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ENStem™-A Human Neural Progenitors provide a user friendly source of human embryonic stem cell derived adherent neural cells

Dave Machacek, Kate Hodges, Carla Sturkie and Steve Stice
Aruna Biomedical and University of Georgia

A renewable source of physiologically relevant human primary neural cells would assist basic researchers and drug discovery by providing material for varied experimental paradigms and assays. Importantly, if these cells could grow in an adherent monolayer, then large numbers of neural cells derived from a single source could be used for characterizing aspects of neuroprotection, neurite outgrowth, synaptogenesis, growth cone collapse, the viability of neural cells (cell health, genotoxicity, cell cycle regulation, DNA repair), pathways (phenotypic differentiation, intracellular signaling) and pharmacology (dose response relationships, receptor desensitization).

Background

Most neurons are produced during embryonic development by neural stem cells or neural progenitor cells. Aruna Biomedical Inc has licensed from the University of Georgia a method to derive neural progenitors from NIH approved human embryonic stem cells (hESCs) (Shin *et al.* 2006). These Neural Progenitor Cells have been exclusively licensed by Millipore. The novelty of this product is that researchers can use a kit containing cryopreserved hESC derived neural progenitors along with required media to propagate and differentiate primary cultures of neural cells. The cells and kit are available in a convenient ready to use format that will be marketed by Millipore under the ENStem-A brand name.

The innovative adherent ENStem-A cell culture system enhances the means to reliably differentiate these cells for quantitative studies of neurons and glial cells, and will help neurobiologists understand the function of these cells. Many existing sources of human neural progenitors are propagated as suspensions of neurospheres that may not be easily adapted for some studies. ENStem-A cells grown in a monolayer are amenable to studies involving electrophysiology, axon guidance and other quantitative analysis mentioned above or for use in high-throughput, and high-content screens for therapeutic compounds or for tests of neurotoxicity.

Uniformity of ENStem-A cells

The adherent ENStem-A cells were derived from WA09 (H9) hESC and these lines are greater than 90 % positive for neural stem cell markers and less than 5 % of the cells remained hESC-marker positive. Both flow cytometry (Table 1) and microscopy were used to quantify these levels (Figure 1). After differentiation to neural progenitors, these cells had a normal compliment of chromosomes (euploidy) following ten passages (Figure 1). Although adult, fetal and hESC-derived neurospheres can be differentiated into primary cultures of neurons and

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Inside

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glial cells the 3-dimensional aspects of the spheres complicate access to many experimental treatments. The ENStem-A Neural Progenitor Expansion kit (Cat. No. SCR055) contains cells and proliferation media to propagate the cells. This is a defined media containing LIF and FGF2, however, BMP4 inhibitor factors (Noggin) or conditioned media are not required. These cells have a population doubling time of approximately 24 hours when grown at high density (Figure 2). The uniformly adherent cultures of ENStem-A cells are likely to respond reproducibly to a wide variety of experimental conditions.

Differentiation of ENStem-A cells

The ENStem-A cells are useful for researchers who may want to or have already developed their own differentiation protocols (*i.e.* induction of dopaminergic cells using SHH and FGF8 or motoneurons using SHH and RA). ENStem-A cells can produce multiple neuronal phenotypes (Figure 3) and glial phenotypes (Figure 4) (also see Shin, *et al.*, 2006) that would be relevant to multiple disease models of the nervous system (Table 2). Use of growth factors and/or genetic markers will no doubt be useful for enrichment of neural cell phenotypes and ENStem-A cells are amenable to gene insertions. We have previously published that SHH and RA can be used to induce motoneuron differentiation in our ENStem-A cells[2]. Finally, we have maintained differentiated cultures for up to 4 months and demonstrated that these cultures begin to form a plexus of cells, demonstrating that they behave similar to primary cell types. We have used immunofluorescence analysis to quantify recovery of neurons after using ENStem-A differentiation medium. ENStem-A cells were plated in 4-well chamber slides coated with poly-ornithine and laminin for 7 and 14 days in differentiation medium without bFGF and LIF. LIF was removed to attempt to preferentially differentiate the cells to a neural phenotype. Projected images of 5 random fields of ~15 cells

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Table 1: % positive ENStem-A cells using immunofluorescence or flow cytometry

	Using immunofluorescence (>1000 cells counted)			Using flow cytometry (>10,000 cells counted)	
Cell Line	**% nestin^{+ve}**	**% SOX2^{+ve}**	**% OCT4^{+ve}**	**% nestin^{+ve}**	**% OCT4^{+ve}**
ENStem-A	98 ± 1	94 ± 2	2 ± 0.51	99.4	0.34
Renal				.5	NA
HUVEC				NA	0.01

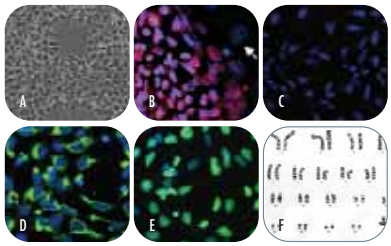

Figure 1: *ENStem-A cells demonstrate the expected imunoreactivity and chromosome number. (A) Adherent ENStem-A cells at 95 % confluency. (B) WA09 human ESC are Oct-4 positive, while mouse feeder cells are negative (arrows). (C) ENStem-A cell line are Oct-4- negative. (D) ENStem-A cell line labeled for Nestin immunoreactivity. (E) ENStem-A cell line labeled for Sox2 immunoreactivity. (F) Karyotype from ENStem-A cell line.*

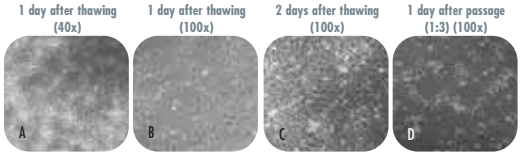

Figure 2: *Phase contrast images at 40x and 100x of ENStem-A Neural Progenitors after thawing. In this case the cells were ready for passage 2 days after thawing (90-100 % confluency, see (C)). Cells should not be left for a long time after confluency is reached, or spontaneous differentiation may occur. Cells will do best if not plated more sparsely than a 1:2 dilution of a confluent plate.*

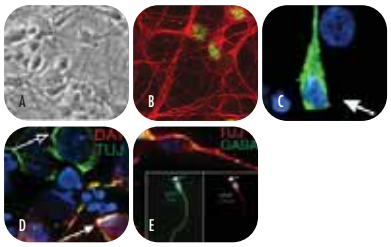

Figure 3: *Neural phenotypes derived from ENStem-A cell lines. (A) Phase contrast image of differentiated culture. (B) Network including post-mitotic motoneurons (HB9). (C) Cholingergic neuron. (D) βIII Tubulin (Tuj-1) positive cells that are DAT-positive (dopamine transporter; closed arrow) and DAT-negative (open arrow). (E) Gabaergic neurons, inset illustrates GABA in axon but not the dendrites (arrow).*

