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Packaging functionally-important plasma proteins into the α -granules of human induced pluripotent stem cell-derived megakaryocytes

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Abstract

The contents of platelet α -granules arrive via a number of pathways; some are synthesized by megakaryocytes (MKs), e.g., von Willebrand Factor (VWF), while others are endocytosed from plasma, e.g., fibrinogen (Fgn), Factor V (FV). Currently, almost all *in vitro* iPSC-derived MKs are generated under serum-free conditions, their α -granule cargo lack components that would normally be taken up from plasma during the course of megakaryopoiesis. How this might affect the ability of *in vitro*-derived platelets to contribute fully to hemostasis is not known. The purpose of this investigation was to examine whether “feeding” human plasma to iPSC-derived MKs might result in loading their α -granules with physiologically important proteins. iPSCs were differentiated to CD41⁺/CD42b⁺ MKs using a serum-free protocol. The resulting MKs were polyploid, expressed a number of platelet-specific surface receptors and spread on Fgn or collagen-coated surfaces. RT-PCR analysis detected mRNA transcripts for FV and VWF, but not Fgn chains. Fluorescence immunocytochemistry and confocal microscopy confirmed constitutive VWF distribution in granule-like structures in MKs cultured under plasma-free conditions, and the granules became positive for Fgn upon incubation with human plasma. iPSC-derived MKs showed a low level of constitutive FV expression that increased dramatically upon incubation with human plasma. Taken together, these data suggest that human iPSC-derived MKs are capable of endocytosing and storing plasma components in their α -granules. Incorporating this methodology into current protocols for producing *in vitro*-derived MKs should provide novel insights into MK biology and lead to the generation of large numbers of MKs and platelets with improved functionality.

Keywords

iPSCs; megakaryocytes; α -granule; fibrinogen; factor V

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AUTHOR CONTRIBUTION

N.Z. and P.J.N. designed the study and wrote the manuscript. N.Z. conducted experiments and analyzed data.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

1 | INTRODUCTION

In addition to their essential role in primary hemostasis and thrombosis, platelets also play important roles in other pathophysiological processes, including inflammation, innate immunity, angiogenesis, wound healing and cancer metastasis (Mancuso & Santagostino, 2017). These phenomena are often mediated through the release of their granular contents, mostly from dense granules and α -granules. Platelet granules are formed in their precursor cells, termed megakaryocytes (MKs), prior to transport into platelets during the maturation process. Dense granules contain small molecules, such as calcium, polyphosphates, adenosine diphosphate (ADP), adenosine triphosphate (ATP) and serotonin that are important for recruiting platelets and augmenting coagulation. α -granules are the most abundant platelet granule, and store several hundred different proteins with diverse functions, including coagulation factors such as factors V (FV), XI and XIII, adhesion molecules such as von Willebrand factor (VWF) and fibrinogen (Fgn), immunologic molecules such as immunoglobulins and complement factors, chemokines such as platelet factor IV (PF4) and β -thromboglobulin, and growth factors such as bFGF, VEGF, IGF-1 and TGF- β (Burnouf, Strunk, Koh, & Schallmoser, 2016; Maynard, Heijnen, Horne, White, & Gahl, 2007). Some of the α -granule contents, such as PF4, thrombospondin-1 (TSP-1) and VWF, are synthesized by translation of mRNAs present in MKs and packaged into granules during biosynthesis (Cramer et al., 1989). Other proteins, present in large amount in the plasma, are endocytosed and incorporated into α -granules either through a receptor-independent pathway, such as albumin and IgG (George, 1990; P. J. Handagama, Shuman, & Bainton, 1989), or through a receptor-dependent pathway like Fgn and FV (Bouchard et al., 2008; Bouchard, Williams, Meisler, Long, & Tracy, 2005; Camire, Pollak, Kaushansky, & Tracy, 1998; Cramer et al., 1989; P. Handagama, Scarborough, Shuman, & Bainton, 1993; Harrison et al., 1989; Rendu et al., 1985).

Recently, rapid progress has been made in the *in vitro* generation of MKs and platelets from induced pluripotent stem cells (iPSCs). This field has been driven, in part, by the possibility that specialty units of *in vitro*-generated platelets might be used to complement donor-derived platelets clinically. However, generating sufficient numbers of fully functional MKs and platelets remains a major challenge in the field. Currently, iPSCs are considered an optimal source for large-scale *in vitro* MK and platelet production because they offer the advantages of unlimited expansion in culture, are amenable to genetic manipulation, and can be generated from somatic cells of any individual. Several protocols have been established in the literature for generating MKs from human iPSCs. Some groups developed directed differentiation procedures by sequentially providing external cytokines and growth factors in the culture to mimic embryonic development *in vitro* (Borger et al., 2016; Eicke et al., 2018; Feng et al., 2014; Liu et al., 2015; Mills, Paluru, Weiss, Gadue, & French, 2014). Nakamura et al. generated immortalized MK progenitor cell lines (imMKCLs) by sequentially introducing genes encoding MYC and BMI1, followed by BCL-XL into human iPSC-derived hematopoietic progenitor cells (Nakamura et al., 2014). The resulting imMKCLs were expanded in culture over five months to produce functional platelets when the transgenes were turned off. Most recently, imMKCLs have been cultured in a turbulent flow-based bioreactor to generate > 100 billion platelets in an eight liter culture (Ito et al., 2018).

