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Inducible expression of chimpanzee prion protein (PrP) in murine PrP knock-out cells

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ABSTRACT

In transmissible spongiform encephalopathy (TSE) pathogenesis the cellular prion protein (PrP^C) is converted into its pathogenic PrP^{Sc} isoform. Prion protein gene (*Prnp*) deficient mice ($PrP^{0/0}$) are resistant to PrP^{Sc} infection, but following reconstitution of *Prnp* they regain their susceptibility to infection. Therefore, it is challenging to simulate this natural situation in a cell culture model. We have previously reported the inducible stable expression of a human PrP^C in murine 3T3 cells. In this study, we used murine $PrP^{0/0}$ cells stably expressing exemplarily the chimpanzee *Prnp* under the control of inducible tetracycline (Tet) system. The *Prnp* was integrated using a lentiviral vector. Its expression in the engineered $PrP^{0/0}$Chimp1/Tet-Off cell line was analyzed by Western blot (Wb) and fluorescence activated cell sorting (FACS) analyses. PrP^C was partially purified by using immobilized metal affinity chromatography (IMAC). Compared to all the other cell systems which possess an endogenous PrP^C expression, here described cell line contains only an overexpressing species specific PrP^C expression which is tightly regulated and can be turned-off at any time without showing any endogenous host PrP^C expression. Consequently, a contamination of the isolated PrP^C is impossible. This cell line potentially offers a new tool for simulation of mice bioassays widely used in TSE infection studies.

TSE diseases can be found in several mammalian species. For example, scrapie in sheep and goat, transmissible mink encephalopathy (TME)[3] in mink, and chronic wasting disease (CWD) in deer. TSE or prion diseases are invariably fatal and differ in their incubation time and intra- and inter-species infectivity. The origin of TSE's appears to be either sporadic, genetic or iatrogenic. Several distinct human TSE's include Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker (GSS) syndrome, fatal familial insomnia (FFI), Kuru, and a new variant of CJD (vCJD) [1]. In the last two decades TSE became a matter of particular interest, because of suspected BSE zoonotic potential. There are several hypotheses with regard to the origin of BSE outbreak. Thus, one hypothesis suggests that the feeding of uninfected cattle with bone meal nutritional supplements contaminated with scrapie-infected sheep carcasses could be the origin of the BSE prions, while the other one states a spontaneous mutation of bovine PrP^C into an infectious and protease-resistant form (sporadic BSE) as the possible source of the BSE prions. Nevertheless, the commonly accepted cause for more than 200 vCJD cases worldwide seems to be the transfer of BSE prions to humans via dietary exposure to BSE infected tissues [2]. Recently, we reported efficient transmission of BSE to non-human primates by feeding of natural BSE infected stem brain material [3,4].

The PrP^{Sc} (Sc – scrapie) protein is the main, if not a sole, component of the prion agent that is found in different TSE diseases [5]. PrP^{Sc} is an isoform of PrP^C (C – cellular). PrP^C is ubiquitously expressed in neuronal cells and several other cells types. It is conserved in all mammalian species examined [6] and found also in reptiles and amphibians [7]. PrP^C occurs in three different glycosylation forms of 27–35 kDa [8–10]. The most common form is the di-glycosylated one. The protein is anchored by a glycosyl phosphatidyl inositol (GPI) anchor in the cholesterol-rich raft regions of the outer cell membrane [11]. PrP^C interacts with numerous proteins of the cell membrane, apoptotic pathways, and endocytic compartments. Its subcellular trafficking is complex and PrP^C

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[3] *Abbreviations used:* TME, transmissible mink encephalopathy; TSE, transmissible spongiform encephalopathy; PrP^C, cellular prion protein; Tet, tetracycline; Wb, Western blot; FACS, fluorescence activated cell sorting; IMAC, immobilized metal affinity chromatography; CWD, chronic wasting disease; FFI, fatal familial insomnia; GPI, glycosyl phosphatidyl inositol; PMCA, protein misfolding cyclic amplification assay; SCA, scrapie cell assay.

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displays a high metabolic turnover rate. The biological function of PrP^{C} [12,13] as well as the pathogenic mechanisms underlying PrP^{Sc} infectivity are still under investigation.

The two PrP isoforms can be discriminated according to their biochemical properties, since anti-PrP isoform specific antibodies of the IgG subtype are not available [14]. Unlike PrP^{Sc}, PrP^{C} is able to bind copper under physiological conditions [15,16], contains predominantly alpha helices and can be completely digested using proteinase K (PK) [17]. In addition, one can test the sample for infectivity either by using *in vitro* assays such as protein misfolding cyclic amplification assay (PMCA) [18], scrapie cell assay (SCA) [19], or animal bioassays (mainly transgenic mice).

According to Prusiner's prion only hypothesis [20] the PrP^{Sc} isoform is the etiologic agent of the TSE and following infection initiates PrP^{C} conversion into PrP^{Sc}. This conversion step is a self-catalytic exponential reaction as described in the seeding hypothesis [21]. An external factor may also be required for the conversion reaction [22,23].

