Megakaryocyte Polyploidization and Proplatelet Formation in Low-Attachment Conditions

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Abstract

In vitro-derived platelets (PLTs), which could provide an alternative source of PLTs for patient transfusions, are formed from polyploid megakaryocytes (MKs) that extend long cytoplasmic projections, termed **proplatelets (proPLTs)**. In this study, we compared polyploidization and proPLT formation (PPF) of MKs cultured on surfaces that either promote or inhibit protein adsorption and subsequent cell adhesion. A megakaryoblastic cell line exhibited increased polyploidization and arrested PPF on a low-attachment surface. Primary human MKs also showed low levels of PPF on the same surface, but no difference in ploidy. Importantly, both cell types exhibited accelerated PPF after transfer to a surface that supports attachment, suggesting that pre-culture on a non-adhesive surface may facilitate synchronization of PPF and PLT generation in culture.

1. Introduction

In vitro-derived platelets (PLTs) could provide an alternative source of PLTs for patient transfusions. PLTs are formed when polyploid megakaryocytes (MKs) extend long, cytoplasmic extensions from the bone marrow and into small blood vessels (sinuses). PLT components are shuttled and packaged along these extensions, termed proplatelets (proPLTs), before being sheared off by flowing blood [1]. MKs and their PLT progeny have been successfully derived *in vitro* from CD34⁺ hematopoietic stem and progenitor cells (HSPCs) from mobilized peripheral blood (mPB) [2, 3] and umbilical cord blood [4-8], as well as MK progenitor immortalized cell lines [9], induced pluripotent stem cells [10-12], and embryonic stem cells [13-15].

Although MKs undergo endomitosis and extend proPLTs during *in vitro* culture, the factors responsible for initiating these events are not well understood. MKs express receptors for different extracellular matrix (ECM) components during development [16] and are found near fenestrated endothelium during terminal maturation, making it likely that MKs interact with proteins and/or glycosaminoglycans (GAGs) at this blood-bone marrow interface. Several studies have examined the role that cell-substrate interactions play in polyploidization and proPLT formation (PPF). Chemokine-mediated localization of MKs to the bone marrow vascular niche promotes platelet production [17]. Cultures supplemented with soluble dermatan sulfate show higher MK ploidy [18], and several different covalently immobilized GAGs, including heparan sulfate and heparin, significantly increase the percentage of MKs with PPF and promote PLT release [19]. MKs can also form proPLTs on several immobilized ECM components, including fibronectin, fibrinogen, and von Willebrand factor, although the kinetics of PPF vary across different substrates [20]. Although cell adhesion is important, a number of studies suggest that formation of mature stress fibers and focal adhesions downregulates polyploidization and PPF. Type I collagen supports MK spreading [21, 22] and inhibits PPF in human MKs [20, 23], while focal adhesion kinase-null mice produce a greater percentage of high-ploidy MKs [24]. Similarly, inhibition of myosin light chain kinase or non-muscle myosin II, by way of blebbistatin treatment or Myh9 knockout, has been shown to increase ploidy and PPF [25-27]. Upstream of myosin II, inhibitors against RhoA and ROCK enhance both ploidy and PPF [26-29].

While several studies have characterized the effect of specific receptor-ligand engagement on MK polyploidization and PPF, the effect of inhibiting MK adhesion has yet to be assessed. In

this study, we compared polyploidization and PPF of MKs cultured on surfaces that either promote or inhibit protein adsorption and subsequent cell adhesion. A megakaryoblastic cell line exhibited increased polyploidization and arrested PPF on a low-attachment surface. Primary human MKs also showed low levels of PPF on the same surface, but no difference in ploidy. Importantly, both cell types exhibited accelerated PPF after transfer to a surface that supports attachment, suggesting that pre-culture on a non-adhesive surface may facilitate synchronization of PPF and PLT generation in culture.

2. Material and Methods

Unless otherwise noted, all reagents were from Sigma Aldrich (St. Louis, MO) and all cytokines were from Peprotech (Rocky Hill, NJ).

