TECHNICAL BULLETIN

Generation of Human Blood Cells and Microglia from Human Pluripotent Stem Cells at Scale

Human pluripotent stem cells (hPSCs) are an emerging alternative source for generating hematopoietic progenitor cells (HPCs) for applications in disease modeling, drug discovery, and the development of cell and gene therapies for hematological disorders. hPSC-derived hematopoietic cells offer several advantages over primary cells, including an unlimited supply, reduced donor dependency, and compatibility with genetic modification. These features are highly beneficial for drug testing and cell/gene therapy workflows, as evidenced by the use of hPSCs in numerous clinical trials in blood transfusion and chimeric antigen receptor (CAR) immunotherapy.

This technical bulletin provides comprehensive instructions for generating HPCs using a scalable 3D suspension culture system. It covers recommendations on seeding densities, culture conditions, and vessel selection, along with methods for evaluating HPC quality. Additionally, this bulletin discusses data demonstrating the comparability of HPCs generated in the 3D suspension system and the standard 2D monolayer protocol, including efficiency of downstream differentiation into myeloid and erythroid lineage.

Background

During human embryonic development, hematopoietic cells arise through three consecutive and overlapping waves.[1,2] The first wave generates predominantly primitive blood cells, including nucleated erythrocytes and low-ploidy megakaryocytes, which support the early needs of the growing embryo. The second wave produces HPCs, including erythro-myeloid progenitors (EMPs) and lympho-myeloid progenitors (LMPs), which can differentiate into functional blood cells such as erythrocytes, megakaryocytes, and mature myeloid and lymphoid cells. The third wave gives rise to definitive hematopoietic stem cells (HSCs) with multilineage differentiation and long-term engraftment potential, capable of sustaining hematopoiesis throughout adult life. Due to current limitations in understanding developmental precursors and intermediates, as well as the scarcity of HSCs during embryogenesis, most existing hematopoietic differentiation protocols cannot efficiently generate large numbers of engraftable HSCs in vitro.[3-5] Nevertheless, current culture methods can produce HPCs that differentiate into functional, mature blood cells, offering therapeutic potential in areas such as transfusion support, disease modeling, drug screening, and immunotherapy.[6]

Current hPSC hematopoietic differentiation protocols can be broadly categorized into 2D monolayer and 3D embryoid body (EB)-forming suspension approaches. Each method has distinct advantages and limitations.[7] The 2D protocol is easier to implement, allows quicker adoption, and generates high numbers of floating cells that are easy to harvest. It does not require cell dissociation or specialized equipment such as low-attachment plates. In contrast, the 3D protocol is more amenable to scale-up because cell production is not limited by the surface area of the culture vessel and does not require surface-coating materials. For instance, large-scale vertical-wheel bioreactors occupy a smaller footprint than multilayer cell factories. Recently, an improved 3D approach—the swirling EB method—was introduced. This method allows EBs to continuously release floating CD34+ cells into the culture medium, which are then ready for immediate downstream applications, removing the need for EB dissociation. These CD34+ cells obtained via the swirling EB method have demonstrated long-term engraftment with multilineage reconstitution, marking a major milestone in the field.[8]

STEMdiff™ Hematopoietic Kit (Catalog #05310) efficiently generates HPCs from hPSCs maintained either in 2D adherent culture (in mTeSR™ Plus; Catalog #100-0276/100-1130) or in 3D suspension culture (in TeSR™-AOF 3D; Catalog #100-0720). This differentiation process occurs via an endothelial-to-hematopoietic (EHT) transition in both 2D monolayer and 3D swirling EB suspension cultures (Figure 1). The kit's chemically defined, animal component-free (ACF) formulation ensures lot-to-lot consistency and reproducible results across diverse hPSC lines, making it suitable for preclinical cell and gene therapy applications.

The following protocol outlines the commonalities and differences between the 2D monolayer and the 3D suspension differentiation methods. It highlights key process parameters for achieving robust hematopoietic differentiation in the 3D system using various culture vessels, including PBS-MINI bioreactors. A comprehensive comparison follows the protocol, evaluating the yield of generated HPCs and their multilineage differentiation efficiency between the standard 2D method (performed in Matrigel®-coated 12-well plates) and the 3D protocol (performed in 6-well plates/Nalgene™ bottles on orbital shakers and in PBS-MINI bioreactors). The downstream differentiation and functionality of the mature progeny, such as microglia, erythroid cells, and megakaryocytes, were also evaluated using the corresponding downstream STEMdiff™ kits. Additionally, the colony-forming potential of the HPCs was evaluated using serum-free MethoCult™ medium.

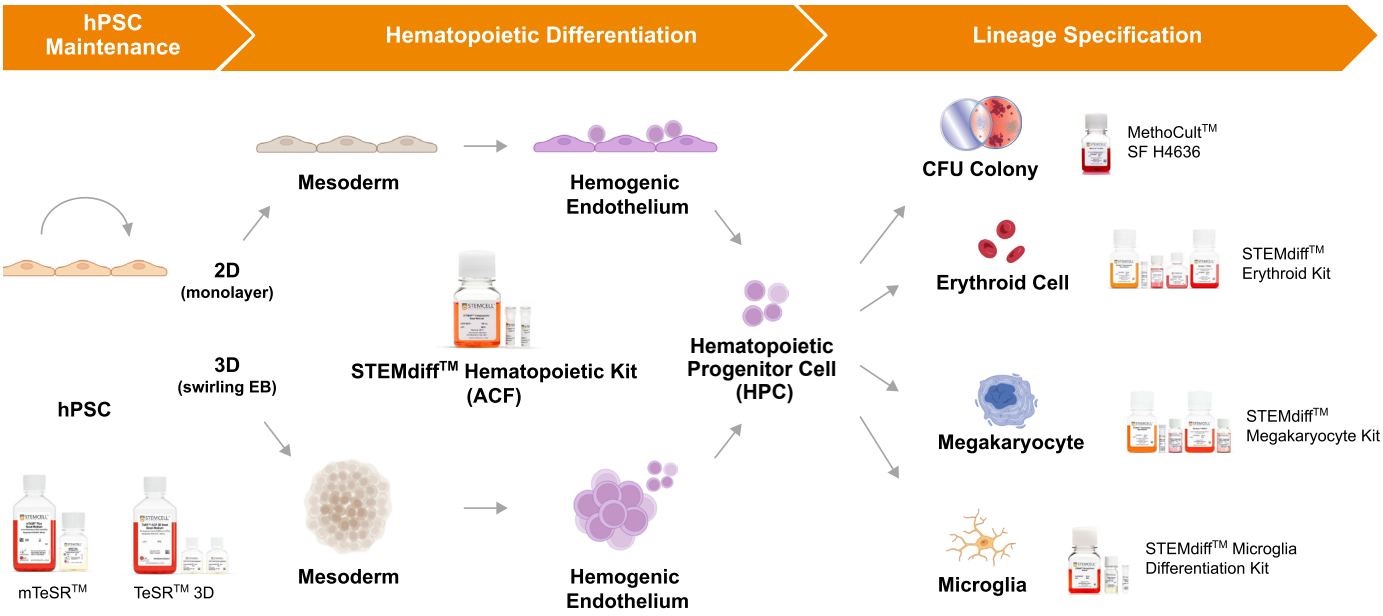


Figure 1. Workflow for Hematopoietic Differentiation from Human Pluripotent Stem Cells

