell culture approaches have provided further insight into the physiological drivers of PLT release, they have been unable to recreate the entire BM microenvironment, exhibiting limited individual control of extracellular matrix (ECM) composition\(^19,20\), BM stiffness\(^21\), endothelial cell contacts\(^22,23\), and
vascular shear stresses\textsuperscript{24,25}; and have been unsuccessful in synchronizing proPLT production, resulting in non-uniform PLT release over a period of 6-8 days\textsuperscript{26}. Moreover, the inability to reproduce the BM microenvironment ex vivo, and resolve physiological proPLT extension and release by high-resolution live-cell microscopy, has significantly hampered efforts to study the cytoskeletal and signaling mechanics of PLT production. Biomimetic platforms that model human BM are needed to support drug development and establish new treatments for thrombocytopenia.

This manuscript describes the development of a scalable, third-generation, human induced pluripotent stem cell-derived MK-compatible PLT bioreactor that employs biologically-inspired engineering to fully integrate the major chemical and physical components of the BM stroma under high-resolution live-cell microscopy. This work constitutes a major advance in PLT biology by providing a window into the complex physiology of thrombopoiesis, and a biomimetic platform to elaborate the physiological drivers of proPLT production, accelerate PLT release, and generate a donor-independent source of functional human PLTs for infusion.

Methods:

**Microfluidic Bioreactor Design and Fabrication.** To ensure efficient gas exchange and support high-resolution live-cell microscopy during cell culture, microfluidic bioreactors were constructed from transparent polydimethylsiloxane (PDMS, a cell-inert silicon-based organic polymer)\textsuperscript{27} bonded to glass slides. The microfluidic bioreactor consists of two inlet channels containing passive filters used to trap air bubbles and dust, followed by fluid resistors used to dampen fluctuations in flow rate arising during chip operation. The inlet channels meet in a central channel 1300 µm long and 130 µm wide, separated by a series of columns (10 µm wide
and 90 µm long) spaced 2 µm apart. For the purposes of clarity the “upper channel” should be construed to represent the top-most channel in the original bioreactor (Fig. 1A-C) and the central channel in the scaled bioreactor (Fig. 1D). Likewise, the “lower channel” should be construed to represent the bottom-most channel in the original bioreactor (Fig. 1A-C) and the side channels in the scaled bioreactor (Fig. 1D). Primary MKs become trapped in these gaps while moving from the upper channel into the lower channel. Two outlet channels collect the effluent.

Rectangular microfluidic channels 30 µm deep for mFLC-MKs and 10 µm deep for hiPSC-MKs were fabricated using soft lithography as previously described28. AutoDesk software in AutoCAD was used to design the desired 2D pattern and printed on a photolithography chrome mask. The silicon wafer (University Wafers, Boston, MA) was spin coated with SU-8 3025 photoresist (Michrochem, Newton, MA) to a 30 µm film thickness (Laurell Technologies, North Wales, PA), baked at 65°C for 1 minute and 95°C for 5 minutes, and exposed to UV light (~10 mJ·cm²) through the chrome mask for 30 seconds. The unbound SU-8 photoresist was removed by submerging the substrate into propylene glycol monomethyl ether acetate for 7 minutes. PDMS was poured onto the patterned side of the silicon wafer, degassed, and cross-linked at 65°C for ~12 hours. After curing, the PDMS layer was peeled off the mold and inlet/outlet holes were punched with a 0.75 mm diameter biopsy punch. The microchannels were sealed by bonding the PDMS slab to a glass coverslide (#1.5, 0.17x22x50 mm, Dow Corning, Seneffe, Belgium) following treatment with oxygen plasma (PlasmaPrep 2, GaLa Instrumente GmbH, Bad Schwalbach, Germany). Samples were infused into the microfluidic bioreactor via PE/2 tubing (Scientific Commodities, Lake Havasu City, AZ) using 1-mL syringes equipped with 27-gauge needles (Beckton Dickinson, Franklin Lakes, NJ). Flow rates of liquids were controlled by syringe pumps (PHD 2000, Harvard Apparatus, Holliston, MA).
**Microfluidic Bioreactor Operation.** Bioreactors were coated with a 0.22 µm filtered (Millipore, Billerica, MA) 10% BSA solution for 30 minutes to prevent direct cell contact with glass. Primary MKs and media were infused in the top-left and bottom-left inlets, respectively, at a rate of 12.5 µL/hour using a two-syringe microfluidic pump (Harvard Apparatus, Holliston, MA). When the top-right outlet is closed, both input solutions are redirected out the bottom-right outlet causing primary MKs to trap in the gaps separating the two middle channels.

