NutriStem: a Defined, Low-Growth Factor, Xeno-Free Medium for the Long Term Culture of Undifferentiated Human ES Cells

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SUMMARY

The most widely-used culture systems for the maintenance and expansion of pluripotent stem cells utilize undefined components such as serum or serum-replacers along with feeder layers of mouse embryonic fibroblasts (MEFs). However, the use of these products for pluripotent stem cell culture is not optimal, as their performance can vary from batch-to-batch, and their use precludes the stem cells from being suitable for clinical applications. To address these issues, Stemgent® has introduced NutriStem™ XF/FF Culture Medium, a xeno-free, feeder-free culture medium for the long-term culture of pluripotent stem cells. Stem cells cultured in NutriStem Medium maintain proper morphology, karyotype and pluripotency, as measured by pluripotent marker expression and the ability to differentiate into cells from all three germ layers. Cells transition rapidly from feeder-based culture to NutriStem Medium.

INTRODUCTION

Since their first isolation in 1998, human embryonic stem (ES) cells have been routinely maintained in culture conditions that include feeder layers of inactivated mouse embryonic fibroblasts (MEFs), and serum or serum-replacers in the culture medium[1,2,3]. Such components introduce a host of undefined variables into a sensitive and highly complex system. First, even after a number of recent studies using proteomics and other methods, the specific contributions of MEFs to human ES cell pluripotency are not well understood[4]. Second, the exact composition of serum and serum-replacers is unknown, and recent work has shown that they contain factors that by themselves act to cause human ES cells to differentiate[5].

MEFs, serum, and serum-replacers add considerable time and expense to human ES cell culture. The process of isolating, expanding and banking MEFs is labor intensive, and the alternative of buying them commercially is generally expensive. The performance of MEFs can vary significantly from batch to batch, and therefore additional labor and time are required to evaluate each new lot. The same is true of serum and serum-replacers; each new lot must be evaluated over several weeks in culture, and it is not uncommon for researchers to purchase entire lots of these components in order to achieve consistent results. Such difficulties caused by variable, undefined components are magnified in drug discovery efforts, where conditions must be particularly consistent.

MEFs and serum or serum-replacers do not meet the requirements of culture systems for therapeutic applications of stem cells. Not only should all components of such systems be completely defined in order to remove unknown variables, but they also cannot contain components of nonhuman origin in order to prevent complications by nonhuman pathogens upon transplantation of the cultured cells or their derivatives. Such “xeno-free” systems would also eliminate nonhuman antigens that could trigger an immune response after transplantation, such as the nonhuman sialic acid Neu5Gc that is taken up and expressed on the surface of human ES cells after culture with MEFs[6].

Some advances towards a defined, xeno-free culture system have recently been made, but these are not without their drawbacks. Most xeno-free advances have been made through the identification of specific cytokines and growth factors that allow human pluripotent stem cells to self-renew *in vitro* without MEF feeder layers. Use of these growth factors has also enabled the substitution of serum and serum replacers with reduced sets of components that are better defined. Such factors include basic Fibroblast Growth Factor (bFGF), a key molecule that maintains human stem cells in the pluripotent state *in vitro*, and Activin A, a signaling molecule of the TGFb/Nodal/Activin A family[7,8,9]. However, the most commonly used of these formulations contain relatively high levels, as much as 100 ng/ml, of one or more of these single factors[10]. Such levels are far higher than what is present physiologically, and highlight the artificial constraints placed on pluripotent stem cells cultured under these conditions.

