A Novel Serum-Free and Animal Component-Free Culture Medium for the Propagation and Maintenance of Human Embryonic Stem Cells

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ABSTRACT

Human embryonic stem cells (hESCs) are viewed as a valuable resource for both cell-based drug discovery and potential therapeutics for a number of currently intractable human diseases. However, many of the currently derived and available hESC lines being propagated are directly or indirectly exposed to reagents which are serum-free but ill-defined and/or contain animal components. This is problematic for their potential future use in therapeutics for a number of reasons; the risk of rejection in transplantation due to the presence of non-human sialic acid, and also the possible transfer of non-human pathogens.

HEScGRO™ is a serum-free and animal-component-free medium that is suitable for the culture and propagation of hESCs. All components in this medium are either recombinant or derived from human sources; this medium does not contain bovine serum albumin (BSA) or BSA-derived fractions, such as are found in many commonly used hESC culture media. This medium was designed for use with a feeder layer of mitotically-inactivated human fibroblasts.

We tested HEScGRO with five different hESC lines: H1, H9, MEL-1, MEL-2 and MEL-4. We found that the medium is able to support propagation of each of these lines, with the vast majority of cells remaining in the pluripotent state beyond 25 passages (approximately 125 population doublings); pluripotency was demonstrated by expression of appropriate markers (alkaline phosphatase, OCT-4, TRA-1-60, TRA-1-81). In addition, hESCs cultured in this medium were able to efficiently differentiate via embryoid bodies, and expression of appropriate markers from all three germ layers was observed. Finally, hESCs cultured in this medium maintained a normal karyotype throughout the culture period. These results demonstrate that HEScGRO allows for hESCs to be successfully maintained in culture conditions that preserve their suitability for therapeutic use.

INTRODUCTION

Human embryonic stem (ES) cells are derived from a pluripotent cell population (the interstitial cell mass, or ICM), they can replicate indefinitely in the embryonic state, they maintain a normal karyotype, and under appropriate conditions, can differentiate into cells representative of all three germ layers in teratomas or *in vitro*[1]. Human ES cells were initially derived and cultured using the same culture conditions that were being used at that time to culture mouse ES cells[2-5]; these conditions included the use of feeder layers made up of mouse embryonic fibroblasts. These culture conditions were clearly not optimal for human ES cell culture, and since that time, a number of challenges have been driving the development of improved methods in this area. These challenges include: 1) the need for defined conditions in which all components are known; 2) the need to greatly expand a human ES cell population, particularly for high-throughput applications; 3) the need for "xeno-free" culture conditions in which all components are human-derived or synthetic, in order to have human ES cells in a suitable state for therapeutic applications (including cells derived from ES cells).

While significant progress has been made towards meeting some of these challenges in recent years, the development of xeno-free conditions has lagged. For example, the use of a serum-replacer instead of fetal bovine serum (FBS) has removed some of the variability associated with FBS, and led to human ES cell media that can be called "serum-free"[6,7]; however, this serum-replacer contains bovine serum albumin and is thus inappropriate for culturing cells intended for therapeutics. Several growth factors have been identified that promote pluripotent growth of human ES cells in culture, most notably basic fibroblast growth factor (bFGF)[7-9]. However, the use of bFGF for human ES cell culture, particularly at the high levels used by some for "feeder-free" culture, must be considered carefully, as bFGF may push ES cells towards differentiation through known positive effects upon ectodermal and mesodermal marker expression[10]. Thus a need has remained for a xeno-free medium that does not rely upon high levels of bFGF.

Recently, a novel medium for xeno-free, serum-free culture of human ES cells has been developed. This medium, called HEScGRO, is a proprietary formulation that contains only humanized or synthetic components, including bFGF that is manufactured under animal-free conditions; in addition, HEScGRO contains human serum albumin rather than its bovine counterpart. HEScGRO was designed for use with human fibroblast feeders and has been validated with two different commercially-available lines, Detroit 551[11] and WS1; the use of human feeders maintains the xeno-free nature of the culture system, and allows the use of low levels of bFGF (20 ng/ml).