2.1. Differentiation of human megakaryoblastic cell lines

The human megakaryoblastic CHRF-288-11 (CHRF) and myelogenous leukemia K562 cell lines were cultured in Iscove's Modified Dubelcco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Waltham, MA). On day 0, cells were resuspended in IMDM+10% FBS to a final concentration of 100,000/mL and seeded in tissue culture-treated (TC) polystyrene, Ultra Low Attachment (ULA; Corning, Tewksbury, MA), or poly(2-hydroxyethyl methacrylate) (polyHEMA)-coated well plates. Cells were seeded such that an entire well could be harvested for each analysis time point. Seeded cells were treated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA; Calbiochem, Whitehouse Station, NJ) to induce MK differentiation [30]. In select experiments, CHRF cells were also treated with various combinations of 12.5 mM nicotinamide (Nic), 0.5 μ M H-1152 (Calbiochem) rho-associated protein kinase (ROCK) inhibitor, and 10 μ M (-)-blebbistatin (active enantiomer) myosin IIa inhibitor.

2.2. Harvest of PMA-treated CHRF and K562 cells

The supernatant from each well was transferred to conical tubes, then a PBS rinse was performed. Each well was incubated at 37 °C for 15 minutes with prewarmed Accutase (Millipore, Billerica, MA). The Accutase was pipetted up and down several times to dislodge any loosely-adherent cells before a final PBS rinse was performed. Both rinses and the Accutase

were collected in the respective conical tube. Any remaining cell aggregates were easily broken up via repeated pipetting or vortexing.

2.3. Preparation of polyHEMA-coated, non-adhesive culture surfaces

TC well plates and T-flasks were treated with a solution of 10% polyHEMA in 95% ethanol with 10 mM NaOH, such that the bottom and walls were coated. Excess solution was removed and the surfaces were allowed to dry in a biosafety cabinet overnight. Prior to use, the surfaces were rinsed with PBS.

2.4. Primary MK culture

Cryopreserved CD34⁺ HSPCs from mPB were purchased from the Fred Hutchinson Cancer Research Center with Northwestern University Institutional Review Board approval. Cells were obtained from healthy donors undergoing granulocyte-colony-stimulating-factor (G-CSF) mobilization following informed consent. Cultures of CD34⁺ cells were initiated in TC T-flasks at 50,000 cells/mL in IMDM + 20% BIT (78% IMDM [Gibco, Carlsbad, CA], 20% BIT 9500 Serum Substitute [STEMCELL, Vancouver, BC, Canada], 1% Glutamax [Gibco], 1 µg/mL low-density lipoproteins [Calbiochem], 100 U/mL Pen/Strep) supplemented with 100 ng/mL thrombopoietin (Tpo), 100 ng/mL stem cell factor (SCF), 2.5 ng/mL interleukin (IL)-3 (R&D Systems, Minneapolis, MN), 10 ng/mL IL-6, and 10 ng/mL IL-11. Cells were cultured in a fully humidified chamber at 37 °C, 5% CO₂, and 5% O₂ for 5 days. On day 5, cells were pelleted and resuspended in fresh IMDM + 20% BIT supplemented with 100 ng/mL Tpo, 100 ng/mL SCF, 10 ng/mL IL-3, 10 ng/mL IL-9, and 10 ng/mL IL-11. Cells were cultured at 20% O₂ until day 7.

MKs were enriched on day 7 using anti-CD61-conjugated magnetic beads (Miltenyi, Bergisch Gladbach, Germany), then resuspended in fresh IMDM+20% BIT supplemented with 100 ng/mL Tpo, 100 ng/mL SCF, +/- 6.25 mM Nic. Cells were seeded on TC or polyHEMA-coated surfaces, as described. Cells were transferred from polyHEMA to TC surfaces at day 9 or 11.

2.5. Flow cytometric analysis of MK ploidy

Cells were washed with cold PBS containing 2 mM EDTA and 0.5% BSA (PEB). For primary MKs, FITC-conjugated anti-CD41 antibody (BD Biosciences, San Jose, CA) was added for 30 minutes at 4°C. Cells were fixed with 0.5% paraformaldehyde (Polysciences, Warrington, PA) in PBS for 15 minutes at room temperature, permeabilized with cold 70% methanol for 1 hour at 4°C, treated with RNase for 30 minutes at 37°C, then incubated with 50 µg/mL propidium iodide to stain DNA prior to analysis with an LSR II flow cytometer (BD Biosciences).