Finally, Moreau et al. established a strategy called ‘forward programming’ to generate expandable MKs from iPSCs (Moreau et al., 2016) in which lentiviral vectors were used to introduce three transcription factors: GATA1, FLI1 and TAL1 into human iPSCs to drive megakaryocyte differentiation. Forward-programmed MKs proliferate and differentiate in culture for several months to produce large numbers of MKs, as well as platelets.

Currently, almost all the *in vitro* iPSC-derived MKs are generated under human serum- and plasma-free conditions. Thus, their α -granule cargo lack components that would normally be taken up from plasma during the course of megakaryopoiesis. How this might affect the ability of *in vitro*-derived platelets to contribute fully to hemostasis is not known. The purpose of this investigation, therefore, was to examine whether “feeding” human plasma to iPSC-derived MKs might result in loading their α -granules with physiologically important proteins. We generated an iPSC line from human peripheral blood mononuclear cells (PBMCs) and differentiated the cells to CD41⁺, CD42b⁺ MKs using a serum-free, feeder-free, two-dimensional monolayer protocol. The resulting MKs showed no endogenous Fgn synthesis, and expressed only low levels of FV. Importantly, when cultured with 10% human C5-deficient plasma, the iPSC-derived MKs endocytosed Fgn and FV from the plasma and stored them in α -granules. These findings demonstrate that incorporating human plasma into current *in vitro* MK production protocols should complete generation of megakaryocytes with fully-loaded α -granule components.

2 | MATERIALS AND METHODS

2.1 Generation of iPSCs from PBMCs

Human buffy coats were obtained from the Blood Services Division of BloodCenter of Wisconsin. PBMCs were separated by density gradient centrifugation with Ficoll-paque Plus (GE Healthcare) and used for iPSC reprogramming with a nonintegrating method as described previously (Okita et al., 2013). PBMCs were transfected with an episomal vector mixture containing pCXLE-hOCT3/4-shp53, pCXLE-hSK, pCXLE-hUL and pCXWB-EBNA1 using a Nucleofector 2 device, and cultured under T-cell-stimulating conditions described previously (Okita et al., 2013).

2.2 iPS cell culture and differentiation

The human iPSC control line PBWT3–1 was a kind gift from Dr. Deborah L. French (Children’s Hospital of Philadelphia). Human iPS cells were cultured either on murine embryonic fibroblasts (MEFs) in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (DMEM/F12) supplemented with 20% knockout serum (Thermo Fisher Scientific), a non-essential amino acids solution (Corning), L-glutamine (Corning), and penicillin/streptomycin (Corning) or on Matrigel (Corning)-coated plates in mTeSR1 medium (Stemcell Technologies) at 37 °C in 4% O₂/5% CO₂. iPS cells were differentiated to HPCs as previously described (Mills et al., 2014; Paluru et al., 2014). Briefly, cells were plated on Matrigel for differentiation. The medium and cytokine changes were followed as described with the following modification. The GSK-3 β inhibitor, CHIR99021 (Tocris) (1 μ M) was used instead of Wnt3a. Cells were cultured at 37°C with 4% O₂/5% CO₂ for 9 days and loosely adherent HPCs were collected by carefully removing the supernatant. Cells were

analyzed by flow cytometry to confirm the surface expression of CD41a and CD235a. The HPCs were further differentiated to MKs in serum-free differentiation (SFD) medium: Iscove's Modified Dulbecco's Medium (IMDM) (Thermo Fisher Scientific) containing 25% Ham's F12 (Corning), 0.5% N2 (Thermo Fisher Scientific), 1% B27 without Vitamin A (Thermo Fisher Scientific), 0.05% BSA (Sigma), 2mM L-glutamine and penicillin/streptomycin supplemented with 50 ng/ml SCF (R&D systems) and 50 ng/ml TPO (R&D systems) at 37 °C, 5% CO₂ for 7 days. To load MKs with proteins present in human plasma, 10% heparinized type AB human complement C5-deficient plasma (Assaypro) was added to culture medium on day 6.

2.3 Alkaline phosphatase activity assay

iPS cells were cultured on MEFs and fixed with 4% paraformaldehyde for 2 min at room temperature. The alkaline phosphatase activity was detected using Alkaline Phosphatase Detection Kit (Millipore) according to the manufacturer's protocol.

2.4 Immunostaining and imaging

iPSCs were fixed in a culture dish with 2% paraformaldehyde for 20 min at room temperature, permeabilized with ice cold 0.5% Triton X-100 in PBS for 15 min, and blocked with 3% BSA in PBS for 15 min. Cells were incubated with Alexa Fluor 488-conjugated anti-OCT4 and Alexa Fluor 594-conjugated anti-SOX2 antibodies (Biolegend) at room temperature for 2 hours. Images were taken with a Nikon Eclipse TE200 inverted fluorescent microscope.

To monitor MK spreading, fibrillar collagen type I (50 µg/ml) or fibrinogen (25 µg/ml) were incubated on coverslips overnight at 4 °C. After washing, the coverslips were blocked with 1% BSA in PBS at room temperature for 1 hour and washed with PBS. Cultured MKs were seeded on collagen-coated coverslips for 2 hours, or fibrinogen-coated coverslips for 45 min at 37 °C. The cells were then fixed with 2% paraformaldehyde for 20 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and incubated with TRITC-phalloidin and Alexa Fluor 488-conjugated anti-vinculin antibody (Affymetrix eBioscience) at room temperature for 1 hr.

