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Stem Cellutions

ESGRO Complete™ Products for Mouse Embryonic Stem Cell Culture in Serum-Free and Feeder-Free Conditions

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A major challenge in harnessing the potential of embryonic stem (ES) cells for both *in vitro* and *in vivo* use is the controlled and reproducible self-renewal of ES cells in the laboratory. Currently, the routine culture of mouse ES cells largely relies on the addition of serum and in some cases the presence of a feeder layer. Serum is an inherently heterogeneous, undefined product that has batch to batch variability, while the use of feeder cells is cumbersome and adds extra variability. A fully defined and easy to use culture system would lead to more systematic and uniform approaches to large scale screening projects. We have developed a fully defined, serum and feeder free system based on the work of Ying *et al.*[1] that allows for the efficient self-renewal of mouse ES cells. This system enables the researcher to successfully passage, freeze and thaw ES cells in a serum free environment while maintaining their pluripotency. Unlike other systems, ES cells grow efficiently at clonal density making this system suitable for transfection and targeting experiments. Here we describe observations on ES cell growth characteristic of this system and the differences with those cells grown in serum.

Background

Stem Cell Sciences plc (SCS) is a leading biotechnology company in the embryonic stem cell field, working in close collaboration with the Institute for Stem Cell Research (ISCR) in Edinburgh Scotland. The publication of the work of Ying *et al.*[1] demonstrates the role of STAT3 and SMAD signaling as revealed in serum free culture conditions. SCS has developed and optimized this serum-free and feeder-free system for the general maintenance of self-renewal of mouse ES cells in their pluripotent state that has universal utility in the stem cell field. This technology has been exclusively licensed by Chemicon International. It is available in a convenient ready to use format and marketed under name ESGRO Complete™.

The Serum-Free and Feeder-Free System Clonal Assay Test

The efficiency of ESGRO Complete™ Clonal Grade Medium to support the self-renewal of mouse ES cells without the presence of feeders is routinely tested in a clonal assay performed over 5 days. This type of assay shows the efficiency of this culture system in the maintenance of the clonal growth of the cells compared to other systems, creating an ideal environment to conduct reproducible and comparable genetic manipulations between different laboratories and researchers. For example, it is very easy to pick individual colonies after transfection of the cells in targeting experiments and lends itself to the development of high throughput screening procedures.

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Figure 1
Undifferentiated ES cells colonies with a distinct cytoplasmic and nuclear morphology (day 4 of a clonal assay).

***(A)** Tight round colony, **(B)** flatter colony*

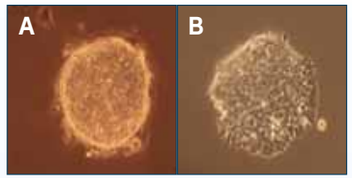


Figure 2
Alkaline phosphatase staining allows for an easy distinction between undifferentiated ES cells (red) and differentiated cells (unstained) on day 5 of the clonal assay.

***(A)** ES cells colony with no differentiation **(B)** differentiated cells at the edge of an ES cells colony*

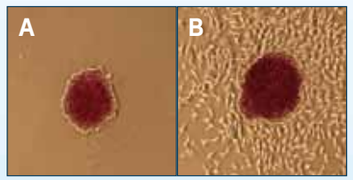


Figure 3
*Standard Trypsin use causes the ES cells to lift off the plates in serum free conditions **(A)** whereas the use of Accutase™ allows for an efficient and gentle way to routinely passage the cells **(B)**.*

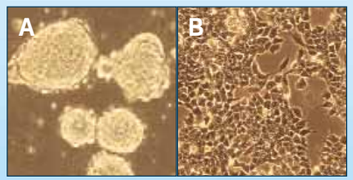
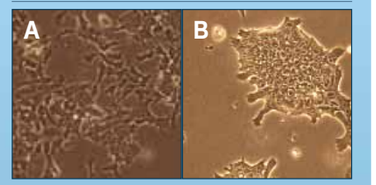


Figure 4
*In serum containing medium, ES cells spread out with spaces between cells **(A)**; whereas in serum free conditions ES cells grow as tight colonies with cells closely packed together **(B)**. Less differentiation is seen in serum free conditions when compared to serum containing medium.*



In this test, we use early passage mouse E14Tg2a ES cells that were previously grown in serum. These cells are directly plated into serum free medium at a clonal density (1 x 10^3 cells) in a 10 cm tissue culture dish pre-coated with ESGRO Complete™ Gelatin. Each single cell gives rise to a "pickable" colony within 5 days of plating. Colony formation is observed as undifferentiated cells often as tight round colonies (Figure 1A) or somewhat flatter colonies (Figure 1B) but all with a distinct cytoplasmic and nuclear morphology. More rarely, some colonies may have differentiation at their edges in the form of large flat cells which we call a "skirt". If the colonies are left for more than 5 days, many will overgrow and start to differentiate. However, if the colonies are picked prior to overgrowth, then self-renewal of the cells from the colonies is easy to maintain on subsequent passage. In those colonies with a differentiated "skirt," any differentiated cells gradually become less and less noticeable during passage as the self renewing cells predominate.

The standard assay to distinguish self renewing cells from any differentiated colonies is to fix and stain the colonies on day 5 for alkaline phosphatase. This marker is expressed in ES cells and, although it is not a specific marker for self renewal, it is an easy and economical test to perform on a routine basis. The ES cell colonies stain intensely red with this assay (Figure 2A), while any differentiated cells do not stain (Figure 2B). When alkaline phosphatase positive colonies are counted during this clonal assay, the cloning efficiency is around 20%, so typically we count around 200 alkaline phosphatase positive colonies per plate.