2.6. Flow cytometric analysis of MK viability

Cells were washed with PEB, then incubated with DAPI (Life, Carlsbad, CA) for 15 minutes at room temperature prior to analysis with an LSR II flow cytometer.

2.7. Flow cytometric analysis of MK apoptosis

Cells were washed with PBS, then with 1X Annexin V binding buffer (BD Biosciences), incubated with Cy5-conjugated Annexin V for 15 minutes at room temperature, and washed with 1X Annexin V binding buffer. DAPI was added for 10 minutes at room temperature prior to analysis with an LSR II flow cytometer.

2.8. Transfer of CHRF cells from ULA to TC surfaces

PMA-treated CHRF cells on a ULA surface were pipetted several times to create a homogeneous suspension. An aliquot of cells was pelleted at 300g for 5 minutes, the supernatant was aspirated, and the cell pellet was resuspended in fresh IMDM+10% FBS. The cells were reseeded on a TC surface and treated with PMA to a final concentration of 10 ng/mL.

2.9. Quantification of CHRF cell PPF and proPLT length

Supernatant was removed from TC wells with PMA-treated CHRF cells and the wells were washed once with 37°C PBS + Ca^{2+} + Mg^{2+} . Fresh 37°C PBS + Ca^{2+} + Mg^{2+} was gently added to each well, being careful not to dislodge any adherent cells. The cells were observed using a DM IL LED inverted microscope (Leica, Wetzlar, Germany), and imaged with a QICAM digital camera (QImaging, Surrey, BC, Canada). The percentage of proPLT forming cells was measured as the number of proPLT-forming adherent cells divided by the total number of adherent cells. ProPLT length was measured using the ‘Segmented Line’ tracer in ImageJ [31]. To limit error in missing cells and/or double-counting cells, proPLT extensions were measured starting in one corner of the image and working across the image, dividing the image into three rows. The total length of all main shafts and proPLT branches was determined and then divided by the total number of cells in the image.

2.10 Quantification of primary MK PPF

For quantification of the percentage of PPF in CD41-selected, primary MK cultures, cells were imaged as described above (Section 2.9). The percentage of PPF was determined by dividing the number of proPLTs (both released and still attached to a cell body) by the total number of cell bodies in each image. Approximately 100 cells were analyzed at each time point for each condition.

2.11 Immunocytochemistry of cytoskeletal proteins

CHRF cells were seeded in uncoated or polyHEMA-coated cell-culture-treated Lab-Tek II glass chamber slides (Nunc, Penfield, NY) and treated with 10 ng/mL PMA on day 0. Cells were permeabilized with 0.3% Triton X-100 in PBS for 5 minutes. Slides were blocked with Image-iT FX signal enhancer (Life), then incubated with mouse anti- β -tubulin antibody (BD Biosciences) overnight. Slides were then incubated with FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA), TRITC phalloidin, and DAPI before being mounted and imaged using a 100X oil objective on a SP5 II Laser Scanning Confocal Microscope (Leica, Wetzlar, Germany).

2.12. Statistical analysis

Results are expressed as mean $\pm$ standard error (SE), unless otherwise indicated. Statistical significance was determined using a paired two-tailed t-test for a particular day in culture, unless otherwise indicated. p-values < 0.05 were considered significant.

3. Results

3.1. Culture on a non-adhesive, ULA surface inhibits CHRF cell PPF, increases polyploidization, and improves viability

Tissue culture-treated (TC) polystyrene is produced via corona-discharge treatment or vacuum-plasma grafting of highly energetic oxygen ions to the culture surface. These modifications create a hydrophilic and negatively-charged surface that readily adsorbs proteins from the culture medium to which cells can then adhere. In contrast, an Ultra Low Attachment (ULA) surface consists of polystyrene that has been coated with a neutral, hydrophilic polymer, rendering it incompatible for protein adsorption and therefore inhibitory for cell attachment.

