Integrative genomic and functional analyses reveal neuronal subtype differentiation bias in human embryonic stem cell lines

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The self-renewal and differentiation potential of human embryonic stem cells (hESCs) suggests that hESCs could be used for regenerative medicine, especially for restoring neuronal functions in brain diseases. However, the functional properties of neurons derived from hESC are largely unknown. Moreover, because hESCs were derived under diverse conditions, the possibility arises that neurons derived from different hESC lines exhibit distinct properties, but this possibility remains unexplored. To address these issues, we developed a protocol that allows stepwise generation from hESCs of cultures composed of ≈70–80% human neurons that exhibit spontaneous synaptic network activity. Comparison of neurons derived from the well characterized HSF1 and HSF6 hESC lines revealed that HSF1- but not HSF6-derived neurons exhibit forebrain properties. Accordingly, HSF1-derived neurons initially form primarily GABAergic synaptic networks, whereas HSF6-derived neurons initially form glutamatergic networks. microRNA profiling revealed significant expression differences between the two hESC lines, suggesting that microRNAs may influence their distinct differentiation properties. These observations indicate that although both HSF1 and HSF6 hESCs differentiate into functional neurons, the two hESC lines exhibit distinct differentiation potentials, suggesting that they are preprogrammed. Information on hESC line-specific differentiation biases is crucial for neural stem cell therapy and establishment of novel disease models using hESCs.

microRNA | neural differentiation | neural stem cells | synapse formation

Human embryonic stem cells (hESCs) are thought to be capable of unlimited proliferation (i.e., self-renewal), and of *in vitro* and *in vivo* differentiation into cell types originating from all three germ layers including neurons, in short, to be pluripotent (1–3). This makes hESCs perhaps the only stable and genetically tractable source of human neurons, which, besides being potentially useful for therapeutic purposes, could be invaluable for studying the function of neurological disease genes in a human genetic background (4). However, different hESC lines were established under diverse culture conditions from embryos with distinct genetic backgrounds which might endow the hESCs and their derivative neurons with distinct epigenetic and cellular properties, with obvious implications for their use in regenerative medicine. The relative properties of neurons derived from different hESC lines, however, are unknown, prompting the present study.

Here, we have developed methods that allow for controlled, stepwise conversion of hESCs into cultures that contain >95% human neural stem/progenitor cells (hNPCs). The hNPCs are in turn differentiated into cultures containing 70–80% human neurons that form functional synaptic networks when cocultured with astrocytes. Using such methods, we compared neuronal sublineage differentiation and network properties of neurons derived from two NIH-registered hESC lines, HSF6 [XX, 46, National Institutes of Health (NIH) no. UC-06] and HSF1 (XY, 46, NIH no. UC-01; see http://stemcells.nih.gov/stemcell). Our findings suggest that these two lines are differentially preprogrammed already at the hESC stage, indicating that not all hESCs are created equal, consistent with previous gene-expression profiling of these cells (5). A thorough understanding of the differentiation bias and/or epigenetic preprogramming of different human ESC lines will facilitate using hESCs in cell replacement therapies in developing human disease models and using hESC-derived cells for high through-put drug screenings.

NEUROSCIENCE

Results

Derivation of hNPCs and Neurons from the HSF6 hESC Line. We stably maintained HSF6 hESCs in a genetically stable and biochemically and morphologically undifferentiated state on γ-irradiated mouse embryonic fibroblast feeder cells [Fig. 1*a*, supporting information (SI) Fig. 6, and *SI Text*], and differentiated the hESCs into human neural stem/progenitor cells (hNPCs), using embryoid body formation or matrigel-supported mono-layer cultures (see *SI Text*). In the presence of basic FGF, undifferentiated hNPCs were then expanded as ≈95% NESTIN- and SOX2-positive cells (Fig. 1*a* and SI Fig. 7).

In the developing mammalian central nervous system, NPCs first differentiate into neurons and then into glial cells (6–8). The sequential neuron-glia lineage differentiation is preserved in hESC-derived hNPCs, because we found that hNPCs initially differentiate into neurons within 30–40 days after hNPC conversion, whereas gliogenic properties emerge after prolonged culturing of hNPCs (Fig. 1 *b* and *c*). Based on the sequential neuron-glia differentiation properties of hNPCs, we developed a stepwise neuronal differentiation protocol that reproducibly generates large quantities of highly enriched human neurons and avoids the generation of heterogeneous, complex cultures of neurons on a lawn of progenitor cells that occurs in most published protocols (9, 10) (Fig. 1*d*). Heterogeneous cultures make quantitative analyses, such as cell counting and population-based biochemical analyses, difficult (SI Fig. 8). Thus, to convert hNPCs into human neurons, hNPCs are

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Abbreviations: EPSC, excitatory postsynaptic current; hNPCs, human neural stem/progenitor cell; hESC, human embryonic stem cell; IPSC, inhibitory postsynaptic current.

