DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELL DERIVED NEURAL PROGENITORS INTO FUNCTIONALLY RESPONSIVE POPULATIONS IN THE ABSENCE OF EXOGENOUS EGF

D.W. Machacek[1], S. K. Dhara[3], C. Sturkie[1,3], K. Hasneen[3], D. Carter[1], L. Murrah Hanson[1], P.R. MacLeish[2], M. Benveniste[2], S.L. Stice[1,3]
Aruna Biomedical Inc[1], Morehouse School of Medicine[2] and University of Georgia[3]

INTRODUCTION

Here we demonstrate that hESC- derived neural progenitors (ENStem-A, Millipore) do not require exogenous EGF for either long-term maintenance of the proliferative progenitors or differentiated functionally responsive cultures. Thus hESC derived neural progenitor cells can be maintained and differentiated into diverse functionally responsive phenotypes in the absence of exogenous EGF.

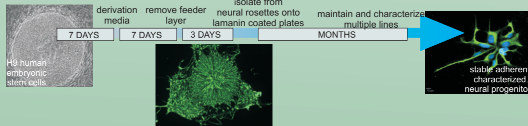
① Nestin positive human neural progenitors can be maintained in a proliferative state in the absence of exogenous EGF and differentiate after removal of bFGF.

② Under basal differentiation conditions we have an up-regulation of genes consistent with a diverse range of phenotypes.

③ Electrophysiolgical recordings 20-25 days following withdrawal of bFGF demonstrate cells with TTX sensitive inward sodium currents which can fire action potentials.

④ Two weeks following removal of bFGF glutamatergic responses can be recorded and the diversity of glutamate receptor subunit expression is increased.

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).

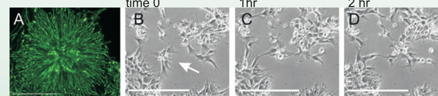
Cell Culture: Cells were cultured in neurobasal media supplemented with penicillin/streptomycin, L-glutamine, B27, bFGF and LIF. Differentiation occurred after removal of bFGF.

Immunohistochemistry: Cells were fixed in 2% paraformaldehyde and stained using standard immunofluorescence protocols. Antibodies against the following proteins used were: nestin (1:200, Neuromics), 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 ΔΔCt quantification method. GAPDH was used as an endogenous control and neural progenitors in their proliferative state were used as a normalizer sample.

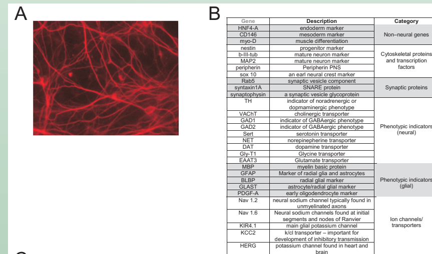
① NEURAL PROGENITOR PROPERTIES



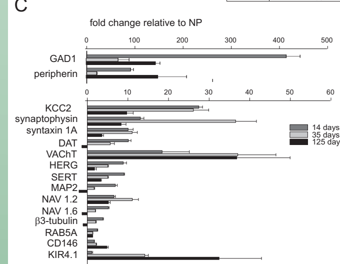
(A) Initial rosette structure from which progenitors are isolated (nestin staining).

(B-C) Time lapse phase images 1 hour apart. Progenitors can be passaged in the absence of EFG and are highly proliferative. Small 'rossette-like' structures can be observed in the cultures but these are transitory (arrow in B).

② DIFFERENTIATION OF MULTIPLE NEURAL PHENOTYPES



Gene	Description	Category
HNF4-A	endoderm marker	Non–neural genes
CD146	mesoderm marker	
myo-D	muscle differentiation	
nestin	progenitor marker	Cytoskeletal proteins and transcription factors
b-III-tub	mature neuron marker	
MAP2	mature neuron marker	
peripherin	Peripherin PNS	
sox 10	an earl neural crest marker	
Rab5	synaptic vesicle component	Synaptic proteins
syntaxin1A	SNARE protein	
synaptophysin	a synaptic vesicle glycoprotein	
TH	indicator of noradrenergic or dopmaminergic phenotype	Phenotypic indicators (neural)
VAChT	cholinergic transporter	
GAD1	indicator of GABAergic phenotype	
GAD2	indicator of GABAergic phenotype	
Sert	serotonin transporter	
NET	norepinepherine transporter	
DAT	dopamine transporter	
Gly-T1	Glycine transporter	
EAAT3	Glutamate transporter	
MBP	myelin basic protein	Phenotypic indicators (glial)
GFAP	Marker of radial glia and astrocytes	
BLBP	radial glial marker	
GLAST	astrocyte/radial glial marker	
PDGF-A	early oligodendrocyte marker	
Nav 1.2	neural sodium channel typically found in unmyelinated axons	Ion channels/ transporters
Nav 1.6	Neural sodium channels found at initial segments and nodes of Ranvier	
KIR4.1	main glial potassium channel	
KCC2	k/cl transporter – important for development of inhibitory transmission	
HERG	potassium channel found in heart and brain	