Day 7-treated MKs that had been incubated with human complement C5-deficient plasma were fixed with 2% paraformaldehyde for 20 min at room temperature before centrifugation at 500 × g for 5 min onto poly-D-lysine-coated coverslips. The cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, and double stained with mouse-anti-Fgn (Santa Cruz), sheep-anti-FV (Affinity biological, Inc.), or rabbit-anti-VWF (DAKO) antibodies at 4 °C overnight. After washing, the cells were incubated with Alexa Fluor 594-conjugated goat-anti-rabbit IgG, DyLight 488-conjugated goat-anti-mouse IgG, or DyLight 488-conjugated donkey-anti-sheep IgG (Jackson ImmunoResearch Laboratories) secondary antibodies at room temperature for 1 hour. After washing, the cells were counter stained with DAPI for 5 min, and imaged on an FV1000 laser-scanning confocal microscope (Olympus) using a 100× objective lens.

2.5 Flow cytometric analysis

Cultured cells were incubated with fluorescently-labeled antibodies for 20 min at room temperature. The antibodies used were Alexa Fluor 488-conjugated anti-TRA-1–60-R, Alexa Fluor 488-conjugated anti-SSEA-3, Alexa Fluor 647-conjugated anti-SSEA-4, FITC-conjugated anti-CD61, APC-conjugated anti-CD42b, FITC-conjugated anti-CD49b (Biolegend), and PE-conjugated anti-CD41a, FITC-conjugated anti-CD42a (BD Biosciences), and Alexa Fluor 647-conjugated anti-GPVI (11A12), as described previously (Chen, Locke, Liu, Liu, & Kahn, 2002). Flow cytometry was performed using a BD LSRII flow cytometer. Flow cytometry data were analyzed using FlowJo software (Tree Star Inc.).

2.6 Karyotyping

Karyotyping of the iPS Cells was performed by Wisconsin Diagnostic Laboratories, Milwaukee, WI.

2.7 DNA content analysis

iPSC-derived day 6 MKs were incubated with FITC-conjugated anti-CD41a (Biolegend) at room temperature for 30 min before fixing with 70% ethanol at –20°C overnight. The cells were then stained with 20 µg/ml propidium iodide (Sigma-Aldrich) and 50 µg/ml RNase A (Sigma-Aldrich) in PBS buffer at room temperature for 1 hour. Cellular DNA content was analyzed on a BD LSRII flow cytometer.

2.8 RT-PCR analysis

Total RNA was extracted from MKs or HepG2 cells using an RNeasy Plus Mini Kit (Qiagen). Reverse transcription was performed with SuperScript III First-Strand (Invitrogen). cDNA was amplified by PCR using the primers listed in Table S1. All the primer sequences are separated by introns in their respective gene; therefore, the length of the genomic DNA amplification products was readily distinguishable from that of cDNA.

3 | RESULTS

3.1 Generation of iPSCs from human PBMCs

We generated an integration-free iPSC line, termed OT1–1, from human PBMCs derived from a healthy volunteer anonymous blood donor. The cells were reprogrammed with a mixture of episomal vectors under T-cell-stimulating conditions, as described (Okita et al., 2013). The resulting OT1–1 cells displayed the characteristic morphology of pluripotent stem cells, with large nuclei and scant cytoplasm (not shown) and formed compact colonies on both MEFs (Figure 1a) and Matrigel-coated plates (not shown). The cells also expressed the intracellular pluripotent markers SOX2 and OCT4, and possessed alkaline phosphatase activity (Figure 1a). In addition, flow cytometry analysis revealed that the cells expressed the surface pluripotent markers TRA-1–60, SSEA-3 and SSEA-4 (Figure 1b), indicating that the OT1–1 cells had been fully reprogrammed. G-banding of chromosomal DNA exhibited a normal karyotype (Figure 1c). Since all the vectors in the reprogramming cocktail contain an Epstein-Barr nuclear antigen 1 (EBNA1) element, we performed PCR analysis of total DNA derived from OT1–1 cells as a reporter of residual vector sequence in cells. As shown in

Figure S1, the EBNA1 sequence was not detectable at passage 15, indicating complete loss of episomal vectors after reprogramming and the footprint-free nature of the OT1–1 iPS cell line

3.2 Characterization of iPSC-derived MKs

OT1–1 iPSCs were differentiated into MKs using a serum-free, feeder-free, adherent monolayer differentiation protocol published previously (Mills et al., 2014; Paluru et al., 2014). OT1–1 cells showed a similar differentiation pattern, including mesoderm development, intermediate differentiation into CD41/CD235 hematopoietic progenitor cells (HPCs), and terminal differentiation into CD41/CD42b MKs when compared with a well-characterized control iPSC line (PBWT3–1 – kind gift of Debra French, Children’s Hospital of Philadelphia) (Figure S2). iPSC-derived CD41a⁺ MKs showed ploidy levels ranging from 2N–16N (Figure 2a), with most cells 2–4N, suggesting that they are more primitive in nature than what is typically observed *in vivo* (Mazur, Lindquist, de Alarcon, & Cohen, 1988). The low ploidy level is consistent with *in vitro* iPSC-derived MKs previously reported by other groups (Liu et al., 2015; Moreau et al., 2016; Wang et al., 2015). As shown in Figure 2b, MKs derived from the OT1–1 iPS cell line also displayed other characteristic platelet surface markers, including CD61, CD42a, GPVI, and CD49b. CD49b expression is known to vary significantly among subjects, thus the low expression of CD49b in OT1–1-derived MKs might be the consequence of the CD49b polymorphism (Kunicki, Orzechowski, Annis, & Honda, 1993; Lagrue-Lak-Hal et al., 2001).

