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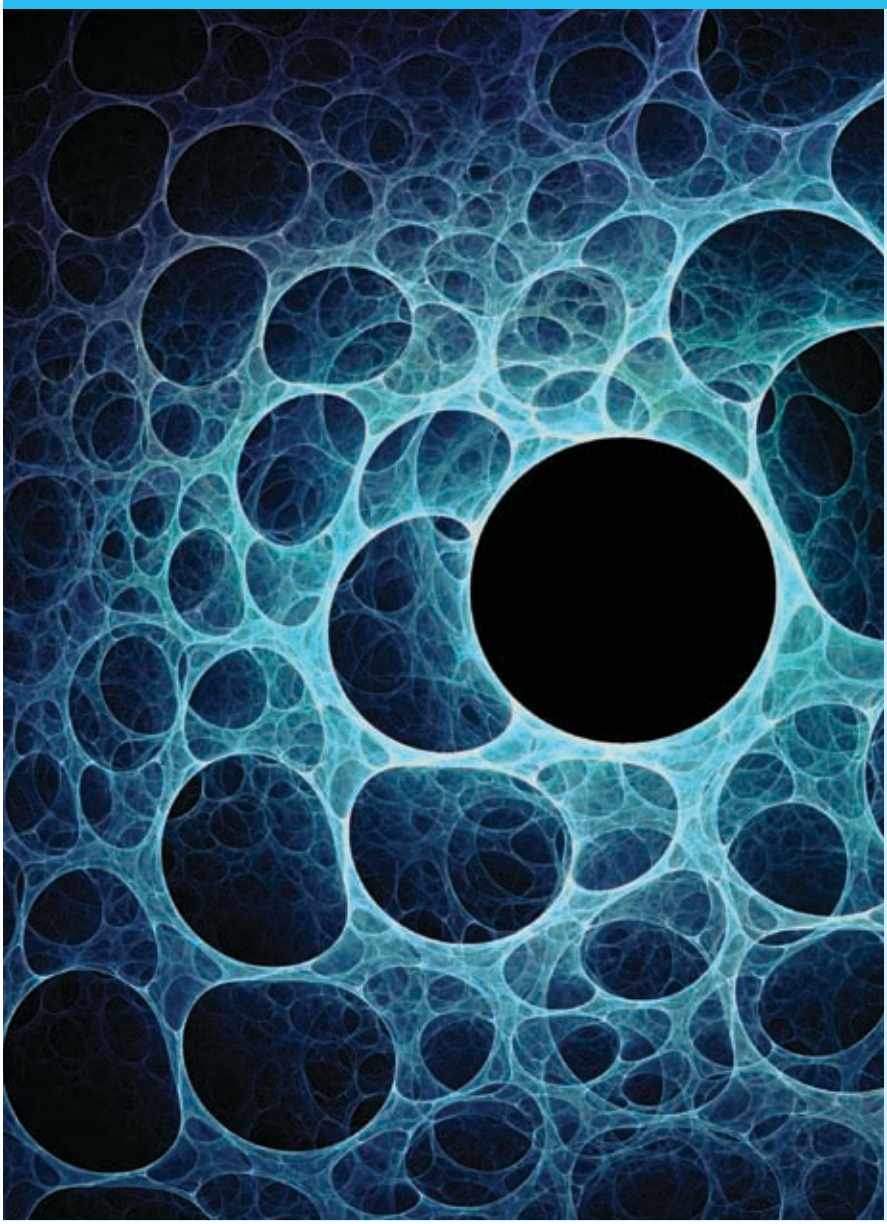

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ReNcell Human Neural Progenitors: Renewable and Consistent Supply of Human Functional Neurons

Erik A. Miljan, Ph.D., ReNeuron group plc

Abstract

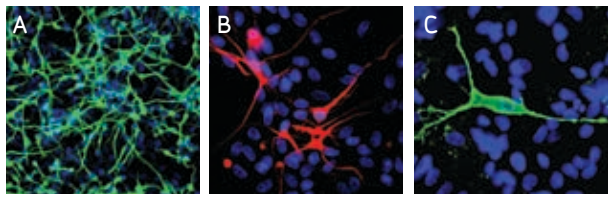
ReNcell VM and ReNcell CX are two well-established neural stem cell lines derived from developing human brains. ReNcell VM and CX cells are generated from the ventral mesencephalon and cortical regions of the brain, respectively, and transduced with the myc transcription factor. Both cell lines offer phenotype and genotype stability, in addition to the multipotential neuronal differentiation capacity, over long-term culture. This article describes the characteristics and differentiation of ReNcell lines. Results of calcium and membrane potential changes in response to various ligands are also shown. The convenience to maintain in culture and flexibility to differentiate to individual scientists' needs make ReNcell lines the ideal platform for research and discovery.

Introduction

Neural stem cells (NSCs) were first described in the rodent brain by Reynolds and Weis in 1992[1]. Seven years later, the isolation of human NSCs was documented by Vescovi *et al.*[2]. Since those initial findings, NSCs have been valuable tools for neuroscience, signal transduction and developmental biology, to name but a few.

STANDARDIZED PLATFORM

Early studies using human NSCs were limited by the short-term stability of genotype and phenotype in culture. However, immortalization of human NSCs with the myc transcription factor has proven highly effective at overcoming these challenges. ReNcell VM and CX lines were immortalized using myc technology. Myc is believed to drive and sustain self-renewal and proliferation of the stem cell, thus keeping differentiation at bay until desired. An upregulation of telomerase activity is observed in myc transduced ReNcell, which lends itself to a stable genotype in culture. Traditionally thought of as a proto-oncogene, it now appears that myc may be a "stemness" gene. An exciting discovery was made when Takahashi and Yamanaka demonstrated that a fibroblast cell could be transformed into a stem cell by using only four genes: c-Myc, Oct4, Klf4, and Sox2[3]. This finding has since been corroborated by independent research groups.



Multipotentiality of ReNcells: Both cell lines readily differentiate into all three neuronal phenoytpes: neurons (βIII-tubulin, green, 20X, A); astrocytes (GFAP, red, 40X, B) and oligodendrocytes (Gal C, green, 60X, C); all counterstained with Hoechst nuclear stain (blue).

ReNcell lines are regularly tested to ensure that they are free from any adventitious agents or contaminants (e.g. mycoplasma), thus guaranteeing their safety. ReNcell VM and CX cell lines are easily maintained as monolayer cultures in serum-free ReNcell maintenance medium (Cat. No. SCM005) using laminin coated T-culture flasks. Passaging the ReNcell cell line is carried out every 3-4 days with typical yields of approximately 4×10^6 cells per T75 cm^2 flask. Both cell lines can be maintained long-term in culture and readily cryopreserved without any adverse effects on genotype or phenotype. The consistent and renewable supply of NSCs generated by ReNcell VM and CX cell lines make them an excellent tool in stem cell research.

DIFFERENTIATION AS EASY AS 1, 2, 3

The advantages of myc transduction on cell growth and genotype have been described. However, it is important to note that myc transduction does not interfere with differentiation. ReNcell VM and CX cell lines retain the ability to differentiate into all three neuronal subtypes - neurons, astrocytes, and oligodendrocytes. Indeed, the proteomics[4] and electrophysiology[5] of ReNcell cell lines have been reported in the undifferentiated and differentiated state. These data indicate that both ReNcell lines are true neural stem cells by their ability to expand in culture and their multipotent capacity to generate all three neuronal subtypes.

Differentiation of the cells occurs when growth is arrested by removal of growth factors bFGF and EGF from the tissue culture medium. ReNcells are compatible with all sizes of monolayer tissue culture plasticware for differentiation. We typically use 96-well plates because both cell lines perform well in this format and it allows high-throughput experimentation. Differentiation of both ReNcell lines can be carried out in three steps. In the first step the cells are passaged as normal and plated at 10,000 cells per well into a laminin coated 96-well plate. ReNcell Maintenance Medium containing growth factors EGF and bFGF is used at 100-200 µL per well and the cells are grown to confluency over 2-3 days. The second step involves inducing differentiation by replacing the medium with ReNcell maintenance medium without EGF and bFGF. In as little as three days after induction, the cells go from a cobblestone stem cell morphology to cells containing extensive processes and mature neuronal morphologies. Analysis comprises the final step of the differentiation process. Immunocytochemistry, Western blot, and PCR are some of the most straightforward methods to demonstrate successful differentiation.

FLEXIBILITY AND DISCOVERY

The standard three-step differentiation method described above provides a framework to tailor the experiment to novel research applications. Each stage can be modified depending on the researcher's needs and imagination. For example, prior to seeding cells in step 1 of the assay, the ReNcell cell line can be seeded at the same density on uncoated plasticware in growth factor containing medium to form neurospheres over 5-7 days. The neurospheres can then be transferred onto laminin coated tissue cultureware in medium containing growth factors and the three differentiation steps carried out. The formation of neurospheres prior to initiation of the differentiation protocol has been shown to enhance electrophysiologically active neural differentiation in ReNcell VM[5] cell line. As a further modification to step 1 in the differentiation protocol, the cells can be "pre-treated" with agents of interest during the growth phase. These agents may include development or regional specificity signals that prime the cells before differentiation. Growth factor withdrawal in step 2 induces differentiation in its simplest form; however, the addition of agents that stimulate differentiation are ideally added at this stage. In our laboratory, we have found that the addition of 1mM dibutyrl-cAMP to the differentiation medium significantly enhances the dopaminergic differentiation of the ReNcell VM cell line.