The efficiency of this system is demonstrated by comparative clonal assays: in standard ES cell medium containing 10% Fetal Calf Serum (FCS), and in ESGRO Complete™. Cells plated in 10% FCS containing medium tend to form colonies with a more irregular morphology and have more colonies that are clearly differentiated. When the plates are stained with alkaline phosphatase, the colonies grown in 10% FCS, in general, show less intense staining with many colonies displaying different levels of alkaline phosphatase staining (data not shown).

Passaging the Cells

While colony formation has been used as a routine test for clonal growth, we also routinely test the media during serial passage of ES cells. Mouse ES cells have a high metabolic rate and, therefore, we find the best results when the media on the cells is changed every 2 days. We also find that the cells prefer to be passaged prior to reaching 70 - 80% confluence, as overgrowth of the cells leads to differentiation. Standard trypsin used in passaging ES cells grown in standard serum containing media is not advised. Over trypsination causes the cells to lift off the plates to form "ball" like structures reminiscent of embryonic bodies (Figure 3A). This is probably due to over trypsination affecting cell adhesion. To avoid this, we assayed for the best method of passaging the cells. ESGRO Complete™ Accutase™ was found to be ideal in giving a rapid dissociation of cells from the plates without any detrimental effects on cell morphology (Figure 3B). In addition, we developed ESGRO Complete™ Trypsin by the titration of porcine trypsin activity in a proprietary formulation and obtained results similar to the use of Accutase™. We also developed ESGRO Complete™ Enzyme Free Dissociation Solution to avoid exposure to any enzymatic based products. This system takes longer for the cells to dissociate from the plastic, but the plating efficiency is also very good (not shown).

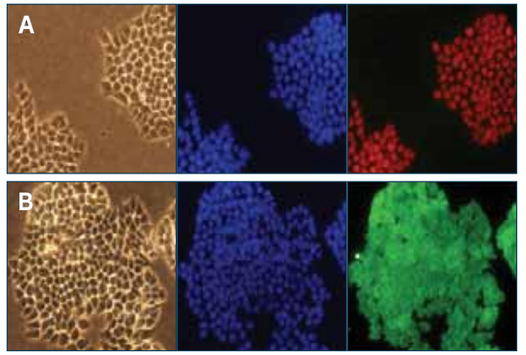
When cells passaged in ESGRO Complete™ Clonal Grade Medium are compared to cells passaged in standard ES cell media containing 10% FCS, we find that cell morphology was again more irregular with irregular cell spacing and high levels of spontaneous differentiation (Figure 4A). When the same cells are grown in ESGRO Complete™ Clonal Grade Medium, they remain as tight colonies with the ES cells closely packed together. Very little if any differentiation was noticed within colonies with any spontaneous differentiation being restricted to the outer edges of the colonies (Figure 4B).

To confirm pluripotency of ES cells after many passages in ESGRO Complete™ Clonal Grade Medium, cells were stained for alkaline phosphatase positivity and antibody stained for Oct-4 (Figure 5A) and Nanog (Figure 5B). Karyotype and germ line competency were confirmed (not shown).

Freezing and Thawing the Cells

We have optimized standard freezing solutions for the cryopreservation of the mouse ES cells to optimize recovery of cells grown in ESGRO Complete™ Clonal Grade Medium. ESGRO Complete™ Freezing Medium was devised by comparison of a number of different freezing protocols and was selected for its ease of use and high recovery rate. It is based on standard DMSO freezing techniques but is serum-free. Sub-confluent mouse ES cells were dissociated using ESGRO Complete™ Cell Dissociation Solution, resuspended in ESGRO Complete™ Clonal Grade Medium and counted. Cells were then spun down and resuspended in

Figure 5
Immunostaining of mouse ES cells grown in ESGRO Complete™ Clonal Grade Medium. From left to right for the top panel: phase contrast, Hoechst staining and Oct-4 staining. From left to right for the bottom panel: phase contrast, Hoechst staining and Nanog staining.



ESGRO Complete™ Freezing Medium at approximately 1 x 10^6 cells/ mL per cryotube and frozen in liquid nitrogen. Cells were thawed gently and washed in ESGRO Complete™ Clonal Grade Medium and plated into a small tissue culture flask precoated with ESGRO Complete™ Gelatin. We observed again that the cells prefer to grow as tight colonies, with the ES cells closely packed together (Figure 6).

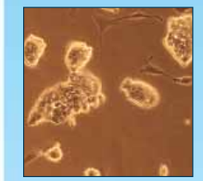
Conclusion

ESGRO Complete™ Clonal Grade Medium is an innovative serum-free cell culture medium that provides a convenient method to grow mouse ES cells. The ability to form clones makes it suitable as a universal serum-free system for genetic manipulations and growth of these cells. Cells have different growth characteristics than in serum being tighter and more closely packed together with less differentiation. Cells can be successfully passaged, frozen and thawed while maintaining their pluripotency (Oct-4 and Nanog expression) with a normal karyotype and germ line competency.

Reference:

1. Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell*. 2003 Oct 31; **115(3)**:281-92.

Figure 6
Mouse ES cells previously frozen in ESGRO Complete™ Freezing Medium and thawed in ESGRO Complete™ Clonal Grade Medium (4 days post- thawing).



Derivation of Mouse Embryonic Stem Cells in Serum-Free and Feeder-Free Conditions

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The availability of murine embryonic stem (ES) cells has revolutionized the study of mammalian development and disease. A culture medium has been developed from the work of Ying *et al.*,[1] that has enabled the identification of the essential signaling pathways that are required for maintenance of pluripotency *in vitro*. We have successfully derived and propagated mouse ES cells in the absence of feeder cells and serum using this medium. Here we describe some observations during derivation of new ES cell lines from mouse embryos in these serum and feeder free conditions. This work was used to develop the ESGRO Complete™ Derivation Kit by Stem Cell Sciences and is available from Chemicon International.

