Directed Differentiation of Human Embryonic Stem Cells into Corticofugal Neurons Uncovers Heterogeneous Fezf2-Expressing Subpopulations

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Abstract

Understanding how neuronal diversity is achieved within the cerebral cortex remains a major challenge in neuroscience. The advent of human embryonic stem cells (hESCs) as a model system provides a unique opportunity to study human corticogenesis in vitro and to identify the mechanisms that promote neuronal differentiation to achieve neuronal diversity in human brain. The transcription factor Fezf2 is necessary and sufficient for the specification of subcerebral projection neurons in mouse. However, its function during human corticogenesis is poorly understood. This study reports the differentiation of a hFezf2-YFP hESC reporter line into corticofugal projection neurons capable of extending axons toward the spinal cord upon transplantation into neonatal mouse brains. Additionally, we show that triple inhibition of the TGFß/BMP/Wnt-Shh pathway promotes the generation of hFezf2-expressing cells in vitro. Finally, this study unveils the isolation of two novel and distinct populations of hFezf2-YFP expressing cells reminiscent of the distinct Fezf2-expressing neuronal subtypes in the developing mouse brain. Overall our data suggest that the directed differentiation of hESCs into corticofugal neurons provides a useful model to identify the molecular mechanisms regulating human corticofugal differentiation and survival.

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Introduction

Hundreds of different neuronal subtypes are generated in the central nervous system during development. Among these, cortical projection neurons are essential for high order cognitive and sensory functions in the human brain. Within the six-layered cerebral cortex, subcerebral projection neurons are located in layer 5 and extend their axons to the midbrain (corticocortical projection neurons), brain stem (corticobulbar neurons) or spinal cord (corticospinal motor neurons, or CSMNs). CSMNs are clinically important, as their degeneration has been implicated in upper motor neuron diseases such as Amyotrophic Lateral Sclerosis (ALS) [1,2]. Neurons from layer 6 project their axons to the thalamus (corticothalamic neurons), and together with CSMNs are referred to as corticofugal projection neurons. Due to their high relevance in human diseases, the molecular mechanisms that underlie the specification and differentiation of subcerebral projection neurons have been the subject of intense investigation.

Much progress has been made toward understanding the molecular mechanisms regulating the development of subcerebral projection neurons in mouse. Several transcription factors, including Fezf2, Bcl11b, Tbr1, Sox5, and Bhlhb5 are part of an interconnected gene network that regulates cortical neuron fate specification and differentiation [3–9]. Among them, the zinc-finger transcription factor Fezf2 (Forebrain Embryonic Zinc Finger 2) is both necessary and sufficient for the specification, differentiation and axon targeting of CSMNs in mouse [10–12]. During mouse brain development, Fezf2 is expressed in progenitor cells as early as embryonic (E) day E8.5, and continues to be expressed at high level in subcerebral projection neurons. Fezf2 null mice show a lack of subcerebral projection neurons and projections to the spinal cord [10,11]. Strikingly, the mutant neurons adopt the identity of other cortical projection neuron subtypes [4,10,11,13]. Furthermore, misexpression of Fezf2 in other neuron subtypes directs their axons to project toward the spinal cord [4,14,15]. Ultimately, these studies demonstrate that in mouse, the identity and differentiation of subcerebral neurons is achieved through repression of alternate neuronal subtype identities. Despite these advances, the molecular mechanisms regulating the development of human subcerebral neurons have not been directly investigated due to the lack of an appropriate model system in which to study human cortical neuron differentiation. However, the high conservation of FEZF2 protein between mouse and human, and the similar expression patterns of Fezf2 during human fetal brain development [16,17] suggest that Fezf2 is a specific marker for human subcerebral neurons.

The cerebral cortex is generated from the dorsal portion of the anterior neural tube. The signaling pathways that promote anterior neural fate and dorsal cell identities are thus likely to promote the differentiation of hESCs into cortical neurons. During early development, inhibition of the BMP signaling pathway by secreted molecules such as Noggin induce neuronal fate [18–20]. SB431542, a small molecule inhibitor of Smads 2, 3 in the nodal...
pathway [21], has been successfully used in conjunction with Noggin, referred to as dual Smad inhibition, in directing differentiating hESCs towards a dorsal neural fate [22]. During the formation of the nervous system, Sonic hedgehog (Shh) mediates the induction of ventral neurons [23], while its inhibitor, cyclopamine [24,25], has been shown in mouse embryonic stem cells (mESCs) to increase dorsal while repressing ventral identities [26]. In human cells, however, it is not known whether cyclopamine has the same effect.

Wnt genes encode a highly conserved family of secreted glycoproteins and play an essential role in the formation of the vertebrate nervous system [27]. However, the role of the Wnt/β-catenin signaling pathway in the generation of cortical neurons from hESCs is largely unknown. Conflicting reports in mESCs either claim that Wnt enhances neural differentiation [28,29], or that it inhibits neural fate [30,31]. Activating Wnt in mESCs has been associated with self-renewal [32]. In hESCs, the inhibition of Wnt was reported to convert hESCs-derived dorsal telencephalic progenitors to ventral progenitors [33], while its activation through GSK3β inhibition, has been reported to maintain hESCs pluripotency [34].