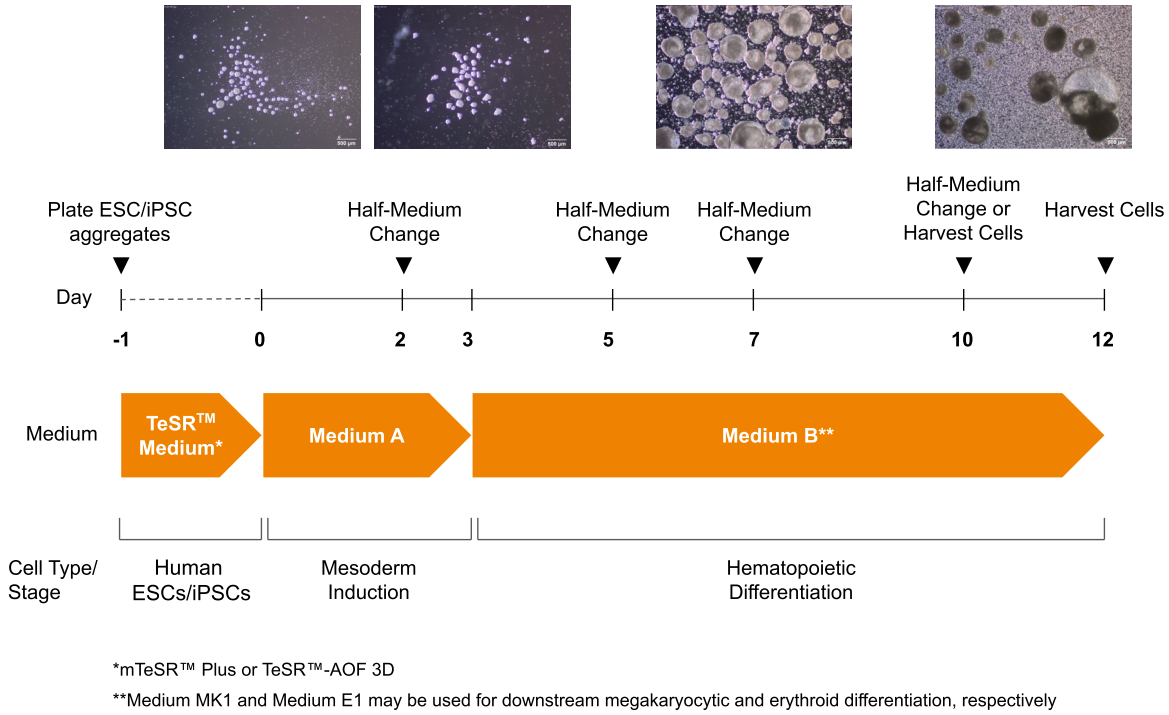
hPSCs can be maintained and expanded in 2D adherent culture in mTeSR™ media (mTeSR™1 or mTeSR™ Plus) or in 3D suspension culture in TeSR™ 3D media (TeSR™-AOF 3D or mTeSR™3D). Using STEMdiff™ Hematopoietic Kit, both 2D- and 3D-maintained hPSCs can be efficiently differentiated via successive steps into mesoderm, hemogenic endothelium, and HPCs. STEMdiff™ Hematopoietic Kit is compatible with both 2D monolayer and 3D swirling EB workflows, and both workflows support similar differentiation efficiency and yield of HPCs. The generated HPCs display multilineage differentiation potential, as they can generate colony-forming unit (CFU)-derived colonies in the methylcellulose-based MethoCult™ medium and can be further differentiated into downstream cell types, including erythroid cells, megakaryocytes, and microglia using the corresponding downstream STEMdiff™ kits.

Materials Required

Product	Catalog #
STEMdiff™ Hematopoietic Kit	05310
STEMdiff™ Microglia Differentiation Kit	100-0019
STEMdiff™ Erythroid Kit	100-0074
STEMdiff™ Megakaryocyte Kit	100-0900
MethoCult™ SF H4636	04636
mTeSR™ Plus or TeSR™-AOF 3D	100-0276/100-1130 or 100-0720
Gentle Cell Dissociation Reagent or ReLeSR™	100-0485 or 100-0483
37 µm Reversible Strainer - Large (> 2 mL culture media)	27250
70 µm Reversible Strainer - Small (≤ 2 mL culture media)	27216
70 µm Reversible Strainer - Large (> 2 mL culture media)	27260

Product	Catalog #
Y-27632	72304/72308
IMDM	36150
6-Well Flat-Bottom Plate, Non-Treated	38040
Nalgene™ Rapid-Flow™ Sterile Filter Storage Bottles	Thermo Fisher 455-0250
PBS-MINI Bioreactor Base Unit	100-1005
PBS-MINI 0.1 MAG Single-Use Vessel	100-1006
PBS-MINI 0.5 MAG Single-Use Vessel	100-1007

Fundamental differences exist between 2D adherent and 3D suspension culture that impact the behavior of the cells, notably the presence or absence of a matrix and culture agitation. To account for these, process parameters were modified to develop a robust 3D hematopoietic differentiation protocol (Figure 2). Commonalities and differences between the 2D monolayer and 3D suspension differentiation protocol are outlined in Table 1.