As a final modification to the protocol, investigators may wish to incorporate survival factors following the induction of differentiation (e.g. GDNF at 2 ng/mL). Due to the flexibility of these cells, testing your compound of interest in ReNcell VM and CX cell lines couldn't be easier.

PHARMACOLOGY OF ReNCell LINES

We used a range of ligands to monitor the cytosolic calcium and membrane potential changes in an application of ReNcell cell lines. Cells were grown in 96-well plates to generate undifferentiated or differentiated cells, and functional responses to agonists were monitored using a Flex Station® Fluorometer (Molecular Devices, Reading, UK). Both ReNcell VM and CX cell lines responded to a range of stimuli including carbachol, ATP, and histamine (weakly). ReNcell CX also showed a robust response to thrombin while 5-HT was inactive. Depolarisation of ReNcell VM and CX membrane potential was seen with KCL. To date, only small hyperpolarization or depolarization responses to receptor ligands have been observed in ReNcell cell lines, which suggests ion channel activity in a small sub-population of cells.

In summary, ReNcell VM and CX cell lines are ideal tools for discovery. Optimize your research output by working with human cell lines. ReNcell maintenance and freezing media, along with our full technical support, guarantee you the best results.

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Millipore Products

Description	Cat. No.
ReNcell CX Kit (ReNcell CX cells and ReNcell Maintenance Medium)	SCC009
ReNcell VM Kit (ReNcell VM cells and ReNcell Maintenance Medium)	SCC010

HEScGRO Xeno-Free Medium for Human Embryonic Stem Cell Culture

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[1] Millipore Corporation, [2] Stem Cell Sciences pty ltd Australia, [3] Stem Cell Sciences Pty Ltd UK

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).

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.

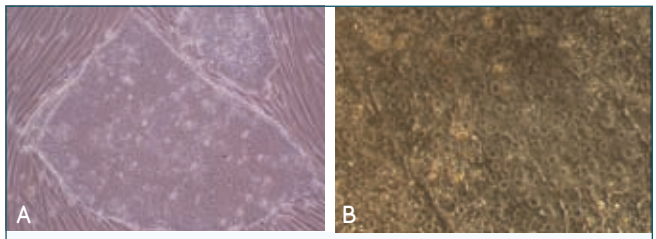

Figure 1. Human ES cells grown in HEScGRO xeno-free, serum-free medium have appearance and morphology typical of human ES cells grown on human feeders. A. H9 (WA09) cells, with well-defined borders and homogenous appearance within the colony. B. MEL-4 cells (shown at higher magnification) display high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

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).

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.

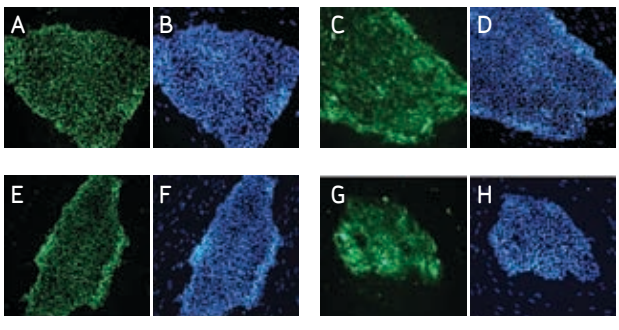

Figure 2. H1 (WA01) and H9 (WA09) cells grown for multiple passages in HEScGRO medium continue to display markers of pluripotency. (A-D) H1 cells after 17 passages in HEScGRO medium show expression of Oct-4 (A) and TRA-1-60 (C). Cells throughout each colony express these markers, as shown by comparison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

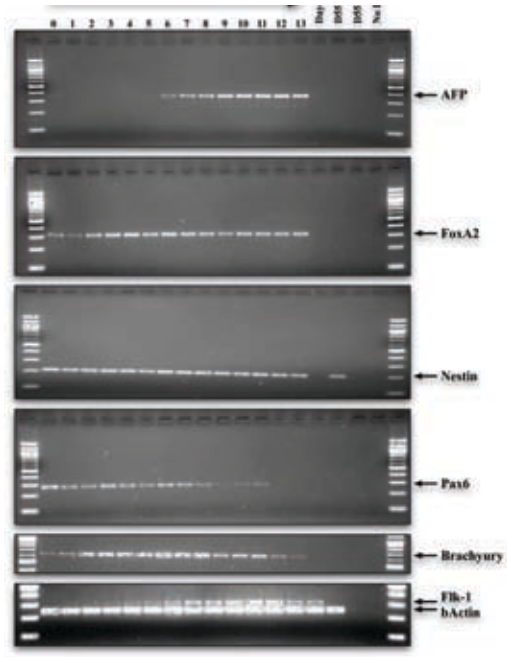

Figure 3. Human ES cells grown in HEScGRO medium can differentiate to all three germ layers. MEL-4 cells grown in HEScGRO medium were differentiated via embryoid body (EB) formation in HEScGRO medium lacking bFGF. Samples were taken every day for 13 days and processed for RT-PCR of the following markers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

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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Maximal Recovery of Hematopoietic Stem Cells from Bone Marrow Endosteum

David Haylock, Ph.D., and Susie Nilsson, Ph.D., Australian Stem Cell Centre, Clayton, Victoria, Australia

Background

The vast majority of studies involving hematopoietic stem cell (HSC) isolation and subsetting have been performed using mouse bone marrow (BM) obtained by flushing the marrow from bones using a needle and syringe. Although this methodology is widely used, there can be considerable variation in the recovery of total cells and HSC, which is attributed to the gauge of the needle and the amount of force and volume of phosphate buffered saline (PBS) used for flushing. In fact, often the exact details of marrow harvesting by the flushing method are frequently not given in the published scientific literature. Another more important consideration with the flushing method is that it results in collection of varying numbers of HSC that reside within the endosteal region. Recent studies demonstrate the significance of the endosteal region for hematopoiesis. The endosteal region contains a high number of HSC and moreover HSC isolated specifically from this site have greater hematopoietic potential than HSC isolated from the central marrow core[1].

OPTIMAL RECOVERY OF HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells (HSC) are frequently located in close proximity to bone lining cells within the endosteum. Accordingly, it is unlikely that the traditional method for harvesting bone marrow (BM) from mice by simply flushing long bones would result in optimal recovery of HSC. Researchers at the Australian Stem Cell Centre have developed improved methodologies based on sequential grinding and enzymatic digestion of murine bone tissue to harvest higher numbers of BM cells and HSC from the endosteal and central marrow regions. This methodology resulted in up to a six-fold greater recovery of primitive hematopoietic cells (lineage- Sca-1+ c-kit+ [LSK] cells) and HSC as shown by transplant studies. HSC from different anatomical regions of the marrow exhibited important functional differences. Compared with their central marrow counterparts, HSC isolated from the endosteal region (a) had 1.8-fold greater proliferative potential, (b) exhibited almost twofold greater ability to home to the BM following tail vein injection and to lodge in the endosteal region, and (c) demonstrated significantly greater long-term hematopoietic reconstitution potential as shown using limiting dilution competitive transplant assays[1]. Working in close collaboration with the Australian Stem Cell Centre, Millipore has developed the Bone Marrow Harvesting and HSC Isolation Kit (Cat. No. SCR051). This kit permits the optimal recovery of total cells and HSC from murine bone tissue. A combination of mechanical fragmentation by grinding of bones and enzymatic digestion is used for this purpose.

Results

HSC isolated from the endosteal region and transplanted into non-ablated recipients had a significantly higher homing efficiency to the bone marrow after 15 hours compared to HSC isolated from the central marrow core ($p<0.05$) (see Figure 1A). In addition, HSC isolated from the endosteal region and transplanted into non-ablated recipients had a significantly higher affinity to re-lodge within the endosteal region of the bone marrow after 15 hours compared to HSC isolated from the central marrow core ($p<0.05$) (see Figure 1B). Data are the mean ± SEM from both endosteal and central marrow HSC isolated from 15 animals and transplanted into 3 individual recipients. Sorted HSC (100 per well) isolated from the endosteal region (light blue bar) and the central marrow core (dark blue bar) were grown in serum free media in the presence of 4 stimulatory growth factors (see Figure 2). After 6 days there was a significant ($p=0.02$, students t-test) increase in the number of cells generated from endosteal HSC compared to those isolated from the central marrow core. Data are from a representative experiment (n=4) showing the mean ± SEM from quadruplicate wells.