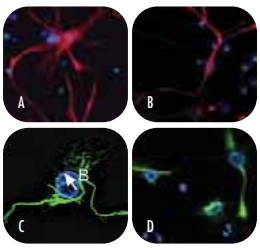

Figure 4: *Glial cells. (A and B) GFAP-positive cells. (A) represents the morphological characteristics of astrocytes where as (B) represents the structure of radial glia. (C and D) mature oligodendrocytes are defined by expressing myelin basic protein.*

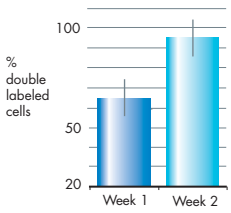

Figure 5: *βIII Tubulin (Tuj-1) positive (neural) cells after 1 week and 2 weeks without proliferative factors (% double labled cells = Tuj-1 and DAPI positive).*

from each replicate were scored by inspection for the frequency of cells showing immunostaining of the indicated neuronal marker βIII Tubulin (Tuj-1). The average percentage of Tuj-positive cells was 65 % after 7 days and 96 % after 14 days (Figure 5). Further refinements should lead to methods that generate an enriched population of neurons for research. However, these enriched neural populations do have a high cell death rate and this can be reduced by adding growth factors such as LIF, GDNF and BDNF to the differentiation media.

Conclusion

ENStem-A cell monolayer cultures are uniform, robust, easily propagated and can differentiate into many neural lineages. Millipore, in conjunction with Aruna Biomedical Inc., offers neurobiologists a normal, non-transformed hESC derived source to study the diverse function, growth and differentiation of human neuronal cells.

References

1. Shin *et al.* (2005). *Stem Cells* **24** 125-38.
2. Shin *et al.* (2006). *Stem Cells Dev* **14** 66-9.

Marker for cellular phenotype	Pathology related to phenotype
ChAT (Choline Acetyltransferase) – synthetic enzyme	Alzheimer's disease
HB9 - transcription factor marker in motoneurons (cholinergic)	ALS,spinal injury, SMA
Dopamine transporter (DAT)	Parkinson's disease, schizophrenia, depression, addictive disorders
GABA – transmitter	Pain processing, conscious state (anesthesiology), alcohol research

Table 2: *Neural phenotypes derived from ENStem-A cell lines and their relevance to disease research.*

Low Bovine Pituitary Extract (BPE) supplementation of Progenitor Cell Targeted (PCT) media further enhances colony formation, growth and *in vitro* longevity of epithelial cells

Jim Johnson
CELLnTEC advanced cell systems AG

Abstract

Progenitor Cell Targeted (PCT) culture media by CELLnTEC provide more cell colonies at isolation and improved growth of epithelial cells by specifically mimicking the environment of their stem cell niche, and thereby retaining progenitor cells in their undifferentiated phenotype.

Previous experiments have shown that fully defined PCT media provide significantly better colony formation and cell growth than alternative defined media, and can even match the performance of competitor's traditional non-defined (high Bovine Pituitary Extract (BPE)) media.

New results have now shown that the addition of low levels of BPE to defined PCT media improves colony formation, growth and *in vitro* longevity of a variety of epithelial cells, thereby creating the most effective epithelial media currently available.

These recent results also demonstrated that the addition of traditional (high) levels of BPE brought no further improvements in cell performance, confirming the already highly effective nature of defined PCT media.

As a result of this work, several new PCT Low-BPE media have now been developed which outperform competitor's defined and non-defined media while containing 2–8 times less BPE. These PCT Low-BPE media provide the absolute best *in vitro* performance, while minimizing any potential variability due to the use of a non-defined component.

Progenitor Cell Targeted (PCT) culture media

The enhanced progenitor cell isolation of CELLnTEC's PCT media results from our understanding of the microenvironment of the epithelial adult stem cell niche. PCT media increase progenitor cell isolation efficiency by maintaining more of these valuable cells in their undifferentiated state (Figure 1), and then continue to select and enrich these cells over several passages (Figure 2).

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PCT culture media
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- Airway
- Cornea
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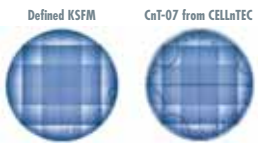

Figure 1: *More colony forming progenitor cells. CnT-07, a fully defined PCT medium, delivers 4x more human keratinocyte colonies at isolation than a competitor.*

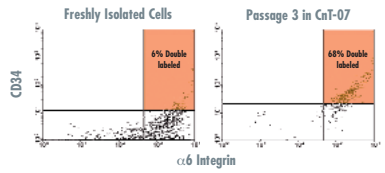

Figure 2: *Enrichment of stem/progenitor cells. FACS analysis showing the percentage of double labelled (CD34/α6-integrin) keratinocyte progenitor/stem cells increases from 6 % at isolation up to 68 % after 3 passages in CnT-07, a fully defined PCT medium.*

In addition to improved colony formation and growth rate, morphology is also improved and more uniform in PCT media than major common competitors (Figure 3).

Figure 3. *CnT-07, a defined PCT medium from CELLnTEC, provides improved growth rate and morphology relative to EMEM (supplemented with serum and fibroblast conditioned medium) or KGM® (a high-BPE keratinocyte medium from Cambrex). Mouse keratinocytes, passage 1.*

Low Bovine Pituitary Extract (BPE) can improve the performance of PCT media

The non-defined supplement BPE has been used successfully to improve cell growth in serum free media for many years. Traditionally, high concentrations (often up to 52 µg/mL) have been required to obtain an effective concentration of growth factors, thereby exacerbating the potential variability associated with the use of non-defined components.

BPE Concentration of Epithelial Culture Media:

CELLnTEC
Low-BPE CnT-57:
6 µg/mL

Cambrex/Invitrogen
Various media:
25-52 µg/mL

Recent tests have revealed that the addition of BPE to a defined PCT medium can also bring improvements. However, interestingly, it was found that the addition of traditional (high) concentrations of BPE either brought no additional benefits or was detrimental to cell growth, thereby confirming the already high effectiveness of the defined PCT formulations.

Epidermal Keratinocytes

The new PCT Low-BPE medium CnT-57 (6 µg/mL BPE) was found to offer improvements in colony formation, growth rate, and longevity. When compared with CnT-07 and non-defined KSFM (Invitrogen), CnT-57 was found to deliver 2–3 times more colonies from isolation up to passage 5 (seeding density 2000 cells per flask, Figures 4 and 5).

As shown in Figure 5, CnT-57 (PCT Low-BPE medium) provided up to 3 times more colonies than non-defined KSFM during the first 5 passages of culture, despite having approximately 5 times less BPE. CnT-07 (defined PCT medium) provided similar initial colony forming efficiency to non-defined KSFM during the first 1–2 passages, but exhibited improved CFE at passage 3 and above.