To test if the extracellular matrix receptors on OT1–1 iPSC-derived MKs are functional, they were incubated on collagen- or fibrinogen-coated coverslips and stained for F-actin and vinculin to visualize focal adhesions. Confocal analysis showed both collagen and fibrinogen induced cell-spreading, and displayed actin filament bundles anchored at the cell periphery as well as vinculin-containing focal adhesions (Figure 2c). Taken together, our *in vitro* iPSC-derived MKs exhibit the major characteristics of human MKs.

3.3 Human plasma treatment to load MK α -granule components

Platelet α -granule components like VWF, TSP-1 and PF4 are de novo synthesized by MKs, while others, like Fgn and FV, are endocytosed from plasma. Fgn mRNA is not present in normal human bone marrow MKs and platelets (Louache, Debili, Cramer, Breton-Gorius, & Vainchenker, 1991; Rowley et al., 2011), although it has been detected in MKs from patients with the high-grade T-cell lymphomas (Podolak-Dawidziak, Hancock, Lelchuk, Kotlarek-Haus, & Martin, 1995). Although human MKs, but not platelets, contain residual FV mRNA (Gewirtz, Shapiro, Shen, Boyd, & Colman, 1992; Rowley et al., 2011; Suehiro et al., 2005), multiple lines of evidence suggest that the vast majority of platelet-derived FV is derived via endocytosis of the plasma molecule by MKs (Bouchard et al., 2005; Camire et al., 1998; Gould et al., 2005; Suehiro et al., 2005; Thomassen et al., 2003). Because both α -granule Fgn and FV are thought to contribute to the overall hemostatic effectiveness of platelets, we sought to determine whether *in vitro* iPSC-derived MKs might constitutively express these two proteins as reporters of general plasma protein uptake. Semi-quantitative RT-PCR showed strong expression of VWF mRNA in iPSC-derived MKs, confirming the MK lineage specification during differentiation (Figure 3a). In contrast, the mRNAs encoding the

Fgn α , β , or γ chains were not detectable, indicating the lack of endogenous Fgn synthesis in iPSC-derived MKs. Relatively low levels of FV mRNA were detected in iPSC-derived MKs, consistent with previous findings in bone marrow MKs and cord blood CD34⁺ cell-derived MKs (Gewirtz et al., 1992; Suehiro et al., 2005).

To determine whether iPSC-derived MKs can take up plasma proteins and package them into their α -granules, we added normal heparinized human type AB plasma to the culture media. Unfortunately, this resulted in significant cell death (Figure S3). Heat inactivation at 56 °C for 30 minutes abolished the cytotoxic effect of human plasma (Figure S3), suggesting that the Complement system might be contributing to cell lysis. Indeed, depleting complement C5 from plasma protected the cells from death (Figure S4). Therefore, complement C5-deficient plasma was used in all future experiments.

As shown in Figure 3b, cells cultured in the absence of human plasma expressed abundant levels of VWF in a punctate pattern typical of MK, but no detectable Fgn, as expected. After one day of incubation with human C5-deficient plasma, however, cultured MKs showed Fgn immunolabeling in granule-like structures. Interestingly, Fgn and VWF exhibited only limited co-distribution, consistent with previous reports in human platelets (Italiano et al., 2008; Kamykowski, Carlton, Sehgal, & Storrie, 2011; Sehgal & Storrie, 2007). Similar to Fgn, iPSC-derived MKs cultured in the absence of added human plasma showed only a low level of constitutive FV expression (Figure 3c), consistent with the expression of low levels of FV mRNA (Figure 3a). FV immunostaining increased dramatically upon MK incubation with human plasma, and staining of FV colocalized substantially with VWF (Figure 3c). Taken together, these data demonstrate that human iPSC-derived MKs are capable of endocytosing and storing plasma components in their α -granules.

4 | DISCUSSION

Megakaryopoiesis is a complex stepwise process that occurs in bone marrow. It involves key molecular changes that induce the commitment of the multipotent stem cells to the MK lineage, MK progenitor proliferation and maturation. During this process, MKs undergo endomitosis, cytoplasmic maturation, expansion and acquisition of all components necessary for production of functional platelets. MKs do not synthesize all the components by themselves. Many of the important cargo components present in their α -granules are endocytosed from plasma and packaged, e.g. immunoglobulins, Fgn and FV. However, the list of human platelet α -granule cargoes that are not synthesized by MKs, but are taken up from plasma, remains incomplete. Although the actual transcriptome of human bone marrow MKs has not been elucidated, the transcriptome from human platelets may provide valuable clues. Genome-wide RNA-seq analysis of human platelets showed no mRNA expression for many of the α -granule cargoes, including factor XI, HGF, IGF-1, angiostatin, endostatin, BMP-2 and BMP-4 (Rowley et al., 2011), suggesting the plasma origin of these proteins.

