Immortalized Human Neural Progenitor Cells from the Ventral Telencephalon with the Potential to Differentiate into GABAergic Neurons

Haiyan Zhang,1 Yisong Wang,2 Yongmei Zhao,3 Yanling Yin,4 Qiuyan Xu,3 and Qunyuan Xu2*

1Department of Cell Biology, Capital Medical University, Beijing, China
2Beijing Institute for Neuroscience, Capital Medical University, Beijing Center of Neural Regeneration & Repairing, Key Laboratory for Neurodegenerative Diseases of Ministry of Education, Beijing, China
3Key Laboratory for Neurodegenerative Diseases of Ministry of Education, Xuanwu Hospital, Capital Medical University, Beijing, China
4Institute for Biomedical Science of Pain, Capital Medical University, Beijing, China

Human neural progenitor cells (hNPCs) are believed to have important potential in clinical applications and basic neuroscience research. In the present study, we created a new immortalized human neural cell line, hSN12W-TERT, derived from human fetal ventral telencephalon, using IRES-based retroviral overexpression of human telomerase reverse transcriptase. We showed that after more than 40 passages, hSN12W-TERT cells possess high telomerase activity, maintain a normal diploid karyotype, and retain the characteristics of hNPCs. Under proliferative conditions, these cells remained undifferentiated, expressing the neural progenitor cell markers nestin, vimentin, and Sox2. The cells were able to differentiate into neurons, astrocytes, and oligodendrocytes after a significant decrease in the level of telomerase following withdrawal of growth factors. The neurons were postmitotic and achieved electrophysiologic competence. Furthermore, we showed that most neurons were GABAergic, especially on differentiation induced by bone morphogenetic protein–2 (BMP2). RT-PCR analysis also confirmed that hSN12W-TERT cells expressed mammalian achaete-scute homolog 1 (Mash1) and Dlx2, genes associated with the development of GABAergic cortical interneurons. BMP2 exposure may activate a positive-feedback loop of BMP signaling in hSN12W-TERT cells. Our data indicated that this hSN12W-TERT cell line could be a valuable experimental model with which to study the regulatory roles of intrinsic and extrinsic factors in human neural stem cell biology and that it would be useful in basic research and in research seeking to discover novel drug targets for clinical candidates. © 2008 Wiley-Liss, Inc.

**Key words:** neural progenitor cells; immortalized; telomerase; human fetal; ventral telencephalon

Recent progress in stem cell research suggests that neural progenitor cells from the human brain are crucial for the successful development of approaches to cell therapy for neurodegenerative diseases such as Huntington’s disease, Parkinson’s disease, and Alzheimer’s disease (Goldman, 2005; Lindvall and Kokaia, 2006). However, these clinically important progenitor cell types have limited capacity for mitotic expansion, which has constrained the therapeutic application of human neural progenitor cells (hNPCs). Several approaches have been developed to overcome this problem, including epigenetic expansion by stimulation of cells with mitogens (Svendsen et al., 1998; Carpenter et al., 1999; Vescovi et al., 1999; Zhang et al., 2005) and a combination of genetic and epigenetic immortalization strategies: cells are transduced with an oncogene (like v-myc) by replica-

The first two authors contributed equally to this work.

Contract grant sponsor: National Program of Basic Research; Contract grant number: 2006CB500700; Contract grant sponsor: National Natural Science Foundation of China; Contract grant number: 30430280; Contract grant sponsor: Beijing Municipal Science & Technology Commission Project; Contract grant number: Z0005187040311; Contract grant sponsor: Beijing Natural Research Foundation; Contract grant number: 7011001; Contract grant sponsor: Scientific Research Common Program of Beijing Municipal Commission of Education; Contract grant number: KM200510025011.

*Correspondence to: Qunyuan Xu, Beijing Institute for Neuroscience, Capital Medical University, Beijing Center for Neural Regeneration & Repairing, Key Laboratory of Neurodegenerative Diseases of Ministry of Education, Beijing 100069, China. E-mail: xuyq@ccmu.edu.cn

Received 1 May 2007; Revised 4 July 2007 and 24 August 2007; Accepted 16 September 2007

Published online 11 January 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21581

