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# Stable Culture of hTERT-transduced Human Embryonic Neural Stem Cells Holds All the Features of Primary Culture

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## Abstract

Human neural progenitor cells promise to be the source for cell therapy of variety of human neurological diseases and trauma. But the proliferation of these cells in vitro is severely limited. Via lentiviral-mediated introduction of telomerase reverse transcriptase gene (hTERT) into the cells of primary culture of human embryonic neural stem cells (NSC) we obtained the culture with extended proliferative potential. Under the conditions of low oxygen (3%) original cells ceased to proliferate after 42 population doublings (PD), NSC-hTERT cells achieve the level of 78 PD for 430 days and are proliferating further with constant rate. The culture of NSC-hTERT contains as differentiated cells (β-III-tubulin or GFAP-positive) as well the non-differentiated (most cells are nestin-positive) ones. Only a small portion of cells expresses high level of hTERT-protein. The culture can grow as monolayer (in the presence of 2% of serum) or as floating spheres (without serum). The cells preserve diploid karyotype. They have ability to form two kinds of colonies under conditions of low density. One type with usual for NSC structure contains different cell types. Cells of other colonies resemble the senesced ones with flattened shape. This study confirms that telomerized cells can be a valuable source for research and clinical application.

**Keywords:** Telomerase; cells senescence; neural stem cells.

## 1. Introduction

The human central nervous system (CNS) has very limited regenerative potential. Patients with severe injuries in the CNS such as spinal cord injury frequently endure lifelong disability. From the other hand various neurodegenerative diseases are accompanied by the death of own neurons because of unclear reasons. It seems that both conditions: trauma and neurodegenerative diseases need to be treated to replace the cells losses.

There are different sources of cells for transplantation, but all of them have own drawbacks. Neurons derived from human embryonic stem (ES) cells can efficiently generate dopaminergic neurons in vitro, when they are grafted, but they have a propensity to form teratomas. Moreover, survival of grafted neurons derived from human ES cells have, so far, been poor. To avoid histocompatibility problems is better to use patient's own cells. But it is very difficult to expand human neural progenitor cells *in vitro* because they have a limited replication life-span and gradually lose their differentiation potential in that state. Recent studies have shown that ectopic expression of the hTERT gene extends the life span of a number of human cell types. In our laboratory we successfully obtained the number of telomerized immortal cells including fibroblasts, stromal cells from lipoaspirate, dermal papilla cells and bone marrow stromal stem cells [1]. Telomerized cells have ability to differentiate. They preserve normal mechanisms of proliferation [2]. In addition telomerized cells have increased ability to survive that may be necessary in the process of grafting [3, 4].

## 2. Methods

### Cells Isolation and Cultures.

Establishment of culture from first trimester human forebrain tissue was performed by Dr. Saburina (Moscow State University) and has been described previously [2]. Cells derived from embryonic brain tissue from 8 weeks human fetus was acquired after an abortion under compliance with Russian government guidelines and the local ethics committee. Briefly, the tissue was mechanically minced, cells were dissociated with 0.05% trypsin/0.04% EDTA and the resulting cell suspension was plated in cultural T25 flasks (Corning, USA) in the presence of 10 ng/ml EGF and 10 ng/ml bFGF (both human recombinant) in an DMEM/F12 medium supplemented with 2% of fetal bovine serum replacement (Fetal Clone III, Hyclone, USA), 2mM glutamine, N2 supplement, 0,11 mg/ml

sodium pyruvate, and 40 unit/ml gentamycin at 5% $CO_2$, 92% $N_2$ and 3% $O_2$. At that conditions cells formed near-monolayer culture. Cells were replated with 0.05% trypsin/0.04% EDTA upon they achieves monolayer density. The same media without serum replacement was used for obtaining of floating neurospheres. After 10-20 days, sphere formation could be observed.

**Karyotyping**

The cultures were exposed to colcemid (0,1 µg/ml) for two hours. The cells were detached from the surface by trypsin/EDTA, treated in a 0.06 M KCl hypotonic solution for one hour, subjected to repeated fixation in methanol/acetic acid (3:1), and dropped on to wet slides. The chromosomes were stained with 4,6-diamidino-2-phenylindole (DAPI) and 7-aminoactinomycin D [5].