Over decades, solid groundwork has been laid for the *in vitro* production of MKs, and the field is advancing steadily. Currently, multiple protocols have been established to generate MKs from iPSCs (Borger et al., 2016; Eicke et al., 2018; Feng et al., 2014; Ito et al., 2018; Liu et al., 2015; Mills et al., 2014; Moreau et al., 2016; Nakamura et al., 2014), and together

these hold exciting promise for the eventual production of large numbers of functional platelets *in vitro*. However, almost all of these protocols use serum- or plasma-free culture conditions, making it likely that the resulting MKs will lack important α -granule cargo proteins that would *in vivo* normally be taken up from plasma. Because lacking important α -granule cargo in Grey Platelet Syndrome (GPS) is known to significantly impair the function of human platelets *in vivo* (Nurden & Nurden, 2007), the purpose of the present study was to examine whether *in vitro* generated MKs could take up and store plasma proteins, especially Fgn - the major adhesive ligand for the α Ib β 3 integrin. Interestingly, an IpSC→megakaryocyte differentiation protocol driven by lentiviral-mediated overexpression of GATA1, FLI1 and TAL1 does result in some fibrinogen being synthesized and stored in α -granules (Moreau et al., 2016), likely due to GATA1-driven, ectopically-induced expression of IL-6 (Cole et al., 2010), a major inducer of Fgn gene expression in the liver (Fish & Neerman-Arbez, 2012).

Here, we used a cytokine- and growth factor-directed differentiation protocol to mimic embryonic development *in vitro*. As with human bone marrow MKs (Gewirtz et al., 1992; Louache et al., 1991), we found the MKs produced with this method expressed no endogenous Fgn, and only minor amounts of FV (Figure 3), but importantly retain the ability to take up reporter plasma proteins like Fgn and FV and store them in α -granules (Figure 3b and 3c). Our data suggest that incorporating human plasma into current protocols for *in vitro* MK production may complete MK the complement of normal α -granule constituents, and lead to the generation of platelets with improved hemostatic properties compared to those being generated using current methods. Alternatively, purified proteins with targeted functions could be used to load MK α -granules for generating platelets to treat patients with special needs, e.g. wound healing and tissue regeneration. For example, platelets harbor and secrete a large number of growth factors, including HGF and IGF-1. In patients with chronic liver disease and cirrhosis, platelet transfusion and splenectomy has been used to promote liver regeneration (Matsuo et al., 2008), with positive effects on hepatic function (Maruyama et al., 2013; Ushitora et al., 2011). Other possibilities for specialty *in vitro*-generated platelet products can readily be envisioned.

The complement system consists of various plasma proteins and membrane-bound proteins and provides an important line of defense against foreign and altered host cells. Initiating complement activation through different pathways-classical, lectin, and alternative pathway all leads to the activation of a common terminal pathway and eventually the formation of cell lytic membrane attack complex (MAC) (Merle, Church, Fremeaux-Bacchi, & Roumenina, 2015). To protect themselves from autologous complement-mediated damage, normal human tissues express cell membrane-associated complement regulatory proteins, such as CD35 (CR1), CD46 (MCP), CD55 (DAF) and CD59 (MACIF).

We were surprised to find that incubation of iPSC-derived MKs with human plasma resulted in significant cell death. Inactivation of complement by heating or depleting C5 from the plasma markedly increased MK viability (Figures S3 and S4), suggesting that activation of the complement system was the major cause of plasma-induced cell death. Removing IgG and IgM from the plasma was by itself not protective, implicating the alternative, rather than the classical pathway, of complement activation. Interestingly, although our iPSC-derived

MKs expressed high levels of the complement regulatory proteins CD46 and CD55, only a small amount of CD59, which inhibits the formation of the complement membrane attack complex (Merle et al., 2015), was expressed on the cell surface, suggesting that insufficient CD59 might be the cause of plasma-induced lysis. Since sheer stress has been shown to promote expression of CD59 on HUVECs and human endothelial progenitor cells (Cui et al., 2017; Kinderlerer et al., 2008), introducing sheer to the MK differentiation protocol might prevent complement damage. Alternatively, generating a CD59 transgenic founder iPSC line could be a potential solution to protect *in vitro*-generated MKs and platelets from complement attack both during generation and upon transfusion into patients.

5 | CONCLUSIONS

In vitro production of platelets from iPSCs has the potential to develop blood donor-independent and genetic manipulation-improved specialty products to complement current transfusion practice. Platelet products are qualitatively and quantitatively approaching a clinically-applicable level owing to advances in expandable MK lines, platelet-producing bioreactors, and novel reagents. However, insufficient attention has been paid to the missing α -granule components that are normally present in normal human platelet products. In this study, we showed that iPSC-derived MKs are capable of endocytosing and storing plasma components in their α -granules. Incorporating this methodology into current protocols for producing *in vitro*-derived MKs should provide novel insights into MK biology and lead to the generation of large numbers of MKs and platelets with improved functionality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Borger AK, Eicke D, Wolf C, Gras C, Aufderbeck S, Schulze K, ... Figueiredo C (2016). Generation of HLA-universal iPSCs-derived megakaryocytes and platelets for survival under refractoriness conditions. *Mol Med*, 22, 274–285. doi:10.2119/molmed.2015.00235 [PubMed: 27262025]
- Bouchard BA, Meisler NT, Nesheim ME, Liu CX, Strickland DK, & Tracy PB (2008). A unique function for LRP-1: a component of a two-receptor system mediating specific endocytosis of plasma-derived factor V by megakaryocytes. *J Thromb Haemost*, 6(4), 638–644. doi:10.1111/j.1538-7836.2008.02894.x [PubMed: 18194421]
- Bouchard BA, Williams JL, Meisler NT, Long MW, & Tracy PB (2005). Endocytosis of plasma-derived factor V by megakaryocytes occurs via a clathrin-dependent, specific membrane binding event. *J Thromb Haemost*, 3(3), 541–551. doi:10.1111/j.1538-7836.2005.01190.x [PubMed: 15748245]

