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Human Motor Neuron Differentiation from Human Embryonic Stem Cells

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ABSTRACT

The therapeutic potential of embryonic stem (ES) cells is promising, but in many cases limited by our inability to promote their differentiation to specific cell types, such as motor neurons. Here we provide the first report of the successful differentiation of human ES cells to cells of a motor neuron phenotype. A renewable source of neuroepithelial cells was generated from human ES cells. Extracellular signals were then employed to induce motor neuron differentiation and related gene expression by these cells. OLIG2 and HLXB9 gene expression increased upon the addition of basic fibroblast growth factor, retinoic acid, and sonic hedgehog, as a motor neuron phenotype expressing Islet1 and choline acetyltransferase (ChAT) developed. This study demonstrates that neuroepithelial cells derived from human ES cells are renewable progenitors capable of generating motor neurons at levels that may be therapeutically useful. Sonic hedgehog, basic fibroblast growth factor, and retinoic acid differentially influence human motor neuron differentiation by mechanisms that remain to be defined.

INTRODUCTION

An in vitro source of human motor neurons derived from human embryonic stem (ES) cells will be of use to study unique aspects of human motor neuron diseases, including spinal muscular atrophy, a human disease that affects two genes, SMN1 and SMN2. We derived a relatively high proportion of cells (20–30%) possessing a motor neuron phenotype using a multistep process initiated from human ES cells. This process simultaneously eliminates contaminating residual pluripotent human ES cells. Therefore, these adherent cultures containing motor neural phenotypes may be an ideal source for cell-based assays, understanding neural developmental cues, and potential regenerative cell therapies.

MATERIALS AND METHODS

Derivation and culture of neuroepithelial cells

Neuroepithelial cells were derived from BG01 and BG02 human ES cells lines maintained and mechanically subcultured as described (1). Briefly, after 1 week of culture on a mouse feeder cell layer, human ES cells were fed with the derivation medium, which is Dubecco's modified Eagle medium (DMEM)/F12 medium (Gibco) supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, N2 (Gibco), and 4 ng/ml of basic fibroblast growth factor (bFGF; Sigma) for 7 days. The mouse feeder layer was then removed, allowing neuroepithelial cells to attach to the culture dish and develop rosettes after 3 days in derivation medium. Derived neuroepithelial cells were propagated further on polyornithine and laminin-coated dishes in neurobasal medium (Gibco) supplemented with L-glutamine, penicillin, streptomycin, B27 (Gibco), 20 ng/ml of bFGF (Sigma), and 10 ng/ml of leukemia inhibitory factor (LIF; Chemicon) and continuously passaged either by mechanical triturating or by trypsin.

Gene expression analysis

To investigate factors involved in the motor neuron induction, neuroepithelial cells were cultured on the polyornithine and laminin-coated dishes with or without extrin-

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sic factors. Neuroepithelial cells were cultured for 7 days with or without sonic hedgehog (1 μg/ml) and retinoic acid (2 uM) and then fully differentiated by culture in neurobasal medium (Gibco) supplemented with L-glutamine for 14 days. At this time, HLXB9 and motor neuron phenotype marker expression was determined. Prior to that OLIG2 expression was determined after only 1 day of exposure to factors, because this is an early marker for motor neuron progenitor cells. For quantitative gene expression, real time RT-PCR was carried out (ABI 7900 system). From each sample, total RNA was extracted using Trizol and 1 μg of total RNA from each sample was treated with DNase (Promega); 500 ng was converted to cDNA by using the Superscript III kit (Invitrogen) with oligo(dT) as a primer and the other 500 ng was prepared without reverse transcriptase (no-RT) to serve as control for genomic amplification. Twenty five nanograms of resulting cDNAs were subjected to real-time RT-PCR using specific primer and probe for OLIG2 (Hs_00377820_m1, Applied Biosystems), HLXB9 (Hs_00232128_m1, Applied Biosystems), and human β-actin (4326315E, Applied Biosystems).

Gene expression was detected as an amplification curve using ABI 7900 sequence detection system, and cycle number was obtained when amplification exceeded threshold (C_t values). All target gene C_t values in each parameter were normalized by reference gene C_t value to determine the ΔC_t value (target gene C_t − reference gene C_t). Relative gene expression was shown as fold change (ratio) in gene expression using the comparative C_t method. For statistical analysis, ΔC_t values of control and treatment group were subjected to one-tailed t-test. Significant differences between the treatments were defined as $p < 0.01$. Each experiment included three identical replicates and two independent experimental replicates.