Overall, the specific signaling pathways promoting the generation of human subcerebral neurons including CSNs are largely unclear. In this study, we utilized a genetically modified hESC line in which a YFP reporter was targeted into the endogenous hFezf2 locus to investigate the differentiation of hESCs into cortical projection neurons [35]. We demonstrate that hESCs can differentiate into corticofugal neurons, including CSNs and corticothalamic neurons in vitro. Upon transplantation into neonatal mouse brains, the differentiated human cells can extend axons toward the spinal cord. Our in vitro hESC differentiation reveals two distinct hFezf2-YFP* subpopulations, similar to the Fezf2-expressing populations in the developing mouse cortex. Interestingly, these two cell populations are molecularly analogous to mouse corticothalamic and subcerebral neurons. Overall, our study show that hESCs-derived cortical projection neurons can be used as an effective model system to investigate the molecular pathways that regulate human CSMN differentiation, axon extension and survival.

Materials and Methods

All the human embryonic stem cell experiments and animal studies were performed in accordance with protocols approved by the IRB committees and IACUC at the University of California, Santa Cruz and performed in accordance with institutional and federal guidelines.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Quantitative Polymerase Chain Reaction (qRT-PCR)

RNA was extracted using RNasy (Qiagen) kit. RNA quality was evaluated using Agilent 2100 Bioanalyzer (Agilent technologies). The RNA samples were reverse-transcribed into cDNA using SuperScript III first strand synthesis system (Life Technologies). Quantitative PCR (qPCR) was performed using the Viia 7 real-time PCR (Applied Biosystems). Expression values (CT values) were normalized with two ubiquitously expressed endogenous reference genes, Beta-2-microglobulin (B2M) and Beta-actin. The normalized expression levels of the genes in the hFezf2-YFP<sup>hi</sup> and hFezf2-YFP<sup>neg</sup> cells were compared to those of the hFezf2-YFP<sup>neg</sup> cells according to the ΔΔCT method [36]. Each sample was run in triplicate and at least three biological replicates were performed for each gene. The primers for RT-PCR and qRT-PCR are listed in Table S1 and Table S2.

Stem Cell Culture

H9 (WA-09, passages 28 to 50) [Wicell Research Institute] cells were maintained on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) as described [37]. MEFs were plated onto coated growth factor reduced Matrigel plates (BD Biosciences) diluted 1:20 into DMEM-F12 medium (Life Technologies). H9 were cultured in Dulbecco’s modified Eagle’s medium-F12 (DMEM-F12) supplemented with 20% Knockout serum replacement, 0.1 mM MEM nonessential amino acids (MEM NEAA), 2 mM GlutaMAX, 0.55 mM, 2-mercaptoethanol (all from Life Technologies) and 0 ng/ml of human recombinant basic fibroblast growth factor (Fgf2) (Peprotech). Cells were fed daily and passaged every 4 days with 200 units/ml of collagenase IV (Life Technologies).

The hFezf2-YFP HUES-9 cells were generously provided by Drs. Binhai Zheng and Katherine Ruby from the University of California at San Diego, and used between 46-52 passages [35]. Both the hFezf2-YFP HUES-9 cells and the HUES-5 cells (passages 19 to 30) (Harvard University) were cultured in knockout Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 10% Knockout Serum Replacement, 10% Plasmanate (Bayer HealthCare), 0.1 mM MEM nonessential amino acids, 2 mM GlutaMAX, 0.55 mM, 2-mercaptoethanol (all from Life Technologies) and 15 ng/ml human basic Fgf added fresh to the medium daily. Cells were passaged every 4 days by trypsinization with 0.5% trypsin/EDTA (Life Technologies) or digestion with collagenase IV at 200 units/ml (Life Technologies) for embryoid body (EB) formation.

Neural Differentiation from hESCs

Embryoid bodies (EBs) were formed by gentle dissociation of undifferentiated hESCs from mouse feeder layer using 200 μg/ml of collagenase IV (Life Technologies). Dissociated cells were washed three times with hESC media and were allowed to settle by gravity to form EBs in N2 medium in an ultra low attachment plate overnight (Day 0). The next day (Day 1) different growth factors or their inhibitors: SB431542 (10 μM) (Sigma-Aldrich), recombinant Human Noggin (500 ng/ml), recombinant human Wnt3α (200 ng/ml) (both from R&D Systems), recombinant human DKK1 (250 ng/ml) (Peprotech), Cyclopamine (4 μM) (Biomol) were added to the N2 media. Feeding with growth factors was performed daily with a no growth factor condition used as a control. N2 media was composed of Dulbecco’s modified Eagle’s medium-F12 (DMEM-F12) medium supplemented with 0.1 mM MEM nonessential amino acids (MEM NEAA), 2 mM GlutaMAX, 1× N2 supplement (all from Life Technologies), Heparin (1 mg/ml) (Sigma-Aldrich) and human basic Fgf (10 ng/ml) (Peprotech). EBs remained for 6 days in suspension and were plated down on day 7 onto a growth factor reduced Matrigel coated plate (BD Biosciences). Feedings continued every day or every other day for 12 days. On day 12 cells were scraped from their support, resuspended in N2 media and plated down onto a new growth factor reduced Matrigel (BD Biosciences) coated plate. The next day, N2 media was replaced with freshly made differentiation media. The differentiation media was composed of Neurobasal medium supplemented with 2 mM GlutaMAX, 1× N2 supplement, 1× B-27 supplement minus vitamin A (all from Life Technologies), cAMP (1 μM), L-ascorbic acid (100 μM) (both from Sigma-Aldrich), and Human Recombinant Brain-Derived Neurotrophic Factor (BDNF) (10 ng/ml), Human Recombinant Gial-Derived Neurotrophic Factor (GDNF) (10 ng/ml) and
Insulin-like Growth Factor-I (IGF-I) (10 ng/ml) (all from Peprotech). During days 13–32, cells were fed daily or every other day with differentiation medium to induce differentiation. Differentiated cells were passaged on day 21 onto a growth factor reduced Matrigel (BD Biosciences) coated plates at 1:20 dilution and fed as described above.

