Expansion and Differentiation Potential of Human Pluripotent Stem Cells Maintained in cGMP Animal Origin-Free Medium

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INTRODUCTION

Successful transition of human pluripotent stem cell (hPSC)-based therapies to the clinic is reliant on demonstrating the safety of the cell product and the components in contact with the product throughout the manufacturing process. To ease this transition, TeSR™-AOF a robust hPSC expansion medium, is manufactured under relevant cGMPs and was developed with animal component-free raw materials with traceability to the secondary level of manufacturing. We investigated key cell quality parameters of hPSCs cultured for at least 10 passages in TeSR™-AOF, and found that these cells had a higher expansion and plating efficiency compared to low-protein media without compromising cell quality. We also assessed the regenerative potential and differentiation efficiency of hPSCs maintained in TeSR™-AOF by evaluating compatibility with several clinically-relevant directed differentiation protocols, and demonstrated performance equivalent to cells maintained in mTeSR™1 or mTeSR™ Plus.

METHODS

Human embryonic stem (ES) cell lines (H9, H1) and induced pluripotent stem (iPS) cell lines (STiPS-F016, STiPS-M001) were cultured on Vitronectin XF™ for up to 20 passages in TeSR™-AOF with restricted feeding schedules, or in TeSR™-E8™ with daily medium changes. In addition, H9- and STiPS-B004 cells were maintained on Corning® Matrigel® for 10 and 5 passages, respectively, in the above conditions. hPSC cultures were passaged as aggregates using ReLeSR™ cell dissociation reagent on a 6- or 7-day passaging schedule. Compatibility of hPSCs expanded in TeSR™-AOF with gene editing was confirmed using the ArciTect™ CRISPR-Cas9 genome editing system. Differentiation to megakaryocytes (MKs), hepatocyte-like cells, cardiomyocytes, intestinal organoids, and dopaminergic neurons was achieved with TeSR™-AOF maintained cells (H9, H1, STiPS-F016, STiPS-M001, WLS-1C) according to the protocols supplied with their respective STEMdiff™ differentiation kits.

Cell Culture and Medium Performance

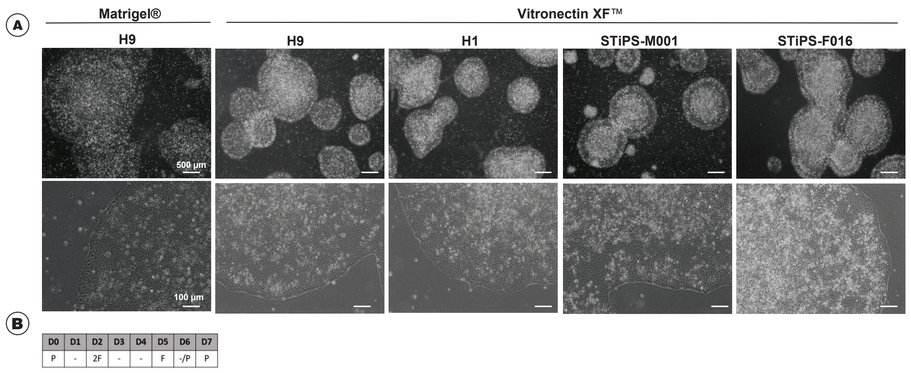

FIGURE 1. hPSCs Cultured in TeSR™-AOF with Restricted Feeding Maintained Excellent Colony Morphology

(A) hPSCs maintained in TeSR™-AOF exhibit hPSC-like morphology, forming round, densely packed colonies with smooth edges. Homogeneous cell morphology characteristic of hPSCs is observed, including large nucleoli and scant cytoplasm. **(B)** Restricted feed schedule followed. P = passage, 2F = double-volume feed, F = single-volume feed, dash = no feed step required.

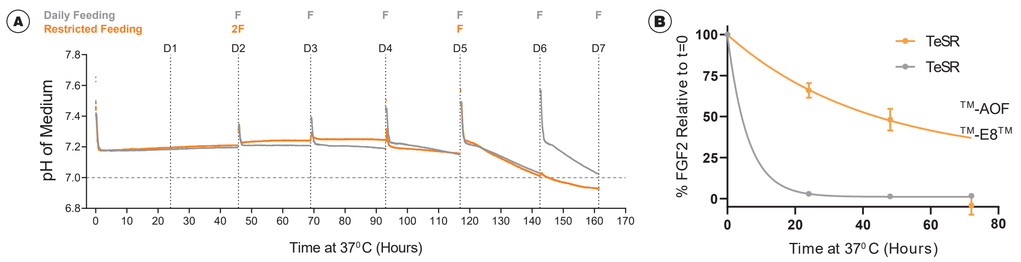

FIGURE 2. Buffering Capacity of TeSR™-AOF was Optimized to Maintain pH of Spent Medium and Native bFGF Levels were Stabilized at 37°C in TeSR™-AOF