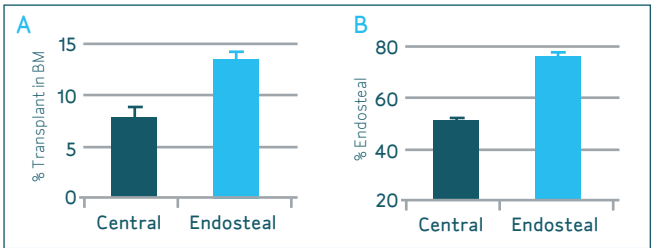

Figure 1. HSCs within the endosteal region have significantly increased homing efficiency to the bone marrow (A) and increased ability to lodge within the endosteal region (B).

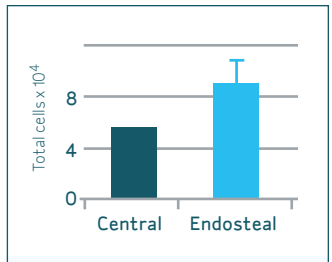

Figure 2. HSCs within the endosteal region have significantly increased proliferative potential *in vitro*.

Conclusion

The Bone Marrow Harvesting and HSC Isolation Kit provides the materials and reagents for ten separate isolations by mechanical fragmentation and enzymatic digestion of murine bone tissue. Two detailed protocols are included in the kit: (1) method for harvesting total bone marrow, and (2) method for harvesting bone marrow from the endosteal region. This kit enables researchers to harvest maximal numbers of total cells and HSC from the endosteal and central marrow regions of murine bone tissue. This isolation method was developed by Haylock, *et al.* (2007), who demonstrated that the HSC isolated from the endosteal region have a greater proliferative potential than their central marrow counterparts. Furthermore, HSCs isolated from the endosteum exhibit a greater ability to home to the BM following tail vein injection, and remarkably show an enhanced ability to return to the endosteal region. Moreover, they have been shown to display greater hematopoietic potential than phenotypically identical LSK cells isolated from the central marrow area. This method of isolation is ideal for the analysis of molecules important in the HSC niche, transcriptional and proteomic profiling, and functional studies, including transplantation, gene marking, and *ex vivo* expansion[1].

Reference

1. Haylock, D. *et al.* (2007) Hematopoietic Stem Cells with Higher Hematopoietic Potential Reside at the Bone Marrow Endosteum. *Stem Cells*, **25**: 1062-1069.

Millipore Products

Description	Cat. No.
Bone Marrow Harvesting & Hematopoietic Isolation Kit, 10 isolations	SCR051

Stem Cell Testing of PES Membrane Containing Stericup Filters

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Summary

Though microporous filtration is the method of choice to prepare and minimize contamination of cell culture media and reagents, there still remains some concern that the filtration step will remove valuable factors and/or add deleterious components to the growth media. This concern is especially true for stem cell culture where factors can be expensive, the cells can be very sensitive to additives, and culture durations may be very long. Here we show that a PES membrane-containing Stericup filter, even after many multiple filtration steps, provides a worry-free alternative for the pluripotent expansion of embryonic stem cells.

Background

Maintaining sterility is vital during the culture of cells; therefore, great care is taken to prevent contamination. Several methods have been routinely used for the sterilization of liquid reagents, including autoclaving and microporous filtration. Autoclaving has many limitations, in that it cannot be used with liquid growth media containing heat labile ingredients such as proteins and other growth factors, and it is time consuming for other more basic buffers. For these reasons, filtration is the method of choice for sterilization of media components and reagents for cell culture.

Filtration through a membrane with 0.2 µm or smaller pore size allows for the efficient removal of contaminates including bacteria, mold and yeast. There are several filter materials typically used in the sterilization of liquid components including nylon, polycarbonate, cellulose acetate, polyvinylidene fluoride (PVDF, Durapore® membrane), and polyethersulfone (PES). These filter materials differ greatly in their protein retention, flow speed, and the presence of leachable materials. PVDF and PES are both very low protein binding materials without leachables that have wide applications in filtering biological liquids. PES filters are generally preferred for media filtration due to their faster flow rate, especially with more viscous, serum-containing media.

As cell media becomes more defined with expensive factors and cells are cultured for longer and longer durations, the need for filtration to assure sterility and retain essential factors is more important than ever. In addition, filtration must not leach any materials into the media that may be toxic or cause other deleterious effects. This is particularly true with stem cell cultures that are very sensitive to the concentrations of growth factors in the media for both long term pluripotent maintenance and differentiation procedures. For these reasons, it is common practice to add these factors after the sterile filtration of the media due to the fear of removing these factors. Here we demonstrate that the loss of important factors due to filtration is minor and that filtration — even multiple filtrations–does not have any deleterious effects on the growth media.

This study investigated the retention of growth factors required for the expansion of pluripotent embryonic stem cells after multiple sterile filtrations. Although media is typically only filtered once before use, we show that the Stericup filters can be used to filter media multiple times (up to ten times sequentially through new filters) without removal of important factors or the addition of deleterious materials. This observation is shown quantitatively by examining the retention of Leukemia Inhibitory Factor (LIF, a factor required for the maintenance of pluripotency of murine embryonic stem cells, or mESCs) as a function of multiple filtrations through a PES membrane Stericup filter device. In addition, the ability to maintain proper expansion of mESCs for up to five passages in multiply filtered media is shown by appropriate colony morphology of low density colonies and immunohistochemical analysis for markers of plurpotency.

Results

Leukemia Inhibitory Factor (ESGRO®, mLIF medium supplement) has been shown to be a critical media additive for maintaining mouse embryonic stem cells in an undifferentiated state when feeder layers of mouse embryonic fibroblasts are not used. We tested the LIF retention of Stericup filtration devices with PES membranes during filter sterilization.

ESGRO media supplement containing media was filtered through multiple new PES Stericup filters. Media samples from each filtration were saved for both retention testing and the ability to maintain pluripotent expansion. Up to ten filtrations through separate PES Stericup filters allowed for a 'worst case scenario', even though 10 filtrations are unlikely in general work.

Relative LIF retention was determined through the use of LIF-specific sandwich ELISA assays (R&D Systems) as per kit instructions within a linear range of LIF concentration response. All samples were tested in duplicate using unfiltered media as the 100 % control. A negative control of media lacking ESGRO media supplement was additionally included for background determination. Sample concentrations were determined by comparison with the standard curve. Results (Figure 1) show that roughly 90 % of LIF is retained after ten filtration steps through new PES Stericup filters at a rate of loss of approximately 1 % per filtration. This loss allows for acceptable levels of LIF to remain present in the media to assure stem cell pluripotency.

Functional testing of the samples was performed to assure that multiple filtration did not affect the activity of LIF or add deleterious leached components into the media. 500mL stocks of media were filtered once each, or were filtered 5 times through successive new PES filters. Three lots of PES Stericup filters were tested per filtration point for a total of 6 stocks. Each stock of media was then used for feeder-free maintenance of mESCs for a total of 5 passages on gelatin coated plastic tissue culture plates. After five passages, mESC colonies were observed for pluripotency by alkaline phosphatase staining and Oct-4 (a transcription factor marker for pluripotency) immunohistochemical analysis. Cultures expanded and passaged at low density in all media samples tested (different PES-Stericup filter lots with one or five filtrations) show colony morphology indicative of pluripotent expansion as well as express appropriate levels of the pluripotency markers alkaline phosphatase (Figure 2) and Oct-4 (Figure 3).

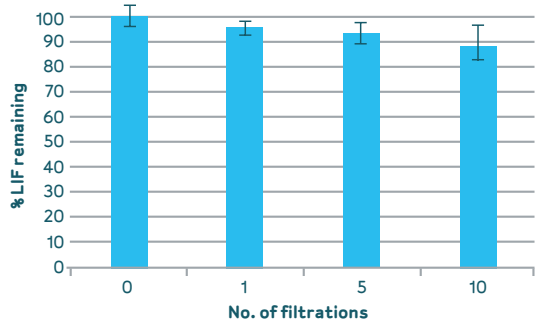


Figure 1. Leukemia Inhibitory Factor (LIF) retention as a function of multiple sequential filtrations through new PES-membrane Stericup Filtration units. LIF concentration in ESGRO supplement containing treated media was determined by sandwich ELISA assays within a predetermined linear range of response. Percentages are normalized to unfiltered media as 100 %.

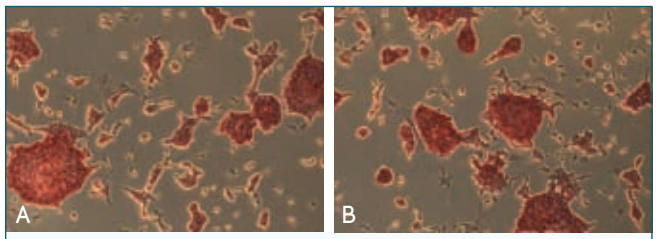


Figure 2. Alkaline Phosphatase staining of mESC colonies. mESC colonies were cultured on 0.1 % gelatin coated solid bottom plastic plates with media filtered through PES-Stericup filters once (a) or 5 times (b). Colonies were fixed with 3.7 % formaldehyde in PBS for 2 minutes. Napthol/ Fast Red Violet solution was added to each well and incubated for 15 minutes before rinsing with TBST buffer.