- Burnouf T, Strunk D, Koh MB, & Schallmoser K (2016). Human platelet lysate: Replacing fetal bovine serum as a gold standard for human cell propagation? *Biomaterials*, 76, 371–387. doi: 10.1016/j.biomaterials.2015.10.065 [PubMed: 26561934]
- Camire RM, Pollak ES, Kaushansky K, & Tracy PB (1998). Secretable human platelet-derived factor V originates from the plasma pool. *Blood*, 92(9), 3035–3041. [PubMed: 9787136]
- Chen H, Locke D, Liu Y, Liu C, & Kahn ML (2002). The platelet receptor GPVI mediates both adhesion and signaling responses to collagen in a receptor density-dependent fashion. *J.Biol.Chem*, 277(4), 3011–3019. [PubMed: 11723134]
- Cole SW, Arevalo JM, Takahashi R, Sloan EK, Lutgendorf SK, Sood AK, ... Seeman TE (2010). Computational identification of gene-social environment interaction at the human IL6 locus. *Proc Natl Acad Sci U S A*, 107(12), 5681–5686. doi:10.1073/pnas.0911515107 [PubMed: 20176930]
- Cramer EM, Debili N, Martin JF, Gladwin AM, Breton-Gorius J, Harrison P, ... Vainchenker W (1989). Uncoordinated expression of fibrinogen compared with thrombospondin and von Willebrand factor in maturing human megakaryocytes. *Blood*, 73(5), 1123–1129. [PubMed: 2649167]
- Cui X, Zhang X, Bu H, Liu N, Li H, Guan X, ... Cheng M (2017). Shear stress-mediated changes in the expression of complement regulatory protein CD59 on human endothelial progenitor cells by ECM-integrinalphaVbeta3-F-actin pathway in vitro. *Biochem Biophys Res Commun*, 494(1–2), 416–421. doi:10.1016/j.bbrc.2017.09.019 [PubMed: 28943429]
- Eicke D, Baigger A, Schulze K, Latham SL, Halloin C, Zweigerdt R, ... Figueiredo C (2018). Large-scale production of megakaryocytes in microcarrier-supported stirred suspension bioreactors. *Sci Rep*, 8(1), 10146. doi:10.1038/s41598-018-28459-x [PubMed: 29977045]
- Feng Q, Shabrani N, Thon JN, Huo H, Thiel A, Machlus KR, ... Lanza R (2014). Scalable generation of universal platelets from human induced pluripotent stem cells. *Stem Cell Reports*, 3(5), 817–831. doi:10.1016/j.stemcr.2014.09.010 [PubMed: 25418726]
- Fish RJ, & Neerman-Arbez M (2012). Fibrinogen gene regulation. *Thromb Haemost*, 108(3), 419–426. doi:10.1160/TH12-04-0273 [PubMed: 22836683]
- George JN (1990). Platelet immunoglobulin G: Its significance for the evaluation of thrombocytopenia and for understanding the origin on a-granule proteins. *Blood*, 76, 859–870. [PubMed: 2203482]
- Gewirtz AM, Shapiro C, Shen YM, Boyd R, & Colman RW (1992). Cellular and molecular regulation of factor V expression in human megakaryocytes. *J Cell Physiol*, 153(2), 277–287. doi:10.1002/jcp.1041530207 [PubMed: 1429848]
- Gould WR, Simioni P, Silveira JR, Tormene D, Kalafatis M, & Tracy PB (2005). Megakaryocytes endocytose and subsequently modify human factor V in vivo to form the entire pool of a unique platelet-derived cofactor. *J Thromb Haemost*, 3(3), 450–456. doi:10.1111/j.1538-7836.2005.01157.x [PubMed: 15748232]
- Handagama P, Scarborough RM, Shuman MA, & Bainton DF (1993). Endocytosis of fibrinogen into megakaryocyte and platelet a-granules is mediated by α IIB β 3 (glycoprotein IIb-IIIa). *Blood*, 82, 135–138. [PubMed: 8391871]
- Handagama PJ, Shuman MA, & Bainton DF (1989). Incorporation of intravenously injected albumin, immunoglobulin G, and fibrinogen in guinea pig megakaryocyte granules. *J Clin Invest*, 84(1), 73–82. doi:10.1172/JCI114173 [PubMed: 2738161]
- Harrison P, Wilbourn B, Debili N, Vainchenker W, Breton-Gorius J, Lawrie AS, ... Cramer EM (1989). Uptake of plasma fibrinogen into the alpha granules of human megakaryocytes and platelets. *J Clin Invest*, 84(4), 1320–1324. doi:10.1172/JCI114300 [PubMed: 2677051]
- Italiano JE Jr., Richardson JL, Patel-Hett S, Battinelli E, Zaslavsky A, Short S, ... Klement GL (2008). Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. *Blood*, 111(3), 1227–1233. doi: 10.1182/blood-2007-09-113837 [PubMed: 17962514]
- Ito Y, Nakamura S, Sugimoto N, Shigemori T, Kato Y, Ohno M, ... Eto K (2018). Turbulence Activates Platelet Biogenesis to Enable Clinical Scale Ex Vivo Production. *Cell*, 174(3), 636–648 e618. doi:10.1016/j.cell.2018.06.011 [PubMed: 30017246]

