A Comparative Analysis of Human Embryonic Stem Cells Cultured in a Variety of Media Conditions

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Abstract

Human embryonic stem cells (hESCs) hold great promise for therapeutic applications. However, their use in regenerative medicine is hindered by the presence of non-human and/or undefined components typically included in the maintenance media. The development of HEScGRO™ medium is a step towards establishing humanized and defined conditions for hESC growth. For this study, we performed direct, side-by-side comparisons between the growth characteristics of hESCs in HEScGRO medium and traditional serum replacement conditions. Short- and long-term growth parameters were examined in detail. Specifically, general morphology and karyotype was followed, proliferation and apoptosis assays were performed, the expression of pluripotency markers by immunocytochemistry and FACS analysis was investigated, and the differentiation potential was analyzed. Culture of hESCs in HEScGRO medium maintained the cells in a pluripotent state, and the characteristics of these hESCs were similar to those grown in serum replacement media conditions. HESCs grown in humanized and defined media such as HEScGRO medium will be useful for basic research and also, significantly, for downstream applications that include cell-based therapies.

Introduction

Since the initial isolation and characterization of human embryonic stem cells[1], there has been rapid growth in research on hESCs. This field offers a valuable system for the study of human development and disease and has significant potential applications for regenerative medicine. However, obstacles remain for the efficient and consistent culture of hESCs, as culture of the cells can be technically challenging. There is a movement towards increasingly defined growth media and conditions as well as removal of non-human components in hESC culture to assist in the development of therapeutic applications.

HEScGRO medium is a defined, humanized media that has been developed for the long-term culture of hESCs. The medium has a proprietary formulation that uses only humanized or synthetic components, including bFGF that is manufactured under animal-free conditions. Additionally, HEScGRO medium contains human serum albumin rather than its bovine counterpart. HEScGRO medium was designed for use with human fibroblast feeders and has been validated with two different commercially-available lines, Detroit 551 and WS1.

To date, there have been no side-by-side, detailed, comparative studies on the growth characteristics of hESCs in HEScGRO medium to hESCs grown in traditional

Knockout™ Serum Replacement (KOSR) medium conditions (method reviewed in reference 2). For this study, we have compared the long- and short-term growth characteristics of hESCs on HEScGRO medium and serum replacement medium conditions. For short-term studies, cells were passaged both enzymatically[3] and manually; for long-term studies, enzymatic expansion was performed. Analysis parameters included the examination of colony morphology, karyotype stability, expression of pluripotency markers by immunocytochemistry and FACS analysis, proliferation, and differentiation potential. HESCs grown in HEScGRO medium were shown to exhibit similar short- and long-term characteristics to hESCs grown in serum replacement conditions. Hence, hESC cultures that are grown in HEScGRO medium are of high quality and have the advantage of being grown in humanized conditions which is beneficial for eventual therapeutic applications.

Materials and Methods

hESC CULTURE

H9 (WiCell) hESCs at passage 34 were grown on Matrigel® coated dishes. Feeder cells were either mitomycin C-treated Detroit 551 cells (ATCC) at a density of 60,000 cells/cm^2 or mitomycin C-treated MEFs (CF-1 strain) (Millipore) at 18,000 cells/cm^2. HESCs were maintained in either KOSR medium (20% Knockout Serum Replacement (Invitrogen) and DMEM-F12, 1X NEAA, 1X BME, and bFGF at 10 ng/mL (Millipore)) or HEScGRO medium (Millipore) with media changes every 1-2 days and passaged every 6-7 days, depending on cell density. For manual passaging, approximately 30-40 pieces of colonies were transferred per well of a 6-well plate. For enzymatic passaging, KOSR grown cells were passaged using collagenase IV at a ratio of 1:3 while HEScGRO cells were enzymatically expanded using Accumax™ Solution (Millipore) at a ratio of 1:3-1:6.