**Results:**

**PLT bioreactor models the physiological characteristics of human BM.** PLT bioreactors were designed to recapitulate the dimensions of human venules in the BM (Fig. 1A), and are comprised of upper and lower microfluidic channels separated by a series of columns spaced 2 µm apart (Fig. 1B-C) to model proPLT extension through gaps in vascular endothelium under controlled flow conditions. To scale PLT production the bioreactor was lengthened and upper and lower microfluidic channels were mirrored as outlined in Figure 1D. Precise bioreactor dimensions are outlined in the supplementary material.

Our bioreactor was designed to support integration of micro-channel size, ECM composition, BM stiffness, endothelial cell contact, and hemodynamic vascular shear stress within a single platform device. The upper and lower channels can be selectively coated with ECM proteins to reproduce the biochemical composition of the BM and blood vessel microenvironments (Fig. 2A). By blocking the upper right channel output to direct flow across the bioreactor, primary mouse MKs infused along the top channel become sequentially trapped between the columns and extend proPLTs into the lower channel (Fig. 2B), recapitulating physiological proPLT extension (white arrow) . To model 3D ECM organization and
physiological BM stiffness (250 Pa)\(^3\) (Figs. S1A-D), primary mouse MKs were infused in a 1% alginate or 3 mg/mL growth factor reduced matrigel solution. Alginates are naturally-derived polysaccharides that are cell-inert and can be modified with RGD-containing cell adhesion ligands to specifically reproduce MK-matrix interactions in the BM, such as GPIIbIIIa receptor binding to fibrinogen\(^3\). Matrigel is a solubilized basement membrane preparation that is comprised primarily of laminin and collagen type IV, which are both known agonists of proPLT production, and is preferred over collagen type I-based hydrogels, which is a known inhibitor of proPLT production\(^1\)\(^9\)\(^2\)\(^0\). Hydrogels were polymerized within the microfluidic bioreactor, selectively embedding the MKs in a 3D gel within the upper channel while retaining vascular flow in the lower channel (0.02 \(\mu\)m fluorescent bead streaking, FITC-dextran fluorescence, Fig. S1C). MK distance from the lower channel could be tightly controlled by regulating infusion rate and starting/stoping flow on the microfluidic pump (Figs. S1A/B). Alginate did not inhibit proPLT production (Fig. S1E). To further reproduce BM blood vessel physiology HUVECs were selectively seeded on fibronectin (Fn) in the lower channel, and grown to confluency (Fig. 2C). HUVECs were positive for CD31 (biomarker) and DAF-2 DA (Nitric Oxide, data not shown). MK trapping, BM stiffness, ECM composition, micro-channel size, hemodynamic vascular shear stress, and endothelial cell contacts can be combined to reproduce the BM vascular niche in vitro, as is represented with primary mouse MKs in Figure 2D.

In the BM, MKs extend proPLTs into sinusoidal blood vessels 20-60 \(\mu\)m in diameter (comparable in size to small arterioles) where they are thought to experience low wall shear rates of \(~100\ s^{-1}\)\(^3\)\(^5\)\(^-\)\(^7\). To reproduce vascular shear stress of BM sinusoids, MKs were infused in the upper channel of the scaled microfluidic bioreactor and flow was directed across the scaled PLT bioreactor. MK behavior was monitored by 10x-150x magnification, high-resolution live-cell
microscopy, and released PLTs were collected from the effluent. Shear rates were characterized by computational fluid dynamics within the central region of the scaled microfluidic bioreactor (Fig. 2E), and were tightly controlled using two syringe pumps (one for each channel). Shear rates within the bioreactor were linearly proportional to infusion rates and were adjusted to span well below and above the physiological range (Fig. 2F, 100-2500 s⁻¹)³⁸. Peaks in this figure represent sequential gap junctions through which shear is highest. MKs infused in the upper channel occupy each gap sequentially, beginning with the furthest gap (position closest to the output channel) and moving inward toward the inlet channel. Local shear stress was determined by multiplying the shear rate by the media viscosity at 37°C (1.2 mPa·s, which is equivalent to human plasma). While shear rates at empty gaps increased with distance from the inlet channel, on MK trapping flow is redirected through the next available gap such that individual MKs continued to experience physiological (912-936 mPa) shear stresses at their site of trapping. This effect is independent of number of blocked sites and ~1/3rd of gaps remained open throughout the course of the experiment (Fig. 2G, S2).

