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Establishment of the cell line expressing human prion protein on PrP0/0 background

M. PRČINA, P. FILIPČÍK, E. KONTSEKOVÁ

Institute of Neuroimmunology, Slovak Academy of Sciences, 845 10 Bratislava, Slovak Republic

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Summary. – In this work we described preparation of the novel cell lines expressing human prion protein on $PrP^{0/0}$-background. Prepared cell lines originated from the Nagasaki mice ($PrP^{0/0}$) and showed a fibroblast phenotype. The expression level of human prion protein in the developed cell lines was comparable to the physiological expression measured in GT1-7 cells. A great advantage of the prepared cell lines was their short doubling time that allowed obtaining of a large amount of cells for the proteomic experiments. Newly established cell lines open a broad spectrum of applications in the prion research. Besides the study of physiological function of the prion protein or its interactome, the new cell lines could be successfully employed as a unique tool for the better understanding of key events in the pathogenesis of prion diseases.

Keywords: cell line; human prion protein; knockout; stable transfection

Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are lethal neurodegenerative disorders. TSEs include Creutzfeldt–Jakob disease, Gerstmann-Sträussler-Scheinker syndrome, fatal familiar insomnia and kuru in humans, scrapie in sheep and goats, bovine spongiform encephalopathy in cattle, and chronic wasting disease in North-American cervids (Prusiner, 1998). Clinically, the prion diseases manifest as dementia and motor dysfunction and neuropathologically as spongiform degeneration of the brain.

A defining feature of this group of disorders is the conformational change of normally expressed prion protein (PrP^{C}) into the insoluble misfolded, β-sheet rich conformer resistant to the proteolytic degradation that is toxic to the neurons (PrP^{Sc}) (Prusiner, 1998). PrP^{Sc} is believed to be the only component of infectious agents causing prion diseases. Expression of PrP^{C} is necessary for the PrP^{Sc} replication and development of the disease. PrP-knockout mice are completely resistant to the prion infection, while $PrP^{+/-}$ mice are intermediately susceptible (Sakaguchi *et al*., 1995). The physiological role of PrP^{C} remains unclear, though several functions are suggested like a role in the copper metabolism, anti-apoptotic activity, or cytoprotection against oxidative stress (Bounhar *et al*., 2001; Pauly and Harris, 1998; Brown *et al*., 1997).

Experimental cellular models are unique tools in many aspects of the prion research. They have been used for the study of physiological role of the prion protein and for the elucidation of prion protein conversion leading to the neurodegeneration. Cell model exhibits a great advantage compared to the animal model such as a fast growth, shorter incubation time after the prion infection, and a lower price. PrP-knocked out cell lines are used mainly for study of the physiological role of prion protein. After transfection with the various wild-type or mutant prion protein genes, they can serve as a unique model for the study of protein function in cellular context. Several stable cell lines derived from $PrP^{0/0}$ mice were established predominantly of neuronal, glial, or fibroblast origin (Nishimura *et al*., 2008; Kim *et al*., 2005; Holme *et al*., 2003; Kuwahara *et al*., 1999; Satoh *et al*, 1998). Currently, there are described only two cell lines stably expressing a prion protein from the natural host on the $PrP^{0/0}$

E-mail: michal.prcina@savba.sk; fax: +421-2-54774276.

Abbreviations: MAb = monoclonal antibody; PrP = prion protein; PrP^{C} = cellular prion protein; PrP^{Sc} = scrapie-associated prion protein; TSE = transmissible spongiform encephalopathy

background. Both cell lines were derived from Zürich mice expressing the VRQ allele of ovine prion protein (Archer *et al*, 2004; Cronier *et al*., 2004). Until now, no cell line expressing the human prion protein on $PrP^{0/0}$ background has been established. Currently available cellular models for the research of human prion diseases include various cell lines transfected with a relevant form of human PrP (Lawson *et al*., 2008; Xu *et al*., 2008).