Introduction

ES cells are invaluable as an *in vitro* model for the study of development and as a means to create genetic modifications in mice for the study of gene function and disease. The derivation of ES

Figure 1
ES cell colonies form 5 days after dissaggregation of outgrowths ***(A)****. Undifferentiated ES cells have a distinct nuclear morphology that is easy to identify* ***(B)****.*

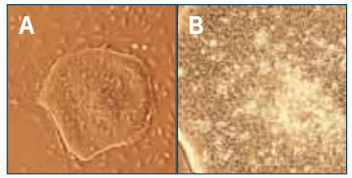

Figure 2
Chimeras produced after microjection of ES cells derived in serum-free conditions ***(A)****. Confirmation of germline transmission was seen as gray pups born to outbred mice* ***(B)****.*

cell lines is predominately achieved using the inbred 129 strain, as it is the most permissive to derivation. In this system, derivation of ES cells involves subjecting the embryos to delayed implantation or diapause; allowing the embryos to attach to the substrate for a few days, and then disrupting the cell contacts by disaggregation and replating. A major limiting factor in determining the efficiency of ES cell derivation is the quality of the serum used in the derivation process. The composition of serum is ill defined and variable between batches and must be prescreened before use. Also, reliance upon its inclusion in media for derivation and propagation of ES cells does not facilitate study of the minimal requirements and molecular responses involved in self-renewal and differentiation. There is, therefore, a requirement for a system that is reliable and free from batch to batch variation in order to obtain the most efficient procedures for mouse ES cell derivation. Here we describe ES derivation using a serum and feeder-free system developed from the work of Ying *et al.*[1].

Delayed Implantation

Isolation of mouse ES cells is most efficient if the embryos are subjected to delayed implantation[2]. Delayed implantation can be induced in mice either by ovariectomy or by intraperitoneal injection of tamoxifen. 129 mice at 2.5 d post coitum (dpc) were subcutaneously injected with Depo-Provera (1-3 mg/mouse -as a source of progesterone) and tamoxifen (10 mg per mouse). The delayed embryos were flushed from the uterus of these mice 4 or 5 days after ovariectomy/tamoxifen injection using ESGRO Complete™ Basal Medium. Embryos were then transferred into ESGRO Complete™ Delayed Blastocyst Incubation Medium, a serum-free media containing LIF only, in gelatinized 4-well plates pre-equilibrated to 6% CO_2 and 37°C.

Disaggregation

After 3 to 5 days in culture, outgrowths were disaggregated by gently detaching each cell clump. This was achieved by using a mouth controlled finely drawn plugged Pasteur pipette whose tip diameter was just bigger than the outgrowth. The clumps were transferred to a small (approximately 5 µL) drop of trypsin at 37°C for a few minutes until they began to dissociate. Taking care to minimize the amount of trypsin, the outgrowths were separated individually in a small amount of culture medium using a finely drawn plugged Pasteur pipette. The dissagregated cells were transferred to a new well of a 4-well plate containing ESGRO Complete™ Clonal Grade Medium as small clumps of about 1-5 cells.

ES cell colonies should subsequently become identifiable after about 5 d (Figure 1). ES cell colonies were expanded into 96-well plates by trypsinization of the well, centrifugation (5 min at 1200 rpm) and resuspended in 50 µL of ESGRO Complete™ Clonal Grade Medium.

Routine Passaging of Serum-Free Media Derived ES Cells

To passage the cells from a gelatinized small flask (T25), we added ESGRO Complete™ Trypsin to cover the cells (about 1 mL) and incubated them at 37°C for a few minutes. We then added 19 mL of ESGRO Complete™ Basal Medium (without cytokines), to dilute the trypsin; centrifuged, aspirated off the medium completely, and resuspended in 0.5 mL ESGRO Complete™ Clonal Grade Medium (containing LIF and BMP4) and replated 1-2 x 10^6 cells into a new gelatinized T25 flask pre-equilibrated to 7% CO_2 and 37°C. ES cells were routinely passaged every 2-4 days.

Injection of Serum-Free Media Derived ES Cells into Blastocysts to Confirm Germline Competency

To confirm that ES cells derived in this serum free media are pluripotent, chimeras were made and checked for germline transmission. ES cell lines were trypsinized and the cells resuspended in a small volume (0.5 mL for a slightly sub-confluent T25 flask) of serum-containing medium (the addition of serum to the medium for cells for blastocyst injection reduces stickiness). 10-20 cells were injected into each C57BL/6 host blastocyst. Chimeras were monitored by coat color chimerism and were mated to outbred mice. Successful germline transmission was monitored by the presence of gray mice in subsequent litters (Figure 2).

Conclusion

The ESGRO Complete™ serum free media conditions offer an improved system for the efficient derivation, growth and passage of mouse ES cell lines, which avoids problems associated with the use of serum. These ES cell lines maintain their germline competency making this system suitable for gene targeting experiments. In addition, the fully defined conditions allow for a detailed and profound understanding of the conditions and factors needed for derivation of mouse ES cells which may affect the ability to generate ES cells from other strains of mice or other animals.

