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Inside

ReNcell VM and CX: Human neural stem cell lines
Characterization and functional differentiation

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Advantages

- Immortalized human neural stem cell lines
- Readily expandable in proprietary, serum-free, defined medium
- Derived from developing cortex and ventral mesencephalon, showing region-specific phenotype potential
- Differentiation *in vitro* – multipotential
- Screened for adventitious agents
- Fast growing – doubling time < 48hrs
- Stable normal diploid cells

Abstract

ReNcell VM and CX are two established human neural stem cell lines, derived from ventral mesencephalon and cortex brain regions respectively. These cell lines have rapid growth rates and are easily maintained as monolayers on laminin coated tissue cultureware in a proprietary serum free defined medium available from Millipore. Differentiation of ReNcells into multiple lineages is convenient and flexible, therefore these cells are ideally suited for a wide range of applications in both basic stem cell biology and neuroscience, as well as applied CNS drug-discovery fields. Here we discuss the characterization, differentiation and physiology of ReNcell VM and CX. A number of applications are presented, from simple differentiation assays to more complex electrophysiological experiments.

Introduction

The use of neural stem cells in medicine and research is becoming increasingly popular. The stem cell represents a renewable platform to study the activity of cellular events, normal or abnormal, within an *in vitro* controlled environment mirroring, for example, normal development or pathological change.

Continued on pg 2

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Rodent neural stem cells have provided a wealth of basic knowledge. However, in today's more applied biomedical research climate, analysis of human samples is necessary. Unfortunately human neural stem cells are notoriously slow growing, have a finite life *in vitro* and are not as readily available, unlike rodent neural stem cells. To meet this demand, ReNeuron has created two immortalized stablilized human neural stem cell lines, designated ReNcell VM and CX. These are versatile and potent tools for basic biology, assay development and neural stem cell research.

Undifferentiated cell lines

The ReNcell lines are transduced with the transcription factor *myc* to promote continuous growth and maintain a stable genotype and phenotype. ReNcell VM was derived from the midbrain of 10-week gestation human tissue sample whereas ReNcell CX was cloned from brain cortex extracted from a 14-week human sample. Extensive testing of the cells in culture has been carried out to ensure that they are safe and free from any adventitious agents or contaminants (eg. mycoplasma). Both cell lines are easily grown and monitored as monolayers on laminin coated tissue cultureware. The cells have a typical undifferentiated stem cell "cobblestone" morphology expanding as islands (Figure 1A). Growth of the cell lines is rapid, with approximate doubling times of 24-48hrs (Figure 1B). When growing the cells, it is important to note that the cell lines should always be maintained subconfluent to retain cells in log-phase growth. In addition, both cell lines are positive for the neural stem cell marker Nestin (Figure 1C). One target of *myc* is telomerase, which lends itself to maintaining a normal karyotype (Figure 1D).

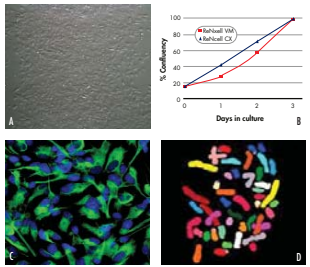

Figure 1
ReNcells grow as monolayer islands with an undifferentiated "cobblestone" morphology on laminin in serum free defined medium, (ReNcell VM shown, A). Both cell lines grow rapidly in the undifferentiated state (growth curve, B) and express the marker Nestin (ReNcell CX shown, Nestin, green; Hoechst nuclear stain, blue; 60X C). A normal male karyotype is observed in both cell lines (Spectral Karyotype shown for ReNcell VM, D[1].)

1. Acknowledge Constance Chua and Dr. Carol Tang, National Neuroscience Institute, Singapore, in generating this data.

Differentiation of ReNcell lines

The ReNcell lines have been selected for their robust growth and differentiation characteristics. Both cell lines are readily differentiated into all three neuronal phenoytpes: neurons, astrocytes and oligodendrocytes using two simple differentiation paradigms (Figure 2A, B).

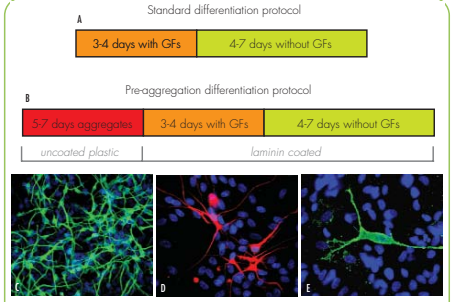

Figure 2
Multipotentiality of ReNcells, differentiation into multiple neuronal lineages. Schematic diagrams of timelines for two differentiation protocols used for ReNcells (A,B). Preparatory phases are shown in red and orange colours and differentiation phases in green. GF denotes growth factors (EGF and FGF) Both cell lines are readily differentiated into all three neuronal phenoytpes: neurons (βIII-tubulin, green, 20X, C); astrocytes (GFAP, red, 40X, D) and oligodendrocytes (Gal C, green, 60X, E); all counterstained with Hoechst nuclear stain (blue).