*mTeSR™ Plus or TeSR™-AOF 3D

**Medium MK1 and Medium E1 may be used for downstream megakaryocytic and erythroid differentiation, respectively

Figure 2. Hematopoietic Differentiation Protocol in 3D Suspension Culture Using STEMdiff™ Hematopoietic Kit

In the 3D swirling EB workflow, the same reagents, seeding method, culture length, and feeding strategy can be applied as in the standard 2D workflow described in the STEMdiff™ Hematopoietic Kit (Catalog #05310) Product Information Sheet (PIS). On Day -1, 2D or 3D-maintained hPSCs are plated as clumps (≥ 50 µm diameter, ~100 cells per clump) at a density of 1.5 - 2 x 10^4 cells/mL (~150 - 200 clumps/mL) in mTeSR™ Plus (Catalog #100-0276/100-1130) or TeSR™-AOF 3D (Catalog #100-0720). For small-scale expansion, cells are cultured in 6-well flat-bottom plates on an orbital shaker (70 RPM for 2.5 cm orbital diameter); large-scale cultures use PBS-MINI 0.1 L vessels (Catalog #100-1005/100-1006) at 40 RPM. A constant agitation speed in RPM is maintained throughout the protocol to support continuous EB formation. After one day, TeSR™ medium is replaced with Medium A (STEMdiff™ Hematopoietic Basal Medium + STEMdiff™ Hematopoietic Supplement A) to induce mesoderm differentiation. On Day 3, the medium is changed to Medium B/MK1/E1 (STEMdiff™ Hematopoietic Basal Medium + STEMdiff™ Hematopoietic Supplement B/Megakaryocyte Supplement MK1/Erythroid Supplement E1) for induction of endothelial-to-hematopoietic transition (EHT) and hematopoietic specification. During this phase, hPSC-derived HPCs emerge from the surface of EBs and are released into suspension. By Day 10 - 12, large numbers of floating single cells can be harvested from the culture supernatant with reversible strainers prior to evaluation.

Table 1. Recommended Protocol Modifications to Use the STEMdiff™ Hematopoietic Kit in 3D Suspension Culture

Process Parameter	2D Monolayer Protocol	Recommended Modification for 3D Suspension Culture
Seeding Density	4 - 8 x 10^3 cells/mL (~40 - 80 clumps/mL)	1.5 - 2 x 10^4 cells/mL (~150 - 200 clumps/mL)
Seeding Method	Clump (≥ 50 µm diameter)	Clump (≥ 20 µm diameter)
Seeding Supplement	N/A	10 µM Y-27632
Media Change Strategy	100% change when switching media; 50% change otherwise	100% change when switching media; 50% change otherwise
Differentiation Initiation Day	1	0 - 1*
Length of Differentiation (days)	12	10 - 12
Agitation	N/A	Reference TeSR™-AOF 3D Technical Manual: Table 1. Suspension Culture Vessels

** To skip the initiation day, seed cells directly into Medium A instead of TeSR™ medium and use a higher density of 1.5 - 2 x 10^5 cells/mL.*

A. Preparation of Media

For complete instructions on preparation of media and storage conditions, refer to the following Product Information Sheets (PIS):

- mTeSR™ Plus Product Information Sheet
- TeSR™-AOF 3D Product Information Sheet
- STEMdiff™ Hematopoietic Kit Product Information Sheet
- STEMdiff™ Microglia Differentiation Kit Product Information Sheet
- STEMdiff™ Megakaryocyte Kit Product Information Sheet
- STEMdiff™ Erythroid Kit Product Information Sheet

B. Passaging Aggregates and Differentiation Setup (hPSC Seeding Day -1)

This protocol is for hPSCs maintained in 2D in mTeSR™ Plus medium on Corning® Matrigel®-coated plates or in 3D suspension culture in TeSR™-AOF 3D. It is critical to start with high-quality hPSC cultures (i.e. high expression of markers of the undifferentiated state, such as OCT4 and TRA-1-60) for efficient hematopoietic differentiation.

1. Prepare enough mTeSR™Plus + 10 µM Y-27632 OR TeSR™-AOF 3D + 10 µM Y-27632 to resuspend and seed all conditions. Warm medium to 37°C in an incubator with the cap slightly opened to allow for CO_2 equilibration.

2. Passage hPSCs as small clumps (~20 - 100 µm in diameter), resuspending in mTeSR™Plus + 10 µM Y-27632 OR TeSR™-AOF 3D + 10 µM Y-27632, using one of the following protocols:

    a. 2D hPSC maintenance in mTeSR™Plus:

        i. Gentle Cell Dissociation Reagent: Clump passaging protocol as described in the technical manual for mTeSR™ Plus.

        ii. ReLeSR™: Clump passaging protocol as described in the Product Information Sheet (PIS; Document #10000008733) for ReLeSR™.

    b. 3D hPSC maintenance in TeSR™-AOF 3D:

        i. Gentle Cell Dissociation Reagent: Clump passaging protocol as described in the technical manual for TeSR™-AOF 3D.

3. Gently flick the tube to resuspend clumps. Use a portion of a sample for viable cell counts and calculate the volume of cell suspension required for seeding at a density of 1.5 - 2 x 10^4 cells/mL (~150 - 200 clumps/mL).

    Note: *It is recommended to use automated viable cell counts (e.g. A100-B Assay on the ChemoMetec NucleoCounter® NC-250™) to reduce seeding variability.*

4. Add required volume of clump suspension to the culture vessel, then top up with warm mTeSR™Plus + 10 µM Y-27632 OR TeSR™-AOF 3D + 10 µM Y-27632 to desired total volume.

5. Incubate for 24 hours at 37°C at the recommended agitation rate (RPM) as detailed in Table 2.

    Note: *As the Nalgene™ Rapid-Flow™ Sterile Filter Storage Bottles do not have vented caps, open the caps slightly when placing the bottles in the incubator to allow gas exchange.*

Table 2. Agitation Rate for Recommended Culture Vessels for Suspension Culture

Culture Vessel	Culture Volume	Recommended Agitation Rate	Relative Centrifugal Force (RCF)
6-Well Flat-Bottom Plate, Non-Treated	2 mL	70 RPM (2.5 cm orbital diameter)*	0.06848
Nalgene™ Rapid-Flow™ Sterile Filter Storage Bottles	15 mL 30 mL 60 mL	40 RPM 55 RPM 65 RPM (2.5 cm orbital diameter)*	0.02236 0.04227 0.05904
PBS-MINI 0.1 MAG Single-Use Vessel	100 mL	40 RPM	N/A
PBS-MINI 0.5 MAG Single-Use Vessel	500 mL	45 RPM	N/A

** For orbital diameters other than 2.5 cm (1.25 cm orbital radius), use the following equation to calculate the adjusted RPM. Further RPM optimization may be required.*

$$RPM_{Adjusted} = \sqrt{\frac{RCF}{1.118 \times 10^{-5} \times (\text{Orbital Radius in cm})}}$$

C. Hematopoietic Differentiation (Media Changes Days 0 - 10)

For preparation of Medium A and B, refer to the Preparation of Media section.

For Day 0 to 10, perform medium-changes as detailed in Table 3.