No plate coatings, feeder cells or other additives required

CELLnTEC's advanced media development techniques mean that time consuming and expensive plate coatings, feeder layers or conditioned media are no longer required in addition to the PCT media themselves.

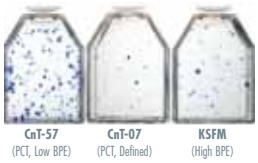

Figure 4: *CnT-57 generates significantly more human keratinocyte colonies in primary culture than non-defined KSFM (Invitrogen). Cells in T25 flasks at passage zero, initial seeding density of 2000 cells, fixed and stained at day 12.*

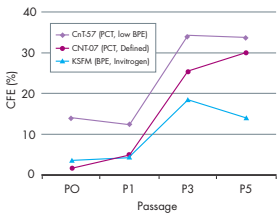

Figure 5: *Colony forming efficiency (% of cells seeded) of human keratinocytes isolated and maintained in different culture media. CnT-57 (PCT Low-BPE) delivers 2-3x more colonies than non-defined KSFM (Invitrogen).*

PCT media therefore offer peak performance and convenience, at optimal cost-effectiveness.

Primary human keratinocyte progenitors now available!

Cells isolated directly into PCT medium experience maximum progenitor cell retention, which generates peak isolation and growth performance.

To combine all the benefits of PCT media with the convenience and reliability of established primary cultures, frozen

Continued on pg 5

human foreskin keratinocyte progenitors (HPEK) isolated in PCT media are now available from pooled or single donors.

The *in vitro* longevity of cells established and maintained in PCT media is enhanced as a result of increased retention of undifferentiated progenitor cells. PCT media have been found to retain human keratinocyte progenitors on average for more than 35 population doublings before cells enter senescence, significantly more than the 20–25 doublings common in traditional media (Figure 6).

Summary

CELLnTEC's progenitor cell targeted media specifically target and retain progenitor cells, and, as a result, offer increased colony forming efficiency, growth rate, and longevity. PCT media are now available as either fully defined or Low-BPE formulations, and are therefore suitable for all potential needs and applications. BPE levels in PCT media are 2–8 times lower than those of the competition, yet PCT media offer even better performance due to the synergy of the BPE with the existing PCT formulation. In this way, peak performance can be obtained while minimizing any potential variability due to the use of an undefined component.

PCT media require no additional plate coatings, feeder cells or additional additives, and are also available as part of a kit containing primary human keratinocyte progenitors. In addition, PCT media are available for a wide range of other epithelia (including cornea, airway, prostate and mammary plus others).

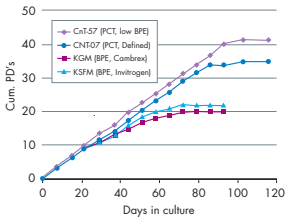

Figure 6: *Cumulative population doublings of human foreskin keratinocytes isolated and maintained in 4 different media.*

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

Tissue	Species	PCT (Defined)	PCT (Low-BPE)	Non-PCT (Differentiation)
Epidermis	**H-M**	CnT-07	CnT-57	CnT-02
	R	CnT-03	-	CnT-33
	D	-	-	CnT-09*
Prostate	**H**	CnT-12	CnT-52	CnT-12**
	R	CnT-11	-	CnT-11**
Airway	**H**	CnT-17	-	CnT-23
	R	CnT-14	-	CnT-34
	R	CnT-15	-	CnT-35
Mammary	**H**	CnT-27	CnT-54	CnT-22
Cornea	**H**	CnT-20	CnT-50	CnT-30
Oral	**H**	CnT-24	-	CnT-32
Vaginal	**H**	CnT-19	CnT-55	CnT-39
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
** For isolation, growth and differentiation

Description	Species	Medium	Quantity*	Cat. No.
Epidermal keratinocyte progenitors, pooled donors (>0.5 x 10^6 cells)	Human	CnT-57	Kit	HPEKp.05
Epidermal keratinocyte progenitors, pooled donors (>1.5 x 10^6 cells)	Human	CnT-57	Kit	HPEKp.15
Epidermal keratinocyte progenitors, single donor (>0.5 x 10^6 cells)	Human	CnT-57	Kit	HPEKs.05
Epidermal keratinocyte progenitors, single donor (>1.5 x 10^6 cells)	Human	CnT-57	Kit	HPEKs.15

*Kits include cells plus 1 bottle of CnT-57, protocols, and guaranteed longevity.

Accumax™ Dissociation Reagent
for the Serial Passaging of Human Embryonic Stem Cells

Hanh Huang and Matthew Singer, Millipore

Abstract

Currently, there are two widely accepted methods for dissociating human embryonic stem cells (hESCs) for passaging: (1) manual passaging, which is the gentlest way to dissociate hESCs, but is also very tedious and time-consuming, and (2) enzymatic passaging, which is much easier than manual passaging and can be applied to higher throughput processing of hESCs, but is associated with concerns of cell health and genomic stability[1]. For enzymatic passaging, many researchers use a type IV collagenase derived from the bacterium *Clostridium histolyticum*. Here we show that the product, Accumax reagent (Cat. No. SCR006) is suitable for passaging human embryonic stem cells in cultures using feeder layers. After cells went through fifteen consecutive serial passages using Accumax reagent, they were shown to maintain pluripotency by morphology and expression of appropriate markers; they also maintained a normal karyotype. In addition, it was noted that there were several advantages to using Accumax reagent versus the type IV collagenase derived from *Clostridium histolyticum*.

Methods

General hESC culture

H9 human ES cells were maintained on CF1 mouse embryonic fibroblasts (Cat. No. PMEF-CF) seeded at a density of 40,000 fibroblasts/cm^2 on tissue culture plates coated with growth factor-reduced Matrigel® EHS matrix (BD Biosciences). hESCs were maintained in a medium containing 20 ng/mL basic FGF (Cat No. GF003); media was replaced every 1–2 days. Cultures were passaged every 6 or 7 days regardless of the dissociation method used (see below).

Dissociating hESCs for passaging with Accumax reagent

Cells growing in adherent culture as described above were rinsed once with Dulbecco's PBS (Cat. No. BSS-1006), then overlaid with Accumax reagent at a volume of approximately 0.05-0.1 mL/cm^2. Cultures were incubated in Accumax reagent for exactly 5 minutes at room temperature, then cells were quickly dislodged from the culture surface (using a serological pipet) and the suspension removed to a tube containing culture media at 5 times the volume of Accumax reagent used. Cells were briefly agitated by gentle pipetting, and were then centrifuged at 75 x g for 5 minutes. After the supernatant was aspirated, cells were gently resuspended in culture media and split at a ratio of 1:3.