After fixation and air-drying, the slides were scanned under a phase contrast microscope. The presence of metaphase spreads; their quality and position on the slide were noted. Two black and white images were collected on an Olympus microscope BX61 using 60x 1.4 N.A. oil immersion lens. Adobe Photoshop 5, 5 software was used to produce color and contrast images. 20 chromosomal plates were analyzed.

**Cell Infection**

Human hTERT cDNA, including full coding region [6], was cloned under CMV-promoter into lentivirus vector pLU-PL3 [7] were gifts from Dr. Chumakov. The medium in monolayer culture of NSC was removed and cells washed twice with PBS. Then the 1:2 mixture of the lentiviral stock containing $10^8$ particles per ml and the growth medium supplemented with 8 µg/ml of polybrene was added. After overnight incubation cells were washed twice and fresh medium was added.

**Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde in PBS. Immunocytochemistry was carried out using standard protocols. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Antibodies and dilutions were as follows: hTERT monoclonal, 1:5000 (Abcam, UK); β-III-tubulin monoclonal, 1:500; GFAP monoclonal, 1:500 (all from Sigma); nestin monoclonal, 1:500 (BD, USA). Fluorescein or Cy-3-labelled secondary antibodies (Sigma, USA) were then applied.

**RNA extraction and RT-PCR.**

Total cellular RNA was extracted from NSC and NSC-hTERT using Total RNA Isolation System (Promega, USA). A poly-A mRNA was reverse transcribed into cDNA by using Oligo(dT)15 cDNA first chain Synthesis kit (Sileks, Russia). For polymerase cycle reactions we used JumpStart™ Taq ReadyMix™ kit (Sigma, USA), and amplifier Tercik (DNA-Technology, Russia). Primer sequences (forward, reverse) and lengths of the amplified products were as follows:

GAPDH (Human glyceraldehyde-3-phosphate dehydrogenase) (5'-TGT TGC CAT CAA TGA CCC CTT-3', 5'-CTC CAC GAC GTA CTC AGC G-3', 202); NEST (Nestin) (5'-GAA ACA GCC ATA GAG GGC AAA-3', 5'-TGG TTT TCC AGAGTC TTC AGT GA-3', 167); NSE (Neuron specific enolase) (5'-ACA AAC AGC GTT ACT TAG GCA A-3', 5'-CTC CAC CAC AGA GAG ACC TGA-3', 101); TH (Tyrosine hydroxylase) (5'-GCA CCT TCG CGC AGT TCT-3', 5'-ACA GCG TGG ACA GCT TCT CA-3', 84); CHAT (Choline acetyltransferase) (5'-TGC CGC CTA CTG AGA GCA-3', 5'-TGG CAG GAG TCA AGG TTG GT-3', 83); GFAP (Glial fibrillary acidic protein) (5'-CCT CTC CCT GGC TCG AAT G-3', 5'-GGA AGC GAA CCT TCT CGA TGT A-3', 161); PLP (Myelin proteolipid protein, lipophilin) (5'-TGA GCG CAA CGG TAA CAG G-3', 5'-GGG ATG TCC TAG CCA TTT TCC-3', 110);

## 3. Results and Discussion

Soon after replating in the presence of 2% serum the culture of NSC forms the pseudo-monolayer (Figure 1b) that consist of the various cell types (Figure 1a). With passing time after replating some proliferation occurs and the cells have tendency to aggregate (Figure 1c).