- Kamykowski J, Carlton P, Sehgal S, & Storrie B (2011). Quantitative immunofluorescence mapping reveals little functional coclustering of proteins within platelet alpha-granules. *Blood*, 118(5), 1370–1373. doi:10.1182/blood-2011-01-330910 [PubMed: 21622648]
- Kinderlerer AR, Ali F, Johns M, Lidington EA, Leung V, Boyle JJ, ... Mason JC (2008). KLF2-dependent, shear stress-induced expression of CD59: a novel cytoprotective mechanism against complement-mediated injury in the vasculature. *J Biol Chem*, 283(21), 14636–14644. doi:10.1074/jbc.M800362200 [PubMed: 18362151]
- Kunicki TJ, Orzechowski R, Annis D, & Honda Y (1993). Variability of integrin $\alpha_2\beta_1$ activity on human platelets. *Blood*, 82, 2693–2703. [PubMed: 8219222]
- Laguerre-Lak-Hal AH, Debili N, Kingbury G, Lecut C, Le Couedic JP, Villeval JL, ... Vainchenker W (2001). Expression and function of the collagen receptor GPVI during megakaryocyte maturation. *J.Biol.Chem*, 276(18), 15316–15325. [PubMed: 11278467]
- Liu Y, Wang Y, Gao Y, Forbes JA, Qayyum R, Becker L, ... Wang ZZ (2015). Efficient generation of megakaryocytes from human induced pluripotent stem cells using food and drug administration-approved pharmacological reagents. *Stem Cells Transl Med*, 4(4), 309–319. doi:10.5966/sctm.2014-0183 [PubMed: 25713465]
- Louache F, Debili N, Cramer E, Breton-Gorius J, & Vainchenker W (1991). Fibrinogen is not synthesized by human megakaryocytes. *Blood*, 77(2), 311–316. [PubMed: 1985697]
- Mancuso ME, & Santagostino E (2017). Platelets: much more than bricks in a breached wall. *Br J Haematol*, 178(2), 209–219. doi:10.1111/bjh.14653 [PubMed: 28419428]
- Maruyama T, Murata S, Takahashi K, Tamura T, Nozaki R, Ikeda N, ... Ohkohchi N (2013). Platelet transfusion improves liver function in patients with chronic liver disease and cirrhosis. *Tohoku J Exp Med*, 229(3), 213–220. [PubMed: 23459612]
- Matsuo R, Ohkohchi N, Murata S, Ikeda O, Nakano Y, Watanabe M, ... Ozaki M (2008). Platelets Strongly Induce Hepatocyte Proliferation with IGF-1 and HGF In Vitro. *J Surg Res*, 145(2), 279–286. doi:10.1016/j.jss.2007.02.035 [PubMed: 17688880]
- Maynard DM, Heijnen HF, Horne MK, White JG, & Gahl WA (2007). Proteomic analysis of platelet alpha-granules using mass spectrometry. *J Thromb Haemost*, 5(9), 1945–1955. doi:10.1111/j.1538-7836.2007.02690.x [PubMed: 17723134]
- Mazur EM, Lindquist DL, de Alarcon PA, & Cohen JL (1988). Evaluation of bone marrow megakaryocyte ploidy distributions in persons with normal and abnormal platelet counts. *J Lab Clin Med*, 111(2), 194–202. [PubMed: 3276801]
- Merle NS, Church SE, Fremaux-Bacchi V, & Roumenina LT (2015). Complement System Part I - Molecular Mechanisms of Activation and Regulation. *Front Immunol*, 6, 262. doi:10.3389/fimmu.2015.00262 [PubMed: 26082779]
- Mills JA, Paluru P, Weiss MJ, Gadue P, & French DL (2014). Hematopoietic differentiation of pluripotent stem cells in culture. In a. KDB Qu C-K (Ed.), *Methods in Molecular Biology* (Vol. 1185, pp. 181–194). New York: Springer Science+Business Media. [PubMed: 25062629]
- Moreau T, Evans AL, Vasquez L, Tijssen MR, Yan Y, Trotter MW, ... Ghevaert C (2016). Large-scale production of megakaryocytes from human pluripotent stem cells by chemically defined forward programming. *Nat Commun*, 7, 11208. doi:10.1038/ncomms11208 [PubMed: 27052461]
- Nakamura S, Takayama N, Hirata S, Seo H, Endo H, Ochi K, ... Eto K (2014). Expandable megakaryocyte cell lines enable clinically applicable generation of platelets from human induced pluripotent stem cells. *Cell Stem Cell*, 14(4), 535–548. doi:10.1016/j.stem.2014.01.011 [PubMed: 24529595]
- Nurden AT, & Nurden P (2007). The gray platelet syndrome: clinical spectrum of the disease. *Blood Rev*, 21(1), 21–36. doi:10.1016/j.blre.2005.12.003 [PubMed: 16442192]
- Okita K, Yamakawa T, Matsumura Y, Sato Y, Amano N, Watanabe A, ... Yamanaka S (2013). An efficient nonviral method to generate integration-free human-induced pluripotent stem cells from cord blood and peripheral blood cells. *Stem Cells*, 31(3), 458–466. doi:10.1002/stem.1293 [PubMed: 23193063]
- Paluru P, Hudock KM, Cheng X, Mills JA, Ying L, Galvao AM, ... Gadue P (2014). The negative impact of Wnt signaling on megakaryocyte and primitive erythroid progenitors derived from

