IMPROVED CELLULAR MAINTENANCE OF HUMAN PLURIPOTENT STEM CELLS IN A SERUM-FREE, XENO-FREE CULTURE SYSTEM FOR CLINICAL APPLICATIONS

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INTRODUCTION

Xeno-free culture environments for clinically compliant cells

The potential of human pluripotent stem cells (hPSCs) continues to show incredible promise, rapidly progressing through clinical trials for use in treatment of a multitude of diseases. However, historical methods of hPSC culture have remained a barrier of entry into the clinic even with relative success within in vitro cellular research. While traditional protocols often require the use of animal-derived feeder layers and supplements, a defined and carefully formulated microenvironment for hPSC culture becomes increasingly critical for entry to the clinic. Ultimately, for the therapeutic application of clinically relevant cells to be more standardized, media formulations and culture conditions must be refined and optimized while fully supporting proper phenotype and genetic stability over long-term culture.

Currently, the recommendation for transitioning hPSC research to clinic is to maintain cells in xeno-free culture conditions. While determining the ideal xeno-free environment remains challenging for technical and biological reasons, there are multiple commercially available formulations for hPSC maintenance that are either conventional and serum-free with animal-derived components, or completely xeno-free. Each available media has varying levels of growth factor supplementation intended to maintain pluripotency and prevent differentiation, but not all formulations are created equally and thus may cause variation in pluripotency potential and stability.

This study aims to examine the fundamental variations in morphology and pluripotency of hPSCs in commercially available culture systems, to better understand the effects of media formulations on hPSC characteristics. Findings demonstrate that the xeno-free, serum-free NutriStem® hPSC Medium yields the highest levels of pluripotency and retains viability over long-term cultures. Furthermore, transitions from each media into this supportive environment are explored to validate efficient methods of obtaining high-performing, clinically compliant hPSCs for therapeutic applications.

MATERIALS & METHODS

PRODUCT	SUPPLIER	CAT. NO.	SERUM-FREE	XENO-FREE
NutriStem® hPSC Medium	Biological Industries USA	05-100-1A	•	•
Vitronectin XF™	Biological Industries USA	S2153-500UG	•	•
mTeSR™1	STEMCELL Technologies	85850	•	
Essential 8™ Medium	ThermoFisher Scientific	A15169-01	•	•
StemFlex™ Medium	ThermoFisher Scientific	A33493-01	•	
Matrigel®, hESC-Qualified	Corning	354277	•	

Human induced pluripotent stem cells (hiPSCs) were cultured for approximately 6 weeks in multiple conditions utilizing either completely xeno-free culture media on a xeno-free substrate, conventional hPSC media on an animal-derived substrate, or some combination of these elements. Cells from all culture conditions were then transitioned to the most supportive xeno-free media, with or without further bFGF supplementation, while examining the cells' ability to adapt from traditional, poorly defined, animal-containing media to a cleaner, more clinically applicable condition. Throughout the duration of these cultures, analyses were performed to determine cellular pluripotency and stability, including marker expression by flow cytometry and fluorescent imaging.

Abbreviations used to describe media transitions are as follows:

ABBREVIATION	INITIAL MEDIUM	FINAL MEDIUM
NS-NS	NutriStem® hPSC Medium	NutriStem® hPSC Medium
TSR-NS	mTeSR™1	
E8-NS	Essential 8™ Medium	
SF-NS	StemFlex™ Medium	

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EXPERIMENTAL RESULTS

Initial adaptation into commercially available systems

Commercially available hiPSCs were plated in each of the 6 available hPSC culture systems and stabilized over 6 weeks of culture. While daily phase contrast imaging depicts generally successful adaptation and areas of normal morphology throughout, flow cytometric and immunocytochemical analysis show varying levels of pluripotency marker expression per system.

Results show that the xeno-free NutriStem hPSC Medium, with the lowest levels of added growth factors (specifically <10 ng/mL bFGF when compared to the high >75 ng/mL bFGF in some systems), presents the highest maintained pluripotency levels of all systems tested. Furthermore, as long-term exposure to excessive levels of growth factors such as bFGF has the potential to alter the cells' ability to differentiate downstream, low bFGF levels become increasingly critical in therapeutic workflows.