**Vascular shear stress triggers proPLT production, physiological extension, and release.** In vivo, BM MKs extend proPLTs in the direction of blood flow and release PLTs, proPLTs, large cytoplasmic fragments (prePLTs), and even whole MKs into sinusoidal blood vessels¹⁵. These cells may become trapped in the pulmonary microvascular bed¹⁶,³⁹, and are believed to mature in the circulation⁴⁰. To determine the effect of physiological shear stress on PLT production, mouse fetal liver culture-derived (mFLC) MKs (hereafter described as ‘primary mouse MKs’) were isolated on culture day 4 and characterized by size and ploidy before being infused into the bioreactor (Fig. 3A, S3A/B). One of the major challenges in producing transfuseable PLTs in
vitro has been identifying factors that trigger proPLT production. Under static conditions primary mouse MKs begin producing proPLTs ~6 hours post-isolation, and reach maximal proPLT production at 18 hours (Fig. 3B). By comparison, primary mouse MKs under physiological shear stress (~600 mPa) began producing proPLTs within seconds of trapping, reaching maximal proPLT production and bioreactor saturation within the first 2 hours of culture (Fig. 3C). Primary mouse MKs cultured under physiological shear stress produced fewer, longer proPLTs that were less highly branched relative to static cultures (Mov. S1 and S2). ProPLTs in shear cultures were uniformly extended into the lower channel and aligned in the direction of flow against the vascular channel wall, recapitulating physiological proPLT production (Mov. S2). The percent of proPLT-producing primary mouse MKs under physiological shear stress were doubled over static cultures to ~90% (Fig. 3D).

ProPLTs are the assembly lines of PLT production, and another major challenge in generating clinical numbers of PLTs for infusion has been that in vitro cultures extend proPLTs at a significantly slower rate than what has been observed in vivo. Application of physiological shear stress in our microfluidic bioreactor increased the proPLT extension rate by an order of magnitude above static culture controls (Fig. S6I) to ~30 µm/min (Fig. 3E), which agree with physiological estimates of proPLT extension rates from intravital microscopy studies in living mice and support increased PLT production in vitro.

Early histological studies in both humans and mice have predicted that whole MKs, as well as MK fragments may be squeezing through gaps or fenestrations in the vascular endothelium lining BM blood vessels to trap in the pulmonary circulatory bed. Large PLT intermediates called prePLTs were recently discovered in blood, and venous infusion of mBM- and FLC-derived MKs and prePLTs into mice produced PLTs in vivo. In the present study,
≥100 µm-diameter primary mouse MKs were routinely observed squeezing through 2-µm gaps (Fig. 4A, Mov. S3A/B), or extending large MK fragments (Fig. 4B, Mov. S4), supporting a model of vascular PLT production. While rarely observed under static conditions, abscission events were routinely captured by high-resolution live-cell microscopy and occurred at variable positions along the proPLT shaft, releasing both prePLT-sized intermediates (3-10 µm diameter) (Mov. S5) and PLTs (1.5-3 µm diameter) (Fig. 4C). Figure 4D and Movie S6 represent an earlier bioreactor design in which gaps are spaced 45 µm apart. Following each abscission (yellow arrow), the resulting proPLT end formed a new PLT-sized swelling at the tip, which was subsequently extended and released, repeating the cycle (Fig. 4E, Mov. S7).