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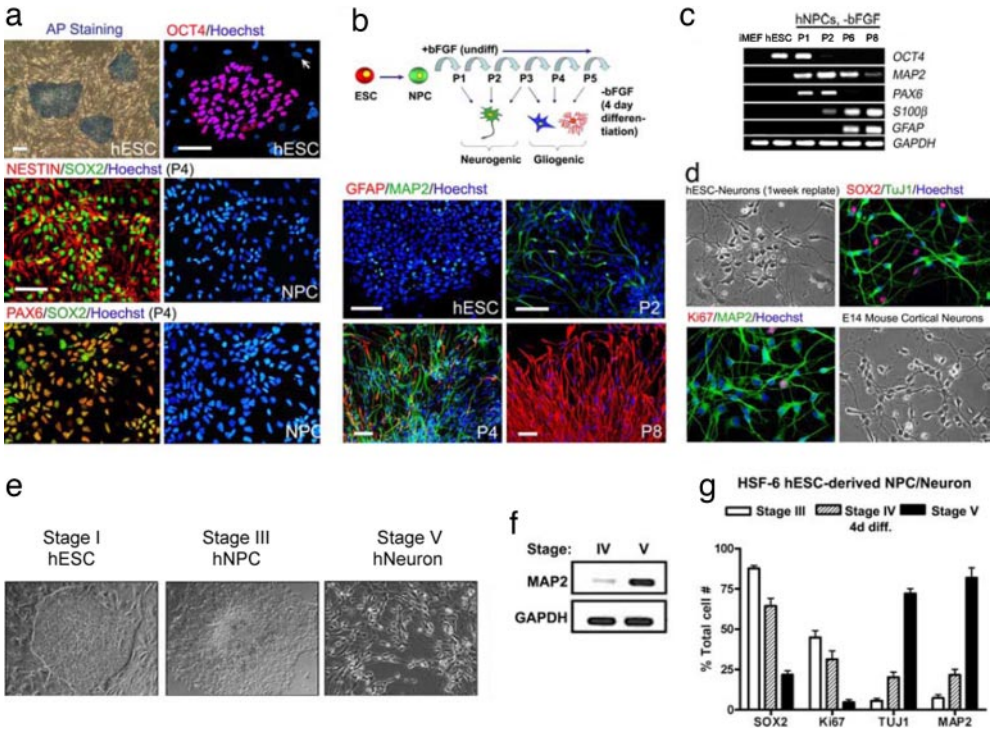

Fig. 1. Derivation of hNPCs and neurones from hESCs. (*a*) Conversion of alkaline phosphatase (AP)- and OCT4-positive HSF6 hESCs (Top) into NESTIN- and SOX2-positive NPCs that are also largely PAX6-positive (*Middle* and *Bottom*). (Scale bars, 100 μm.) (*b*) Sequential neuronal-glial differentiation of hNPCs. The top drawing schematically illustrates the differentiation sequence, whereas the four bottom images depict undifferentiated hESCs and hNPCs differentiated for 4 days at passage 2, 4, or 8 (P2, P4, and P8, respectively). Cells were triple-immunolabeled for GFAP, MAP2, and the nuclear dye Hoechst. (Scale bars, 100 μm.) (*c*) RT-PCR analysis of the expression of the markers indicated on the right in HSF6 hESCs and hNPCs at the indicated passage numbers, using irradiated mouse embryonic fibroblasts (iMEFs) as a negative control. (*d*) Phase-contrast and immunofluorescence images (stained for the indicated markers) of cultured mouse cortical neurons (*Lower Right*) and HSF6-derived neurons at stage V (*Upper Left*). (*e*) Representative phase-contrast images for the data shown in Table 1. (*f*) Semiquantitative RT-PCR analyses of MAP2 and GAPDH mRNAs at stages IV and V. (*g*) Quantitation of the percentage of Ki67-, SOX2-, MAP2-, and TuJ1-positive cells in NPC and neuronal cultures at stage III, IV, and V.

cultured at early neurogenic passages (P2 or P3) in hNPC medium lacking basic FGF for 2–3 weeks, and then treated with papain that dissociates neurons into single cells but keeps most hNPCs as cell aggregates. Finally, dissociated neurons are passed through a cell strainer to remove NPC aggregates, and are replated onto laminin-coated plates in neuronal culture medium (Basal Media Eagle with 5% serum, B27, and penicillin/streptomycin). Immunocytochemical analyses indicated that ≈70–80% of the resulting cells are neurons (Fig. 1 *d–g* and Table 1), with the remaining cells being SOX2-positive hNPCs. With this protocol, we routinely generate ≈10–20 million human neurons (one 100-mm dish of human neuronal culture) from ≈2 million hESCs (one 35-mm dish of hESC culture).