Table 3. Medium Change Workflow

Day	Medium	% Medium-Change
0	Medium A	100
2	Medium A	50
3	Medium B	100
5	Medium B	50
7	Medium B	50
10	Medium B	50

The following are instructions for performing media changes in different culture vessels.

6-well plate:

1. Aliquot required volume of fresh medium for the medium change into a tube or bottle and warm to 37°C in an incubator with the cap slightly opened to allow for CO_2 equilibration. Once warmed, proceed to step 2.
2. Swirl plate to center the aggregates, then place the plate at a 30 - 45° angle and allow aggregates to settle for ~ 1 minute.
3. Remove required volume of spent media using a P1000 pipette without disturbing the aggregates (e.g. 2 mL for a 100% medium change; 1 mL for a 50% medium change).

   Note: *It may be challenging to remove 2 mL per well without aspirating any aggregates. If needed, reduce to a 95% medium change instead of a 100% medium change in the 6-well plate.*
4. Replace with an equal volume of warmed fresh medium.

   Note: *To maximize yields, a 50% volume top up can be performed instead of the Day 10 50% medium change. Alternatively, the single cells removed during the 50% medium change can be collected by centrifugation and added back into the culture.*

Nalgene™ Bottle:

1. Aliquot required volume of fresh medium for the medium change into a tube or bottle and warm to 37°C in an incubator with the cap slightly opened to allow for CO_2 equilibration. Once warmed, proceed to step 2.
2. Remove required volume of spent media with a serological pipette and pass over a Large 37 µm Reversible Strainer with the arrow pointing up to collect removed aggregates (50% or 100% of the seed volume).
3. Flip strainer back over the Nalgene™ Bottle such that the arrow is pointing down, and rinse aggregates back in with an equal volume of warmed fresh medium.

   Note: *To maximize yields, a 50% volume top up can be performed instead of the Day 10 50% medium change. Alternatively, the single cells removed during the 50% medium change can be collected by centrifugation and added back into the culture.*

PBS-MINI:

1. Aliquot required volume of fresh medium for the medium change into a bottle and warm to 37°C in an incubator with the cap slightly opened to allow for CO_2 equilibration. Once warmed, proceed to step 2.
2. Remove required volume of spent media with a serological pipette and pass over a Large 37 µm Reversible Strainer with the arrow pointing up to collect removed aggregates (50% or 100% of the seed volume).
3. Add 80% of the required volume of warmed fresh media into the PBS-MINI.
4. Flip strainer back over the PBS-MINI such that the arrow is pointing down and rinse aggregates back in with the remaining 20% of the required volume of fresh medium.
5. This eliminates the “death drop” of the aggregates being rinsed back into an empty vessel and colliding with the wheel.

   Note: *To maximize yields, the single cells removed during the Day 10 50% medium change can be collected by centrifugation and added back into the culture.*

D. Harvest of Hematopoietic Progenitor Cells (Harvest Day 10 - 12)

The following are instructions for harvesting the shed single cell HPCs from different culture vessels.

6-Well Plate:

1. Place plate at a 30 - 45° angle and pipette well content up and down 2 - 5 times with a 2 mL serological pipette (on SLOW speed).
2. Allow aggregates to settle for 15 - 30 seconds.
3. Collect supernatant with a P1000 pipette and dispense over a Small 70 µm Reversible Strainer with the arrow pointing up into a 15 mL conical tube.
4. Add 1 mL IMDM to each well and triturate as in step 1.

5. Filter out the aggregates by passing the entire well contents over the strainer using a serological pipette.
6. Rinse well with 1 mL IMDM and dispense rapidly over the strainer.
7. Discard strainer with aggregates.
8. Optional: Centrifuge conical tube at 300 x g for 5 minutes. Aspirate supernatant and resuspend cells in desired media at the required cell concentration. Flick or pipette to resuspend.
9. Remove a sample for viable cell counts.

   Note: *It is recommended to use the AODAPI Assay on the ChemoMetec NucleoCounter® NC-250™ for single cell suspension cell counts.*

Nalgene™ Bottle / PBS-MINI

1. Pipette vessel contents up and down 2 - 5 times with a serological pipette (on SLOW speed).
2. Filter out the aggregates by passing the entire vessel contents through a Large 70 µm Reversible Strainer with the arrow pointing up into a 50 mL conical tube or sterile collection bottle.

   Note: *Multiple strainers may be required for the PBS-MINI cultures.*
3. Rinse culture vessel 1 - 2 times with 5 - 10 mL IMDM and dispense rapidly over the strainer.
4. Discard strainer with aggregates.
5. Optional: Centrifuge samples at 300 x g for 5 minutes. Aspirate supernatant and resuspend cells in desired media at the required cell concentration. Flick or pipette to resuspend.
6. Remove a sample for viable cell counts.

   Note: *It is recommended to use the AODAPI Assay on the ChemoMetec NucleoCounter® NC-250™ for single cell suspension cell counts.*

Evaluation of Multilineage Differentiation Potential of hPSC-Derived HPCs Generated in 3D Suspension Culture

To assess the functionality of HPCs generated in 3D suspension culture using STEMdiff™ Hematopoietic Kit, we first compared the marker expression and cell yield of HPCs generated using three different culture platforms: the standard 2D monolayer protocol in Corning® Matrigel®-coated 12-well plates, the 3D protocol in 6-well plates/Nalgene™ bottles on orbital shakers, and the 3D protocol in PBS-MINI 0.1 L vessels. All three culture methods were performed with the same reagents and feeding strategy as described in Figure 2. These comparisons were performed across multiple hPSC lines, including human embryonic stem cell (hESC) line H9 and human induced pluripotent stem cell (hiPSC) lines WLS-1C, SCTi003-A (Catalog #200-0511), and SCTi004-A (Catalog #200-0769).

HPCs generated with all three platforms consistently expressed high levels of pan-hematopoietic marker CD43, with expression exceeding 90% (Figure 3A, B). While CD34 and CD45 co-expression levels varied among platforms, all three methods successfully generated high yields of CD34+CD45+ cells. On average, each platform produced nearly one million CD34+CD45+ cells per mL of culture volume. In one PBS-MINI 0.1 L experiment, this is equivalent to a total output of 100 million CD34+CD45+ HPCs.

We next assessed the functional potential of the generated HPCs using a colony-forming unit (CFU) assay. The cells were plated in a serum-free, methylcellulose-based medium, MethoCult™ SF H4636 (Catalog #04636), and after 14 days colonies were enumerated and classified as CFU-GEMM colonies (representing multi-potential progenitors), BFU-E colonies (derived-from-erythroid progenitor cells), and CFU-GM colonies (generated by granulocyte/macrophage progenitor cells). All three platforms produced colonies with comparable morphology, size, total CFU yield, and colony subtype distribution (Figure 3C, D). These results confirm the HPCs generated across these platforms are functional and can differentiate into myeloid and erythroid lineages.