METHODS

hESC Propagation: MEL-1, MEL-2 (data not shown), MEL-4, H1 and H9 are routinely cultured in HEScGRO™ on Mitomycin C-treated Detroit551 or WS1 fetal skin fibroblasts feeders (ATCC; D551/CCL-110, WS1/CRL-1502). hESCs were passaged manually except for the cells described in Figure 7, which were passaged with Accumax™ (Millipore SCR006; protocol available).

Alkaline Phosphatase Staining: Colonies were fixed with 4% paraformaldehyde in D-PBS for 20 minutes, and were subsequently processed with an alkaline phosphatase detection kit (Millipore SCR004). D-PBS was added to each well for cell visualization at phase contrast.

Pluripotent Marker Expression of hESCs by Immunocytochemical Analysis: Colonies were fixed with absolute ethanol (MEL-4) or 4% paraformaldehyde (H1, H9) and permeablized with 0.1% Triton X-100. Primary antibodies mouse anti-OCT4 (Millipore MAB4401), mouse anti-SSEA-1 (Abcam #ab16285), mouse anti-TRA-1-60 (Millipore MAB4360) or mouse anti-TRA-1-81 (Millipore MAB4381) were followed by secondary antibody goat anti-mouse Alexa Fluor® 555 (MEL-4) or goat anti-mouse-FITC (H1, H9).

hESC Differentiation: MEL-4 human embryonic stem cells were bulk cultured for 3 weeks in HEScGROTM medium on D551 fibroblast feeders. hESC colonies were then dissociated with 0.25X TrypLETM and the single cell suspension seeded into 96-well plates (U-bottom) with 4000 cells/well, centrifuged at 478xg (1500rpm) for 5mins. 'Spin3' EBs[12] were cultured for 13 days in HEScGRO minus bFGF with media changes on days 3, 5, 7, 9 and 11. Cells collected for 8x pooled EB wells (above) and 8x single EB wells (data not shown) for day 0-13. cDNA isolation for PCR analyses from TrizolTM preparation and Oligo-dT primed RT-PCR.

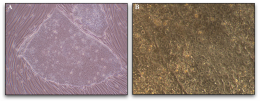

Figure 1. Human ES cells grown in HEScGRO xeno-free, serum-free medium have appearance and morphology typical of human ES cells grown on human feeders. A. H9 cells, with well-defined borders and homogenous appearance within the colony. B. MEL-4 cells (shown at higher magnification) display high nuclear-to-cytoplasmic ratio and visible nucleoli.

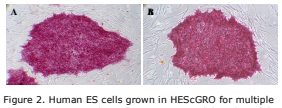

Figure 2. Human ES cells grown in HEScGRO for multiple passages maintain their pluripotency. H1 (A) and H9 (B) cells grown for 17 passages in HEScGRO, and subsequently reacted for alkaline phosphatase activity.

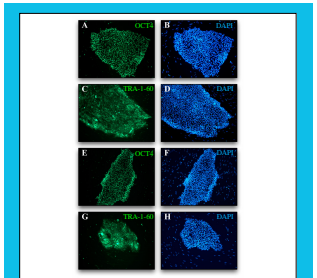

Figure 3. H1 (WA01) and H9 (WA09) cells grown for multiple passages in HEScGRO continue to display markers of pluripotency. (A-D) H1 cells after 17 passages in HEScGRO show expression of OCT4 (A) and TRA-1-60 (C). Cells throughout each colony express these markers, as shown by comparison to the corresponding DAPI staining in B, D. (E-H) H9 cells after 17 passages showing expression of OCT4 (E) and TRA-1-60 (G), with corresponding DAPI images in F, H. All cells maintained a normal karyotype (not shown).

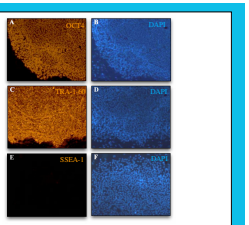

Figure 4. MEL-4 human ES cells grown for 20 passages in HEScGRO have high expression of pluripotency markers and low expression of a differentiation marker. Expression of OCT4 (A) and TRA-1-60 (C) in passage 20 MEL-4 cells is high, while expression of SSEA-1 (E) is low. Corresponding DAPI images shown in B, D, and F. All cells maintained a normal karyotype (not shown).