While shear stress was kept constant, proPLT extension rates varied at different positions along the shaft, predictive of a regulated cytoskeletal driven mechanism of proPLT elongation. While these studies were performed with primary mouse MKs, this was also true of hiPSC-MKs (Fig. 4C, hiPSC-MK culture shown). Increasing shear stress within the physiological range did not affect the median proPLT extension rate or the distribution of proPLT extension rates in primary MK culture (Fig. 4F), and proPLT projections in primary mouse MKs retrovirally transduced to express GFP-β1 tubulin were comprised of peripheral MTs that formed coils at the PLT-sized ends (Fig. 4G, Mov. S8). ProPLTs reached lengths exceeding 5 mm (Fig. S4A), and resisted shear stress up to 1200 mPa in vitro; recapitulating physiological examples of proPLT production from intravital microscopy11, and demonstrating that abscission events were not caused by membrane tethering. To confirm that shear stress-induced proPLT extension was cytoskeletal-driven, primary mouse MKs were incubated with 5 µM Jasplakinolide (Jas, actin stabilizer) or 1 mM erythro-9-(3-[2-hydroxynonyl] (EHNA, cytoplasmic dynein inhibitor) prior to infusion into the microfluidic bioreactor. Inhibition of cytoskeletal reorganization significantly
reduced shear stress-induced proPLT production from 90% to 12% (Jas) and 26% (EHNA), respectively (Fig. 4H/I, Mov. S9/10), and inhibited proPLT extension under static culture conditions (Fig. S4B). These studies validate the use of our PLT bioreactor as a platform technology to study physiological proPLT production under vascular shear stress.

Bioreactor-derived PLTs manifest structural and functional properties of blood PLTs. To establish PLT yield, biomarker expression, and forward/side scatter and relative concentration of glycoprotein (GP) IX+ primary mouse MKs were measured by flow cytometry immediately before infusion into the bioreactor on culture day 4 (Fig. 5A). Effluent was collected from the bioreactor 2 hours post infusion and compared to primary mouse MK input (Fig. 5B). Input MKs and effluent PLTs both expressed GP IX and IIbIIIa on their surface, and displayed characteristic forward/side scatter. The application of shear stress shifted the cellular composition of the effluent toward more PLT-sized GPIX+ cells relative to static culture supernatant isolated on culture day 5 (Fig. 5C). 85±1% of primary mouse MKs were converted into PLTs over 2 hours, which agreed with our quantitation of percent proPLT production (Fig. 3D) and constitutes a significant improvement over static cultures (Fig. 5D). Continuous media perfusion at ~500 s⁻¹ over 2 hours in our microfluidic bioreactor yielded an additional 24 ±7 PLTs per MK over static primary mouse MK culture PLT yields (18 PLTs/MK, Fig. S3C); more than doubling PLT production rates over static cultures, and resulting in a total PLT yield of ~42 PLTs per MK from 5.7x10⁴ ±3.4 x10⁴ MKs per mL. This constitutes a major advance in PLT production rate over existing culture approaches that generate comparable PLT numbers over a longer period of time (6-8 days).⁴³,⁴⁴.
To quantify the morphological composition of our product, the bioreactor effluent was probed for β1 tubulin (PLT-specific tubulin isoform) and Hoechst (nuclear dye), and analyzed by immunofluorescence microscopy (Fig. S5A). Cells were binned according to their morphology and size, (Fig. S5B); and compared to static primary mouse MK culture supernatants. The application of shear stress shifted the cellular composition of the effluent toward more PLT-sized β1 tubulin⁺ Hoechst⁻ cells (Fig. 5E), which agreed with flow cytometry data (Fig. 5C) and resulted in a product that was more similar in composition to the distribution of PLT intermediates in whole blood⁴⁰. Quantitation of free nuclei in the effluent (Fig. 5E, insert) confirmed increased bioreactor-mediated PLT production versus static culture and established PLT yields of ~20 ±12 PLTs per MK above static culture yields, which agree with flow cytometry data.

Bioreactor-PLTs were ultrastructurally indistinguishable from mouse blood PLTs by electron microscopy; and contained a cortical MT coil, open canalicular system, dense tubular system, mitochondria, alpha- and dense-granules (Fig. 5F). Bioreactor-PLTs and PLT intermediates displayed comparable MT and actin organization to mouse blood PLTs by immunofluorescence microscopy (Fig. 5G), and spread normally on contact activation with glass, forming both filpodia and lamellipodia (Fig. 5H, Mov. S11).