Differences in the Neuronal Differentiation Potential Between HSF1 and HSF6 hESCs. We differentiated HSF1 hESCs (XY, 46, NIH no. UC01) into hNPCs as described in ref. 9 for H1, H7, and H9 hESCs. Like HSF6-derived hNPCs, HSF1-derived hNPCs are NESTIN- and SOX2-positive, and largely express PAX6 (Fig. 2*a*). HSF1-derived hNPCs, however, contain rosette structures (Fig. 2*a*) and express the forebrain marker FOXG1B (BF1), whereas HSF6-derived hNPCs lack these features (Fig. 2*b*). Moreover, noggin treatment during ESC to NPC conversion in monolayer cultures enhanced forebrain neuron formation from HSF1 hESCs, but eliminated expandable hNPCs from HSF6 hESCs.

Analysis of HSF6-derived neurons indicated that ≈33% are PAX2- and GAD67-positive nonforebrain GABAergic neurons,

Table 1. Stepwise hESC neural differentiation protocol

Stage	Substrate	Medium	Growth factor	Dissociation method	Culture duration
I. hESC	iMEF	DMEM/20% KSR	FGF2	Collogenase IV/Dispase	Unlimited (≈5 d per pass)
II. Neural induction	Laminin or EB	DMEM-F12/B27	FGF2 or Noggin	Collogenase IV	7–10 d
III. hNPC	PO/fibronectin	DMEM-F12/B27	FGF2	HBSS	Up to 3 passages (7 d per pass)
IV. hNPC + hNeuron	PO/fibronectin	DMEM-F12/B27	NT3, BDNF	Papain	2–3 weeks
V. hNeuron	PO/laminin or astrocytes	BME/5% FBS/B27	Astrocyte CM (optional)	NA	Up to 4 weeks

Representative phase-contrast images are shown in Fig. 1e. iMEF, irradiated mouse embryonic fibroblast; KSR, knockout serum replacer; PO, polyornithine; CM, conditioned medium; d, days; NA, not applicable.