The CHRF human megakaryoblastic cell line resembles an early MK phenotype and exhibits transcriptional features of MK differentiation following PMA treatment, according to gene expression microarray analysis [30]. Phenotypically, PMA-simulated CHRF cells undergo a process that closely resembles primary MK differentiation. When cultured on TC plastic in serum-containing medium, PMA-stimulated CHRF cells adhere to the surface within 30 minutes of treatment, spread, undergo polyploidization, and extend proPLT-like extensions (Figure 1A, top) [30]. In contrast, we found that PMA-treated CHRF cells cultured on a ULA surface remain in suspension, but tend to clump together, retain a rounded shape, and do not form proPLT-like extensions (Figure 1A, bottom). CHRF cells cultured on a TC-like, cell-culture-treated glass surface have F-actin distribution characteristic of stress fiber formation (Figure 1B, top, *) and display acetylated α -tubulin, indicative of stable microtubules, along proPLT-like extensions (Figure 1B, bottom, #). In contrast, CHRF cells cultured on a ULA-like, polyHEMA-coated, glass surface have diffuse F-actin staining and acetylated α -tubulin only at their center. (The

rationale for using a polyHEMA-coated surface as a substitute for a ULA surface is provided in section 3.6.) Importantly, PMA-treated CHRF cells cultured on a ULA surface also undergo polyploidization, and do so to a greater extent than when cultured on a TC surface. This is evidenced by a higher percentage of $\geq 8N$ cells (Figure 1C) and increased mean ploidy (Figure 1D) during ULA culture. ULA culture also results in a small population of 128N cells, which does not appear during TC culture (Figure 1E), as well as a 5-10% increase in the fraction of viable cells throughout the culture (Figure 1F).

Figure 1. ULA culture results in different morphology as well as increased polyploidization and viability of CHRF cells

CHRF cells were seeded on a TC, ULA, TC-like, cell-culture-treated glass surface (TC-like glass), or ULA-like, polyHEMA-coated glass (ULA-like polyHEMA) surface and treated with PMA on day 0. (A) Representative day-9 images of cell morphology. Scale bar = 20 μm . (B) Representative day-5 images of cells were stained for F-actin (red), acetylated α -tubulin (green), and DNA (blue). Scale bar = 20 μm . (C, D) Percentage of high-ploidy ($\geq 8N$) CHRF cells and mean ploidy values $\pm$ SE for 3 or 4 independent experiments. (E) Representative day-11 ploidy histograms. (F) Percentage of viable (DAPI-) cells $\pm$ SE for 3 or 4 independent experiments. * indicates $p < 0.05$.

3.2. Culture on a ULA surface results in decreased CHRF cell apoptosis

After observing higher viability for PMA-treated CHRF cells on a ULA surface, compared to a TC surface, we hypothesized that a longer lifespan may allow cells on the ULA surface to undergo additional rounds of endomitosis, thereby increasing mean ploidy. To explore this further, we examined apoptosis in cultures on the two surfaces via Annexin V labeling of extracellular plasma membrane phosphatidylserine. At both day 5 and 10 of PMA-treated CHRF cell culture, there were fewer apoptotic cells on the ULA or PolyHEMA-coated surface (non-adhesive surface), as evidenced by the ratio of non-adhesive apoptosis to TC apoptosis being less than 1 (Figure 2), supporting the idea that prolonged cell viability may play a role in increased ploidy.

Figure 2. Fewer CHRF cells are apoptotic on a non-adhesive surface. CHRF cells were seeded on TC or non-adhesive (either ULA or polyHEMA-coated) surfaces and treated with PMA on day 0. On day 5 and 10, cells were harvested and stained with Annexin V (AnV) and DAPI. (A) Representative AnV vs. DAPI density plots. (B) Ratio of the percentage (mean $\pm$ SE) of apoptotic cells in the viable fraction for non-adhesive vs. TC for 3 independent experiments. The reduction in percent apoptotic cells was statistically significant (p-values 0.044 and 0.026 for day 5 and day 10, respectively), as determined using a one-tailed t-test with unequal variance.