Flow Cytometry and Cell Sorting

Cells were gently dissociated using 0.5% Trypsin/EDTA (Invitrogen) followed by a neutralization step with hESC culture media containing serum as described above. Cells were then resuspended into 1XPBS, 0.5 M EDTA and 2% FBS (all from Life Technologies). Prior to sorting, propidium iodide (1 μg/ml, Molecular Probes, Invitrogen) was added to each sample at 1:5,000 dilution to distinguish between live and dead cells. Cells were sorted on a fluorescence-activated-cell sorter FACS ARIA II (BD Biosciences) with a 100 μm nozzle at 22 PSA referred to as “gentle FACS” [38]. Sorted cells were collected in 500 ul of HBSS media supplemented with 20 mM glucose, 10% FBS, 1x penicillin/streptomycin (Invitrogen). Gating was performed using unstained, or negative controls that were not expected to express Fezf2. Data Analysis was performed using the FlowJo software (Tree Star, Inc). All experiments, including analysis and sorting were repeated at least three times.

Immunohistochemistry

Dissociated Fezf2-YFP cells and H9 cells were fixed with 4% paraformaldehyde in PBS for 15 minutes, washed twice in PBS, permeabilized with 0.1% Triton X-100 in PBS for 20 minutes, and then blocked in 5% horse serum for 1 hour. Cells were incubated overnight at 4°C with primary antibodies. After 3 PBS washes, cells were incubated in the appropriate secondary antibodies for 1 hour at room temperature protected from light. Cells were washed 3 times in 1XPBS and stained with DAPI. The primary antibodies used in this study for mouse and human cells (Table S3) were the following: chicken anti-GFP (Life Technologies), rat anti-BCL11B (Abcam), rabbit anti-TBR1 (Abcam), rabbit anti-NFIA (Active Motif), rabbit anti-NFIB (Active Motif), mouse anti-HuNu (Millipore), mouse TuJ1 antibody (Covance), rabbit anti-TBR2 (Abcam), rabbit anti-PAX6 (Covance), mouse anti-Nestin (Millipore). Secondary antibodies were as follows: Alexafluor 488, 594 and 647 (Life Technologies).

Analysis of Fezf2-GFP Mouse Cells

The brains of Fezf2-GFP BAC transgenic mice (GENSAT) were dissected at P0 (n = 3) and immediately dissociated using Papain dissociation system (Worthington Biochemical Corporation). GFP positive and negative populations were sorted using a FACS ARIA II (BD Biosciences). 50,000–75,000 cells were sorted and processed for RNA isolation using RNAeasy Plus mini kit (Qiagen) and the quality assessed using a Bioanalyzer 2100 (Agilent Technologies). cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen).

**Fezf2 In situ Hybridization**

The in situ hybridization using Fezf2 probe was performed as described [39].

Transplantation

Neural rosettes from day 12–20 were mechanically isolated from in vitro differentiated hFezf2-YFP cells, dissociated using 0.5% Trypsin/EDTA (Invitrogen) and resuspended in serum containing media. Dissociated cells were transplanted (500–2,000 cells) using ultrasound guided imaging (VisualSonics) targeting the deep layers of the mouse motor cortex. Transplants were performed using postnatal day 0 (P0) CD-1 wild-type mice (n = 40) (Charles River Laboratories, Hollister, CA). Mice were sacrificed at P90, intracardiac perfusion were performed with fresh 4% PFA and embedded in O.C.T compound (Sakura Finetek) and kept at −80°C until sectioning was performed using a Microme sliding microtome (ThermoScientific).

**Results**

**Differentiation of hESCs into Cortical Projection Neurons**

To generate cortical neurons from hESCs, we developed a differentiation protocol based on both current knowledge of early cortical development in mice and established hESC neural differentiation approaches [22,40]. A schematic of our neural differentiation strategy is shown in Figure 1A, and involves three steps spanning 30 days. First, neural induction was initiated by the generation of embryoid bodies (EBs), in which undifferentiated H9 or HUES5 cells were allowed to aggregate in suspension culture, mimicking the spatial organization of the morula stage of development. Then, neural rosette formation was initiated by plating down EBs onto matrigel, supplemented with SB431542 and Noggin [20,22,41] for the first 12 days of culture. In the final neural differentiation stage, cells were grown in a culture medium that promoted cell survival and allowed neuronal differentiation.

Expression of forebrain progenitor markers Pax6, Emx2, Nestin, Otx2, Foxg1, Tbr2 and Fezf2 [42–45] was confirmed using reverse transcriptase-polymerase chain reaction (RT-PCR) (Figure 1B) and immunohistochemical staining (Figure 1C–E). Pax6, Emx2 and Foxg1 showed a progressive increase in expression throughout differentiation until day 32 as assayed by RT-PCR (Figure 1B). The intermediate progenitor marker Tbr2 was expressed concurrently with the immature neuronal marker βIII tubulin (TuJ1) as determined by immunohistochemistry staining (Figure 1E). Subcortical neuron markers Fezf2 and Nb6 [46], corticothalamic neuron marker Tbr1, and CSMN marker Bcl11b were robustly expressed at day 32 (Figure 1B, F–H). Our neural differentiation protocol did not solely generate cortical neurons, as the expression of ventral cell marker Ascl1 [47] was detected at day 32 (Figure 1B). Expression of the pluripotent marker Nanog [48] was also maintained throughout the neuronal differentiation procedure, suggesting that some cells retained their stem cell characteristics throughout differentiation (Figure 1B). However, another pluripotency gene, Pou5F1 [49] (also known as Oct4) showed significant down-regulation upon differentiation (Figure 1B).