References:

1. Ying *et al.* (2003). *Cell.* **115(3)**:281-92.
2. Nichols *et al.* (2001). *Development.* **128(12)**:2333-9.

ESGRO Complete™ Clonal Grade Medium: Serum-Free Medium for Mouse Embryonic Stem Cell Culture

Matt Singer, Ph.D., *Chemicon International, Inc.*

Abstract

ESGRO Complete™ Clonal Grade Medium is a fully defined, serum-free media for mouse ES cell culture. It is equivalent to serum-containing media for the maintenance of murine embryonic stem cells in the pluripotent state, even in feeder-free culture. ESGRO Complete™ Clonal Grade Medium is suitable for all standard transgenic protocols from single-cell cloning to the efficient generation of chimeric animals, and thus can replace the need for serum-containing media for multiple mouse ES cell applications. Also noteworthy are the utility of two additional reagents for serum-free culture of murine ES cells, ESGRO Complete™ Accutase™ for cell dissociation and ESGRO Complete™ Freezing Medium for serum-free cryopreservation.

Introduction

Fetal bovine serum (FBS) has remained an indispensable component of standard culture media for murine embryonic stem cells, despite several drawbacks. First, FBS is very expensive, and may not always be available. Second, lot-to-lot variability in the performance of murine ES cells is common with FBS; therefore, multiple lots of FBS must be pre-tested prior to purchase of each new lot. Finally, the composition of FBS is unknown; it is certainly preferable that any media used for ES cell culture be completely defined.

Here we introduce ESGRO Complete™ Clonal Grade Medium, a serum-free and fully defined media for multiple applications of mouse ES cell culture. We show that mouse ES cells cultured for multiple passages remain pluripotent and maintain a normal karyotype. In addition, ESGRO Complete™ Clonal Grade Medium is superior to standard FBS-containing media for the generation of single mouse ES cell clones, particularly in that pluripotent clones arise earlier than with media containing FBS. Finally, mouse ES cells cultured in this medium yield high-percentage chimeras. Thus, ESGRO Complete™ Clonal Grade Medium is suitable for multiple applications of mouse ES cell culture, and bypasses the need for FBS.

Results

Murine ES cells [(strains E14Tg2a.IV (hereafter referred to as "E14") originally at passage 16, and 129/S6, originally from Specialty Media at passage 11)] were maintained without a feeder layer in gelatinized tissue culture dishes in 7.5% CO_2. Cells were grown in parallel cultures in either ESGRO Complete™ Clonal Grade Medium or standard mouse ES cell media (with 15% FBS, prescreened for optimal growth, HyClone) supplemented with nonessential amino acids, sodium pyruvate, 2-mercapto-ethanol and 1000 U/mL recombinant murine LIF (ESGRO®, Chemicon International). Media was changed once per day. Cells were maintained at standard densities (i.e., between 5 x 10^4/cm^2 and 5 x 10^5/cm^2), and were passaged every 2-3 days with ESGRO Complete™ Accutase™ (Chemicon International), a dissociation reagent that does not require quenching with serum.

After 10 successive passages in either media, cells in culture were examined for their morphology and pluripotency. Cultures of E14 had normal morphology (Figure 1A); most cells were in rounded clusters, with scattered individual cells (often larger than cells in clusters and with a flattened shape) appearing between clusters. This morphology is typical of mouse ES cells cultured without a feeder layer, and was also observed with the 129/S6 cells, as well as with either strain cultured in standard FBS-containing media (not shown).

Mouse ES cells that had been cultured for 10 successive passages in ESGRO Complete™ Clonal Grade Medium were also examined for expression of pluripotency markers. E14 cells maintained expression of alkaline phosphatase[1], as determined by colorimetric activity staining (Figure 1B). In addition, E14 cells maintained expression of the pluripotency marker Oct4[2], as shown by indirect immunofluorescence for Oct4 protein (Figure 1C,D). Similar results were observed with 129/S6 cells, as well as with either strain in standard FBS-containing media (not shown).

Cells were examined for chromosomal abnormalities after 12 successive passages in ESGRO Complete™ Clonal Grade Medium. Karyotype analysis (GTW banding method) demonstrated a normal karyotype (data not shown).

To examine the suitability of ESGRO Complete™ Clonal Grade Medium for the generation of single mouse ES cell clones (such as for clone selection during standard murine transgenic protocol), cells of either the E14 or 129/S6 strains were plated in either ESGRO Complete™ Clonal Grade Medium or standard FBS-containing media at a density of 25 cells/cm^2 (on gelatinized TC plastic; note that cells plated into a particular media had already been passaged 5 times at standard densities in that media). Media was changed daily beginning the third day after plating. By day 4 (day 0 = plating), colonies were already visible in the ESGRO Complete™ Clonal Grade Medium for both strains, and by day 6 these colonies were of a sufficient size to be picked; these colonies were composed of undifferentiated cells, as shown by alkaline phosphatase activity staining (Figure 2A). In contrast, colonies in standard FBS-containing media were not easily observed until day 6, and were not of a sufficient size until day 9 or 10; these colonies also contained pluripotent cells (Figure 2B). Thus, not only is ESGRO Complete™ Clonal Grade Medium suitable for single-clone generation, but pluripotent clones arise more rapidly in ESGRO Complete™ Clonal Grade Medium than in standard FBS-containing media.

To examine the competency of mouse ES cells cultured in ESGRO Complete™ Clonal Grade Medium for the generation of transgenic mice, E14 cells that had gone through 11 passages (and were therefore at passage 27) were injected into mouse blastocysts, and the resulting chimeras examined (Table 1); it should be noted that between passages 10 and 11, these cells were cryopreserved in ESGRO Complete™ Freezing Medium, which is also serum- free, and subsequently thawed for injection. 40 blastocysts injected (16 live births) resulted in 6 males that were greater than 85% chimeric by coat color, indicating that mouse ES cells cultured in ESGRO Complete™ Clonal Grade Medium can successfully contribute to adult tissues, and result in the efficient generation of chimeric animals.