CELL CHARACTERIZATION

For ICC and FACS analysis the following antibodies were used: Oct-4, SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81 (All Millipore). A Cy3-conjugated goat anti-mouse antibody was used for ICC and a PE goat anti-mouse antibody was used for FACS. For alkaline phosphatase staining, an Alkaline Phosphatase Detection Kit (Millipore) was used. For proliferation studies, a BrdU Cell Proliferation Assay Kit (Millipore) was used. Finally, the ApopTag® Plus Fluorescein *In Situ* Apoptosis Detection Kit (Millipore) was used to measure apoptosis.

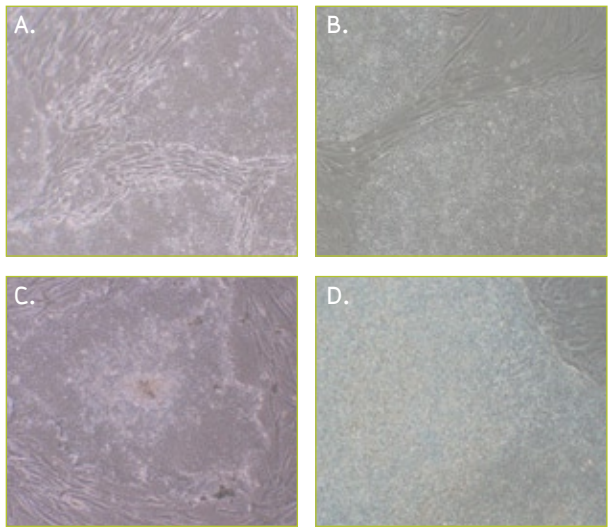


Figure 1. Morphology of hESCs grown in HEScGRO medium as compared to KOSR medium conditions at passage 5. H9 hESCs grown in A) HEScGRO and B) 20% KOSR medium and enzymatically passaged. H9 hESCs grown in C) HEScGRO and D) 20% KOSR media and manually passaged. Cells are at day 6 following passaging.

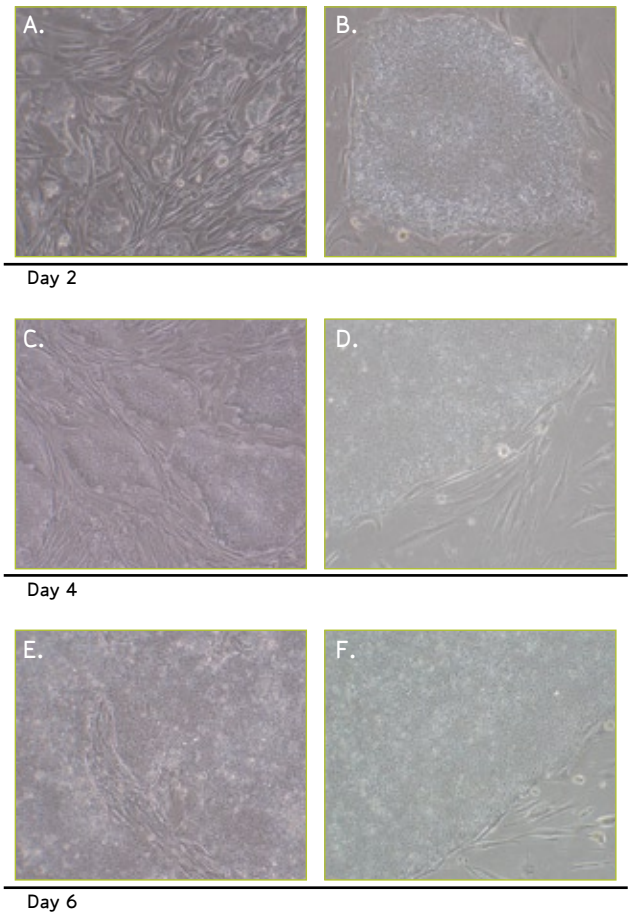


Figure 2. Morphology of hESCs grown in HEScGRO as compared to KOSR medium conditions at passage 22. H9 hESCs grown in HEScGRO (A, C, E) and 20% KOSR medium (B, D, F) at days 2, 4, and 6 following enzymatic expansion.

