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Differentiation of human embryonic stem cell (hESC) derived pyramidal neurons

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Abstract

The mammalian cerebral neocortex is a complex six-layered structure containing multiple types of neurons. Pyramidal neurons of the neocortex are formed during development in an inside-out manner, by which deep layer (DL) neurons are generated first, and upper layer (UL) neurons are generated last. Neurons within the six-layered neocortex express unique markers for their position, showing whether they are subplate, deep layer, upper layer, or Cajal-Retzius neurons. The sequential generation of cortical layers, which exists in vivo, has been partially recapitulated in vitro by differentiation of mouse embryonic stem cells (Gaspard et al., 2008) and human embryonic stem cells (hESC) (Eiraku et al., 2008). The timeline of generation of cortical neurons from hESC is still not well defined, and could be very important in the future of cell therapy. In this study we will define timeline for UL and DL neurons for our experimental paradigm as well as test the effects of fibroblast growth factors (FGF) 2 and 8 on this neuronal differentiation. Recent papers suggest that FGFs are critical for forebrain patterning (Storm et al., 2003). Neuronal differentiation after treatment with either FGF2 or FGF8 from hESCs will be examined and the proportion of specific neuronal markers will be analyzed using immunocytochemistry. Our results show that the generated pyramidal neurons will express DL and UL laminar markers in vitro as they do in vivo and that the presence of FGF8 in induction media creates a proliferative effect, while FGF2 induces hESC to differentiate at a higher rate.

Introduction

The study of human embryonic stem cells (hECS) their pluripotent potential is leading to immense possibility in the future of medicine. hESCs have the ability to differentiate into a variety of tissue cells when indicated to do so, including neurons. To be able to differentiate into neurons, hESCs must be induced into a neuronal fate in vitro. This could be achieved by subjecting the cells to a serum-free induction media with growth factors that allow them to take on the potential for neuronal differentiation. To start neuronal induction and differentiation, hESCs grown on irradiated mouse embryonic fibroblast (irrMEF) feeder cells should be mechanically
scraped off and placed in a suspension of induction media, in which they create clusters called embryoid bodies (EBs). After proliferating as EBs, they should be plated on an adhesion surface to flatten and form neural tube shaped groups of cells termed ‘rosettes’ that express early neuroectodermal markers such as Pax6 and Sox1 (Elkabetz et al., 2008). Once at rosette stage, the cells are at their highest potential for wide variety differentiation. It has been shown that by implanting rosette cells in vivo, they incorporate themselves into the brain and will test positive for β3-tubulin and nestin, two neurogenenic markers (Zhang et al., 2001) In vitro, these neuronal precursor cells have the ability to develop into different types of neurons when placed in environments with specific growth factors. It is important to isolate rosette cells and either freeze or differentiate them at this point (Elkabetz et al., 2008).

We introduced fibroblast growth factor 2 (FGF2) to embryoid bodies and then divided them into two groups. The cultures are then plated as rosettes on poly-D-lysine and laminin substrates and treated with either FGF2 or FGF8. Fibroblast growth factors are proteins that control embryonic neurogenesis by binding receptor tyrosine kinases in high-affinity, which subsequently activates multiple signal transduction pathways (Storm et al., 2001). FGF2 has been shown to increase the dendritic growth, migration and survival of neural stem cells while developing (Vergaño-Vera et al., 2009), while also being crucial to the proliferation of neuroepithelial cells (Zhang et al., 2001.)

At rosette stage, cells will be treated with either FGF2 or FGF8. Because of the inclusion of FGF2 or FGF8 in the medium, the majority of these progenitors are expected to produce forebrain pyramidal cells. FGF2 has been proven to allow an improvement of cell growth and colony quality (Zhang et al., 2001). It also directs developing neurons to be more forebrain specified. FGF8 is essential to the normal development of the forebrain; it is mainly expressed along the rostral-most point of the neural ridge, where most pre-anterior forebrain progenitors reside (Storm et al. 2003). Both FGF-2 and FGF-8 will be compared by observing the effect they have on the differentiation phase of the neuronal maturation.