Discussion

Here we have demonstrated the utility of ESGRO Complete™ Clonal Grade Medium, a serum-free, fully defined media for the culture of mouse embryonic stem cells. This medium is suitable for multiple applications involving mouse ES cells, including general culture at standard densities, single-cell cloning and generation of transgenic mice. Mouse ES cells cultured in ESGRO Complete™ Clonal Grade Medium, maintain their pluripotency as well as normal karyotype and the capacity to differentiate. ESGRO Complete™ Clonal Grade Medium performs as well as, or superior to, serum-containing media in all applications tested.

The use of ESGRO Complete™ Clonal Grade Medium thus bypasses the need for FBS for mouse ES cell culture. This provides the user with several benefits, including being able to avoid the expense and the lot-to-lot variability of FBS as well as the effort necessary to qualify media prior to purchase. Finally, it should be noted that two additional Chemicon products, ESGRO Complete™ Accutase™ and ESGRO Complete™ Freezing Medium, are particularly suited to serum-free mouse ES cell culture.

References:

1. Pease, S., Braghetta, P., *et. al., Dev. Biol.* **141:** 344 (1990).
2. Nichols, J., Zevnik, B., *et al., Cell* **95:** 379-391 (1998).

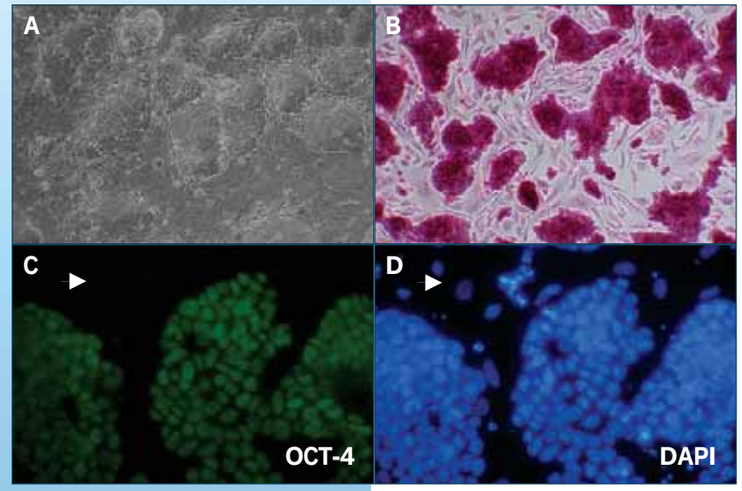

Figure 1
Murine ES cells passaged with ESGRO Complete™ Clonal Grade Medium maintain normal morphology and expression of pluripotency markers. All cells are E14Tg2a.IV that have undergone 10 successive passages.

(A) *ES cells appear morphologically normal. Most cells are in rounded clusters, and look typical of mouse ES cells cultured without a feeder layer.* ***(B)*** *Cells in rounded clusters express alkaline phosphatase, a marker for pluripotency.* ***(C), (D)*** *Cells in rounded clusters express Oct-4* ***(C)****, another pluripotency marker, as shown by indirect immunofluorscence for the Oct-4 protein (arrow denotes isolated cells in* ***(D)****, revealed by DAPI staining, that do not express Oct-4).*

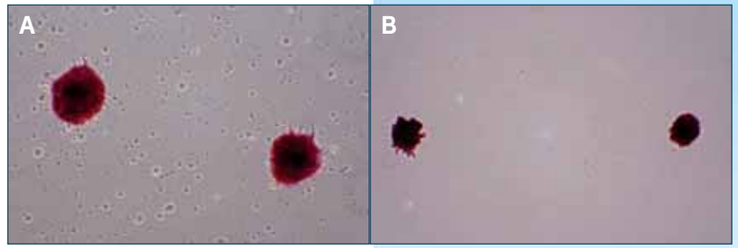

Figure 2
ES cells grown at clonal density in ESGRO Complete™ Clone Grade Medium yield pluripotent colonies more rapidly than FBS-containing media.

(A) *E14Tg2a.IV colonies grown in ESGRO Complete™ Clone Grade Medium at day 6, labeled for alkaline phosphatase activity.* ***(B)*** *E14Tg2a.IV colonies grown in standard 15% FBS media at day 9, labeled for alkaline phosphatase activity. Note that the colonies in* ***(B)*** *are smaller than those in* ***(A)****, even with three additional days of culture.*

Table 1
Generation of High-Percentage Male Chimeras from E14Tg2a.IV cells cultured for 11 passages with ESGRO Complete™ Clonal Grade Medium.

Blastocysts Injected	Live Births	% Male Chimeras
40	16 (40%)	6 (15%)

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Progenitor Cell Targeted (PCT) Media Deliver Ten-fold Enriched Progenitor Cell Cultures and Improved Isolations

Jim Johnson, *CELLnTEC advanced cell systems AG*

ADVANTAGES

- *Improved isolation efficiencies*
- *Extended culture lifespan*
- *Reduced cost – no need for time consuming feeder layers, conditioned media or plate coatings*
- *Reduced variability – serum-free, defined medium*

Abstract

Adult stem cells, also known as progenitor cells, represent around 5-10% of the total number of cells in some tissues. When using traditional culture media for isolation, the majority of these cells quickly differentiate and die and are lost from the culture. In contrast, by mimicking the environment of the adult stem cell niche, progenitor cell targeted (PCT) media retain many more progenitor cells in culture than regular media, thus delivering large numbers of these valuable colony forming cells for further experimentation.

Introduction

Adult stem cells drive all tissue renewal *in vivo*. These progenitor cells have the ability to grow without further differentiation, which combined with their unlimited colony forming capability makes them the ideal cell type for *in vitro* studies.