A total of 250 WLS-1C iPS cell aggregates were seeded into two Matrigel®-coated 6-well plates in TeSR™-AOF. The first plate was fed daily and the second plate was fed on the restricted feed schedule. **(A)** pH measurements were obtained every 30 seconds for 7 days in culture using a PyroScience GmbHoptical pH, oxygen, and temperature meter (FireSting®-PRO [4 Channels] or FSPRO-4) for real-time monitoring of medium pH in culture conditions. **(B)** TeSR™-AOF and TeSR™-E8™ were incubated at 37°C for 24, 48, and 72 hours. FGF2 levels were measured by Meso Scale Discovery (MSD) immunoassay; data were normalized to t = 0 levels for TeSR™-E8™ and TeSR™-AOF, respectively. FGF2 levels in TeSR™-AOF remained at 36.7 ± 5.61% of t = 0 levels at 72 hours when incubated at 37°C in polypropylene microtubes. Data representative of n = 3 biological replicates ± SD.

Cell Quality

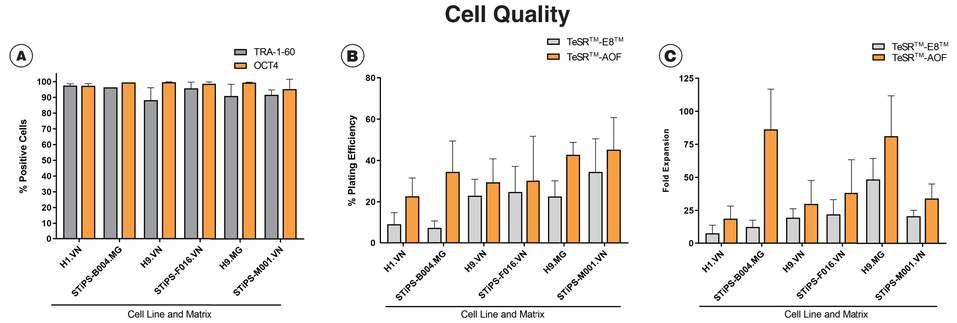

FIGURE 3. hPSCs Maintained in TeSR™-AOF Expressed Markers of the Undifferentiated State and Exhibited Improved Attachment and Higher Overall Expansion Compared to Low-Protein Medium

(A) hPSCs maintained in TeSR™-AOF exhibit high levels of markers of the undifferentiated state (TRA-1-60 and OCT4) as determined by flow cytometry at passage 5 and 10. Across n = 5 cell lines, the average (± SD) expression of TRA-1-60 and OCT-4 was 92.8 ± 3.77% and 98.1 ± 1.79%, respectively. Data shown represent an average of passage 5 and 10 flow cytometry results for each cell line. MG = Matrigel®; VN = Vitronectin XF™. **(B)** hPSCs cultured in TeSR™-AOF demonstrated a higher plating efficiency compared to hPSCs maintained in low-protein medium (TeSR™-E8™). Plating efficiency is calculated by comparing the number of aggregates seeded to the number of established colonies on day 7. **(C)** hPSCs maintained in TeSR™-AOF exhibited a higher average fold expansion per passage compared to those maintained in TeSR™-E8™. Data are represented as mean plating efficiency or fold expansion across 10 passages ± SD.

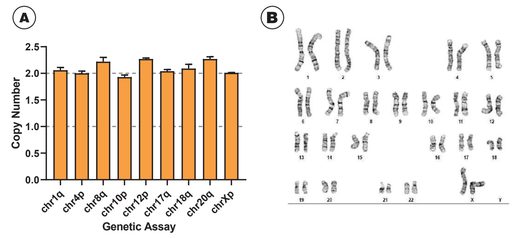

FIGURE 4. hPSCs Maintained in TeSR™-AOF Maintained a Normal Karyotype

A total of two ES (H9, H1) and two iPS (STiPS-M001, STiPS-F016) cell lines cultured in TeSR™-AOF were screened for chromosomal abnormalities using **(A)** the hPSC Genetic Analysis Kit and by G-banding at ≥ 20 passages. MG = Matrigel®; VN = Vitronectin XF™. **(B)** Representative karyogram of H9 ES cells displaying a normal karyotype by G-banding at passage 20.