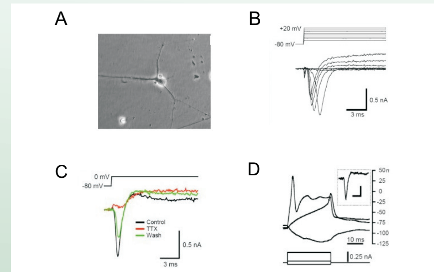


D

	NP	14 days			35 days		125 days
Gene	Prolif	diff	+ serum	+BDNF	diff	+ serum	diff
TH	X	✓	✓	✓	✓	✓	✓
EAAT3	X	✓	✓	✓	✓	✓	✓
GAD2	X	X	✓	X	✓	✓	✓
GFAP	X	X	X	X	X	✓	✓
PDGFRA	X	X	X	X	X	✓	X
MYO-D	X	X	X	X	X	X	✓
MOBP	X	X	X	X	X	X	X
SOX 10	X	X	X	X	X	X	X
NET	X	X	X	X	X	X	X
HNF4A	X	X	X	X	X	X	X

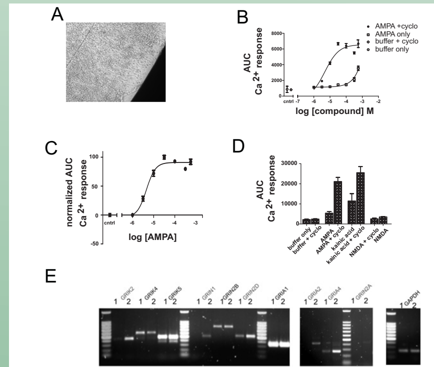
(A) Removal of bFGF results in Tuj-1 positive cells with neuronal morphology.
(B) 30 genes related to cellular phenotypes of the culture examined with real time PCR.
(C) Relative fold changes in mRNA expression comparing proliferative neural progenitors to cells 14, 35 and 125 days following removal of bFGF. Genes shown had a more than 5 fold up-regulation.
(D) Relative fold changes could not be calculated for genes where expression was not detected after 40 cycles of PCR in the neural progenitor population. However, some genes were detectable following differentiation under different conditions, including glial genes GFAP and PDGFRA.

③ Development of inward sodium currents



(A) Phase image of differentiated cell 23 days after removal of bFGF.
(B) Voltage gated inward and outward currents elicited from cell shown in A with depolarizing voltage steps.
(C) Inward currents were reversibly abolished by application of 1 μM tetrodotoxin.
(D) Action potential upon current injection under whole cell current clamp. Inset: Voltage clamp recording in the same cell to a step from -80 mV to -10 mV. Peak current of 457 pA. Scale bars for inset: 5 ms, 0.2 nA. All recordings made with potassium gluconate solution.

④ Development of functional glutamatergic receptors



(A) Cells were differentiated for 2 weeks and then plated at high densities into 96 well dishes.
(B) $[Ca^{++}]_i$ 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 does-response relationship of $[Ca^{++}]_i$ with addition of AMPA in the presence of cyclothiazide (Ec50=4.5 μM).
(D) 100 μM AMPA and kainic acid produce increased $[Ca^{++}]_i$ that are potentiated in the presence of cyclothiazide (50 μM) in cultures differentiated for 4 weeks. No detectable changes in $[Ca^{++}]_i$ were recorded with the addition of 100 μM NMDA .
(E) RT-PCR shows increase in expression of glutamatergic receptor subunits. Lane 1 is from the progenitor population, lane two is from cells two weeks after withdrawal of bFGF.

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 in the absence of exogenous EGF.

Differentiate into neural cultures containing diverse cellular phenotypes.

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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