

Development of Neural Cultures From Human Embryonic Stem Cell Derived Neural Progenitors

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Part 1 of 2



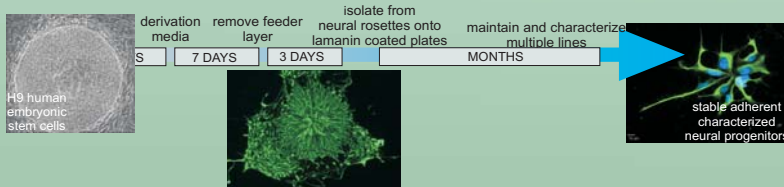
INTRODUCTION

The differentiation of neural cell types from human embryonic stem cells provides an *in vitro* model of human neural development. These cells and their derivatives can serve as a developmental model as well as produce clinically relevant tissue to screen compounds for toxicity and therapeutic properties. To meet this need we have derived a line of neural progenitors which met the following criteria:

- ① Display immunoreactive properties consistent with neural precursors and can be maintained in a proliferative state in monolayer cultures.
- ② Differentiate under serum-free conditions into neuronal phenotypes with functionally responsive transmitter receptors *in vitro*.
- ③ Maintain multiple neural phenotypes in long term serum-free culture.
- ④ Can be directed to alter phenotypic characteristics under different media conditions.

Our goal: To derive neural progenitors from H9 human embryonic stem cells and to characterize the populations of cells which they differentiate into.

METHODS



Derivation of neural progenitors: Neuroprogenitors were isolated from H9 human embryonic stem cells as previously described (Shin et al., Stem Cells Dev. 2005, 14(3):266-9). These cells are commercially available as EnstemTM neural progenitors (Millipore).

Cell Culture: Cells were cultured in neurobasal media supplemented with penicillin/streptomycin, L-glutamine, B27, bFGF and LIF. Differentiation occurred after removal of bFGF.

Immunohistochemistry: Cells were fixed in 2% paraformaldehyde and stained using standard Immunofluorescence protocols. Antibodies against the following proteins used were: nestin (1:200, Neuromics), oct4 (1:200, Santa Cruz), tuj-1 (1:500, Neuromics).

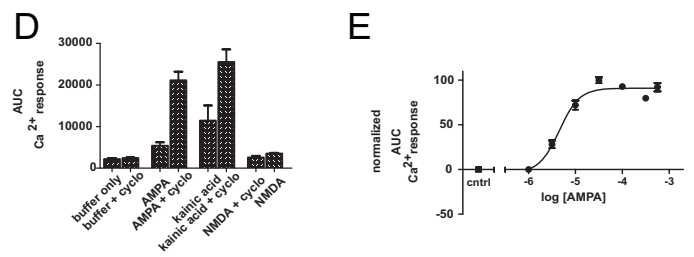
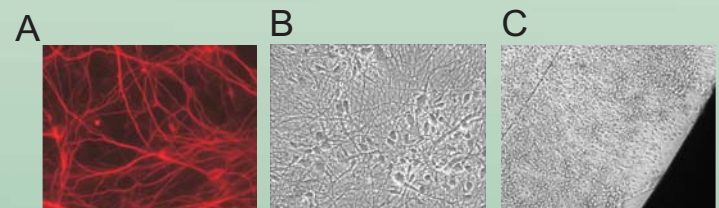
Ca⁺⁺ Imaging: Cells differentiated for 2-4 weeks and plated into 96 well plates. Assays were run on a flexstation 3 (Molecular Devices) plate reader using a FLIPR calcium 4 assay kit (Molecular devices).

Real time PCR: Real time PCR was run on an Applied Biosystems 7900HT system. Gene expression data (3 replications) were acquired and SDS software was used to estimate relative fold change values using $\Delta\Delta Ct$ quantification method. GAPDH was used as an endogenous control and either neural progenitors in their proliferative state or two weeks differentiated were used as a normalizer sample (defined on y-axis of graphs).

① NEURAL PROGENITOR PROPERTIES

- A. oct4 negative
- B. nestin positive stable karyotype
- (A) Neural progenitors do not express the transcription factor oct4, a stem cell marker.
- (B) More than 90% of neural progenitors show immunoreactivity to nestin, a cytoskeletal protein commonly used to identify neural progenitors. A stable karyotype was maintained for over 10 passages.