Gene Editing and Cloning

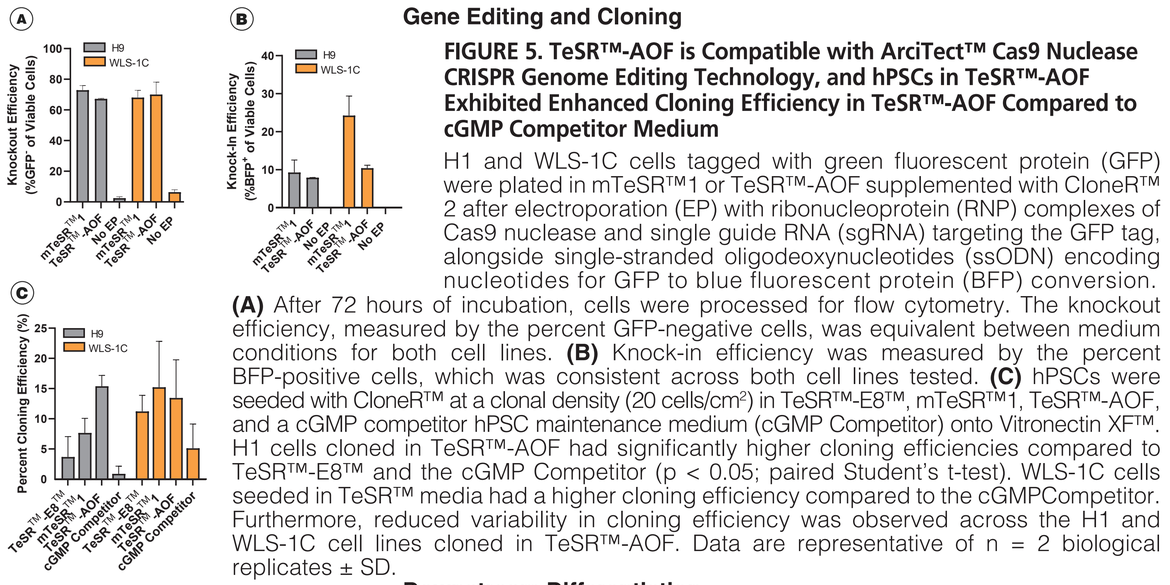

FIGURE 5. TeSR™-AOF is Compatible with ArciTect™ Cas9 Nuclease CRISPR Genome Editing Technology, and hPSCs in TeSR™-AOF Exhibited Enhanced Cloning Efficiency in TeSR™-AOF Compared to cGMP Competitor Medium

H1 and WLS-1C cells tagged with green fluorescent protein (GFP) were plated in mTeSR™1 or TeSR™-AOF supplemented with CloneR™ 2 after electroporation (EP) with ribonucleoprotein (RNP) complexes of Cas9 nuclease and single guide RNA (sgRNA) targeting the GFP tag, alongside single-stranded oligodeoxynucleotides (ssODN) encoding nucleotides for GFP to blue fluorescent protein (BFP) conversion. **(A)** After 72 hours of incubation, cells were processed for flow cytometry. The knockout efficiency, measured by the percent GFP-negative cells, was equivalent between medium conditions for both cell lines. **(B)** Knock-in efficiency was measured by the percent BFP-positive cells, which was consistent across both cell lines tested. **(C)** hPSCs were seeded with CloneR™ at a clonal density (20 cells/cm²) in TeSR™-E8™, mTeSR™1, TeSR™-AOF, and a cGMP competitor hPSC maintenance medium (cGMP Competitor) onto Vitronectin XF™. H1 cells cloned in TeSR™-AOF had significantly higher cloning efficiencies compared to TeSR™-E8™ and the cGMP Competitor ($p < 0.05$; paired Student's t-test). WLS-1C cells seeded in TeSR™ media had a higher cloning efficiency compared to the cGMPCompetitor. Furthermore, reduced variability in cloning efficiency was observed across the H1 and WLS-1C cell lines cloned in TeSR™-AOF. Data are representative of n = 2 biological replicates ± SD.

Downstream Differentiation

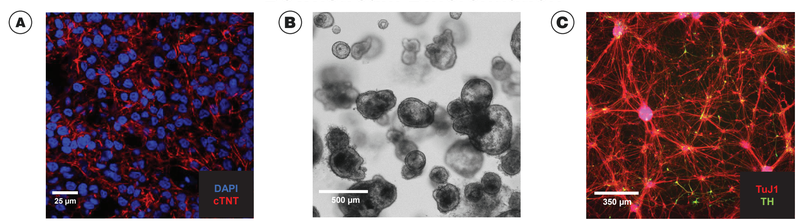

FIGURE 6. hPSCs Maintained in TeSR™-AOF with Restricted Feeding Differentiated to the Three Germ Layers