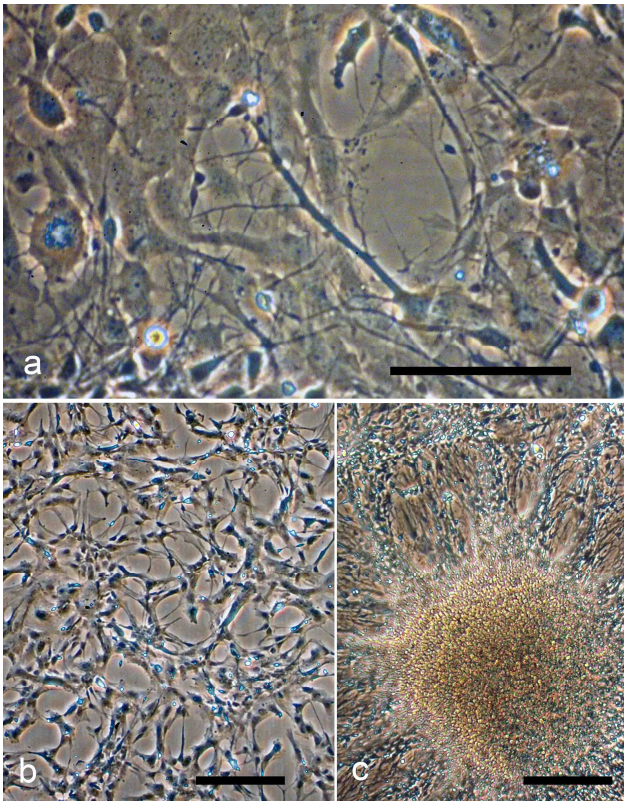


**Figure 1**. Original monolayer NSC culture. a. different cell types, b. common view of monolayer , c. tendency to form spheres. Bars: a - 100 µm, b and c - 250 µm. Phase-contrast, digital contrast.

The lenti-viral construct pLA-CMV-hTERT containing human telomerase reverse transcriptase gene (hTERT) was introduced in the cells of the NSC culture at the level of 9 population doublings (PD). The efficacy of transfection with similar construction containing GFP was around 75%. There were no morphological differences in both NSC and NSC-hTERT cultures during the long time. Original NSC decreased proliferation around 40 PD and proliferation completely ceased at the level of 42 PD (Figure 2).

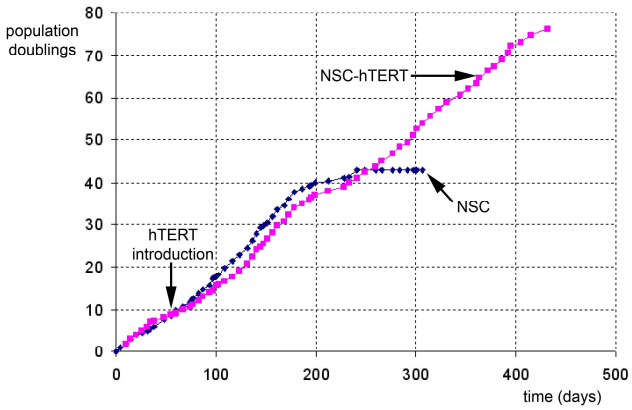


**Figure 2**. Growth curve of NSC and NSC-hTERT.

NSC-hTERT cells continued to proliferate with previous rate. At present time they achieve the level of 78 PD for 430 days and are proliferating further with constant rate. The cells of both cultures have the tendency to aggregate in the presence of 2% of serum replacement (Figure 3a,3c). Cells aggregates connect each other by numerous neurites, stained for β-III-tubulin (Figure 3b).

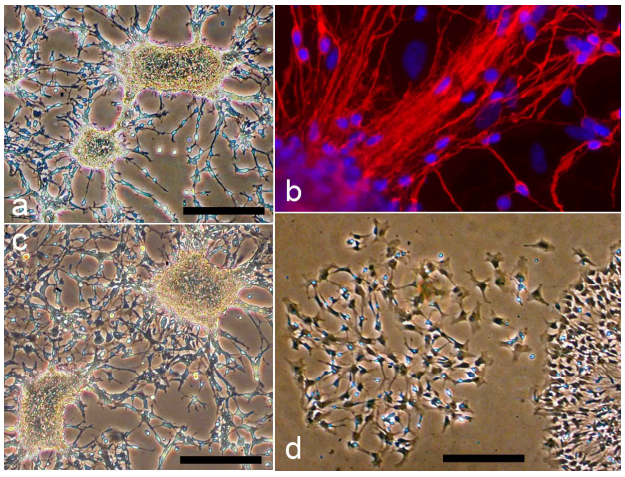


**Figure 3**. Morphological features of NSC and NSC-hTERT. a. NSC-hTERT, b. β-III- tubulin in NSC-hTERT, c. NSC, d. flattened morphology of NSC-hTERT after transfer of floating spheres to the media with 10% of serum. Bars: a, c, d - 250 μm. Phase-contrast, digital contrast.