3.3. ULA culture increases CHRF cell polyploidization more rapidly than nicotinamide and synergizes with nicotinamide to further increase ploidy

We have previously shown that nicotinamide (Nic) increases the polyploidization of PMA-treated CHRF cells during culture on a TC surface [32]. ULA culture in the absence of Nic increased CHRF cell ploidy to a similar extent by day 11 as TC culture in the presence of Nic, but the rate of ploidy increase was much greater for ULA culture (Figure 3A-C). Culture on ULA together with Nic combined the benefits of ULA and Nic on polyploidization. Interestingly, the fraction of polyploid cells with ULA plus Nic plateaued on day 7, although the mean ploidy continued to increase until day 11. The additive effects suggest that Nic and ULA increase CHRF cell ploidy via distinct mechanisms. The higher ploidy on ULA surfaces in the absence of Nic resulted in a lower percent increase in ploidy after Nic addition (Figure 3D).

Figure 3. Nic treatment and ULA culture additively increase CHRF cell polyploidization.

CHRF cells were seeded on a TC or ULA surface and treated with PMA $\pm$ 12.5 mM Nic on day 0. Percentage of high-ploidy ($\geq 8N$) cells (A), representative day 11 ploidy histograms (B), mean ploidy (C), and percent increase in mean ploidy over the PMA-only condition (D) are shown. (A), (C), and (D) show the mean $\pm$ SE for 3 or 4 independent experiments. * indicates $p < 0.05$.

3.4. Inhibitors of cytoskeletal rearrangement increase CHRF cell polyploidization

Integrin binding to an immobilized ligand often results in cell spreading through phosphorylation of FAK, recruitment of Src, and an initial decrease in the level of active RhoA-GTP [33]. Late in spreading, activated guanine nucleotide exchange factors (GEFs) increase the level of Rho-GTP, which in turn leads to activation of rho-associated protein kinase (ROCK). ROCK-mediated inhibition of myosin light chain (MLC) phosphatase activity results in an increase in phosphorylated myosin IIa, which has ATPase activity that results in contraction of actin bundles and stress fiber formation. Several small molecule inhibitors targeting different parts of this signaling cascade have been discovered, including H-1152 [34] and blebbistatin [35], which inhibit ROCK and myosin IIa ATPase activity, respectively.

Because ULA culture inhibits ligand immobilization and subsequent surface receptor binding, we hypothesized that ROCK-myosin IIa signaling may be affected. In an effort to further understand the mechanism by which ULA culture results in increased polyploidization, PMA-treated CHRF cells were cultured on TC and ULA surfaces in the presence of either H-1152 or (-)-blebbistatin (active enantiomer). Both inhibitors increased the maximum mean ploidy achieved on the TC and ULA surfaces, compared to the PMA-only condition on the same surface, although blebbistatin was more potent than H-1152 on both surfaces (Figure 4). The inhibitors were also used concurrently with Nic. H-1152 and Nic synergized on both surfaces to give higher maximum mean ploidy than that attained with either Nic or H-1152 alone (Figure 4B). In contrast, blebbistatin and Nic synergized on the TC surface, but not on the ULA surface, where the maximum mean ploidy with combined treatment was less than with blebbistatin alone

(Figure 4B). In addition, blebbistatin treatment in the absence of PMA increased CHRF ploidy, while H-1152 and Nic did not produce that effect (Figure S1).

Figure 4. Inhibitors against cytoskeletal rearrangement increase CHRF cell polyploidization on TC and ULA surfaces. CHRF cells were seeded on TC or ULA surfaces and treated with PMA $\pm$ 12.5 mM Nic, 0.5 μ M H-1152 ROCK inhibitor (ROCKi), and/or 10 μ M blebbistatin (Bleb) on day 0. Cells were harvested and analyzed for ploidy on days 3, 5, and 7. Mean ploidy on days 3, 5, and 7 (A) and maximum mean ploidy normalized to the PMA-only condition on the corresponding surface (B) are shown as the mean $\pm$ SD for 2 independent experiments. * indicates $p < 0.05$.