© 2008 Wiley-Liss, Inc.
tion–deficient retroviral vectors, and their proliferation is also supported with growth factors (Sah et al., 1997; Villa et al., 2000; Cacci et al., 2007). However, telomeric shortening occurs with decreased telomerase, which acts as a brake on cell division and expansion (Harley et al., 1990; Masutomi et al., 2003); hNPCs typically show down-regulated telomerase activity to undetectable levels by a gestational age of 16 weeks (Wright et al., 1996; Ulaner and Giudice, 1997) or during early passage in vitro (Ostenfeld et al., 2000). The developmental decline in telomerase activity reflects in part transcriptional inactivation of telomerase reverse transcriptase, the rate-limiting component of the telomerase enzyme complex (Wright et al., 1996). Human telomerase reverse transcriptase (TERT) has been overexpressed in several neural progenitor cell types as a means of maintaining mitotic competence (Bai et al., 2004; Roy et al., 2004). Telomerase overexpression permits the generation of stable, nontransformed lines of human neural progenitor cells whose progeny is able to differentiate into mature cells of relatively restricted and uniform phenotypes (Roy et al., 2004). The advent of such cell lines would provide cellular substrates for the rational structural restoration of the damaged brain. However, the potential of hNPCs to differentiate may vary according to their region of origin (Hitoshi et al., 2002; Ostenfeld et al., 2002; Jain et al., 2003). Thus, the positional source of donor cells may be crucial to their use as therapeutic vectors, both in terms of homeodomain codependent responses to positional cues and transmitter repertoires (Sawamoto et al., 2001). In the ventral telencephalon, major subgroups of cerebral cortical interneurons originate in the medial ganglionic eminence. GABAergic interneurons, which comprise 20–30% of cortical neurons and their functional diversity provides the essential regulatory drive for output control in pyramidal cells. Studies of how these interneurons are born and become neurochemically and functionally specified and how they might be replenished in many neurological conditions (for example, epilepsy, Parkinson’s disease, and Huntington’s disease) are in progress (Berghuis et al., 2004).

Based on this consideration, we generated a new human neural progenitor cell line by immortalizing hNPCs from human ventral telencephalon of a gestational age of 12 weeks by overexpression of telomerase. This human TERT–immortalized cell line divided in several neural progenitor cell types as a means of maintaining mitotic competence (Bai et al., 2004; Roy et al., 2004). Telomerase overexpression permits the generation of stable, nontransformed lines of human neural progenitor cells whose progeny is able to differentiate into mature cells of relatively restricted and uniform phenotypes (Roy et al., 2004). The advent of such cell lines would provide cellular substrates for the rational structural restoration of the damaged brain. However, the potential of hNPCs to differentiate may vary according to their region of origin (Hitoshi et al., 2002; Ostenfeld et al., 2002; Jain et al., 2003). Thus, the positional source of donor cells may be crucial to their use as therapeutic vectors, both in terms of homeodomain codependent responses to positional cues and transmitter repertoires (Sawamoto et al., 2001). In the ventral telencephalon, major subgroups of cerebral cortical interneurons originate in the medial ganglionic eminence. GABAergic interneurons, which comprise 20–30% of cortical neurons and their functional diversity provides the essential regulatory drive for output control in pyramidal cells. Studies of how these interneurons are born and become neurochemically and functionally specified and how they might be replenished in many neurological conditions (for example, epilepsy, Parkinson’s disease, and Huntington’s disease) are in progress (Berghuis et al., 2004).

Based on this consideration, we generated a new human neural progenitor cell line by immortalizing hNPCs from human ventral telencephalon of a gestational age of 12 weeks by overexpression of telomerase. This human TERT–immortalized cell line divided in the presence of EGF and bFGF in adherent cultures, maintained high telomerase activity, with contact and density inhibition, and could be maintained for more than 40 passages in vitro, with an estimated population doubling time of about 4.5 days. It showed karyotypic normalcy without replicative senescence. This cell line retained its capacity to generate glia and physiologically mature neurons with persistent expression of mammalian achaete-scute homolog 1 (Mash1), an important regulator of neurogenesis in the ventral telencephalon (Casarosa et al., 1999). The production of specialized differentiated neurons derived from stem cells has been proposed as a revolutionary technology for use in regenerative medicine. However, few examples of specific neuronal cell differentiation have been described to date (Muotri and Gage, 2006). Thus, our cell line may be a valuable experimental tool with which to study the neuronal diversification of the regulatory roles of intrinsic and extrinsic factors in human neural stem cell biology and should be useful for basic research and for the discovery of novel drug targets for the treatment of neurodegenerative diseases.

**MATERIALS AND METHODS**

**Isolation and Immortalization Neural Progenitor Cells**

Human neural progenitor cells isolated from the ventral telencephalons of first trimester embryos were plated on poly-L-lysine (Sigma)–coated culture plates and cultured in a NPC medium consisting of DMEM/F12, N2 supplement [1% (v/v); Gibco], 0.6% glucose, 20 μg/mL human insulin (Sigma), 2 mM l-glutamine, 3 mM sodium bicarbonate, 1% bovine serum albumin (Gibco), 20 ng/mL human recombinant EGF (hrEGF; Gibco), and 20 ng/mL human recombinant basic FGF (hrbFGF; Gibco; Zhang et al., 2005).