In the absence of serum NSC-hTERT forms free floating spheres (Figure 4). These spheres can be cultivated long time as spheres. After addition of 10% of serum neurospheres attach to the plastic and cells acquire flatten morphology (Figure 3d).

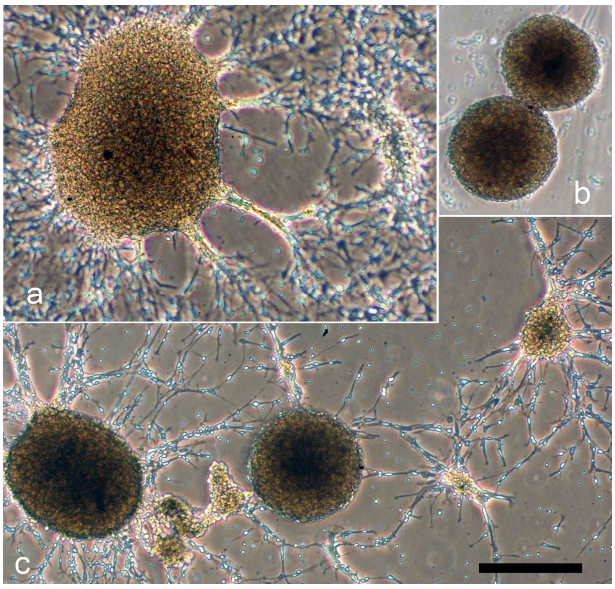


**Figure 4**. Formation of floating spheres in the culture of NSC-hTERT in the absence of serum. Bars: a, b, c – 250 μm. Phase-contast, digital contrast.

Most of NSC-hTERT expresses nestin (Figure 5c), about 8-18% - β-III-tubulin (Figure 5b,5d), and 2-10% - GFAP (Figure 5b,5d). The proportion of these markers is similar in NSC and NSC-hTERT.

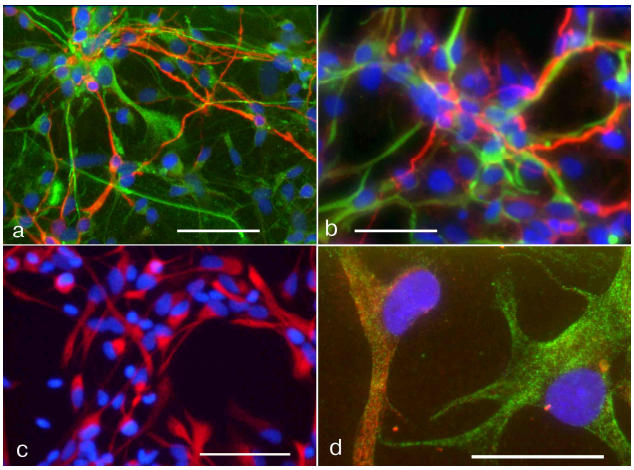


**Figure 5**. Protein markers of NSC and NSC-hTERT. a. NSC, β-III- tubulin (red), GFAP (green); b and d. NSC-hTERT, β-III- tubulin (red), GFAP (green), c. NSC-hTERT; nestin (red). Bars: a, b, c – 150 μm, d – 50 μm.

Despite of nestin, GFAP, and NSE mRNA expression, NSC-hTERT does not express mRNA of more mature neuronal marker - tyrosine hydroxylase. Also, expression level of choline acetyltransferase decreases (Figure 6, arrowhead).