3.5. ULA-to-TC transfer results in more rapid and increased CHRF cell PPF and curbs polyploidization

Because PMA-treated CHRF cells cultured on a non-adhesive, ULA surface did not form proPLTs, we were interested to determine whether re-introducing an adhesive surface would initiate PPF. To explore this, CHRF cells were seeded on a ULA surface and treated with PMA on day 0, then transferred to a TC surface on day 3 or 5. ULA-to-TC transferred cells generated proPLTs (Figure 5A) more rapidly and had a higher maximum percentage of proPLT-forming cells than cells seeded on a TC surface on day 0 for the same period of time (Figure 5B). ULA-to-TC transferred cells also had 50% (day 3) and 100% (day 5) longer total proPLT length, on average, per proPLT-forming cell (Figure 5C) when compared to cells cultured on a TC surface from day 0. For the aforementioned metrics, day-5 transfer was better than day-3 transfer. ULA-to-TC transfer also resulted in slowed polyploidization, compared to continued culture on a ULA surface (Figure S2). Together, these results suggest that adhesion-mediated proPLT formation is accompanied by arrested polyploidization.

Figure 5. ULA-to-TC transfer of CHRF cells results in more extensive PPF. CHRF cells were seeded on TC or ULA surfaces and treated with PMA on day 0. (A) Cells treated with PMA and cultured on ULA for 3 days (i) then transferred to TC and imaged 48 hours later (ii). Scale bar = 25 μm . (B) Percentage of adherent cells extending proPLTs. (C) Average total proPLT length per proPLT-forming cell. Results are shown as the mean $\pm$ SE for a single experiment where proPLT length was quantified for at least 30 cells per condition, per time point.

3.6. Culture on a ULA-like surface does not affect primary MK CD42b expression or polyploidization,

Corning sells its proprietary ULA culture surface in a limited number of well plate formats and T-flask sizes. In order to increase the variety of culture surface formats that we could use for culturing cells in the absence of adhesion, we hypothesized that we could form our own ULA-like culture surface by coating TC polystyrene with a thin film of the neutral, hydrophilic polymer polyHEMA. This approach has been previously described in the literature [36]. We found that coating TC polystyrene with polyHEMA resulted in a surface comparable to the ULA surface, as evidenced by the similar rounded shape, lack of PPF (Figure S3A), and ploidy profile (Figure S3B) of PMA-treated CHRF cells cultured on the two surfaces.

To determine if ULA-like culture would affect primary human MK maturation and PPF, mPB CD34⁺ HSPCs from 2 different donors were first differentiated to MKs on a TC surface, as previously described [37]. On day 7, the culture was selected for CD41⁺ MKs, which were then reseeded on either a TC or polyHEMA-coated surface in the presence of Nic. We have previously demonstrated the generation of functional PLTs from selected MKs treated with Nic [3, 37]. Primary MKs matured to express the CD42b surface receptor at a similar rate (Figure 6A) and underwent polyploidization to a similar extent (Figure 6B) on the TC and polyHEMA surfaces. Viability was also similar between the two surfaces (80.5% vs. 73.7%, 65.4% vs 59.6%, and 77.3% vs 76.8% for TC vs polyHEMA day 11 viability for 3 different donors, respectively). Nic treatment also similarly increased polyploidization, over Tpo and SCF alone, on both surfaces (data not shown).

Figure 6. PolyHEMA culture results in normal primary MK CD42b expression and polyploidization. CD34⁺ HSPCs from mPB were cultured with cytokine cocktails from day 0 to induce MK differentiation. On day 7, CD41⁺ MKs were selected, resuspended in media with Tpo, SCF, and Nic, and reseeded on either a polyHEMA-coated or TC surface. (A) Percentage of MKs expressing the mature MK surface marker CD42b on day 11. (B) Day 11 MK ploidy histograms.