The primary cultures were immortalized using VSVg-pseudotyped retrovirus encoding hTERT in the MGIN vector (Fig. 1), in which an internal ribosome entry (IRES) was used to coexpress the hTERT, truncated human nerve growth factor receptor (tNGFR), and enhanced green fluorescent protein (EGFP) genes in a bicistronic transcript emanating from the MSCV LTR (Cheng et al., 1997). The retrovirus-producing cell line PG13/JH1-hTERT was kindly provided by Dr. Alex Zhang (Xuanwu Hospital, Capital Medical University, China). For immortalization, hNPCs were plated at density of 2 × 10^5 cells/cm², grown as a monolayer (5 days in vitro after plating), and then treated with a mixture of 1 volume of viral supernatant and 2 volumes of growth medium for 20 hr with 8 μg/mL polybrene (Gibco). A repeated-infection procedure was performed over a period of 72 hr. After retroviral infection for 1 week, neural progenitor cells were expanded and passaged by trypsinization (using 0.025% trypsin and 0.05 mM EDTA) and replated at 8 × 10^3 cells/cm².

**Differentiation of Immortalized Cultures**

For differentiation, immortalized cells were plated on 24-well plates coated with poly-L-lysine/laminin (Sigma), and the medium was switched to a differentiation medium, NPC medium, which lacked growth factors hrEGF and hrbFGF. Cells were induced to differentiate by adding RA (1 μM; Sigma) in 0.01% dimethyl sulfoxide (DMSO; Sigma) and

![Fig. 1. Schematic representation of the hTERT–encoding retroviral vector. The NGFR and EGFP genes were placed downstream of an IRES, which potentially allows for coexpression of an upstream gene, hTERT, in bicistronic transcripts that also encodes the transmembrane domains of NGFR and EGFP reporters.](image-url)
incubating cells for 1, 3, 5, 7, and 14 days. In some experiments, the following factors were included in the differentiation medium alone: bone morphogenetic protein–2 (BMP2, 1 ng/mL; Peprotech EC), BDNF (10 ng/mL; Peprotech EC), and GDNF (10 ng/mL; Peprotech EC).

Telomerase Assay

Telomerase activity was measured using a TRAPEZE ELISA Telomerase Detection Kit S7750 (Chemicon) according to the standard telomeric repeat amplification protocol (TRAP) based on a previously published method (Kim and Wu, 1997). An ELISA assay was used for the nonquantitative detection of telomerase activity.

Immunofluorescence

Cultures were fixed with 4% buffered paraformaldehyde and stained using previously described procedures (Zhang et al., 2005). Representative fields from at least two independent experiments were imaged using a DFC300 digital camera and FW4000 imaging software (LEICA, Germany) and then scored. 4',6-Diamidino-2-phenylindole (DAPI; Sigma) counterstaining of nuclei was used to determine the total number of cells in a field. Primary antibodies included antibodies for glial fibrillary acidic protein (GFAP, 1:200; Sigma), or Alexa Fluor 488–conjugated chicken antirabbit IgG (1:400; Molecular Probe), or Alexa Fluor 488–conjugated goat anti-rabbit IgG (1:200, Sigma), Cy3-conjugated goat antirabbit IgG (1:400; Molecular Probe), or Alexa Fluor 488–conjugated goat anti-rabbit IgG (1:400; Molecular Probe). The specificity of all primary and secondary antibodies was confirmed in appropriate positive and negative control cultures. The secondary antibodies used for immunofluorescent detection were Cy3–conjugated goat antimouse IgG (1:200, Sigma), Cy3–conjugated goat antirabbit IgG (1:200; Sigma), Alexa Fluor 594–conjugated goat antirabbit IgG (1:400; Molecular Probe), or Alexa Fluor 488–conjugated chicken antirabbit IgG (1:400; Molecular Probe). The specificity of all primary and secondary antibodies was confirmed in appropriate positive and negative control cultures. The 5’–bromo-2’–deoxyuridine (BrdU) incorporation assay was carried out using a Cell Proliferation BrdU kit (Roche) according to the manufacturer’s instructions.

RT-PCR

Total RNA from the cultured cells was extracted by acid phenol extraction (Trizol Reagent, Invitrogen) according to the manufacturer’s protocol. Reverse transcription (RT) was performed with an oligo-dT primer using the Super-Script™ first-strand synthesis system (Invitrogen) and equal amounts of RNA from each group. One microliter of cDNA was subjected to metaphase mitotic arrest using colcemide and then harvested by trypsin dispersal, hypotonic shock with 0.075 M KCl, fixation with methanol/acetic acid (3:1 in volume) fixative, and staining with Giemsa. Cell slide preparations were taken from human lymphocytes. Data were processed using ModFitLT V2.0 software. Analysis of each sample was replicated an average of three times with a minimum of two cultures on independent days.

Flow Cytometry

Cell samples (5 × 10^5 cells) were stained with propidium iodide (PI) for DNA content measurement and analyzed as previously described (Zhang et al., 2005). Control samples were taken from human lymphocytes. Data were processed using ModFitLT V2.0 software. Analysis of each sample was replicated an average of three times with a minimum of two cultures on independent days.