In this work, we describe the establishment of permanent cell line expressing the human prion protein on $PrP^{0/0}$ background for the first time. Immortalized $PrP^{0/0}$ cells were derived from the Nagasaki mice using chemical mutagen 3-methylcholanthrene (3-MC) and transfected with the vector coding human prion protein.

Material and Methods

Cell lines. Mouse hypothalamic neuronal cell line GT 1-7 and mouse embryonic fibroblast cell line NIH 3T3 were used as the controls.

Development of immortalized embryonic PrP0/0cell line from Nagasaki mouse. Four 14–18-day-old embryos from the pregnant PrP-deficient females were used for the preparation of a stable PrP-knockout ($PrP^{0/0}$) cell line (Sakaguchi *et al*., 1995). After removal of fetal membranes, the embryos were rinsed 3x in sterile PBS and their viscera and limbs were removed. The embryos were cut into small pieces and incubated 3 x 10 mins at 37°C in accutase (PAA). The released cells were strained off after each 10 mins into the tube with fetal calf serum (Gibco BRL) and centrifuged at 1000 rpm for 10 mins. The pellet was resuspended in 2 ml of Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 15% FCS and loaded into 6-well tissue culture plates. Embryonic PrP-knockout cells were maintained in a 5% CO_2 atmosphere at 37°C. For immortalization, isolated embryonic cells were incubated at various cell concentrations in DMEM in 6-well tissue culture plates for 3 days. The cells with a confluent monolayer were treated with 3-methylcholantrene (3-MC, Sigma) at a final concentration of 1 μg/ml, 2.5 μg/ml, and 5 μg/ml for 48 hrs. Thereafter, 3-MC medium was exchanged for the fresh medium without 3-MC and the cells were cultivated for 3 weeks. Transformed PrP-knockout embryonic cells were then stabilized by cloning in agarose (Kontseková *et al*., 1991). The isolated $PrP^{0/0}$ subclones were cultivated in DMEM containing 10% FCS, 2 mmol/l L-glutamine and the growing parameters of individual cell subclones were evaluated.

Gene expression of the stabilized PrP0/0 cell line. To examine a gene expression of the immortalized knock-out embryonic cells, the genomic DNA was isolated (Laird *et al*., 1991). PCR was performed using the following primers: mPrP-F: 5'-CCCAAGCTT GGGATATGGCGAACCTTGGCTAC-3' and mPrP-R: 5'-GGAAT TCCTCATCCCACGATCAGGAAG-3'.

Estimation of growth and morphological parameters of the established PrP0/0 cell line. 5.10^4 cells were seeded on a 24-well plate, counted every 24 hrs for 3 or 4 days. Their doubling time was estimated using the equation $T_{(d)} = \ln 2*T/(\ln N - \ln N_0)$, where $T_{(d)}$~doubling time, T ~ time of growth, N ~ final number of cells, and N_0 ~ number of seeded cells. For morphological analysis, the cells were seeded at a low density on Lab-Tek chambers (Nalge Nunc) and observed with an inverted microscope (Zeiss Axiovert 200M).

Vector construction and stable transfection of PrP0/0 cells. Human DNA was isolated from the blood using QIAamp DNA Blood Midi Kit (Qiagen). The coding sequence of the prion gene was amplified by PCR using the primers huPrP-S-AflII (5'-AAT TCTTAAGGAGCAGTCATTATGGCGAAC-3') a huPrP-AS-NheI (5'-ATATTATTGCTAGCCTCCCTCAAGCTGGAAAAAGA-3'). The product was digested with AflII and NheI restriction enzymes, gel-purified, and ligated into the vector pIREShyg3 (Clontech) digested with the same enzymes, yielding the plasmid pHuPrP. The accuracy of the construct was confirmed by the restriction fragment analysis and sequencing. The plasmid pHuPrP was transfected into $PrP^{0/0}$ cells using the Nanofectin reagent (PAA) according to the manufacturer´s instructions. Stable transfectants were selected in the presence of 400 μg/ml hygromycin and tested for the expression of human prion protein with Western blot analysis using anti-prion monoclonal antibody (MAb) SAF 32.