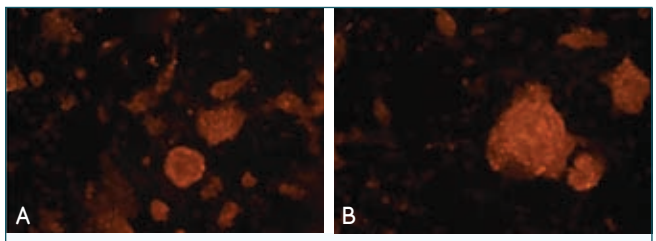


Figure 3. Immunocytochemistry Analysis of mESC colonies. mESC colonies were cultured on 0.1 % gelatin coated solid bottom plastic plates with media filtered once (a) or 5 times (b). Colonies were fixed with 3.7 % formaldehyde and permeabilized with 0.1 % saponin. Expression of Oct-4 was determined with primary anti-Oct-4 antibody followed by visualization with a Cy3 conjugated secondary antibody.

Millipore Products

Description	Cat. No.
Stericup-GP Express Plus, 500 mL	SCGPU05RE
ESGRO mLIF Medium Supplement	ESG1106

Recombinant Growth Factors

Description	Cat. No.
N BAFF, human	GF136
N Epidermal Growth Factor, human	GF144
N Epidermal Growth Factor, mouse	GF155
FGF Basic, human	GF003
IGF-I, human	GF006
Leukemia Inhibitory Factor, human	LIF4010
Leukemia Inhibitory Factor, mouse	LIF2010
Leukemia Inhibitory Factor, rat	LIF3010
PDGF-AA, human	GF017
PDGF-BB, human	GF018
Stem Cell Factor, human	GF021
Stem Cell Factor, mouse	GF049
N VEGF, human, 165aa isoform	GF094
N VEGF, mouse, 165aa isoform	GF140
Wnt3a, mouse	GF145
Wnt5a, mouse	GF146

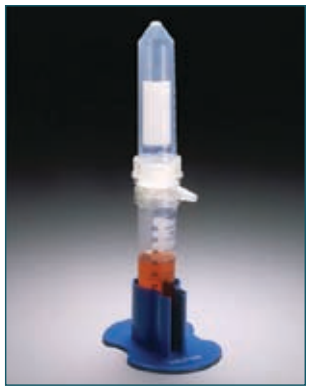
N = New

Isolation of Cardiac Stem Cells and their Differentiation into Cardiomyocytes

Stephen Kendall, Ph.D., and Vi Chu, Ph.D., Millipore Corporation, Temecula, CA

Abstract

Adult murine cardiomyocytes provide an excellent model system for the study of cardiovascular diseases, because the heart's striated muscle cells represent the functional contractile unit of the circulatory system. Methods to isolate cardiomyocytes have traditionally relied upon mechanical mincing and enzymatic treatment of ventricular tissues, followed by the use of cell strainers, but this risks introducing contamination to the sample. These methods often yield mixed populations of cells making analyses of discrete populations difficult. Here we report an improved method for the isolation and purification of cardiomyocytes from endogenous cardiac stem cells (CSCs). This technology provides a reliable and reproducible method for the isolation and culture of adult CSCs, and should be suitable for a wide array of biological applications.



Introduction

The isolation and expansion of cardiac stem cells opens new opportunities to the field of cardiac regenerative medicine. CSCs have recently been isolated from human and murine tissues based upon cell biomarkers (Sca-1, TERT, c-Kit, side population), demonstrating the presence of a non-circulating stem cell niche within the myocardium that is estimated to account for 11-14% of the total cell population[1]. These cells appear to be bi-potent in their capacity to form cardiomyocytes and vascular endothelial cells. Moreover, preliminary engraftment studies suggest that these cells are ideal candidates for future research on cardiac regeneration. However, the rarity of CSCs coupled with the complex isolation procedure is a significant challenge for the advancement of the field. To overcome this obstacle, we have developed an easy-to-use cell isolation kit capable of obtaining a high yield, pure population of CSCs. This advancement allows investigators to obtain a significantly greater number of CSCs for their studies without the need for time consuming, complex protocols and expensive cell sorting equipment.

Results

Isolation and purification of murine CSCs using the Millipore CSCs Isolation Kit (Cat. No. SCR061) begins with surgical resection of the heart from the thoracic cavity. Heart tissue is minced and pooled from five separate mice per collection and a cell suspension is created through gentle agitation in our dissociation buffer. A novel application of our isolation kit is the use of the self-contained Steriflip® filtration device, which allows for the separation of larger volumes of cellular material with the added advantage of maintaining a sterile environment ensuring sample sterility, an option that open top gravity flow filter devices can not guarantee.

The study used a 100 µm nylon mesh vacuum driven Steriflip device that allows the passage of cells while retaining large tissue clumps. This improves recovery and lessens time to collect the isolated cells.

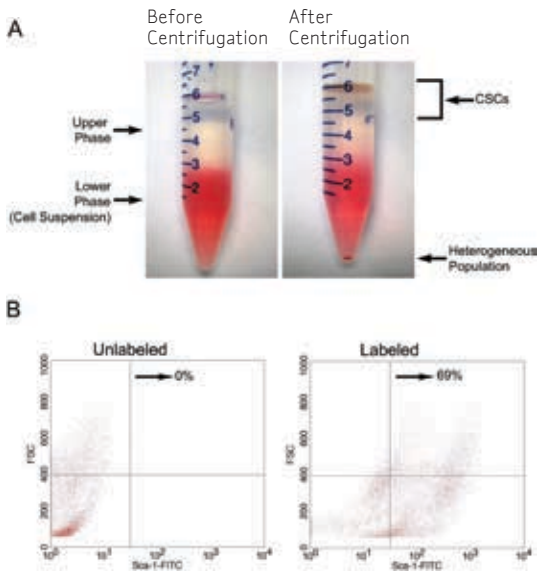


Figure 1. Discrete cell populations can be isolated from ventricular heart tissue through differential gradient centrifugation. (A) Representative photos depicting heterogeneous cell populations present in the lower phase before centrifugation and a pure CSC population present in the upper phase after centrifugation. (B) Purity of differential gradient isolated CSCs as determined by flow cytometry analysis for the stem cell marker Sca-1.

A major advantage that our system provides is the rapid purification of Sca-1 positive CSCs without the need for expensive cell sorting equipment. This is accomplished through the use of differential gradient centrifugation (Figure 1A). To validate the purification method, we harvested five C57/BL6 mouse hearts per triplicate isolation and performed the collections as described above. Purified samples were subsequently labeled with Sca-1-FITC conjugated antibodies and cells were analyzed using a FACSCalibur flow cytometer. Isolations yielded an averaged 1.2 x 10^6 cells/mL with a Sca-1 purity of 69 % (Figure 1B). This method appears far superior to the traditional method of dissociation and filtration[2] that yield a heterogeneous population composed of only 11-14 % Sca-1 positive cells.

Purified CSCs can maintain a mesenchymal stem cell like phenotype (Figure 2A) during *in vitro* culture. Moreover, when regular growth media is replaced with differentiation media, CSCs undergo a phenotypic change indicative of mature cardiomyocytes (Figure 2A). Labeling cells for filamentous actin (F-Actin) highlights the transition from stem cell to cardiomyocyte, with long striated myofibrils present in the differentiated population (Figure 2B). Both isolation of adult cardiomyocytes and differentiation of embryonic stem cells into cardiomyocytes have been problematic due to the limited number of cells one can obtain. The Millipore CSC isolation kit provides the advantage of not only purifying greater number of cells, but also allowing expansion of the original population as needed. Isolated cells can be expanded in culture using defined growth media for periods of up to two weeks before quiescence (Figure 2C), providing the opportunity for additional experimental analysis.

Isolated cells were cultured for 1 week and characterized. Immunocytochemical staining demonstrated a ubiquitous Sca-1 labeling, as previously shown through FACS analysis. Moreover, these cells concurrently stained for the stem cell marker, telomerase, suggesting the retention of their stem cell characteristics (Figure 3A). Furthermore, CSCs maintained in culture continued to proliferate and stained positive for Ki67 (Figure 3A).

To establish the differentiative capacity of isolated CSCs, we plated cells on poly-L-ornithine coated slides and maintained them in differentiation media for a period of 12 days. Samples were then fixed and stained with markers for mature cardiomyocytes. Cells showed strong immunoreactivity to cardiomyocyte markers (Cat. No. SCR059), Troponin I, Desmin, and Actinin (Figure 3B). Collectively, these results demonstrate an efficient isolation of CSCs that are capable of expansion differentiation when treated with the appropriate media *in vitro*.

