Engineering of Embryonic Stem Cell Neurogenic Differentiation for Neurodegenerative Diseases

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ABSTRACT

Embryonic stem cells (ESCs) have the potential to become a limitless source of tissue for cell therapies and drug testing. Specifically, efficient generation of neuronal cells from human ESCs (hESCs) will have a major impact in transplantation therapies to replace aged or diseased neural tissue. This study focuses on better understanding the specific signals directing the differentiation of ESCs to neural progeny. We further examine the differentiation of hESCs in a bioreactor as a way of producing neuronal cells in quantities suitable for clinical therapies. The integrative nuclear fibroblast growth factor (FGF) signaling (INFS) has been show to mediate the activation of genes thereby coaxing the differentiation of umbilical cord blood cells and human neuronal precursors to neuronal cells. Here, we investigated if a similar mechanism can be utilized for the neurogenic commitment of mouse ESCs (mESCs). We found that treatment of mESCs with agents such as retinoic acid (RA) and cyclic adenosine monophosphate (cAMP) activates INFS, in part by nuclear translocation of an FGF receptor. Immunopositivity for glial fibrillary acidic protein (GFAP) revealed an increased propensity of mESCs for astrocyte differentiation upon stimulation with cAMP. In contrast, RA prompted the differentiation towards neurons as shown by enhanced expression of tyrosine hydroxylase and β3-tubulin. To that end, the localization of the RA receptor in differentiating stem cells in various stages of neuronal differentiation was also studied. These findings may lead to the development of methods for directed differentiation of ESCs to neuronal cells with high efficiency. For such methods to be utilized in clinical therapies, ESC-derived neurons must be generated in high quantities. Hence, we also examine if neuronal differentiation of hESCs can be performed in stirred suspension bioreactors. Cells formed neurosphere-like aggregates in the bioreactor and in a serum-free medium. A transition was noted to neuroepithelial cells and then radial glial cells. Finally, neuron-like cells emerged upon plating of the neurospheres. Differentiated cells displayed increased gene/protein levels of Pax6, Nestin, β3-tubulin, MAP2 and GABA (Fig. 1) compared to undifferentiated hESCs, and similar levels of ectoderm genes when compared to cells differentiated exclusively in static culture. This scalable system has the ability to produce mass quantities of neurospheres. In conjunction with an INFS-based method for directing the differentiation of these cells further to neuronal cells, this stem cell culture modality may facilitate the production of cellular material suitable for therapies against Parkinson and Alzheimer diseases.

INTRODUCTION

There are several neuronal diseases that afflict millions of Americans, including Alzheimer's, Huntington's, Parkinson's, Bell's Palsy and Neuromuscular disorders. Of these Parkinson's disease, which is a progressive disorder of the central nervous system affecting more than 1.5 million people in the United States, has a precedence of been treating using fetal stem cells.[1] Due to the inadequate amount and the moral issues of the use of fetal tissue, embryonic stem cells could perhaps be used to produce larger quantities of these cells for the treatment of Parkinson's disease one day. Since their creation, mouse and human embryonic stem cells have demonstrated the ability to proliferate in an undifferentiated state for long periods of time[2,3]. Due to this growth potential, these stem cells provide an ideal source for cell based therapies which require large quantities of cells, for a large number of patients. Producing a scalable process for the development of therapeutically effective tissue from embryonic stem cells could be very valuable in the future.

In addition to the ability to proliferate indefinitely, embryonic stem cells also possess the capability to differentiate to all three germ layers, including the ectoderm[4]. Functional neurons have been differentiated from mESCs for over a decade with the addition of RA and creating suspension aggregates of the mESCs termed embryoid bodies (EBs), and then let grow on surfaces[5]. The exact mechanism by which RA stimulates the differentiation is unknown, but the INFS pathway functions in association with cell differentiation and gene regulation[6], and could have a role. Cyclic AMP has shown the ability to elevate the gene expression levels of an important neuronal gene tyrosine hydroxylase[7,8] which may also coax the differentiation to mature neurons. Recently neurospheres have been produced by suspending hESCs aggregates on Petri dishes in a neuronal differentiation media and then placed on tissue culture surfaces for further maturation[9]. Bioreactors have been employed in the expansion of undifferentiated mESCs in a scalable process[10]. Human ESCs were recently differentiated hematopoietic progenitors via cultivation in stirred tank bioreactors[11].