Antibodies and immunocytochemistry

Cells plated on polyornithine and laminin-coated permonox slides were washed in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde and 4% sucrose. Permeabilization and blocking in 0.1% Triton, 5% fetal bovine serum (FBS) in Tris buffer for 40 min was followed by primary antibodies (2 h) at room temperature and washed three times in blocking buffer before secondary antibody application. The secondary antibodies, goat anti-mouse Alexa-conjugated, donkey anti-goat Alexa-conjugated, and goat anti-rabbit Alexa-conjugated (Molecular Probes), were applied to cells for 40 min at room temperature. For negative controls, first antibodies were omitted and the same staining procedures were followed. Primary antibodies and dilutions used were mouse anti-Islet 1 (1:100; DSHB), rabbit anti-nestin (1:200; Chemicon), goat anti-choline acetyltransferase (ChAT) (1:100; Chemicon), rabbit anti-Tuj1 (1:500; Covance), and mouse anti-Oct4 (1:200; Santa Cruz biotech).

RESULTS AND DISCUSSION

National Institutes of Health approved human ES cells (BG01) were first committed to a neuroepithelial cell phenotype (Fig. 1A). These cells are self-renewing and can give rise to all the cells that comprise the mammalian central nervous system, including various types of neurons and glial cells (2). We cultured neuroepithelial cells for 10 months in neurobasal medium supplemented with B27 (1×), LIF (10 ng/ml), and bFGF (20 ng/ml) in serum-free medium without overt changes in expression of Nestin, Musashi1, or SOX 1, 2, and 3, or in differentiation potential to neuron and glial phenotypes. Importantly, these cultured adherent human neuroepithelial cells lose expression of OCT4 (Fig. 1B,C).

Retinoic acid and sonic hedgehog are well-known molecules involved in motor neuron differentiation. When mouse ES cells were induced to neural fate and exposed to sonic hedgehog and retinoic acid, the differentiating population was preferentially directed to motor neuron fate (Islet 1, HB9, and Tuj1 positive) (3). In addition, bFGF alone induced long-term cultured fetal human neural stem cells to form cholinergic neurons (4), suggesting that bFGF may also be an effective growth factor for in vitro differentiation to human motor neurons. Thus, we predicted that subjecting adherent human neuroepithelial cell cultures to these three factors would lead to relatively high proportions of motor neuron phenotypes.

First, we examined gene expression of PTCH, the sonic hedgehog receptor, to determine whether this portion of the sonic hedgehog signaling pathway was present in our propagating neuroepithelial cells. Gailani and Bale showed that the vertebrate homolog of *Drosophila* PTCH is expressed in all known target tissues of sonic hedgehog and that expression of this receptor can be a useful biological marker in screening tissue for sonic hedgehog treatment (5). We found PTCH gene expression in cultured neuroepithelial cells; however, PTCH expression alone does not necessarily represent responsiveness to sonic hedgehog for motor neuron cell specification, which must be determined by examining the effect of sonic hedgehog and other factors on motor neuron lineage gene expression and induced phenotype.

The motor neuron progenitor gene OLIG2 is expressed early in motor neuron induction (6). Using real-time RT-PCR, we found that bFGF significantly increased OLIG2 expression, and when bFGF was combined with retinoic acid and sonic hedgehog, OLIG2 expression was further increased (Fig. 2A). However, the combination of sonic hedgehog and retinoic acid alone had little effect on OLIG2 expression, suggesting a critical role for bFGF in motor neuron induction. Subsequently, we examined expression of the motor neuron gene HLBX9 to investigate the effect of sonic hedgehog and retinoic acid on motor