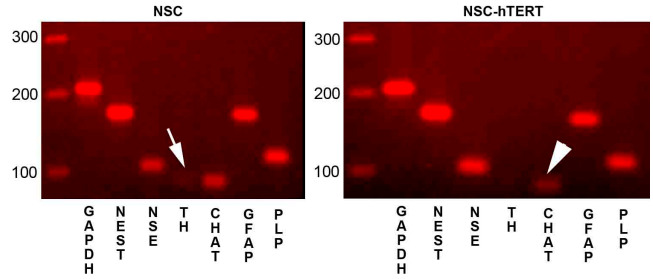


**Figure 6**. RNA expression in NSC-hTERT. Arrow indicates weak signal from TH

Karyotype analysis reveals the presence of diploid set of chromosomes in the NSC-hTERT cells. Chromosome banding is corresponding to R-banding of human chromosomes (Figure 7).

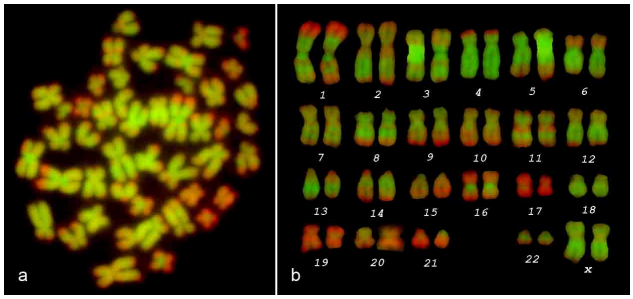


**Figure 7**. Chromosomes of NSC-hTERT. a. chromosome plate, b. karyotype.

When original NSC stopped proliferation their morphology became very similar with morphology of senescent fibroblasts (Figure 8).

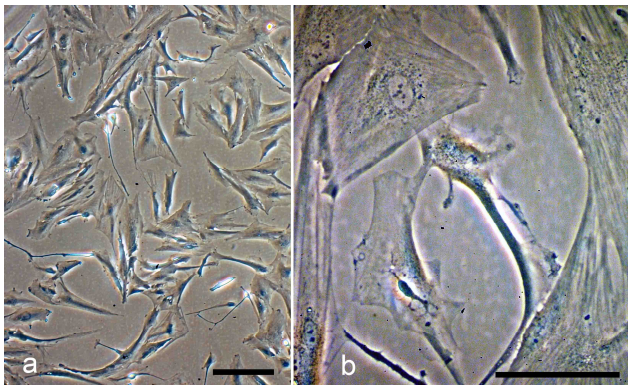


**Figure 8**. Senescent culture of NSC. Bars: a – 250, b – 100 μm. Phase-contrast, digital contrast.

Plating at very low density NSC-hTERT during 30 days form colonies with different cells types. There are at least two types of colonies: the colonies with usual for NSC culture structure contained all cell types (Figure 9a). Another colonies forms by non-dividing epithelial-shape cells (Figure 9b,9c).

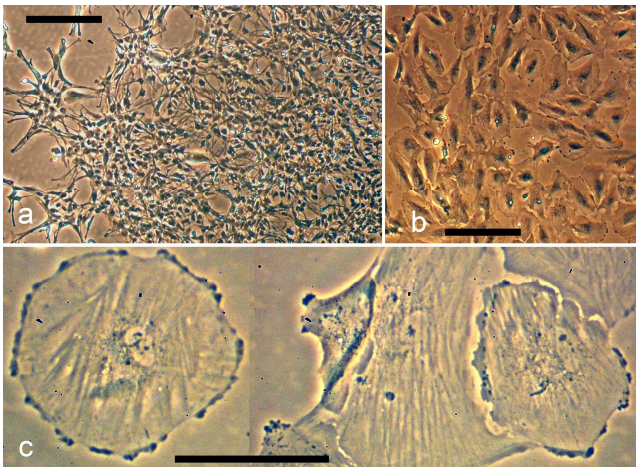


**Figure 9**. Two types of colonies formed by NSC-hTERT. Bars: a, b – 250 μm, c – 100 μm. Phase-contrast, digital contrast.