**Application of bioreactor to human PLT production.** Can PLT production be recapitulated with human MKs? To generate human PLTs, primary mouse MKs in our bioreactor were replaced with hiPSC-derived MKs, which (unlike CD34⁺ human cord blood- or embryonic stem cell-derived MKs) provide a virtually unlimited source of MKs for infusion. hiPSC-MKs were isolated on culture day 15, once they had reached maximal diameter (20-60 µm) (Fig. 6A), and
were ultrastructurally similar to primary human MKs (Fig. 6B). The detailed generation and characterization of these hiPSC-MKs is outlined in another manuscript (submitted, Feng et al. Nature Methods, 2014). In static culture, our hiPSC-MKs began producing proPLTs at 6 hours post-isolation, and reached maximal proPLT production at 18 hours (Fig. 6C). By comparison, hiPSC-MKs under physiological shear stress (~600 mPa) began producing proPLTs immediately upon trapping, and extended/released proPLTs within the first 2 hours of culture (Fig. 6D, Mov. S12-14). Percent proPLT-producing hiPSC-MKs under shear stress were increased significantly (~90% of total MKs) over static cultures (~10%) (Fig. 6E). ProPLT extension rates were slightly lower than primary mouse MKs (~19 µm/min versus 30 µm/min) (Fig. 6F) and more closely approximated physiological controls11(Fig. S6I). hiPSC-MK proPLT production and PLT release was otherwise morphologically identical to primary mouse MKs. hiPSC-MK-derived bioreactor-PLTs displayed forward and side scatter, and surface biomarker expression characteristic of human blood PLTs (Fig. 6G), were ultrastructurally indistinguishable from human blood PLTs by electron microscopy 45(Fig. 6H), were anucleate and displayed comparable morphology and MT expression to human blood PLTs by immunofluorescence microscopy (Fig. 6I), and spread normally upon contact-activation with glass, forming both filpodia and lamellipodia (Fig. 6J). Continuous media perfusion at ~500 s⁻¹ over 2 hours in our microfluidic bioreactor yielded a total of ~30 PLTs per MK from 1.9x10⁴ ±1.3x10⁴ MKs per mL (Fig. S3D). Taken together these data demonstrate that hiPSC-MKs can be applied to our biomimetic microfluidic bioreactor to model physiological human PLT production and generate potentially unlimited numbers of functional human PLTs for infusion. Representative data for mouse and human platelet controls are included in Supplementary Figure 6.
Discussion:

This study capitalizes on a novel microfluidic design to recapitulate human BM and blood vessel physiology ex vivo, to dramatically and significantly improve the rate of platelet production over static cultures, thereby paving the way for additional improvements in platelet yield with the ultimate goal of generating a donor-independent source of functional human platelets for infusion. Two major quantitative roadblocks persist in the development of donor-independent PLTs for therapeutic use: (1) generating sufficient numbers (~3x10^8) of human MKs to support the production of one PLT transfusion unit (~3x10^{11} PLTs), and (2) generating physiological numbers of functional human PLTs (~10^3) per MK. The development of human embryonic stem cell cultures (hESC)\textsuperscript{44,46}, and more recently, human induced pluripotent stem cell cultures (hiPSC)\textsuperscript{47}(submitted, Feng et al. Nature Methods, 2014), offer a physiologically relevant and potentially unlimited source of progenitor cells that can be differentiated into human MKs in vitro to address the first quantitative roadblock\textsuperscript{48}. Indeed, because PLTs are anucleate, PLT bioreactor-derived units could be irradiated prior to infusion, addressing concerns that cellular products derived from hESC or hiPSCs could be oncogenic or teratogenic\textsuperscript{49}.

Nevertheless, attempts to study the environmental drivers of PLT production have fallen short. A major limitation of 2D liquid cultures has been their inability to account for 3D BM composition and stiffness, directionality of proPLT extension, and proximity to venous endothelium\textsuperscript{15,24,25}. Alternatively, intravital microscopy studies have provided physiologically accurate examples of proPLT production, however poor resolution and limited control of the microenvironment has prohibited detailed study of how the BM microenvironment contributes to PLT release\textsuperscript{11,15}. Nakagawa et al. recently published three bioreactor designs to produce human PLTs at scale from hiPSC- and ESC-derived MKs \textsuperscript{50}. While all three designs intend to use flow...
to trigger PLT production, direct visualization of proPLT production is severely restricted, shear rates are not modeled, and the profile of flow within the devices is unknown. Although there are superficial similarities to our bioreactor, there are major limitations to all three of Nakagawa’s bioreactor designs that are worth noting. Bioreactor Design 1 traps MKs against HUVECs within 23 µm-diameter porous channels. While the authors do not show evidence of the HUVECs within the bioreactor and it is unclear whether HUVECs are confluent, viable, or necessary for proPLT production in their bioreactor system, HUVECs are undesirable in a PLT product, and may constitute a significant transfusion risk. PLT purity and yield from this device is not reported. Bioreactor Design 2 reduces pore size to 8 µm and removes HUVEC co-culture but achieves marginal (<1.5-fold) improvements in PLT yield over static culture for two hESC-MKs replicates. Bioreactor Design 3 decreases pore size further to 4 µm and infuses MKs at a 60°-angle to lateral flow. Nakagawa et al. report 71,755 PLTs from 120,000 hESC-MKs, constituting a total PLT yield of 0.59 PLTs per MK. hiPSC-MK PLT yields are not reported. Neither bioreactor supports visualization of live PLT production at high resolution in real-time to resolve the effect of physiological stimuli on PLT formation, and PLT characterization is significantly limited.