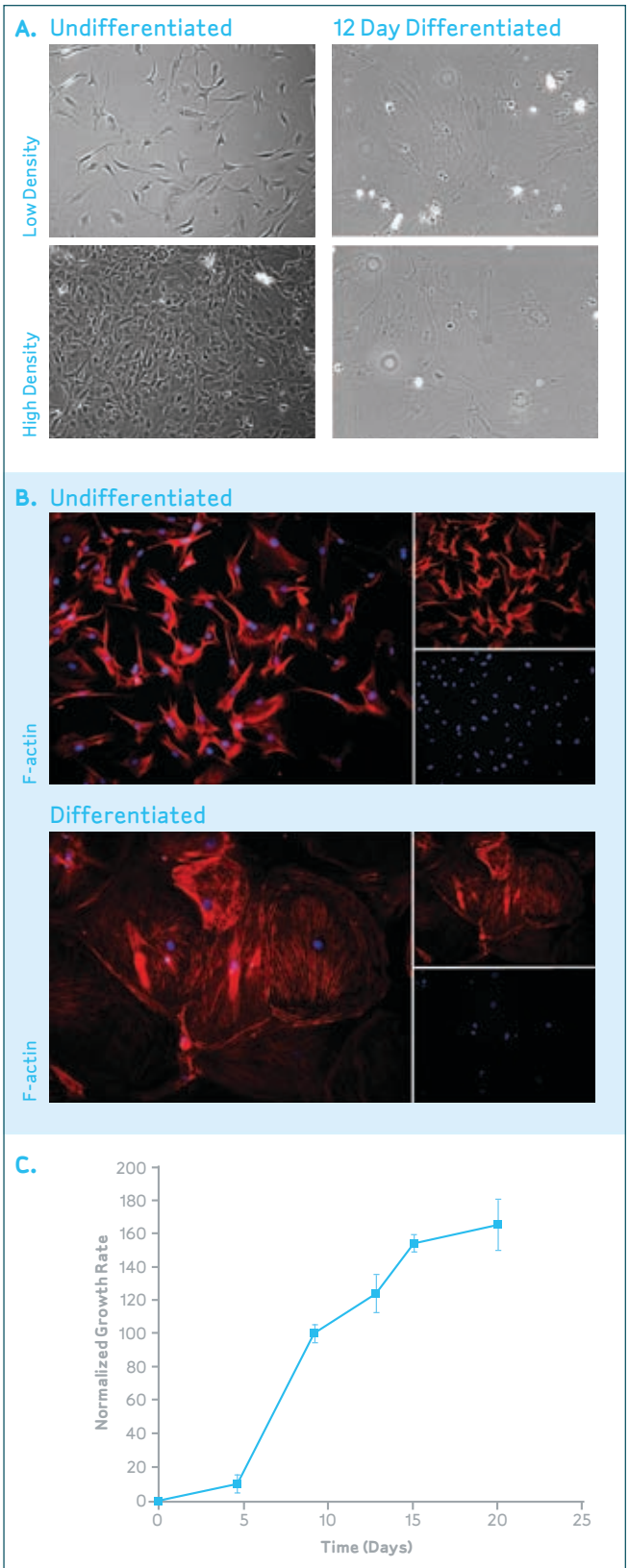


Figure 2. Cardiac stem cells can be cultured *in vitro* and differentiated through selected use of defined growth or differentiation media. (A) Representative photomicrographs of low and high density cultures of purified CSCs and 12-day differentiated cardiomyocytes. (B) Fluorescent microscopy images of cultured CSCs and differentiated cardiomyocytes stained for F-Actin. Note the presence of striated myofibrils present in the differentiated cells. (C) Normalized growth curve demonstrating the expansion ability of CSCs in culture.

Conclusion

Efficient high throughput isolation of cardiac stem cells has remained elusive. This obstacle has slowed progress on the development of therapeutic applications of endogenous CSCs. We show here how Millipore's CSC Isolation Kit enables researchers to obtain significantly greater numbers of cells for use in their experimental applications. Our novel isolation and purification approach, coupled with defined growth media, permits pure CSCs to be isolated from ventricular tissue and expanded as needed. Using our defined differentiation media, cardiomyocytes can be efficiently generated. The dynamic capabilities of this kit enable advancement of cell and molecular biology related to both cardiac stem cell and cardiomyocytes.

References

1. Oh, H., Bradfute, *et al.* (2003) Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc. Natl. Acad. Sci.* **100**:12313-12318.
2. Barile, *et al.* (2007) Endogenous cardiac stem cells. *Prog. Cardiovasc. Dis.* **50**:31-48.

Millipore Products

Description	Cat. No.
Cardiac Stem Cell Isolation Kit, 5 isolations	SCR061
Cardiomyocyte Characterization Kit, 1 kit	SCR059
Steriflip 100 µm Nylon Net, 25/pk	SCNY00100
Steriflip 60 µm Nylon Net, 25/pk	SCNY00060
Steriflip 40 µm Nylon Net, 25/pk	SCNY00040

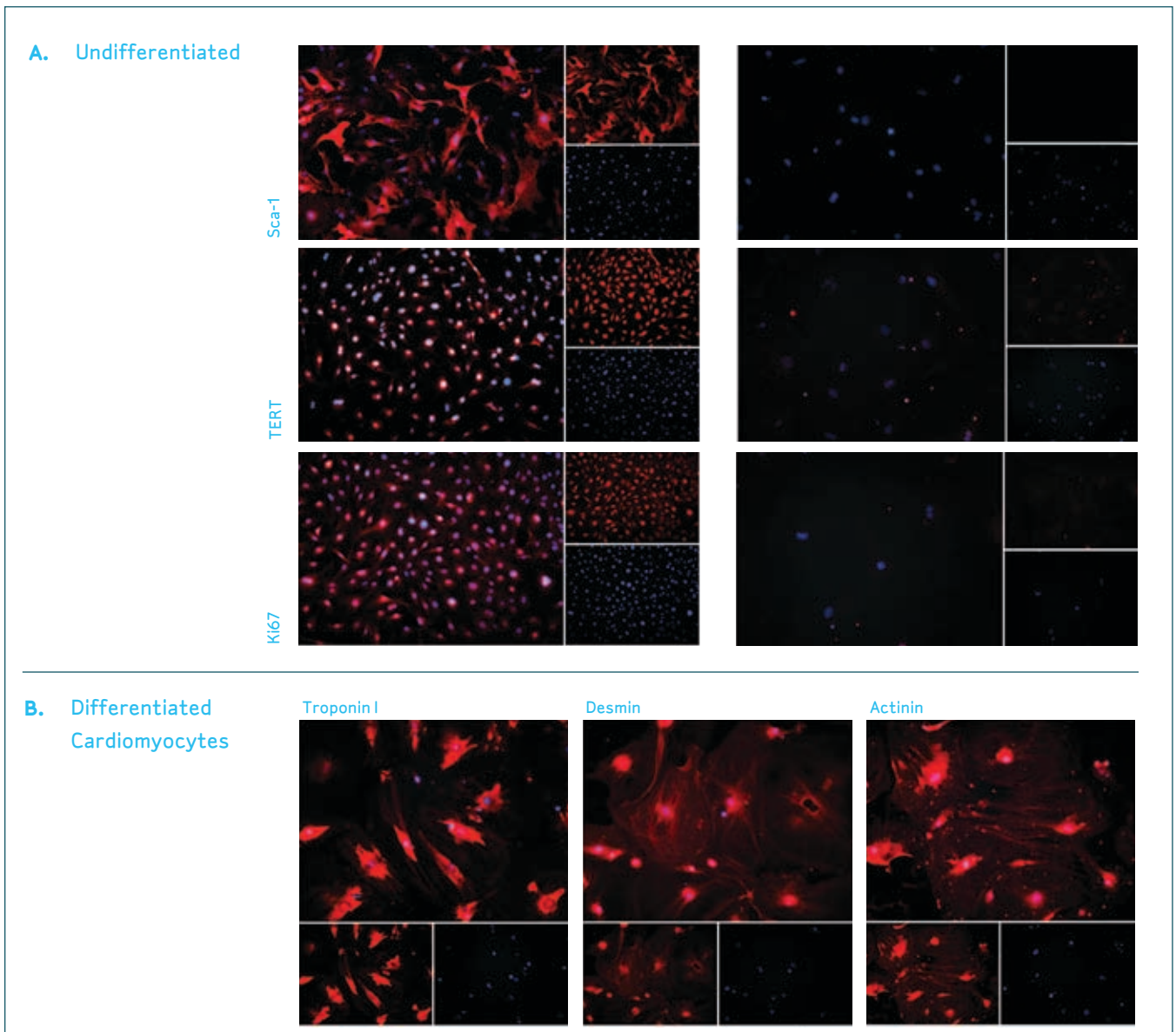


Figure 3. Cultured CSC retain their stem cell characteristics and efficiently differentiate into cardiomyocytes. (A) One-week cultures of purified CSCs ubiquitously express stem cell markers Sca-1 and telomerase, while remaining in a proliferative state as determined by Ki67 immunoreactivity. (B) Differentiated CSCs express mature markers for cardiomyocytes (Troponin I, Desmin, and Actinin).