Western blot and quantification of human PrP expression. 50 μg of total cellular proteins were separated by PAGE in a 12% gel under reducing conditions and electrophoretically transferred onto a PVDF membrane in 10 mmol/l N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) (pH 11). The blot was blocked in 5% non-fat milk in PBS for 1hr and incubated with the MAb SAF32 (SPI Bio; diluted 1:2,000 in PBS) overnight at 4°C, followed by polyclonal horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000; DAKO). The blot was developed with Super Signal West Pico Chemiluminescent Substrate (Pierce) and scanned with a LAS3000 imaging system (FUJI Photo Film). Relative levels of PrP expression in the individual cell lines expressing human prion protein were determined from blots using Advanced Image Data Analyzer (Aida) software (Raytest, Straubenhardt). The expression level of human PrP was compared to the physiological expression of mouse PrP in the hipothalamichippocampal neuronal cell line GT1-7.

Immunofluorescence. Cells were seeded onto poly-D-lysine/laminine coated cover slips and cultivated for 24 hrs. Then, the cells were fixed with 3% paraformaldehyde for 12 mins at 37°C, permeabilized with 0,1% Triton X-100 in PBS for 1.5 min, and blocked with 3% BSA in PBS/Triton X-100 at room temperature for 30 mins. For the detection of prion protein the same procedure without Triton X-100 permeabilization was used. After blocking, the cells were incubated for 1 hr with several primary antibodies: (i) against tau protein (polyclonal antibody against human tau protein; diluted 1:2,000) as a neuronal marker, (ii) against fibronectin (dilution 1:1000; Abcam) as a fibroblast marker, and (iii) against prion protein (6H4 antibody; dilution 1:500, Prionics) diluted in blocking solution. Next, the cells were washed three times for 10 min (0.1% Tween 20 in PBS) and incubated with the Alexa 488-conjugated secondary antibody. After washing, the cells were dried in 96% ethanol and mounted in Vectashield H-1000 mounting medium for the fluorescence (Vector). Preparations were examined using the inverted microscope (Zeiss Axiovert 200M).

Results and Discussion

The main objective of this study was to develop a mouse cell line stably producing the human prion protein on the $PrP^{0/0}$ background. The initial step was the establishment of immortalized cell line from the prion-deficient mice. For the isolation of mouse cells lacking endogenous PrP, the 14-day-old embryos from pregnant $PRNP^{0/0}$ females were used. The embryonic cells with fine growth properties and cell morphology were used for the immortalization by 3-MC. Transformation of cells with 3-MC at the final concentration of 1 μg/ml for 48 hrs resulted in the formation of 7 transformed clones. Two of these clones were successfully grown without any noticeable change in phenotype for more than 85 passages and therefore, they were considered as immortalized (Gudjonsson *et al*., 2002). Moreover, these two cell clones were stabilized by cloning in soft agar and two sub-cloned cell lines $PrP^{0/0}$/1 and $PrP^{0/0}$/3 were selected. It was observed that the cell line $PrP^{0/0}$/1 showed better growth characteristics than cell line $PrP^{0/0}$/3 and consequently, the experiments described here were carried out with the cell line $PrP^{0/0}$/1. Three different experimental approaches were used with the aim to verify PrP-knockout phenotype of developed cell line $PrP^{0/0}$/1. The absence of PRNP coding sequence in the selected cell line $PrP^{0/0}$/1 was confirmed by the PCR technique (Fig. 1a). Cell line $PrP^{0/0}$/1 unlike the cell lines NIH 3T3 and GT1-7 was the only one lacking the prion protein gene. This cell line was also tested for the prion protein expression. Consistent with our prediction, no PrP was detected in the $PrP^{0/0}$/1 cell extract (Fig. 1b). Moreover, identical data were obtained by the immunocytochemistry (Fig. 1c). No PrP staining was visible on the cell surface and intracellular compartments of the $PrP^{0/0}$/1 cells. These results confirmed PrP-knockout phenotype of the established $PrP^{0/0}$/1 cell line.