DIFFERENTIATION

For differentiation of hESCs, cells were manually scraped and plated on low adhesion plates in FBS containing Human Embryonic Stem Cell Embryoid Body Formation Medium (Millipore). Embryoid bodies (EBs) were cultured in suspension for 7-21 days and plated onto 0.1% gelatin (Millipore) coated dishes for a variable number of days. For RT-PCR analysis, RNA was isolated using the Absolutely RNA® Miniprep Kit and reverse transcribed using SuperScript™ II Reverse Transcriptase. Platinum™ Taq DNA Polymerase was used for the reactions. For ICC, Pax-6, cTNI, and SMA (All Millipore) and AFP were utilized. Secondary antibodies were Cy3 conjugated goat anti-mouse antibody and Alexa Fluor® 555 goat anti-rabbit.

Results and Discussion

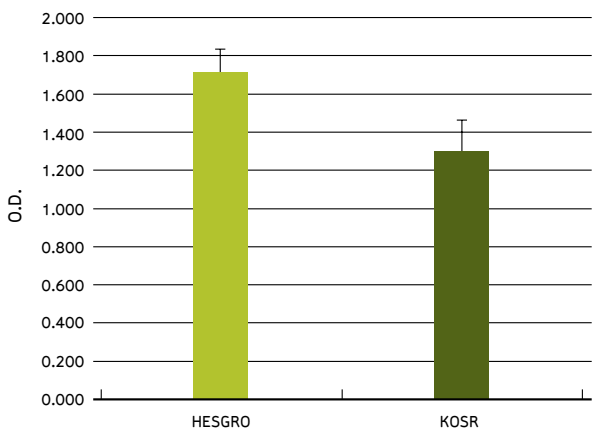
A comparative, side-by-side comparison was performed on hESCs grown in KOSR medium or HEScGRO animal component-free medium. Low passage, karyotypically-stable H9 hESCs were passaged both manually and enzymatically in the two media conditions for five passages. Cells cultured in KOSR medium were enzymatically expanded using collagenase IV, whereas cells grown in HEScGRO medium were expanded using Accumax. Collagenase IV was not used to enzymatically passage hESCs grown in HEScGRO since it causes cells to rapidly differentiate (data not shown). Long-term passage data (over 21 passages) was collected only from cells that were enzymatically expanded.

The morphology of hESCs was compared in the different media. HESC colonies passaged in HEScGRO medium had slightly flatter morphology when compared to their KOSR medium counterparts (Figure 1A, C versus Figure 1B, D) but grew as compact colonies with distinct borders and displayed a high nuclear-to-cytoplasmic ration. The morphology of hESCs expanded for over 22 passages in both conditions had similar morphology to short-term cultures (Figure 2). Enzymatic passaging with Accumax solution resulted in smaller colony sizes upon passaging when compared with collagenase IV. This discrepancy of colony size is not reflective of proliferation rates. HESCs expanded with Accumax solution had slightly higher proliferation rates (Figure 3A) and an increase in cell yield (Figure 3B) when compared to hESCs grown in KOSR and passaged with collagenase IV. A normal karyotype was also maintained in long-term enzymatic expansion culture (passage 21) in both media conditions (data not shown).

To further characterize the pluripotent status of hESCs grown in both conditions, cultures were examined for the expression of multiple pluripotency markers. All cells expressed high levels of alkaline phosphatase in both short (Figure 4) and long-term expansion (Figure 5). Expansion of cells with Accumax solution often led to a dense monolayer of hES cells that maintained high levels of alkaline phosphatase expression (Figure 4B). Cells were also strongly immunoreactive with markers common to hESCs in both short term (data not shown) and long term growth conditions (Figure 6). Specifically, HEScGRO medium maintained hESCs expressed high levels of Oct-4, TRA-1-60, and TRA-1-81 (Figure 6a) as shown by immunocytochemistry. Additionally, cells grown in HEScGRO medium and either enzymatically or manually passaged expressed levels of pluripotency markers that were comparable to cells grown in KOSR medium as determined by flow cytometry (Figure 7a). FACS analysis on extended culture of hESCs in HEScGRO and KOSR media also showed comparable levels of pluripotency marker expression (Figure 7b). Levels of apoptosis were similar in both media and both passaging methods (data not shown).