Differentiation conditions have been optimized for a 96 well plate, but can be scaled to suit. Standard differentiation (StD) involves plating cells at 10,000 cells per well in EGF/bFGF on laminin coated plates and allowing to grow to confluency over a 2-3 day period, followed by mitogen withdrawal for 5-7 days to differentiate (Figure 2A). As a modification to the StD, the preaggregate differentiation (PreD) assay begins by plating the cells on uncoated plastic for 5-7 days to form neurospheres. The neurospheres are then transferred to laminin-coated plates in a 1:1 ratio and the StD carried out. The spheres reform monolayer cultures prior to differentiation. Differentiated cells stained positively for βIII-tubulin, GFAP and Gal C indicating the presence of the three main neuronal types of cell - neurons, astrocytes and oligodendrocytes (Figure 2C-E).

While multi-lineage differentiation occurs spontaneously in these assay conditions, the power in this differentiation system is that factors or differentiation agents can easily be introduced. During the growth phase of the differentiation assay, pretreatment or priming agents can be incubated with the cells. With a simple media change during the differentiation phase, differentiation-inducing agents can be assessed. We found that optimal differentiation of ReNcell VM into tyrosine hydroxylase (TH) positive neurons was achieved using the PreD assay combined with dopamine (<0.1mM) pretreatment of the cells followed by 1mM dibutyrl-cAMP, 2ng/ml GDNF to induce differentiation (Figure 3A,B). High numbers of TH positive neurons can be differentiated from ReNcell VM, as shown by Western blot (Figure 3C). Conversely, the same conditions only produce low levels of TH positive neurons from ReNcell CX with a different morphology (data not shown). The brain region from which the lines were derived appears to influence the differentiation properties of each cell line. In addition to βIII-tubulin, the neurons are also positive for MAP2, Neurofilament light chain, Neurofilament 150 and Neurofilament 200 (data not shown). It is important to note the multipotentiality of these lines; in addition to the dopaminergic phenotype, we have also found a number of other neural subtypes in the differentiated cultures, for example serotinergic and glutaminergic neurons (data not shown).

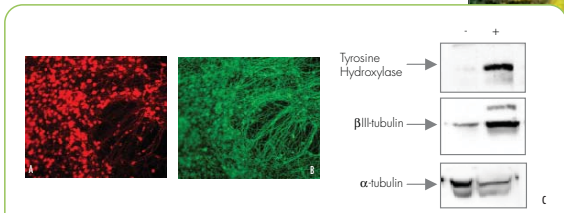

Figure 3
ReNcell VM can be differentiated into high levels of tyrosine hydroxylase positive neurons, a marker for dopaminergic neurons (TH, red, A; colabelled with βIII-tubulin, green, B; 10X). Western blot analysis of one well of a 96 well plate from ReNcell VM samples shown in A and B, an upregulation of neuron specific makers in differentiated samples is demonstrated (- undifferentiated, + differentiated; C).

Physiology

The differentiated ReNcells may be positive for neuronal markers but are they functional neurons? Electrophysiological analysis of the undifferentiated cell lines showed that undifferentiated ReNcell VM and CX displayed only a passive change in membrane potential under current clamp, and no changes in current were observed in voltage clamping experiments, consistent with the lack of neuronal morphology and markers observed in these cells. In the literature, it is rare to find cultured human neural stem cells that can differentiate into functional neurons. Differentiated ReNcell VM consistently displayed active currents; and injecting current steps generated action potentials in 18 out of 25 cells tested using the PreD method [1]. These action potentials were blocked in the presence of 0.6mM tetrodotoxin (TTX) indicating the primary involvement of sodium channels (data not shown). Surprisingly using the same differentiation paradigms, differentiated ReNcell CX did not appear to develop sodium channels but only displayed spontaneous channel activity during hyperpolarization. In addition to current and patch-clamp studies, ReNcells are ideal models to study ion channel regulation and function using Ca^{2+} imaging and FLEX-station (Molecular Devices, Reading, UK). Both ReNcell VM and CX responded to a range of stimuli including carbachol, ATP and weakly to histamine. In summary, the differentiated ReNcells look morphologically like mature neurons and they also contain functional ion channels and receptors.