To address the drawbacks of current culture media for pluripotent stem cells, Stemgent offers **NutriStem™ XF/FF Culture Medium (Cat. No. 01-0005)**. NutriStem Medium contains human serum

albumin and low amounts of key factors such as bFGF and TGFb1. Here we show that NutriStem Medium is suitable for the long term culture of human ES cells under feeder-free conditions. Human ES cells cultured for multiple passages in this medium maintain good morphology and proliferate at a rate that is equivalent or superior to other media formulations, including culture on MEF feeder layers. Human ES cells cultured in NutriStem Medium for multiple passages also maintain their pluripotency by strict criteria, including expression of pluripotency markers by more than 98% of the cells in culture, maintenance of a normal karyotype, and the ability to differentiate into cells from all three germ layers by two different methods. Thus NutriStem Medium provides a robust way to culture pluripotent stem cells in a defined medium without variables such as feeder cells, serum, or serum-replacement. In addition, NutriStem Medium is a xeno-free medium that can be suitable for investigations with regards to clinical applications.

RESULTS

To assess NutriStem Medium for long-term culture of pluripotent stem cells, human ES cells (hES cells) of the H1 line (passage 37) were transferred from culture with 20% Knockout™ Serum Replacement on MEFs to culture with NutriStem Medium on Matrigel™ without a feeder layer. Transition between these two culture conditions was accomplished in one step; no adaptation protocol involving gradual weaning off MEFs or transition between culture media was performed. Attachment of the hES cells with NutriStem Medium was excellent, as over 90% of cell clumps were observed to be attached after 24 hours post-seeding (data not shown).

In general, colonies of hES cells in NutriStem Medium showed robust growth. Colonies had well-defined boundaries with little to no observable differentiation (**Figure 1**). Within colonies, hES cells were slightly more separated from one another as compared to control colonies being cultured on MEFs (not shown), but did not take on a differentiated appearance. Furthermore, this morphology was uniform across the colonies, suggesting that there were no significant heterogeneities present in the colonies.

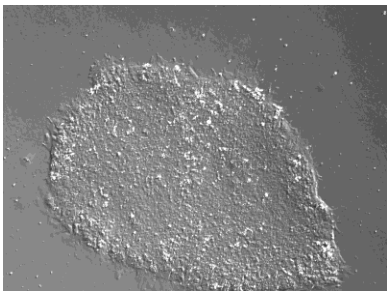

Figure 1. Morphology of hES cells grown in NutriStem Medium. Bright-field photomicrograph of H1 human ES cells cultured for 20 passages in NutriStem Medium. The colony is a densely-packed monolayer of cells with sharp colony boundaries.

After five passages and again after ten passages, the cells cultured in NutriStem Medium were examined for their expression of known markers of pluripotency. Cells cultured in NutriStem Medium were also compared to cells grown in another commonly used media formulation. Virtually all cells at either passage showed expression of Oct4 and SSEA-4 by standard immunocytochemistry (**Figure 2**). This was confirmed by flow cytometry, where labeling for Oct4 and SSEA-4 expression showed that over 98% of cells were positive for these markers (**Figure 3**). The proliferation rate of hES cells in NutriStem Medium was analyzed at passage five, and was found to be equivalent to that of the other media formulations (**Figure 4**).

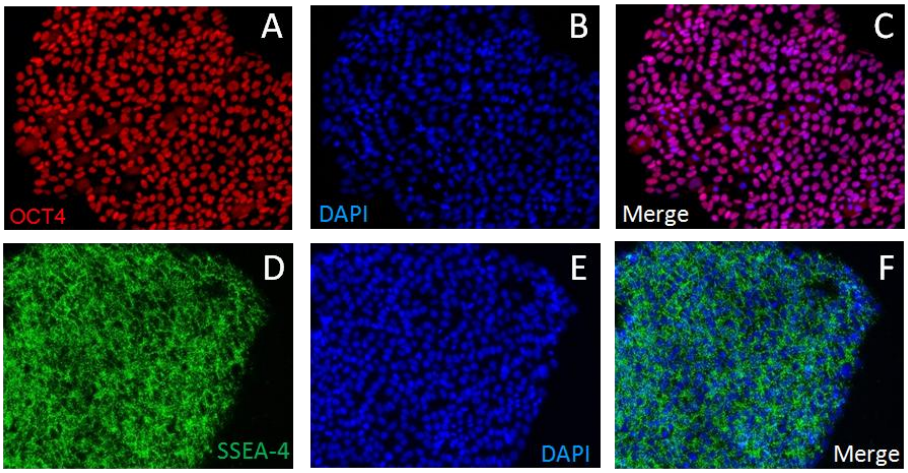

Figure 2. Expression of pluripotency markers after culture in NutriStem Medium. Expression of the pluripotency markers Oct4 (A-C) and SSEA-4 (D-F) by H1 human ES cells after 5 passages in NutriStem Medium.