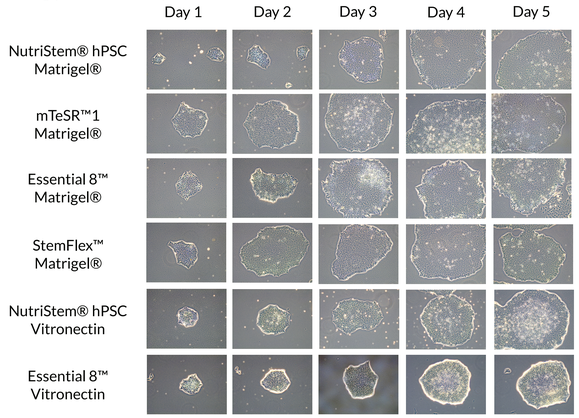

Figure 1. Representative cell morphology of hiPSCs plated and cultured over 5 days in various commercially available hPSC media and substrates. Images at 10x magnification.

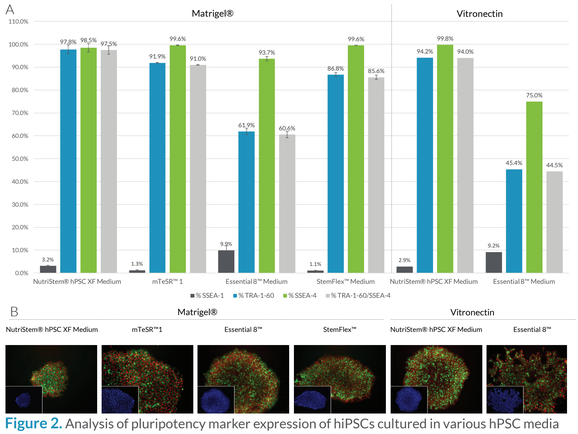

Figure 2. Analysis of pluripotency marker expression of hiPSCs cultured in various hPSC media systems after 6 weeks, depicted by (A) flow cytometric staining for TRA-1-60, SSEA-4, and Double TRA-1-60/SSEA-4 expression (SSEA-1 performed to measure differentiation) as well as (B) representative immunocytochemical staining for TRA-1-60 (green), Nanog (red), and Hoechst (blue) nuclei stain. Images at 10x magnification.

Transition into clinical-quality, xeno-free culture media

Upon completion of initial cellular stabilization in all culture systems, hiPSCs were independently transitioned from each preliminary system into the culture medium yielding the highest overall levels of pluripotency after 6 weeks of culture. Specifically, the highest performing media formulation was the clinically relevant NutriStem hPSC Medium, in which cells were transitioned on their respective substrates either without any additional supplementation, or with varying amounts of bFGF (either 20 ng/mL bFGF or 100 ng/mL bFGF). Upon this initial transition it was determined that each of the 4 alternative media systems could be transitioned to the xeno-free media and sustain relatively high levels of pluripotency after an additional 3 weeks of culture using a bulk passaging technique. Culture optimization with additional bFGF demonstrated that further bFGF supplementation could assist in the maintenance of culture morphology and higher pluripotency levels as determined both quantitatively and qualitatively.

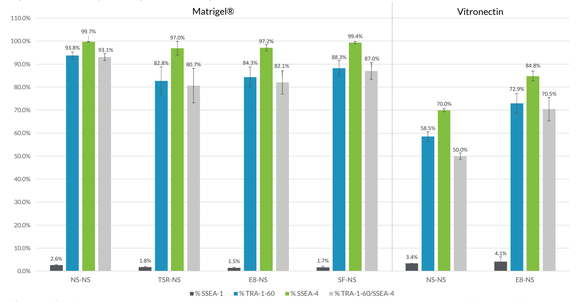

Figure 3. Pluripotency analysis of hiPSC cultures after transition into the NutriStem hPSC medium from the previous hPSC media, and after 3 weeks of additional culture, as depicted by flow cytometric staining for TRA-1-60, SSEA-4, and Double TRA-1-60/SSEA-4 expression (SSEA-1 performed to measure differentiation).