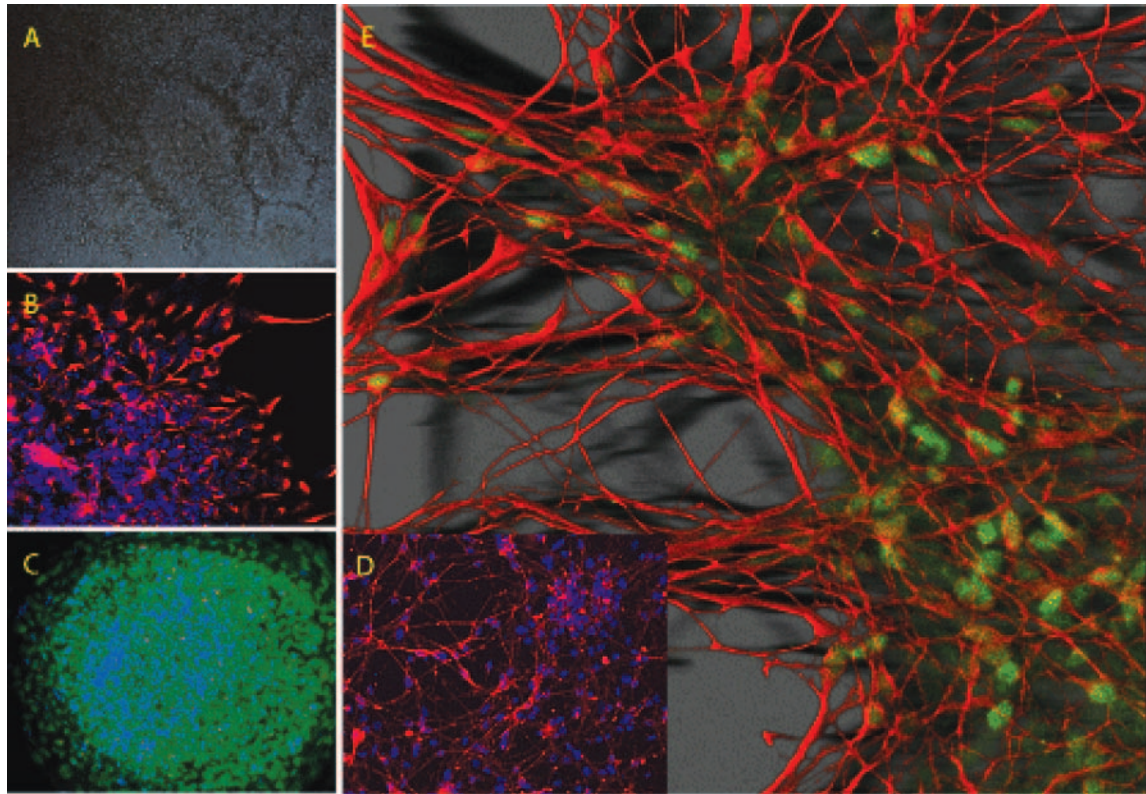
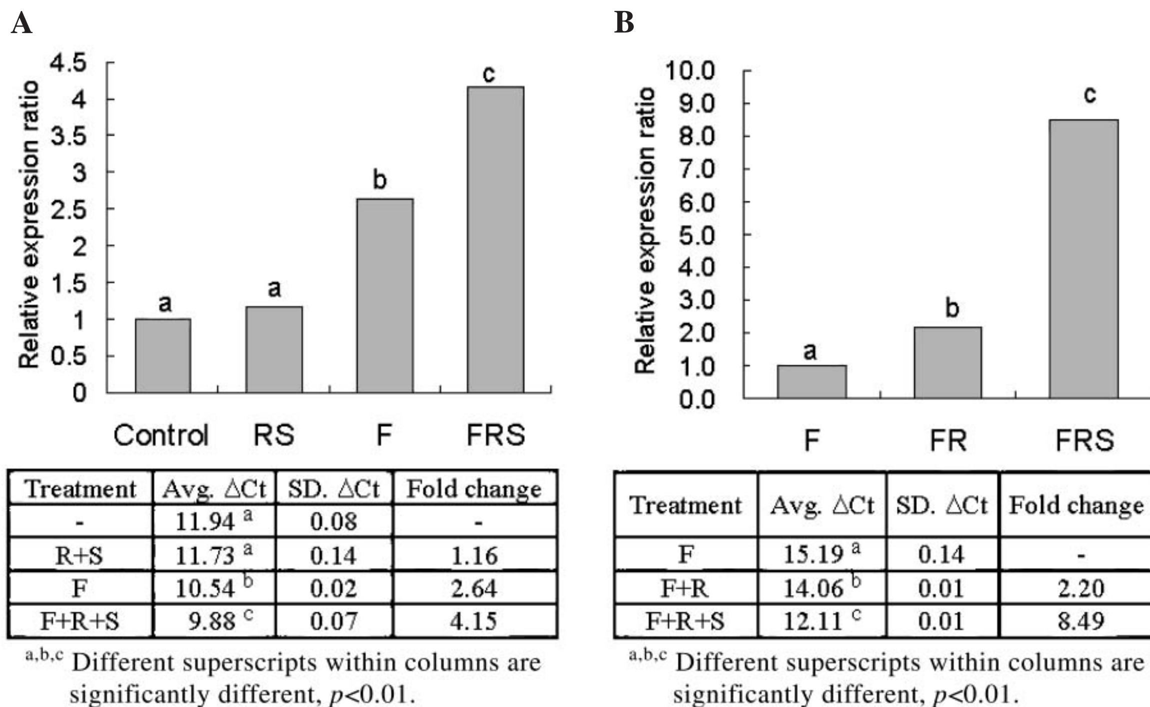