Karyotype Analysis

To establish whether the lines were diploid, cells were subjected to metaphase mitotic arrest using colcemide and then harvested by trypsin dispersal, hypotonic shock with 0.075 M KCl, fixation with methanol/acetic acid (3:1 in volume) fixative, and staining with Giemsa. Cell slide preparations were then analyzed for polyplody.

Tumorigenicity Assay

To examine the tumorigenicity of the immortalized cells, 2 × 10^6 cells were injected subcutaneously into the right flanks of 4-week-old female athymic Balb/c nude mice. As a control, athymic nude mice received transplantation of 2 × 10^6 human neuroblastoma–derived SK-N-SH cells. Animals were examined weekly for the presence of tumors over a 10-month period.

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Primer sequence (5’–3’)</th>
<th>Size (bp)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP2</td>
<td>CCACCATGAAGAATCTTTGG</td>
<td>575</td>
<td>55</td>
</tr>
<tr>
<td>BMP2RIA</td>
<td>GGACATTGTCTTTGCCATATA</td>
<td>424</td>
<td>55</td>
</tr>
<tr>
<td>BMP2RII</td>
<td>TAATGCACAATGCCAAGGAGG</td>
<td>187</td>
<td>55</td>
</tr>
<tr>
<td>Dlx2</td>
<td>TGGCTGATATGCACTCGCA</td>
<td>261</td>
<td>55</td>
</tr>
<tr>
<td>EGFP</td>
<td>ATGGTGACCAAGGGCGAGGA</td>
<td>710</td>
<td>58</td>
</tr>
<tr>
<td>GAD67</td>
<td>CGAGGACCTCTCGAGCAGGAGG</td>
<td>182</td>
<td>55</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCACCAGAAGAAGCTTGGAT</td>
<td>187</td>
<td>55</td>
</tr>
<tr>
<td>GAT-1</td>
<td>AGTGCTCCACCTGACAGCTT</td>
<td>122</td>
<td>55</td>
</tr>
<tr>
<td>hTERT</td>
<td>GAGAGAGAGCGACAGCCAC</td>
<td>289</td>
<td>58</td>
</tr>
<tr>
<td>Mash1</td>
<td>GTCGAGTACATCCGCAGCGT</td>
<td>220</td>
<td>65</td>
</tr>
<tr>
<td>nestin</td>
<td>AGAGTGGAGTGGAGTGGAG</td>
<td>266</td>
<td>55</td>
</tr>
<tr>
<td>Sox2</td>
<td>AGTCTCAAGGCCAGAGAAA</td>
<td>410</td>
<td>55</td>
</tr>
<tr>
<td>vGAT</td>
<td>ACAGACCTGACATTTGAGCAG</td>
<td>358</td>
<td>55</td>
</tr>
</tbody>
</table>

control for the efficiency of mRNA isolation and cDNA synthesis. The primers used for transcription factor PCR are listed in Table I.

TABLE I. Sequences of PCR primers used

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Primer sequence (5’–3’)</th>
<th>Size (bp)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP2</td>
<td>CCACCATGAAGAATCTTTGG</td>
<td>575</td>
<td>55</td>
</tr>
<tr>
<td>BMP2RIA</td>
<td>GGACATTGTCTTTGCCATATA</td>
<td>424</td>
<td>55</td>
</tr>
<tr>
<td>BMP2RII</td>
<td>TAATGCACAATGCCAAGGAGG</td>
<td>187</td>
<td>55</td>
</tr>
<tr>
<td>Dlx2</td>
<td>TGGCTGATATGCACTCGCA</td>
<td>261</td>
<td>55</td>
</tr>
<tr>
<td>EGFP</td>
<td>ATGGTGACCAAGGGCGAGGA</td>
<td>710</td>
<td>58</td>
</tr>
<tr>
<td>GAD67</td>
<td>CGAGGACCTCTCGAGCAGGAGG</td>
<td>182</td>
<td>55</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCACCAGAAGAAGCTTGGAT</td>
<td>187</td>
<td>55</td>
</tr>
<tr>
<td>GAT-1</td>
<td>AGTGCTCCACCTGACAGCTT</td>
<td>122</td>
<td>55</td>
</tr>
<tr>
<td>hTERT</td>
<td>GAGAGAGAGCGACAGCCAC</td>
<td>289</td>
<td>58</td>
</tr>
<tr>
<td>Mash1</td>
<td>GTCGAGTACATCCGCAGCGT</td>
<td>220</td>
<td>65</td>
</tr>
<tr>
<td>nestin</td>
<td>AGAGTGGAGTGGAGTGGAG</td>
<td>266</td>
<td>55</td>
</tr>
<tr>
<td>Sox2</td>
<td>AGTCTCAAGGCCAGAGAAA</td>
<td>410</td>
<td>55</td>
</tr>
<tr>
<td>vGAT</td>
<td>ACAGACCTGACATTTGAGCAG</td>
<td>358</td>
<td>55</td>
</tr>
</tbody>
</table>
Electrophysiology