Discussion

The ability to grow for extended periods in culture, self-renew, and differentiate into all three neuronal subtypes, establishes both ReNcell VM and CX as neural stem cells. Rather than transforming the cells, *myc* appears to maintain normality of the cells in culture. Traditionally thought of as a proto-oncogene, it has been recently reported that *myc* may be a 'stemness' gene maintaining multipotentiality in stem cells [2]. It certainly appears to be the case that in the presence of growth factors over a multitude of passages, *myc* maintains stable growth rates, karyotype, phenotype and multipotentiality, including specific neuronal phenotypes. Remarkably upon removal of growth factors, within days the cells morphology radically changes to contain elongated processes or "telegraph wires" throughout - indicating that *myc* does not interfere with differentiation. Furthermore, this differentiation results in the development of fully functional neurons.

The ease of growing and differentiating the ReNcells makes human stem cell research within reach and adaptable to any laboratory with minimal tissue culture facilities. Recently published is an extensive proteomic analysis of ReNcell VM during differentiation [3]. This illustrates how scientists can readily study human neural development that would otherwise be practically very difficult or impossible.

Standardize your research output and target human biology with ReNcells, VM and CX cells. Optimized ReNcell maintenance and Freezing Media provide a serum-free, defined environment for eliminating variables and ensuring the successful culture of ReNcells. For more information visit *www.millipore.com/chemicon*.

References

1. Donato, R., Miljan, E.A. *et al.* (2006) *Manuscript in preparatio.*
2. Takahashi, K. and S. Yamanaka, (2006) *Cell.*
3. Hoffrogge, R., Mikkat, S. *et al.* (2006) *Proteomics*, **6(6)**: p. 1833-47.

Description	Cat. No.
ReNcell VM Immortalized Cell Line	SCC008
ReNcell CX Immortalized Cell Line	SCC007
ReNcell Maintenance Media	SCM005
ReNcell Freezing Medium	SCM007
ReNcell VM Kit (SCC008, SCM005, SCM007)	SCC010
ReNcell CX Kit (SCC007, SCM005, SCM007)	SCC009

Expansion of Embryonic Stem Cell Clones by Membrane Based Co-Culture

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Abstract

One of the many advantages to culturing cells on porous membrane substrates is that it provides a surface that better recapitulates *in vivo* growth and tissue organization. In addition, porous mebranes allow for the co-culture of cells from different origins in order to study how cells interact through indirect signaling or by providing a conditioned niche for the proper growth and identity of cell type. In this study, we examine the ability of mouse embryonic fibroblasts (MEFs) to condition the media for the growth and clonal isolaton of undifferentiated embryonic stem cells (ESCs) in a multiwell porous membrane based indirect co-culture system. Variations in this theme can easily be adapted for the study of indirect cell-cell interactions to direct differentiation of stem cells as well as screening of factors that may interfere with these processes.

Background

Embryonic stem cells are essential tools in the development of genetically modified mouse models. The requirement that the ESCs are maintained in the undifferentiated state is a general challenge during clone isolation and expansion. Current methods typically accomplish this by direct co-culture of ESCs on a MEF feeder layer in 96 well tissue culture treated plates. The traditional workflow to expand ESC clones on MEFs after transgenic manipulation is a multi-step process that involves inactivating the MEFs by gamma irradiation or mitomycin C treatment (to prevent their overgrowth), and subsequent removal of the MEFs before using the ESCs for blastocyst injection. In addition to the many steps involved, ESC–MEF co-cultures are metabolically very active requiring frequent media changes due to the smaller media volumes in the 96 well format.

We have evaluated the ability to co-culture ESCs and MEFs in a Millicell® 96 well filter-based cell culture plate. The configuration of the co-culture setup involves growing the MEFs in a feeder tray below wells containing ESCs separated by a porous membrane filter. This arrangement allows for a physical separation between the two cell types eliminating the need for mitotically inactivating the MEFs, while continuing to allow the MEFs to condition the media for maintenance of pluripotent ESCs. Since the ESC containing wells share a larger volume of media, media change frequency can be reduced. In addition to improving the culturing and expansion of the ESC clones, the separation of the cell types during this filter based co-culture eliminates the requirement for removing the MEFs before blastocyst injection.