Mounting evidence that cell-cell contacts, ECM composition, and stiffness, vascular shear stress, pO2/pH, soluble factor interactions, and temperature contribute to proPLT formation and PLT release have suggested that recapitulating key components of BM and blood vessel microenvironments within a 3D microfluidic culture system is necessary to achieve clinically significant numbers of functional human PLTs. Our microfluidic bioreactor design expands control and resolution of the microenvironment, and has allowed us to drastically improve time to PLT release (2 from 18 hours) and more than double
PLT yield. Moreover, application of vascular shear stress within our microfluidic bioreactor increased proPLT production (90% from 10%), and reproduced physiological proPLT extension and release. This study demonstrates the minimum system components required to support efficient and reproducible human platelet production in a commercial setting, and represents the critical next step in creating an alternative source of functional human platelets for infusion.

Acknowledgements: This work was supported in part by the BRI Translational Technologies and Innovation grant and National Institutes of Health grant R01HL68130 (J.E.I.). J.E.I. is an American Society of Hematology Junior Faculty Scholar. J.N.T. is an American Society of Hematology Scholar. L.M. is supported by the Marie Curie Actions – International Outgoing Fellowship (300121). We acknowledge the staff at Advanced Cell Technologies for access to their hiPSC- -derived MKs; Drs. Byungwook Ahn and Wilbur Lam at Emory University for their advice and support with the endothelialization of our PLT bioreactors; and Drs. Markus Bender and John Hartwig at Harvard Medical School for GFP-β1 tubulin viral supernatants.

Author contributions: J.N.T. designed and performed the experiments, analyzed the data and wrote the manuscript; L.M. and D.A.W. designed and fabricated the microfluidic bioreactors and contributed to writing the manuscript; S.W. performed many of the microfluidics, microscopy, and flow cytometry experiments. J.L.S. and K.B.N. performed the vascular shear rate modeling; A.E. performed the BM stiffness modeling. K.R.M. performed the GFP-β1 tubulin retroviral transfection; Q.F., S.L., and R.L. designed and performed the human induced pluripotent stem cell cultures; J.E.I. helped design the experiments, analyze the data, and contributed to writing the manuscript.
Disclosure of Conflicts of Interest: Q.F., S.L., and R.L. are employees of Advanced Cell Technology, a biotechnology company in the field of stem cells and regenerative medicine. J.N.T., L.M., and J.E.I. are founders of, and have financial interest in, Platelet BioGenesis, a company that aims to produce donor-independent human platelets from human induced pluripotent stem cells at scale. J.N.T., L.M., and J.E.I. are inventors on this I.P. J.N.T. and J.E.I.’s interests were reviewed and are managed by the Brigham and Women’s Hospital and Partners HealthCare in accordance with their conflict of interest policies. L.M.’s interests are managed by Harvard University in accordance with their conflict of interest policies. The remaining authors declare no competing financial interests.

References:


**Figure Legends:**

**Fig. 1.** PLT bioreactor design. (A) PLT bioreactors are based on custom-built polydimethylsiloxane (silicon-based organic polymer) bonded to glass slides, and are comprised of an upper and lower microfluidic channel separated by a series of columns. (B) 2 µm gaps separate columns to trap MKs entering the upper channel from crossing into the lower channel upon fluid withdrawal from the outlet of the lower channel. Scale bar for B is 50 µm. (C) Scanning electron micrograph of bioreactor central channels. Scale bar for C is 5 µm. (D) Scaled bioreactor design showing typical device operation.