**Fezf2 is a specific marker for corticofugal neurons in mouse and human [16,17]. To further investigate the hESC-derived subcerebral neurons, we differentiated a genetically modified hESC line (Fezf2-YFP HUES9) in which the YFP reporter was inserted into the endogenous human Fezf2 gene locus [35]. Following our neural differentiation protocol, many Fezf2-YFP-expressing cells extended long neuronal processes by day 40 (Figure 1F, J and Figure 2). In addition to H9 (Figure 1B–H) and Fezf2-YFP HUES9 (Figure 1I, J) lines, our neuronal differentiation protocol also successfully generated cortical projection neurons, including CSMNs, from HUES 5 cells (Figure S1).

Transplanted hESCs-derived Fezf2-YFP-expressing Neurons Extend Axons in the Mouse Brain

To determine whether in vitro differentiated corticofugal neurons could integrate and project their axons to appropriate targets, we tested their ability to extend axons subcortically...
upon transplantation into neonatal mouse cortices. We dissociated neural rosettes from day 12 cultures and injected them using ultrasound guided imaging into the P0 cortex of wild-type mice. Ninety days after transplantation, the recipient mice were sacrificed and their brains analyzed with GFP and human nuclei (HuNu) antibodies by immunohistochemistry (Figure 2). The expression of the human nuclear antigen confirmed the human origin of the transplanted cells (Figure 2B). Consistent with corticofugal identity, many Fezf2-YFP+ cells were observed extending axons from the cell injection site in the cortex to subcortical targets (Figure 2A). Fezf2-YFP+ axons were observed in the white matter (Figure 2C), and turning into the internal capsule (Figure 2D). YFP+ axons were also observed at the base of the ventral forebrain (Figure 2E), and in the pyramidal decussation on their way to the spinal cord (Figure 2F). Injected Fezf2-YFP cells in the cortex expressed the CSMN marker BCL11B (Figure 2G). These experiments demonstrated that transplanted hCSMs integrated into the host brains and extended axons toward subcortical targets, similar to endogenous Fezf2-expressing projection neurons.

Figure 1. In vitro hESC neural differentiation yields cortical neurons. (A) Schematic representation of the strategy for differentiating hESCs into cortical neurons, displaying culture and treatments conditions. (B) RT-PCR of H9 cells shows differential expressions of pluripotent markers (Pou5F1, Nanog), dorsal progenitors (Pax6, Emx2, Otx2, Foxg1), cortical neurons (Fezf2, Nfib, Tbr1), and ventral marker (Ascl1) at neural differentiation day-6, 12 and 32. (C–E) Immunofluorescence staining of differentiated H9 cells shows expression of neural progenitor markers NESTIN, PAX6, TBR2 and immature neuronal marker Tuj1. (F–H) Immunohistochemistry shows that differentiated H9 cells express cortical neuron marker BCL11B (F), Nfib (G) and TBR1 (H). Scale bars in C, D, 50 μm; E, 25 μm; F–H, 100 μm. (I) Immunofluorescence staining of differentiated Fezf2-YFP-HUES 9 reporter line on day 40 with GFP antibody. Scale bar, 200 μm. (J) Differentiated Fezf2-YFP reporter line expressing GFP and the human specific nuclear antigen. Scale bar, 25 μm. Abbreviations: DAPI, 4′,6′-diamidino-2-phenylindole; YFP, yellow fluorescent protein; GFs, growth factors; doi:10.1371/journal.pone.0067292.g001
Differentiated hESCs Contain Two Distinct Fezf2-YFP+ Sub-populations

To identify the culture conditions that efficiently promote hESC differentiation into CSMNs, we differentiated Fezf2-YFP HUES9 cells under different growth factor conditions. We subsequently performed Fluorescence Activated Cell Sorting (FACS) analysis at day 30 of differentiation to quantify the percentage of Fezf2-expressing cells. Our goal was to identify the culture conditions most efficient at promoting the generation of Fezf2-YFP-expressing neurons. Treatment with SB431542, Noggin, DKK1 and cyclopamine, as described in Figure 1, resulted in an increase in the percentage of Fezf2-expressing cells compared to the no growth factor control (Figure 3C). Interestingly, under these differentiation conditions, in addition to a Fezf2-YFP2 population, we consistently observed two distinct Fezf2-YFP+ cell populations (Figure 3A,C): cells expressing high levels of Fezf2-YFP (YFPHi) and cells expressing low levels of Fezf2-YFP (YFPLow) (Figure 3A). To confirm the presence of these Fezf2-YFP+ populations, we performed qRT-PCR on each sorted population to test the expression levels of the human Fezf2 transcript. As expected, Fezf2 was highly expressed in the YFPHi subpopulation compared to the Fezf2-YFP2 cell population F(2,6) = 8.655; p = 0.0171; n = 3) (Figure 3B), whereas expression of Fezf2 in the YFPLow population was intermediate between Fezf2-YFP2 and Fezf2-YFPHi populations (Figure 3B). Interestingly, both YFPHi and YFPLow populations were observed even when differentiation occurred in the absence of growth factors (Figure 3C, first panel). Thus, our Fezf2-YFP HUES9 neural differentiation protocol generated two novel and distinct Fezf2-YFP+ populations (Figure 3A,C), suggesting they might exhibit different molecular characteristics.

