A rosette-type, self-renewing human ES cell-derived neural stem cell with potential for in vitro instruction and synaptic integration

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An intriguing question in human embryonic stem cell (hESC) biology is whether these pluripotent cells can give rise to stably expandable somatic stem cells, which are still amenable to extrinsic fate instruction. Here, we present a pure population of long-term self-renewing rosette-type hESC-derived neural stem cells (lt-hESNSCs), which exhibit extensive self-renewal, clonogenicity, and stable neurogenesis. Although lt-hESNSCs show a restricted expression of regional transcription factors, they retain responsiveness to instructive cues promoting the induction of distinct subpopulations, such as ventral midbrain and spinal cord fates. Using lt-hESNSCs as a donor source for neural transplantation, we provide direct evidence that hESC-derived neurons can establish synaptic connectivity with the mammalian nervous system. Combining long-term stability, maintenance of rosette-properties and phenotypic plasticity, lt-hESNSCs may serve as useful tool to study mechanisms of human NSC self-renewal, lineage segregation, and functional in vivo integration.

human embryonic stem cells | neural differentiation | regionalization | synapse formation

Human embryonic stem cells (hESCs) are derived from the human blastocyst and have the potential for almost unlimited self-renewal (1). Similar to cells of the inner cell mass, they can differentiate into cells and tissues derived from all 3 germ layers. During the in vitro-differentiation process, they may pass the state of tissue-specific somatic stem cells. Biomedical applications of hESCs will critically depend on the ability to differentiate them into defined and purified somatic cell types in vitro. So far, most hESC differentiation paradigms represent "run-through" procedures where undifferentiated ES cells are exposed to a variety of extrinsic factors, cocultures with stromal cells, or genetic modifications to enrich for the desired cell type [for review see (2)]. Major challenges of such an approach are the removal of unwanted cell types and batch-to-batch variations due to often lengthy and complex differentiation protocols. The results of recent studies suggest that rosette-forming cells isolated from early hESC differentiation stages retain a broad differentiation potential while their subsequent expansion in the presence of growth factors results in an increased gliogenic bias and resistance to in vitro regionalization (2, 3). These observations have led to the notion that it is not possible to derive bona fide long-term expandable hESC-derived neural stem cells, which still retain sufficient plasticity to be recruited into different neuronal subtypes in a controlled manner.

Here, we report the derivation of a long-term, self-renewing neuroepithelial stem cell population from hESCs (lt-hESNSCs). While these cells retain a constant neuro- and gliogenic potential even after long-term proliferation, they undergo a pronounced restriction of their phenotypic and regional identity. Yet, these cells maintain the ability to form neuroepithelial rosette architectures in vitro, express genes recently identified as rosette-specific and remain responsive to extrinsic morphogens even after extensive passaging. Importantly, neurons derived from lt-hESNSCs are functional and can undergo synaptic integration into a host brain.

Results

HESCs were neuralized to generate neural rosettes as described (4, 5). At the stage where rosettes start to form 3-dimensional neural islands these island were manually isolated, taking special care to minimize the content of contaminating other cell types, which can rapidly overgrow the cultures later on. The isolated clusters were kept as floating spheres for at least 24 h. Spheres were dissociated to single cells by trypsin digestion and plated as a monolayer under defined conditions (see Material and Methods and *SI Methods*). Since high-cell density was found crucial for propagating the cells through the initial passages, cultures were split at a 1:2 ratio up to passage 5. These conditions yielded a population with homogeneous morphology forming typical rosette-like patterns (Fig. 1*A* and *B*) and without detectable contamination by undifferentiated ESCs or derivatives of other germ layers (Fig. S1 and *SI Methods*). The cells could be extensively propagated (>150 passages/450 doubling times) while retaining their characteristic morphological and immunocytochemical properties. Derivation of this population was repeated >25 times with 3 different hESC lines (H9.2, I3, I6; Fig. S2). Proliferating cells expressed high levels of the neuroepithelial marker Pax6 (Fig. 1*H*). In addition to nestin (Fig. 1*C*) and Sox2 (Fig. 1*D*), they showed homogeneous Sox1 expression (Fig. 1*E* and *H*) and, unlike stable mESC-derived precursors, did not convert in a Sox2(+)/Sox1(-) phenotype (6). Proliferating cells adopted a specific growth rate with doubling times of ≈38 h (Fig. S3; H9.2: 37.6 h; I3: 38.8 h; and I6: 38.2 h) independent of the passage number and growth rates of the parental hESC lines (H9.2: 52.4 h; I3: 76.8 h; I6: 81.2 h). Using RT-PCR and telomere repeat amplification protocols, we found that telomerase is highly expressed within this population (Fig. 1*H* and Fig. S3), an important prerequisite for the maintenance of a stem cell fate (7). The cells could be frozen and thawed without detectable alteration in proliferation or differentiation and retained a stable karyotype for at least 100 passages ($2n = 46$; Fig. 1*F* and *G*).