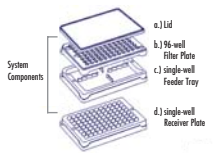

Figure 1
Schematic of Millicell 96 well cell culture plate assembly

Millicell® Multiwell Filter Plate Based Co-Culture

In this study, 96 well Millicell culture plates with either polyester (PET) or polycarbonate (PCF) membrane with pore sizes of 1.0 µm and 0.4 µm respectively were used. Though the co-culture presented here can also be performed in 24 well culture plates or individual filter inserts that fit into 6 to 24 well plate formats with the same membrane selection, the 96 well filter plates were chosen in this study to examine the high density clonal selection typical of transgenic targeting experiments. The Millicell cell culture plate is designed to support cell attachment, growth and differentiation for many cell applications. All procedures are designed to be carried out in a single device and can be performed using manual or automated methods for cell seeding, feeding, washing, and experimental procedures. The filter plates are composed of three parts (Figure 1): a.) a single-well feeder tray with baffles to reduce media leakage and contamination that contains approximately 32 mL of media, b.) a filter plate with individually isolated wells with membrane bottoms and c.) a lid to prevent contamination and evaporation during incubation. In addition, the plate assembly features a main basolateral access port to allow for media exchange in the feeder tray without disassembling the cell culture plate and feeder tray. In this configuration, all 96 wells are able to share the same reservoir of media and allow a simple way to exchange media either manually or automated. The greater shared volume of media in the feeder tray of this assembly allows for an extension of the time and a decrease in the level of metabolic byproducts accumulated in the media between feedings. In addition to the single well feeder tray, receiver plates with individual feeder wells can be used when different culture conditions are desired for different wells (Fig 1d.). For these applications, the filter plate is designed with individual basolateral access ports for each individual well to allow for individual media exchange and sampling without cross contamination between wells.

Indirect Co-culture with MEFs Promotes Undifferentiated ESC Colony Formation

Typical co-culture of MEFs and ESCs involves the direct co-culture of the two cell types. In order to investigate the ability of indirect co-culture to expand clonal colonies in a Millicell 96 cell culture plate, MEFs (Chemicon strain CF-1) were seeded onto fibronectin treated feeder trays and cultured to confluence. MEFs were then mitotically inactivated by mitomycin C treatment. ESCs (Chemicon strain 129/S6) were seeded at cell densities from 50 to 1500 cells per filter plate well directly to the untreated membrane surface. ES cells were cultured in medium containing 1000 units/mL ESGRO® for 5 days before alkaline phosphatase analysis to assess undifferentiated state of the colonies. Clonal colonies developed in this manner showed distinct morphology with well formed boundaries and stained strongly for alkaline phosphatase activity (Figure 2). In order to isolate the conditioning effects of the MEFs from the presence of ESGRO in the media, co-cultures were set up to evaluate the individual contributions. Figure 3 shows that either the presence of ESGRO in the media or MEFs in the feeder tray are capable of maintaining proper colony morphology and alkaline phosphatase activity of ESCs grown on the membrane. Cell seeded into plates in the absence of either MEFs and ESGRO quickly differentiated into a monolayer of heterogeneous cells without alkaline phosphatase activity. The conditioning of the media by co-culture of MEFs in the feeder tray was independent of mitotic inactivation, since the results observed were indistinguishable when MEFs were not mitomycin C treated (data not shown). Though ESCs differentiated in the absence of ESGRO and MEFs, as determined by loss of distinct colony morphology and alkaline phosphatase activity after 6 days post seeding, the clonal colonies cultured on membrane initially show a more undifferentiated phenotype when cultured for a shorter period (4 days) (Figure 4). Alternatively, cells seeded in parallel onto plastic bottom TC plates never form colonies and disperse into heterogeneous monolayers lacking alkaline phosphatase activity. These observations suggest that the surface structure of the membrane, and the ability for media exchange though the pores under the colonies, have a beneficial impact on the health and maintenance of the ESCs.

Clonal Selection of Embryonic Stem Cell Colonies by Serial Dilution

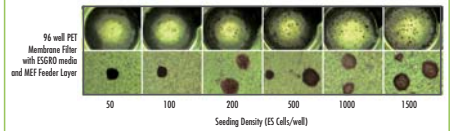

Figure 2
Serial dilution clonal selection of ES cells in Millicell 96 well cell culture plate. Single well feeder tray was coated with 25 µg/mL fibronectin and seeded with MEFs (1.67x10⁴ cells per tray). ES cells were seeded (seeding density as indicated) directly to untreated 1 µm PET membrane surface of culture plate wells. ES cells were cultured in medium containing 1000 units/mL ESGRO for 6 days. Alkaline phosphatase staining was performed by Napthol/Fast Red Violet treatment of fixed colonies according to kit instructions (Chemicon ES Cell Characterization Kit, Cat. No. SCR001).

Conclusions

Embryonic stem cell colonies can be grown on membrane filters by co-culture with mouse embryonic fibroblasts without the need for direct interactions. Co-cultured ESC colonies show high alkaline phosphatase activity suggesting maintenance of pluripotency. Colonies can be derived from single cells (cloning) by serial dilution via the filter based co-culture method. Since the ESC colonies and MEF feeder layer are not in direct contact, this culture method does not require mitotic inactivation of the MEFs grown in feeder tray. Early embryonic stem cell colonies cultured on filter membranes maintain some pluripotent characteristics even in the absence of MEFs and ESGRO, suggesting a supporting role for the structure of the porous membrane.

Embryonic Stem Cell Colony Morphology and Alkaline Phosphatase Expression

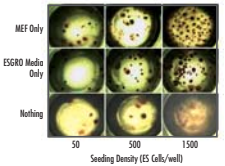

Figure 3
Dependence of serially cloned ESC colony morphology and alkaline phosphatase expression on presence of co-cultured MEFs and/or ESGRO on 1 µm PET Millicell cell culture plates.

