

Scale-up of Human Pluripotent Stem Cells and Differentiation to Megakaryocytes or Neural Crest Cells in 3D Suspension Culture

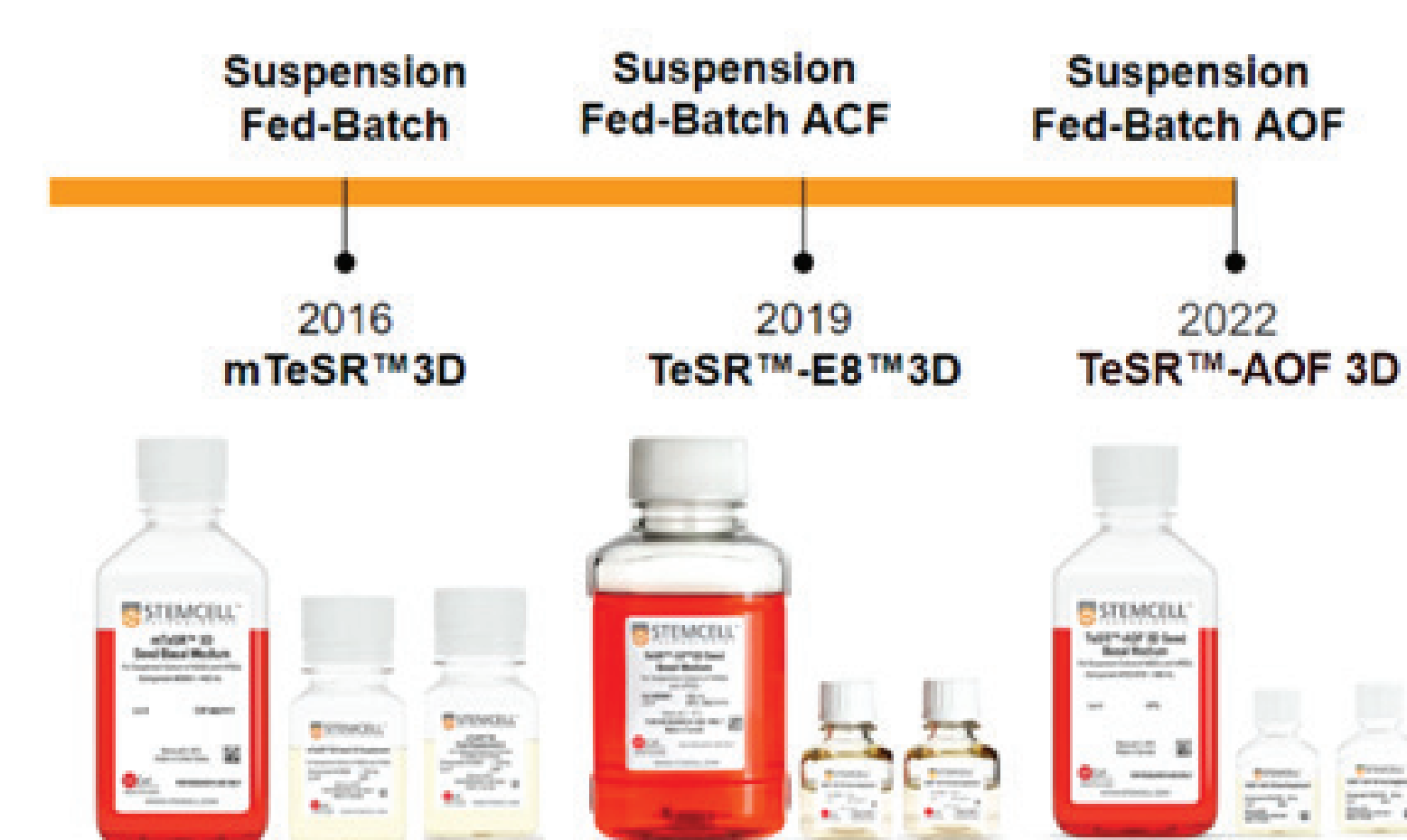
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INTRODUCTION