Inhibiting Wnt Signaling Promotes the Generation of Fezf2-YFP-expressing Cells

Next we sought to determine whether activating or inhibiting specific signaling pathways during the first 12 days of the neural differentiation protocol affected the efficiency of generating Fezf2-YFP+ cells. Interestingly, the frequency of Fezf2-YFP2 cells (Figure 4B) on day 30 was consistently higher (range: 23–26.7%, n = 4 per differentiation condition) than the frequency of Fezf2-YFPHi cells (range: 5–10.8%, n = 4 per condition) (Figure 4A). In addition, Fezf2-YFPHi and Fezf2-YFPLow subpopulations respond-

Figure 2. Human ESCs-derived Fezf2-YFP-expressing neurons extend axonal projections in the mouse brain at P90. (A) Schematic of sagittal mouse brain indicates the location of panels B–F. YFP+HuNu+ cells were present in the injection site in the cortex (B), some YFP+ axons were present in the white matter (C, D), at the base of the forebrain (E), and pyramidal decussation (F). (G) Transplanted hFezf2-YFP cells at the injection site in the deep layer of the cortex expressed the CSMN marker BCL11B. Mouse layer 5 neurons expressing BCL11B are indicated by white arrow. Scale bars: 100 μm (B, C, F, G); 200 μm (D, E).

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ed differently to the 5 treatments conditions tested, which included 1) no growth factor, 2) dual SMAD inhibition, 3) dual SMAD inhibition plus Dickkopf-1 (DKK1, inhibiting Wnt signaling pathway), 4) dual SMAD inhibition plus cyclopamine (inhibiting Shh pathway), and 5) dual SMAD inhibition plus DKK1 and cyclopamine.

The generation of Fezf2-YFP\textsuperscript{Hi} cells was more sensitive to inhibition from the TGFß/BMP/Wnt-Shh signaling pathways as compared to the Fezf2-YFP\textsuperscript{Low} population (Figure 4A). Inhibition of the Wnt pathway using the secreted protein DKK1 induced a 2-fold increase in the Fezf2-YFP\textsuperscript{Hi} subpopulation over the no growth factor control (Figure 4A). A similar increase in induction of the Fezf2-YFP\textsuperscript{Hi} subpopulation was observed upon inhibition of Shh signaling with cyclopamine. However, we did not observe further enhanced induction of the Fezf2-YFP\textsuperscript{Hi} population when using both DKK1 and cyclopamine, suggesting a lack of synergism between inhibiting these two pathways. In contrast to the YFP\textsuperscript{Hi} population, generation of YFP\textsuperscript{Low} cells was not significantly influenced by inhibition of TGFß/BMP, Shh or Wnt signaling pathways (Figure 4B). Despite the ability to induce YFP\textsuperscript{Hi} cells by growth factor treatment, the percentage of YFP\textsuperscript{Hi} expressing cells was consistently lower compared to the YFP\textsuperscript{Low} cells. The difference in response to inhibition of signaling pathways, as well as the difference in population sizes suggested that Fezf2-YFP\textsuperscript{Low} and Fezf2-YFP\textsuperscript{Hi} cells represented two distinct populations.

To further determine the effects of activating and inhibiting Wnt signaling on generating Fezf2-YFP-expressing cells from hESCs, we next assessed the effect of Wnt activation by addition of Wnt3a to the media. We observed an almost complete loss of both Fezf2-YFP\textsuperscript{Hi} and Fezf2-YFP\textsuperscript{Low} populations upon addition of Wnt3a (Figure 4C, D). Compared to inhibition of Wnt signaling with DKK1, the percentage of Fezf2-YFP\textsuperscript{Hi} cells at day 30 showed a 39.8 fold decrease (n = 3) and the percentage of Fezf2-YFP\textsuperscript{Low} cells showed a 10.65 fold decrease (n = 3) when Wnt3a was added to the media (Figure 4C,D). Moreover, when we substituted Wnt3a with the small molecule Wnt agonist CHIR99021, the generation of Fezf2-YFP-expressing cells was completely inhibited (Figure 4E). Wnt activation therefore seems to have strong inhibitory effect on the generation of both Fezf2-YFP\textsuperscript{Hi} and Fezf2-YFP\textsuperscript{Low} populations. Collectively, these experiments demonstrate that the
Figure 4. TGFβ/BMP/Wnt-Shh triple inhibition activates Fezf2 expression in hESCs. (A) Quantification by FACS of differentiated hFezf2-YFP cells under different conditions shows a 2.2 fold increase in efficiencies in generating hFezf2-YFP^{Hi} subpopulation using SB+NG+DKK1 and SB+NG+Cyclo treatments compared to a no growth factor control F(4,20) = 13.74) p = 0.0235. Error bars represent standard error of mean, sem (n = 4). p and F values were assessed by one-way ANOVA with Tukey’s post-test). The efficiency of generating Fezf2-GFP^{Hi} cells was in the range: 5–10.8%, n = 4 per condition, quantified by FACS. (B) FACS analysis of differentiated hFezf2-YFP cells shows that the efficiencies to generate hFezf2-YFP^{Lo} subpopulation were similar under different conditions (range: 23–26.7%, n = 4) but yielded an overall higher percentage than the Fezf2-GFP^{Hi} population. (C) Representative FACS histograms and FACS dot plots showing differentiated hFezf2-YFP cells under Wnt activation (Wnt3a) and Wnt inhibition (DKK1). (D) Quantification of the efficiencies of generating hFezf2-YFP^{Hi} and hFezf2-YFP^{Lo} subpopulations under Wnt activation and Wnt inhibition conditions. Student t-test, unpaired, two-tailed, Welch’s corrected t = 3.91; p = 0.0298 (for low) and t = 6.329; p = 0.0080 (for high). (E) FACS histogram showing inhibition of both hFezf2-YFP^{Hi} and hFezf2-YFP^{Lo} populations under treatment with the Wnt agonist CHIR99021 at day 30. doi:10.1371/journal.pone.0067292.g004
differentiation of hFezf2-YFP-expressing populations and Fezf2 expression was robustly activated by the triple inhibition of TGFβ/BMP/Wnt-Shh signaling.