Telomerase expression investigated by immunocytochemistry revealed that only small portion (a few percents) of NSC-hTERT express hTERT at relatively high level exceeding the level of hTERT protein in NSC or diploid human fibroblasts (Figure 10). It is interesting, but some cells contain hTERT in the cytoplasm (Figure 10e). These cells usually have flattened shape resemble a senescent fibroblast

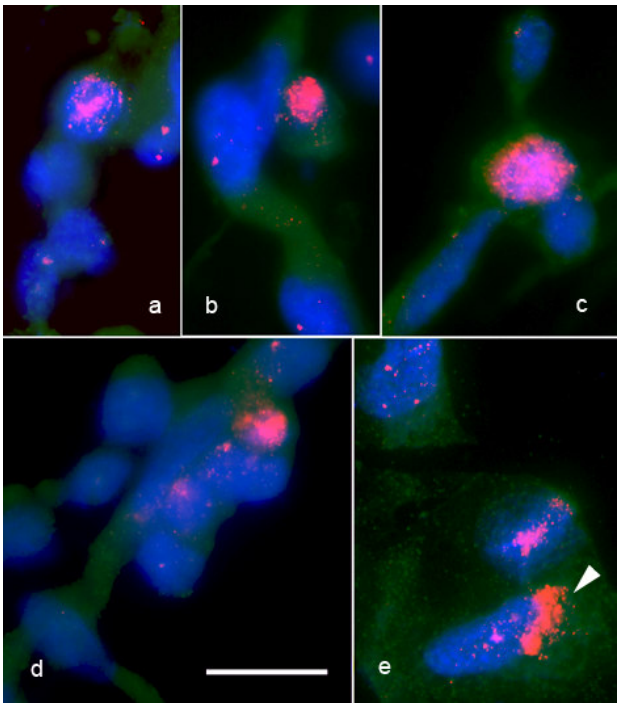


**Figure 10**. hTERT expression in NSC-hTERT culture. Only small portion of cell nuclei have hTERT staining. Arrowhead indicates hTERT in the cytoplasm. Bar - 25 μm.

## 4. Conclusions

Present work describes embryonic human NSC immortalization by hTERT transfection. From the best of our knowledge it is the second case after the work of Chinese scientists [8]. All described properties of our NSC-hTERT cells demonstrate the preservation of growth regulated properties. The cultures of NSC and NSC-hTERT cannot be distinguished by morphology, they contain similar portions of β-III tubulin and GFAP-positive cells. Both cultures can form spheres, and these spheres equally respond to 10% of serum.

Expression of telomerase doesn't prevent the processes of differentiation. It is interesting that original NSC culture at the final steps of the growth lost the signs of differentiation. All the cells became very similar with the culture of senescent fibroblasts (fig. 8). In contrast, NSC-hTERT during colony-forming assay can differentiate into the non-dividing cells, but that cells have marked epithelial morphology (fig. 9b,c). The absence of TH expression and decreased level of CHAT mRNA (fig. 6) may be consequences of long-time proliferation of NSC-hTERT (about 70 PD) in contrast with the level of around 20PD in case of NSC. TH and CHAT participate in the interneuronal communication and our culture condition (frequent passing of the cells) prevents this process.

Our NSC-hTERT culture grow relatively slowly, one PD for 5,5 days, because part of cells constantly exit cell cycle and differentiate. High expression of hTERT exists only in small portion of cells. Their morphology (absence of neurites or glial outgrowths and small size) resembles non-differentiated state. This possible indicates that stemness is maintained only in the little portion of cells.

There are many ways to produce immortal NSC cells, for example by transfection v-*myc* oncogene, as described previously [9]. But the oncogenes change regulation of proliferation of cells and increase the probability of tumor formation. In case of hTERT expression growth regulation is not perturbed [1, 2].

We think that the problem of telomerization (immortalization via telomerase expression) is consisting of the selection of appropriate culture conditions. Some cells like epithelial cells need other cells or their products (basal membrane) to proliferate [10]. So they can be immortalized by hTERT only in the appropriate culture conditions. The presence of some differentiated cells in the culture of NSC should provide these conditions.

Telomerized cells have another advantage over original cells except increased proliferation potential. They have decreased level of reactive oxygen species (ROS) and increased stress resistance [11]. That should together with telomerase activity preserve genome from the damage, especially after transplantation in the diseased tissues, which are generally characterized by the increased ROS levels.

## Acknowledgements

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