After rosette isolation, differentiation will be initiated. Addition of neuronal growth factors, neurotrophin 3 (NT3) and brain derived neurotrophic factor (BDNF) in the cell culture serum-free media induces differentiation. Neuronal progenitor cells have intrinsic signaling, allowing them to follow natural corticogenesis in vitro (Gaspard et al., 2008). Pyramidal neurons, anterior and posterior, are characterized by their monoaxonal shape, excitable behavior and specific markers (Gaspard et al., 2008). They develop from progenitors in the ventricular zone (VZ), and then migrate to deeper and upper cortical layers. When mature, neurons are found in six different cortical layers. DL neurons express signaling genes such as CTIP2 and Sox5, while upper layer neurons...
express SATB2 and CUX1 (Fig. 1)(Gaspard et al., 2008, Leone et al., 2008)(Fig. 1). Using \textit{in vitro} studies to observe presence of the layer-unique markers allows the possibility create and quantify a natural model of corticogenesis. This marking method gives the ability to identify a timeline of pyramidal neuron positioning. Our goal is to create layer specific pyramidal neurons from hESC and quantify them using immunocytochemistry with the important laminar markers (Fig. 1).

Reproducing this natural timeline of hESCs through pyramidal neurons includes the difficulties of cell culture as well as the precise timing by which the hESCs and subsequent developing neurons must be in the presence of specific brain factors in order to mirror corticogenesis \textit{in vivo}. The intrinsic neocorticogenesis patterning has been found, but mechanisms are still largely unknown. By following the induction and differentiation neuronal media process with these neuronal factors as well as FGF2 and FGF8, it will be possible to observe the fate of neurons derived from stem cells leading to mechanistic clues and methods for future therapy. In the future, this study will allow a better understanding of cortical layer mapping \textit{in vitro} and the effects of fibroblasts growth factors 2 and 8 on the process of differentiation.

\textbf{Methods}

hESC culture and Induction of neuronal cell fate

Human Embryonic Stem Cell line (H9, University of Connecticut Health Center Stem Cell Core) was grown on top of irrMEF for approximately 1 week (n=3). The colonies were then scraped off and grown as a suspension in hESC media [(DMEM/F12, knockout serum replacer (KSR), β-mercaptoethanol, 2mM L-glutamine, nonessential amino acids (NEAA)] to form free-floating aggregates. After 4 days, the aggregates were switched into high concentration FGF2 containing media (8ng/mL). After the total
EB propagation for 8 days, the cell aggregates were transferred onto poly-D lysine and laminin coated plates for adhesion and rosette formation. At this point, the cells were switched into neuronal induction media (DMEM/F12, N2 supplement, B27 supplement, 2mM L-glutamine, 4ng/mL FGF) to start priming the cells for neuronal fate. Half of the cells were placed in NIM FGF2 (4ng/mL) and the other half in NIM FGF8 (4ng/mL). Cells were proliferated until rosette-shaped colonies were formed (Fig 2).

Differentiation of neurons from hESC

After 7 days in NIM with FGF2 or FGF8, the rosettes were digested with trypsin and plated as monolayer cultures on laminin-coated plates in NIM with FGF2 or FGF8 with Y-27632 (4ng/mL), a ROCK inhibitor (Fig 2). After one day to adhere, the monolayer cultures were switched to NT3 differentiation medium (DMEM/F12, 2mM L-glutamine, N2 and B27 supplements, NT3 [20ng/mL], BDNF [20ng/mL]). These cells were then fixed and stained on day 7, 14 and 21 from the start of differentiation.

Immunocytochemistry

Cell cultures (n=3) were fixed with 4% paraformaldehyde every 7, 14 and 21 days. After being rinsed with PBS, a blocking solution containing 5% normal goat serum (NGS) and 0.5% Triton-100 was placed on the coverslips for 30 minutes followed by incubation with primary antibodies (overnight, RT) and secondary antibodies (2h, dark RT). The coverslips were then mounted using Vectasheild mounting serum. The primary antibodies are presented in Table 1:
After incubation with primary antibodies, cells were washed with PBS, and the secondary antibodies were added. The secondary antibodies consisted of either goat anti-rabbit IgG Alexa 488 conjugated with fluorescein (1:200, Invitrogen) or goat anti-mouse IgG Alexa 568. For nuclear staining cells were incubated with ToPro (1:1000, Invitrogen) or/and DAPI.