**Fig. 2.** PLT bioreactor models major components of BM. Primary mouse MKs are shown. (A) The upper and lower channels can be selectively coated with ECM proteins to reproduce osteoblastic and vascular niche composition. (B) MKs trap at gaps and extend proPLTs into the lower channel (white arrow). MKs can be selectively embedded in alginate or matrigel gels, modeling 3D ECM organization and physiological BM stiffness (250 Pa). Vascular flow is retained in the lower channel as demonstrated by 0.02 µm fluorescent bead streaking and FITC-dextran fluorescence. (C) HUVECs can be selectively cultured in ECM-coated channels to reproduce blood vessel physiology. (D) MK trapping, BM stiffness, ECM composition, micro-channel size, hemodynamic vascular shear stress, and endothelial cell contacts can be combined to reproduce human BM *in vitro*. (E) Shear rate distribution along the length of the channel. Arrows indicate the magnitude and direction of the velocity field. (F) Fluid shear rates are well characterized, and can be tightly regulated across the bioreactor as a function of flow rate. (G) Regardless of the number of occupied slits, trapped MKs experience physiological shear stresses at gap junctions. Media viscosity is 1.20 mPa·s. Scale bars are 50 µm.
**Fig. 3.** Physiological shear stress induces proPLT production in primary mouse MKs. (A) Primary mouse MKs range between 20-85 µm in diameter on culture day 4, and become larger (40-100 µm) if they do not form proPLTs. (B) MKs in static culture begin producing proPLTs at 6 hours post-purification, and reach maximal proPLT production at 18 hours. (C) Primary mouse MKs under physiological shear stress (~600 mPa) begin producing proPLTs immediately upon trapping and extend/release proPLTs within the first 2 hours of culture. (D) Percent proPLT-producing primary mouse MKs under physiological shear stress are increased significantly to ~90% over static cultures (~50%)\(^2\). (E) ProPLT extension rates under physiological shear stress are increased significantly to ~30 µm/min over static cultures (0.85 µm/min)\(^4\). Scale bars are 50 µm.

**Fig. 4.** Shear stress-mediated proPLT extension is a cytoskeleton-driven process. (A) ~100 µm diameter primary mouse MK squeezing through 2 µm gap. (B) Release of large MK fragment from primary mouse MK into the lower channel resulting in prePLT formation. (C) ProPLT extension rates vary at different positions along the shaft, predictive of a regulated cytoskeletal driven process. hiPSC-derived MKs are shown. (D) Individual release events (yellow arrow) are routinely captured by high-resolution live-cell microscopy at different positions along the proPLT shaft. White arrow denotes proPLT end. Scale bars for A-D are 50 µm. Primary mouse MKs are shown in an earlier bioreactor design in which gaps are spaced 45 µm apart. (E) PLTs form at new proPLT ends following each abscission event (yellow arrow). Primary mouse MKs are shown. (F) Increasing shear stress from 100 to 1000 s\(^{-1}\) does not increase proPLT extension rate in primary mouse MKs. Data is represented as a box-and-whisker plot where light grey indicates the upper quartile and dark grey indicates the lower quartile. (G) Primary mouse MKs
retrovirally transduced to express GFP-β1 tubulin show proPLT extensions are comprised of peripheral MTs that form coils at the PLT-sized ends. (H) 5 µM Jasplakinolide (Jas, actin stabilizer) and 1 mM erythro-9-(3-[2-hydroxynonyl] (EHNA, cytoplasmic dynein inhibitor) inhibit shear stress-induced proPLT production in primary mouse MKs. (I) Representative images of drug-induced inhibition of proPLT production under physiological shear stress from Fig. 4H. Scale bar for E is 5 µm.