This study delves into the mechanisms of neuronal differentiation using the mESC as a model, and then looks into creating neurons from hESCs in a more scalable process. The purpose is to better understand what is involved in the differentiation of ESCs to mature neurons, and create a system that can better produce large numbers of mature neurons to meet a large demand that might be needed for cell therapies for patients of Parkinson's disease, or other neuronal disorders.

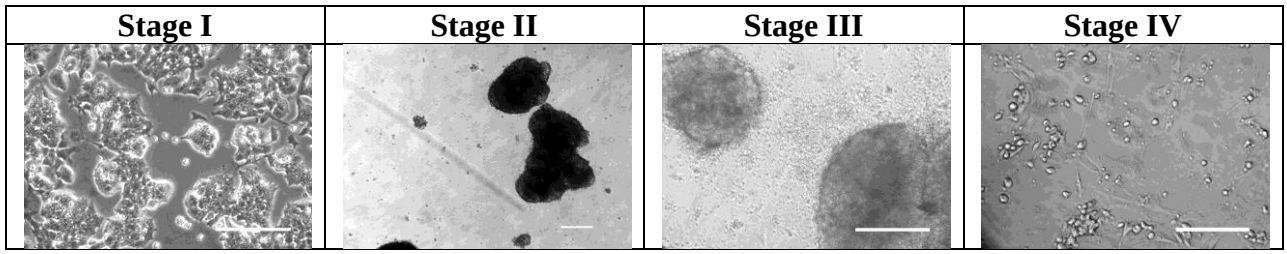
RESULTS

The first section of this study focuses on the mechanisms, and factors that are important during ESC differentiation. For this work mESCs were coaxed to form neurons, and then investigated, and probed in a number of ways. The location of receptors for Fibroblast Growth Factor (FGF) and RA were studied over the differentiation time course. Different small molecules were added during the last stage of differentiation to see if it impacted cell differentiation. For the second segment of this paper, the differentiation of hESCs to neurons in a scalable bioreactor was assessed. The formation of neurospheres in the stirred suspension was assessed over time. More mature neurospheres were stained for neuronal markers and viewed under confocal microscopy. Finally gene expression was probed via real-time PCR for cells from neurospheres and then compared to undifferentiated hESCs.

Method of differentiation of ESCs to neurons

To produce neurons from mESCs a protocol was adapted from a paper discussed above by Fraichard[12]. Mouse ESCs were first grown undifferentiated on gel in the presence of LIF (Stage I), then they were trypsinized and placed in Petri dishes without LIF, and with 1 μM RA to form suspending EBs for 2 days, then the RA was removed for 2 additional days (Stage II). The EBs were then placed on a tissue culture surface, and let mature for 4 days (Stage III), and finally these cells are trypsinized and placed on slides for viewing, and additional treatment with stimulating molecules (Stage IV). Bright field images of each of these 4 stages are shown below in Figure 1, with the white bar indicating 200 μm.

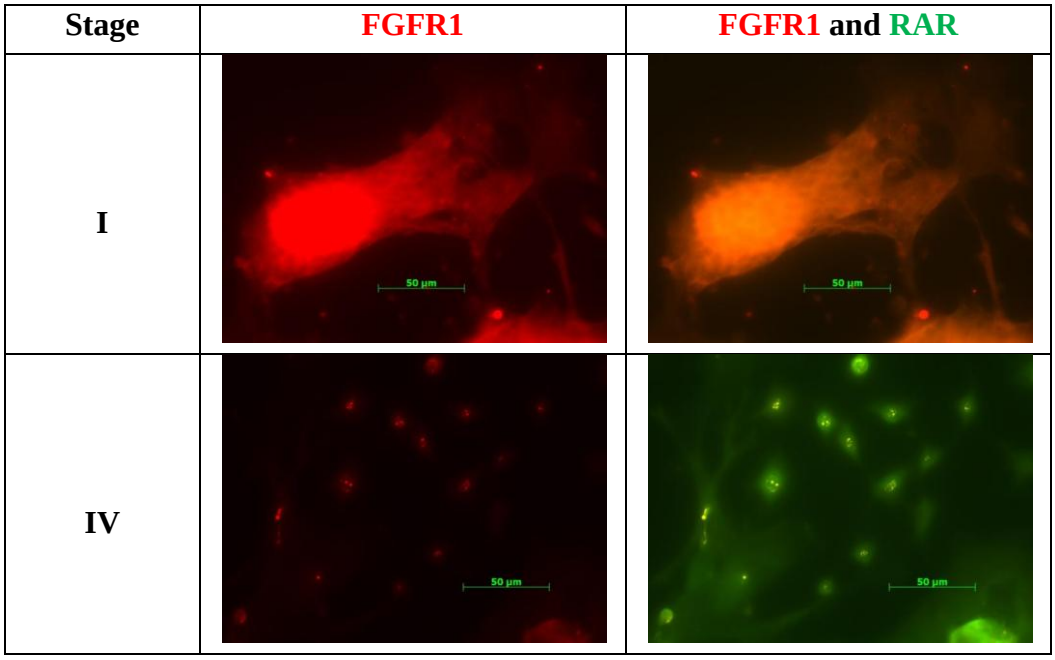
Figure 1. Stages involved for the differentiation of mESCs to neurons (bars: 200 μm)