Successful Isolation and Growth of Human Bladder Epithelial Cells with Progenitor Cell Targeted Culture Media

Jim Johnson, CELLnTEC Advanced Cell Systems, AG

Successful isolation and culture of human urothelial cells is a challenging process. When combined with limited tissue availability, these difficulties have meant that there have been few, and recently, no commercial sources of primary human urothelial cells and suitable culture media. CELLnTEC has addressed this issue, and is now providing both Progenitor Cell Targeted (PCT) media for the efficient isolation and growth of bladder epithelial cells, as well as early passage human bladder progenitor cells isolated in a PCT medium.

Background

CELLnTEC's expertise is in the development of Progenitor Cell Targeted culture media. These media are formulated to mimic the microenvironment of the adult stem cell niche of a number of different epithelial tissues. In this way, these media select and establish more progenitor (colony-forming) cells *in vitro*. By targeting progenitor cells, PCT media establish cell cultures that exhibit high colony forming efficiency, uniform morphology, and extended *in vitro* lifespan--all of which make these cultures ideal for further experimentation.

DEVELOPMENT OF PCT BLADDER EPITHELIUM MEDIA

Primary human bladder epithelium cell cultures were not available commercially for quite some time. To address this shortfall, CELLnTEC has developed a series of culture media for both the isolation and differentiation of primary bladder epithelium cell cultures. As with all CELLnTEC PCT media, no additional supplements, plate coatings, feeder cells or other additives are required. (Fig. 1).

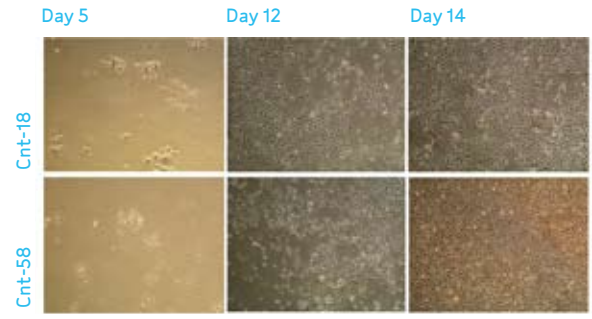


Figure 1. Primary human bladder epithelial cells, isolated and cultured in PCT media. Best isolation and growth was obtained in CnT-58 (a low-BPE medium), although the fully defined CnT-18 also provided good isolation efficiency and proliferation at higher seeding densities. Optimal seeding densities are as follows. CnT-58: 100 cell clumps per cm^2. CnT-18: 300 cell clumps per cm^2. See protocol for details.

Cat. No. CnT-58; PCT Bladder Epithelium Medium, Low BPE.
CnT-58 combines all the progenitor cell retention benefits of a PCT medium with the isolation and early-passage growth boost of BPE (Bovine Pituitary Extract). Due to the already highly efficient PCT formulation, only a very low amount of BPE is added (4 to 5 times lower than what is normally used), thereby minimizing BPE's inherent variability and the influence of unwanted factors. This medium provides the highest isolation efficiency, and also provides strong cell growth at lower seeding densities.

Cat. No. CnT-18; PCT Bladder Epithelium Medium, Defined.
CnT-18 is a fully defined PCT medium which does not require the addition of serum, BPE, or feeder cells. It is effective for isolation and growth, but for optimal performance, a higher seeding density than that for CnT-58 is recommended. Due to its fully defined formulation, CnT-18 provides complete control of your experimental system and is also especially selective for epithelial cells.

Cat. No. CnT-21; Bladder Epithelium Medium, Defined.
CnT-21 is recommended for culture of established cells that are to be induced to differentiate. It shares the same formulation as CnT-18, but does not contain the PCT components. PCT media are specifically designed to retain progenitor cells in an undifferentiated phenotype. Thus, when induced to differentiate, cells in these media may respond more slowly in the presence of the PCT components. For differentiation of both monolayer and 3D cultures, the non-PCT formulation (Cat. No. CnT-21) is recommended. Differentiation experiments are conducted by simply changing to the non-PCT sup medium prior to inducing differentiation (see recommended protocols).

ISOLATION PROTOCOL PLAYS A CRITICAL ROLE

During the course of our development of the PCT bladder media, it became evident that the protocols used for isolation and passaging are critical elements in the successful establishment of these cell cultures. Particularly important steps of the protocol include the tissue dissociation enzyme and the seeding density used.

For isolation and passaging, CELLnTEC recommends Neutral Protease (Dispase). This enzyme provides a mild proteolytic action, making the enzyme especially suitable for bladder tissue dissociation. Commonly used alternatives such as Trypsin or similar enzymes are harsher on the cells, and significantly impair subsequent cell attachment and outgrowth.

CELLnTEC Neutral Protease (Dispase):
- Functionally tested for use with CELLnTEC protocols
- Cat. No. CNT-NPD-01

We tested a number of neutral protease (dispase) preparations on the market and found a wide variety of activities, independent to the stated enzyme unit activity. It was found that this enzyme's unit definition has little relevance to its use in tissue dissociation. Thus CELLnTEC now provides Neutral Protease (Dispase), (Cat. No. CNT-NPD-01) that has been functionally tested for use in our specific isolation and passaging protocols at the indicated concentrations.

Dispase digestion of tissue pieces produces cell clumps, not single cells. Additional digestion (with trypsin, for example) of the cell clumps generated by Dispase digestion has been found to noticeably decrease subsequent attachment and cell growth. Thus another important recommendation in our suggested digestion protocol is that cell clumps be seeded directly, without further digestion with an additional enzyme to break the cell clumps into single cells.

A final consideration when culturing primary human bladder cells is the timing of passaging. Allowing cultures to become too confluent has a significant, detrimental effect on the attachment and growth of the cells in subsequent passages. In particular, it is important that cell colony size is monitored, and that cultures are passaged before cells in the center of the colonies begin to change morphology and differentiate. We recommend that cultures are passaged when colonies reach approximately 500 cells in size, which will typically fall in the range of 60-90 % confluence. Frequency of media changes should also be monitored carefully as confluence increases.

EARLY PASSAGE BLADDER EPITHELIUM PROGENITOR CELLS

The purchase of frozen, early passage cells from bladder epithelium is a convenient way for researchers to avoid time-consuming and often challenging processes of tissue sourcing, virus testing, isolation, amplification, and quality control. CELLnTEC's Human Bladder Progenitor cells are unique because they are isolated and grown in a PCT Bladder medium. In addition to the colony-forming and morphology benefits during early passages, these cells also represent excellent value due to the extended lifespan provided by the progenitor cell retention effect of the PCT media.

CELLnTEC's primary bladder epithelium cells provide further value as they are packaged with a 500 mL kit of PCT culture medium. No additional supplements, plate coatings or feeder layers are needed. For those wanting to use an animal model, Rat Long Term Bladder Cell Systems are available.

CELLnTEC Human Bladder Progenitors:
- Cat. No. HBEP-05
- Cat. No. HBEP-15

Kit containing
- Frozen cells
- **Plus** PCT Bladder Epithelium Medium

Summary

PCT bladder cell culture media provide significant improvements in isolation and growth of human urothelial cells as a result of specific formulations designed to target the progenitor cell population in the starting bladder tissue samples, and an optimized isolation and passage protocol using CELLnTEC's Neutral Protease (Dispase). These media are available in either low-BPE or fully defined formulations. Early passage bladder progenitor cells from CELLnTEC provide excellent growth and longevity, in addition to convenience and value. Bladder cultures isolated and grown in PCT media allow more passages than cultures in traditional culture media, which, when combined with the isolation and growth benefits, provide a range of new experimental possibilities for bladder researchers.

CELLnTEC Products for Bladder Research

Product Category	Cat. No.	Product Name	(Species)	Pack Size
Media - Bladder	CnT-18	**PCT** Bladder Epithelium Medium, Defined	(Human)	500 mL Kit*
	CnT-58	**PCT** Bladder Epithelium Medium, Low BPE	(Human)	500 mL Kit*
	CnT-21	Bladder Epithelium Medium, Defined	(Human)	500 mL Kit*
	CnT-16	**PCT** Bladder Epithelium Medium, Defined	(Rat)	500 mL Kit*
	CnT-36	Bladder Epithelium Medium, Defined	(Rat)	500 mL Kit*
Primary Cell Systems	**Cat. No.**	**Product Name**	**Quantity**	**Pack Size**
	HBEP-05	Bladder Epithelium Progenitors, Human	1 x ›5 x 10^4 Cells	Cells + Media Kit
	HBEP-15	Bladder Epithelium Progenitors, Human	3 x ›5 x 10^4 Cells	Cells + Media Kit
Long-Term Cell Systems	**Cat. No.**	**Product Name**	**Quantity**	**Pack Size**
	RBLAK-WIS	Bladder Urothelium Progenitors, Wistar, Rat	1 x › 6.5 x 10^5 Cells	Cells + Media Kit
	RBLAK-SD	Bladder Urothelium Progenitors, Sprague Dawley, Rat	1 x › 6.5 x 10^5 Cells	Cells + Media Kit
Related Products	**Cat. No.**	**Product Name**	**Quantity**	**Pack Size**
	CnT-ABM	Antibiotic/Antimycotic Solution (100X)		100 mL
	CnT-ABM10	Antibiotic/Antimycotic Solution (200X, ready to use single aliquots)		10 x 2.5 mL
	CnT-ABM20	Antibiotic/Antimycotic Solution (200X, ready to use single aliquots)		20 x 2.5 mL
	CnT-NPD-01	Neutral Protease (Dispase), Functionally tested		1 gm

*CELLnTEC media are supplied as a kit consisting of a 500 mL bottle of basal media with frozen supplements to be added prior to use.
** CELLnTEC Cell Systems are supplied with a vial (or vials for the larger package size) of frozen cells with a kit of the appropriate medium.

