A case study on eliminating the use of Y-27632 in thawing and maintenance of hiPS cells.

Xeno-Free hiPS Cell Culture using NutriStem hPSC XF Medium on a Human Vitronectin Substrate without a need for ROCK Inhibitors

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INTRODUCTION

NutriStem® hPSC XF Medium is a defined, xeno-free, serum-free cell culture medium designed for optimal growth and proliferation of undifferentiated human pluripotent stem cells (hPSCs). NutriStem hPSC XF Medium has been validated to support the long-term maintenance of human embryonic stem (hES) cells in both feeder-dependent culture on mouse embryonic fibroblasts (MEFs), as well as in feeder-free culture on Matrigel®, a protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells. However, the use of a xeno-free (XF) or completely humanized cell culture system would be ideal for future stem cell-based therapies and clinical trials. Multiple xeno-free media formulations have been developed that typically replace animal-derived components, such as fetal bovine serum (FBS) and bovine serum albumin (BSA), with human serum proteins, and replace MEFs and Matrigel substrates with human recombinant extracellular matrix proteins, such as human vitronectin and laminin proteins.

While these feeder-free cell culture systems demonstrate improved clinical compliance, in general, many have been found to be less efficient in cell survival after thawing, clonal propagation, and even routine passaging in culture[1], likely owing to a lack of key components present in the undefined FBS, BSA, and/or MEFs. To address these issues, many protocols recommend the addition of a Rho-associated coiled-coil protein kinase (ROCK) inhibitor, specifically the small molecule Y-27632, when cell cultures are thawed or passaged. Likewise, several of the commonly used hPSC media include very high concentrations of bFGF (up to 100 ng/mL), a critical growth factor to maintain adequate cell proliferation and pluripotency. Unfortunately, both prolonged use of ROCK inhibitors and excessive

bFGF supplementation hold the potential to cause undesired consequences in pluripotent cell cultures, including transcriptional and epigenetic modifications, and a biased differentiation potential.[2–5]

Taking the points above into consideration, the ideal xeno-free culture medium is one that promotes proliferative hPSC growth and survival when thawing and passaging without the dependency of ROCK inhibition or high concentrations of bFGF. Biological Industries' NutriStem hPSC XF Medium meets this need by requiring very low concentration of bFGF to maintain pluripotency and is not dependent on the supplementation of a ROCK inhibitor for efficient cell growth.

The following experiments demonstrate that NutriStem hPSC XF Medium can be used to efficiently maintain human induced pluripotent stem (hiPS) cells in a completely xeno-free system using a human vitronectin substrate. Additionally, we show that hiPS cells can be thawed directly into a NutriStem hPSC XF Medium and vitronectin culture and passaged over time without requiring the ROCK inhibitor Y-27632 to maintain characteristic hPSC morphologies or proliferative cell growth.

The use of NutriStem hPSC XF Medium in human pluripotent cell culture reduces the need for broad or prolonged use of ROCK inhibitors or a disproportionately high concentration of bFGF. This not only reduces lab costs by decreasing the amount of additives needed to maintain healthy hPSC cultures, but also potentially lessens the likelihood of generating undesired genotypic or phenotypic abnormalities associated with excessive chemical manipulation.

MATERIALS & METHODS

Stem Cell Culture

All human stem cell culture work was carried out at the University of California, Irvine in accordance with appropriate hSCRO and IBC protocols. Human pluripotent cell lines were provided courtesy of Dr. Brian Cummings (UC Irvine). Cells used in this experiment were previously maintained in TeSR-E8 (STEMCELL Technologies) before transitioning to NutriStem hPSC XF Medium (Biological Industries). In these experiments, cells were cultured side-by-side in either TeSR-E8 or NutriStem hPSC XF Medium using human recombinant vitronectin, Vitronectin XF™ (STEMCELL Technologies) as the substrate. Unless specifically noted, all cell culture methods were performed as outlined in the TeSR-E8 manufacturer protocols to maintain consistency.