Generating large quantities of high-quality human pluripotent stem cells (hPSCs) for downstream applications is challenging. Many conventional 2-dimensional (2D) culture methods are not conducive to large-scale expansion, and while 3D suspension culture is a more scalable approach, progress has been hindered by the concurrent shear stress sensitivity and aggregation properties of hPSCs. To address this, we have developed TeSR™ 3D-based media and protocols for robust and reproducible expansion of hPSCs as aggregates in suspension culture. We observed a 1.5- to 1.9-fold expansion per day (cell line-dependent; n = 8 lines) with > 85% viability and > 90% OCT4+ and TRA-1-60+ marker expression, while maintaining a stable karyotype. The downstream differentiation potential of 3D-expanded hPSCs was assessed in 3D suspension cultures using two STEMdiff™ kits previously optimized for 2D differentiation of adherent hPSC monolayers. Following expansion, hPSC aggregates were differentiated into polyploid megakaryocytes (MKs; n = 2 lines) or neural crest cells (NCCs; n = 3 lines) in suspension culture. The initiation and length of differentiation stages were optimized in small-scale cultures using non-treated 6-well plates mixed on a one-inch throw orbital shaker at 70 RPM. For 3D hPSC-generated MKs, a 10-day endothelial-to-hematopoietic transition phase and a 4-day progenitor-to-mature MK stage maximized MK marker expression and yields. For 3D hPSC-derived NCCs, maximum marker expression and yields were achieved by pre-forming hPSC aggregates in TeSR™-AOF 3D media for 48 hours before 6 days of NCC differentiation. When 3D hPSC-derived NCC aggregates were dissociated and seeded on Corning® Matrigel®-coated plates, further differentiation and maturation to sensory neurons expressing peripherin and BRN3A was achieved. 3D hPSC differentiation to NCCs was also performed at a larger scale in a PBS-MINI 0.1 L Bioreactor, yielding 170 million CD271+SOX10+ cells with comparable marker expression to the small-scale control.

Flexible Family of Fed-Batch Optimized Media



3D Suspension Media Design Objectives

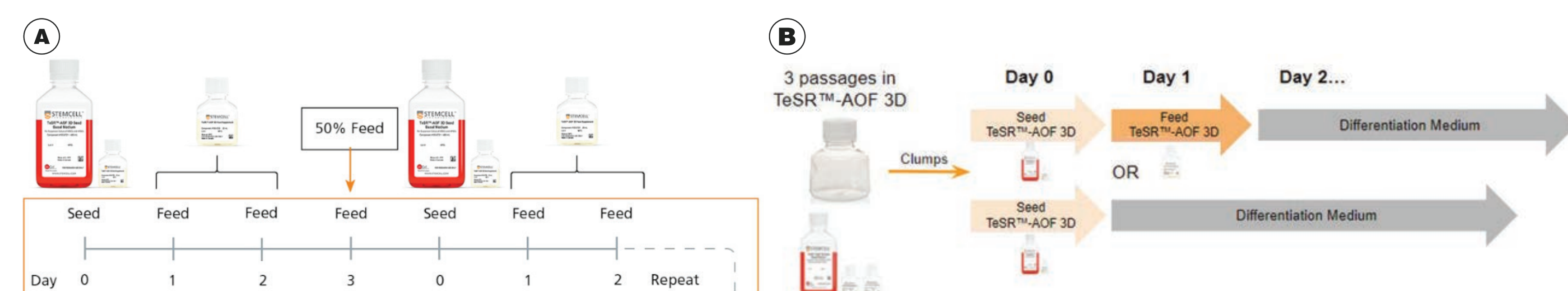
- ✓ No adaptation step from 2D to 3D suspension culture
- ✓ Cells exhibit a sustained daily expansion with viability > 85%
- ✓ Fed-batch protocols minimize culture handling and disruption
- ✓ Non-enzymatic filter based passaging
- ✓ Protocols maintain high glucose and low lactate concentrations
- ✓ After 5 passages in suspension: high expression of markers of undifferentiated hPSCs; functional pluripotency as measured by trilineage differentiation; stable karyotype
- ✓ Lower cost per cell produced compared to traditional 2D culture media