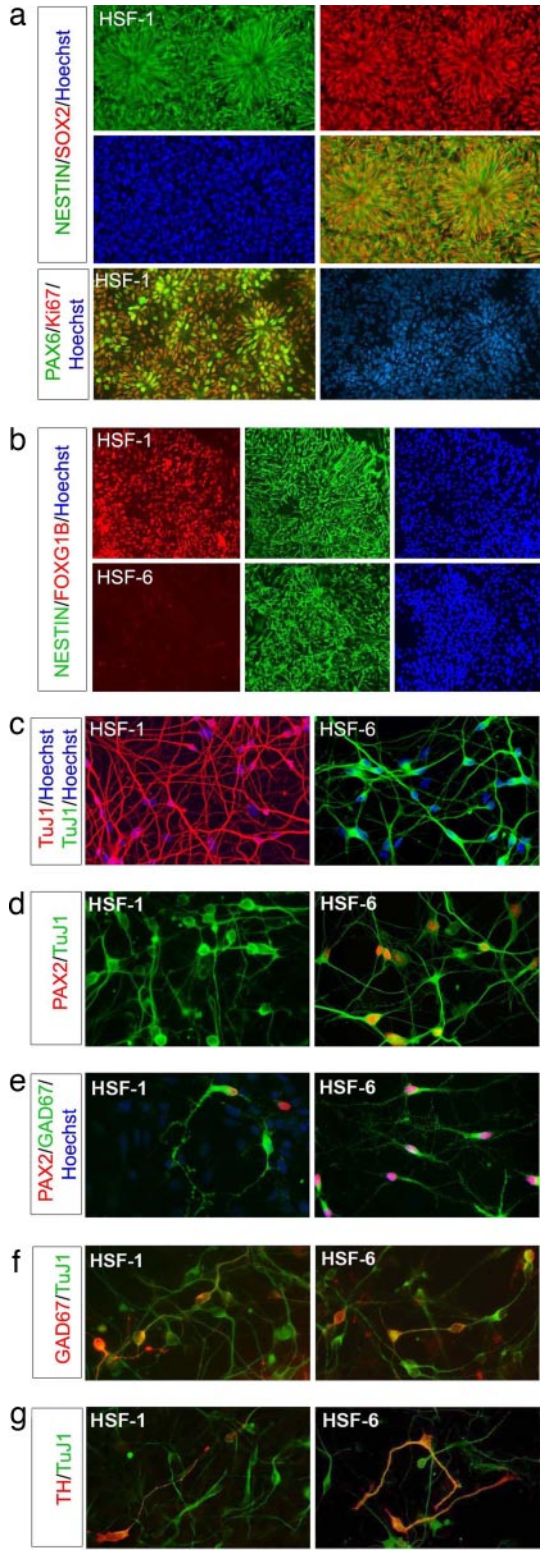

Fig. 2. Distinct neuronal sublineage differentiation of HSF1 and HSF6 hESCs. (*a*) Immunostaining of HSF1-derived hNPCs for NESTIN, SOX2, Ki67, and PAX6. Note that HSF1 hNPCs are prone to forming rosette-like structures. (*b*) Immunofluorescence comparison of FOXG1B and NESTIN expression in HSF1- and HSF6-derived hNPCs. (*c–g*) Comparative immunofluorescence labeling of HSF1- and HSF6-derived neurons for the pan-neuronal marker TuJ1 (*c*), the nonforebrain marker PAX2 (*d*), the GABAergic marker GAD67 (*e* and *f*), and the dopaminergic marker tyrosine hydroxylase (TH) (*g*). Note that neurons in *c* and *e–g* were derived by embryoid body conversion, and neurons in *d* were derived by monolayer conversion.

whereas HSF1-derived neurons generated through embryoid body formation include some GAD67-positive but few PAX2-positive neurons, and HSF1-derived neurons generated through noggin-treated monolayer conversions include no PAX2-positive neurons (Fig. 2 *c–f*). The remaining HSF6-derived neurons are composed of tyrosine hydroxylase-positive and dopamine β-hydroxylase-negative dopaminergic neurons (≈33%), and a mixture of HB9-positive cholinergic motor neurons, serotonergic neurons, and vGLUT1/2-positive glutamatergic neurons as suggested by RT-PCR analyses (≈33%; Fig. 2*g* and data not shown). mRNA array analyses showed that HSF1-derived hNPCs express more anterior markers, including EMX2, OTX1/2, LHX2, FOXG1B (BF1), and SIX3, whereas HSF6-derived hNPCs express higher levels of more posterior markers, including many genes in the HOX gene clusters (SI Fig. 9). In addition, the expression level for HB9 is higher in HSF6 than in HSF1 neurons (SI Fig. 9). Taken together, these data show that HSF1 and HSF6 hESCs both differentiate into morphologically apparently normal neurons, whereas HSF1 hESCs predominantly form forebrain neurons, and HSF6 hESCs predominantly hindbrain/cervical/thoracic neurons.

HSF1- and HSF6-Derived Neurons Form Functional Synaptic Networks with Distinct Properties. To study the electrical properties of hESC-derived neurons, we used whole-cell patch-clamp recordings. Already 3 days after replating, both HSF1- and HSF6-derived neurons fire sodium channel-dependent action potentials (SI Fig. 10), but fail to exhibit spontaneous or evoked synaptic activity even after 2–3 weeks in culture, suggesting that no functional synapses are formed (data not shown). Because astrocytes can promote synapse formation (11–14), we replated the papain-dissociated human neurons onto mitotically inhibited monolayers of astrocytes from newborn mice. Strikingly, spontaneous synaptic events became apparent in HSF1- and HSF6-derived neurons 7 days after replating, indicating that astrocytes promote synapse formation (Fig. 3).

We examined the membrane properties of the HSF1- and HSF6-derived neurons at 7, 14, and 21 days after replating. During this time period, the neuronal input resistance decreased 40–50%, the membrane capacitance increased 70–100%, the resting membrane potential declined ≈15%, the action potential amplitude increased 10–20%, and the sodium channel amplitude grew 50–60% (SI Table 2). The overall changes in HSF1- and HSF6-derived neurons were similar, but the resting membrane potential was significantly less negative in HSF6- than in HSF1-derived neurons (SI Table 2).

We further studied the synaptic properties of the human neurons. Increasingly more HSF1- and HSF6-derived neurons exhibited spontaneous excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) at 7, 14, and 21 days after replating onto astrocytes, with increasingly higher frequencies (Fig. 3 *c–h*). However, the relative properties of synaptic activity differed dramatically between the two types of neurons. HSF1-derived neurons exhibited more spontaneous IPSCs than EPSCs, whereas HSF6-derived neurons exhibited more spontaneous EPSCs than IPSCs (Fig. 3*c*). Moreover, the frequency of spontaneous synaptic events was generally higher in HSF6- than in HSF1-derived neurons, and the ratio of the frequency of excitatory to inhibitory events was greater in HSF6- than in HSF1-derived neurons (Fig. 3 *f–h*). We also measured evoked synaptic responses in HSF1- and HSF6-derived neurons, using a standard local stimulation protocol (15, 16) (Fig. 4 *a* and *b*). Synaptic responses could be reliably elicited with similar success rates and amplitudes in HSF1- and HSF6-derived neurons (Fig. 4 *c–e*). The percentage of responsive neurons exhibiting EPSCs was slightly higher in HSF6- than in HSF1-derived neurons (Fig. 4*c*), and the synaptic amplitudes of the EPSCs was almost twice as large in HSF6-derived neurons as in HSF1-derived neurons (Fig. 4*e*). Viewed together, these results indicate that HSF6-