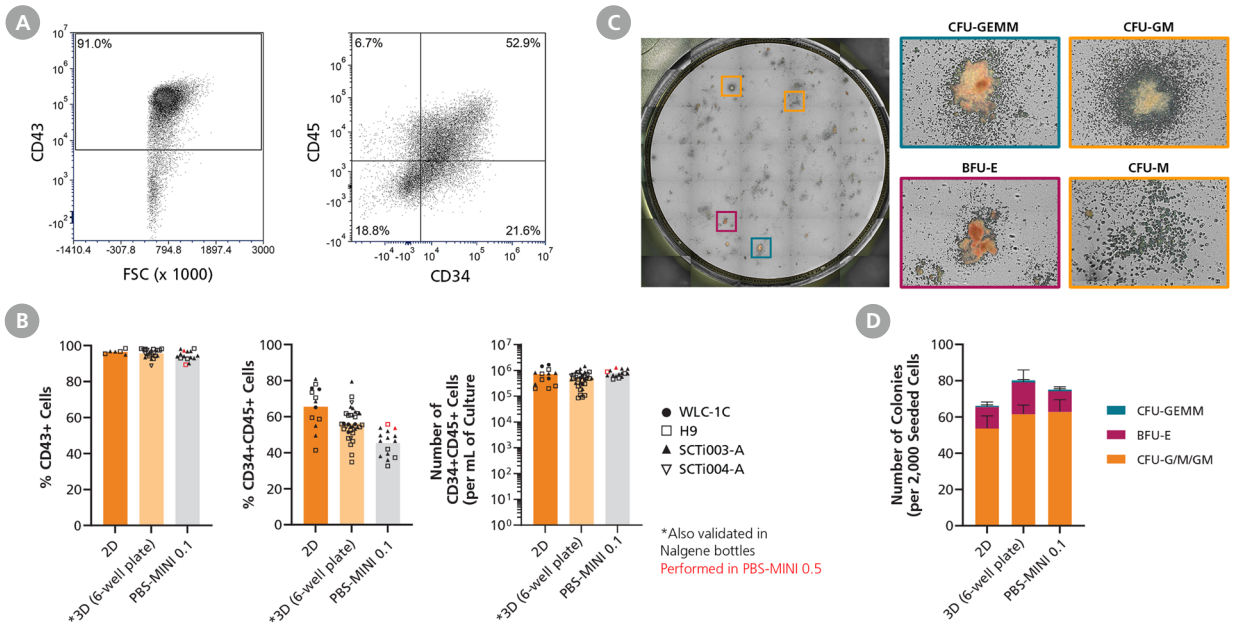


Figure 3. The Scalable 3D Workflow Generates High Numbers of Hematopoietic Progenitor Cells (HPCs) with Multilineage Colony Forming Unit (CFU) Potential

Multiple hPSC lines, including human embryonic stem cell (hESC) line H9, and human induced pluripotent stem cell (hiPSC) lines WLS-1C, SCTi003-A (Catalog #200-0511), and SCTi004-A (Catalog #200-0769) were induced to differentiate to HPCs following the standard 2D monolayer protocol in Corning® Matrigel®-coated 12-well plates or the 3D suspension culture protocol in both 6-well plates on orbital shakers and PBS-MINI 0.1 L vessels as described in Figure 2. On Days 10 - 12, cells were harvested from the supernatant and analyzed for expression of CD34, CD45, and CD43 markers by flow cytometry. Dead cells were excluded by light scatter profile and propidium iodide (PI) staining. On Day 12, cells were also assessed in colony-forming unit (CFU) assays using methylcellulose-based MethoCult™ SF H4636 (Catalog #04636) medium. (A) Representative flow cytometry plots of Day 12 hiPSC-derived (SCTi003-A) HPCs generated using PBS-MINI 0.1 L vessels. (B) Frequencies of CD43+ cells, CD34+CD45+ cells, and yield of CD34+CD45+ cells per mL of media generated from the standard 2D protocol or from the 3D suspension culture protocol (including both 6-well plate and PBS-MINI 0.1 L vessel formats). Each dot represents one biological replicate. While CD34 and CD45 co-expression levels varied among the platforms, all three platforms consistently produced high levels of pan-hematopoietic marker CD43 and generated high yields of CD34+CD45+ cells. On average, each platform produced nearly one million CD34+CD45+ cells per mL of culture volume (total output of 100 million CD34+CD45+ cells in one PBS-MINI 0.1 L experiment). (C) Representative CFU colony images of hESC-derived (H9) HPCs generated from PBS-MINI 0.1L vessels. Colonies were imaged with the STEMvision™ instrument and counted manually from digital images. The progenitor cell types observed included multi-potent progenitor (CFU-GEMM) colonies, erythroid progenitor (BFU-E) colonies, and granulocyte/macrophage progenitor (CFU-M, CFU-G and CFU-GM) colonies. (D) CFU output expressed as the total number of colonies per 2,000 hPSC-derived HPCs plated. Data are shown as mean ± SEM (n = 2 - 4 for H9, SCTi003-A, and SCTi004-A). Across all three platforms, robust colony formation with comparable morphology, size, total CFU yield, and colony subtype distribution was observed.

We next evaluated whether HPCs generated using the STEMdiff™ Hematopoietic Kit in 3D suspension culture can be differentiated into functional microglia, the tissue-resident macrophages of the brain. Microglia possess a unique transcriptional signature and play essential roles in brain development and immune defense, making them valuable tools for modeling neurodegenerative and neuroinflammatory disorders.[9,10] All three platforms produced abundant floating single cells with over 90% CD43 expression (Figure 3A, B), enabling these cells to proceed directly to microglia differentiation using STEMdiff™ Microglia Differentiation Kit (Catalog #100-0019). This workflow is adapted from the Blurton-Jones Laboratory protocol[11] but does not require isolation of CD43+ cells prior to microglia differentiation. The HPCs generated in 3D suspension culture from both small-scale 6-well plates and PBS-MINI 0.1 L vessels differentiated efficiently into microglia that exhibited strong expression of macrophage markers CD11b and CD45, as well as microglia-specific markers P2RY12 and CX3CR1, comparable to those derived from the standard 2D protocol (Figure 4A, B). Cell yields were consistently high across all platforms and hPSC lines tested.