METHODS

hPSC Maintenance: (A) High-quality adherent hPSC cultures are dissociated non-enzymatically to clumps using Gentle Cell Dissociation Reagent (GCDR). Cell clumps are resuspended at 0.5 - 1 x 10⁵ viable cells/mL in seed medium + 10 μM Y-27632. Fed-batch feed supplement is added on days 1 and 2, starting 24 hours after inoculating cell clumps. A half-medium change is performed on day 3 of a 4-day passage. After 3 or 4 days, aggregates are recovered using a 37 μm strainer, then incubated in GCDR at 37°C for 6 minutes. GCDR is removed and aggregates are resuspended in seed medium + 10 μM Y-27632. Immediately after resuspension, aggregates are forced through a 37 μm strainer to generate cell clumps. Clumps are re-seeded into a fresh culture vessel at 0.5 - 1 x 10⁵ viable cells/mL. Cells in 3D suspension culture maintain high expression of markers of the undifferentiated state OCT4 and TRA-1-60.

3D Megakaryocyte Differentiation: (B) After three passages in 3D suspension culture, the aggregates are passaged and seeded as 2 mL suspension cultures in TeSR™-AOF 3D Complete Medium. Megakaryocyte differentiation is initiated after 24 hours using the off-the-shelf STEMdiff™ Megakaryocyte Kit, beginning with Medium A for 3 days followed by Medium MK1 for 7 days at which point the hematopoietic progenitor cells are harvested and reseeded into Medium MK2 for 4 days. Mature megakaryocytes are harvested after 14 days of differentiation.

3D Neural Crest Differentiation: (B) After three passages in 3D suspension culture, the aggregates are passaged and seeded as 2 mL suspension cultures in TeSR™-AOF 3D Complete Medium, followed by a fed-batch feed with TeSR™-AOF 3D Feed Supplement on Day 1. Neural crest differentiation is initiated on Day 2 using the off-the-shelf STEMdiff™ Neural Crest Differentiation Kit, followed by 6 days of differentiation with daily 75% medium changes with the Neural Crest Differentiation Medium. NCCs are harvested on Day 8.