Differentiated hFezf2-YFP<sup>Hi</sup> and hFezf2-YFP<sup>Low</sup> Populations Recapitulate Fezf2 Expression during Mouse Cortical Development

To determine whether the hFezf2-YFP<sup>Hi</sup> and hFezf2-YFP<sup>Low</sup> populations arose from a cell culture artifact or were biologically relevant, we analyzed Fezf2 expression in the developing mouse cortex to determine if these two populations were present in vivo. Since a FEZF2 antibody suitable for immunohistochemistry is not available, we performed in situ hybridization to detect Fezf2 expression at P0 in the mouse cortex (Figure 5A). As previously reported [10], Fezf2 mRNA expression was observed in two different cortical layers: a higher level of Fezf2 expression was detected in layer 5, while a lower Fezf2 expression level was detected in layer 6 (Figure 5A). To further investigate whether there were two distinct Fezf2<sup>Hi</sup> and Fezf2<sup>Low</sup> populations in the developing mouse cerebral cortex, we analyzed cell populations in the Fezf2-GFP BAC transgenic mouse containing a modified bacterial artificial chromosome in which an EGFP open reading frame was inserted at the start codon of the mouse Fezf2 gene.

We dissected cortices from P0 Fezf2-GFP BAC mice, dissociated the cells, and analyzed GFP-expressing cells by FACS (Figure 5B). Dissociated cells from the cerebellum of Fezf2-GFP BAC mice, which do not express Fezf2, were used as the negative control. We found that two distinct Fezf2-GFP-expressing populations existed in the PO mouse cortex (Figure 5B). To directly examine the expression levels of endogenous Fezf2 mRNA in these populations, we sorted Fezf2-GFP<sup>Neg</sup>, Fezf2-GFP<sup>Hi</sup>, and Fezf2-GFP<sup>Low</sup> populations, and performed qRT-PCR (Figure 5C). Fezf2 was not expressed in Fezf2-GFP<sup>Neg</sup>, but was highly expressed in Fezf2-GFP<sup>Hi</sup> population with a 33-fold increase (n = 3) over the Fezf2-GFP<sup>Low</sup> population (Figure 5C). These results demonstrated that two Fezf2-expressing populations existed in the developing mouse cortex and could be successfully isolated by FACS, therefore suggesting that the Fezf2-YFP<sup>Hi</sup> and Fezf2-YFP<sup>Low</sup> cells generated from hESCs were biologically relevant, and likely recapitulated the two populations of Fezf2-expressing neurons present during mammalian cortical development.

Differentiated Fezf2-YFP<sup>Hi</sup> and Fezf2-YFP<sup>Low</sup> Cells Express Corticofugal Neuron Markers

Next, we sought to determine the molecular identities of the differentiated Fezf2-YFP<sup>Hi</sup> and Fezf2-YFP<sup>Low</sup> cell populations by comparing their gene expression. We first investigated whether

![Figure 5. Fezf2-GFP BAC transgenic mice contain two distinct Fezf2-GFP<sup>+</sup> populations. (A) In situ hybridization shows that a P0 wild-type mouse cortex contains layer 5 neurons expressing Fezf2 at a high level and layer 6 neurons expressing Fezf2 at a low level. (B) Representative FACS histograms and dot plots of P0 Fezf2-GFP BAC transgenic mice show that the cerebellum contains a mFezf2-GFP<sup>Neg</sup> population, and the cerebral cortex contains mFezf2-GFP<sup>Hi</sup> and mFezf2-GFP<sup>Low</sup> populations. (C) Quantitative RT-PCR analysis of mFezf2 mRNA expression levels in sorted mFezf2-GFP<sup>Hi</sup>, mFezf2-GFP<sup>Low</sup> and mFezf2-GFP<sup>Neg</sup> sub-populations.](http://www.plosone.org/figure/10.1371/journal.pone.0067292.g005)
these populations differed in their progenitor versus post-mitotic neuronal states by performing qRT-PCR analysis on the hFezf2-YFP-expressing populations at day 30. Indeed, Fezf2 is a gene expressed during early mouse development [E8.5] and continues to be expressed in post-mitotic neurons in subcortical projection neurons [11]. Expression levels of neural progenitor markers Pax6 and Nestin were not significantly different between Fezf2-YFP<sup>Hi</sup> and Fezf2-YFP<sup>Low</sup> populations at day 30 (Figure 6A). Cell cycle analysis based on DNA content by FACS confirmed that Fezf2-YFP<sup>Hi</sup> and Fezf2-YFP<sup>Low</sup> cell populations shared similar cell cycle kinetics and that approximately 20% of the cells were proliferating in both populations (Figure 6B).