Investigation of the location of receptors of interest during differentiation

To delve into the mechanisms of the differentiations a closer examination of the location of the RA receptor (RAR), and another receptor of note, the FGF receptor 1 (FGFR1). Prior to the differentiation (Stage I), both receptors seem to be localized within the cytoplasm, but after differentiation and treatment with RA (Stage IV) the receptors were located in the nuclei of the cells, as shown in Figure 2 shown below.

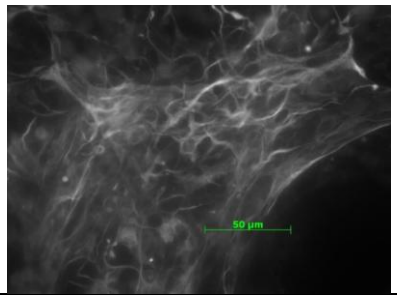
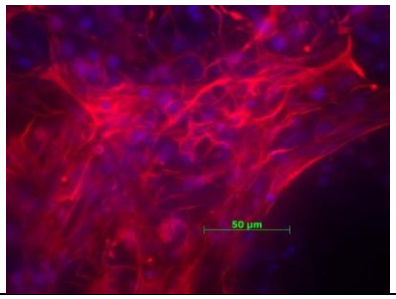
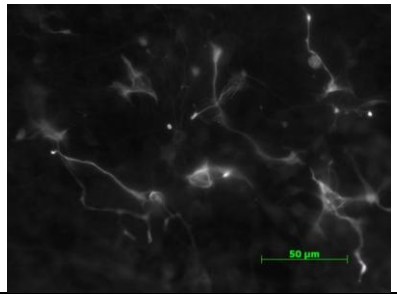
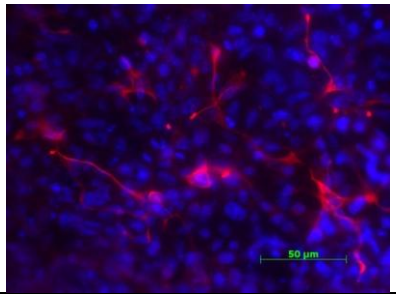
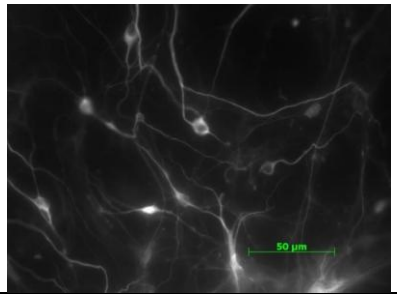
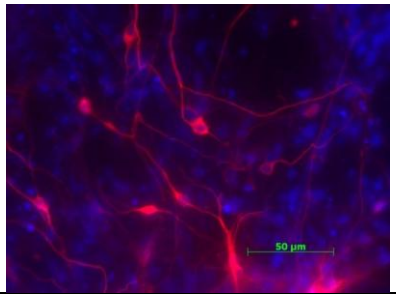
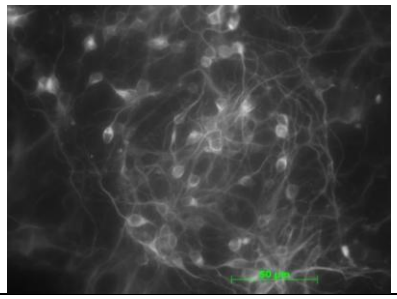
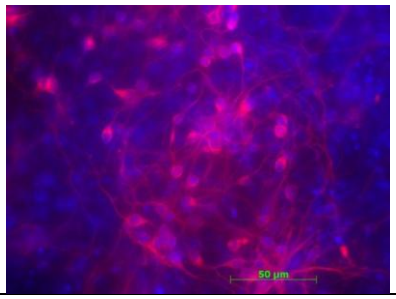
Figure 2. Translocation of the FGF and RA receptors upon cell differentiation (bars: 50 μm)