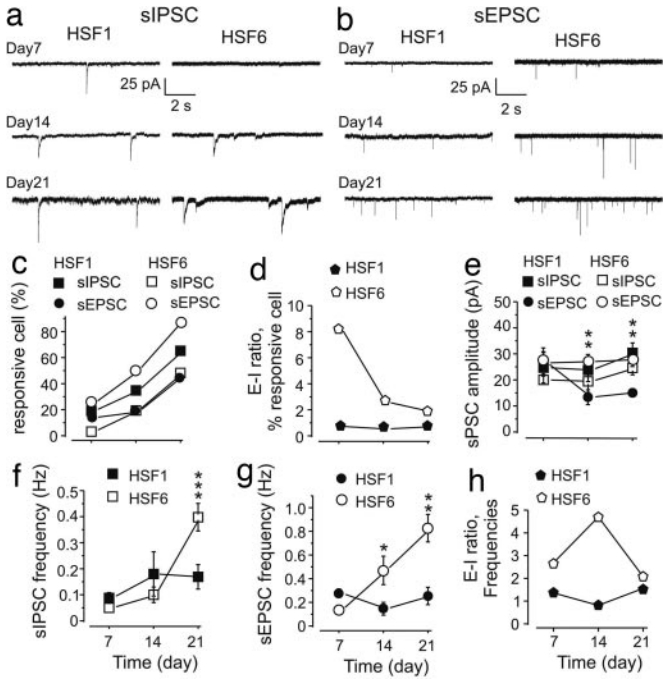

Fig. 3. Spontaneous synaptic activity of human neurons derived from hESCs. (*a* and *b*) Representative traces of spontaneous inhibitory and excitatory synaptic responses (sIPSCs and sEPSCs) in HSF1- and HSF6-derived neurons 7, 14, and 21 days after replating on a monolayer of mouse astrocytes. Recordings were performed in 50 μM 2-amino-5-phosphonovaleric acid and 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione for sIPSCs, and in 30 μM Bicuculline and 3 μM CGP55845 for sEPSC. (*c*) Percentages of recorded neurons that exhibited spontaneous IPSCs or EPSCs in cultures of HSF1- and HSF6-derived neurons on day 7, 14, and 21 after replating. (*d*) Ratio of the percentages of neurons that exhibited spontaneous excitatory inhibitory synaptic activity (E-I ratio). (*e*) Amplitudes of spontaneous IPSCs and EPSCs 7, 14, and 21 days after replating. (*f* and *g*) Frequencies of spontaneous IPSCs and EPSCs in responsive cells as a function of time after replating. (*h*) Ratio of the frequencies of spontaneous excitatory and inhibitory synaptic events in HSF1- and HSF6-derived neurons as a function of time after replating. Data are mean ± SEM ($n \geq 3$ independent cultures; all numerical values, including the number of neurons analyzed per condition, from electrophysiological recordings are listed in SI Tables 2 and 3; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