Previous studies had shown that FGF2/EGF-expanded neural precursors change their responsiveness to growth factors and their differentiation potential during in vitro expansion (3, 8). To address this issue we differentiated our neural precursors at passages 10, 25, 50, and 75, and determined percentages of resulting progeny 28 days after growth factor withdrawal. Remarkably, the fractions of the individual neural lineages remained stable over the passages with a strong propensity toward neuronal differentiation. More than 60% of the cells exhibited neuronal markers, whereas glial antigens were

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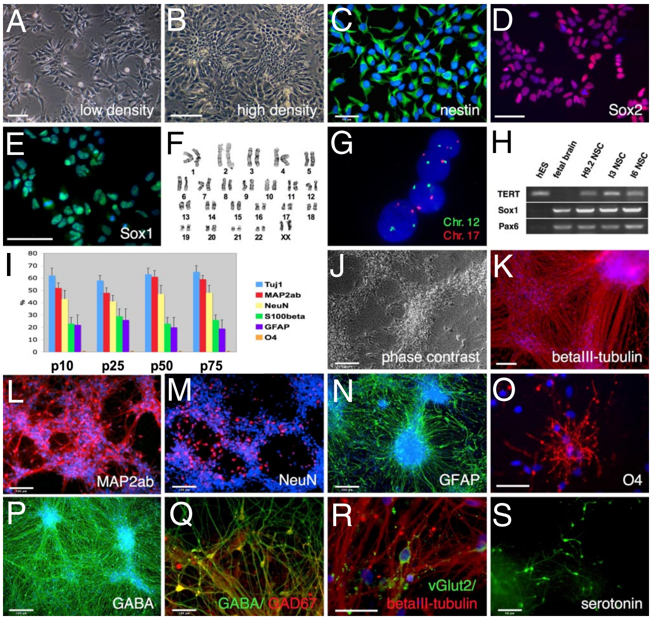

Fig. 1. Long term self-renewing neural stem cells generated from human embryonic stem cells (lt-hESNSCs). These cells can be continuously propagated as a highly homogenous population forming rosette-like patterns (*A*, low density; *B*, high density). In addition to nestin (*C*), lt-hESNSCs express Sox2 (*D*), and Sox1 (*E*). (*F*) Representative karyotype from lt-hESNSCs (derived from H9.2) in passage 50. Fluorescence in situ hybridization (FISH) studies were performed to screen for trisomy of chromosomes 12 and 17, alterations frequently observed during long-term propagation of undifferentiated hESC (*G*). RT-PCR analysis reveals high levels of telomerase expression and expression of the neuroepithelial markers Sox1 and Pax6 (*H*). Upon growth factor withdrawal, lt-hESNSCs give rise to a dominant fraction of neurons expressing beta III-tubulin (*I*–*K*), MAP2ab (*I* and *L*) and NeuN (*I* and *M*). Prolonged differentiation (>2 weeks) promotes differentiation into astrocytes positive for GFAP (*I* and *N*) or S100 beta (*I*) and oligodendrocytes expressing 04 (*I* and *O*). Despite continuous passaging, lt-hESNSCs retain a stable neurogenic differentiation potential (*I*, bar graph depicts percentages of immunoreactive cells in passages 10, 25, 50, and 75 after 28 days of differentiation). The large majority of lt-hESNSC-derived neurons display a GABAergic phenotype, staining positively for GABA (*P* and *Q*) and GAD67 (*Q*). Only occasional neurons exhibit glutamatergic (*R*) or serotoninergic (*S*) phenotypes. Immunofluorescence data show representative pictures of lt-hESNSCs (passages 21–51) derived from H9.2. Representative RT-PCR data are from lt-hESNSCs at passages 24–34. (Scale bars, *A*–*E*, *O*, *R*, and *S* 50 μm; *J*–*M*, and *Q*: 100 μm; *N* and *P*: 200 μm.)

detectable in ≈20% of the cells (Fig. 1*I*–*O*). Oligodendrocytes were detected only occasionally after 4 weeks of differentiation but became slightly more frequent (yet <1%) after longer differentiation times (Fig. 1*O*). In summary, long-term propagation (> 225 doubling times) did not affect lineage differentiation of our neural precursor population.

While continuous self-renewal and multipotency strongly support a stem cell nature of this cell population, clonal analysis is required to demonstrate that these properties are reflected at the single cell level. Single neural precursors derived from lines H9.2 (passages 25–75) and I3 (passages 20–60) were deposited on gamma-irradiated astrocytes derived from murine ESCs using the CytoClone system (9, 27) (Fig. S4). Some of the spotted cells had been engineered to express EGFP under control of the phosphoglycerate kinase (PGK) promoter element (10). In this coculture paradigm, 99 out of 1,071 deposited cells (9.2%) generated clones containing >20 cells within 2 weeks. After 4 weeks of growth factor withdrawal-induced differentiation, all clones investigated at this time point (n = 35) had generated beta III-tubulin-positive neurons (40–70% of all cells). A majority (74.3%) of the clones contained GFAP-expressing cells, and no oligodendrocytes

Table 1. Clonal analysis of lt-hESNSC

Cell line	Weeks of diff	Cells deposited	Clones received	β-III tubulin	GFAP	O4
H9.2	4	236	21	21	15	0
I3		164	14	14	11	0
H9.2	8	86	9	9	9	4
I3		57	6	6	6	1
H9.2	12	158	14	14	14	11
I3		164	17	17	17	13

After a 2-week expansion of single cells and subsequent differentiation in the absence of growth factors, lt-hESNSC-derived clones give rise to beta-III tubulin-positive neurons, GFAP-positive astrocytes and, after prolonged differentiation, 04-positive oligodendrocytes. diff, differentiation.

were detectable at this stage of differentiation. By 8 and 12 weeks of differentiation, all clones investigated contained both beta III-tubulin-positive neurons and GFAP-positive astrocytes. By these time points, O4-positive oligodendrocytes were observed in 33.3% and 83.9% of the clones, respectively (Table 1). These data demonstrate that our neural precursors are indeed able to generate multipotent clones at a single-cell level. Subsequent experiments showed that single cell-derived clonal lines could be propagated for at least 50 passages while retaining multipotent differentiation and stable rates of neuro- and gliogenesis (Fig. S5). Together with their capacity for extensive self-renewal, this property qualifies them as long-term self-renewing neural stem cells (lt-hESNSCs).