Coaxed differentiation of ESCs to neurons or astrocytes

The addition of small molecules cAMP and RA during the last stage of differentiation, for a duration of three days showed difference in the protein expression levels of several neuronal markers. Cyclic AMP treated stage IV differentiated mESCs showed higher levels of astrocytic marker GFAP, when compared to RA treatment (shown in the upper four images of Figure 3). On the other hand, RA stimulated differentiation caused an increase in the neuronal protein level of β-3-Tubilin, as shown below in the second half of Figure 3.

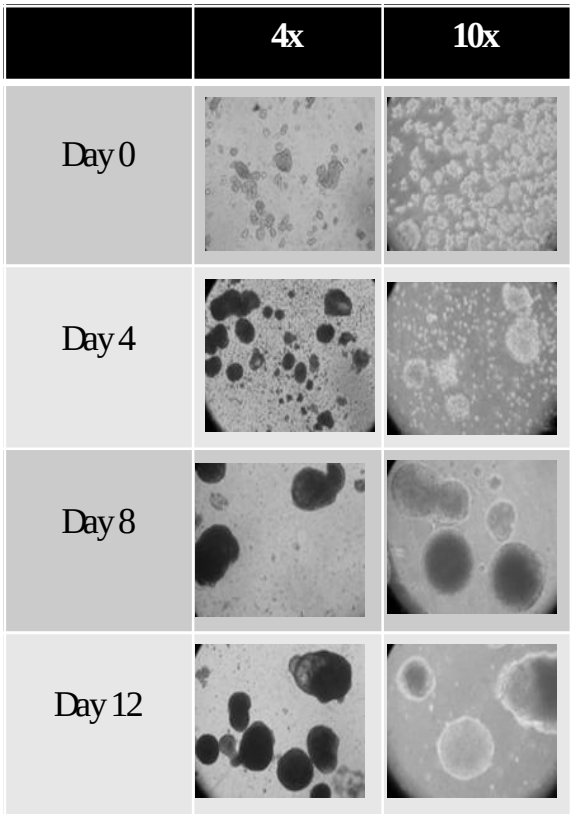
Figure 3. Stimulated differentiation of mESCs to astrocytes or neurons (bars = 50 um)

Treatment	Black and White	Merge with DAPI
cAMP	GFAP 	GFAP and DAPI 
RA	GFAP 	GFAP and DAPI 
cAMP	Beta-3-Tubulin 	Beta-3-Tubulin and DAPI 
RA	Beta-3-Tubulin 	Beta-3-Tubulin and DAPI 

Method of differentiation of ESCs to neurospheres in the bioreactor

To produce neurospheres from hESCs in a bioreactor, a protocol was adapted from a study that produced neurospheres from hESCs in suspension[13]. Below in Figure 4, bright field images show how hESC aggregates from the bioreactor mature in the stirred tank. These cells were taken from matrigel coated tissue culture surfaces on day 0, and dissociated into small clumps of cells using collagenase, and then seeded into the bioreactor using neuronal proliferation media for the first four days, followed by neuronal differentiation media.