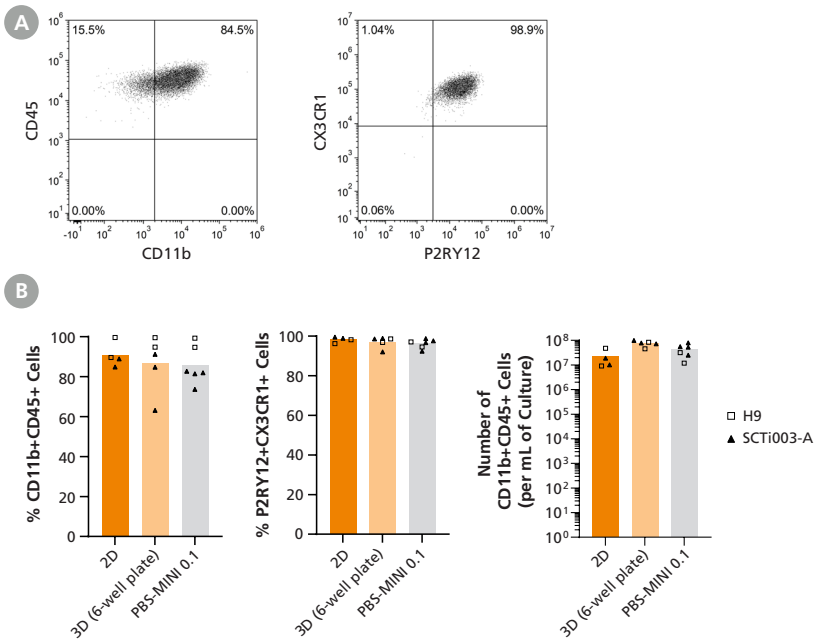


Figure 4. The HPCs Generated from the 3D Workflow Display Microglia Differentiation Potential

The Day 12 HPCs generated using STEMdiff™ Hematopoietic Kit from all three platforms (the standard 2D monolayer protocol, the 3D protocol in 6-well plates, and the 3D protocol in PBS-MINI 0.1 L vessels) were differentiated into microglia for an additional 24 days using STEMdiff™ Microglia Differentiation Kit (Catalog #100-0019) following the PIS protocol. At the end of the protocol, cells were harvested and analyzed by flow cytometry for expression of macrophage markers CD11b and CD45, and microglia-specific markers P2RY12 and CX3CR1. Dead cells were excluded by light scatter profile and propidium iodide (PI) staining. (A) Representative flow cytometry plots of hiPSC-derived (SCTi003-A) microglia generated from PBS-MINI 0.1. (B) Frequencies of CD11b+CD45+ cells and P2RY12+CX3CR1+ cells, and numbers of CD11b+CD45+ cells derived from the Day 12 HPCs generated from the standard 2D protocol and from the 3D suspension culture protocol (including both 6-well plate and PBS-MINI 0.1 L formats). Each marker represents one biological replicate. The HPCs generated from both small-scale 6-well plates and large-scale PBS-MINI 0.1 L vessels efficiently differentiated into microglia, exhibiting strong expression of macrophage and microglia markers and high cell yields, comparable to those obtained with the standard 2D protocol.

Functionality of the hPSC-derived microglia was evaluated by measuring responses to inflammatory stimuli such as lipopolysaccharide (LPS). After 24 hours of LPS treatment, microglia generated from all tested platforms exhibited a morphological shift from a homeostatic, ramified resting state, to an activated state characterized by enlarged, rod-shaped morphology (Figure 5A). This LPS response was also reflected by the release of pro-inflammatory cytokines TNFα and IL-6, as measured by ELISA (Figure 5B). The functionality of these hPSC-derived microglia was further evaluated using a phagocytosis assay, where pH-sensitive bioindicator particles were added in cultures and particle uptake was monitored over a 24-hour period using live-cell imaging. As the particles are phagocytosed and taken up by microglia, the particles would fluoresce, allowing for real-time visualization of phagocytic activity. Similar to microglia derived from the 2D-generated HPCs, microglia derived from the 3D-generated HPCs (from both 6-well plates and PBS-MINI vessels) exhibited robust phagocytosis in both the H9 SCTi003-A lines, demonstrating conserved functional capacity across these platforms (Figure 5C).

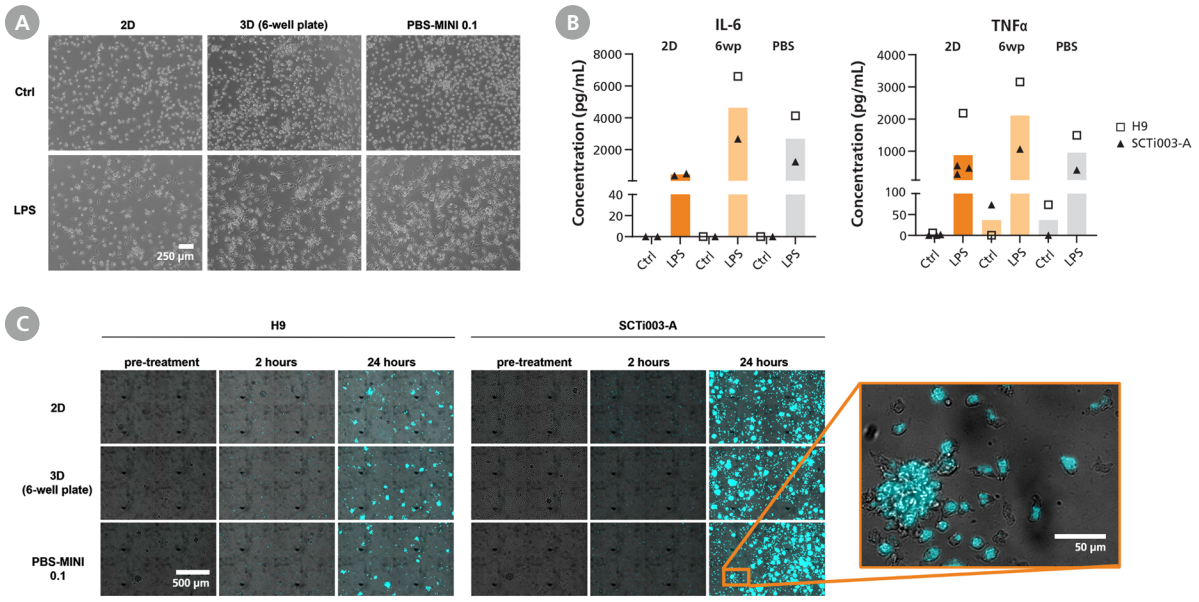


Figure 5. The hPSC-Derived Microglia Respond to Inflammatory Signals and Show Phagocytic Activity