We next asked whether neurons derived from lt-hESNSCs can functionally mature in vitro. Analysis of whole-cell currents revealed a gradual maturation of voltage-dependent inward and outward currents facilitating repetitive action potential generation upon depolarization (Fig. S6). Immunohistochemical analysis revealed that under standard in vitro differentiation conditions, the vast majority of the cells acquired an interneuron-like phenotype with coexpression of GABA and the GABA-producing enzyme glutamic acid decaboxylase (GAD67; Fig. 1*P* and *Q* and Fig. S2). GABA immunoreactivity could be detected in 86 ± 7% of the beta III-tubulin-positive cells of which ≈60% also expressed GAD67. We could not detect vesicular glutamate transporter 1 (vGlut1) as a marker for glutamatergic neurons in later passages even though few vGlut1-positive cells were present in lower (<5) passages. A small fraction of the cells expressed vGlut2, comprising 4.8 ± 2.6% of the beta III-tubulin-positive cells by 6 weeks of differentiation (Fig. 1*R*). Differentiated cells also included small populations of neurons expressing choline acetyl transferase [ChAT; ≈1/10,000 beta III-tubulin(+) cells], serotonin [≈1–2/1,000 beta III-tubulin(+) cells; Fig. 1*S*], and very occasional tyrosine hydroxylase (TH)-positive cells. These data indicate that neuronal differentiation of lt-hESNSCs is strongly biased toward a GABAergic phenotype.

We wondered whether lt-hESNSCs also exhibit a biased regional differentiation. To that end we analyzed the expression of region-specific transcription factors in lt-hESNSCs cultured for >15 passages. This analysis revealed that lt-hESNSCs express a highly restricted transcription factor code, mostly compatible with a ventral anterior hindbrain fate (Fig. 2*A* and *B*). Among the markers used for partitioning the anterior–posterior axis, we could detect expression of the mid-hindbrain marker En1, the anterior hindbrain markers Gbx2, Nkx6.1, HoxA2, and HoxB2, as well the rhombomeric marker Krox20. Transcripts of the more posterior markers HoxA1, HoxB1, or HoxB4 were detected in decreasing amounts, and there was no expression of transcription factors indicating even more posterior fates (HoxB6 and HoxC5). Markers representative for an anterior regional identity (FoxG1, Emx1, Emx2, Nkx2.1, Gsh2, or Otx2) could not be detected (Fig. 2*A*). In the dorso-ventral axis, lt-hESNSCs expressed transcription factors indicative of a