The technique of isolating epithelial progenitor cells from tissue to establish a cell culture is challenging and variable. Further complicating the issue is that isolation efficiencies can vary widely from tissue to tissue, between species/ strains and decreases with the age of the donor. Traditionally, non-defined supplements such as Bovine Pituitary Extract (BPE) have been used to optimize difficult isolation procedures.

However, in recent years there has been an increasing drive toward the replacement of non-defined additives in cell culture media. In particular, the replacement of Fetal Calf Serum (FCS) and BPE has been difficult to achieve for many cell types without a loss of isolation efficiency and *in vitro* growth. By specifically targeting and retaining progenitor cells, the new PCT media by CELLnTEC represent the first range of defined epithelial media to equal or exceed the performance of non-defined alternatives.

Chemicon is proud to be the worldwide distributor of CELLnTEC PCT products for epithelial cell growth. These media have been used successfully to increase the number of progenitor cells in primary cultures, to remove serum and feeders from the culture, and to provide a defined environment that fosters the growth of progenitor cells.

SUPPORTING EXPERIMENTS

Enrichment of Epidermal Stem Cells in Early Passage Culture

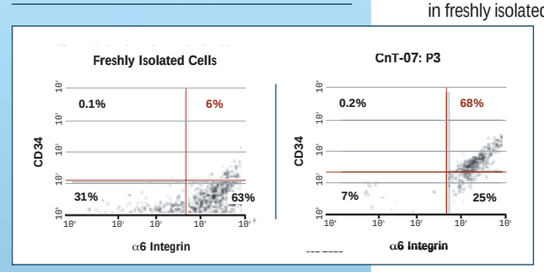
CnT-07 PCT Epidermal Keratinocyte Medium enhances the isolation of epidermal progenitor cells by mimicking the environment of the adult skin stem cell niche. To evaluate its ability to isolate and enhance growth of epidermal stem cells, we conducted FACS analysis using two proposed epidermal stem cell markers, CD34 and α6 integrin.

METHODS: Primary mouse keratinocytes were isolated from the epidermis of neonatal mice using a Dispase/rProtease method[1]. Cells were plated at 3 x 10^4 cells per cm^2. Cells were allowed to grow to confluency and then split using rProtease[2]. FACS analysis was performed comparing newly isolated keratinocytes to cells grown to confluency after passage three. Our finding of 6% progenitor cells in the newly isolated keratinocytes falls within the reported range of 5% to 10% in freshly isolated cell populations.

RESULTS: This FACS experiment (Figure 1) shows that CnT-07 PCT Keratinocyte medium provides a 10-fold increase in double-labeled (progenitor) cells over 3 passages.

CONCLUSION: CELLnTEC's CnT-07 medium mimicks the adult stem cell niche micro-environment and enhances the retention and subsequent growth of progenitor cells.

Figure 1
FACS analysis of double-labelled cells comparing freshly isolated cells to cells grown to passage three in CnT-07 showing 10-fold enrichment of progenitor cells.



Growth Rate Comparisons: Defined CnT-07 vs. Non-defined KSFM

Non-defined (BPE containing) KSFM by Gibco/Invitrogen has been a dominant epithelial culture media for over ten years. Although a groundbreaking formulation at its launch, recent advances in our understanding of both the stem cell niche in different tissues and the signaling pathways that control differentiation and retention of adult stem cells now enable the development of novel, improved formulations.

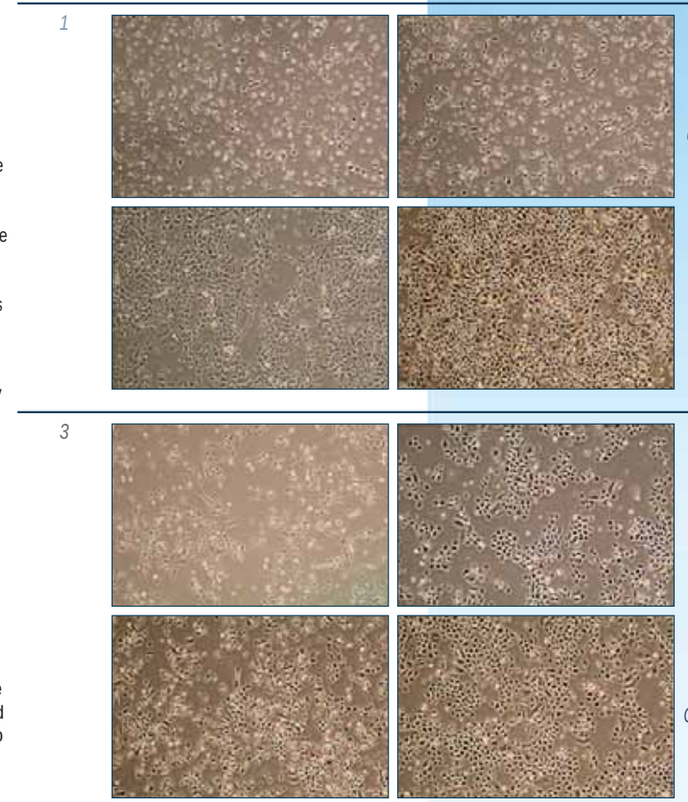
In this experiment, the isolation and early passage growth of human keratinocytes was compared in non-defined KSFM and defined CnT-07, PCT Epidermal Keratinocyte Medium by CELLnTEC.

METHODS: Cells were isolated from the epidermis of 2 human donors. Isolates were then pooled and seeded at 9 x 10^3 cells per cm^2 into each of the two culture media. Uncoated, T-25 culture flasks were used for all treatments. Cultures were then photographed at equal times post isolation, to enable comparison of the respective growth rates.