The microglia derived from HPCs generated using all three platforms (the standard 2D monolayer protocol, the 3D protocol in 6-well plates, and the 3D protocol in PBS-MINI 0.1 L vessels) were assessed for functionality, including the ability to respond to inflammatory stimuli such as lipopolysaccharide (LPS), and the ability to phagocytose. At the end of the STEMdiff™ Microglia Differentiation Kit workflow, microglia were stimulated with 100 ng/mL LPS or treated with 5 µg/mL pH-sensitive bioindicator particles for 24 hours. Upon LPS stimulation, (A) bright-field images were taken to examine microglia morphology, and (B) an enzyme-linked immunosorbent assay (ELISA) was performed to measure the concentrations of pro-inflammatory cytokines IL-6 and TNFα in cultures. Each marker represents one biological replicate. The hiPSC-derived (SCTi003-A) microglia generated across platforms exhibited a morphological shift from a homeostatic ramified resting state to an activated state characterized by enlarged, rod-shaped morphology. This LPS response was accompanied by the release of pro-inflammatory cytokines TNFα and IL-6, as observed in the microglia derived from the two hPSC lines (H9 and SCTi003-A) tested across all platforms. Upon pH-sensitive bioindicator particle treatment, (C) live-cell fluorescence imaging was used to monitor particle uptake over a 24-hour period. As the particles are phagocytosed by microglia, the particles fluoresce and concentrate within the cells. Similar to the microglia derived from the 2D-generated HPCs, microglia derived from the 3D-generated HPCs exhibited robust phagocytic activity in both H9 and SCTi003-A lines.

The HPCs generated with the 3D suspension culture method produce BFU-E colonies in the methylcellulose-based CFU assay. To further evaluate their differentiation potential, the generated HPCs were differentiated into erythroid cells or megakaryocytes using STEMdiff™ Erythroid Kit (Catalog #100-0074) and STEMdiff™ Megakaryocyte Kit (Catalog #100-0900), respectively. hPSC-derived erythrocytes and platelets can be used in disease modeling, drug screening, and drug delivery, and hold significant potential for transfusion therapy, addressing critical supply shortages in treating hematological disorders such as sickle cell disease, thalassemia, and thrombocytopenia.[12] Thus, development of cost-efficient, scalable production methods for hPSC-derived erythrocytes and platelets is crucial for realization of their clinical potential.[13] Using STEMdiff™ Erythroid Kit, the HPCs generated from all three platforms efficiently differentiated into CD71+GlyA+ erythroid cells, with yields reaching up to 150 million cells per mL of culture (Figure 6A, B). In one PBS-MINI 0.1 L run, this equates to a total output of 15 billion erythroid cells. Similarly, STEMdiff™ Megakaryocyte Kit robustly differentiates HPCs into CD41a+CD42b+ megakaryocytes across platforms, yielding an average of 1.8 million CD41a+CD42b+ cells per mL of culture volume (Figure 6C, D).

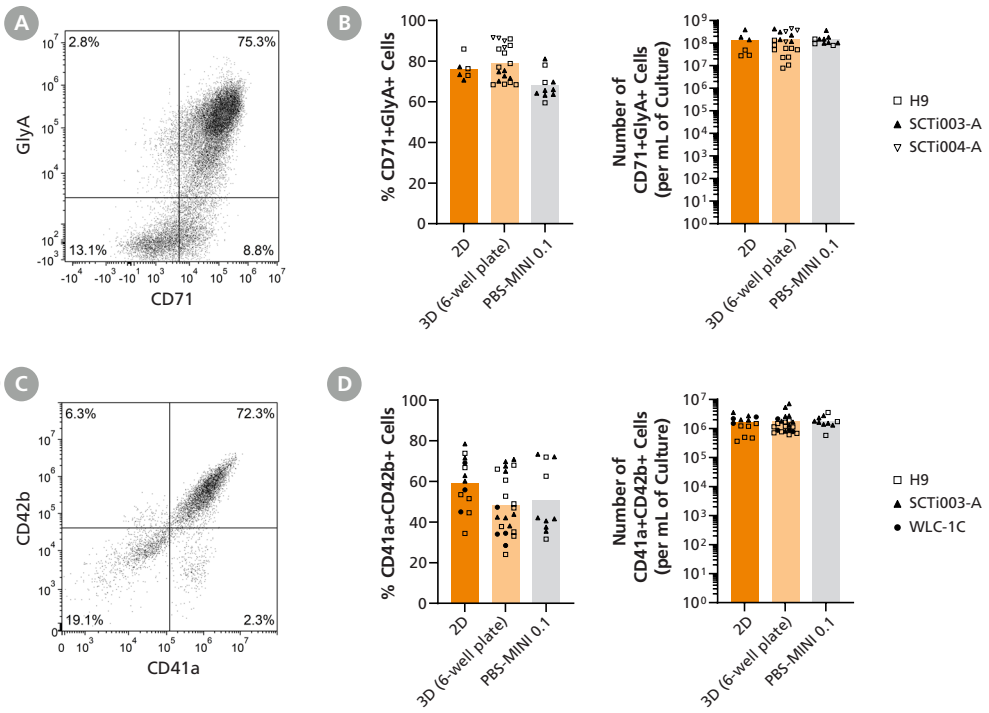


Figure 6. The Scalable 3D HPC Generation Method Is Compatible with Downstream Erythroid and Megakaryocytic Differentiation Workflows

The Day 12 HPCs generated using STEMdiff™ Hematopoietic Kit and all three platforms (the standard 2D monolayer protocol, the 3D protocol in 6-well plates, and the 3D protocol in PBS-MINI 0.1 L vessels) were differentiated into erythroid cells and megakaryocytes for additional 14 or 5 days using STEMdiff™ Erythroid Kit (Catalog #100-0074) or STEMdiff™ Megakaryocyte Kit (Catalog #100-0900), respectively. At the end of the protocols, cells were harvested and analyzed by flow cytometry for expression of erythroid markers CD71 and GlyA, and megakaryocyte markers CD41a and CD42b. Dead cells were excluded by light scatter profile and propidium iodide (PI) staining. (A, C) Representative flow cytometry plots for iPSC-derived (SCTi003-A) erythroid cells and megakaryocytes generated from PBS-MINI 0.1. (B, D) Frequencies and numbers of CD71+GlyA+ cells and CD41a+CD42b+ cells derived from the HPCs generated from the standard 2D protocol and the 3D suspension culture protocol (including both 6-well plate and PBS-MINI 0.1 L vessel formats). Each dot represents one biological replicate. The HPCs generated from all three platforms efficiently differentiated into CD71+GlyA+ erythroid cells and CD41a+CD42b+ megakaryocytes, yielding an average of 150 million erythroid cells and 1.8 million megakaryocytes per mL of culture, respectively. In one PBS-MINI 0.1 L run, this equates to a total output of 15 billion erythroid cells and 180 million megakaryocytes.