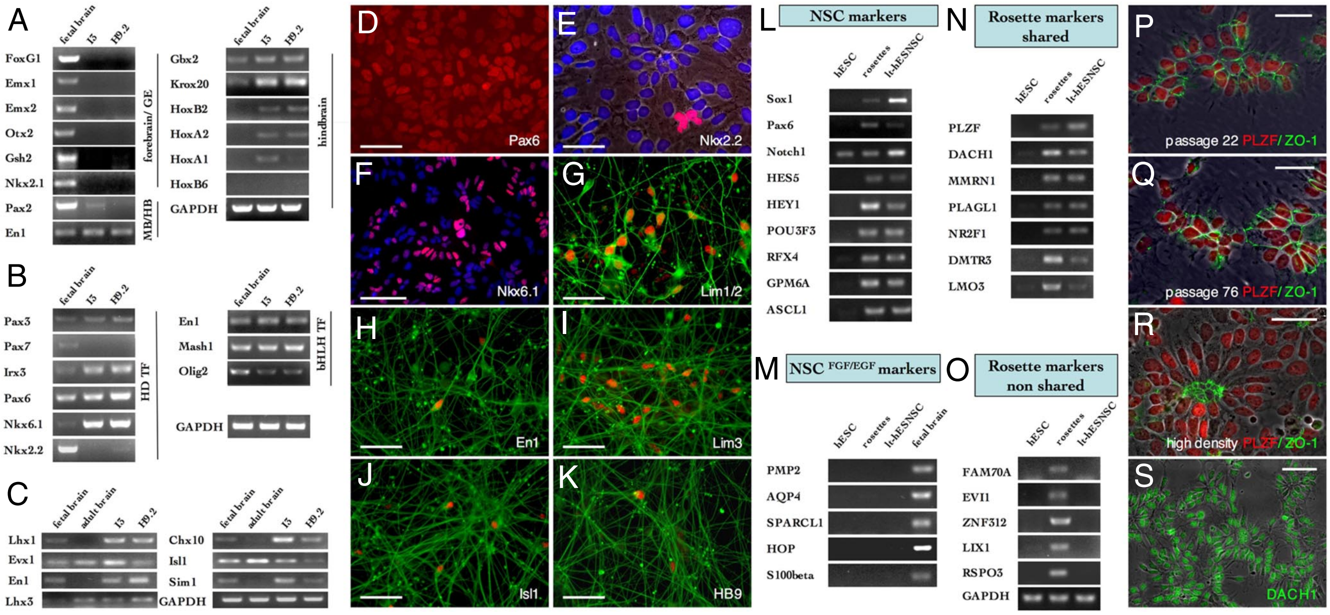

Fig. 2. lt-hESNSCs express transcription factors compatible with an anterior ventral hindbrain fate and maintain rosette-properties. lt-hESNSCs cultured for >15 passages exhibit a posterior identity corresponding to an anterior hindbrain location. (*A*) Telencephalic markers FoxG1, Emx1, Emx2, Otx2 Gsh2, and Nkx2.1 are not detectable by RT-PCR. Instead, lt-hESNSCs show prominent expression of the anterior hindbrain markers Gbx2, HoxA2, HoxB2, and Krox20. More posterior markers (e.g., HoxB6) are absent. In the dorso-ventral axis, lt-hESNSCs express markers compatible with a ventral hindbrain localization (*B*, *D*–*F*), that is, Irx3, Pax6 (*B* and *D*), Nkx6.1 (*B* and *F*) and Nkx2.2 (*B* and *E*); note lacking expression of the dorsal marker Pax7. Differentiated neurons express transcription factors found in V0–V3 interneurons and pMN-derived motoneurons (*C*, *G*–*K*) including Lim1/2 (*G*), En1 (*H*), Lim3 (*I*), Isl1 (*J*), and HB9 (*K*). Neuronal identity was confirmed by costaining for beta III-tubulin (green, *G*–*K*). (*L*) Rosettes generated from plated EBs and lt-hESNSCs (passage 48) express NSC markers at comparable levels. (*N*) lt-hESNSCs and rosettes share expression of PLZF, DACH1, MMRN1, PLAGL1, NF2F1, DMTR3, and LMO3, genes previously described as "rosette-specific". (*M*) lt-hESNSCs do not express PMP2, AQP4, SPARCL1, S100beta, or HOP, genes proposed to be characteristic of FGF/EGF-expanded cells with limited patterning potential. (*O*) Some rosette-specific genes were no longer expressed in lt-hESNSCs, including FAM70A, EVI1, ZNF312 LIX1, and RSPO3. (*P* and *Q*) lt-hESNSCs in passage 22 (*P*) or passage 76 (*Q*) show nuclear expression of PLZF, a columnar growth pattern and apical nuclei as well as ZO-1 expression, which was particularly accentuated at the apical and lateral surface of clustered lt-hESNSCs. At high density, lt-hESNSCs form typical rosette-like structures with pronounced central expression of ZO-1 (*R*). (*S*) lt-hESNSCs show homogeneous nuclear expression of DACH1 [cells in (*R*) and (*S*) are from passage 52]. (Scale bars, *D*, *E*, *G*–*K*, and *S*: 50 μm; *F*: 100 μm; *P*–*R*: 25 μm.)

ventral hindbrain identity, such as Irx3, Pax6, and Nkx6.1, and, to a lesser extent, the very ventral markers Olig2 and Nkx2.2. Dorsal markers such as Pax7 and Pax3 were expressed only occasionally (< 1/10,000 cells and only weak PCR bands). We conclude that our lt-hESNSCs are largely devoid of dorsal progenitors (Fig. 2*B* and Fig. S7). Immunocytochemical staining confirmed that lt-hESNSCs were mainly positive for Pax6 (H9.2 > 95% and I3 > 98%; Fig. 2*D*) and Nkx6.1 (H9.2 38 ± 9% and I3 42 ± 10%) (Fig. 2*F*). Only few cells showed nuclear expression of Nkx2.2 (H9.2 < 0.2% and I3 < 1/10,000; Fig. 2*E*). This expression pattern corresponds well to the interneuronal progenitor regions V0–V3. Along the same line, we found that differentiated neurons express the transcription factors Evx1/2, En1, Chx10, Lhx1 (Lim1), Isl1, and Sim1, markers typically found in ventral hindbrain interneurons (Fig. 2*C* and Fig. S7) (11, 12). On a protein level, 46 ± 10% (I3) and 53 ± 8% (H9.2) of the neurons expressed Lim1/2 (Fig. 2*G*) whereas 0.5–3% (both cell lines) were positive for En1 (Fig. 2*H*). Nuclear expression of Lim3 could be detected in 27 ± 6% (I3) and 30 ± 9% (H9.2) of all neurons (Fig. 2*I*), and Isl1 was expressed in 3–6% (I3) and 4–7% (H9.2) of neuronal cells (Fig. 2*J*). We detected HB9 expression in ≈0.05% of all neurons (Fig. 2*K*), indicating that the population also includes occasional motoneurons. No significant differences in the expression of region-specific transcription factors were observed between passages 20 and 70 (Fig. S8). Taken together, these data suggest that lt-hESNSCs propagated under standard conditions exhibit a remarkably restricted and stable regionalization profile, which corresponds well to a ventral anterior hindbrain location.

Similar to normal development neural cells derived from hESCs develop via an obligate anterior regional identity (13, 14). We therefore asked whether the restricted posterior identity of lt-hESNSCs represents the result of the in vitro expansion process. During embryonic development, posterior neural identities are generated through the reprogramming of cells with an initial anterior character. This process is supposed to be mediated by molecules including members of the FGF family and retinoic acid (15). FGF2 is also a key factor for the expansion of our NSCs. Interestingly, freshly isolated neural precursors proliferated in the presence of FGF2 for only 2 passages still showed prominent expression of anterior markers such as Otx2 and FoxG1 (Fig. S9). After consolidation (passages ≥ 10), these markers were consistently absent. Similar observations were made for the dorsal marker Pax7. Acquisition of a posterior fate was independent from the retinoic acid present in traces in the B27 supplement detectable in media containing B27 without retinoic acid (Fig. S9). Taken together, these data suggest that restriction of regional transcription factor expression is an early event in the lt-hESNSC derivation process. Once consolidated, these cells maintain a stable transcription factor expression profile (Fig. S8).