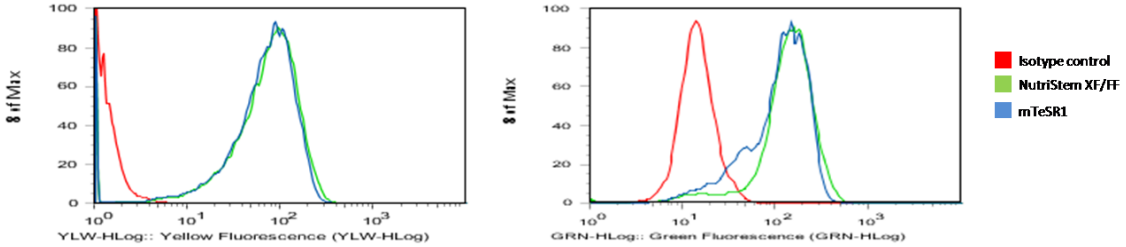

Figure 3. Flow cytometry analysis. H1 human ES cells cultured for 5 passages in either NutriStem Medium or mTeSR1 (Stem Cell Technologies) were subjected to flow cytometry analysis for the pluripotency markers Oct4 (left panel) and SSEA-4 (right panel). Greater than 98% of the cells cultured in NutriStem Medium express Oct4 and SSEA-4, similar or better than what is observed with mTeSR1.

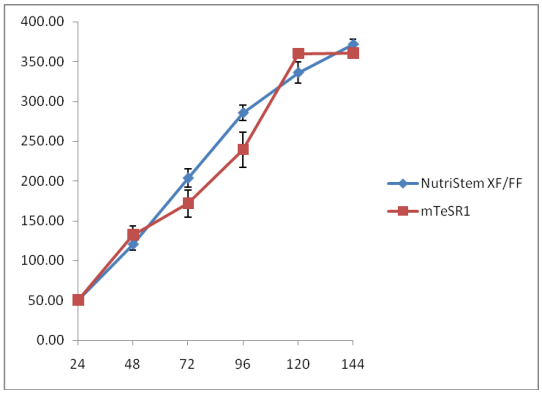

Figure 4. Proliferation data. The proliferation rate of H1 human ES cells grown for 6 passages in either NutriStem Medium or mTeSR1 was examined at 24-hour intervals over six days. The growth rate of cells in NutriStem Medium is similar to that of cells grown in mTeSR1.

At passage 14, the karyotype of the hES cells cultured in NutriStem Medium was analyzed by standard G-band analysis. No chromosomal abnormalities were found in any of the 20 nuclei analyzed (data not shown).

The pluripotency of the cells after long term culture in NutriStem Medium was examined by two different methods. After 15 passages, the hES cells were spontaneously differentiated as embryoid bodies (EBs). Cells cultured in NutriStem Medium readily formed EBs (**Figure 5A**), and were cultured in suspension for eight days before being plated for eight additional days in adherent culture. Cells differentiated by this protocol showed expression of genes from all three germ layers by RT-PCR analysis (**Figure 5B**). In addition, after 17 passages, hES cells were injected subcutaneously into SCID mice for teratoma formation; five million cells were injected into each of five sites on three mice. Tumors were observed after four weeks at two of the five injection sites, and these two tumors were harvested after nine weeks. Cells from each of the three germ layers were found in both of the tumors (**Figure 6**).