Long-term cell cultures in both media were assayed for the *in vitro* ability to differentiate into three embryonic

A.



B.

Medium Condition	Cell Number per 6-Well Dish
HEScGRO Accumax	1.7 x 106
KOSR Collagenase	1.3 x 106
HEScGRO Manual	0.5 x 106
KOSR Manual	1.6 x 106

Figure 3. Proliferation and cell numbers of cells of cells grown in HEScGRO and KOSR medium conditions. a) Proliferation as measured by BrdU incorporation in H9 hESCs in HEScGRO and KOSR medium that have been enzymatically expanded for 12 passages and b) yield of cells grown in various media conditions.

germ layers through formation of embryoid bodies (EBs). EBs grown in HEScGRO medium had a slightly smaller morphology and a less cystic quality (Figure 8A-D) than those grown in KOSR (Figure 8E-H). After variable times in suspension, EBs were transferred to adherent conditions and further cultured. Immunostaining and RT-PCR were performed for lineage specific markers. By RT-PCR, the expression levels of pluripotent markers (Nanog and Oct-4) declined more slowly in the cells cultured in HEScGRO medium compared to cells cultured in KOSR medium (Figure 9). Higher ectodermal (Pax-6) marker levels were observed in EBs from hESCs cultured in HEScGRO medium in comparison to their KOSR counterparts. This possibly indicates than hESCs maintained in HEScGRO medium may have a greater ability to differentiate towards ectodermal lineages than hES cells grown in KOSR medium. Immunostaining of differentiated hESCs grown for eighteen passages in HEScGRO medium conditions detected markers specific for endoderm (AFP), ectoderm (Pax-6), and mesoderm

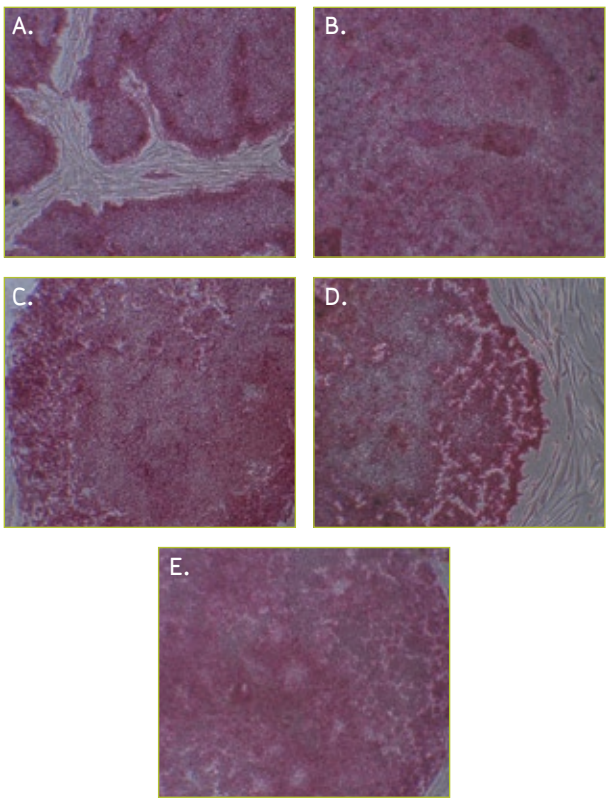


Figure 4. Alkaline phosphatase staining of hESCs grown in HEScGRO as compared to KOSR medium at passage 5. H9 hESCs grown in a-b) HEScGRO and c) 20% KOSR medium and enzymatically expanded. H9 hESCs grown in d) HEScGRO and e) 20% KOSR medium and manually expanded.

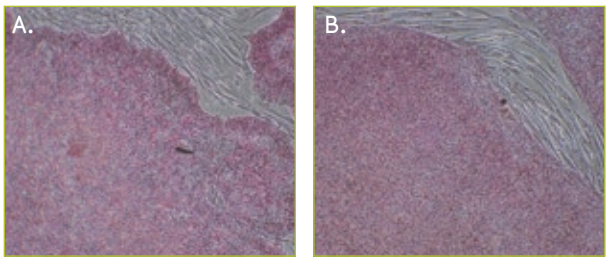


Figure 5. Alkaline phosphatase staining of hESCs grown in HEScGRO medium as compared to KOSR medium at passage 22. H9 hESCs grown in A) HEScGRO medium and B) 20% KOSR medium and enzymatically expanded.