Dissociating hESCs for passaging with collagenase

As a control for the use of Accumax reagent, separate cultures of hESCs were maintained under identical conditions, except that they were dissociated for passaging using the type IV collagenase derived from *Clostridium histolyticum*. Cultures were rinsed once with Dulbecco's PBS, then overlaid with approximately 0.1 mL/cm^2 of a 1 mg/mL collagenase solution. Cultures were incubated for 10 minutes at 37 °C, or until the edges of colonies began to separate from the culture surface. Cells were then scraped off of the culture surface and transferred to a tube containing culture media at 3 times the volume of collagenase used. Cells were briefly agitated by gentle pipetting, and were then centrifuged at 75 x g for 5 minutes. After the supernatant was aspirated, cells were gently resuspended in culture media and split at a ratio of 1:3.

Results

Parallel cultures of human ES cells were passaged using either Accumax reagent or type IV collagenase from *Clostridium histolyticum*, but were otherwise maintained under identical conditions. Different effects upon the cells were observed between the two enzymatic treatments. After 5 minutes of treatment with Accumax reagent, clusters of cells tended to show some fragmentation into individual cells at the edges of colonies, but otherwise clusters remained largely intact; in contrast, after treatment with collagenase for 10 minutes, clusters showed separation of the edge from the culture surface, but few individual cells were observed (not shown). The colonies resulting from Accumax reagent passaging were smaller and more numerous than those resulting from passaging with collagenase, indicating that Accumax reagent dissociates human ES cell clusters to smaller fragments than does collagenase. Finally, clusters of cells passaged by Accumax reagent treatment

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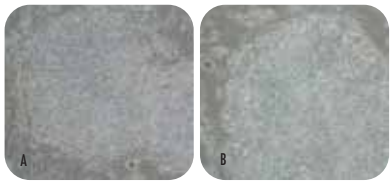

Figure 1: *Morphologies of H9 human ES cells after 15 passages in either Accumax (A) or type IV collagenase (B) are highly similar. H9 cells were grown on a CF1 mouse embryonic fibroblast feeder layer.*

tended not to be as compact as those passaged by collagenase during the first few days of culture. However, by day 6 or 7 of culture, clusters passaged by the two treatments could not be distinguished from one another by morphology (Figure 1).

After 15 consecutive passages by either one or the other treatment, cultures were analyzed for pluripotency and karyotypic stability. Pluripotency was examined by use of an alkaline phosphatase activity staining kit (Cat. No. SCR004), as shown in Figure 2, and by indirect immunofluorescent labeling with antibodies to OCT4 and SSEA-4 (Cat. Nos. MAB4401 and MAB4304), as shown in Figure 3; data is shown for cells treated with Accumax reagent, but similar results were observed for cultures passaged with type IV collagenase. As shown, cells passaged 15 times in Accumax reagent retained expression of pluripotent markers. In addition, a normal karyotype was maintained (data not shown).

Discussion

This work demonstrates the utility of Accumax reagent for the dissociation and passaging of human ES cells. Like collagenase, Accumax reagent saves time and effort over manual passaging. Furthermore, cells passaged multiple times with Accumax reagent maintain expression of pluripotency markers as well as their normal karyotype.

There are some differences between Accumax reagent and collagenase that should be noted. In terms of ease of use of product, Accumax reagent is supplied as a sterile, ready-to-use 1X liquid, while collagenase is supplied as a powder that must be weighed out, put into solution and then filter-sterilized. Also, once thawed, Accumax reagent can be stored at 4 °C for up to two months (it can also be aliquotted and refrozen, if desired), whereas reconstituted collagenase must be immediately frozen, and subsequently thawed aliquots are generally discarded immediately after use. The differences between the two enzyme treatments in their effect upon human ES cells is also of note. By creating a greater number of smaller colonies (*i.e.*, in comparison to collagenase), Accumax reagent is more useful for the expansion of cultures, including higher-throughput applications.

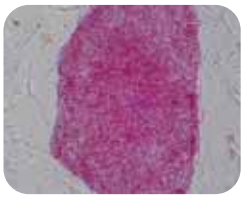

Figure 2: *Alkaline Phosphatase expression in H9 human ES cells after 15 passages using Accumax reagent to dissociate the cells.*

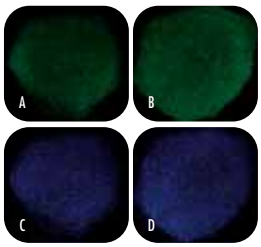

Figure 3: *Expression of OCT4 (A) and SSEA-4 (B) pluripotency markers in H9 human ES cells after 15 passages using Accumax reagent to dissociate the cells. Corresponding DAPI images are shown in (C) and (D).*

References

1. Hanson, C., Caisander, G. Human embryonic stem cells and chromosome stability. *APMIS* 2005;**113**: 751–5.

Description	Quantity	Cat. No.
Accumax Reagent	100 mL	SCR006
Primary Mouse Embryonic Fibroblasts, Strain CF1, mytomycin C treated, passage 3	5 vials	PMEF-CF
DMEM/F12, with L-Glutamine	500 mL	DF-042-B
Basic FGF, Recombinant Human	50 µg	GF003
Alkaline Phosphatase Detection Kit	1 kit	SCR004
Oct-4 Monoclonal	100 µg	MAB4401
SSEA-4 Monoclonal	100 µg	MAB4304

Integrin Profiling of Stem Cells and Differentiated Progeny

Matthew Hsu, Ph.D.
Millipore Corporation, Bioscience Division

Abstract

To identify integrin subunits that are specifically and/or differentially expressed during proliferation and differentiation, adult rat neural stem cells and their differentiated progeny, neurons and astrocytes, were profiled using Millipore's arrayed panel of α and β integrin antibodies. Relative quantification of the cells captured by specific immobilized integrin antibodies revealed that αvβ5 integrin is highly expressed in both adult rat neural stem cells and differentiated astrocytes that are cultured on laminin. Additionally, αvβ5 integrin has been implicated in the VEGF-induced angiogenesis pathway, providing a possible mechanistic link between angiogenesis and neurogenesis in the central nervous system. Further investigations into the role of integrins in neural stem cell biology are merited, and the differential expression of integrins on different types of stem cells will shed light on the selection of appropriate ECM for the cell culture plate coating and cell adhesion studies in a more efficient manner.

Introduction

Cell adhesion plays a major role in cellular communication and regulation, and is of fundamental importance in the development and maintenance of tissues. A family of cell surface glycoproteins, the integrins, mediate cell adhesion to proteins of the extracellular matrix (ECM). Integrins act as receptors for ECM proteins (collagen, laminin, vitronectin, or fibronectin) or for membrane-bound counter-receptors on other cells. They play an important role in regulating gene expression, cell cycle, programmed cell death, cell migration and proliferation. Integrins exist as heterodimers containing an α and β subunit. Each subunit has a large extracellular domain, a single membrane-spanning region and a short cytoplasmic domain (Figure 1). There are 16 distinct α subunits and 8 or more β subunits, which form more than 20 distinct integrins. The α/β pairings specify the ligand-binding abilities. The extracellular domains contribute to formation of the binding site. Activated integrins often form multimers, which have higher affinities to cell adhesion molecules.