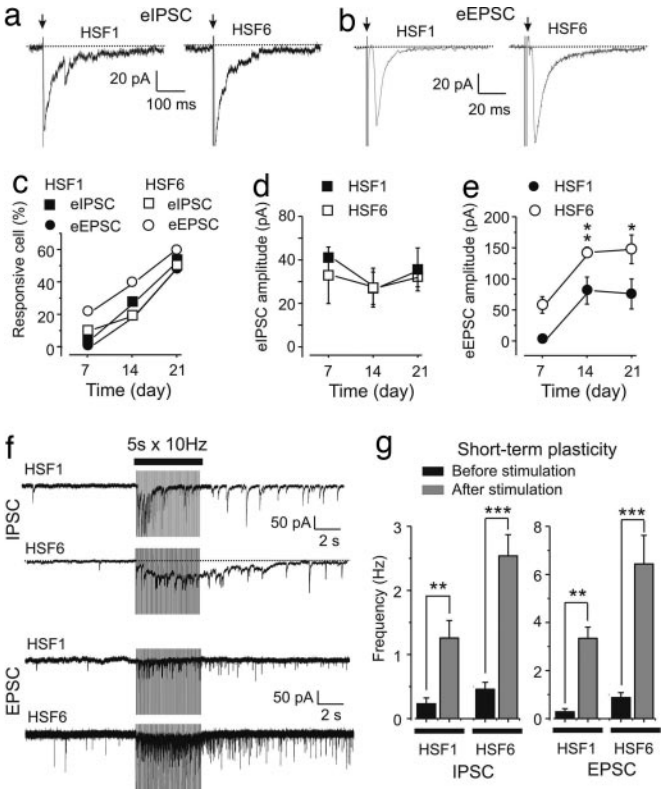

Fig. 4. Evoked synaptic responses in human neurons derived from hESCs. (*a* and *b*) Representative traces of evoked IPSCs and EPSCs in the presence of 50 μM 2-amino-5-phosphonovaleric acid and 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (for IPSCs) or 30 μM bicuculline and 3 μM CGP55845 (for EPSCs). (*c*) Percentage of neurons showing evoked synaptic activity in both HSF1 and HSF6 derived neurons at 7, 14, and 21 days after replating. (*d* and *e*) Amplitudes of evoked IPSCs and EPSCs in HSF1 and HSF6 derived neurons 7, 14, and 21 days after replating. (*f* and *g*) Short-term synaptic plasticity in hESC NPC-derived neurons. Representative traces (*f*) and summary graphs of the mean frequency (*g*) of synaptic responses (IPSCs and EPSCs) 10 s before, during, and 10 s after a 5-s stimulus train applied at 10 Hz. Data shown are pooled from cultures maintained 14 and 21 days after replating.

derived neurons form relatively more excitatory synapses and HSF1-derived neurons more inhibitory synapses.

Finally, we examined short-term synaptic plasticity. After a 5-s stimulus train applied at 10 Hz, the frequencies of excitatory and of inhibitory spontaneous synaptic responses are dramatically increased in HSF1- and in HSF6-derived neurons (Fig. 4*f*). The average frequency of spontaneous IPSCs increased ≈5-fold in both HSF1- and HSF6-derived neurons, whereas the average frequency of spontaneous EPSCs increased ≈12-fold in HSF1-derived neurons, but only ≈7-fold in HSF6-derived neurons (Fig. 4*g*).

Differential microRNA Expression in HSF1 and HSF6 hESCs, hNPCs, and Human Neurons. Because noncoding microRNA-mediated regulation of gene expression is a major epigenetic mechanism (17–20), we examined the microRNA expression patterns in HSF1 and HSF6 cells at the hESC, hNPC, and human neuron stages, using Taqman-based real-time PCR to profile for 184 miRNA species (Applied Biosystems, Foster City, CA). The results show that, during hESC differentiation, miRNA expression patterns change dramatically and can be classified into five groups (Fig. 5*a*): miRNAs with increased (Group I; ≈50% of miRNAs) or with decreased (Group II; ≈28% of miRNAs) expression upon conversion of hESCs to hNPCs and human neurons; miRNAs with increased expression upon conversion of hESCs to hNPCs, but decreased expression upon differentiation of hNPCs into human neurons (Group III; ≈5%); miRNAs with decreased expression in HSF1-derived NPCs, but increased expression in HSF6-derived NPCs (Group IV; ≈7%, including hsa-miR-133a, hsa-miR-133b, and hsa-miR-206); and miRNAs that do not change (Group V; 10%). U6 snoRNA was used as an internal control for sample loading. These results were generally confirmed in the HSF6 line using the NCode miRNA microarray from Invitrogen (Carlsbad, CA), but direct comparisons suggest that the Taqman-based quantitative PCR assays are more quantitative and sensitive (SI Fig. 11 and SI Table 4). Moreover, they confirm previous assignments of ESC-specific and brain-specific miRNAs (21–27) with some important exceptions. We find that hsa-miR-134, a microRNA with a supposed neuronal function (27), is highly expressed in hESCs but decreases >100-fold upon neuronal differentiation. Hsa-miR-134 is expressed at >1,000-fold lower levels in neurons than typical brain-specific miRNAs (e.g., hsa-miR-124a), suggesting that at least in human neurons, hsa-miR-134 is unlikely to have a universal central function.

A comparison among HSF1 and HSF6 hESCs, hNPCs, and human neurons reveals that on average, 40% of miRNAs exhibit a >2-fold line-to-line difference in expression. Upon conversion into hNPCs and neurons, HSF6 cells tend to express higher miRNA levels than HSF1 cells, but overall only four miRNAs (e.g., hsa-

