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Journal of Neuroscience Methods 170 (2008) 262–276

JOURNAL OF NEUROSCIENCE METHODS

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Immortalization and characterization of lineage-restricted neuronal progenitor cells derived from the porcine olfactory bulb

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Received 14 February 2007; received in revised form 27 December 2007; accepted 23 January 2008

Abstract

Crucial aspects in the development of *in vitro* neuropathogenic disease model systems are the identification, characterization and continuous mitotic expansion of cultured neuronal cells. To facilitate long-term cultivation, we immortalized porcine olfactory neuronally restricted progenitor cells by genomic insertion of a cDNA encoding the catalytic subunit of the human telomerase reverse transcriptase (hTERT) yielding a stable neuroblast subclone (OBGF400). The altered cells exhibited progenitor-cell-like morphology and mitotic competency based on sustained subpassaging, prevalence in the cell cycle G0/G1 phase and an overall lack of cellular senescence as compared to primary cultures. An OBGF400 neuronal phenotype was indicated by the recognition of a transfected neuronal progenitor-cell-specific tubulin-α1 gene promoter, intracellular presence of early neuronal markers (TuJ1, neuregulin-1, doublecortin and SOX2) and enhanced expression of neuronal- and progenitor lineage-active genes (MAP2, nestin, ENO and Syn1) compared to that of porcine epithelial cells. These OBGF400 neuroblasts are likely dependent on telomerase to prevent terminal differentiation as subcultures with a predominance of neuronally differentiated members had less enzymatic activity. Based on its susceptibility to a porcine alphaherpesvirus infection, this novel neuroblast cell line may be useful for exploring neuronal cell–pathogen interactions *in vitro*.

Keywords: Olfactory bulb; Porcine neuroblasts; Porcine neuronal progenitor cells; Neural transcriptome analysis; hTERT; Alphaherpesvirus

1. Introduction

Biomedical research benefits from the use of natural host–pathogen systems, a situation inherently limited with respect to humans, thereby necessitating animal models. In this regard, swine fulfill the need for a suitable alternative that accurately and precisely emulates many aspects of human structure and physiology. For instance, these animals are similar to humans in terms of organ size, digestive physiology, pulmonary and coronary vasculature, social behaviors, dietary habits and propensity to obesity (reviewed in Tumbleson and Schook, 1996). Their consequential use for medical studies has provided significant advances even to the field of neurology, such as in the understanding of the neurological dysfunctions of Alzheimer's (Smith et al., 1999) and Parkinson's (Mikkelsen et al., 1999) disorders. Furthermore, infectious disease investigations have been advanced most effectively by the direct use of naturally occurring host–pathogen systems. As viral disease outcomes are dependent on interactions of the pathogen with the intracellular macromolecular environment and its surrounding milieu, the pig is an excellent host for studying disturbances of host–viral equilibria that create circumstances conducive for the emergence of lytic and latent viral infections in the nervous system. In this regard, pseudorabies virus (PrV), a natural pathogen of pigs, offers an attractive yet underutilized *in vivo* and *in vitro* system for the direct study of viral infections in the context of the natural host (Scherba and Zuckermann, 1996). Clearly, the development of a suitable *in vitro* neuronal model system will ultimately facilitate the exploration of the multifaceted viral–cell interactions during viral neurotropic pathogenesis. The gained understandings may be relevant to evaluating the impact of other neurotropic viruses on their hosts and applicable to *in vivo* situations. Interestingly, thus far *in vitro* porcine neural cell lines suitable for such model systems are not available due in large part to the low frequency of tumor formation in the porcine central nervous system; an intriguing situation in and of itself. Accord-

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doi:10.1016/j.jneumeth.2008.01.028

ingly, current *in vitro* models must utilize primary porcine neural cultures, and thus would benefit from the ability to propagate neuronal cells for extended periods of time, an intricate endeavor at best. A major obstacle to this long-term cultivation has been the limited capacity of neuronally restricted progenitor cells for mitotic expansion (Kirschenbaum et al., 1994; Reynolds and Weiss, 1992; Roy et al., 2004).

The loss of mitotic capacity during subpassaging of primary cell cultures can be attributed in some measure to decreased telomerase activity and concomitant shortening of the 3′ ends of the telomeres, a process known to trigger cell crisis through chromosomal fusion and mitotic degradation (Counter et al., 1992). In contrast to primary cells, immortalized cells undergo continuous division, a process enabled by telomerase activity that maintains the length of chromosomal telomeres. Generally, somatic cells exhibit little if any telomerase gene expression (Kilian et al., 1997), whereas multipotent stem cells sustain high telomerase activity and retain their self-renewal potency. In human progenitor cells, such enzymatic activity is typically reduced by the 16th week of gestation or during early subpassaging of cultured cells (Ulaner and Giudice, 1997; Wright et al., 1996). Interestingly, this diminution is partially the result of transcriptional silencing of the catalytic subunit of the telomerase reverse transcriptase (TERT). Thus compensatory ectopic expression of TERT prevents telomere shortening thereby enabling the generation of stable and non-oncogenic cell lines (Counter et al., 1992). Pertinent to our research interests, a recent study by Roy et al. (2004) observed that ectopically induced over-expression of human TERT (hTERT) in neuronal-restricted progenitor cells derived from the human fetal spinal cord significantly extended their life span without compromising responses to mitogenic factors or altering phenotypic characteristics. Moreover, the cells retained karyotype normalcy during serial passaging.