The morphology of the $PrP^{0/0}$/1 cell line was similar to that of mouse fibroblast NIH 3T3 cell line and indicated a fibroblast-like rather than neuronal phenotype (Fig. 2a). To verify this supposal, we used several antibodies recognizing markers specific to the neurons (tau protein) and to fibroblasts (fibronectin) for cell-type characterization. Cell lines NIH 3T3 and GT 1-7 were used as positive controls for the fibroblast and neuronal markers, respectively. Immunocytochemistry results showed that the $PrP^{0/0}$/1 cell line was negative for the neuron-specific tau protein, whereas a positive staining was obtained using anti-fibronectin antibody (Fig 2c). Thus, the established cell line showed a fibroblast phenotype. A previously described fibroblast cell line lacking prion protein gene was derived from the Nagasaki mice (Satoh *et al*., 1998). Since those cells were prepared from the abdominal skin and differed from the $PrP^{0/0}$/1 cell line morphologically, we could assume that the developed fibroblast cell line $PrP^{0/0}$/1 did not originate from the skin. Next, we estimated the growth cycle of the immortalized PrP-knockout cell line and compared it with fibroblast cell line NIH 3T3 as a control. The average doubling time of $PrP^{0/0}$/1

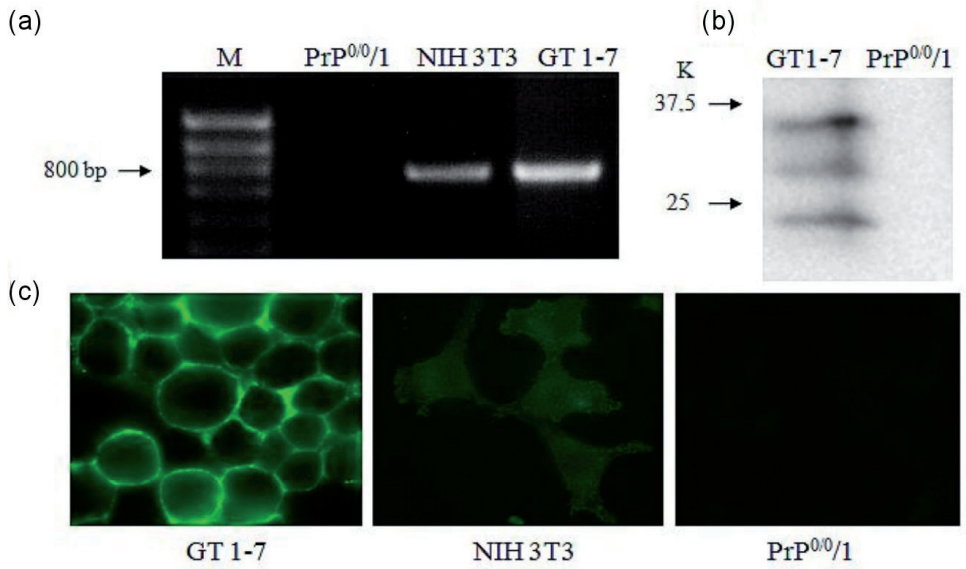

Fig. 1

PrP-knockout phenotype of the PrP0/0/1 cell line

a) PCR analysis of $PrP^{0/0}$/1 cells. NIH 3T3 and GT1-7 cells were used as the positive controls. 100 bp DNA ladder (lane M). b) Western blot analysis $PrP^{0/0}$/1 cells. GT1-7 cells were used as the positive control. Size markers on the left. c) Immunofluorescent staining of $PrP^{0/0}$/1 cells. GT 1-7 and NIH 3T3 cells were used as the positive controls with different levels of PrP expression.