Data from recent publications suggest that early-derived rosette-forming cells retain a broad differentiation potential while this potential is subsequently lost in the presence of growth factors such as FGF2 and EGF (3). However, these rosette-type cells could not be easily maintained over many passages. Because we observed rosette-like structures even after extensive passaging in FGF2 and EGF (Fig. 1*A* and *B*) we asked whether genes typically expressed in rosette-cells are detectable in our cultures. Indeed we could show that many genes suggested to be rosette-specific are expressed at equivalent levels in early rosettes picked from spontaneously differentiating EBs and lt-hESNSCs propagated for >50 passages (Fig. 2*L*–*S*) In addition to the expression of PLZF, DACH1, MMRN1, PLAGL1, NR2F1, LMO3, and DMTR3 (Fig. 2*N* and *P–S*), lt-hESNSCs exhibited expression of ZO-1, a marker typically found in rosette-stage NSCs (3). Within rosette structures, ZO-1 labeling was accentuated at the apical and lateral membrane and, at higher cell densities, localized to the center of the rosettes (Fig. 2*P–R*). Moreover, lt-hESNSCs lacked expression of PMP2, AQP2, SPARCL1, HOP, or S100beta (Fig. 2*M*), markers recently proposed to be characteristic of FGF2/EGF-expanded cells with limited patterning potential (3). This specific expression pattern was stable for at least 74 passages (Fig. 2*P* and *Q*). Furthermore, expression of the Notch-related genes Hes5 and Hey1 was found in lt-hESNSCs (Fig. 2*L*). However, some previously described "rosette-markers" such as FAM70A, EVI1, ZNF312, LIX1, or RSPO3 were lacking in our lt-hESNSCs (Fig. 2*O*). Together with the data on regional determination this expression profile supports the view that lt-hESNSCs represent a stem cell population that partially maintains properties of early rosette-type NSCs despite long-term expansion in FGF2 and EGF.

We next became interested in whether these cells, similar to early rosette cells, remain responsive to instructive regionalization cues known to induce other regional phenotypes such as midbrain TH-positive neurons (localized anterior and ventral to the putative regional phenotype of our population) and more posterior hindbrain and spinal cord fates (Fig. 3*A*) (14, 16). For induction of midbrain fates we used lt-hESNSCs (passages 12–53) and cultured them in the presence of sonic hedgehog (SHH) and FGF8b for at least 8 days. Indeed we observed strong induction of the midbrain marker En1 on the protein level (>50% of the cells; Fig. 3*B* and *C*) associated with an induction of En1, Lmx1a, and Lmx1b on the RNA level (Fig. 3*H*). Two weeks after growth factor withdrawal we detected a prominent population of TH-positive cells (31.8 ± 7% of beta III- tubulin positive cells: Fig. 3*D*). Those cells coexpressed the dopamine transporter DAT (Fig. 3*E*) and were negative for GABA (Fig. 3*F*). Many of the TH-positive neurons coexpressed En1 (Fig. 3*G*). This phenotypic shift was accompanied by an induction of Lmx1b, En1, Pax2, Nurr1, TH, Ptx3, and aromatic L-amino acid decarboxylase (AADC; Fig. 3*I*). Remarkably, the induction of dopaminergic neurons was independent of the passage number of the lt-hESNSCs (Fig. 3*J*). To explore the potential induction of more posterior fates, we exposed the cells to 1 μM retinoic acid for 6 days. This treatment resulted in enhanced transcription of the weakly expressed Hox genes HoxA1 and HoxB1 and a strong induction of additional and more posterior Hox genes, including HoxB4, HoxB6, and HoxC5 (Fig. 3*L*). Along this line, immunofluorescence analysis showed an increase in HoxB4 expression from 0.01% to 76 ± 6% of the cells (Fig. 3*K*). When exposed to both SHH and RA, many of the cells (up to 60% vs. < 1% in the control) expressed the pMN progenitor marker Olig2, some of which were also positive for Nkx2.2 (Fig. 3*M*). Independent of their passage number, these cells gave rise to large numbers of Isl1(+) neurons (Fig. 3*N*). A significant fraction of the neurons (15.5 ± 2% from passages 12–16 and 17 ± 0.7% from passages 48–53) showed nuclear expression of HB9, indicative of a motoneuron fate (Fig. 3*O* and *P*). These data indicate that the restricted differentiation of lt-hESNSCs observed upon mere growth factor withdrawal can still be modulated toward different neuronal phenotypes using defined morphogens.

We next addressed whether lt-hESNSCs retain their neurogenic potential after transplantation in vivo. To address this issue, 10^5 cells expressing the EGFP gene under control of the PGK promoter element (10) (passage 33, 46, or 54) were injected bilaterally into the telencephalon of newborn SCID-beige immunodeficient mice (n = 35). Independent of the passage number, engrafted lt-hESNSCs were found to differentiate almost exclusively into neurons with consecutive expression of maturation-associated neuronal proteins (Fig. S10). Also in vivo, inhibitory phenotypes were predominant. No neural overgrowth or formation of teratomas was noted up until at least 24 weeks after transplantation (latest time point investigated). Functional maturation of lt-hESNSCs was monitored by patch-clamp technique in acute brain slices. To avoid recording of synaptic interaction between the engrafted cells, care was taken to