3.7 Day 11 polyHEMA-to-TC transfer of primary MKs results in a rapid burst of PPF

To examine the effect of re-introducing an adhesive surface, similar to experiments performed with the CHRF megakaryoblastic cell line, primary MKs originally reseeded on a polyHEMA surface on day 7 were transferred to a TC surface on day 9 or 11. Up to day 11, little PPF was observed on either the TC or polyHEMA surface (Figure 7A; Figure 7B first TC and polyHEMA data points on each graph). Day 9 transfer resulted in an increase in PPF at a similar rate to that observed for cells seeded on a TC surface on day 7 (data not shown). However, MKs transferred from polyHEMA to TC on day 11 exhibited faster PPF than that observed for cells that had been transferred to a TC surface on day 7 (Figure 7A iii; Figure 7B second TC and polyHEMA data points on each graph). The morphology of proPLTs generated by polyHEMA-to-TC MKs was indistinguishable from those generated by MKs that had been cultured on TC only (Figure 7C). This result may be due, in part, to the fact that the MKs were more mature at day 11 than day 9, with respect to CD42b expression (Figure 6A) and ploidy (Figure 6B), and therefore were perhaps more poised to generate proPLTs at the later transfer day.

Figure 7. Similar morphology but more rapid formation of proPLTs from cells transferred from polyHEMA to TC. CD34⁺ HSPCs from mPB were cultured with cytokine cocktails from day 0 to induce MK differentiation. On day 7, CD41⁺ MKs were selected, resuspended in media with Tpo, SCF, and Nic, and reseeded on either a polyHEMA or TC surface. On day 11 (~96 hours post CD41 selection), cells on the polyHEMA surface were transferred to a TC surface. (A) Representative day 11 images of CD41⁺ MKs that had been reseeded on a TC surface (i) or polyHEMA surface (ii) on day 7, as well as CD41⁺ MKs 8 hours post day-11 polyHEMA-to-TC transfer (iii). Scale bar = 20 μ m. (B) Percentage of PPF formation for 3 different donors (i, ii, and iii), as determined by dividing the sum of released proPLTs and cells with proPLTs by the

total number of cell bodies. (C) Representative images of proPLT morphology for MKs cultured on TC only (i) or transferred from polyHEMA-to-TC (ii). Scale bar = 30 μm .

4. Discussion

Several studies have evaluated MK polyploidization and PPF on surfaces coated with different ligands that can be bound by MK surface receptors. However, the effect of preventing MK adhesion has yet to be explored. In this study, we cultured two megakaryoblastic cell lines, as well as primary MKs derived from CD34⁺ HSPCs, on either a TC or ULA surface. The negatively-charged TC surface adsorbs proteins and therefore facilitates cell adhesion to the surface, while the neutral ULA surface allows little protein adsorption, keeping cells primarily in suspension. CHRF cells, which mimic an early MK progenitor and undergo MK-like maturation with PMA treatment, adhered to a TC surface, spread, and formed proPLTs, but remained in suspension and devoid of proPLTs during culture on a ULA surface. CHRF cells on the ULA surface also underwent more rounds of polyploidization, resulting in the emergence of cells with a 128N ploidy class (Figure 1E) and overall higher mean ploidy (Figure 1D). K562 cells, which resemble a bipotent, erythrocyte/MK progenitor that can be induced to differentiate toward the MK lineage with PMA treatment [38] also remained in suspension on a ULA surface (Figure S4A), but ploidy and viability was similar on the two surfaces (Figure S4B-E). Primary MKs cultured on a ULA-like, polyHEMA surface displayed less PPF, but, as for K562 cells, polyploidization was largely unaffected (Figure 6B).

Although CHRF cells, K562 cells, and primary MKs derived from CD34⁺ HSPCs all adhered to a TC surface, the CHRF cells exhibited the strongest, most transformative kind of adhesion. CHRF cell adhesion involves stress fiber formation and cytoskeletal rearrangement, resulting in a spread, flat cell (Figure 1A, B). In contrast, both K562 cells and primary MKs mainly undergo loose adhesion to a TC surface with minimal spreading, such that they retain a fairly rounded