Despite their versatility and importance, little is known about the expression pattern and regulation of integrins in stem cells and their differentiated progeny. Furthermore, the intracellular signaling cascades initiated by integrins in response to the binding of laminin, an ECM molecule commonly used to culture neural stem cells, remain poorly understood.

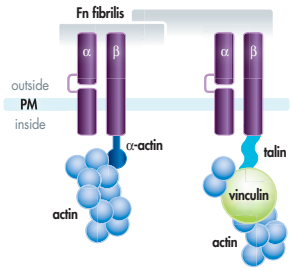

Figure 1: *Structure of α/β integrin dimers. Cytoplasmic domains are key nexus of interaction between the extracellular environment and intracellular structures and signaling cascades. Contribute to cytoskeletal organization, cell motility, signal transduction and modulation of integrin affinity for ligands.*

Historically, individual antibodies have been used to determine the integrin profiles of cells by immunoprecipitation, immunofluorescence, immunoblotting, and flow cytometry. These methods are time-consuming, laborious or require sophisticated equipment. Millipore's α/β integrin-mediated cell adhesion array kit is designed as a cost effective, efficient method for identification of cell surface integrins.

Methods, Results and Discussion

Millipore offers a variety of stem cells and characterization kits as research reagents, including embryonic and adult stem cells from different tissues and species. Neural stem cells (NSC) and their differentiated progenies were used in this integrin profiling study, although they require ECM proteins such as

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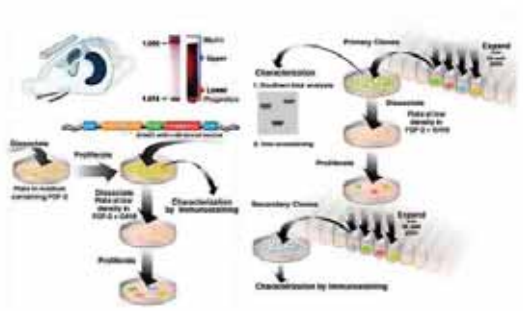

Figure 2: *Schematic for isolating and propagating adult NSCs*

laminin and fibronectin for culture, the mechanism and signaling cascades initiated by the integrins in response to the binding of the ECM remain poorly understood. NSCs are self-renewing cells with the capacity to differentiate into neurons, astrocytes and oligodendrocytes.

In this study, we isolated rat adult NSC (Cat. No. SCR022) from discrete regions of the hippocampus and the subventricular zone (Figure 2). Approximately 20 adult rats were sacrificed and their hippocampi were surgically removed. The tissue was then enzymatically digested to dissociate the cells. The cell mixture was subjected to a Percoll density gradient column and the presumptive NSCs were extracted after high speed ultracentrifugation. The extracted cells were grown in media containing FGF-2 and allowed to proliferate.

Using a series of assays, the NSC population was immunostained for stem cell markers and assessed for their ability to differentiate towards specific cell lineages. The Neural Stem Cell Marker Characterization Kit (Cat. No. SCR019) was used to further characterize and QC the purity of the cells. Each lot of primary adult rat hippocampal neural stem cells was validated for a high level of expression of Nestin and Sox 2 and for their self-renewal and multi-lineage differentiation capacities. The NSCs were negative for differentiated markers (β-Tubulin, Map2ab, GFAP, and O1). Cells also displayed normal karyotypes as assessed by chromosome spread and tested negative for mycoplasma (Figure 3). While pluripotent embryonic stem (ES) cells are capable of producing most tissues of an organism and over 200 cell types, adult stem cells possess a limited potential to differentiate. In most cases, adult stem cells can only give rise to specialized cells of the organ system from which they were derived. The isolated adult rat NSCs were then differentiated into neurons, astrocytes and oligodendrocytes (Figure 4). The differentiated lineages were characterized with selective antibodies (Figure 5).

Since the culture of NSCs requires ECM proteins, various integrins must be expressed on the cell surface. Millipore's α/β integrin-mediated cell

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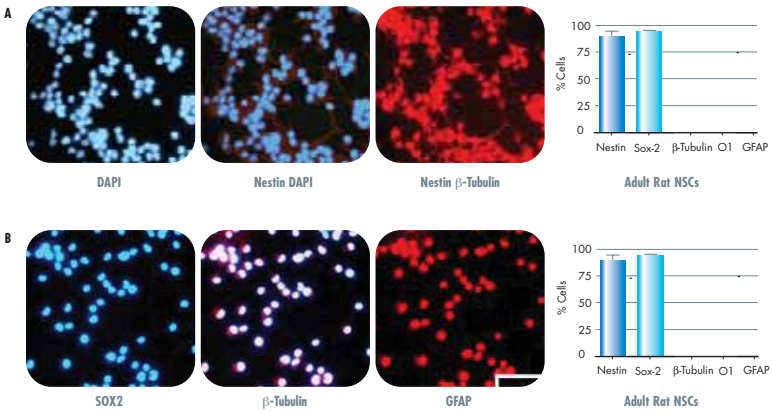

Figure 3: *(A) Adult Rat NSCs are Nestin+, βIII Tubulin-, O1- and GFAP-. (B) Adult Rat NSCs are Sox2+, βIII Tubulin-, O1- and GFAP-.*

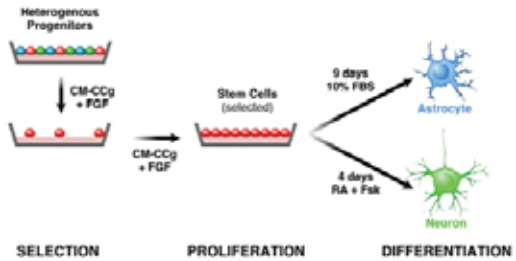

Figure 4: *Differentiation of NSCs into neurons and astrocytes.*

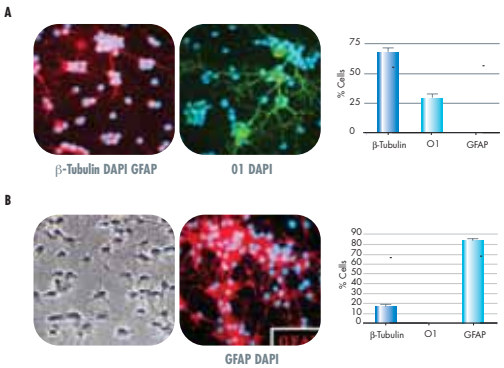

Figure 5: *(A) Differentiated neurons and oligodendrocyte express β-Tubulin and O1. (B) Differentiated astrocytes express GFAP.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	β1	β1	β1	β1	β1	β1	β1	β1	β1	β1	β1	β1
B	β2	β2	β2	β2	β2	β2	β2	β2	β2	β2	β2	β2
C	β3	β3	β3	β3	β3	β3	β3	β3	β3	β3	β3	β3
D	β4	β4	β4	β4	β4	β4	β4	β4	β4	β4	β4	β4
E	β6	β6	β6	β6	β6	β6	β6	β6	β6	β6	β6	β6
F	αVβ5	αVβ5	αVβ5	αVβ5	αVβ5	αVβ5	αVβ5	αVβ5	αVβ5	αVβ5	αVβ5	αVβ5
G	α5β1	α5β1	α5β1	α5β1	α5β1	α5β1	α5β1	α5β1	α5β1	α5β1	α5β1	α5β1
H	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Figure 7: *The layout of Millipore's β integrin arrays.*