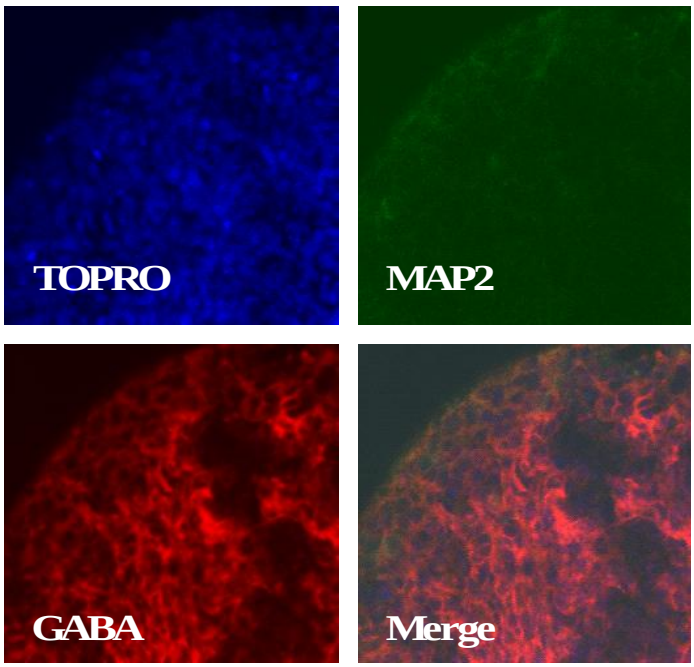
Figure 4. Bright field images of the maturation of neuropheres in a bioreactor



Analysis of neuronal proteins from neurospheres produced in the bioreactor

After 16 days in the bioreactor the neurospheres were removed and assessed for neuronal markers. Whole aggregates were stained with neuronal markers GABA, and MAP2, and as shown in Figure 5, when imaged on the confocal microscope, the neurosphere aggregates were stained positively for those markers.

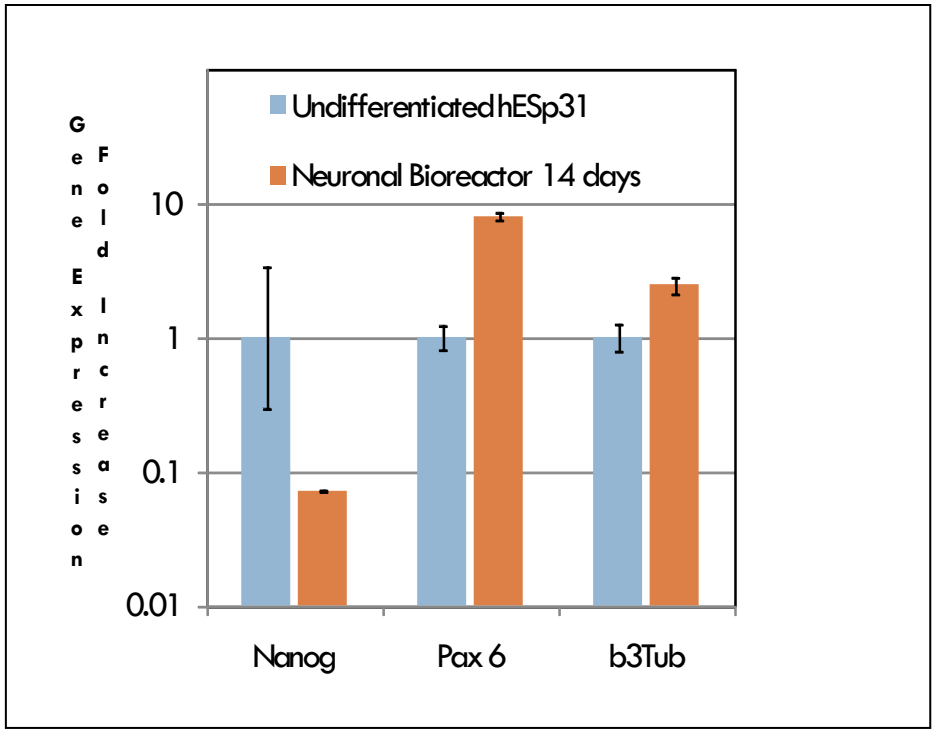
Figure 5. Confocal images of neuropheres taken from bioreactor



Analysis of neuronal RNA levels from neurospheres produced in the bioreactor

After 16 days in the bioreactor RNA was isolated from and then cDNA was produced and neuronal primers for PAX6 and β-3-Tubulin were tested along with stem cell marker NANOG. Levels of the stem cell marker decreased as the cells differentiated, while neuronal markers PAX6 and β-3-Tubilin increased on the log scale graph shown below in Figure 6.

Figure 6. Real-time PCR results of cDNA from Neurosphere produced in bioreactor.



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