RESULTS

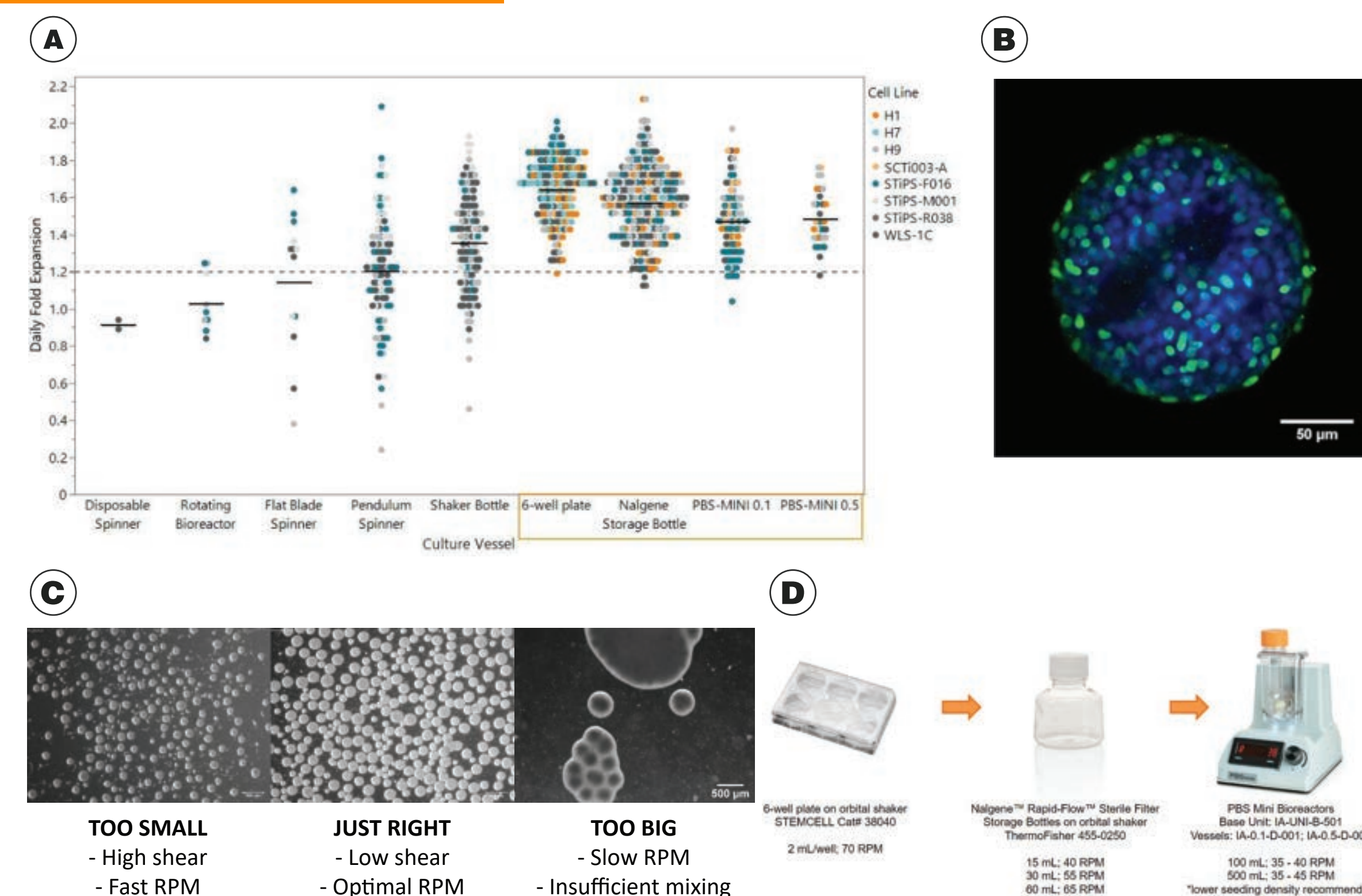


Figure 1. Optimizing Suspension Culture Operation

(A) Daily fold expansion of 8 cell lines in culture vessels tested, with vessels that had consistent daily fold expansion above 1.2 outlined in orange. (B) The immunofluorescent image shows an optically cleared confocal cross-section through a typical PSC aggregate showing these void spaces (blue: DAPI; green: OCT4). (C) 'Good' and 'Bad' aggregate morphology in different culture environments. Optimal aggregates are uniform in size and below 500 μm in diameter at day 4 of culture. They have a dimpled morphology in bright-field images resulting from multiple small voids distributed throughout the aggregate. (D) Cells can be readily scaled up from 2D cultures to 2 mL suspension cultures in 6-well plates, from there up to 60 mL in an orbital shaker bottle, and then into 100 and 500 mL cultures in PBS-MINI MagDrive Bioreactors (Catalog #100-1006 and #100-1007, respectively).