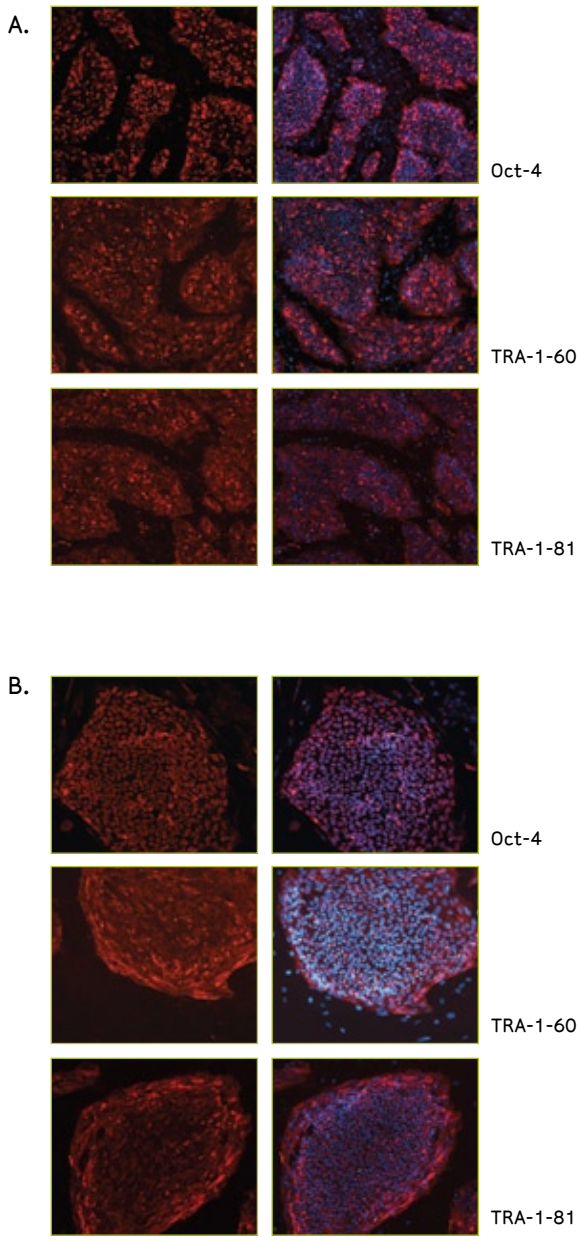


Figure 6. Stem cell marker expression in hESCs grown in HEScGRO and KOSR medium conditions. H9 hESCs were grown for 20 passages in A) HEScGRO medium and B) KOSR medium conditions and analyzed for the expression of the pluripotency markers Oct-4, TRA-1-60, and TRA-1-81 by ICC at day 3 following passaging. Staining for the pluripotency markers alone is shown in the left panels with the right panels depicting a merge of antibody staining with DAPI counter-staining.

