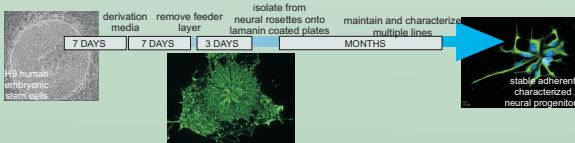


INTRODUCTION

Human embryonic stem cells and their progeny can provide a novel tissue source for understanding developmental pathways, pharmaceutical screening and tissue replacement therapies. Drug screening using human embryonic stem cell (hESC) derived tissue uniquely offers several advantages over traditional cell lines: Large quantities of cells can be produced due to the proliferative nature of the cells, repeated studies can be done on a common genetic background and signaling can be studied in endogenously expressed receptors in non-transformed cells. We have isolated neural progenitors which can be maintained in their proliferative state for multiple passages and differentiated by removal of bFGF. These cells are commercially available (ENStem-A™, Millipore). Here we demonstrate that under basal differentiation conditions these isolated hNP cells:

- ① Up-regulate expression of genes consistent with a diverse range of neural phenotypes
- ② Up-regulate multiple Na⁺ and Ca⁺ channel subunits
- ③ Up-regulate multiple glutamate receptor subunits
- ④ Functional glutamatergic responses can be detected in differentiated cells using a FLIPR Ca⁺ assay.

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 ENstem™ neural progenitors (Millipore).

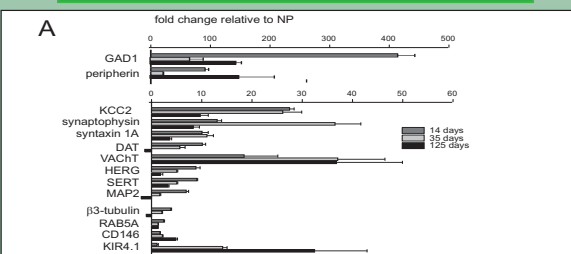
Cell Culture: Cells were cultured in neurobasal media supplemented with penicillin/streptomycin, L-glutamine, B27, bFGF and LIF. Differentiation occurred after removal of bFGF.

Immunohistochemistry: Cells were fixed in 2% paraformaldehyde and stained using standard immunofluorescence protocols. Antibodies against the following proteins used were: nestin (1:200, Neuromics), tuji-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).

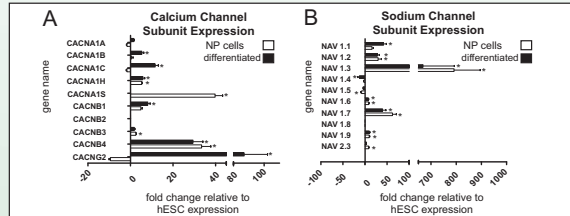
Real time PCR: Real time PCR was run on an Applied Biosystems 7900HT system. Gene expression data (3 replications) were acquired and SDS software was used to estimate relative fold change values using $\Delta\Delta Ct$ quantification method. GAPDH was used as an endogenous control and neural progenitors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

① DIFFERENTIATION OF MULTIPLE NEURAL PHENOTYPES



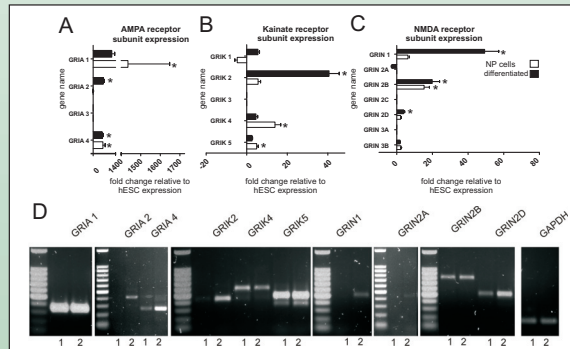
(A) Relative fold changes in mRNA expression comparing proliferative neural progenitors to cells 14, 35 and 125 days following removal of bFGF. Genes shown had a more than 5 fold up-regulation.

② EXPRESSION OF NA⁺ AND CA⁺ CHANNEL SUBUNITS



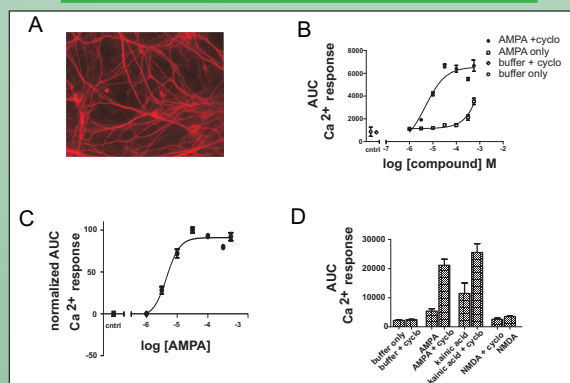
(A) Real-time PCR results showed that differentiated cells had significant up-regulation of CACNA1B, 1C and 1H consistent with N-type, L-type and T-type Ca⁺ currents. (B) Real-time PCR results showed that both the progenitor and differentiated cells expressed multiple sodium channel subunits. NAV 1.3 showed the highest up-regulation. NAV 1.1 was up-regulated in the differentiated cells. Note: * denotes significantly different expression relative to hESCs (p<.05)

③ EXPRESSION OF GLUTAMATE RECEPTOR SUBUNITS



(A) Real-time PCR demonstrated up-regulation of AMPA receptor subunits GRIA1,2 and 4. (B) Real-time PCR demonstrated up-regulation of Kainate receptor subunits GRIK2,4 and 5. (C) Real-time PCR demonstrated up-regulation of NMDA subunits GRIN1,2A and 2D. (D) RT-PCR with independent primers confirmed our real-time results (lane 1 are hNP, lane 2 are cells differentiated for 2 weeks). Note: * denotes significantly different expression relative to hESCs (p<.05)

④ DEVELOPMENT OF FUNCTIONAL GLUTAMATERGIC RESPONSES



(A) Removal of bFGF for two weeks results in TUJ1 positive cells with a neuronal morphology. (B) [Ca⁺] increased in response to glutamatergic agonists using a FLIPR assay. Response to AMPA was potentiated by the AMPA potentiator cyclothiazide. (C) Pooled data from 3 separate experiments run in triplicate demonstrate a dose-response relationship of [Ca⁺] with addition of AMPA in the presence of cyclothiazide (EC₅₀=4.5 μM). (D) 100 μM AMPA and kainic acid produce increased [Ca⁺] that are potentiated in the presence of cyclothiazide (50 μM) in cultures differentiated for 4 weeks. No detectable changes in [Ca⁺] were recorded with the addition of 100 μM NMDA.

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 neural cultures containing diverse cellular phenotypes.

mRNA expression is up-regulated for a variety of Na⁺, Ca⁺ channel subunits and glutamate receptor subunits.

Differentiate into cells which are functionally responsive to glutamatergic agonists

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

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