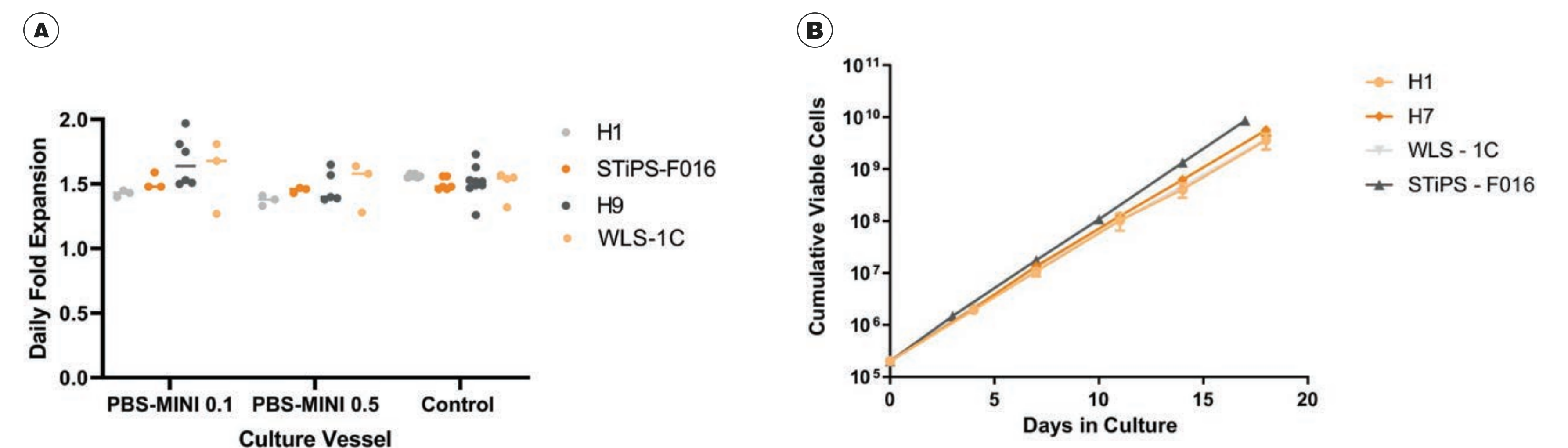


Figure 2. Reproducible Growth in the PBS-MINI MagDrive Bioreactors

(A) Daily fold expansion of hPSC aggregates in mTeSR™3D and TeSR™-AOF 3D in the PBS-MINI alongside control cultures in Nalgene™ Storage Bottles. (B) Consistent expansion of hES and hiPS cell lines as aggregates in TeSR™-AOF 3D across multiple culture vessels. Greater than 10⁹ cells produced after 5 passages in suspension culture with no adaptation passage.