The whole-cell patch-clamp technique was used to record current. Patch electrodes of thick-walled borosilicate glass were pulled using a PP-83 micropipette puller (Narishige). The typical resistance of the glass electrodes was 3–5 MΩ when filled with intracellular pipette solution. The range of resistance of the whole-cell series was 10–15 MΩ. Data were collected using an Axopatch 200B amplifier (Axon Instruments) and acquired and analyzed using pCLAMP 9 software (Axon Instruments). The extracellular solution contained 150 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES (pH 7.4). For the recording of evoked sodium currents, cell membrane potential was held at −70 mV and stepped up to +30 mV. The patch-pipette solution contained 153 mM CsCl, 1 mM MgCl₂, and 10 mM HEPES (pH 7.2). In some experiments, tetrodotoxin (TTX, 10 μM) was added to block the inward current. To detect current through potassium channels, the patch-pipette solution contained 153 mM KCl, 1 mM MgCl₂, and 10 mM HEPES (pH 7.2).

Calcium Imaging

To identify neurons physiologically, cells in selected plates were challenged with a depolarizing stimulus of 60 mM KCl, during which their cytosolic calcium level was observed. Cells were loaded with fluo-3-acetoxymethylester (Fluo-3-AM; Molecular Probes) by incubation with 2 μM Fluo-3-AM for 30 min at room temperature (20°C–22°C) in Tyrode’s medium containing 136 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1.9 mM CaCl₂, 5 mM HEPES, and 5.6 mM glucose. After loading with Fluo-3-AM, the cells were washed with Tyrode’s medium and incubated for an additional 20 min to allow complete de-esterification of the dye. Ca²⁺ imaging was performed using LEICA-NT confocal microscopes (LEICA, Germany), generating images 512 × 512 pixels in size at an acquisition rate of 0.5 frames/sec. Fluo-3-AM was excited using a 488-nm argon ion laser, and emitted light >505 nm was detected. The Ca²⁺ imaging data in the present study were obtained at 20°C–22°C. We required a threefold increase in calcium in response to depolarization to assign neuronal identity.

Cell Counting and Statistical Analysis

All cell counts were performed on blind-coded samples. We counted both total cell number, based on DAPI-positive nuclei, and number of cells immunoreactive for different markers in the same field in three independent experiments. One-way analysis of variance (ANOVA) followed by Fisher’s post hoc test was used to assess differences between groups. Data are expressed as means ± SEMs, and differences were considered significant at P < 0.05.

RESULTS

Immortalization of Human Neural Progenitors by Overexpression of hTERT

Ventral telencephalon tissue from human fetal brains was mechanically and enzymatically dissociated to a single-cell suspension and then plated and transduced with hTERT retrovirus. The heterogeneous cell culture was further expanded with low-density replating, thus allowing for the transduced cells to generate polyclonal cell lines. Single-cell clones were then isolated using cloning discs (Sigma), deposited in each well of 24-well plates, and propagated in NPC medium with hrEGF and hrbFGF. Most selectants showed multipolar morphology, with uniform distribution on the surface of the flasks (Fig. 2a), a morphology similar to that of primary neural progenitors. One of the colonies, which we designated hSN12W-TERT (human striatum, neural, 12 weeks g.a., hTERT-immortalized), was established and underwent full characterization.

As an initial step toward studying the utility of hTERT overexpression as a means of immortalizing neural progenitor cells, we examined the expression of hTERT and the marker genes EGFP and tNGFR in...
hSN12W-TERT cells. Immunoreactivity for NGFR was clearly present on the membranes of cells (Fig. 2b). RT-PCR analysis also showed that hSN12W-TERT cells coordinated the expression of EGFP and hTERT genes at different passages (Fig. 2c). To further confirm the expression of hTERT, the telomerase activity in these cells was assessed with untransfected neural progenitors and SK-N-SH cells serving as controls. A high level of telomerase activity was found in hSN12W-TERT cells at passage 18, but this was dramatically reduced in differentiated cells. Relatively weaker activity was also detected in untransfected neural progenitors (represented as hSNs) at passage 2 (Fig. 2d). These experiments confirmed the expression of the transfected hTERT gene in hSN12W-TERT cells.