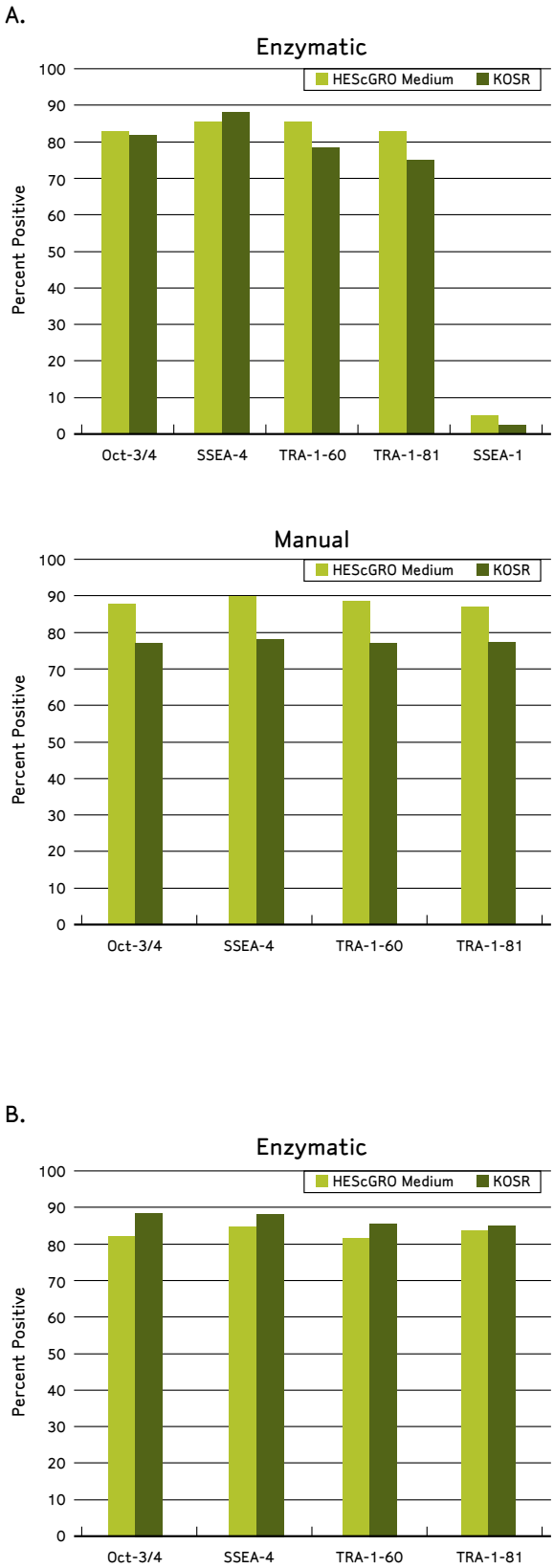


Figure 7. FACs analysis of hESCs. Percentage of positive cells for various stem cell markers of H9 hESCs expanded A) 5 times enzymatically and manually in HEScGRO and KOSR and B) 10 times enzymatically in HEScGRO and KOSR medium.

(cTNI and SMA) (Figure 10). Additionally, hESCs cells grown in HEScGRO medium were able to differentiate to beating cardiomyocytes (data not shown). Hence, cells grown in HEScGRO medium show a similar capability for differentiation to cells grown in KOSR medium conditions.

Conclusion

The characteristics of hESCs expanded for both short and long-term in the animal component free medium HEScGRO medium in comparison to KOSR medium are very similar (summarized in Table 1). Morphologically, hESCs grown in HEScGRO medium are compact and express high levels of various pluripotency markers. When enzymatically expanded, cells grown in HEScGRO medium have a slightly higher rate of proliferation and a higher cell yield when compared to enzymatically-passaged KOSR medium cells. Both cell populations maintain a stable karyotype over extended passages and display similar levels of apoptosis. The differentiative capacity of hESCs grown in both media are similar, with a slight increase in ectodermal differentiation potential (as measured by Pax-6 expression) in HEScGRO media conditions. Hence, hESCs grown in HEScGRO medium are similar to cells grown in KOSR medium conditions. HEScGRO medium, however, has the benefit of being a defined, animal-free medium with increased value for downstream therapeutic applications.