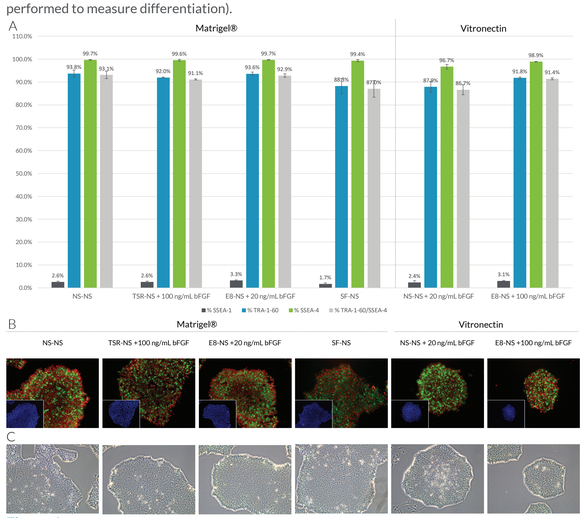

Figure 4. Analysis of pluripotent marker expression of hiPSCs cultured after transition into the optimized NutriStem hPSC Medium formulation from the previous hPSC media, and after 3 weeks of additional culture, as depicted by (A) flow cytometric staining for TRA-1-60, SSEA-4, and Double TRA-1-60/SSEA-4 expression (SSEA-1 performed to measure differentiation), (B) representative immunocytochemical staining for TRA-1-60 (green), Nanog (red), and Hoechst (blue) nuclei stain, as well as (C) phase contrast depicting morphology. Images at 10x magnification.

bFGF supplementation in already streamlined formulations

Although the addition of bFGF was determined to aid in the maintenance of cultures that originated in media with high levels of bFGF, cells that were consistently cultured in NutriStem hPSC Medium (with <10 ng/mL bFGF) on Matrigel were found to maintain high levels of pluripotency markers with or without further supplementation with bFGF.

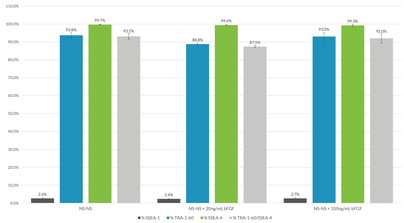

Figure 5. Pluripotency of hiPSCs cultured in NutriStem hPSC Medium on Matrigel supplemented with various concentrations of bFGF after 3 additional weeks of culture depicted by flow cytometric staining for TRA-1-60, SSEA-4, and Double TRA-1-60/SSEA-4 expression (SSEA-1 performed to measure differentiation).

Selecting a completely xeno-free culture system

After 9 weeks of culture, or passage 10 for this system, Essential 8 cultures had poor pluripotency levels, poor morphology, and increased levels of differentiation. Conversely, when samples of these cultures were transitioned to NutriStem hPSC Medium (with various levels of bFGF supplementation), results show that cultures were rescued and subsequently recovered appropriate pluripotency characteristics through the supplementation of NutriStem with 100 ng/mL bFGF.

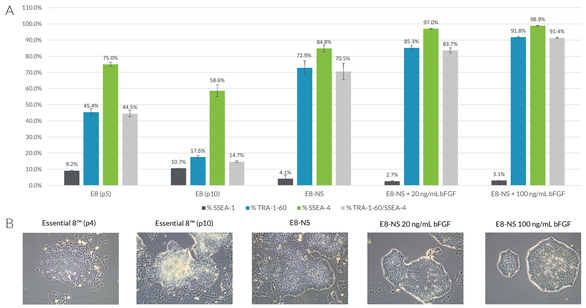

Figure 6. Analysis of pluripotent marker expression of hiPSCs cultured in Essential 8 on Vitronectin at p5 and p10, compared to NutriStem cultures on Vitronectin with varying levels of bFGF supplementation transitioned from Essential 8, as depicted by (A) flow cytometric staining for TRA-1-60, SSEA-4, and Double TRA-1-60/SSEA-4 expression (SSEA-1 performed to measure differentiation), and (B) phase contrast depicting morphology. Images at 10x magnification.

SUMMARY

While finding the ideal xeno-free culture environment for hPSCs has historically been a challenge, this study validates the ability of such a system to promote highly pluripotent and viable hiPSCs for therapeutic applications. Not only did hiPSCs cultured in NutriStem hPSC Medium on Matrigel show high levels of pluripotency in continuous culture, but hiPSCs cultured in NutriStem hPSC Medium on xeno-free or XF Vitronectin transitioned from alternative systems also resulted in enhanced overall levels of measured pluripotency markers. However, it is important to note that not all cells respond to culture environments in the same manner.

- While many serum-free, xeno-free culture systems are commercially available, some maintain cellular stability and pluripotency more efficiently than others.
- Transitions to clinically compliant culture conditions can be seamlessly achieved with minor adjustments to bFGF levels, facilitating stable, long-term cultures that retain high levels of pluripotency and without over-exposure to high amounts of bFGF.
- The completely xeno-free culture system consisting of NutriStem hPSC Medium and Vitronectin can easily support the maintenance of hPSC qualities for over 3 months in culture.