To assess the proliferation capacity of hSN12W-TERT cells, we carried out flow-cytometry analysis of the cell cycle. The proportion of cells in the G0/G1 pool was 89% at passage 12 and 77.25% at passage 30; the proportion of cells in the G2/M/S pool was 10.35% at passages 12 and 21.38% at passage 30 (Fig. 3a,b). To test the retention of mitotic competence, cultured cells at passages 12 and 30 were exposed to BrdU for different lengths of time (24, 48, 72, and 96 hr). The percentage of BrdU+ cells was correlated with the length of time to incorporate ($r^2 = 0.98$). Forty-eight hours after plating in the presence of hrEGF and hrbFGF, about 22% and 27.3% of the cells were BrdU+ (Fig. 3c,d) and slightly higher, 35% and 37% of the cells were Ki67 immunoreactive at passages 12 and 30, respectively (Fig. 3e,f). The growth rates of these cells remained similar in long-term culture, as shown in Figure 3g. hSN12W-TERT cells have been propagated continuously for more than 36 months and expanded through 40 passages to date, each of which we calculate to have included 2–3 population doublings (PDs) with an estimated population doubling time of about 4.5 days. Thus, we estimate that this line has undergone about 80–120 PDs. These experiments indicated that hSN12W-TERT cells become immortalized after overexpression of hTERT.
hTERT-Immortalized Neural Progenitors Retain Euploidy and Remain Karyotypically Normal

To verify that hTERT had immortalized the neural progenitor cells without causing neoplastic transformation, we first assessed their karyotypes. Metaphases from hSN12W-TERT cells at passages 12 and 30 were prepared and G-banded. Metaphases of both passages were found to be diploid with 23 pairs of human chromosomes (20 of 20; Fig. 3b). Neither numerical nor structural chromosomal abnormalities were detected with extended passage in vitro. We next used flow-cytometric analysis to assess the DNA content of hSN12W-TERT cells and found that the overwhelming majority of cells were diploid, with no evidence of hyperploidy at either passage and with no increase in noneuploidy at passages 12 and 30 (Fig. 3a,b). Finally, we used xenograft analysis to further evaluate the possibility of tumorigenicity. Forty weeks after transplantation of passage 12 hSN12W-TERT cells, no tumors were found at the injection sites in Balb/c nude mice (n = 5) by gross or histological examination at autopsy. In contrast, SK-N-SH cells gave rise to tumors at the dorsal site in five immunodeficient nude mice (>5) by gross or histological examination at autopsy. In contrast, SK-N-SH cells gave rise to tumors at the dorsal site in five immunodeficient nude mice (244 ± 68 pA; n = 10 cells; Fig. 5a,b) was rapid and blocked by 10 μM TTX (5 of 5 cells), indicating that all the current was TTX sensitive. Two kinds of potassium current were detected (2,810 ± 840 pA; n = 10 cells; Fig. 5a,b).

Fig. 4. Immortalized cell line possessing the properties of neural progenitor cells. a: RT-PCR showed expression of the transcription factor Sox2 and nestin by cells at different passages (hSN, passage 2 of primary neural progenitors; 1–4, passages 11, 18, 24, and 40 of immortalized cells). Immunofluorescence showed expression of nestin (b, passage 12) and vimentin (c, passage 40) by immortalized cells under proliferative conditions. Immortalized cells at passage 12 showing expression of GFAP (d), β-tubulin III (f), MAP2a/b (h), and EGFP (i) after treatment with RA (1 μM) for 7 days in vitro. e, g: DAPI counterstaining of nuclei in a field of e and f. j: Percentage of cells immunoreactive for β-tubulin III and GFAP after differentiation for 7 days. Data were obtained from triplicate experiments and are expressed as means ± SEMs (*P < 0.05, **P < 0.01, one-way ANOVA and Fisher’s post hoc test).

hSN12W-TERT Cells Possess the Potential Properties of Neural Progenitor Cells

To determine if hSN12W-TERT cells maintained the properties of neural progenitor cells, we first tested their expression of the transcription factor Sox2 and the cytoskeletal proteins nestin and vimentin, accepted markers for neuroepithelial progenitors. The hSN12W-TERT cells stably expressed mRNA for nestin and Sox2 during long-term proliferation, as shown by RT-PCR (Fig. 4a). They also produced nestin and vimentin as shown by immunocytochemistry (Fig. 4b,c). We then analyzed their differentiation potential, an important aspect of neural progenitor cells. Seven days after the withdrawal of growth factors, most cells had differentiated into GFAP+ astrocytes, with a few differentiating into GalC+ oligodendrocytes (data not shown). A significant percentage of cells differentiated into neurons expressing β-tubulin III+. When the cells hSN12W-TERT were treated with 1 μM RA, they rapidly showed neuronal differentiation. Many phase-bright cells with rounded cell bodies and long processes with β-tubulin III immunoreactivity were observed 3 days after the initiation of differentiation. After 7 days of RA treatment, about 35% of the cells were GFAP+ astrocytes (Fig. 4d,e), 60% cells exhibited β-tubulin III+ positive immunoreactivity (Fig. 4f,g), and a small of percentage were positive for MAP2a/b (Fig. 4h). Two weeks later, the number of cells showing MAP2a/b immunoreactivity cells had risen dramatically, to 45%. EGFP staining (Fig. 4i) was confined to immortalized cells. We also examined the effects of several factors known to promote neuronal differentiation on the hSN12W-TERT cells (Fig. 4j). The addition of RA or BMP2 significantly increased the number of cells differentiating into neurons compared with those in the control medium and the neurotrophins BDNF and GDNF. This result indicates that hSN12W-TERT cells possess the potential properties of neural progenitor cells.

