HEScGRO™ Xeno-Free Medium for Human Embryonic Stem Cell Culture

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Human embryonic stem cells (hESCs) are considered a valuable resource for both cell-based drug discovery and potential therapeutics for a number of human diseases. However, many of the currently 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 due to a number of reasons: the risk of rejection in transplantation caused by the presence of non-human sialic acid[1] and also the possible transfer of non-human pathogens. HEScGRO medium (Cat. No. SCM020) is the first ready-to-use, serum- and animal-component-free medium. Developed by Stem Cell Sciences and commercially available through Millipore, HEScGRO medium is designed to support the undifferentiated growth and expansion of hESCs on mitotically-inactivated human fibroblast feeder cells. It has been rigorously tested and validated to maintain the pluripotency and chromosomal stability of several hESC lines including H1 (WA01) and H9 (WA09; WiCell) , and MEL-1, MEL-2 and MEL-4 (Melbourne IVF, Stem Cell Sciences pty ltd and the Australian Stem Cell Centre) for over 25 passages or approximately 125 population doublings.

Background

Human ES cells were first successfully derived and cultured in 1998, using the same conditions that were being used at that time to grow mouse ES cells[2], including 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, have driven the development of improved methods. The challenges involved 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).

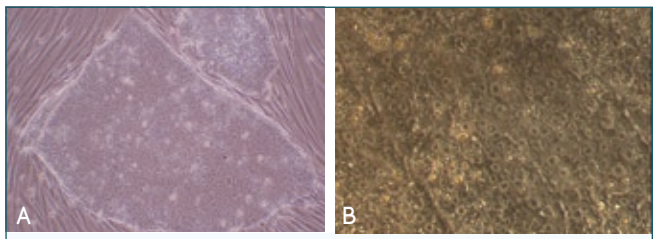

Figure 1. Human ES cells grown in HEScGRO xeno-free, serum-free medium have appearance and morphology typical of human ES cells grown on human feeders. A. H9 (WA09) cells, with well-defined borders and homogenous appearance within the colony. B. MEL-4 cells (shown at higher magnification) display high nuclear-to-cytoplasmic ratio and visible nucleoli.

While significant progress has been recently made towards meeting some of these challenges, 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"[3,4]; however, this serum-replacer contains bovine serum albumin[3] 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)[4,5]. 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[6]. Thus a need has remained for a xeno-free medium that does not rely upon high levels of bFGF.

A XENO-FREE, SERUM-FREE MEDIUM FOR HUMAN ES CELL CULTURE

HEScGRO medium is a proprietary formulation that contains only humanized or synthetic components. These include bFGF that is manufactured under animal-free conditions (Cat. No. GF003-AF) and human serum albumin rather than its bovine counterpart. Because it is a defined medium that is ready-to-use straight from the bottle, users can rely on consistent performance without any need to batch-test components. HEScGRO medium was designed for use with human fibroblast feeders and has been validated with two different commercially available lines, Detroit 551[7] 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).

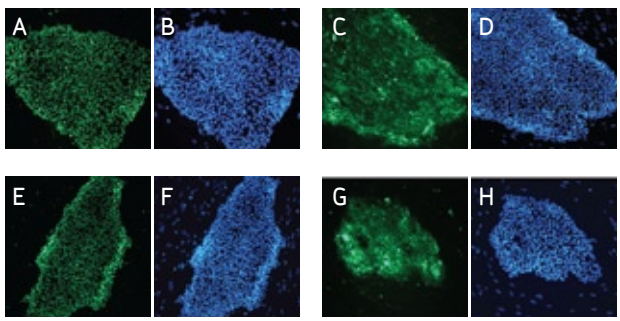

Figure 2. H1 (WA01) and H9 (WA09) cells grown for multiple passages in HEScGRO medium continue to display markers of pluripotency. (A-D) H1 cells after 17 passages in HEScGRO medium show expression of Oct-4 (A) and TRA-1-60 (C). Cells throughout each colony express these markers, as shown by comparison to the corresponding DAPI staining in B, D. (E-H) H9 cells after 17 passages showing expression of Oct-4 (E) and TRA-1-60 (G), with corresponding DAPI images in F, H.

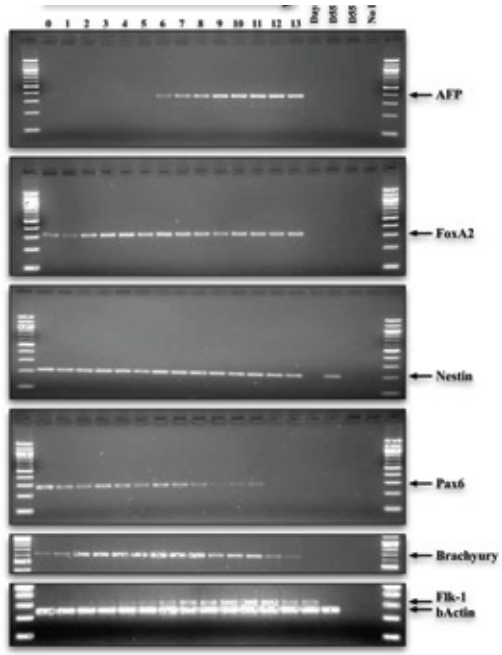

Figure 3. Human ES cells grown in HEScGRO medium can differentiate to all three germ layers. MEL-4 cells grown in HEScGRO medium were differentiated via embryoid body (EB) formation in HEScGRO medium lacking bFGF. Samples were taken every day for 13 days and processed for RT-PCR of the following markers of differentiation: alpha fetoprotein (AFP) and foxA2 (endoderm), nestin and pax6 (ectoderm), brachyury and flk-1 (mesoderm). Expected products are indicated by the arrows.