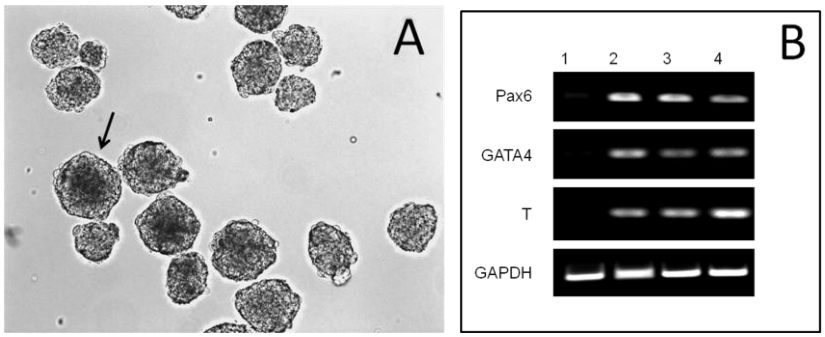

Figure 5. In vitro differentiation after culture in NutriStem Medium. (A) Spontaneous differentiation by embryoid body (EB) formation was performed with H1 hES cells after 14 passages in NutriStem Medium. EBs cultured for 8 days in suspension formed spherical cystic bodies typical of EBs (example, arrow). (B) After 8 days in suspension culture and 8 days in adherent culture, cells were analyzed by RT-PCR for expression of differentiated markers. Unlike the control hES cells in NutriStem Medium (lane 1), differentiated cultures (lanes 2-4) expressed markers from all three germ layers: *Pax6* (ectoderm), *gata4*, and *Brachyury/T* (mesoderm).

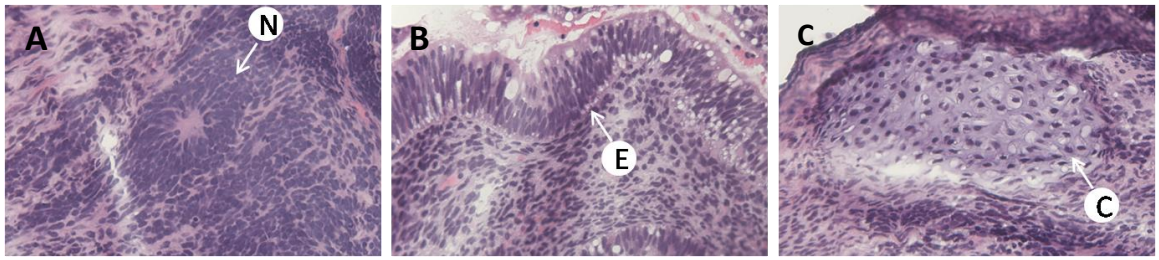

Figure 6. Teratoma formation after culture with NutriStem Medium. H1 human ES cells that had been cultured with NutriStem Medium for 14 passages were injected subcutaneously into SCID mice. Teratomas were harvested after 8 weeks and processed for histological analysis. Tissue types from all three germ layers could be identified in the sections (A-C). (A) ectoderm with neural rosette (arrow marked N); (B) endoderm with columnar epithelium (arrow marked E); (C) mesoderm with cartilage (arrow marked C).

EXPERIMENTAL PROCEDURES

Transferring hES cells from Standard Feeder-Based Culture into NutriStem Medium in a Feeder-Free Environment

hES cells cultured in 6-well plates were washed once with Dulbecco's PBS (DPBS) then dissociated with Dispase (1 mg/ml dissolved in DMEM/F12 medium) for 2 to 5 minutes. After incubation, the Dispase was removed and the wells were washed one time with DPBS. 2 ml of NutriStem Medium was added and the colonies were dislodged by gentle rinsing. Cells were centrifuged at 200 x g for 5 minutes and resuspended in 6 to 12 ml of NutriStem Medium. A 6-well Matrigel-coated plate was warmed to room temperature for 30 minutes. Once warmed, the Matrigel was removed and 2 to 3 ml of the resuspended cells were transferred to each well for a passage ratio of 1:3 to 1:6. Cells were cultured and medium was changed daily with 2 to 3 ml fresh NutriStem Medium until the undifferentiated colonies were big enough to passage (about 5 to 7 days).