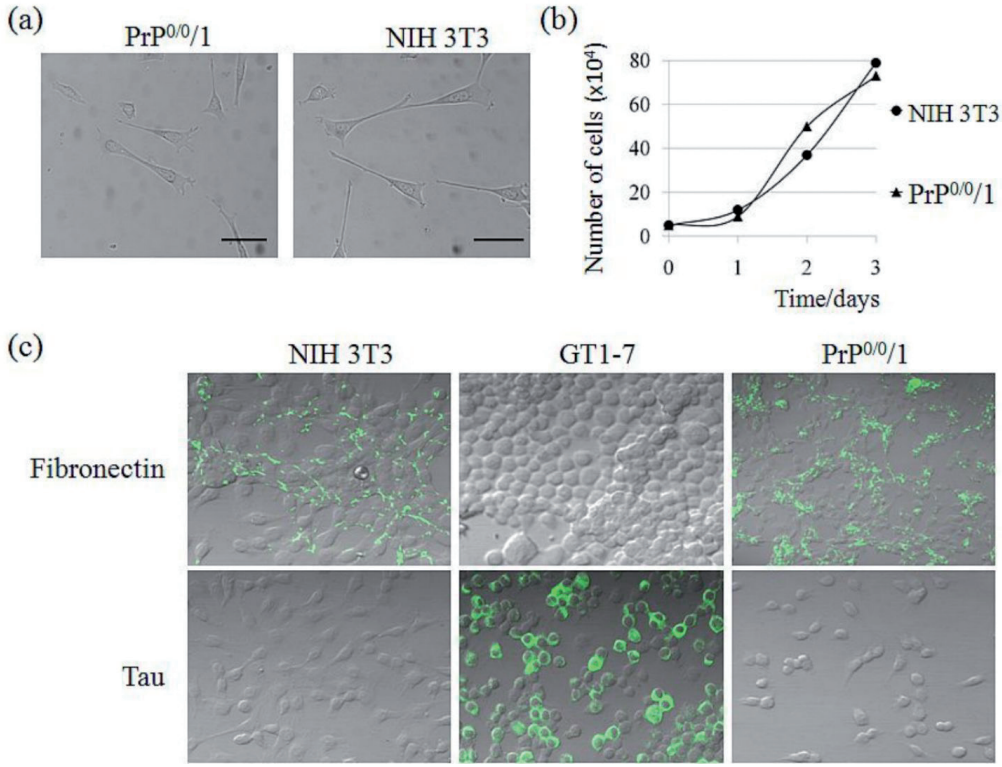

Fig. 2

Cell-type characterization of PrP0/0/1 cell line

(a) Morphology of PrP$^{0/0}$/1 cells. Fibroblast cells NIH 3T3was used as a control. Scale bar 20 μm. (b) Growth rate of the PrP$^{0/0}$/1 and NIH 3T3 cell lines. (c) Immunofluorescent staining of PrP$^{0/0}$/1, GT 1-7, and NIH 3T3 cells. Neuronal cell line GT1-7 and fibroblast cell line NIH 3T3 were used as controls. Tau protein was used as a neuronal marker and fibronectin as a fibroblast marker.

cells determined 72 hrs after seeding was 18 hrs. The growth rate of PrP$^{0/0}$/1 cells was very similar to that of the mouse fibroblast NIH 3T3 cell line (Fig. 2b). The relatively short doubling time and stable phenotype of the established PrP-deficient cell line indicated that the cells could be used for various genetic manipulations. Generally, the development of such a cell line opens a broad spectrum of applications. In addition to the study of physiological function of the prion protein, this cell line provides a versatile basis for the development of new cell models directed at the elucidation of key events in the pathogenesis of prion diseases.