adhesion arrays were used in order to determine which types of integrins are expressed or activated on both adult rat NSCs and differentiated cells that are cultured on ECMs.

The layout of Millipore's integrin-mediated cell adhesion arrays is shown in Figure 7. The integrin adhesion arrays are a cost effective, efficient method for the identification of cell surface integrins. The kits format of 12 x 8-well removable strips in a plate frame allows for convenience and flexibility in designing assays. Integrin profiling using Millipore's α/β integrin-mediated cell adhesion arrays can be completed within 1–2 hours, providing a rapid, sensitive, and quantitative assays for the characterization of α and β integrin surface expression.

The profiling results indicate that both adult rat NSCs and differentiated cells express αVβ5 integrins, but not others, which is consistent with immunocytochemical staining result using anti-αVβ5 integrin antibody (Figure 8). αVβ5 integrin is highly expressed in both adult rat neural stem cells and differentiated astrocytes that are cultured on laminin. αVβ5 integrin has been implicated in the VEGF-induced angiogenesis pathway, providing a possible mechanistic link between angiogenesis and neurogenesis in the central nervous system. Integrin profiling of stem cells will provide a new method for stem cell isolation through Fluorescence Activated Cell Sorting (FACS) using lineage specific anti-integrin antibodies and help identify potentially new ECM coating reagents for the culturing of different types of stem cells.

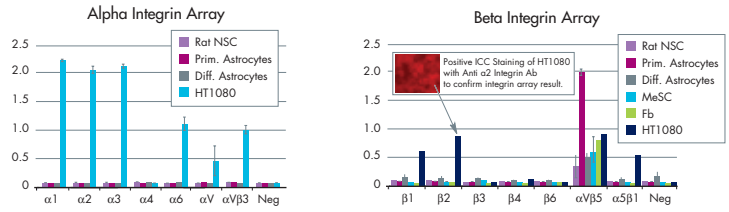

Figure 8: *Both integrin (β and α) array profiling and ICC results suggest that adult rat NSCs and differentiated cells express αVβ5 integrins, but not others.*

Description	Format	Quantity	Cat. No.
α Integrin-mediated Cell Adhesion Array Kit	Colorimetric	96 wells	ECM530
α Integrin-Mediated Cell Adhesion Array Kit	Fluorimetric	96 wells	ECM533
β Integrin-mediated Cell Adhesion Array Kit	Colorimetric	96 wells	ECM531
β Integrin-Mediated Cell Adhesion Array Kit	Fluorimetric	96 wells	ECM534
α/β Integrin-mediated Cell Adhesion Array Combination Kit: 1 each ECM530 / ECM531	Colorimetric	2 x 96 wells	ECM532
α/β Integrin-Mediated Cell Adhesion Array Combination Kit: 1 each ECM533 / ECM534	Fluorimetric	2 x 96 wells	ECM535
ECM Cell Adhesion Array Kit	Colorimetric	96 wells	ECM540
ECM Cell Adhesion Array Kit	Fluorimetric	96 wells	ECM545
Adult Rat Hippocampal Neural Stem Cells		1 x 10^6 cells	SCR022
Neural Stem Cell Marker Characterization Kit		1 kit	SCR019

Rescue, Derive and Expand with RESGRO™ Culture Medium

Novel Mouse ES Cell Culture Medium

Background

RESGRO Culture Medium is a complete ready-to-use cell culture medium that complements traditional mouse Embryonic Stem (ES) cell culture media containing ESGRO® mouse LIF medium supplement.

In contrast to traditional medium, RESGRO Culture Medium is recommended for a number of specialized applications including the rescue of established ES cell lines and the derivation of new ES cell lines.

Rescue of Established ES Cell Lines

RESGRO Culture Medium has the capacity to rescue established ES cell lines that have started drifting and either generate low percentage chimeras or have lost germline transmission capability. Differentiation, when present in ES cell culture but not visible with traditional medium, will become recognizable when using RESGRO Culture Medium. After 2 passages, a clear difference is seen between differentiated and undifferentiated ES cells, at which time undifferentiated cells can be removed by sub-cloning. The application of RESGRO medium to improve the efficiency of mouse ES cell lines that had generated a low percentage of chimeras and germline transmission capability was demonstrated (Table 1). In all cases, the subculturing the cell lines with RESGRO medium resulted in an improved proportion of chimeras born and an increased percentage of chimeric progeny.

Derivation of Mouse ES Cells

Genetically altered mice derived by homologous recombination in 129 ES cell lines may exhibit highly variable phenotypes due to variation in genetic background, indicating that genes unrelated to the targeted genes can markedly affect the observed phenotype. Backcross breeding diminishes overall genetic heterogeneity, but selection for the targeted locus maintains flanking parental genomic DNA, precluding generation of identical congenic experimental and control mice. Elimination of genetic background variability requires derivation of germline competent ES cell lines from inbred mouse strains with specific genetic backgrounds, enabling generation of isogenic gene-targeted and control mice.

The efficiency of ES cell derivation is greatly strain dependent. To date, very few mouse ES cell lines are available from inbred strains other than 129 strains, and those derived have generally been obtained with low success rates. Furthermore, ES cells derived from strains other than 129 are, in general, more difficult to propagate *in vitro*. Especially at high passage number and after genetic manipulation, these cell lines generate chimeras less efficiently and contribute less frequently to the germline.