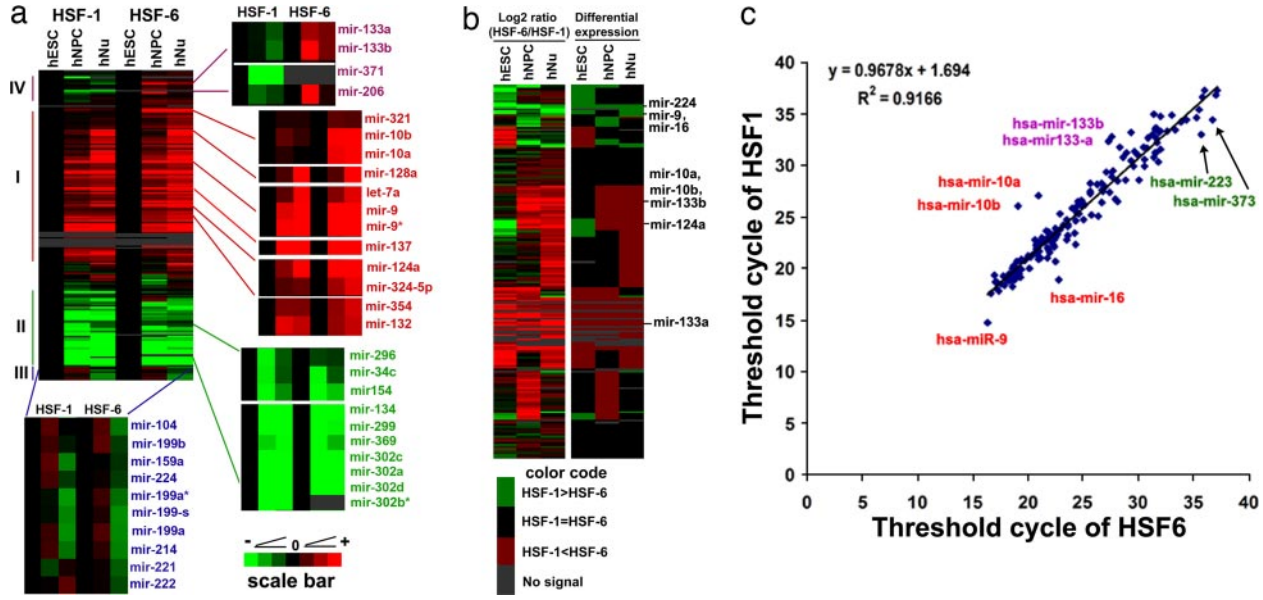

Fig. 5. Differential microRNA expression in HSF1 and HSF6 hESCs, hNPCs, and hNeurons. (*a*) Hierarchical clustering of the stage-dependent expression profiles of 184 miRNAs (with three negative controls for *Caenorhabditis elegans* miRNAs and two water controls) in HSF1 and HSF6 hESCs and their derivative hNPCs and hNeurons. Relative expression levels (heat-map) at three stages are displayed as log2 ratios for hESC/hESC (therefore always 0), hNPC/hESC, and neurons/hESC (red, expression increases; green, expression decreases; see scale bar at bottom). Representative miRNAs are shown in the enlarged panels. See SI Table 4 for a listing of the results for each miRNA. (*b*) Hierarchical clustering of miRNAs with expression differences between HSF1 and HSF6 hESCs and their derivative hNPCs and neurons. Log2 ratios of HSF6/HSF1 are shown at *Left* (see scale bar for *a*), and differentially expressed miRNAs (>2-fold change) are color-coded at *Right* (see color code at bottom). (*c*) Scatter-plot of the threshold cycle of the quantitative miRNA PCR array for all miRNAs in HSF1- and HSF6-derived neurons. Many miRNAs are expressed at similar levels between HSF1 and HSF6 lines, as indicated by a high linear correlation coefficient (R^2). Representative differentially expressed miRNAs are identified.

miR-10a, -10b, -133a, and -133b), were expressed at >4-fold higher levels in HSF6- than in HSF1-derived neurons and might potentially be involved in regulating neuronal sublineage differentiation bias between these two hESC lines. Interestingly, hsa-miR-10a is embedded in a HOX gene cluster that is highly expressed in the brainstem and cervical spinal cord, and its higher expression in the HSF6-derived neurons compared with HSF1-derived neurons is consistent with the notion that the HSF6 line produces more neurons with a more posterior identity, whereas the HSF1 line generates more neurons with anterior identity (Fig. 5 and SI Fig. 9).

Discussion

In this study, we developed a protocol that allows reliable differentiation of hESCs into neurons that form functional synaptic networks when cocultured with astrocytes, and we characterized the properties of the synapses in these networks. We compared the properties of neurons derived from two different, well established hESC lines, HSF1 and HSF6. Based on the analysis of protein and miRNA expression and of synaptic transmission, we demonstrated that neurons derived from these hESC lines exhibit major differences, suggesting that the HSF1 and HSF6 lines are "preprogrammed," by which we mean that although the two hESC lines are fully capable of differentiating into major cell types derived from all three germ layers, they exhibit a differentiation bias within the spectrum of cell types developing from the neural ectoderm, as described for the types of neurons that are formed upon differentiation of the HSF1 and HSF6 hESC lines. These findings have implications for the biology of stem cells and for the use of stem cell lines in effecting tissue repair and in building human cellular disease models.

Neuronal Differentiation of hESCs. Before the derivation of hESCs, the only sources for directly studying human neurons were aborted human fetuses or adult tissues obtained during brain surgery or postmortem. The differentiation protocol described here generates readily available neurons that can be reliably isolated with same properties and same genetic background. Moreover, our protocol produces cultures containing 70–80% human neurons, whereas most of the other available methods produce heterogeneous cell mixtures that are difficult to use in mechanistic studies of cell differentiation or neuronal function. The synaptic networks formed by the human neurons, when cocultured with astrocytes, can be used not only to study the basic synaptic properties of human neurons, but also pathological mechanisms triggered by neurological disease genes, e.g., *Huntingtin*, *Mecp2*, and *α-Synuclein*. In addition, this human neuronal culture system can be adapted for high throughput drug screening to identify molecules that can reverse the cellular phenotypes induced by various disease genes.