Visit **www.millipore.com** for a complete listing of CellnTec products for epithelial cell culture.

The Use of Accumax Solution for Enzymatic Passaging of Human Embryonic Stem Cells Cultured in HEScGRO Medium

Nil Emre, Ph.D., Rhoda Mondeh, Ph.D., and Matthew Singer, Ph.D., Millipore Corporation, Temecula, CA

Abstract

Human embryonic stem cells (hESCs) are fundamental tools in the study of development and disease and hold great promise for therapeutic applications. However, there remain many challenges to hESC work, one of which is the removal of non-human and/or undefined components for the maintenance of hESCs. The development of HEScGRO medium is a step towards establishing humanized and defined conditions for hESC growth. hESCs that are grown in HEScGRO medium can be passaged manually. Although manual dissociation is recognized as the gentlest method to passage hESCs, it can be time-consuming, and it is often difficult to scale up cultures. As an alternative to manual passaging of hESCs grown in HEScGRO medium, we have enzymatically passaged cells using Accumax solution. Cells that were expanded with Accumax maintained a normal morphology. ICC and FACS analysis further confirmed that hESCs grown in HEScGRO medium and passaged with Accumax solution for over 10 passages maintained pluripotency markers. hESCs were also able to form embryoid bodies (EBs) when cultured under these conditions. Additionally, hESCs that were passaged with Accumax solution for 15 passages maintained a normal karyotype. Therefore, Accumax solution can be used to enzymatically passage hESCs grown in HEScGRO medium and allows for ease of passage and an increased yield in a time-efficient manner while simultaneously maintaining hESCs in a pluripotent state.

Introduction

Since the initial isolation and characterization of hESCs[1], research has grown rapidly. This field now constitutes a valuable system for the study of human development and disease and offers significant potential applications to 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 from hESC culture to assist in the development of therapeutic applications. HEScGRO media is a defined humanized media that has been developed for the long-term culture of hESCs.

Traditionally, hESCs are passaged using either mechanical dissection or enzymatic methods. Manual passaging holds the distinct advantage of being a gentler method since cells are not exposed to enzymatic processes. However, manual passaging is labor- and time-intensive, and it can be difficult to scale up cultures for use in experiments such as differentiation or screening studies. Previous studies[2] have highlighted the use of Accumax solution for expansion of hESCs grown in Knockout Serum Replacement (KOSR™) media. Accumax enzymatic expansion on hESCs was investigated and validated in order to scale up hESCs cultures grown in HEScGRO medium and as an alternative to manual passaging.

METHODS

hESC Culture

H9 hESCs were grown on Matrigel™ (BD)-coated dishes on Mitomycin C-treated Detroit 551 feeders (ATCC) that were plated at a density of 60,000 cells per square centimeter. hESCs were maintained in HEScGRO medium (Cat. No. SCM020) with media changes every 1-2 days and passaged every 6-7 days, depending on cell density.

Passaging hESCs with Accumax

At the time of passage, cells were rinsed once with PBS (Cat. No. BSS-1006-B), and overlaid with approximately 1 ml of Accumax solution (Cat. No. SCR006) per well of a 6-well dish. Plates were incubated for 5 minutes in the tissue culture incubator or until the cells began to detach from the plate. HEScGRO media was added onto the cells and the cells were gently pipetted up and down. Most of the cells were detached, although additional gentle scraping with a serological pipette may be required. Wells were rinsed with additional culture medium so that the total amount of culture medium used to quench was 5 times the volume of Accumax solution that was used. The cells were centrifuged at 75 x g for 5 minutes, the supernatant was aspirated, and the pellet resuspended so that the splitting ratio was between 1:6 and 1:8. (Note: When initially transferring cells from HEScGRO manual passage to Accumax enzymatic passage, a split ratio of 1:3 is advised for the initial split).

Cell Characterization

For ICC and FACS analysis, the following antibodies were used: Oct3/4 (Cat. No. MAB4401), SSEA-4 (Cat. No. MAB4304), Tra-1-60 (Millipore, Cat. No. MAB4360), and Tra-1-81 (Cat. No. MAB4381). A FITC conjugated goat anti-mouse antibody (Cat. No. AP130F) was utilized for ICC, while a PE conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories) was used for FACS. For alkaline phosphatase staining, an Alkaline Phosphatase detection kit (Cat. No. SCR004) was used. For differentiation of hESCs, cells were manually scraped and plated on low adhesion dishes (CoStar) in FBS containing differentiation media[3].

RESULTS & DISCUSSION

As an alternative to the manual expansion of hESCs grown in HEScGRO medium, the ability to enzymatically passage hESCs using Accumax solution was evaluated. Accumax is a proprietary solution that contains collagenolytic, proteolytic, and DNAse enzymes and is provided as a ready-to use solution. As an added benefit, Accumax solution does not contain any mammalian or bacterial components. The effects of enzymatic passaging on hESCs were examined by analyzing general colony morphology, karyotype, expression of pluripotency markers by ICC and FACS, and differentiation potential.

Specifically, hESCs were passaged for over 15 passages with Accumax solution and shown to maintain a normal morphology (Figure 1). Upon colony dissociation with Accumax solution, colonies were broken up into numerous small clumps. Discrete hESC colonies were visible by day 2 after passaging (Figure 1A), and cells grew as compact colonies with well defined edges (Figure 1B). Colonies passaged with Accumax solution rarely exhibited signs of differentiation. Cells were split at ratios between 1:6 and 1:8 and an average of 1.4 x 10^6 cells per well of a six-well dish were obtained. Hence, in addition to the ease afforded by enzymatic passaging of hESCs with Accumax solution, there is the added benefit of being able to efficiently scale cultures up for various applications, including high-throughput screening experiments and differentiation studies. hESCs passaged with Accumax solution have been carried out to 15 passages (passage 49 total) and maintain a normal karyotype (data not shown). Besides H9 hESC lines, H1 and MEL-1 hESC lines have also been successfully passaged in HEScGRO medium with Accumax solution. In contrast, the use of Collagenase IV, which is used to passage hESCs grown in media containing Knockout Serum Replacement, causes hESCs grown in HEScGRO medium to rapidly differentiate (data not shown).

To further confirm the undifferentiated status of hESCs passaged with Accumax solution, cells were stained with pluripotency markers (Figure 2). Indicative of an undifferentiated state, cells expressed high levels of alkaline phosphatase (Figure 2A). hESCs passaged with Accumax solution also stained positive for the transcription factor Oct-4 (Figure 2B) and the cell surface antigens TRA-1-60 (Figure 2C) and TRA-1-81 (Figure 2D). In addition, hESCs grown in HEScGRO medium and passaged with Accumax solution express pluripotency markers as determined by FACS

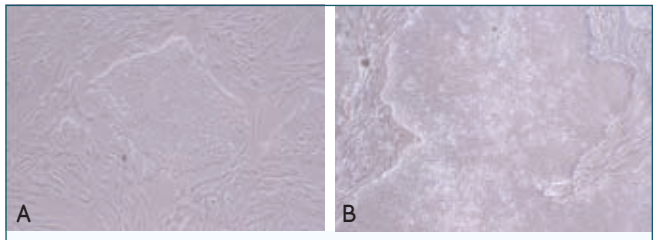


Figure 1. Morphology of hESCs grown in HEScGRO and enzymatically expanded using Accumax solution. H9 hESCs are at passage 45 total and split for 11 passages with Accumax solution on inactivated D551 feeders. Cells are shown at A) day 3 and B) day 6 after passaging.