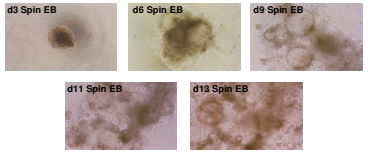

Figure 5. Cells grown in HEScGRO form embryoid bodies (EBs). Representative EBs, formed from hESCs cultured in HEScGRO, are shown at different time points during differentiation.

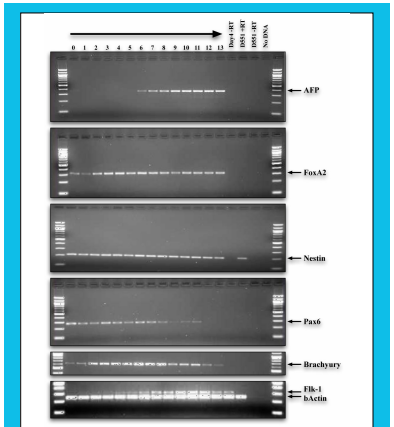

Figure 6. Human ES cells grown in HEScGRO can differentiate to all three germ layers. MEL-4 cells grown in HEScGRO were differentiated via embryoid body (EB) formation in HEScGRO lacking bFGF. Samples were taken every day for 13 days and processed for RT-PCR of the following markers of differentiation: *alpha fetoprotein* (AFP) and *foxA2* (endoderm), *nestin* and *pax6* (ectoderm), *brachyury* and *flk-1* (mesoderm). Expected products are indicated by the arrows.

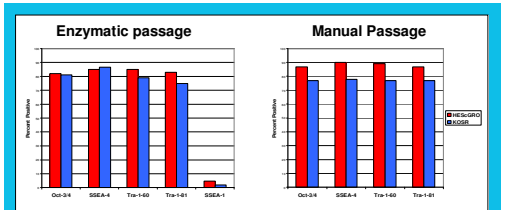

Figure 7. Cultures of cells grown in HEScGRO express levels of pluripotency markers comparable to cells grown in medium containing 20% KOSR as determined by flow cytometry. H9 hESCs were passaged either ezymatically with Accumax (left) or manually (right) 5 times in HEScGRO (red bars) or 20% KOSR medium (blue bars). Cells were then processed for flow cytometry, and labeled with either OCT4, SSEA-4, TRA-1-60, TRA-1-81 or SSEA-1.

Summary of HEScGRO and SR Media Comparison

Cell Integrity
Cells grown in HEScGRO and manually/enzymatically passaged have slightly different morphology than those grown in SR-containing medium.
Cells grown in HEScGRO maintain normal karyotype.

Pluripotency
By FACS and ICC, cultures grown in HEScGRO express pluripotency markers at levels comparable to cultures grown in SR-containing medium.

Proliferation/Cell Number
Manually passaged cells in HEScGRO show decreased cell number/proliferation compared to manually passaged cells in SR-containing medium.
Enzymatically passaged cells in HEScGRO show similar cell numbers/proliferation as cells enzymatically passaged in SR-containing medium.

Apoptosis
Comparable amounts of apoptosis in cells grown in HEScGRO vs. SR-containing medium.

Differentiation Capacity
For EB formation, cells grown in HEScGRO can form EBs but morphologically, EBs are slightly different than those grown in SR-containing medium.

SUMMARY

HEScGRO is the only commercially available serum- and animal component-free human embryonic stem cell medium.
HEScGRO is complete and ready-to-use.
HEScGRO has been validated for long-term (> 20 passages) culture with licenced cell lines (MEL-1, MEL-2, H1, and H9) and MEL-4.
Human embryonic stem cell colonies cultured in HEScGRO retain pluripotency by:
- Alkaline phosphatase activity and expression of the pluripotency markers OCT4, SSEA-3, SSEA-4, TRA-1-61, TRA-1-81 (and negative for SSEA-1).
- Genetic stability as designated by normal karyotype (not shown).
- Ability to form embryoid bodies and express progenitor endoderm, ectoderm and mesoderm genetic markers.

Key benefits of HEScGRO:
- Convenience of a standardized medium eliminating batch testing.
- Reproducibility and consistency.
- Serum-free offers defined environment for significant research settings such as drug discovery and development.
- Animal-free offers ability to grow cells for therapeutic applications.

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