Quantification

Cells were viewed under a tabletop Nikon fluorescent microscope or Laica confocal microscope. Each set of cells from days 7, 14 and 21 were quantified for the neuronal markers expressed at each time point. The number of β-III tubulin, an immature neuronal marker, was expressed as a percentage of total cells, stained with DAPI. Five random fields were quantified under 20X magnification.

Results

Two full rounds of cell cultures were completed allowing us to compare and contrast the effects of FGF2 and FGF8. hECS (H9, University of Connecticut Health Center Stem Cell Core) were successfully grown on irrMEF. They began as sharp edged cells with small colonies (Fig. 3A), grew into large, undifferentiated colonies (Fig. 3B) and were ready to be mechanically lifted and grown as undifferentiated cell aggregates, or embryoid bodies, for 8 days (Fig. 3C).

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Labeling</th>
<th>Type of antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>β3-tubulin</td>
<td>Immature neurons</td>
<td>Anti-mouse IgG</td>
<td>1:200</td>
</tr>
<tr>
<td>Ctip2</td>
<td>Deep layer neurons</td>
<td>Anti-rabbit IgG</td>
<td>1:200</td>
</tr>
<tr>
<td>SATB2</td>
<td>Upper layer neurons</td>
<td>Anti-mouse IgG</td>
<td>1:200</td>
</tr>
<tr>
<td>Sox5</td>
<td>Deep layer neurons</td>
<td>Anti-rabbit IgG</td>
<td>1:200</td>
</tr>
<tr>
<td>Oct3/4</td>
<td>Stem cells</td>
<td>Anti-rabbit IgG</td>
<td>1:200</td>
</tr>
<tr>
<td>Pax6</td>
<td>Neuroectodermal marker</td>
<td>Anti-mouse-IgG</td>
<td>1:200</td>
</tr>
<tr>
<td>Nestin</td>
<td>Neural stem cells</td>
<td>Anti-mouse IgG</td>
<td>1:200</td>
</tr>
</tbody>
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Table 1. Primary antibodies for immunocytochemistry. Table includes: Abbreviated name of protein stained, where this protein is expressed during development, type of immunoglobulin antibody, and working dilution factor.

Figure 3. Human embryonic stem cells in culture. Plated on irradiated mouse fibroblasts, hESCs begin as single cells and then form small colonies (A). (B) is a larger colony of hESCs ready to be transferred into a floating aggregate media. These floating undifferentiated aggregates are shown in (C) called neurospheres (NS).
The embryoid bodies (EB) were then plated on a poly-D lysine substrate to flatten as rosettes in neural induction media. Rosettes showed neural tube morphology (Fig. 4A,B), as well as neuronal precursor staining of nestin (Fig. 4C), Pax 6 (paired box gene), a gene regulating early progenitors to project to telencephalic neocortex (Molyneaux et al., 2007, Eiraku et al., 2008) (Fig. 4D), and Otx2 (orthodenticle homeobox 2)(Fig. 4E).

Figure 4. Immunocytochemical representation of rosettes. (A) and (B) are contrast images showing rosette morphology and neural tube similarity. Nestin expression for staining for neuron precursors is present (C), (D) for Pax6 and (E) Otx2 protein staining.
Laminar marker expression visualized via immunocytochemistry allowed us to track the DL or UL position of the differentiated cells. After one week in neuronal differentiation media with NT3 (20ng/mL) and BDNF (20ng/mL), almost all cells expressed nestin (Fig. 5A,B). Nestin is expressed in neuronal precursor cells and cells that are dividing during early development of the CNS and down-regulates after differentiation. It is clear that these cells are still undifferentiated. β3-tubulin, an immature neuronal marker, which would show differentiation, is expressed very rarely (Fig. 5D). DL laminar markers Ctip2 and Sox5 have begun to express, as shown in Fig. 5 B-D. CTIP2, (COUP-TF) interacting protein 2, plays a dynamic role in the axonal extension and growth of the correct pathway during development of the cerebral cortex (Arlotta et al., 2008). During first week in differentiation media culture, only CTIP2+ neurons are detected (30±3.8%)(Fig. 7). The expression of this protein is strong at one week and Sox 5, another DL marker involved in the neuronal subtype specificity (Lai et al., 2008) is less prevalent and weaker when compared to the cells fixed at two weeks (Fig. 6B,C).