Passaging hES cells Grown in NutriStem Medium

Culture medium was aspirated and cells were washed with 2 ml per well of DPBS. 1 ml per well of Dispase was added and cells were incubated at room temperature until the edges of the colonies started to fold up. After incubating, the Dispase was removed and cells were washed once with 2 ml per well of DPBS. 2 ml of NutriStem Medium was added to each well. Colonies were scraped and transferred to a conical tube. 6 to 12 ml of NutriStem Medium was added. A 6-well Matrigel-coated plate was warmed to room temperature, Matrigel was removed, and 2 to 3 ml of the resuspended cells were transferred to each well for a passage ratio of 1:3 to 1:6. Medium was changed daily with 2 to 3 ml of fresh NutriStem Medium until the undifferentiated colonies were big enough to passage (about every 5 to 7 days).

Immunocytochemistry and Flow Cytometry

Immunocytochemistry and flow cytometry were performed using standard procedures (reference *Protocol: Immunocytochemistry and Protocol: Flow Cytometry* online at www.stemgent.com /support/protocols).

Cell Proliferation Assay

Assay was conducted according to the protocol included with the CyQuant® Cell Proliferation kit.

Embryoid Body Formation

Medium was aspirated and the hES cell culture was washed with 2 ml per well of sterile DPBS. 1 ml per well of Dispase was added and cells were incubated at 37°C until the edges of the colonies started to loosen and slightly fold up. The Dispase was removed, 2 ml per well of NutriStem Medium was added, and colonies were scraped and washed off with a 5 ml pipet. Cells were transferred into a sterile 50 ml suspension culture flask with 20 ml of prewarmed NutriStem Medium. The flask was incubated for 2 days then the culture medium was changed to EB Formation Medium (DMEM/F-12 supplemented with 20% Knockout™ Serum Replacement, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 1% nonessential amino acids). Medium was changed every two days for a total of 8 days. The wells of a 6-well tissue culture plate were coated with 0.1% gelatin the day before attaching the EBs. On day 8, the EBs were collected by transferring them to sterile 50 ml conical tubes and allowing them to settle by gravity for 5 minutes. EBs were resuspended in DMEM supplemented with 10% fetal bovine serum and cultured for an additional 8 to 10 days before ICC and RT-PCR analysis (medium was changed when the color changed to yellow).

Karyotype Analysis

Cells were sent to the Cytogenetics Laboratory, Department of Pathology, Children's Hospital & Research Center, Oakland.

RT-PCR for in vitro Differentiation

Cells were collected and stored at -80°C before RNA extraction. Total RNA was isolated using the Qiagen RNA Isolation Kit, and the reverse transcription reaction was done by following kit protocol. The PCR primers used were: human Pax6, 5′-tcaggcttcgctaatggg-3′ and 5′-aaaaggcctcacacatctg-3′; human GATA4, 5′-ggaagcccaagaacctgaat-3′ and 5′-ctggagttgctggaagcac-3′; human Brachyury(T): 5′-acagcgcatgatcaccag-3′ and 5′-

tttgcaaatggattgtacttaatttt-3'. The PCR amplification conditions were: 94°C 3 minutes denature, followed by 40 cycles of 94°C 30 seconds, 55°C 30 seconds and 72°C 30 seconds, followed by 3 minutes extension at 72°C.

Teratoma Formation

Passage 17 H1 hES cells were harvested and subcutaneously injected into SCID mice. Each mouse received two injections with 5 million cells per injection. Mice were observed weekly for teratoma formation. The tumors were collected after 9 weeks. After blocking and sectioning, the tissue slides were stained with H&E and examined by a pathologist.

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