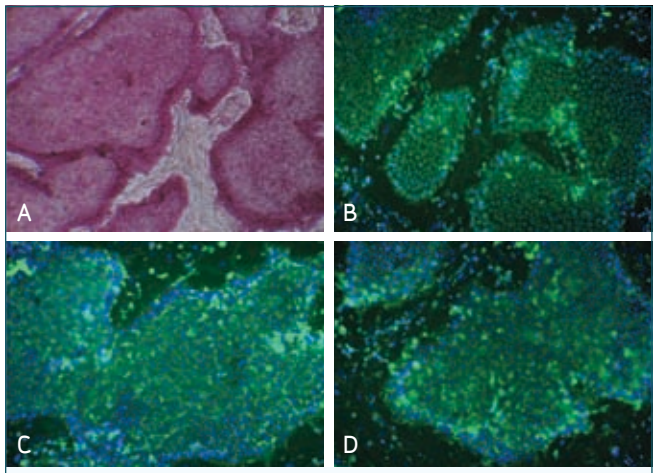


Figure 2. Stem cell marker expression of hESCs passaged using Accumax solution. H9 hESCs are at a total of 44 passages and have been enzymatically passaged 10 times with Accumax solution. Alkaline phosphatase (A), Oct 4 (B), TRA-1-60 (C), and TRA-1-81 (D) staining; (B-C) FITC and DAPI counter-staining.

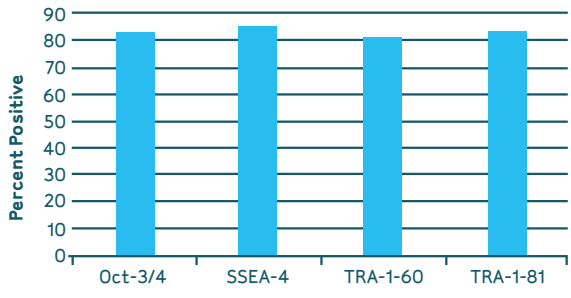


Figure 3. FACS analyis of hESCs enzymatically passaged with Accumax. Percentage of positive cells for various stem cell markers of H9 hESCs expanded 10 times with Accumax solution (Passage 44 total).

(Figure 3). Cells expressed high levels of markers for the undifferentiated state: Oct4 (83 %), SSEA-4 (85 %), TRA-1-60 (81 %), and TRA-1-81 (83 %). Finally, hESCs grown in HEScGRO medium and passaged with Accumax solution were able to form embroid bodies (EBs) when plated on low adhesion plates (Figure 4).

Accumax solution can thus be utilized as an alternative to manual passaging when expanding hESCs grown in HEScGRO medium. Specifically, characterization by ICC and FACS demonstrated that hESCs expanded using Accumax solution expressed pluripotency markers while also maintaining a normal morphology and karyotype. With enzymatic passaging by Accumax, hESC cultures are of high quality and high yield and can be utilized for various downstream manipulations.

Millipore Products

Description	Cat. No.
HEScGRO Medium	SCM020
Accumax	SCR006
PBS	BSS-1006-B
Oct3/4 Antibody	MAB4401
SSEA-4 Antibody	MAB4304
Tra-1-60 Antibody	MAB4360
Tra-1-81 Antibody	MAB4381
FITC anti mouse Antibody	AP130F
Alkaline Phosphatase Detection Kit	SCR004

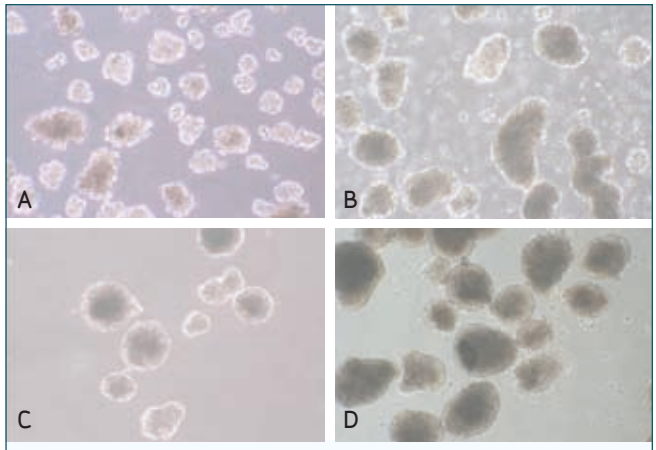


Figure 4. Embryoid bodies formed from hESCs passaged with Accumax solution. EBs were formed from H9 hESCs at passage 10 in Accumax solution (Passage 44 total) and are shown at days 1 (A), 4(B) , 6(C) , and 7(D) after formation.

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Screening Kits to Monitor Cell - Extracellular Matrix Interactions

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Cell adhesion plays a major role in cell communication and regulation, and is of fundamental importance in the development and maintenance of tissues. Cell-extracellular matrix (ECM) interactions have global implications in many disease states through processes such as angiogenesis, apoptosis, and inflammation, and are critical for normal and tumor cell signal transduction pathways. Knowledge of the molecular mechanisms involved in these interactions will facilitate the development of novel therapeutic molecules to benefit patients diagnosed with diseases such as arthritis and cancer.

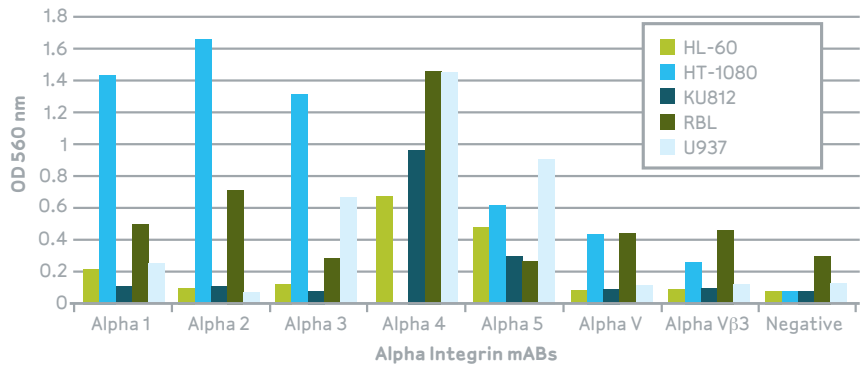
The extracellular matrix is a complex structural and functional network of proteins and proteoglycans that can interact simultaneously with multiple cell surface receptors (Aplin, *et al.*, 1998). The majority of these proteins are glycosylated, including a wide variety of collagens, laminins, fibronectin, and elastins. ECM proteins can influence cellular function through a complex feedback mechanism. A class of cell surface proteins known as integrins mediates the adhesion of cells to ECM proteins and endothelial surfaces. These receptors anchor cells to the ECM leading to the transduction of signaling events that regulate cell survival, proliferation,

and migration. Functional integrins are heterodimeric molecules, containing one alpha and one beta transmembrane glycoprotein subunit that are non-covalently bound together. Different integrin combinations may recognize a single ECM ligand, while others bind several different ECM proteins (Aplin, *et al.*, 1998).

Identification of cellular integrin profiles and ECM binding properties is the first step in understanding the mechanism of action in these protein-protein interactions. Historically, antibodies have been used to determine the integrin profiles on a cell's surface by immunoprecipitation, immunofluorescence, immunoblotting, or flow cytometry; however, these methods are laborious or require the use of sophisticated equipment. As an alternative, Millipore offers Integrin-Mediated Cell Adhesion Array kits and ECM Cell Adhesion Array kits as cost effective and efficient tools to screen cell surface profiles on virtually any human cell. Using the array format reduces variability in experimental conditions; therefore interassay results are comparable in a consistent, controlled protocol. In less than 2 hours, adhesion expression profiles are generated for up to 8 samples in one user-defined experiment.

Millipore's Integrin-Mediated Cell Adhesion Array kit is based on a 96-well plate format, consisting of 12 x 8-well removable strips, for convenience and flexibility in experimental design. Each 8-well strip is composed of seven individual integrin pre-coated wells and one negative control well coated with bovine serum albumin (BSA). After a short incubation period with the cells of interest, integrin protein binding is detected with a stain solution and analyzed using a standard microplate reader. Integrin-Mediated kits available from Millipore include an alpha integrin screening kit, a beta integrin screening kit, and a combination kit containing one plate of each. The array kits are available in either colorimetric or fluorimetric detection formats.

Similarly, the ECM Cell Adhesion Array kit format is a 96-well plate complete with stripwells. Each individual well of the strip is pre-coated with one of seven extracellular matrix proteins (collagens I, II, IV, fibronectin, laminin, tenascin, and vitronectin), and a BSA-coated negative control well. Again, both colorimetric and fluorimetric options are available and all necessary reagents are included.



Alpha Integrin-Mediated Cell Adhesion Array. Multiple cell lines were incubated for 2 hours at 37° C in wells coated with the array of alpha integrin monoclonal antibodies. After incubation, wells were washed, stained, and measured using a standard microplate reader.

Targeting cell-ECM involvement is critical in determining whether a cell proceeds through a normal course of development or transforms its adhesive associations. Defining the process by which integrins modulate the response of cells to the ECM is fundamental to the identification of specific structural and mechanical properties of the ECM environment that influence cell behavior.

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Millipore Products

Description	Cat. No. (Colorimetric)	Cat. No. (Fluorimetric)
α Integrin-Mediated Array	ECM530	ECM533
β Integrin-Mediated Array	ECM531	ECM534
α / β Integrin-Mediated Arrays	ECM532	ECM535
ECM Arrays	ECM540	ECM545

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