We next investigated whether differentiated hFezf2-YFP<sup>Hi</sup> and hFezf2-YFP<sup>Low</sup> populations preferentially expressed markers for cortical projection neurons of layer 5 and/or 6, since in situ hybridization revealed that Fezf2 was expressed in layers 5 and 6 of the mouse cortex, where subcerebral and corticothalamic neurons are respectively located (Figure 5A). Cortical neuron markers expressed at a high level in layer 5 subcerebral neurons, and low level in layer 6 corticothalamic neurons in mice include Nfib, Bcl11b, and Sox5 (Figure S2), in addition to Fezf2 (Figure 5A). When hFezf2-YFP<sup>Hi</sup>, hFezf2-YFP<sup>Low</sup> and hFezf2-YFP<sup>Low</sup> populations were tested for these markers by qRT-PCR, Nfib, Bcl11b and Sox5 showed the general trend of being more highly expressed in hFezf2-YFP<sup>Hi</sup> compared to hFezf2-YFP<sup>Low</sup> cells (Figure 7A, C, I). Conversely, genes expressed at high levels in layer 6 corticothalamic neurons in mice, such as Nfib [50] (Figure S3) showed elevated levels of expression in hFezf2-YFP<sup>Low</sup> cells as compared to Fezf2-YFP<sup>Hi</sup> cells (Figure 7G). These results suggested that hFezf2<sup>Hi</sup> cells were more similar to layer 5 subcerebral neurons whereas the hFezf2<sup>Low</sup> population was more similar to layer 6 corticothalamic neurons. However, expression of another layer 6 corticothalamic marker, Tbr1 (Figure S3), showed a highly elevated expression in the Fezf2-YFP<sup>Hi</sup> population, with a 2 fold increase over Fezf2-YFP<sup>Low</sup> (Figure 7E). In addition, expression levels of Dapsh2 (Figure S3) were comparable between the Fezf2-YFP<sup>Hi</sup> and the Fezf2-YFP<sup>Low</sup> cells (Figure 7J). These results suggested that hFezf2-YFP<sup>Hi</sup> population showed both layer 5 and 6 (corticospinal and corticothalamic) molecular characteristics. The ventral marker Dlx5 [51] was not expressed in either Fezf2-YFP<sup>Hi</sup> or Fezf2-YFP<sup>Low</sup> populations, confirming the enrichment of corticofugal neurons in hFezf2<sup>Hi</sup> cell populations (Figure 7K). Immunohistochemical analysis of differentiated hFezf2-YFP<sup>Hi</sup> cells further confirmed that hFezf2-YFP<sup>Hi</sup> cells expressed Nfib, Bcl11b, Tbr1, and Nfia proteins (Figure 7B, D, F, H). These results suggest that Fezf2-YFP<sup>Hi</sup> is most similar to layer 5–6 projection neurons and Fezf2-YFP<sup>Low</sup> cells more similar to layer 6. Taken together, these data confirm that hFezf2-YFP<sup>+</sup> cells represent deep-layer corticofugal projection neurons.

Discussion

Human embryonic stem cells allow the manipulation of cellular signaling pathways thus providing researchers with an in vitro tool to assess human cortical cell fate specification. While well studied in mouse models [4,5,7,8,10,11,15,52,53], modulation of signaling pathways that regulate projection neuron fate is challenging in human cells largely due to the lack of relevant models. Here we report the successful generation of corticofugal neurons, including corticospinal and corticothalamic neurons from human ESCs that are able to integrate upon transplantation in a manner suggesting corticofugal identity.

Our study identified two distinct Fezf2<sup>Hi</sup> subpopulations in differentiated hESCs that are reminiscent of Fezf2-expressing subpopulations in the mouse cortex. The two Fezf2-expressing cell populations isolated in this study demonstrate that Fezf2-expressing cells exist in phenotypically distinct states, with one subpopulation expressing layer 5, 6 markers, and the other expressing layer 6 markers. While the Fezf2<sup>Hi</sup> population, predominantly expressed layer 5 markers, the corticothalamic marker (layer 6) Tbr1 was also highly expressed in this population. In addition, Dapsh2, a gene highly expressed in layer 6 neurons in mouse (Figure S3), showed comparable expression levels between the human Fezf2-YFP<sup>Low</sup> and Fezf2-YFP<sup>Hi</sup> populations. These results indicate that the Fezf2-YFP<sup>Hi</sup> population was not strictly analogous to CSMN (layer 5), but instead exhibited molecular characteristics of deep layer projection neurons in general. It is also possible that human subcortical neurons are different from the mouse subcortical neurons, and that the different gene expression levels observed between the mouse subcerebral and corticothalamic neurons may not be identical in human subcortical projection neurons. We also investigated the formation of the two Fezf2-YFP<sup>Hi</sup> populations after day 30 and observed whether a potential conversion of Fezf2-YFP<sup>Low</sup> to a Fezf2-YFP<sup>Hi</sup> population was occurring. Our data showed that Fezf2<sup>Hi</sup> subpopulations were still clearly distinct after day 100 (data not shown), suggesting that Fezf2-YFP<sup>Low</sup> is not a precursor of Fezf2-YFP<sup>Hi</sup> but instead a bona fide population. However, since the human ES cell differentiation procedure did not fully recapitulate the in vivo brain development condition, it is possible that even though we were able to generate neurons with subcortical neuron features, chromatin state and gene expression levels in the differentiated neurons were not identical to endogenous human subcortical neurons. Our findings highlight the use of the hESC system not only to dissect signaling pathways affecting neuronal differentiation, but also to identify cell heterogeneity within a population of differentiated neurons.

**Figure 6.** Differentiated hFezf2-YFP cells at day 30 share similar cell cycle kinetics. (A) Quantitative RT-PCR analysis of progenitor markers, Pax6 and Nestin, on differentiated and sorted cells at day 30. (B) Flow cytometry analysis of cell cycle properties of differentiated and sorted hFezf2-YFP cells. The cells were stained with propidium iodide to reveal DNA content. Abbreviation: PI, propidium iodide.