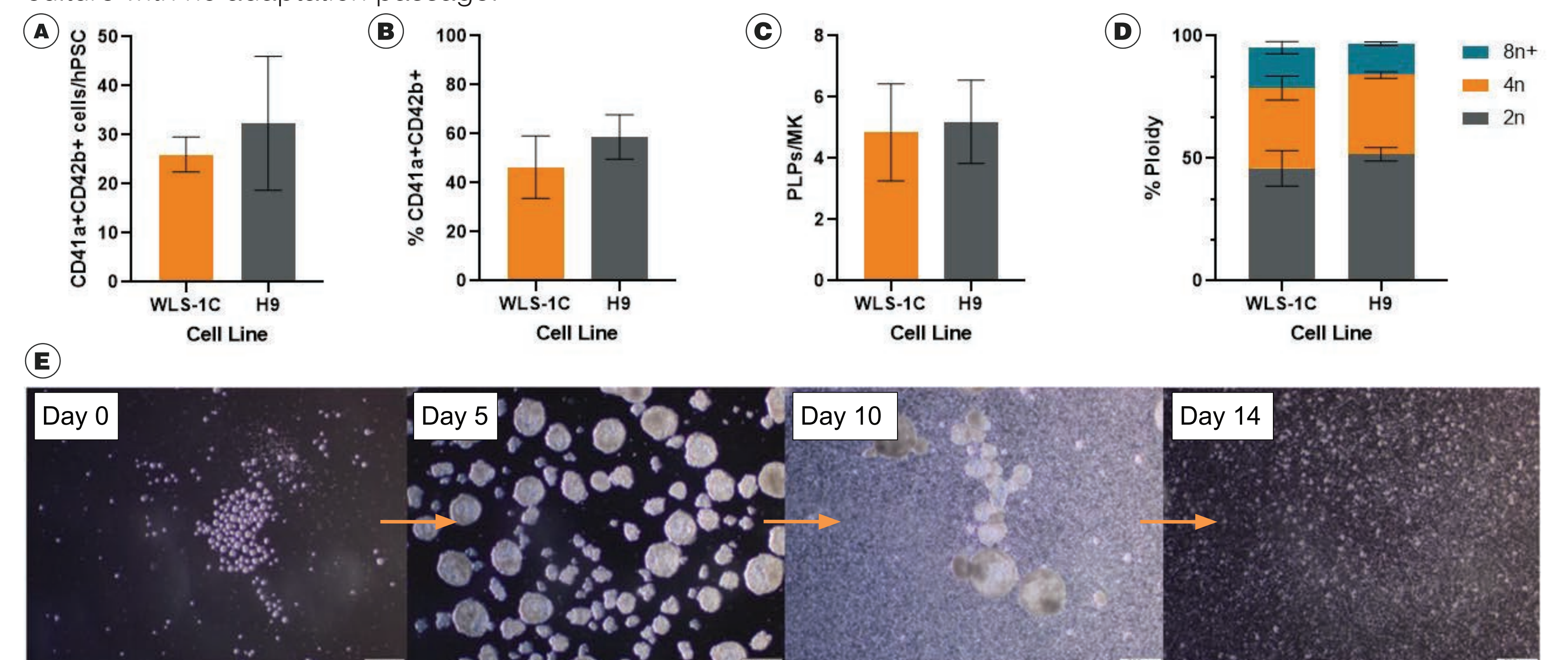


Figure 3. Megakaryocyte Differentiation in Suspension Culture

(A) The number of CD41a+CD42b+ cells generated per input hPSC and (B) the frequency of CD41a+CD42b+ MKs after culturing hPSCs for 14 days in suspension culture using STEMdiff™ Megakaryocyte Kit (n = 4). (C) The number of platelet-like particles (PLPs) generated per MK (gating based on blood platelets; n = 3). (D) The ploidy of the CD41a+CD42b+ cells (n = 3). (E) Morphology evolution of the MK aggregates over the first 10 days and of the reseeded single cells over the last 4 days of the MK differentiation in 3D suspension culture.