HEScGRO medium has been tested with multiple human ES cell lines. These include the H1 (WA01) and H9 (WA09) lines available from the WiCell Research Institute, and the MEL-1, MEL-2 and MEL-4 lines derived at the Australian Stem Cell Centre. Cells from each of these lines grow well in HEScGRO medium, as judged by the following criteria: colony morphology is normal even after multiple passages in HEScGRO medium (Figure 1), colonies have the typical "random" (i.e., non-circular) morphology when human ES cells are grown on human feeders, and colonies have sharp boundaries without signs of differentiated cells at the edges (Fig. 1A). The appearance of individual cells within the colony is also normal; cells are tightly packed, with each cell having a high nuclear-to-cytoplasmic ratio and prominent nucleoli (Fig. 1B).

PLURIPOTENCY OF CELLS CULTURED IN HEScGRO MEDIUM

Human ES cells cultured in HEScGRO medium retain their pluripotency after multiple passages in the medium. This is demonstrated not only by expression of pluripotent markers but also through subsequent differentiation to representatives of all three germ layers. For pluripotent marker expression, H1, H9 and MEL-4 cells were cultured for more than 17 passages in HEScGRO medium before being processed for immunocytochemistry. As shown in Figure 2 for the H1 and H9 lines, cells grown for multiple passages in HEScGRO medium continue to express the pluripotent markers OCT-4 (Cat. No. MAB4401) and TRA-1-60 (Cat. No. MAB4360); the vast majority of cells in a colony express these markers, as can be seen by comparison to the corresponding images of DAPI staining (similar results were obtained for the MEL-4 line; data not shown). Cells cultured in HEScGRO medium also retain tissue-specific alkaline phosphatase activity (not shown; Cat. No. SCR004), another indicator of ES cell pluripotency. Expression of SSEA-1 (Cat. No. MAB4301), which in human ES cultures is expressed by differentiated cells, is low or absent (not shown). Finally, in each of these experiments, these cells maintained a normal karyotype.

DIFFERENTIATIVE CAPACITY OF CELLS GROWN IN HEScGRO MEDIUM

For differentiation studies, cells grown in HEScGRO medium were allowed to spontaneously differentiate via embryoid body (EB) formation. Briefly, cells passaged in HEScGRO medium were detached from the cell surface and re-aggregated to form EBs in suspension according to Ng *et al.*[8]. These EBs were subsequently grown in suspension culture in HEScGRO medium without bFGF as a differentiation medium. EBs were allowed to grow for up to 13 days; each day, samples were removed for analysis by RT-PCR for markers of differentiated lineages. As shown for the MEL-4 line (Figure 3), EBs formed from human ES cells grown in HEScGRO medium can differentiate cells expressing the following markers for all three embryonic germ layers: alpha-fetoprotein (AFP) and foxA2 (endoderm), nestin and pax6 (ectoderm), and brachyury and flk-1 (mesoderm).

SPECIFIC ATTRIBUTES & METHODOLOGIES

There are some particular attributes to cells cultured in HEScGRO medium. Colonies tend to be flatter in HEScGRO medium than in serum- or serum-replacer-containing media (although as mentioned, both overall colony shape and the appearance of individual cells within the colony appear normal). There are also some specific methods to follow when using HEScGRO medium. The human feeders (Detroit 551 or WS1, as mentioned above) should be plated (generally one day prior to plating the ES cells) at a density of 60,000 cells per square centimeter. Once the human ES cells are plated, the media should be changed every day, although every two days may be acceptable. For passaging, we have found that manually dissecting colonies grown in HEScGRO medium culture is best, since this is the gentlest way to handle the cells. As HEScGRO medium is serum-free, one must be careful when attempting to passage HEScGRO medium cultures ezymatically. Trypsin should never be used. We have found that HEScGRO medium cultures can be passaged with the collagenase preparation Accumax (Cat. No. SCR006; see Emre *et al.* in this issue); note that the collagenase type IV commonly used for passaging cells in medium containing serum-replacer will cause cells in HEScGRO medium to readily differentiate.

Summary

HEScGRO medium meets the demands for improved human ES cell culture. It is a ready-to-use formulation that utilizes a low amount of basic FGF. Being serum-free, HEScGRO medium provides a defined medium that does not need to be batch-tested, while its xeno-free composition allows for human ES cells to be cultured without concerns of contaminating animal products. HEScGRO medium has been validated for use with commercially-available human fibroblasts and with several different human ES cell lines. Cells cultured in HEScGRO medium for multiple passages retain their pluripotency, as shown by marker expression as well as by the ability to express markers of all three germ layers upon differentiation.

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