morphology (Figures S1A, 7A, 7C). These differences may help explain the increase in polyploidization that was observed for CHRF cells, but not the other two cell types. Because rounding up of the cell body is required for entering mitosis [39], CHRF cell tight adhesion may impede initiation of endomitosis, thereby resulting in the lower mean ploidy observed on the TC surface. When the same CHRF cells were cultured in suspension on a ULA surface, this physical block towards endomitosis was lifted, thereby allowing the cells to progress through cell cycle more easily. In contrast, the physical state of the K562 cells and primary MKs was not substantially different on the ULA surface compared to the TC surface. The fact that inhibitors against cytoskeletal rearrangement, H-1152 (ROCK inhibitor) and blebbistatin (Myosin IIa ATPase inhibitor), also increased CHRF cell mean ploidy on the TC surface further supports the idea that physical interaction of the cells with the culture surface is inhibitory toward cell cycle progression. Myosin IIa mediates contraction of actin stress fibers in maturing cell adhesions [40], as well as in the contractile ring formed at the onset of anaphase in animal cells [41]. Blebbistatin's ability to dramatically increase CHRF cell ploidy in the absence of PMA-induced differentiation suggests that its inhibitory effect on contractile ring formation may overshadow any effects on differentiation-induced and/or adhesion-mediated signaling. These results correspond with a study from Shin et al. where blebbistatin treatment resulted in polyploid, non-MK cells [27], suggesting that blebbistatin can target non-MK-specific signaling pathways.

PPF was completely inhibited for CHRF cells cultured on a ULA surface, whereas a small percentage of primary MKs formed proPLTs on a ULA-like, polyHEMA surface. This suggests that adhesion is required for initiation of PPF in CHRF cells, but not for primary MKs. This disparity is also likely related to the phenotypical differences between the type cell morphology

displayed during PPF for the two cell types. CHRF cells with PPF are tightly adherent, as evidenced by F-actin formation (Figure 1A, B), whereas primary MK cell bodies display loose adhesion during PPF (Figure 7A, C). This suggests that CHRF cell PPF likely requires stable adhesion, whereas studies have shown that these conditions are inhibitory towards primary MK PPF [20, 23]. In addition, although a few primary MKs formed proPLTs on the polyHEMA surface, the percentage of cells with proPLTs increased substantially after transfer to a TC surface (Figure 7B). This result suggests that adhesion promotes primary MK PPF.

It is important to note that the CHRF cells are cultured in medium supplemented with serum, which contains many different proteins that can be bound by cell surface receptors. Specifically, the RGDS adhesion peptide, which is present in many ECM proteins [42], was found to be important in PMA-treated CHRF cell adhesion [43]. In contrast, the primary MKs in this study were cultured in medium supplemented with the serum substitute BIT, which contains albumin from bovine serum, recombinant insulin, and transferrin from human plasma. Although studies have shown that PLT surface receptors can bind albumin that has unfolded to a certain degree [44, 45], the same studies have not been performed for MKs. However, the higher PPF observed on a TC surface, compared to a ULA surface, in the absence of any added ECM proteins, suggests that MKs may also be able to interact with adsorbed albumin. Alternatively, perhaps MKs secrete a protein that mediates their adhesion to a TC surface.

We typically observe that *in vitro* PLT generation occurs over a period of up to 5 days. In order to preserve the functionality of PLTs produced at the beginning of this period, we have suggested having multiple PLT harvest time points during the culture and have published on the use of an

automated, spinning-membrane-filtration-based, cell processing system for this purpose [37]. In the current study, we observed lessened PPF for primary MKs cultured on a ULA surface, followed by a burst of PPF when MKs were transferred to a TC surface on day 11 (Figure 7B). A similar burst in PPF was observed after transfer of CHRF cells from ULA to TC. These results suggest that staggered MK culture, first on a ULA surface then on a TC surface, could help synchronize PPF and subsequent PLT release, thus reducing the need for as many PLT harvest time points, without compromising overall PLT yield and functionality. It is important to note that despite the burst in PPF, the maximal percentage of PPF was similar to or lower for ULA-to-TC transferred MKs compared to TC only MKs (Figure 7B, compare last time points). Further studies to quantify proPLT length and formation of functional, unactivated PLTs should be performed to better understand the effect of ULA preculture on *in vitro* PLT generation.

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