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Figure 7. Differentiated hFezf2-YFP\textsuperscript{Hi} and hFezf2-YFP\textsuperscript{Low} cells express corticofugal neuron markers. (A) Quantitative RT-PCR analysis for \textit{Nfib} mRNA expression on sorted hFezf2-YFP subpopulations (F(2,6) = 7.405; p = 0.0240). (B) Immunofluorescence staining showing NFIB expression in differentiated hFezf2-YFP\textsuperscript{+} cells at day 35. (C) Quantitative RT-PCR analysis of \textit{Bcl11b} mRNA expression on sorted hFezf2-YFP subpopulations. (D)
Further delineation of the characteristics of these two populations by transplant experiments, which have thus far been difficult to execute due to low live cell recovery rate after FACS, would provide more information on their heterogeneity and distinct functions.

Previous studies have established a role for TGFβ/BMP inhibition using the small molecule SB431542 and the BMP inhibitor Noggin in the differentiation of hESCs towards a neuroectodermal fate [22,54]. Motor neurons have been differentiated from induced pluripotent stem cells (iPS) or hESCs using Shh agonists and retinoic acid [55–58]. Recently, the retinoid-signaling pathway induced by vitamin A treatment was shown to efficiently generate cortical neurons from hESCs and iPS cells [59]. Using hFezf2-YFP as a marker, we investigated the effects of different signaling pathways on generating corticofugal neurons. Our study shows that the inhibition of both Wnt and Shh pathways enriches for hFezf2-YFP-expressing neurons. Landmark studies in zebrafish and mouse model systems have established that Wnt antagonism is required for proper telencephalic patterning early in development [60–63]. Fezf2 is expressed from E8.5 in early mouse development and functions in the rostro-caudal (anterior-posterior or A/P) patterning of the forebrain [64]. The expression of Fezf2 early in development and its function in A/P axis places this gene at the crossroads of important patterning events involving the Wnt pathway, and could explain the complete repression of hFezf2-YFP by Wnt in vitro. Incidentally, Fezf2 was first identified in a DKK1 overexpression screen in zebrafish, and showed low or no expression in embryos overexpressing Wnt8b [65]. This study further demonstrated that Fezf2 overexpression in zebrafish inhibited Wnt1 expression. A separate study showed that Fezf2 was able to rescue a headless (hdl/tcf3) mutant embryo, encoding a Wnt transcriptional repressor [66]. Overall, these studies suggest that Wnt and Fezf2 act in a common pathway and that Wnt signaling might act upstream of Fezf2 expression on sorted hESCs.

**Conclusions**

Overall, this study demonstrates that hESCs are a useful model system to dissect signaling pathways functioning during neuronal differentiation towards corticofugal projection neurons. We demonstrate that triple inhibition of TGFβ/BMP/Wnt-Shh enhances the differentiation of hFezf2-expressing cells greater than 2-fold over no growth factor control condition. Furthermore, we uncover the presence of two novel and distinct hFezf2-YFP-expressing subpopulations suggesting that Fezf2-expressing cells are heterogeneous. Knowledge derived from such studies can be used for in vitro differentiation of patient-derived induced pluripotent stem cells (iPSCs) into corticospinal motor neurons, elucidate disease mechanisms underlying upper motor neuron diseases, as well as provide a platform to discover potential targets for drug development.

**Supporting Information**

**Figure S1** Reverse Transcriptase PCR (RT-PCR) shows expression of cortical cell markers in differentiated HUES 5 cells. Pluripotent markers PuxSFl1 and Nanog are expressed in undifferentiated cells, their expression persists throughout differentiation albeit at lower levels. Radial glia marker, Pax6 is strongly expressed at day 6 and can still be detected at day 60. The neural progenitor marker Emx2 is detected starting at day 6 and its expression increases throughout differentiation until day 60. The corticospinal motor neuron Fezf2 is first detected at day 6 and is strongly expressed at day 60. Corticofugal neuron markers Bcl11b, Nfib and Tbr1 are all robustly expressed at day 60. None of the markers show expression in mouse embryonic fibroblasts (MEFs) control.

**Figure S2** Immunohistochemistry of wild-type mouse cortex showing corticofugal neuron marker expression at P0. (A) Subcortical neuron marker BCL11B (green) is expressed strongly in layer 5 and weakly in layer 6 of the mouse cortex. DAPI indicates nuclear staining (blue) in all sections. (B) Corticofugal neuron marker SOX5 (green) is expressed in layer 5–6 of the mouse cortex. (C) Subcortical neuron marker NFIB (green) is expressed in layer 5–6 of the mouse cortex. Abbreviations: DAPI, 4,6-diamidino-2-phenylidole; Scale bars: 150 μm.

**Figure S3** Immunohistochemistry of wild-type mouse cortex showing expression of layer 6 markers at P0. (A) NFIA (green) is expressed strongly in layer 6 of the mouse cortex. DAPI indicates nuclear staining (blue) in all sections. (B) DARPP32 (green) is strongly expressed in layer 6 of the mouse cortex. (C) Corticothalamic marker TBR1 (green) is expressed strongly in layer 6. Abbreviations: DAPI, 4′,6-diamidino-2-phenylidole; Scale bars: 150 μm.

**Table S1** Human and mouse RT-PCR primers.

**Table S2**

**Table S3** Primary Antibodies used in this study.

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Author Contributions
Conceived and designed the experiments: MK BC. Performed the experiments: MK CG GE. Analyzed the data: MK BC. Contributed reagents/materials/analysis tools: BC. Wrote the paper: KM BC.

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