FIG. 1. (**A**) Phase-contrast image of neuroepithelial cells. (**B**,**C**) Oct4 (green) and Nestin (red) expression in neuroepithelial cells with no Oct4 expression (**B**) and human ES cells (**C**) with Oct4 expression. (**D**,**E**) Motor neuron phenotype marker expression in differentiated neuroepithelial cells. (**D**) ChAT (red) and DAPI (blue), (**E**) Islet1(green) and Tuj1 (red). Magnification, 200×.



A

Treatment	Avg. ΔCt	SD. ΔCt	Fold change
-	11.94 [a]	0.08	-
R+S	11.73 [a]	0.14	1.16
F	10.54 [b]	0.02	2.64
F+R+S	9.88 [c]	0.07	4.15

[a,b,c] Different superscripts within columns are significantly different, $p<0.01$.

B

Treatment	Avg. ΔCt	SD. ΔCt	Fold change
F	15.19 [a]	0.14	-
F+R	14.06 [b]	0.01	2.20
F+R+S	12.11 [c]	0.01	8.49

[a,b,c] Different superscripts within columns are significantly different, $p<0.01$.

FIG. 2. (**A**,**B**) Gene expression fold change by signaling molecules bFGF (F), retinoic acid (R), and sonic hedgehog (S) using real-time RT PCR. (**A**) OLIG2 expression change; (**B**) HLBX9 expression.

neuron differentiation. On the basis of results with OLIG2, bFGF was included at the time of retinoic acid or retinoic acid plus sonic hedgehog exposure. The combination of retinoic acid and bFGF produced a 2- to 20-fold increased in HLBX9 expression level, and combining bFGF, sonic hedgehog, and retinoic acid increased HLBX9 expression 8- to 49-fold higher than bFGF alone in human neuroepithelial cells (Fig. 2B).

In addition to motor neuron-related gene expression, motor neuron phenotype markers were examined. Neuroepithelial cells differentiated to motor neurons that expressed Islet 1, Tuj1 (Fig. 1E), and ChAT (Fig. 1D). On the basis of this criterion, approximately 20–30% of the cultures have a motor neuron phenotype when they are exposed to neural basal medium supplemented with B27 (1×), bFGF (20 ng/ml), sonic hedgehog (1 μg/ml), and retinoic acid (2 μM) for 7 days and then 14 additional days without these growth factors on polyornithine and laminin-coated slides. We expect that it will be possible to obtain an even greater proportion of motor neurons once dose–response experiments have been conducted. However, with our current culture conditions, neuroepithelial cells proliferating adherently in bFGF-containing medium gave rise to motor neurons even without sonic hedgehog and retinoic acid exposure. Although the underlying mechanisms remain to be defined, our propagating neuroepithelial cells may have been primed to differentiate toward a motor neuron phenotype as a result of low level exposure to bFGF during neuroepithelial cell proliferation. This growth factor has previously been shown to induce cholinergic neuron differentiation in fetal neural stem cells (4).

Here we report motor neuron differentiation from human ES cells-derived neuroepithelial cells and the effects of extrinsic factors on motor neuron induction of the neuroepithelial cells. We demonstrated that motor neuron induction was affected not only by the kinds of factors applied, but how they are combined, and that bFGF, sonic hedgehog, and retinoic acid had additive and synergistic effects. We also showed that an established neuroepithelial cell culture system can serve as an in vitro model for the study of human motor neuron development. Detailed examination of motor neurons derived from ES cells will facilitate elucidation of molecular mechanisms that regulate survival of motor neurons. These cells may provide a model system for the screening of factors involved in motor neuron dysregulation and pathology as well as provide the foundation for regeneration of diseased or traumatized tissue in patients.

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