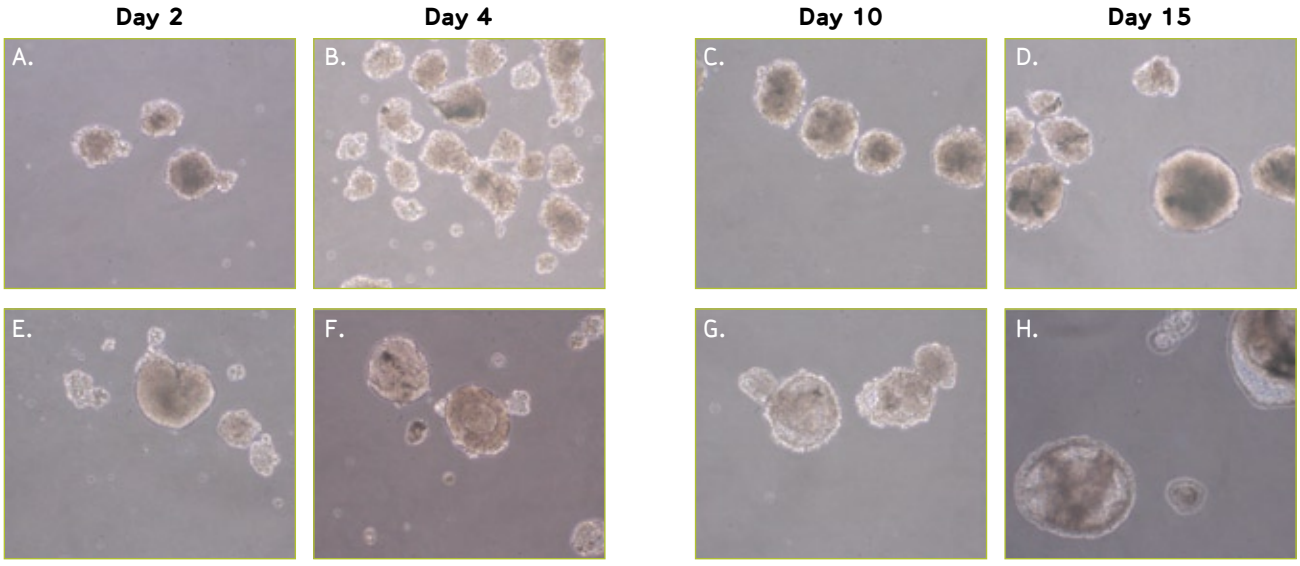


Figure 8. Comparative morphology of EBs formed from hESCs after 19 passages in HEScGRO and KOSR medium conditions. A-D) H9 hESCs grown in HEScGRO and enzymatically passaged. E-H) H9 hES cells grown in KOSR and enzymatically passaged.

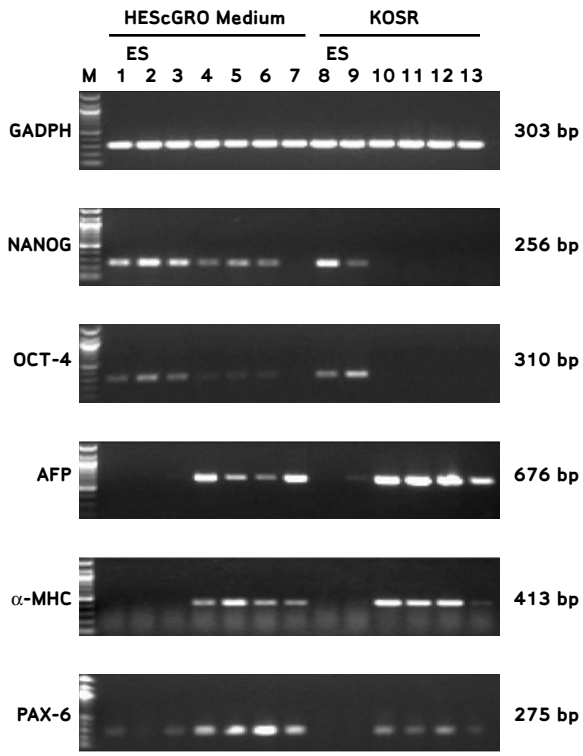


Figure 9. RT-PCR analysis of hESCs cultures in HEScGRO and KOSR medium conditions. RT-PCR analysis of H9 hESCs cultured in HEScGRO medium (1 to 7) and KOSR medium (8-13). (1, 8) ES, 5 passages; (2, 9) ES, 20 passages; (3, 10) 13 day EBs with enzymatic passage; (4, 11) 13 day EBs with manual passage; (5, 12) 17 day EBs; (6, 13) 23 day EBs; (7) 30 day EBs. To characterize gene expression, different markers were amplified via RT-PCR. The level of cDNA in each set of markers has been optimized to reach quantifiable conditions. The size of each amplified fragment in base pairs (bp) is shown on the right of each panel.
M: molecular weight marker.