In the second week of differentiation, the percentage increased (55±14.9%) and Satb2+ upper layer neurons appeared (2.2 ±1.9%)(Fig. 6B, 7) and Sox5, which was not expressed in every cell at week one, is now widespread, showing development (Fig 6C). It is clear that there is strong β3-tubulin in cells from week 2, shown in Figure 6 B-D. The Satb2+ cells along with strong DL neuronal expression
demonstrates temporal appearance of cortical neurons \textit{in vitro} from hESC.

In a previous study, it was shown that FGF8 is critical in development of the anterior forebrain (Storm et al., 2003) and FGF2 is crucial to forebrain neuronal preparation (Zhang et al., 2001). By adding these separate fibroblast growth factors to the induction media, we came across interesting observations. We tested this as well as the fate of neurons based on their layer specifications. Under identical conditions there were general differences in the general health and behavior of the cultured cells. FGF2 cells aggregated much more with fewer cells in culture than FGF8. However, cells with FGF8 administration showed far less differentiated morphology and a smaller number of $\beta_3$-tubulin+ immature neurons was noticed in these cultures (Fig. 8A-D). With induction media containing FGF2, at week 2 in differentiation media there was a average percentage of 7.5\%±4.9 expression of $\beta_3$-tubulin compared to the total number of cells shown by DAPI/ToPro expression (n=3). FGF8 inhibited this
much expression of β3-tubulin, only allowing 2.5%±.7 to express β3-tubulin (n= 3) (Fig. 8D). Furthermore, in week 3, there was an average of 23.3%±12 β3-tubulin expressing cells in FGF2 affected cells (n=4) and only a average of 1.2%±.8 from FGF8 cells (Fig. 8D). These data show that the FGF2 cells differentiated in a higher degree, while FGF8 cells proliferated instead; the cells would still retain the original β3-tubulin expression if they had previously differentiated. FGF2 induced differentiating cells to cluster into small colonies, showing high expression of β3-tubulin (Fig. 8B). In contrast, FGF8 kept cells in a monolayer, singlized culture, with few differentiated cells that had long bipolar processes (Fig. 8A).

![Image of hESC differentiation into pyramidal neurons](image)

**Figure 8.** Effects of FGF2 or FGF8 on hESC differentiation into pyramidal neurons. Cells affected with FGF8 show more proliferation, as with TOPRO cell body staining, and few cells differentiating into neurons stained with β3-tubulin (A). FGF2 (B,C) increases the amount of differentiated cells by showing clumps of neurons in which β3-tubulin is strongly expressed. From week 2 to week 3, the percentage of β3-tubulin expressing cells affected with FGF2 increased from 7.5%±4.9 to 23.3%±12, showing differentiation (D); that percentage increase was not found in FGF8 cells. Cells affected with FGF8 showed a decrease from 2.5%±7 at week 2 to 1.2%±8 at week 3, showing massive undifferentiated cell proliferation (D).

**Discussion**

Human embryonic stem cells are a popular focus of current research studies. Their ability to differentiate into brain cells, bone cells, muscle cells, and epithelial
cells with the proper extrinsic signaling opens wide doors to possibilities of helping devastating diseases without present cures. In this study we described in vitro generation of neocortical neurons from human embryonic stem cells and the effect of two growth factors, FGF2 and FGF8 on their differentiation. Major findings of the study are as follows: 1. Human cortical neurons follow the same temporal pattern of generation \textit{in vitro}, as \textit{in vivo}; 2. Two members of the FGF family, FGF2 and FGF8 have differential effect on neuronal differentiation when added in early phase of cell culture, during neuronal induction.