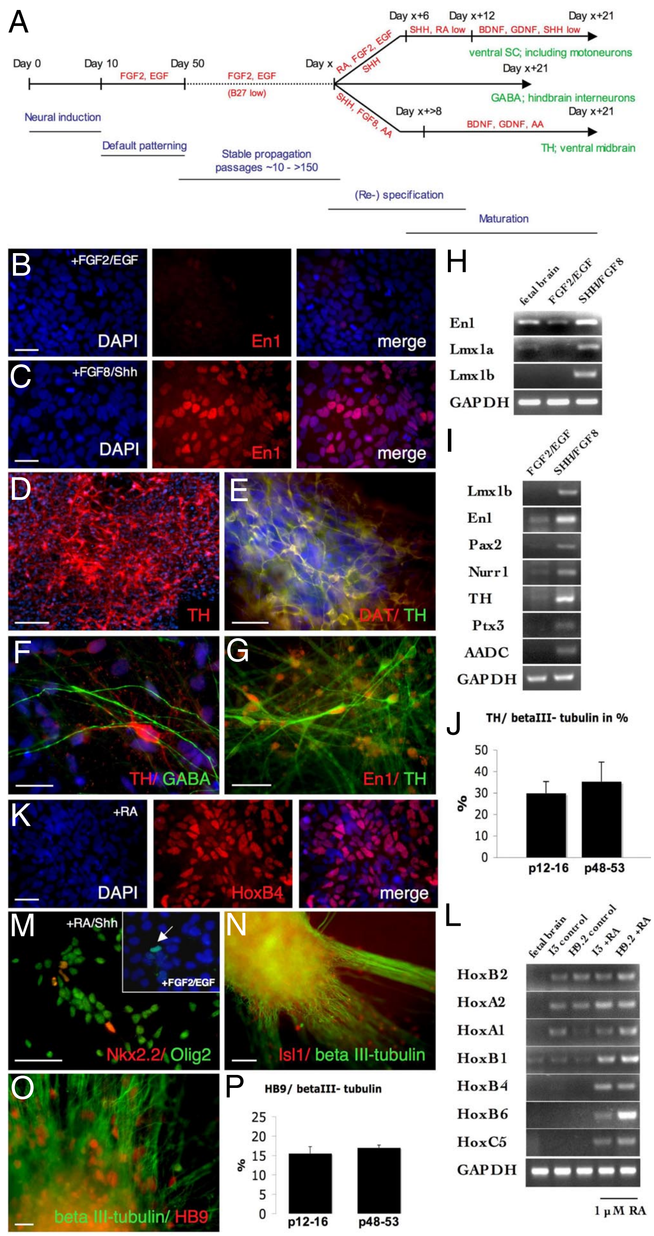

Fig. 3. lt-hESNSCs remain responsive to instructive regionalization cues. Schematic representation of the experimental protocol (*A*). Compared with control cells treated with FGF2/EGF/B27low (*B*), cells treated with Shh/FGF8 show prominent nuclear immunoreactivity for En1 (*C*). After growth factor withdrawal, they give rise to large numbers of TH-positive neurons (*D*), which coexpress DAT (*E*) but lack GABA (*F*). Many of the TH expressing neurons coexpress En1 (*G*). (*H* and *I*) Shh/FGF8-mediated induction of midbrain-specific transcripts for En1, Lmx1a, Lmx1b, Pax2, Nurr1, TH, Pitx3, and AADC in proliferating (*H*) and differentiating (*I*) lt-hESNSCs. Fetal brain and lt-hESNSCs treated with FGF2/EGF/B27low only were used as controls. (*J*) SHH/FGF8-mediated induction of TH-positive neurons is stable across the passages. (*K* and *L*) Retinoic acid (RA)-exposed lt-hESNSCs show induction of more posterior Hox genes including HoxB1, HoxB4, HoxB6, and HoxC5. On the protein level, numbers of cells with nuclear HoxB4 expression increases from <0.05% to >70% (*K*). (*M*) Exposure of RA-treated cells to SHH induces nuclear expression of Olig2 and Nkx2.2 [compared with only single Olig2(+) cells in the control (insert; arrow)]. These cells differentiate into large numbers of Isl1(+) neurons (*N*), many of which express the motoneuron-specific antigen HB9 (*O*). This induction of HB9(+) motoneurons is independent of the passage number (*P*). (Scale bars, *B*, *C*, *F*, *K*, and *O*: 25 μm; *D*: 200 μm; *E*, *G*, *M*, and *N*: 50 μm.)