Intrinsic Differences Between hESC Lines. Our study suggests that different hESC lines may be biased toward distinct cell lineages, possibly because they were obtained under diverse conditions. This conclusion is consistent with previous cDNA microarray analyses of three different hESC lines, HSF1 (XY, 46), HSF6 (XX, 46), and H9 (XX, 46), showing that 30–50% of genes exhibit differential expression patterns (5). We show that HSF6 hESCs generate midbrain/hindbrain neurons but fail to produce forebrain neurons, whereas HSF1 is prone to differentiating into forebrain neurons. Among others, this finding indicates that diseases of the forebrain, such as Huntington's disease, would be better modeled in HSF1 than in HSF6 hESCs, whereas pan-neuronal disorders can be modeled by both HSF1 and HSF6-derived neurons.

It is possible that neuronal subtype differentiation of HSF6 and HSF1 hESC can still be regulated by extrinsic signals, as described for neurons derived from mouse ESCs (28). A number of extracellular molecules and transcription factors regulate neuronal differentiation (10, 28–32), and testing these factors and molecules on the hESC differentiation paradigm will be important.

Epigentic Preprogramming of Different Lines of hESCs. It is likely that the preprogramming of the HSF1 and HSF6 hESCs occurs by epigenetic mechanisms. For example, the differential expression of FoxG1B (BF1) in HSF1 and HSF6 cells may be caused by distinct chromatin states of the FoxG1B gene in these hESC lines, a hypothesis that can be tested by examining the epigenetic code of the FOXG1 gene (i.e., methylation of its DNA, and methylation and acetylation of its associated histones) in HSF1 and HSF6 hESCs. Our mRNA expression microarray analyses suggested that the X-linked gene MECP2 has similar expression levels in HSF1 and HSF6 lines (SI Fig. 9), indicating that HSF6 hESCs likely have proper X-inactivation upon neuronal differentiation. One major mechanism involved in epigenetic gene regulation is the expression of noncoding miRNAs. miRNAs in hESCs may play a critical role in the self-renewal of hESCs through inhibition of differentiation gene expression at the translational level, and function as "master regulators" to coordinate cell lineage differentiation programs. Our miRNA profiling results indicate that, during hESC differentiation into hNPCs and human neurons, there are large-scale dramatic changes in the miRNA expression patterns, consistent with the notion that miRNAs play critical roles in neuronal differentiation and maturation, including process outgrowth and synapse formation. Although many miRNAs exhibit changes, only four miRNAs display >4-fold changes between the two lines at human neuron stages, and might be involved in the regulation of neuronal subtype differentiation.

Together, our results show that two different hESC lines exhibit differential neuronal differentiation biases, potentially because of distinct epigenetic preprogramming. Further investigation of the epigenetic preprogramming including DNA methylation, histone modifications, and miRNAs will not only help us understand the differentiation control of hESCs toward neural lineages but also have strong implications for the use of hESCs to study and treat neurological disorders.

Materials and Methods

Cultures, Array Analyses, and Immunocytochemistry, of hESCs, hNPCs, and Human Neurons. NIH-registered hESC lines HSF1 (XY, 46, NIH no. UC01) and HSF6 (XX, 46, NIH no. UC06) were maintained on CF1 irradiated murine embryonic fibroblasts in DMEM-high glucose supplemented with 20% knockout serum replacer (Invitrogen), basic FGF (10 ng ml^{-1}), 1 mM glutamine, 1% nonessential amino acids, 1% penicillin/streptomycin, and 0.1 mM 2-mercaptoethanol with daily medium changes, and passaged every 4–6 days by incubation with 1 mg ml^{-1} dispase/collogenase IV (Invitrogen) for 10–20 min at 37°C. Differentiation, microarray analyses, and immunocytochemistry of the cells are described in detail in *SI Text*.

Electrophysiological Analyses. Electrophysiology was performed from HSF1- and HSF6-derived neurons after replating without and with astrocytes at days 7, 14, and 21. Synaptic responses were recorded in the whole-cell voltage clamp mode. Evoked synaptic responses were triggered by 1-ms current injection (900 μA) through a local extracellular electrode (FHC, Bowdoin, ME; concentric bipolar electrode, catalog no. CBAEC75) essentially as described (15, 16). For a detailed description, see *SI Text*.

Statistical Analysis. All statistical analyses were performed by using the one-way ANOVA test plus Fisher's post hoc *t* test.

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