② DIFFERENTIATION AND FUNCTIONAL RESPONSES



- (A-C) Neural progenitors cultured in differentiation media for > two weeks form monolayer cultures that are highly β -III tubulin positive (A). These cultures can be maintained for long periods of time in regular culture dishes (B) and plated at high density into 96 well plates (C).
- (D) 100 μ M AMPA and kainic acid produce increased $[Ca^{++}]_i$ that are potentiated in the presence of cyclothiazide (50 μ M) in cultures differentiated for 4 weeks. No detectable changes in $[Ca^{++}]_i$ were recorded with the addition of 100 μ M NMDA.
- (E) Pooled data from 3 separate experiments run in triplicate demonstrate a dose-response relationship of $[Ca^{++}]_i$ with addition of AMPA in the presence of cyclothiazide ($EC_{50} = 4.5 \mu$ M).

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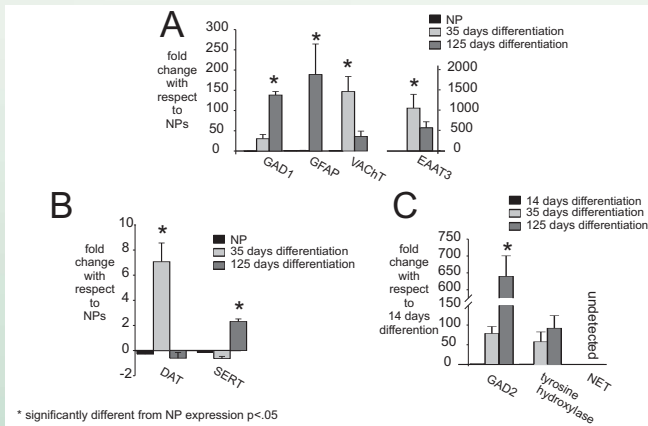
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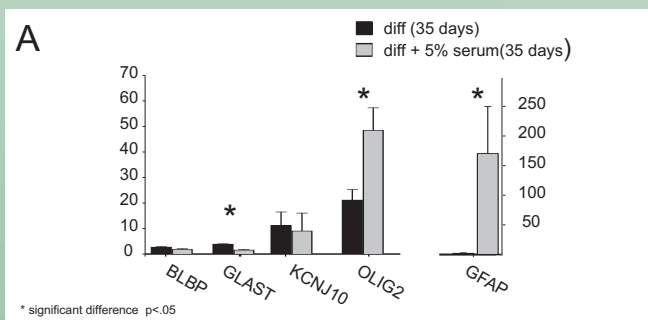


② LONG TERM SERUM-FREE DIFFERENTIATION



- (A & B) Following differentiation for 35 or 125 days there was up-regulation of mRNA expression of GAD1, VAcHT, EAAT3, DAT and SERT consistent with the cultures containing GABAergic, cholinergic, glutamatergic, dopaminergic and serotonergic cells.
- (C) GAD2, tyrosine hydroxylase and NET were not detectable in neural progenitor samples. Signal for GAD2 and tyrosine hydroxylase was detectable after two weeks in differentiation media therefore relative changes were normalized to the 2 week time point. Note: NET expression was still not detectable.

④ EFFECTS OF SERUM ON GLIOGENESIS



- (A) Although GLAST, (a glutamate transporter predominately found on astrocytes) was slightly down regulated GFAP (mainly associated with astrocyte development) and OLIG2 (mainly associated with oligodendrocyte and motoneuron precursors) were highly up regulated consistent with a trend towards gliogenesis for cultures differentiated in the presence of 5% serum.

Gene name	protein encoded	Cell phenotype
GAD 1	glutamic acid decarboxylase 1	GABAergic
GAD 2	glutamic acid decarboxylase 2	GABAergic
VAcHT	vesicular acetylcholine transporter	cholinergic
EAAT3	glutamate transporter	glutamatergic
DAT	dopamine transporter	dopaminergic
SERT	serotonin transporter	serotonergic
NET	noradrenergic transporter	noradrenergic
TH	tyrosine hydroxylase	dopaminergic or noradrenergic or adrenergic
BLPB	fatty acid binding protein 7	radial glia
GLAST	glutamate transporter	astrocyte
KCNJ10	(Kir)4.1 K ⁺ channel	glia
Olig2	bHLH transcription factor Olig2	oligodendrocyte precursor
GFAP	Glial fibrillary acidic protein	astrocytes

CONCLUSIONS

ENStem-A™ neural progenitors derived from human embryonic stem cells are:

Stable for multiple passages, highly proliferative and can be maintained in an adherent monolayer

Differentiate into cells which are functionally responsive to glutamatergic agonists

Differentiate into neural cultures containing diverse cellular phenotypes

Can be maintained differentiated in culture for months

Differentiation in serum can lead to an earlier onset of gene expression related to gliogenesis

These cells represent a novel tissue source for academic and commercial researchers interested in studying human neural disease and development.

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