Acknowledging the fact that a human brain develops slower, this study shows in humans what Gaspard et al., 2008, discovered in rodents. Gaspard’s previous study showed that nestin down-regulated in between day 14 and 21 in mouse. Our images show that the down regulation begins week 2, or day 29 including EB culture (8days) and rosette formation (7days), showing an initiation of differentiation 2 weeks behind rodent down-regulation. Human stem cells are important to research because this difference between mouse and human stem cell development is very different. Humans, because of an extended lifespan, have more delayed dividing of embryonic stem cells than of a mouse. Downstream of slower division means later differentiation and expression of laminar markers, perhaps withholding the expression of Sox5 in human ES cells. So far, are not studies except one (Eiraku et al., 2008) which focused on the laminar specification of neurons derived from hESC.

In cortex deep layers 5 and 6, neurons express Ctip2 and Sox5 (Leone et al., 2008). After week one in differentiation media (NT3, BDNF 20ng/mL), Ctip2 was expressed in 30% of cells, showing that after week 1 of differentiation, 22 days in culture, there was axonal growth from forebrain DL neurons. In comparison, this is earlier than Eiraku et al. Sox5 was weakly expressed at week one, or day 22 after EB formation, and widespread in week 2, day 29. The probable reason for the difference in expression is the difference between human and rodent neuronal development.

The 2008 study of Eiraku et al., which shows DL staining of human neurogenic markers, did not describe UL markers of neurons developed from hESC. Our study shows Satb2 was shown to express after 2 weeks in differentiation media (29 days). Staining at week one for Satb2 (not shown) was negative, showing later expression and the presence of more mature neurons. Neurons that have migrated to the upper layers of the cortex express Satb2 and also Cux1 (not shown.) Satb2 is expressed in neurons in layers 2-4 that have projected into the cortical plate (Leone et al., 2008, Alcamo et al., 2008). This data and our experiment that shows Satb2 was expressed in a few cells after two weeks of differentiation media proves that these differentiated progenitor cells could now be layer 4 neurons. In sequence with Gaspard et al., it
follows that Satb2 would be expressed at 29 days in human as it was expressed in mouse at day 12, taking into account the delayed brain development in humans. This exemplifies the in vivo recapitulation of human ES cell differentiation in vitro.

The growth factors FGF2 and FGF8 have been shown to regulate neocortical patterning in neuronal progenitors. In the study of Storm et al. from 2003, it was found that an elimination or high dosage of FGF8 produced an apoptotic effect, where as lowering the dosage increased cell survival in rodent neuronal progenitors. Here we used a low dosage (4ng/mL) and showed that only 1.2% of cells differentiated into β3-tubulin neurons, in high contrast with the 23.3% from cells treated with FGF2. A more recent study by Storm et al. in 2006 showed that FGF8 mutations led to abnormal telencephalon development due to decreased cell survival and increased cell death, proving that FGF8 does have significant proliferative effects. While FGF2 is expressed throughout the developing CNS, FGF8 is tightly localized to specific regions of the developing brain and is expressed only in the embryo during early phases of proliferation and neurogenesis. FGF8 is expressed first at the rostral most point of the neural plate and then following neural tube closure, in the rostral midline of the telencephalon. FGF8 has a role in initial patterning of mouse telencephalon, but also in anterior-posterior patterning of the brain (Storm et al., 2003, Fukushi-Shigomori et al., 2001). Our future studies will test for this anterior-posterior patterning and progenitor projection when treated with FGF8 by focusing on markers such as BF-1, Otx2, Emx1 in rostral telencephalon, and COUP-TF1 in caudal telencephalon. By treating with varying dosages of FGF2 vs. FGF8, the underlying rostral or caudal fate of neuronal progenitors could be directed for future therapies.

In summary, this study developed a reproducible method for the production of DL and UL neurons in vitro, and shown that FGF2 has a more potent effect for directing neuronal progenitors toward differentiation than FGF8. While not the only significant reason for this study, an example to recreate neurons of specified layers is to assist in the therapy of amyotrophic lateral sclerosis (ALS). UL neurons send projections to subcortical targets, spine, pons, thalamus, and DL neurons send their dendrites of sensory-motor cortex to corticospinal neurons. In ALS, these neurons degenerate. With the specificity of neuronal cortical layering shown, the necessary human stem cell differentiation for the degeneration characteristic of ALS is possible in the future, making this study very relevant to potential therapies for degenerative diseases like ALS.
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