RESULTS

Adaptation of hiPS Cells to NutriStem hPSC XF Medium on Vitronectin XF

The hiPS cells previously maintained in TeSR-E8 medium on human recombinant Vitronectin XF were passaged according to the TeSR-E8 protocol and transitioned directly to NutriStem hPSC XF Medium by simply replacing TeSR-E8 with NutriStem hPSC XF Medium the first day after the cells were passaged. These hiPS cells were cultured for 5 passages in NutriStem hPSC XF Medium on the Vitronectin XF substrate. The hiPS cells cultured in NutriStem hPSC XF Medium retained typical pluripotent cell morphology (Figure 1) and exhibited in vitro growth rates comparable to hiPS cells cultured in TeSR-E8 over the course of the 5 passages.

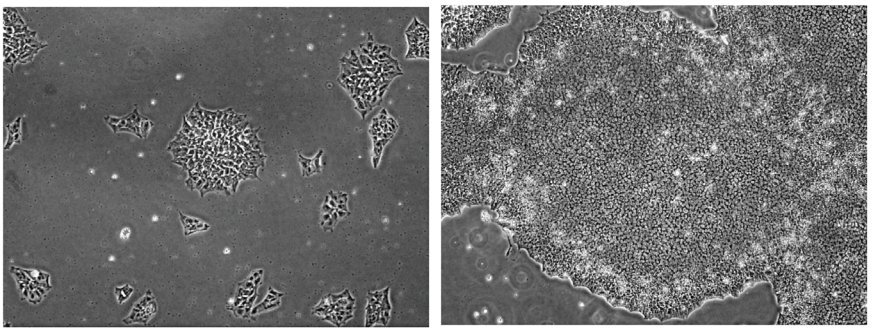

Figure 1. hiPS cells were transitioned directly from TeSR-E8 medium to NutriStem hPSC XF Medium and maintained for 5 passages on Vitronectin XF substrate. The images show that transitioned cells maintain typical pluripotent cell morphology during both the early (Day 1, left image) and late (Day 5, right image) phases of growth post-passage. Images shown at 20X magnification.

Improved Recovery of hiPS Cells Thawed into NutriStem hPSC XF Medium on Vitronectin XF

After observing that hiPS cells cultured in TeSR-E8 on Vitronectin XF could easily be transitioned directly into NutriStem hPSC XF Medium, cryopreserved hiPS cells previously cultured in TeSR-E8 were then thawed and plated directly into NutriStem hPSC XF Medium on the vitronectin matrix in the absence of the ROCK inhibitor Y-27632 (Figure 2). This direct transition from a frozen cryovial eliminated the need to initially establish cells in their previous culture medium (TeSR-E8) prior to culturing in NutriStem hPSC XF Medium.

Xeno-Free hiPS Cell Culture using NutriStem hPSC XF Medium on a Human Vitronectin Substrate without a need for ROCK Inhibitors