Influence of Substrate on ES Cell Colony Morphology

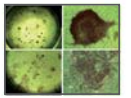

Figure 4
Early embryonic stem cells colonies cultured on filter membranes maintain some pluripotent characteristics. ES cells were plated at 200 cells per well in media without ESGRO and without the presence of a MEF feeder layer then grown for 4 days before alkaline phosphatase staining.

Millipore offers a comprehensive array of cells, kits, markers and cell culture products optimized specifically for neural stem cell research. Kits and cells are highlighted in this newsletter. Please see our Stem Cell Catalog for detailed information, which includes our complete range of monoclonal and polyclonal antibodies for neural stem cell research.

Products Available:

Species	Cell Lines (Derived= D; Isolated = I)	Cell Culture Products	Markers	Characterization Kits	Differentiation Kits
Mouse	YES (I)	YES	YES	YES	YES
Rat	YES (I)	YES	YES	YES	YES
Human	YES (D)	YES	YES	YES	NO

Kits

Human Neural Stem Cell Characterization Kit *(Cat. No.SCR060)* — Kit contains a panel of markers that are frequently used to identify neural stem cells/progenitors. Includes Nestin, Sox-2, Musashi, βIII-tubulin for neurons, GFAP for astrocytes and O1 for oligodendrocytes.

Rodent Neuron Differentiation Kit *(Cat. No. SCR035)* — Kit provides two neuronal inducers that when added to a defined serum-free medium allows for the preferential differentiation of rodent neural stem cells to a neuronal lineage. Other components of the kit include reagents for the fixation and two antibodies for the immunocytochemical characterization of the resulting neuron population.

Rat Neural Stem Cell Blotting Kit *(Cat. No. SCR044)* — Kit provides biotools for the assessment of cell signaling molecules and proteins involved in neural stem cell biology. Each kit contains two protein blots carrying normalized cellular extracts from neural stem cells and their differentiated progenies, neurons, astrocytes and oligodendrocytes. Also included are two neural-specific antibodies (Nestin and GFAP), actin control antibody and the ReBlot™ Plus Kit for multiple reuse of the protein blots.

Adult Rat NSC Expansion Kit *(Cat. No. SCR034)*
Mouse Cortical NSC Expansion Kit *(Cat. No. SCR032)*
Mouse Spinal Cord NSC Expansion Kit *(Cat. No. SCR033)* — These kits provide a multi-component system for the culture and analysis of neural stem cells and their differentiated progenies. These systems include primary neural stem cells, neural stem cell expansion medium, and a panel of antibodies for immunocytochemical staining of neural stem/progenitor cells (Nestin and Sox2) and differentiated neural phenotypes (Map2ab for neurons, GFAP for astrocytes and O1 for oligodendrocytes).

Rat Hippocampal Neural Stem Cell Kit *(Cat. No. SCR021)* — Kit contains ready-to-use primary rat neural stem cells and antibodies for immunocytochemical staining of neural stem/progenitor cells (Nestin and Sox 2) and differentiated neural cells (MAP-2 for neurons, GFAP for astrocytes).

Rat Hippocampal Astrocyte Kit *(Cat. No. SCR007)*
Rat Hippocampal Neuron Kit *(Cat. No. SCR009)* — These kits provide researchers with a convenient means for the culture and analysis of primary rat hippocampal astrocytes and neurons. In addition to viable, cryopreserved cells, these kits also include reagents for the immunostaining of astrocytes (GFAP) and for the detection of neuron formation (Map2ab).

Neural Stem Cell Characterization Kit *(Cat. No. SCR019)* — Kit provides researchers with a convenient means to phenotype neural stem cells using a panel of antibodies, including Nestin and Sox2 to identify neural stem/progenitor cells, along with more differentiated lineage markers including Map2ab for neurons, GFAP for astrocytes and O1 for oligodendrocytes.

Embryonic Stem Cell Derived Neuron Integration and Characterization Kit *(Cat. No. NS140)* — Kit allows for the localization, characterization and analysis of ES cell-derived neuron integration into adult central nervous system (CNS) tissue. The kit includes antibodies for the identification of Glutamate neurons (EAAC1), GABAergic neurons (GAD67), Dopaminergic neurons (TH), and serotonergic neurons (Serotonin). It also includes antibodies for the general identification of synapses (PSD-95) and (Synaptophysin) as well as for GABAergic synapses (GABA A Receptor β-chain).

Kits (continued)

Neuron-Glial Cell Marker Sampler Kit *(Cat. No. NS130)* — Kit contains antibodies for the identification of neurons (βIII-tubulin and Map2ab), astrocytes (GFAP), and oligodendrocytes (RIP).