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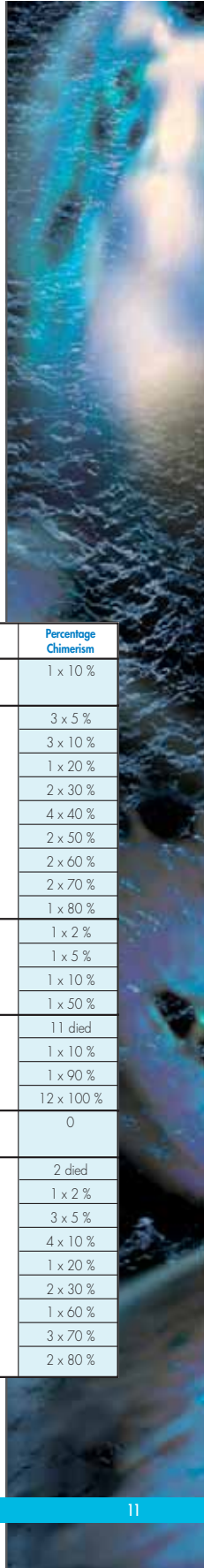
Table 1: Improved efficiency of Murine ES cell lines using RESGRO Culture Medium

ES Cell Line	Medium* & Method Used	Number of embryos transferred	Number of pups born	Number of chimeras born	Percentage Chimerism
R1#19 Knockout clone	Traditional medium Blastocyst injection	56	7	1	1 x 10 %
R1#19 Knockout clone	RESGRO medium Blastocyst injection	64	27	20	3 x 5 %
					3 x 10 %
					1 x 20 %
					2 x 30 %
					4 x 40 %
					2 x 50 %
					2 x 60 %
					2 x 70 %
					1 x 80 %
129SvEv Wild-type clone	Traditional medium Diploid aggregation	40	28	4	1 x 2 %
					1 x 5 %
					1 x 10 %
					1 x 50 %
129SvEv Wild-type clone	RESGRO medium Diploid aggregation	106	25	25	11 died
					1 x 10 %
					1 x 90 %
					12 x 100 %
C57Bl/6 Knockout clone	Traditional medium Blastocyst injection	50	8	0	0
C57Bl/6 Knockout clone	RESGRO medium Blastocyst injection	96	38	19	2 died
					1 x 2 %
					3 x 5 %
					4 x 10 %
					1 x 20 %
					2 x 30 %
					1 x 60 %
					3 x 70 %
					2 x 80 %

*Tradtional medium: basal medium supplemented with ESGRO® mLIF Medium Supplement.



RESGRO medium enables the efficient derivation and maintenance of ES cell lines from several inbred mouse strains, including certain strains that were previously considered to be non-permissive for ES cell derivation (Figures 1 and 2). A recent study demonstrated that RESGRO medium allowed the derivation of ES cell lines from 5 inbred strains other than 129, including FVB, a strain previously considered to be non-permissive for ES cell derivation, and C57Bl/6N, BALB/c, 129/SvEv and DBA/2N mouse strains[1].

ES cell lines were derived from all of 5 inbred mouse strains tested and the efficiency of ES cell line derivation ranged from 38 %–60 % (Table 2). Furthermore, all ES cell lines tested resulted in chimeric offspring, as judged from the contribution to the coat color of the strain from which the ES cell lines were derived (Figure 3). These chimeras had the ability to pass the ES cell genome to the next generation, as judged from offspring with the coat color of the ES cell strain after mating with relevant recipient females.

Table 2: Efficiency of ES Cell Derivation and Germline Competence with RESGRO Culture Medium

Mouse Strain	Blastocysts Cultured (n)	Established ES cell lines (n)	(%)	No. germline competent ES cell lines / no. ES cell lines cultured
C57Bl/6N	35	18	51	10 / 11
FVB/N	20	8	40	6 / 9
BALB/c	34	15	44	7 / 7
129SvEv	10	6	60	4 / 4
DBA-2/N	34	13	38	3 / 3

References

1. Schoonjans L *et al.* (2003). *Stem Cells* **21(1)**:90-7.

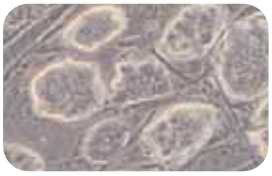


Figure 1: *DBA-2N embryonic stem cells passage 67 on mouse embryonic fibroblast cells.*

Figure 2: *C57Bl/6N ES cells on bare dish.*

Figure 3: *Offspring born after injection of FVB/N #17 passage 18 ES cells into C57Bl/6N blastocysts. Left mouse shows 100 % chimerism, right mouse shows 5 %.*

Description	Quantity	Cat. No.
RESGRO Culture Medium	250 mL	SCM001
RESGRO Culture Medium	500 mL	SCM002

Millipore Products
for Stem Cell Research

New HEScGRO™ - Animal Component-Free Medium for Human ES Cell Culture

No other hES cell media is available as a serum-free, ready-to-use, and complete formulation. HEScGRO medium has been extensively tested on licensed hES cell lines, and has been shown to maintain hES cells in their undifferentiated state for up to 20 passages. The medium is completely animal- free; all components are derived from human or recombinant sources. Feeders are required.

Alkaline Phosphatase stained MEL-1 cells at passage 8 following culture with HEScGRO ACF Medium.

Description	Quantity	Cat. No.
HEScGRO Medium for hES cells	5 x 100 mL	SCM020
HEScGRO Medium for hES cells	100 mL	SCM020-100

New Recombinant Wnt3a for cell culture applications

Wnt3a signaling has been implicated in the control of differentiation of stem cells. Wnt proteins have been shown to regulate cell-to-cell interactions during embryogenesis, more specifically regulating mesoderm differentiation and osteogenesis, and to have putative roles in the regulation of adult stem cells. The recombinant mouse Wnt3a protein has been shown to be bioactive as measured by its ability to induce an increase in alkaline phosphatase activity after incubation with C2C12 myoblast cell line.

Description	Quantity	Cat. No.
Wnt3a, recombinant mouse	10 µg	GF145

New ENStem-A Human Neural Progenitors and Optimized Media

ENStem-A Human Neural Progenitor Cells are derived from NIH approved WA09 (H9) human embryonic stem cells (hESCs). They proliferate as an adherent cell monolayer, maintain karyotype stability up to 10 passages and can readily differentiate into different neuronal subtypes. ENStem-A human neural progenitor cells may be used for a variety of research applications such as studies of neurotoxicity, neurogenesis, electrophysiology, neurotransmitter and receptor functions. They are provided with optimized Expansion Media, containing FGF-2.

Description	Quantity	Cat. No.
ENStem-A Human Neural Progenitor Expansion Kit (1 x 10^6 viable cells, and SCM004 Expansion Media)	1 kit	SCR055
ENStem-A Human Neural Expansion Media	500 mL	SCM004
ENStem-A Human Neuronal Differentiation Media	100 mL	SCM017
ENStem-A Human Neural Freezing Media	50 mL	SCM011

Continued on pg 14

New NDiff® Medium Supplements for Stem Cell Culture

Millipore introduces two new medium supplements for serum-free stem cell culture. The NDiff Neuro-2 Medium Supplement was developed for the *in vitro* differentiation of murine ES cells into post-mitotic neurons particularly via monolayer differentiation[1-3] and for the derivation, propagation and maintenance of mouse neural stem cells[4-6]. The NDiff Neuro-27 Medium Supplement was developed for the *in vitro* propagation and maintenance of undifferentiated murine ES cells[1] and for the differentiation of murine ES cells into post-mitotic neurons particularly via monolayer differentiation[2-3].