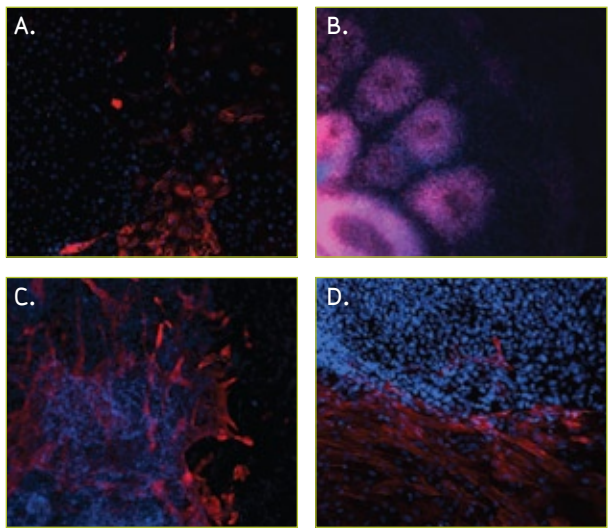


Figure 10. Differentiative capacity of hESCs grown in HEScGRO Medium. Cells grown for 18 passages and expanded enzymatically in HEScGRO medium are able to differentiate to all three germ layers as shown by positive and specific staining for AFP (endoderm), Pax-6 (ectoderm), and CTNI and SMA (mesoderm).

Table 1. Summary of HEScGRO and KOSR Medium Comparison.

Cell Integrity

General Morphology: Cells grown in HEScGRO medium and manually/enzymatically passaged maintain distinct borders and have a high nuclear to cytoplasmic ratio.

Karyotype: Cells grown in HEScGRO medium maintain normal karyotype.

Proliferation/Cell Number

Manually passaged cells in HEScGRO medium show decreased cell number than manually passaged cells in KOSR.

Enzymatically passaged cells in HEScGRO medium show slightly higher cell numbers/proliferation than cells enzymatically passaged in KOSR.

Pluripotency

By FACS and ICC, cells grown in HEScGRO medium express pluripotency markers.

Apoptosis

Comparable amounts of apoptosis in cells grown in HEScGRO medium versus KOSR.

Differentiation Capacity

Cells grown in HEScGRO medium can form EBs that are morphologically slightly different than those grown in KOSR.

HEScGRO medium passaged cells are able to differentiate to mesoderm, endoderm, and ectoderm.

References

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3. Emre, N., Mondeh, R., Singer, M. The Use of Accumax for Enzymatic Passaging of Human Embryonic Stem Cells Cultured in HEScGRO. *Cellutions* **1**:16-18 (2007).

Millipore Products

Description	Catalogue No.
HEScGRO Medium, 500 mL	SCM020
EmbryoMax® Primary Mouse Embryo Fibroblasts, Strain CF1, Mitomycin C treated	PMEF-CF
DMEM-F12	DF-041-B
BME	ES-007-E
NEAA	TMS-001-C
bFGF	GF003
Accumax	SCR006
PBS	BSS-1006-B
Oct-4 Antibody	MAB4401
SSEA-1 Antibody	MAB4301
SSEA-4 Antibody	MAB4304
Tra-1-60 Antibody	MAB4360
Tra-1-81 Antibody	MAB4381
Alkaline Phosphatase Detection Kit	SCR004
BrdU Cell Proliferation Assay	2750
ApopTag Plus Fluorescein *In Situ* Apoptosis Detection Kit	S7111
ApopTag Peroxidase *In Situ* Apoptosis Detection Kit	S7100
ApopTag Peroxidase *In Situ* Oligo Ligation (ISOL) Apoptosis Detection Kit	S7200
ApopTag ISOL Dual Fluorescence Apoptosis Detection Kit (DNase Type I & II)	APT1000
Human Embryonic Stem Cell Embryoid Body Formation Medium	SCM026
Pax-6 Antibody	AB5409
cTNI Antibody	MAB1691
SMA Antibody	CBL171

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