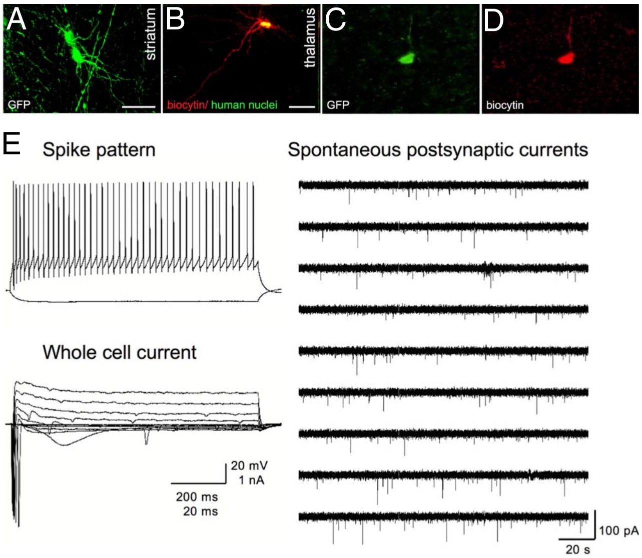

Fig. 4. Functional Integration of lt-hESNSC-derived neurons in vivo. Four months after transplantation lt-hESNSC-derived neurons were found in a variety of brain regions, where they exhibited complex neuronal morphologies. (*A*) Representative example showing GFP-positive cells in the host striatum. (*B*) Biocytin-filled neuron (red) in the host thalamus, double labeled with an antibody to human nuclei (green). (*C–E*) Representative traces from electrophysiological analysis of a lt-hESNSC-derived neuron in the host thalamus, recorded 18 weeks after transplantation into the brain of 1-day-old SCID/beige mice (*E*). Incorporated neurons exhibited large voltage-dependent inward and complex outward currents, and were able to fire repetitive action potentials. Notably, they also received spontaneous synaptic input (right traces). Immunofluorescence revealed colocalization of biocytin (diffused from the patch pipette into the recorded cell) and EGFP, confirming the donor cell nature of the recorded cells (*C* and *D*). (Scale bars: *A* and *B*, 30 μm.)

identify individual donor neurons in areas devoid of other GFP-positive donor cell processes. Recorded biocytin-filled cells were subsequently visualized by rhodamine-avidin immunohistochemistry and could be identified as donor-derived by virtue of their GFP fluorescence (Fig. 4). At 6 to 9 weeks after transplantation, GFP(+) neurons appeared far more mature than those kept in vitro, exhibiting both transient and persistent outward, and large amplitude transient inward currents. All of the neurons recorded (7 at 42 d and 3 at 60 d after transplantation) were able to fire repetitive action potentials upon long-lasting depolarization, but showed no postsynaptic currents (Fig. S11). At 18 to 24 weeks after transplantation, grafted cells displayed different phenotypes ranging from bipolar neurons to more complex morphologies with long, ramified axons and multiple dendrites (Fig. 4*A* and *B* and Fig. S10). Large voltage-dependent inward and outward currents and complex firing patterns could be monitored in all cells investigated. At this time point, spontaneous postsynaptic currents (PSCs) could be recorded in 8 out of 9 neurons (Fig. 4*C*, *D*, and *E*). Those currents exhibited a monoexponential decay with a time constant of 1.79 $\pm$ 0.33 ms compatible with AMPA receptor-mediated glutamatergic input. Taken together, these data indicate that lt-hESNSC-derived neurons can functionally integrate with and receive synaptic input from host brain tissue.

Discussion

The results of this study demonstrate that stably proliferating neural stem cells can be derived from hESCs. The stem cell status of these cells is supported by their ability for continuous self-renewal, their stable neuro- and gliogenic potential across many passages, and, most importantly, by demonstration of their multipotentiality at a clonal level. Under standard conditions lt-hESNSCs differentiate predominantly into GABAergic neurons expressing transcription factors typically observed in the ventral anterior hindbrain. Despite this apparent bias lt-hESNSCs retain expression of genes typically found in neural rosettes, the most primitive form of a neural stem cell (3, 4, 13). Accordingly, they also retain a responsiveness to instructive cues enabling the derivation of, for example, ventral midbrain TH-positive neurons or motoneurons. Finally, we demonstrate neuronal identity also on a functional basis. In particular, we provide first direct evidence for synaptic integration of human ES cell-derived neurons into a mammalian brain at a single cell level. Considering that lt-hESNSCs remain stable across many passages, are amenable to cryoconservation and genetic modification and retain responsiveness to instructive differentiation cues, they should provide a versatile tool for biomedical applications.

The stable expansion of neural stem cells has been a central topic in neurobiology. Over many years, the generation of neurospheres has been used as an important tool for both, long-term expansion and clonal analysis of neural stem and progenitor cells (17–19). However, it became evident that neurospheres are heterogeneous structures containing restricted progenitors and differentiating cells alongside bona fide stem cells (17, 20). Recently, Conti *et al.* described the derivation of adherently proliferating "NS" cells from mouse ESCs (6). While these cells, similar to ours, exhibit a remarkable degree of stability with a constant neuro- and gliogenic output across many passages, they transit through a Sox1-positive into a Sox1-negative state. In contrast, our cells still show sustained Sox1 expression. This observation could suggest that there are different developmental stages of expandable neural stem cells. Recently, Elkabetz and coworkers performed a comparative analysis of early rosette-type cells and their FGF2/EGF-expanded "NS" counterpart and found that there are pronounced differences in gene expression and responsiveness to extrinsic morphogens. Proliferation of these rosette-type NSCs was found to be strictly Notch-dependent and difficult to maintain over many passages (3). Remarkably, lt-hESNSCs exhibit rosette-like patterns even after extensive passaging in vitro. When we compared the gene expression of early rosettes and long-term expanded lt-hESNSCs, we found that many rosette-specific genes (e.g., DACH1 and PLZF) are strongly expressed by our population whereas other genes described in the rosette stage were undetectable. This indicates that lt-hESNSCs represent an FGF2/EGF-expandable population, which partially retains rosette properties. Expression of the Notch downstream genes Hes5 and Hey1 suggests that this pathway might be constitutively active during proliferation of these cells. We found that successful maintenance of lt-hESNSCs depends on the specific composition of the cell culture medium. In particular, a low concentration of B27 (1:1,000) proved to be essential, whereas media without B27 or higher concentrations (e.g., 1:50 according to manufacturer's guidelines) promote spontaneous differentiation and senescence as well as a reduced responsiveness to instructive factors.