Dopaminergic Neuron Integration and Characterization Kit *(Cat. No. NS145)* — Kit includes antibodies for the identification of dopaminergic neurons in different stages of neurotransmitter synthesis and maintenance: Dopa Decarboxylase; Tyrosine Hydroxylase (TH); Dopamine Transporter (DAT). Also included are antibodies for the general identification of neurons (NeuN), astrocytes (GFAP) and markers for the identification of cholinergic (ChAT) and Serotonergic (Serotonin) neurons.

Media

Astrocyte Differentiation Medium *(Cat. No. SCM010)* — Specially formulated medium optimized for the preferential differentiation of rodent neural stem cells to an astrocyte lineage. The medium has been extensively validated on mouse cortical and spinal cord neural stem cells and on rat hippocampal neural stem cells.

Mouse Neural Stem Cell Expansion Medium *(Cat. No. SCM008)*
Rat Neural Stem Cell Expansion Medium *(Cat. No. SCM009)* — These expansion media are provided as two-component systems that are convenient and easy-to-use. Kits include the Neural Stem Cell Basal Medium and the necessary supplements, which when combined allow for the growth and proliferation of mouse and rat neural stem cells.

Neural Stem Cell Basal Medium *(Cat. No. SCM003)* — Defined serum-free, growth factor-free medium that has been optimized for the growth and *in vitro* differentiation of neural stem cells derived from rodents. When used in conjunction with bFGF or bFGF, EGF and heparin, the basal medium allows for the proliferation of rat and mouse neural stem cells, respectively.

Cells

ReNcell VM, derived from ventral mesencephalon *(Cat. No. SCC008)*
ReNcell CX, derived from cortex *(Cat. No. SCC007)* — ReNcell VM and ReNcell CX cell lines are immortalized cells derived from human fetal neural tissue. These cells have been extensively validated, and shown to differentiate into cells of all three major neuronal pathways (astrocytes, neurons and oligodendrocytes). Specially optimized ReNcell maintenance media (SCM005) and freezing media (SCM007) also available.

Mouse Cortical Neural Stem Cells *(Cat. No. SCR029)*
Mouse Spinal Cord Neural Stem Cells *(Cat. No. SCR031)*
Adult Rat Hippocampal Neural Stem Cells *(Cat. No. SCR022)*
Rat Hippocampal Neurons *(Cat. No. SCR010)*
Rat Hippocampal Astrocytes *(Cat. No. SCR008)* — Ready-to-use, primary cells for a variety of research applications, including drug development, studies of neurotoxicity, neurogenesis, electrophysiology, neurotransmitter and receptor functions and CNS diseases and disorders. Each lot of primary cells has been validated for high expression of the appropriate markers, and with respect to adult rat NSC, for their self-renewal and multi-lineage differentiation capacities.

Fully Defined Progenitor Cell Targeted (PCT) Culture Media

provide improved isolation and proliferation, but delay terminal differentiation

Jim Johnson
CELLnTEC advanced cell systems AG
July, 2006

Millipore is a worldwide distributor of CELLnTEC products

Abstract

CELLnTEC's fully defined Progenitor Cell Targeted (PCT) media have been shown to provide excellent isolation and extended proliferation without the need for non-defined components, plate coatings, feeder cells or additional supplements. These benefits result from specific medium formulations that actively select and retain progenitor cells in an undifferentiated state. However, this effective progenitor cell retention is known to delay the onset of terminal differentiation. In order to allow differentiation studies on progenitor cells established in PCT medium, CELLnTEC has developed matching non-PCT, defined media that are recommended for all work in which cells are induced to differentiate.

PCT culture media

Fully defined, Progenitor Cell Targeted media that specifically target and retain progenitor cells by mimicking the environment of the stem cell niche. Ideal for high efficiency isolation and extended proliferation.

Non-PCT culture media

Defined culture media with optimized nutrient profiles. Should be used for differentiation of cells established using a PCT medium.

BPE-containing culture media

Non-defined media that may offer improved isolation in difficult situations, such as poor tissue quality or low seeding density.

Characteristics of cells isolated using PCT media

As more researchers report on their use of CELLnTEC's PCT media, it has become evident that these formulations are very efficient at isolating and retaining the progenitor cells from a number of epithelial tissues. By mimicking the stem cell niche, PCT media enable up to a 10-fold enrichment of progenitor cells during the first 3 passages of culture (see Figure 1). The progenitor cell retention provided by PCT media enables them to match or even outperform both defined and non-defined alternatives. Additionally, the PCT media support cultures with an extended lifespan as compared to other media formulations. This is all accomplished without the need for serum, Bovine Pituitary Extract (BPE), feeder cells or plate coatings.