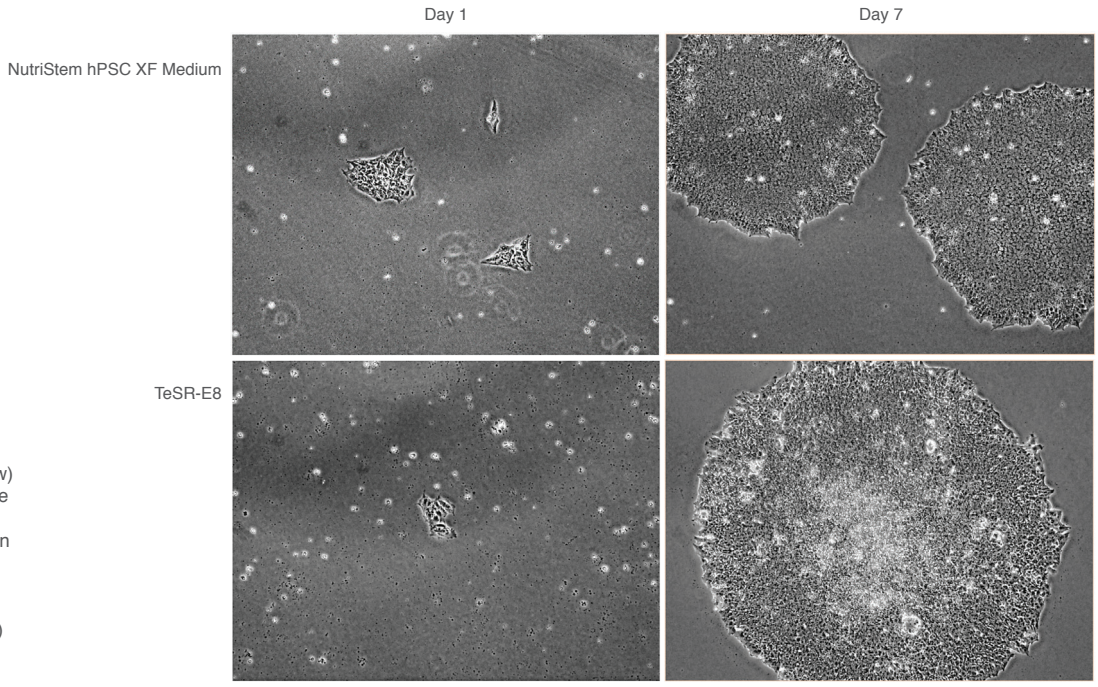

Figure 2. hiPS cells were thawed directly into either NutriStem hPSC XF Medium (top row) or TeSR-E8 medium (bottom row) on Vitronectin XF substrate in the absence of the ROCK inhibitor Y-27632. The images demonstrate thawed cells maintain comparable pluripotent cell morphology during the first passage post-thaw in both media types during both the early (Day 1, left images) and late (Day 7, right images) phases of growth after thawing. Images shown at 20X magnification.

Furthermore, the recovery of hiPS cells was compared when the ROCK inhibitor Y-27632 was omitted from the thawing protocol. Prior to cryopreservation, the hiPS cells had been cultured in TeSR-E8 medium for several passages and frozen at the same timepoint using the same density of cells (1 well of a 6-well plate approximately 90% confluence per cryovial). The resulting thawing efficiencies indicated that NutriStem hPSC XF Medium supported greater post-thaw cell recovery in the absence of Y-27632 compared to TeSR-E8. In each instance, cells were thawed side-by-side into a vitronectin-coated 6-well plate at both a 1:6 thaw ratio (1 cryovial to 6 wells) (4 ± 2 colonies in TeSR-E8 vs 42 ± 4 colonies in NutriStem hPSC XF Medium) and a 1:2 thaw ratio (1 cyovial to 2 wells) (7.5 ± 3.5 colonies in TeSR-E8 vs 262.5 ± 22.5 colonies in NutriStem hPSC XF Medium) (Figure 3).