References 1. Nichols J and Ying QL. (2006) *Methods Mol Biol*. **329**:91-8. 2. Ying QL and Smith AG. (2003) *Methods Enzymol*. **365**:327-41. 3. Ying QL, *et al*. (2003) *Nat Biotechnol*. **21(2)**:183-6. 4. Conti L, *et al*. (2006) *Brain Pathol*. **16(2)**:143-54. 5. Pollard SM, *et al*. (2006) *Cereb Cortex*. *16 Suppl* **1**:i112-20. 6. Conti L, *et al*. (2005) *PLoS Biol*. **3(9)**:e283.

Description	Quantity	Cat. No.
NDiff Neuro-2 Medium Supplement (200x)	5 mL	SCM012
NDiff Neuro-27 Medium Supplement (100x)	10 mL	SCM013
EmbryoMax® DMEM/F12 Medium, with L-Glutamine, without HEPES	500 mL	DF-042-B

New Mesenchymal Stem Cell Culture

Mesenchymal stem cells (MSCs) have the capacity to differentiate into multiple cell types, including osteoblasts, adipocytes, and chondroblasts. Millipore's Mesenchymal Stem Cell Expansion Medium has been optimized and qualified for the growth and expansion of MSCs derived from human and rodent origins. Cells expanded in Mesenchymal Stem Cell Expansion Medium express the correct MSC markers and are capable of differentiating into adipocytes and osteocytes. For consistent cryopreservation and high viability upon thawing and plating, the Mesenchymal Stem Cell Freezing Medium is recommended for the cryopreservation of human and rat MSCs that have been cultured in Mesenchymal Stem Cell Expansion Medium.

Description	Quantity	Cat. No.
Mesenchymal Stem Cell Expansion Medium (1x)	500 mL	SCM015
Mesenchymal Stem Cell Freezing Medium (1x)	50 mL	SCM016

New Pancreatic Islet Cell Characterization Kit

This Pancreatic Islet Cell Characterization Kit provides a convenient set of validated antibodies that allows researchers to reliably identify mature pancreatic islet cells. Along with antibodies generated against discrete hormones secreted by α, β, δ and γ cells of the pancreatic islets, the kit includes antibodies against PDX-1 (pancreatic duodenal homeobox gene-1), a master regulator of islet cell development and GLUT-2, a glucose transporter present in β-islet cells.

Description	Quantity	Cat. No.
Pancreatic Islet Cell Characterization Kit, Human	1 kit	SCR045

New Pancreatic Cell Development Pathway Kit

The Pancreatic Cell Development Pathway Kit provides a collection of antibodies that are unique to key transition points along the developmental pathway of pancreatic cells. Included in the kit are antibodies to critical transcription factors expressed during the program of development (FoxA2, Hes-1, Pax 6, IDX-1) along with two antibodies to hormones secreted by mature islets cells (Glucagon and Pancreatic Polypeptide).

Description	Quantity	Cat. No.
Pancreatic Cell Development Pathway Kit, Human	1 kit	SCR046

Continued on pg 15

New **Pancreatic Cell DTZ Detection Assay**

The Pancreatic Cell DTZ Detection Assay provides the researcher a simple and quick method to identify insulin-producing β cells from a mixed cell culture preparation or from pancreatic tissues, by detecting high levels of zinc (typically contained in pancreatic β cells), with the use of a zinc-chelating agent, DTZ. This kit contains DTZ staining and rinse solutions along with filters and syringes required for live staining reactions.

Description	Quantity	Cat. No.
Pancreatic Cell DTZ Detection Assay	1 kit	SCR047

New **MEL-1 and MEL-2: Early Passage Human Embryonic Stem Cells**

MEL-1 and MEL-2 are human embryonic stem cell lines that have been approved for stem cell derivation by the Australian National Health and Medical Research Council. They are provided at passage 10–12, providing extended research time in a stable, pluripotent state. The MEL cell lines grow as compact colonies with well defined cells displaying high nuclear to cytoplasmic ratios and prominent nucleoli. MEL-1 has a stable XY karyotype, MEL-2 an XX.

Description	Quantity	Cat. No.
MEL-1 Human Embryonic Stem Cell Line (XY)	20-24 colonies	SCC020
MEL-2 Human Embryonic Stem Cell Line (XX)	20-24 colonies	SCC021

New **PluriStem® NZWC1 Murine ES Cells**

New Zealand White (NZW) mice are used in research fields such as hematology, immunology and inflammation. F1 hybrids of NZB (New Zealand Black) and NZW are widely used as a model for autoimmunie diseases resembling human systemic lupus erythematosus. Millipore's PluriStem NZWC1 mouse ES cell line was derived from blastocysts from the mouse strain NZW/LacJ. This novel cell line will enable gene modification in NZW ES cells and the creation of mouse models for autoimmune studies.

Description	Quantity	Cat. No.
PluriStem NZWC1 Murine ES Cells	2 vials 2.5 x 10^6 cells/ vial, passage 11	SCC013

New **Improved, Oct-4 Monoclonal Antibody for Immunocytochemistry**

Millipore's new Oct-4 clone, 10H11.2, shows outstanding nuclear staining of human ES cells, and also works well for western blot applications, ELISA and FMAT. Oct-4 is a critical stem cell marker, vital for the formation of self renewing pluripotent stem cells.

Labeling of H9 human embryonic stem (ES) cells with New Oct-4 (Cat. No. MAB4401) shows its specificity for undifferentiated ES cells; H9 cells in culture on a mouse embryonic fibroblast (MEF) feeder layer were labeled by standard indirect immunofluorescent protocol.

Description	Quantity	Cat. No.
Oct-4 Monoclonal Antibody, Clone 10H11.2	100 µg	MAB4401

Chemicon, Upstate and Linco are now part of Millipore!

Together we are committed to providing even more innovative tools, services and application expertise to enhance your research efforts.

We now offer:

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- An increased number of applications and protocols that facilitate your development process
- Extended global expertise, leadership and presence to serve you more effectively
- Larger support and research development teams to deliver innovative products that cost-effectively impact your research

On behalf of everyone at Millipore, thank you for your support and continued loyalty. We look forward to establishing an even greater partnership with you for many more years of successful research.

New Mouse Embryonic Stem Cell Culture Manual

- Filled with protocols for targeting ES cells
- Traditional and serum-free, feeder-free cell culture methodologies
- Protocols for deriving new ES cell lines
- Formulations and Troubleshooting guide
- Complete range of ES cell optimized media

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