Efficient differentiation to the three germ lineages was demonstrated in one ES and one iPS cell line maintained for > 5 passages in TeSR™-AOF. **(A)** STiPS-M001 cells were processed for immunocytochemistry and stained for cardiac troponin T (cTNT) and DAPI on day 15 following differentiation using STEMdiff™ Ventricular Cardiomyocyte Differentiation Kit. **(B)** Morphology of mid/hindgut H9 spheroids released from the cell monolayer into the culture on day 9 following differentiation to intestinal organoids using STEMdiff™ Intestinal Organoid Kit. **(C)** STiPS-F016 cells were processed for immunocytochemistry and stained for class III β-tubulin (TuJ1) and tyrosine hydroxylase (TH) following differentiation to dopaminergic neurons using the STEMdiff™ midbrain neuron system. Cultures contain a population of cells expressing midbrain markers TuJ1 and TH.

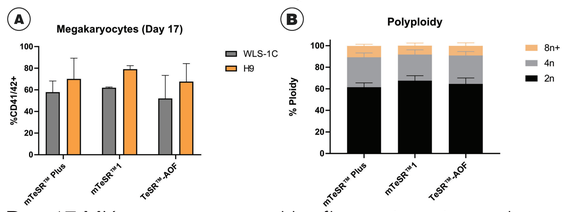

FIGURE 7. hPSCs Maintained in TeSR™-AOF Differentiated to CD41/42+ Polyploid Megakaryocytes

H9 and WLS-1C cells cultured in TeSR™-AOF were differentiated to MK-erythroid-biased HSPCs after 12 days using the STEMdiff™ Megakaryocyte Kit. Cells were further cultured in MK2 maturation medium for 5 additional days to promote differentiation into mature MKs. **(A)** To assess the mature and adult-like features of hPSC-derived MKs, Day 17 MKs were assessed by flow cytometry and were found to co-express CD41 and CD42. On average, 52% of TeSR™-AOF-maintained WLS-1C cells (n = 3), and 67% of TeSR™-AOF-maintained H9 cells (n = 3) co-expressed CD41 and CD42. Data are shown as mean ± SD, n = 3. **(B)** The ploidy profile was assessed by flow cytometry using ethanol-fixed MKs derived from H9 and WLS-1C cells maintained in TeSR™-AOF. DNA content was determined by the quantity of propidium iodide (PI) staining. Ploidy analysis was done on gated CD41+ cells. Bar graph shows ploidy distribution of MKs generated from different media with cell line data averaged together. Data are shown as mean ± SD, n = 6. On average, 64% of TeSR™-AOF-maintained cells were 2n, 26% were 4n, and 9% were 8n or higher which is indicative of mature MKs.

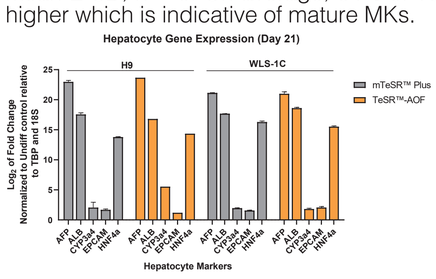

FIGURE 8. hPSCs Maintained in TeSR™-AOF Demonstrated Efficient Differentiation to Hepatocyte-like cells

H9 and WLS-1C cells were first transitioned to CellAdhere™ Laminin-521 matrix for 1 week and differentiated to hepatic progenitors using the STEMdiff™ Hepatocyte Kit before being assessed at day 21 of differentiation Gene expression profiles were indicative of hepatocyte-like cells with increased expression of AFP, albumin (ALB), CY3a4, EPCAM, HNF4α when compared to undifferentiated controls. Data represented are shown as mean ± SD, normalized to undifferentiated control cells relative to TATA-Box Binding Protein (TBP) and 18S reference genes, n = 2.

Summary

- TeSR™-AOF is an animal origin-free hPSC culture medium manufactured with raw material traceability to the secondary level of manufacturing.
- hPSCs maintained in TeSR™-AOF exhibit increased attachment and more consistent expansion when compared to hPSCs maintained in low-protein medium.
- hPSCs maintained in TeSR™-AOF maintain hPSC gene and marker expression, genetic stability, and robust differentiation potential
- Higher cloning efficiency is observed when hPSCs are seeded in TeSR™-AOF with CloneR™ at clonal density compared to a cGMP competitor medium.
- TeSR™-AOF is compatible with CRISPR-Cas9 genome editing technology.
- hPSCs maintained in TeSR™-AOF with restricted feeding are capable of downstream differentiation to cardiomyocytes, intestinal organoids, dopaminergic neurons, hepatocyte-like cells,and polyploid CD41/42+ megakaryocytes.

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