In anticipation of establishing a stable porcine neuronal cell line, the olfactory bulb (OB) should be considered as it is a superior source for the identification and isolation of neuronal lineage-restricted progenitor cells. The OB undergoes neurogenesis even during adulthood and contains multi-lineage as well as lineage-restricted neural progenitors (Liu and Martin, 2003). As a direct extension of the anterior subventricular zone (SVZ) of the lateral ventricle and the rostral migratory stream (RMS), the OB region is the final destination of tangentially migrating neuroblasts. Despite exhibiting biological properties associated with differentiated neurons, these cells maintain their mitotic competence until they reach the more peripheral layers of the OB (Coscun and Luskin, 2002; Lois and Alvarez-Buylla, 1994).

Here we report the extended mitotic competency of such porcine olfactory neuronal progenitor cells after the insertion of a cDNA encoding the catalytic subunit of hTERT into their genomes. This transduced cell line has been stably maintained throughout approximately 100 subpassages with consistent hTERT transcription and activity levels. Moreover, the expression of the tumor suppressor gene p53 and the proto-oncogene c-myc also was found to be unaltered. This neuroblast cell line was phenotypically characterized at the levels of translation by using immunocytochemistry and transcription by utilizing a porcine-specific real-time (r) RT-PCR array. Additionally, to test the suitability of this novel cell line for studies on alphaherpesviruses, their permissiveness to viral infection was determined by monitoring PrV replication and latency-associated transcript (LAT) levels in the immortalized OB neuroblasts. Hence, our approach denotes valuable methodologies for surmounting replicative cellular senescence of committed neuronal progenitor cells and additionally provides comprehensive information on porcine neural gene expression through utilization of a porcine-specific real-time array for a robust transcriptional analysis to characterize this new *in vitro* neuronal cell system.

2. Materials and methods

2.1. Establishment of primary porcine olfactory bulb cell cultures

Olfactory bulbs from 1-day-old pigs were removed and transferred into ice-cold PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]) solution (120 mM NaCl, 5 mM KCl, 25 mM glucose, 200 μM glutamine, 20 mM PIPES pH 6.4, 100 U/ml penicillin and 0.1 mg/ml streptomycin) (Sigma, St. Louis, MO). Tissues were minced and dissociated in PIPES solution containing 3 mg/ml collagenase A (Sigma) for 45 min at 37 °C with gentle tituration every 15 min. Following complete dissociation, the cell suspensions were applied to a 40-μm cell strainer (Becton-Dickinson, San Jose, CA) and subsequently centrifuged at 400 × *g* and 4 °C for 10 min. The pellets were then resuspended through gentle pipetting in growth medium consisting of Dulbecco's modified Eagle medium (DMEM)/F12/HAM (Sigma) supplemented with 10% bovine calf serum (Sigma), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.05 mg/ml gentamicin (Sigma). Approximately 2×10^6 cells/ml were plated into poly-D-lysine precoated 6-well tissue culture dishes (Midwest Scientific, St. Louis, MO) and incubated at 37 °C in 5% CO_2 for 18 h. Medium was then aspirated to remove non-adherent cells and replaced with fresh growth medium every 48–72 h. After at least 10–12 days in culture, the cells were trypsinized, plated by limiting dilution to select for mitotically competent, adherent cells and incubated as described above for at least 4–5 days prior to the immortalization procedure. All resultant cultures displayed similar morphology.

2.2. Cellular immortalization

The established primary heterogeneous porcine OB cultures were infected with a murine leukemia virus-pseudotyped amphotrophic retrovirus designed to express hTERT. Production of the amphotrophic retrovirus was achieved through co-transfection of an hTERT cDNA-containing pBabe-Neo vector (p0196) and a pCL-10A1 (p0467) packaging construct (both generous gifts from Dr. C. Counter, Duke University) into 293TS cells as previously described (Counter et al., 1998). Briefly, 3 μg of each vector and 12 μl Fugene-6 (Roche, Indianapolis, IN) were mixed in 200 μl of serum-free minimum essential medium (Sigma) and incubated at 25 °C for 15 min. The transfection mix-