Generation of Functional Neurons from hSN12W-TERT Cells

To examine whether the differentiated neurons had electrophysiological function, cells differentiated for 1, 3, 5, 7, and 14 days in the presence of RA or for 7 days in the presence of BMP2 that had neuronal morphology were selected for electrophysiological recordings. Substantial voltage-dependent potassium current was observed in most cells from the first day, whereas sodium currents was only observed in cells on the seventh day after treatment. The voltage-gated sodium current (244 ± 68 pA; n = 10 cells; Fig. 5a,b) was rapid and blocked by 10 μM TTX (5 of 5 cells), indicating that all the current was TTX sensitive. Two kinds of potassium current were detected (2,810 ± 840 pA; n =
10 cells), a delayed rectifier-type $K^+$ current with slight inactivation (7 of 10; Fig. 5c) and A-type potassium currents (3 of 10; Fig. 5d), which delayed rectifier inactivation within 10 msec. Some differentiated cells expressed both $I_{Na}$ and $I_K$, but the relative magnitude of the two currents varied widely. Some cells expressed $I_{Na}$ or $I_K$ alone ($n = 6$), whereas other expressed neither.

In addition, neurons generated from hSN12W-TERT cultures exhibited neuronal calcium responses to depolarization. When cultures ($n = 5$) were loaded with the calcium indicator dye Fluo-3-AM and exposed to 60 mM KCl, morphologically mature neuronlike hSN12W-TERT cells displayed a rapid and reversible more than threefold elevation of cytosolic calcium in response to $K^+$, consistent with the activity of neuronal voltage-gated calcium channels (Fig. 5e).

**DISCUSSION**

In this article we report the generation of a new telomerase-immortalized human neural progenitor cell line, hSN12W-TERT. This cell line maintains high telomerase activity and proliferates for more than 40 passages (with at least 80–120 cell population doublings) in vitro without replicative senescence or phenotypic or karyotypic changes in the presence of mitogens. In addition, our progenitor cells are nonanaplastic, with contact inhibition, and do not generate detectable subcutaneous masses in nude mice. The hSN12W-TERT cell line described in this report may be legitimately considered a human neural progenitor cell line that shares some properties with other neural progenitor cell lines (Villa et al., 2000; Bai et al., 2004; Cacci et al., 2007), such as retaining the capacity to divide without a change in its undifferentiated phenotype in the presence of mitogens and its potential to differentiate into neurons and glia following withdrawal of mitogens. However, this cell line also displays distinct properties: (1) it is derived from the embryonic human ventral telencephalon, maintains $Mash1$ expression and has the gene profile characteristics of the ventral telencephalic area after multiple passages in...
vitro; (2) it coexpresses the markers EGFP and NGFR, enabling facile monitoring of cells after transplantation; (3) it gives rise to neurons that achieve electrophysiological competence; (4) it differentiates into neurons that are predominantly GABAergic; and (5) its cells express the BMP2 receptor and maintain competence for BMP2-induced neuronal differentiation. Our findings indicate that hSN12W-TERT cells have NPC properties and could be an interesting source of GABAergic neurons for future basic and applied research.

Telomeric shortening occurs with a fall in the level of telomerase, which acts as a brake on cell division and expansion. Telomeres are synthesized by telomerase, an enzyme composed of RNA and catalytic protein subunits called hTERC and hTERT in humans (Feng et al., 1995; Nakamura and Cech, 1998). Telomerase, acting as a reverse transcriptase, produces telomeric repeats using a template provided by hTERC (Greider and Blackburn, 1989). This enzymatic activity correlates with hTERT expression, implicating this catalytic subunit as the rate-limiting component of the telomerase holoenzyme (Nakamura and Cech, 1998). hNPCs typically down-regulate telomerase activity to undetectable levels by a gestational age of 16 weeks (Wright et al., 1996; Ulaner and Giudice, 1997) or during early passages in vitro (Ostenfeld et al., 2000). Thus, ectopic expression of hTERT in normal hNPCs confers telomerase activity,
stabilizes telomere length, and directly immortalizes some of these hTERT-expressing cells (Natesan, 2005).