human embryonic stem cells. *Stem Cell Res*, 12(2), 441–451. doi:10.1016/j.scr.2013.12.003 [PubMed: 24412757]

Podolak-Dawidziak M, Hancock V, Lelchuk R, Kotlarek-Haus S, & Martin JF (1995). The expression of mRNA for fibrinogen in megakaryocytes isolated from patients with T-cell lymphoma. *Br J Haematol*, 91(2), 362–366. [PubMed: 8547076]

Rendu F, Marche P, Viret J, Daveloose D, Leterrier F, Levy-Toledano S, & Caen JP (1985). Molecular membrane organization in normal and pathological platelets: changes in inositide metabolism and membrane fluidity. *Nouvelle Revue Francaise D Hematologie*, 27, 293–297. [PubMed: 2997707]

Rowley JW, Oler AJ, Tolley ND, Hunter BN, Low EN, Nix DA, ... Weyrich AS (2011). Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. *Blood*, 118(14), e101–111. doi: 10.1182/blood-2011-03-339705 [PubMed: 21596849]

Sehgal S, & Storrie B (2007). Evidence that differential packaging of the major platelet granule proteins von Willebrand factor and fibrinogen can support their differential release. *J Thromb Haemost*, 5(10), 2009–2016. doi:10.1111/j.1538-7836.2007.02698.x [PubMed: 17650077]

Suehiro Y, Veljkovic DK, Fuller N, Motomura Y, Masse JM, Cramer EM, & Hayward CP (2005). Endocytosis and storage of plasma factor V by human megakaryocytes. *Thromb Haemost*, 94(3), 585–592. [PubMed: 16268476]

Thomassen MC, Castoldi E, Tans G, Magdeleyns EJ, Delaunoit C, Debusscher L, ... Rosing J (2003). Endogenous factor V synthesis in megakaryocytes contributes negligibly to the platelet factor V pool. *Haematologica*, 88(10), 1150–1156. [PubMed: 14555311]

Ushitora Y, Tashiro H, Takahashi S, Amano H, Oshita A, Kobayashi T, ... Ohdan H (2011). Splenectomy in chronic hepatic disorders: portal vein thrombosis and improvement of liver function. *Dig Surg*, 28(1), 9–14. doi:10.1159/000321886 [PubMed: 21293126]

Wang Y, Hayes V, Jarocha D, Sim X, Harper DC, Fuentes R, ... Poncz M (2015). Comparative analysis of human ex vivo-generated platelets vs megakaryocyte-generated platelets in mice: a cautionary tale. *Blood*, 125(23), 3627–3636. doi:10.1182/blood-2014-08-593053 [PubMed: 25852052]

FIGURE 1 -

Establishment of integration-free iPSCs from human PBMCs. **(a)** Immuno-fluorescence staining and histochemistry showed expression of SOX2 (red), OCT4 (green) and alkaline phosphatase in newly generated iPSC colonies. **(b)** Flow cytometry analysis demonstrating the expression of TRA-1-60, SSEA-3 and SSEA-4 surface markers of iPSCs. **(c)** Karyotype of iPSCs revealed a normal distribution of 46 chromosomes with XX sex chromosomes.

FIGURE 2 -

Characterization of iPSC-derived MKs. **(a)** DNA ploidy analysis by flow cytometry of iPSC-derived CD41a⁺ MKs. DNA ploidy of up to 16N was observed in these cells. **(b)** Flow cytometry analysis showed surface expression of GPIIb/IIIa, GPIbα/GPIX, GPVI and GPIa on iPSC-derived MKs. **(c)** Confocal images showed iPSC-derived MKs spread on 50 µg/ml collagen I-coated and 25 µg/ml fibrinogen-coated surfaces. The cells were labeled with phalloidin (red) and anti-vinculin antibody (green). Scale bar: 10 µm.

FIGURE 3 -

iPSC-derived MKs take up Fgn and FV from human plasma and package them into α -granules. **(a)** RT-PCR analysis showed no Fgn expression and low level of FV expression, and high level of VWF expression in iPSC-derived MKs. Hep G2 cells were used as a positive control for RT-PCR conditions. **(b)** Immunofluorescence staining and confocal imaging showed iPSC-derived MKs endocytosed Fgn (green) into α -granules from plasma after 1 day incubation. Fgn only partial colocalized with VWF (red). DAPI staining (blue) showed nucleus in the merged images. Scale bar: 5 μ m. **(c)** Immunofluorescence staining and confocal imaging showed iPSC-derived MKs endocytosed FV (green) into α -granules from plasma. FV colocalized substantially with VWF (red). DAPI staining (blue) showed nucleus in the merged images. Scale bar: 5 μ m.