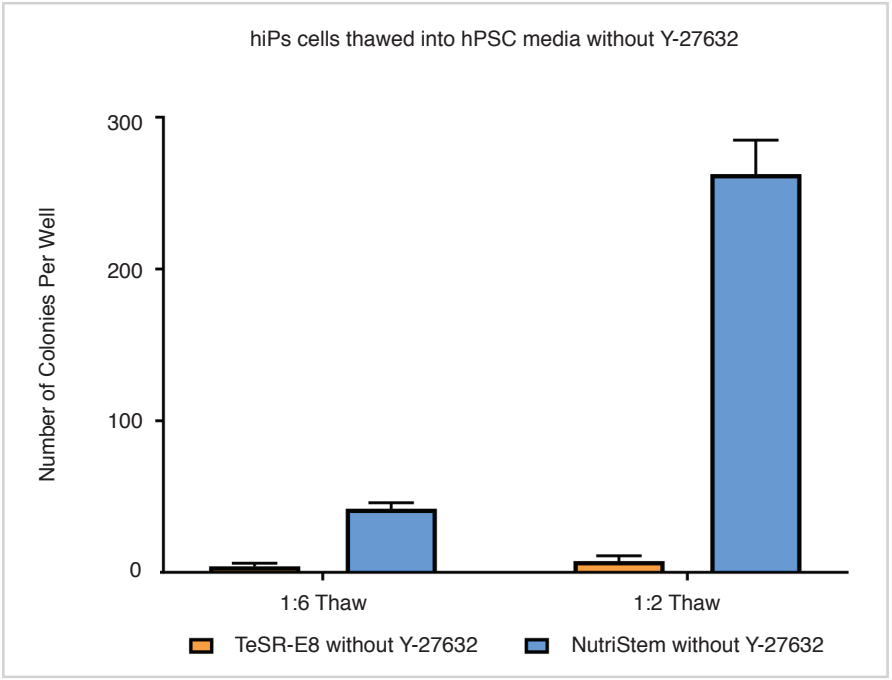

Figure 3. hiPS cells were thawed directly into either TeSR-E8 medium (orange bars) or NutriStem hPSC XF Medium (blue bars) in the absence of the ROCK inhibitor Y-27632. The hiPS cells were thawed at both a 1:6 thaw ratio (1 vial into 6 wells) as well as a 1:2 thaw ratio (1 vial into 2 wells) in Vitronectin XF-coated 6-well plates. Values above represent mean ± SEM from two separate experiments.

Finally, hiPS cells thawed directly into NutriStem hPSC XF Medium were maintained for 5 subsequent passages without the addition of Y-27632 at any point (Figure 4), demonstrating the superior ability of NutriStem hPSC XF Medim to support both post-thaw recovery and long-term maintenance of hiPS cells on human vitronectin without requiring chemically induced ROCK inhibition.

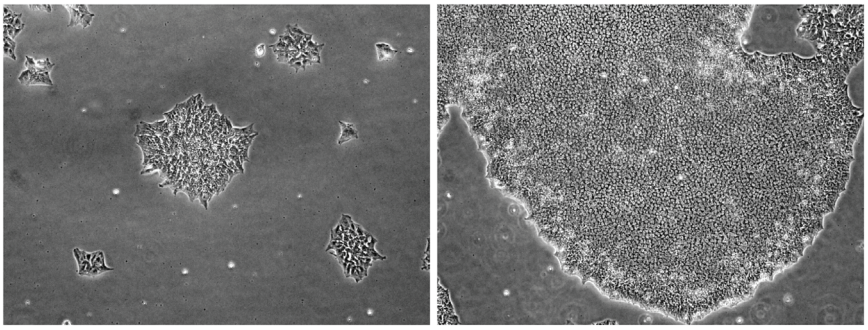

Figure 4. hiPS cells that were previously thawed directly into NutriStem hPSC XF Medium in the absence of the ROCK inhibitor Y-27632 were maintained in culture conditions without Y-27632 for 5 passages on Vitronectin XF substrate. The images show that hiPS cells maintain typical pluripotent cell morphology during both the early (Day 1, left image) and late (Day 5, right image) phases of growth post-passage. Images shown at 20X magnification.

CONCLUSION

This report demonstrates that NutriStem hPSC XF Medium supports the maintenance of hiPS cells on a human recombinant vitronectin substrate without the need for chemical ROCK inhibition. hiPS cells cultured previously in TeSR-E8 medium can be successfully transitioned directly to NutriStem hPSC XF Medium, as well as thawed directly into NutriStem hPSC XF Medium without the need for Y-27632. Furthermore, hiPS cells thawed into NutriStem hPSC XF Medium can be successfully maintained without any additional Y-27632 supplementation.

The field of human pluripotent stem cell research has made significant strides since the initial cultures of hES and hiPS cells were established. Over time, the composition of the cell culture system itself has also changed significantly, as today many researchers have shifted from hPSC culture systems containing bovine- and murine-derived components to systems that are highly defined, and in some cases, completely xeno-free and compliant with clinical regulations. However, the variety of xeno-free hPSC media commonly used are not equal in composition or their requirements for other additives to promote cell health. The risks of cell dependency on ROCK pathway inhibition or high concentrations of bFGF or other growth factors should be taken into consideration, and culture media that do not require these additions, such as NutriStem hPSC XF Medium, should be used for long-term culture of hPSCs in a defined, xeno-free environment.

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