We next assessed the quality of the hPSC-derived megakaryocytes and found that at least 25% of the megakaryocytes exhibited a ploidy of 4N or higher (Figure 7A) and that, on average, 3.5 platelet-like particles (PLPs) were shed per megakaryocyte (Figure 7B). This translates to a total output of more than 0.5 billion platelets generated in a single PBS-MINI 0.1 L experiment. Lastly, we evaluated the functionality of the hPSC-derived megakaryocytes and PLPs generated from both 2D and 3D workflows by performing a flow cytometry-based activation assay measuring CD62P expression. CD62P is normally stored in α-granules within the cytoplasm and translocates to the cell surface upon activation, where it stabilizes CD41/CD61–fibrinogen interactions to promote platelet aggregation.[14] In this assay, we simultaneously measured intracellular and surface CD62P levels in CD42+ gated cells following treatment with platelet activation agonists, including adenosine diphosphate (ADP), thrombin receptor activator peptide (TRAP), collagen-related peptide (CRP), and phorbol 12-myristate 13-acetate (PMA). Compared to the resting control condition, all agonist-treated samples showed a significant increase in surface CD62P expression in both megakaryocytes and the shed PLPs across all platforms (Figure 7C, D). These results confirm that the megakaryocytes and their shed PLPs derived from both 2D and 3D approaches are functionally competent and capable of activation.

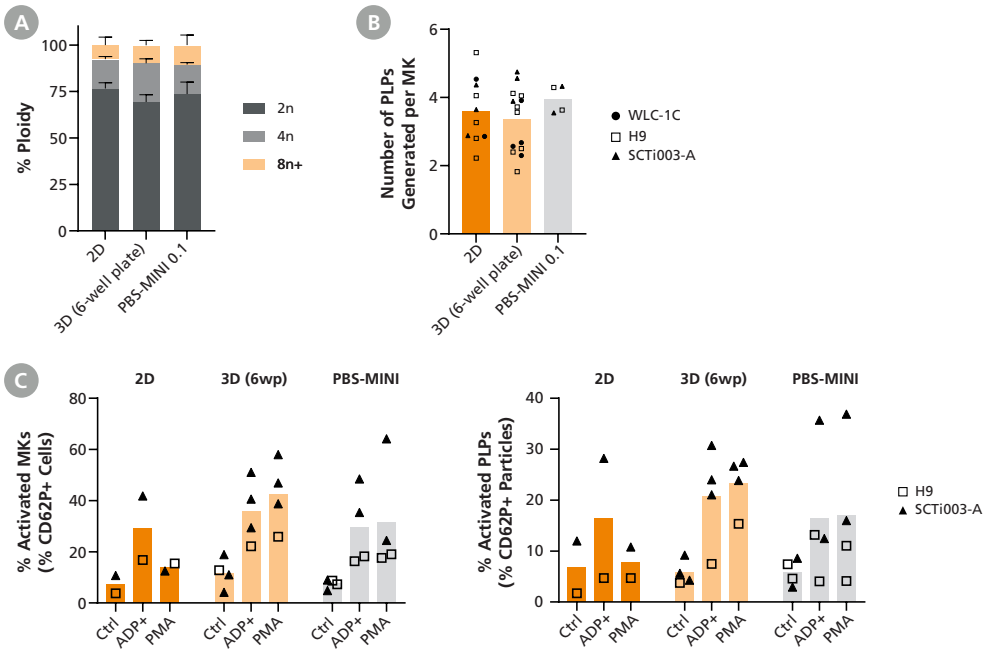


Figure 7. Megakaryocytes Generated Using Either 2D or 3D Workflows Are Polypoid and Functional

The megakaryocytes (MKs) derived from HPCs generated from all three platforms (the standard 2D monolayer protocol, the 3D protocol in 6-well plates, and the 3D protocol in PBS-MINI 0.1 L vessels) were assessed for ploidy distribution, platelet-like particle (PLP) yield, and functionality. At the end of the STEMdiff™ Megakaryocyte Kit protocol, MKs/PLPs were (A) fixed with ethanol and stained with propidium iodide (PI) for DNA ploidy profiling and (B) enumerated based on the size of blood platelets and the numbers of CD41a+CD45-GlyA- PLPs and viable CD41a+ MKs. MKs/PLPs were also assessed in an activation assay (C), in which they were stained with antibodies against cell surface and intracellular CD62P marker following treatment with agonists, including 100 µM adenosine diphosphate (ADP), 100 µM thrombin receptor activator peptide (TRAP), 5 µg/mL collagen-related peptide (CRP), and 0.1 µg/mL phorbol 12-myristate 13-acetate (PMA). Data are shown as mean ± SEM (n = 3-4 for SCTi003-A). Each marker represents one biological replicate. ADP+ includes ADP, TRAP, and CRP. The MKs derived from HPCs generated from all platforms are polyploid (≥ 25% MKs exhibited a ploidy of 4N or higher) and are capable of shedding PLPs (an average of 3.5 PLPs were generated per MK). Both MKs and PLPs generated are functional and capable of activation as they showed an increase in surface CD62P expression in CD42+ gated cells upon agonist treatments. The level of activation is consistent across platforms.

Conclusion

Here we describe an optimized 3D suspension culture protocol using STEMdiff™ Hematopoietic Kit for generating large numbers of hPSC-derived HPCs. This method achieves robust differentiation efficiency and high yields comparable to the standard 2D monolayer protocol. This EB-based 3D workflow is fully scalable and compatible with PBS-MINI bioreactors. A single 0.1 L vessel can produce over 100 million CD34+CD45+ HPCs, which is approximately 100 CD34+CD45+ HPCs per input hPSC. The HPCs generated in this 3D system demonstrate strong colony-forming and multilineage differentiation potential. They give rise to mature cell types including microglia, erythrocytes, and megakaryocytes/PLPs, all of which exhibit functionality comparable to the HPCs generated using the standard 2D monolayer protocol. These data demonstrate that the STEMdiff™ Hematopoietic Kit is adaptable to both 2D and 3D protocols, offering flexibility and support of multiple culture formats, including PSB-MINI bioreactors. Additionally, its chemically-defined, ACF formulation ensures robust and consistent performance across multiple hPSC lines, making it ideal for preclinical research and translational applications such as drug testing, disease modeling, and cell and gene therapy development.

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