RESULTS: CnT-07 provided significantly better isolation efficiency, growth rate and morphology than KSFM (Figure 2). Specifically, CnT-07 enabled the formation of many rapidly growing colonies, prevented virtually all apoptosis and necrosis and yielded 3x more cells than non-defined KSFM.

CONCLUSION: CELLnTEC's defined CnT-07 medium provided improved performance when compared with non-defined KSFM in the critical period following isolation. With a yield of more than 3x more cells than the non-defined medium, CnT-07 will enable scientists to begin experiments more than a week earlier than when using KSFM.

Figure 2
Isolation and early passage growth of human keratinocytes showing improved isolation efficiency and growth rates with better morphology in the CnT-07.



PCT Products

FREE Samples Available

Description	Species	Quantity	Cat. No.	Price
PCT Epidermal Keratinocyte Medium Complete	Mouse, Human, Rabbit	500 mL	CnT-07	$82
PCT Prostate Epithelium Medium Complete	Rat	500 mL	CnT-11	$82
PCT Prostate Epithelium Medium Complete	Human	500 mL	CnT-12	$82
PCT Small Airway Epithelium Medium Complete	Rat	500 mL	CnT-14	$82
PCT Large Airway Epithelium Medium Complete	Rat	500 mL	CnT-15	$82
PCT Bladder Epithelium Medium Complete	Rat	500 mL	CnT-16	$82
PCT Airway Epithelium Medium Complete	Human	500 mL	CnT-17	$82
PCT Bladder Epithelium Medium Complete	Human	500 mL	CnT-18	$82
PCT Vaginal Epithelium Medium Complete	Human	500 mL	CnT-19	$82
PCT Corneal Epithelium Medium Complete	Human	500 mL	CnT-20	$82

Chemicon is proud to be the worldwide* distributor of the complete CELLnTEC media and model cell system product line. To find more information on the Progenitor Cell Targeted product line listed here and other CELLnTEC products for the study of epithelial cells, please visit **www.chemicon.com**.

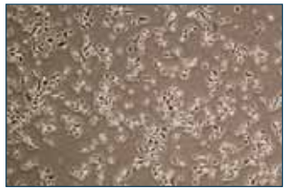
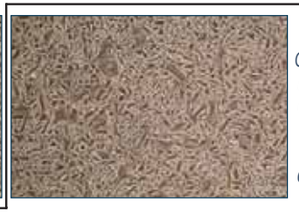
**Not available in Japan*

Comparative Testing: Defined CnT-12 vs. Non-defined PrEGM™

PrEGM™ by Clonetics™ is one of the most commonly used media for culturing human prostate epithelium. It provides good isolation and growth of these cells, due largely to the presence of BPE in its formulation. When the BPE supplement is omitted, PrEGM™ medium does not enable viable establishment of prostate epithelial cultures.

The new defined formulation CnT-12, PCT Prostate Epithelium Medium by CELLnTEC now provides an alternative to the non-defined PrEGM™ formulation. This experiment is designed to compare the early passage growth of these defined and non-defined formulations.

Figure 3
Defined CnT-12 provided significantly faster growth than in non-defined PrEGM™, reaching the end of passage 2 in the time that cells in PrEGM™ took to grow to confluence in passage 1.

Passage #	PREGM™ (containing BPE)	CnT-12	Days after seeding
0			5
			Smaller, more homogeneous cells in CnT-12
1			1
			Faster adhesion after passaging, improved morphology in CnT-12
			4
			Cells past confluency in CnT-12, ~50% in PREGM
2			7 | 3
			Cells in CnT-12 have reached confluency in P2, PREGM™ reach confluency P1

METHODS: Cells were isolated from prostate tissue biopsies taken from 2 human donors. Isolates were then pooled and seeded into each of the culture media at 4 x 10^3 cells per cm^2. Uncoated, T-25 culture flasks were used for all treatments. Cultures were then photographed regularly post isolation to enable comparison of the respective growth rates.

RESULTS: During passage zero, CnT-12 produced larger colonies with smaller, more homogenous cell morphology than PrEGM™ (Figure 3). In passage 1, cells growing in CnT-12 showed significantly faster adhesion after passaging and growth than cells in PrEGM™.

CONCLUSION: The defined CELLnTEC medium CnT-12 for prostate epithelium provided improved performance when compared with the non-defined PrEGM™ by Clonetics™. Cells in the CnT-12 showed improved morphology in primary culture (size and homogeneity), adhered faster following passaging, and then exhibited faster growth rate in the critical early passages during which most research is carried out. With these faster adhesion and growth rates, cells in CnT-12 completed 2 passages in the time PrEGM™ cells completed 1 passage, thereby offering significantly faster expansion, and earlier commencement of experimentation following isolation.

Discussion

CELLnTEC's progenitor cell targeted (PCT) approach to serum free, defined media formulation enables significant improvements in isolation efficiency and subsequent cell culture growth. By enhancing progenitor cell retention and growth, more of these valuable cells are available for experimentation in a shorter period of time. As these experiments demonstrate, this approach delivers benefits for skin and prostate epithelium. In addition, novel PCT media are also available for bladder, airway and vaginal epithelia.

References

1. Isolation of Primary Mouse Keratinocytes; www.cellntec.com/resources
2. Protocol for the cultivation of CELLnTEC mouse keratinocytes, www.cellntec.com/resources.

Mesenchymal Stem Cell Products

Vi Chu, Ph.D., *Chemicon International, Inc.*

Mesenchymal stem cells, also known as marrow stromal cells[1], are defined as a self-renewing population of adherent, bone-marrow-derived multipotent progenitor cells with the capacity to differentiate into several mesenchymal cell lineages. In defined *in vitro* assays, mesenchymal stem cells have been shown to readily differentiate into lineage-specific cells that form bone, cartilage, fat, tendon and muscle tissues[1,2]. Mesenchymal stem cells also provide support and maintenance for the other major stem cell population in the bone marrow, the hematopoietic stem cells[2].