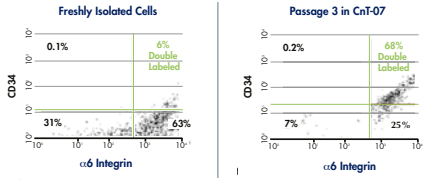

Figure 1
FACS analysis showing a 10-fold enrichment of double-labeled progenitor cells. Culture was grown in CnT-07 PCT Epidermal Keratinocyte Medium from isolation up to Passage 3.

The benefits of defined PCT media:

- Equal or better isolation and proliferation than non-defined alternatives
- No need for serum, BPE, feeder cells or plate coatings
- Extended culture lifespan due to progenitor cell retention

Non-PCT Media for differentiation experiments

The PCT formulation of CELLnTEC's media provides increased isolation efficiency by retaining viable progenitor cells during isolation. Additionally, the progenitor cell phenotype is retained in subsequent passages allowing the growth of an enriched population of undifferentiated cells.

While this feature of the CELLnTEC PCT media benefits the establishment and amplification of the cells for study, the PCT formulation is known to delay the onset of terminal differentiation (see Figure 2). Thus it is recommended that researchers wanting to perform differentiation studies switch their cells to the recommended non-PCT medium (see Table 1) before induction of differentiation. The cells can be immediately changed to the non-PCT formulation without the need to wean them off the PCT supplemented medium. Once in the non-PCT medium, researchers can use their standard procedure to induce differentiation.

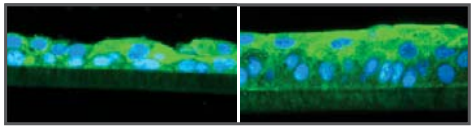

Figure 2
Rat urothelial cells grown on Millipore Millicell/PCF inserts and isolated in PCT medium, then grown in 3D culture and stained for Uroplakin 3 (green) at day 14. Left: cells induced to differentiate in PCT medium (CnT-16), showing 1-2 cell layers. Right: cells induced to differentiate in non-PCT medium (CnT-36) showing 3-4 cell layers.

Seeding density considerations for optimal isolation and proliferation

CELLnTEC's PCT media aim to equal or outperform older formulations that rely on non-defined supplements such as Fetal Bovine Serum (FBS) or feeder cells. To verify that our media meet this goal, we complete a wide range of comparisons with widely used commercial and published formulations.

As part of this comparison work, we standardize a number of variables to ensure that comparisons remain equal. Seeding density is an important variable, for which there is an optimal range in which isolation performance is best. Accordingly, for all CELLnTEC media we recommend particular seeding densities be used following both isolation and passaging. To ensure that you get the most from your PCT medium, please observe the recommended densities, especially during your initial tests of the medium.

BPE-containing media

Bovine Pituitary Extract is a common non-defined supplement used in culture media. For some tissue, its use can be beneficial during isolation and early passage culture in certain situations such as poor tissue quality or low seeding densities. In these situations the benefits of BPE can outweigh its detrimental aspects, namely its unknown and variable composition.

For tissues where CELLnTEC has observed that BPE can improve isolation and early passage growth (particularly in the sub-optimal situations described above), we offer a BPE-containing medium (see Table 1). As with the PCT media, there is a recommended seeding density. In general we suggest that in case of isolation and cultivation difficulties, the BPE-containing media should also be evaluated in parallel with the PCT formulations if a non-defined medium is an option.

Millipore does not distribute CELLnTEC products in Japan.

Table 1:
CELLnTEC Culture Media (Cat. No.)

Tissue	Species	PCT (Isolation & Maintenance)	Non-PCT (Differentiation)	Contains BPE
Epidermis	**H-M**	CnT-07	CnT-02	
	R	CnT-03	CnT-33	
	D	-	CnT-09*	
Prostate	**H**	CnT-12	-	CnT-52
	R	CnT-11	-	
Airway	**H**	CnT-17	CnT-23	
	R	CnT-14	CnT-34	
	R	CnT-15	CnT-35	
Mammary	**H**	CnT-27	CnT-22	CnT-54
Cornea	**H**	CnT-20	CnT-30	CnT-50
Oral	**H**	CnT-24	CnT-32	
Vaginal	**H**	CnT-19	CnT-39	CnT-55
Bladder	**H**	CnT-18	CnT-21	
	R	CnT-16	CnT-36	
Dermis	**H/M/R**		CnT-05*	

H = Human; M = Mouse; R = Rat; D = Dog
*Note: Contains serum, and can be used for isolation and differentiation