With the aim to generate a cell line stably expressing the human prion protein on a PrP-knockout background, the PrP$^{0/0}$/1 cells were transfected with a plasmid pHuPrP containing coding sequence of the human prion gene. Stable transfectants were characterized according to the expression of human prion protein using immunochemical methods (Fig. 3). Immunoblot analysis of prepared cell lines showed that the majority of surviving cells exhibited expression of the human prion protein. However, the levels of prion protein expression markedly varied in the cells (Fig. 3a). The expression level of prion protein was often a determining factor of cell line suitability for the prion research, because it could affect the susceptibility of cell line to the prion infection (Nishida *et al.*, 2000). Therefore, we used a quantitative determination of human prion protein expression in the selected cell lines. Quantitative analysis revealed that lines huPrP1 and huPrP10 expressed slightly lower levels of human prion protein than those observed in the cell line GT1-7 (Fig. 3b). On the contrary, lines huPrP6, huPrP7, and huPrP9 expressed less than half the amount of human PrP, when compared to the PrP expression in GT1-7 cells (Fig. 3b). In addition, an immunocytochemistry approach was used to confirm the expression of human PrP protein in the developed cell lines (Fig. 3c). MAb 6H4 stained human prion protein on the surface of all tested cell lines with different intensity. The levels of human PrP were highest in the cell lines huPrP1 and huPrP10, similar to the results obtained by immunoblot analysis. Expression of the human prion protein affected a proliferation activity of the generated cell lines. We noticed that the proliferation rate of cell lines expressing human PrP was lower than that of the

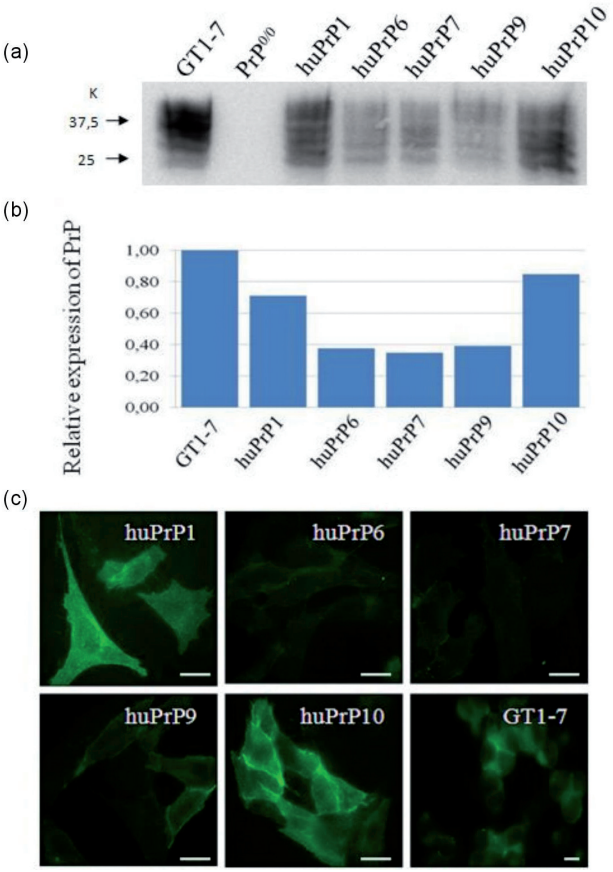

Fig. 3

Expression of human prion protein in transfected cell lines

(a) Western blot analysis of transfected cell lines huPrP1, huPrP6, huPrP7, huPrP9, and huPrP10. GT1-7 and PrP$^{0/0}$/1 cell lines were used as positive and negative control, respectively. (b) Relative expression level of human prion protein in the transfected cell lines. The intensity of signals representing human PrP-expressing cell lines was compared to the signal of GT1-7 cells. Level of PrP expression in GT1-7 cells was set as physiological. (c) Immunofluorescent staining of transfected cell lines expressing human PrP. GT 1-7 cell line was used as the positive control. Scale bar 20 μm.

PrP-knockout cell line and negatively correlated with the level of PrP expression (data not shown). Most likely, this outcome was a consequence of pIREShyg3 vector use. Since prion protein and hygromycin-resistance genes are expressed from the same mRNA, the cell lines with higher expression of PrP also express more hygromycin resistance gene, and consequently they survive easier in the selection medium.

In addition to the study of physiological function of the prion protein, established cell lines can facilitate PrP interactome study or elucidate the key events leading to the pathogenesis of prion diseases. Since the knockout cell line PrP$^{0/0}$/1 was the basis for the development of cell lines expressing human prion protein, it can serve as an especially advantageous control for the proteomic experiments.

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