Mesenchymal stem cells have historically been isolated based on the ability of these cells to form adherent cell layers in culture and the concomitant lack of adherence of other cells in the bone marrow stroma such as hematopoietic stem cells, adipocytes and macrophages[1-3]. While this procedure results in enriched populations of mesenchymal stem cells, the resulting bone marrow derived cell populations are nonetheless, heterogeneous – comprised not only of mesenchymal stem cells, but also of committed lineage-restricted progenitors[1-3].

We have developed stem cell reagents that aid in the accurate identification and characterization of the stem cell population and that allow for the preferential differentiation of mesenchymal stem cells into adipocytes and osteocytes.

The Rat Mesenchymal Stem Cell Kit provides ready-to-use primary mesenchymal stem cells isolated from the bone marrow of adult Fisher 344 rats along with a panel of positive and negative selection markers for characterization of the mesenchymal stem cell population (Figure 1). Positive cell markers include antibodies directed against integrin β1 (Figure 1A) and CD54 (Figure 1B), two cell-surface molecules that are present on mesenchymal stem cells[2,4]. Negative cell markers include antibodies directed against two specific hematopoietic cell surface markers that are not expressed by mesenchymal stem cells: CD14, present on leukocytes and CD45, present on monocytes and macrophages[2,3,4]. Mouse and rabbit immunoglobulins for assessment of background staining are also included. Researchers can purchase the cryopreserved Rat Mesenchymal Stem Cells alone or in combination with the Mesenchymal Stem Cell Kit. All of the antibodies provided in the Mesenchymal Stem Cell Kit (Figure 1) have been tested and optimized for use in immunocytochemistry on rat mesenchymal stem cells. We recommend that the Rat Mesenchymal Stem Cells be used in conjunction with our differentiation assays to demonstrate multipotentiality of the starting cell population (Figure 2). The differentiation assays available are the Mesenchymal Stem Cell Adipogenesis Kit (Figure 2A, B) and Mesenchymal Stem Cell Osteogenesis Kit (Figure 2C, D).

References

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3. Alhadlaq, A. and Mao, J. J. (2004). *Stem Cells and Development* **13**: 436-448.
4. Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R. (1999). *Science* **284**: 143-147.
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Mesenchymal Stem Cell Products

Chemicon offers mesenchymal stem cells from rat and human, as well as a number of stem cell products for the characterization and differentiation of mesenchymal stem cells.

Description	Quantity	Cat. No.	Price
Rat Mesenchymal Stem Cell Kit	1 Kit*	SCR026	$825
Cryopreserved Rat Mesenchymal Stem Cells	1 Vial*	SCR027	$525
Mesenchymal Stem Cell Characterization Kit	1 Kit	SCR018	$495
Mesenchymal Stem Cell Adipogenesis Kit	1 Kit	SCR020	$295
Mesenchymal Stem Cell Osteogenesis Kit	1 Kit	SCR028	$595
LT2 Human Immortalized Pancreatic Mesenchymal Cell Line	5 x 10^5 cells	SCR013	$630
VIT1 Human Primary Pancreatic Mesenchymal Cell Line	5 x 10^5 cells	SCR014	$545

**We guarantee > 1 million viable cells upon thawing*

Figure 1
*Rat Mesenchymal Stem Cells express mesenchymal stem cell markers: integrin β1 **(A)**, CD54 **(B)**, collagen type I **(C)**, and fibronectin **(D)**. Nuclei of the cells were visualized with DAPI (blue).*

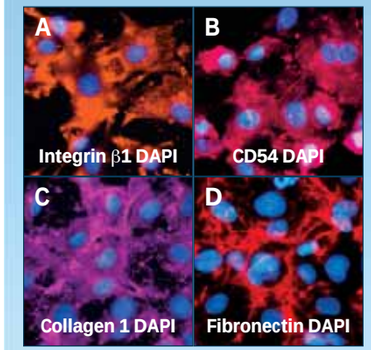
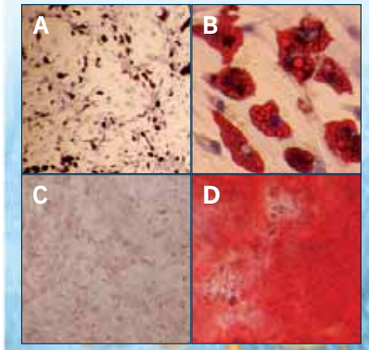


Figure 2
*Rat Mesenchymal Stem Cells are multipotent. Rat mesenchymal stem cells were differentiated in adipogenic **(A, B)** and osteogenic **(D)** differentiation medium. Using the Mesenchymal Stem Cell Adipogenesis Kit, rat mesenchymal stem cells differentiated after 21 days to mature adipocytes as indicated by the accumulation of lipid vacuoles that stain with Oil Red O (**A**, 10X magnification; **B**, 40X magnification). Cell nuclei (purple) were stained with Hematoxylin Solution. Control rat skin fibroblast cells did not contain any lipid droplets (data not shown). Using the Mesenchymal Stem Cell Osteogenesis Kit, rat mesenchymal stem cells readily differentiated to an osteocyte lineage as indicated by Alizarin Red S (ARS) staining **(D)**. ARS staining was not observed in control rat skin fibroblasts that were treated in the same manner **(C)**. Alizarin red S staining demonstrates mineral deposition throughout the culture.*



Stem Cellutions

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