Germline Transmission of Embryonic Stem Cells

Derived in Serum- and Feeder-Free Conditions

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Initial attempts to derive embryonic stem (ES) cells in serum- and feeder-free conditions were conducted using embryos from the most permissive strain of mouse (the 129 strain). Following injection of the first such line into host blastocysts, twelve mice were born, all of which were male chimeras, and eleven of which transmitted the ES cell genome through the germline[1]. This line maintained germline-competence until at least passage eighteen. Additional ES cell lines have subsequently been derived from 129 embryos with very high frequency using a new optimized serum- and feeder-free protocol involving isolated epiblasts (Battle-Morera, Smith and Nichols, in preparation). Two of these have been injected into host blastocysts and have produced chimeras. One of these has proved to be 100% germline. A further two lines were aggregated with host morulae and gave rise to very good chimeras that are currently being tested for germline competence. ES cells have previously been derived from embryos of the C57BL/6 strain of mice, but with much reduced frequency compared to those of the 129 strain. We have been able to derive ES cells from C57BL/6 embryos using our serum- and feeder-free derivation protocol[2]. Several of these lines have been injected into host blastocysts, and contributed extensively to the coat color. Selection of a suitable strain and stage of host embryo has been reported to present some difficulties for creating germline chimeras using C57BL/6 ES cells[3,4]. So far, we have achieved 100% germline transmission from a chimera created by sub-zona injection into a BALB/c morula of C57BL/6 ES cells derived in serum- and feeder-free culture conditions (see Figure 1). Other C57BL/6 lines derived under serum- and feeder-free conditions are still under investigation.

Figure 1
Chimeric male mouse created by injection of C57BL/6 ES cells (black) into BALB/c host morula (albino) with germline pups exhibiting black coat color characteristic of the C57BL/6 strain of mouse.

Continued on page 11

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The Value of Protocols:

The importance of following protocols when culturing mouse ES cells in serum-free and feeder-free conditions

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Mouse embryonic stem (ES) cells that were routinely cultured in medium supplemented with serum and on a feeder layer can now be grown without these cumbersome elements. The development of the serum-free ESGRO Complete™ system allows culturing of mouse ES cells in a totally defined medium on gelatin-coated plates, without the need for serum or feeders in your culture. As these conditions will initially be 'unusual' for cell lines that are accustomed to growing in the presence of serum and feeders, it is essential that protocols are carefully followed, as these have been specifically developed and optimized to achieve the best results.

The serum-free ESGRO Complete Clonal Grade Medium supports the self-renewal of mouse ES cells by providing the basic nutrients normally provided by serum and feeders in the usual culturing method. These include hormones, growth factors and vitamins, as well as mLIF and BMP4. However, there are many other undefined components present in serum, including different proteins and molecules, which create a protective environment for the cells. When mouse ES cells are grown in serum-free conditions, they become more sensitive and are therefore more vulnerable to exogenous proteases added to the culture. These sensitive conditions make it essential to follow the recommendations described in the protocols. Indeed, as specified in the data sheet for the ESGRO Complete Switch Kit, the intended use of the kit is for the "adaptation" of mouse ES cells to their new environment without feeders and serum. As this is an adaptive process, it is crucial to proceed step by step as described to ensure the best response of the cells to these new conditions.

Cells will also notably be more sensitive to the density they are grown at when cultured serum-free, and shouldn't be allowed to become over-confluent, as this will lead to differentiation. It is also important to change the medium regularly (we recommend every 48 hours), to bring fresh nutrients to the cells.

When passaging mouse ES cells, it is also fundamental to use ESGRO Complete Accutase™ from the first passage. Indeed the usual use of Trypsin is inappropriate to passage the cells as it would result in dramatically damaging them. The Accutase is a cell detachment solution of proteolytic and collagenolytic enzymes that has been specially qualified for use for the detachment of mouse ES cells cultured in serum-free conditions with ESGRO Complete Clonal Grade Medium. As shown in Figure 1, mouse ES cells can easily and efficiently be passaged in serum-free conditions using Accutase whereas the use of Trypsin affects the attachment properties of the cells, which form clusters of floating cells (Figure 2).

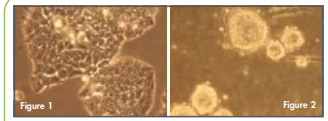

Figure 1
Use of ESGRO Complete Accutase in serum-free conditions preserves the attachment properties of the mouse ES cells.

Figure 2
Use of Trypsin in serum-free conditions induces the formation of floating cells clusters.

The ESGRO Complete Enzyme-Free Cell Dissociation Solution is also a special formulation that has been developed for cells that are too sensitive for enzymatic treatment and allows for gentle dislodgment of adherent cells from a substrate, while preserving the structural and functional integrity of cell surface proteins.

In conclusion, it is vital to follow the prescribed protocols when changing from a serum and/or feeder containing culturing system to a serum-free, feeder-free system. The general protective properties of undefined components within serum are not available to mouse ES cells cultured in a serum-free system, and the protocols for adaptation and growth have been appropriately modified during the product development of ESGRO Complete. Take comfort in knowing that these protocols have been optimized for use with ESGRO Complete products and will guarantee you the best results for your mouse ES cell cultures.

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