A major concern regarding genetic immortalization strategies, such as the one used in the present study, is the safety of the generated cell lines. Although the debate about the role of telomerase in cell transformation continues, our results demonstrate that hSN12W-TERT cells are stable and do not undergo transformation. First, the hSN12W-TERT cells retained a normal karyotype even after more than 40 passages in culture, without numerical or structural chromosomal abnormalities, as shown by our chromosomal analysis. Second, the telomerase activity of hSN12W-TERT cells dramatically decreased when the cells were induced to differentiate by the withdrawal of mitogens, consistent with previous observations in another cell line (Villa et al., 2004; Cacci et al., 2007). Third, no gross or histological tumor formation was observed 40 weeks after transplantation of the hSN12W-TERT cells into five immunodeficient nude mice.

Previous studies have demonstrated that genetically propagated lines of human neural progenitor cells allow for the maintenance of the inherent differentiation potential of hNPCs (Roy et al., 2004; Cacci et al., 2007). Consistent with these observations, characterization of the hSN12W-TERT cells in the present study showed them to possess the properties of NPCs. For example, the hSN12W-TERT cells proliferated rapidly with the expression of nestin, vimentin, and Sox2, without undergoing differentiation, in the presence of the mitogens bFGF and EGF. However, they differentiated into glia and neurons after removal of mitogens. More importantly, the differentiated neurons were electrophysiologically functional, showing inward TTX-sensitive and -resistant Na+ currents and an inwardly rectifying K+ current and global, spontaneous calcium spikes of KCl-induced depolarization. Increasing the number of neurons generated from human neural stem cells is of interest to basic scientists, drug companies, and cell therapy programs. Different neurotrophic factors have been tested for any effect on the differentiation of human neural stem cells (Sah et al., 1997; Caldwell et al., 2001). The differentiation of hSN12W-TERT cells into neurons was accelerated by the treatment with RA or BMP2, which is consistent with the results of previous studies. RA has been described as a potent inducer of neuronal differentiation (Pleasure and Lee, 1993; Sah et al., 1997). BMP2 promotes the survival and differentiation of embryonic rat striatal GABAergic neurons in culture (Hattori et al., 1999). Furthermore, our results suggest that hSN12W-TERT cells retained their regional identity, even after multiple in vitro expansions. Using RT-PCR, we found that the hSN12W-TERT cells expressed the genes encoding the basic helix-loop-helix transcription factor Mash1 and the homeodomain transcription factor Dlx2. The proneural protein Mash1 has already been shown to be essential for the production of neuronal precursor cells in the embryonic ventral telencephalon (Casarosa et al., 1999; Horton et al., 1999). In addition, Mash1 expression in embryonic progenitors from the telencephalon is necessary for the survival and/or differentiation of GABAergic interneurons and may regulate the expression of the Dlx homeodomain protein family, members of which are needed to produce GABA (Fode et al., 2000; Parras et al., 2002). Our data showed that the expression of Mash1 and Dlx2 increased on differentiation induced by BMP2 and that this increased expression was coincident with increasing expression of GAD67, the key enzyme involved in the synthesis of GABA, as well as vGAT and the high-affinity transporter protein GAT-1. Furthermore, about 61% of all cells were GABA immunoreactive following exposure to BMP2 for 7 days, similar to the percentage of GABAergic neurons generated from the immortalized striatal neural stem cell line ST14A (Bosch et al., 2004). In order to explore the mechanism of BMP2-induced differentiation, the expression of BMP2 and its receptors was examined in hSN12W-TERT cells. Our results suggest that hSN12W-TERT cells express two kinds of receptors for BMP2, BMP2RIA and BMP2RII, and that BMP2 exposure may activate a positive feedback loop of BMP signaling by elevating the spontaneous expression of additional BMP2. With regard to the interaction between BMP2 and Mash1 during neuronal differentiation, the study by Lo et al. (1997) points to a feedback interaction between these proteins in a reciprocal manner to promote neurogenesis. BMP2 is required for both the induction and maintenance of Mash1 expression, and continued expression of Mash1 in progenitor cells maintains their capacity to respond to BMP2. This linkage creates a self-reinforcing positive-feedback loop that may help to drive the cells toward a neuronal fate. The basis for this irreversible change found in our study is not understood but will be revealed by studies of a loss-of-function mutation in the future.

In conclusion, the immortalized human CNS cell line described in this article can be readily expanded in proliferative growth conditions, providing a renewable, homogeneous source of cells. These cells can then be differentiated over a relatively short period into glia and/or neurons that express appropriate phenotypic markers. Thus, this human CNS cell line offers significant advantages for CNS drug discovery. Moreover, the reported cell lineage studies provide a basis for investigating the molecular mechanisms that regulate cell fate in the human CNS.

ACKNOWLEDGMENTS

We thank Dr. Qi-Lin Cao at the Spinal Cord Injury Research Center, University of Louisville, for critical reading of the manuscript and Professor Alex Zhang for providing the retrovirus-producing cell PG13/JH1/hTERT.

REFERENCES