Recent data support the notion that cells harvested at an early stage of neural differentiation exhibit an anterior phenotype (2, 3, 13, 14). Our data show that under standard in vitro conditions, lt-hESNSCs exhibit a regional transcription factor code characteristic of anterior hindbrain cells. The mechanisms underlying the pronounced posteriorization of expanded lt-hESNSCs are not understood. It is conceivable that this phenomenon, too, reflects the responsiveness of the cells to developmental cues rather than an ill-defined in vitro artifact. FGF2, which is used for the proliferation of lt-hESNSCs, is well known to exert a posteriorizing effect during nervous system development (15). The fact that freshly isolated neural precursors in passages 1–3 still show prominent expression of anterior markers such as Otx2 or FoxG1 suggests that early hESC-derived neural cells—similar to the first neural precursors appearing during normal development in vivo—exhibit an anterior identity.

At first glance, the regional restriction of lt-hESNSCs appears to limit the biomedical potential of this cell population. However, we also demonstrate that lt-hESNSCs exhibit sufficient plasticity to be recruited into other phenotypes such as the ventral mesencephalic TH-positive neurons or HB9-positive motoneurons. Yet, this plasticity appears to be limited. For example, we have been, so far, unable to "re-anteriorize" this population toward a telencephalic fate, suggesting that the posteriorization acquired during the derivation of lt-hESNSCs might be permanent.

The restricted regional transcription factor profile coincided with a prominent bias toward a GABAergic neurotransmitter phenotype. While preferential differentiation into GABAergic neurons is a well-known phenomenon in long-term expanded primary and ES cell-derived neural precursors (2, 6, 21), the reasons for this phenomenon remain unclear. In our cell culture paradigm, the predominant GABAergic phenotype would be compatible with the observed restriction in regional differentiation, thus representing inhibitory hindbrain interneurons. Interestingly, the small population of detectable glutamatergic neurons was found to express vGlut2, a glutamate transporter present in glutamatergic V2a and V3 ventral interneurons (22). Detection of occasional serotonin- and HB9-positive cells, possibly corresponding to raphe and motoneurons, further supports the notion that our lt-hESNSC-derived neurons exhibit an anterior hindbrain identity. Thus, the pronounced propensity to generate GABAergic neurons may reflect the regional restriction of our cell population rather than a cell culture artifact.

Few data are available on how human embryonic stem cell-derived neurons integrate into host tissues. While xenotransplantation is widely used for the in vivo validation of primary and ES cell-derived human neural cells (23, 24), there is little knowledge on the extent human neurons can synaptically interact with the rodent CNS. Extending previous observations that hESC-derived neurons can generate action potentials in vitro (25), Muotri *et al.* recently demonstrated that this property is maintained after transplantation into a host brain (26). However, the crucial question whether neurons derived from hESCs can indeed functionally connect to host neurons has, so far, not been directly addressed. We show that neurons derived from engrafted lt-hESNSCs not only exhibit mature neuronal phenotypes and membrane properties including the ability of repetitive firing. By detecting clear spontaneous PSCs in lt-hESNSC-derived neurons at >4 months after transplantation we also demonstrate that hESC-derived neurons can electrophysiologically connect to a host brain circuitry. These observations complement data on the morphology and intrinsic electrophysiological properties of transplanted human neurons (23, 26) and provide direct evidence that grafted human neurons in general can receive synaptic input in a mammalian CNS. The fact that this property is conserved upon cross-species grafting further supports the importance of xenogeneic transplantation models. At the same time, it is important to note that the heterotopic transplantation paradigm used in this study merely demonstrates the principal ability of lt-hESNSCs to undergo synaptic integration. Transplantation in homotopic regions and disease models will be required to explore whether these cells can contribute to functional circuit restoration and neural repair. Considering their amenability to large-scale expansion, cryopreservation and genetic modification, lt-hESNSCs should also provide an attractive tool for pharmacological screening and eventually transgenic disease modeling in human neurons.

Materials and Methods

Cell Culture. Human embryonic stem cells (hES cells; lines H9.2 passages 32–61, I3 passages 55–82, and I6 passages 42–55) were maintained according to standard protocols (1). Differentiation to neural rosettes was performed as described previously (4, 5, 10). Lt-hESNSCs were maintained as monolayer culture on polyornithine/laminin (both Sigma–Aldrich) coated plastic dishes in neural stem cell medium (NSCM) containing DMEM/F12, N2 supplement (1:100; both Invitrogen), 20 μg/mL additional insulin (Sigma–Aldrich), 1.6 g/L glucose, 10 ng/mL FGF2 (R&D Systems), 10 ng/mL EGF (R&D Systems), and 1 μL/mL B27 supplement (Invitrogen). Terminal differentiation was performed in DMEM/12 (N2 supplement; 1:100) and Neurobasal (B27 supplement; 1:50) mixed at a 1:1 ratio. cAMP (300 ng/mL Sigma–Aldrich) was added to the media (referred to as differentiation media). Differentiation toward midbrain or spinal cord fates was induced using SHH, FGF8 (both R&D Systems) and ascorbic acid (Sigma–Aldrich) or SHH and retinoic acid (Sigma–Aldrich), respectively (14, 16). For details of the cell cultures protocols see *SI Methods*.

Clonal analysis was performed using the CytocloneTM system (Evotec Technologies) (27).

Fluorescent in situ hybridization and TRAP assay were performed using Vysis alpha satellite DNA (12p11.1-q11/32–132012 and 17p11.1-q11.1/32–130017) and Chemicon Trapeze reagents.

For further details and standard methodology used for karyotype analysis, electrophysiological recordings, lentiviral transduction, immunocytochemistry, and RT-PCR see *SI Methods*.

SI Tables. For information about the primers and antibodies used in this study, see Tables S1–S4.

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