**Fig. 5.** Bioreactor-derived mPLTs manifest structural and functional properties of mouse blood PLTs. (A) Biomarker expression, and forward/side scatter and relative concentration of GPIIX+ primary mouse MKs infused into bioreactor following isolation on culture day 4, and (B) effluent collected from bioreactor 2 hours post infusion. (C) Application of shear stress shifts GPIIX+ product toward more PLT-sized cells relative to static culture supernatant. (D) Comparison of primary mouse MK culture product under static and bioreactor conditions over a period of 2 hours. (E) Application of shear stress shifts product toward more PLT-sized β1 tubulin+ Hoechst− cells (labeled ‘Platelet’) relative to static culture supernatant. Insert shows quantitation of free nuclei in effluent. (F) Bioreactor-mPLTs are ultrastructurally similar to mouse blood PLTs and contain a cortical MT coil, open canalicular system, dense tubular system, mitochondria, and characteristic secretory granules. (G) Bioreactor-mPLTs and PLT intermediates are morphologically similar to mouse blood PLTs (not shown) and display comparable MT and actin expression. (H) Bioreactor-mPLTs form filopodia/lamellipodia on activation and spread on glass surface. Scale bars for D, G and H are 5 µm. Scale bar for F is 1 µm.
Fig. 6. Bioreactor-derived hiPSC-PLTs manifest structural and functional properties of human blood PLTs. (A) hiPSC-MKs reach maximal diameter (20-60 µm) on culture day 15. (B) hiPSC-MKs are ultrastructurally similar to primary human MKs and contain a lobulated nuclei, invaginated membrane system, glycogen stores, organelles, and characteristic secretory granules. Scale bar for B is 1 µm. (C) hiPSC-MKs in static culture begin producing proPLTs at 6 hours post-purification, and reach maximal proPLT production at 18 hours. (D) hiPSC-MKs under physiological shear stress (~600 mPa) begin producing proPLTs immediately upon trapping and extend/release proPLTs within the first 2 hours of culture. Insert shows multiple PLT-sized swellings denoted by white arrows along single proPLT extension. Scale bars for C and D are 50 µm. (E) Percent proPLT-producing hiPSC-MKs under physiological shear stress are increased significantly to ~90% over static cultures (~10%). (F) ProPLT extension rates under physiological shear stress are ~19 µm/min. Data is represented as a box-and-whisker plot where light grey indicates the upper quartile and dark grey indicates the lower quartile. (G) Bioreactor-hPLTs display forward and side scatter, and surface biomarker expression characteristic of human blood PLTs. (H) Bioreactor-hPLTs are ultrastructurally similar to human blood PLTs and contain a cortical MT coil, open canalicular system, dense tubular system, mitochondria, and characteristic secretory granules. Top-right insert shows peripheral MT coil. Scale bar for H is 0.5 µm. (I) Bioreactor-hPLTs are anucleate, morphologically similar to human blood PLTs, and display comparable MT expression. (J) Bioreactor-mPLTs form filopodia/lamellipodia on activation and spread on glass surface. Scale bars for I and J are 5 µm.
Figure 1

A. [Diagram with labels: Input, Center, Output]

B. [Image with measurements: 130 μm, 1300 μm, 10 μm width, 50 μm length, 2 μm gaps]

C. [Image with a scale bar: 30 μm]

D. [Diagram with numbered steps:]

1. Megakaryocytes are generated in the lab and fed into the upper channel (central channel in the scaled bioreactor). The upper effluent channel (central effluent channel in the scaled bioreactor) is blocked to direct flow through column gaps into the lower channel (side channels in the scaled bioreactor).

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

3. Proplatelets are extended as megakaryocytes “filter” through the barrier.

4. Proplatelets transform into platelets in vascular channels and are collected for human infusion.
Figure 2

A. Extracellular Matrix Composition
- Pre-Coating
- FITC-Fg
- Rhod-Fn
- FITC-Matrigel

B. Megakaryocyte Trapping
- No Gel
- Alginate
- Matrigel
- 0.02 μm beads
- Cell Tracker Red
- Hoechst
- FITC-Dextran

C. Endothelialization
- Matrigel
- Rhod-Fn
- FITC-Dextran
- CMFDA
- Hoechst

D. Complete System
- Matrigel
- Cell Tracker Red
- Hoechst
- HUVEC

E. 12.5 μL/hr Infusion
15 μm height (z-axis)

F. Shear rate (s⁻¹)

G. 12.5 μL/hr Infusion
Number of blocked slits
Figure 3

A.

B.

C.

D.

E.
Figure 5

A. 

B. 

C. 

D. Static Culture (2 hrs)  

E. Percent Composition of Product (%)  

F. 

G. 

H. 

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Platelet bioreactor-on-a-chip