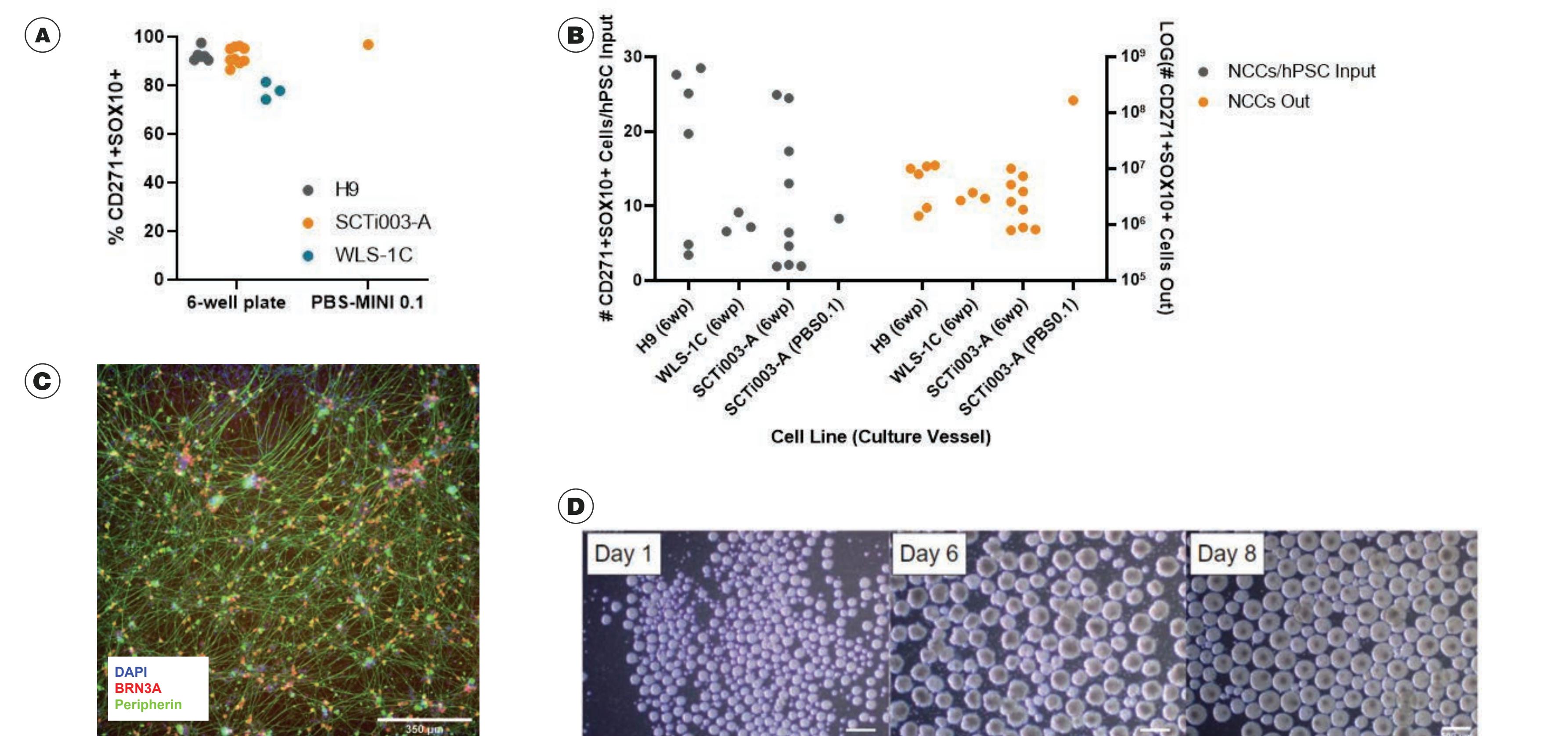


Figure 4. Neural Crest Differentiation in 3D Suspension Culture

(A) The frequency of CD271+SOX10+ cells after 6 days in STEMdiff™ Neural Crest Differentiation Medium (n = 3). (B) The number of CD271+SOX10+ NCCs generated per input hPSC (left) and the total number of CD271+SOX10+ NCCs harvested per well of a 6-well plate or PBS-MINI 0.1 (right; n = 3). (C) Morphology of NCC aggregates during differentiation. (D) Sensory neurons generated after 3D hPSC-derived NCCs were dissociated and seeded on Corning® Matrigel®-coated plates, and cultured with the STEMdiff™ Differentiation and Maturation Kits. Resulting cultures contain a population of cells expressing sensory neuron markers peripherin (green) and BRN3A (red).

Summary

- Using STEMCELL's TeSR™ 3D family of media, hPSC expansion workflows have been optimized for consistent growth and aggregate morphology in 3D suspension culture, starting from 2 mL in a 6-well plate to 500 mL in a PBS-MINI MagDrive Bioreactor.
- STEMdiff™ Megakaryocyte Kit can be used for differentiation of hPSCs maintained in TeSR™-AOF 3D to CD41a+CD42b+ polyploid MKs in 3D suspension culture in 14 days.
- hPSCs maintained in TeSR™-AOF 3D can be differentiated to CD271+SOX10+ NCCs in 3D suspension culture using STEMdiff™ Neural Crest Differentiation Kit in 8 days. These NCCs can be plated and further differentiated into sensory neurons expressing BRN3A and Peripherin.