Cell models for prion replication are an important molecular tool for studying the normal prion function as well as the prion pathogenesis. In order to amplify the pathogenic PrP^{Sc} *ex vivo*, an increasing number of different biochemical *in vitro* conversion assays and biological cell culture models have been established [24,25]. In the cell culture models primary or permanent neuronal cell lines and even cells of non-neuronal origin were infected with host adapted or natural prions. Several of these cell lines were genetically modified by stably inserting an exogenous *Prnp* gene. Some of these models used murine neuroblastoma N2a cells [26–29], ScGT1 hypothalamic neuronal cells, chronically infected with the scrapie isolate [30–32], murine brain Smb cells [33,34], rabbit epithelial Rov cells [35–38], murine epithelial 3T3 cells [39], and murine epithelial 3T3-L1 Tet-Off cells [40]. In addition, a murine myoblast C2C12 cell line was utilized [41]. An up to date list of TSE permissive cell lines was published by Vilette [25]. However, in all so far described cell systems it is difficult to distinguish between the endogenous and the exogenous molecules [42]. Thus, a cell system which enables inducible expression of PrP^{C} in $PrP^{0/0}$ environment would be a potentially useful experimental model for inter-species transmission of PrP^{Sc}. Such a model would eliminate interpretation errors regarding the species barrier by avoiding the influence of endogenous PrP^{C} and simplify the interpretation of the infection experiments. Recently, a murine $PrP^{0/0}$ cell line permanently expressing mutated PrP^{C} which was anti-PrP 3F4 antibody epitope tagged was described [43]. This system was used to study the influence of specific amino acid residues following scrapie infection.

In order to study prion biology a number of PrP knock-out mice strains were created [44]. Furthermore, different $PrP^{0/0}$ cell lines were obtained. In this study we used PrP knock-out kidney fibroblast cells derived from Zürich I strain [45]. These cells do not express host PrP^{C} so that after the integration of a *Prnp* gene the only detectable PrP^{C} is of exogenous origin. Using 3T3-L1 fibroblast cells we previously established a cell system conditionally expressing PrP^{C} [40]. Here we report the adaptation of this system to PrP knock-out cells. Our new cell lines overexpresses only the stably inserted foreign *Prnp* genes of different mammalian species but not the endogenous *Prnp*. In this report we focus exemplarily on the chimpanzee *Prnp* gene. The important advantage of the established cell system compared to common animal models and to other cell models is the adjustable and exact PrP^{C} expression level. This will enable more precise analysis of PrP^{C} biology and inter-species transmission of PrP^{Sc}. In the preliminary infection experiments with a TSE agent (stem brain homogenate of a natural BSE infected cow) the chimpanzee PrP^{C} appeared to interact with bovine PrP^{Sc} suggesting the possibility of prion propagation.

Materials and methods

Tet-Off vector construction

The PrP^{C} expression in $PrP^{0/0}$ cells was regulated by a Tet-system. The tetracycline transactivator (tTA) element from the pTet-Off vector (Clontech) was cloned into the pCMV/Bsd vector (Clontech) via the XhoI and HindIII restriction sites and verified by dideoxy sequencing. This new construct pCMV/Bsd Tet-Off carries a blasticidin instead of a neomycine resistance marker. This step was obligatory due to the preexisting neomycine resistance of the $PrP^{0/0}$ cells. After transfection, cells were selected by blasticidin for Tet-Off protein expression.

Lentivirus vector for Prnp gene transfer

For the genomic introduction of the human (data not shown) and the chimpanzee *Prnp* into $PrP^{0/0}$ cells we used lentiviral vector pRev-TRE (Clontech). The *Prnp* open reading frame (ORF) (NCBI Protein No. NP 001009093) was amplified from chimpanzee genomic DNA by PCR with a forward (5′-GCGGATCCATGGCAAAC CTTGGCTGCT-3′) and a reverse (5′-GCGGATCCTCATCCCACTATCA GGAAG-3′) primer. The PCR was performed as follows: denaturing at 94 °C for 30 s, annealing at 59 °C for 1 min and elongation at 72 °C for 45 s (35 cycles). The primers introduced BamHI restriction sites on each end of the DNA fragment. The amplified fragment was then cloned into the BamHI restriction site of pGEM-T vector (Promega) and subsequently into the lentiviral vector pRev-TRE behind the tetracycline response element (TRE). The sequence of the inserted fragment and its orientation were verified by dideoxy sequencing and found to correspond to the *Prnp* gene [6].

pRev-TRE-Prnp lentivirus amplification

The lentiviral vector with the *Prnp* gene was linearized by SspI. Ten micrograms of the linearized vector were electroporated with a Gene Pulser II (Bio-Rad) at 0.25 kV and 0.975 F into 1×10^{6} cells of the PT67 packing cell line (Clontech). These cells were cultivated at 37 °C, 5% CO_2 supply in Dulbecco's modified Eagle medium (DMEM) with 10% [v/v] FCS, 1% [v/v] penicillin/streptomycin (P/S), 4 mM L-glutamine and 4.5 g/l glucose. Selection of transfected cells was performed with 10 μg blasticidin per ml medium for 3 weeks. The resulting clones were screened for stable lentivirus production by a test infection and virus titer determination. To verify the stable integration cells were frozen, thawed, re-cultivated and monitored for a subsequent virus titer determination.

Transfection of PrP knock-out cells

$PrP^{0/0}$ cell line was kindly provided by Weissmann [45]. The cells were cultivated at 37 °C, 5% CO_2 supply in DMEM with 10% [v/v] FCS and 1% [v/v] P/S. Five-hundred micrograms pCMV/Bsd Tet-Off vector linearized with ScaI were transfected into 5×10^{6} cells by electroporation (Gene Pulser II, Bio-Rad) at 0.25 kV and 0.975 F. Cells resistant to blasticidin concentration of 5 μg/ml were selected by limiting dilution and screened for transactivation by tetracycline (Tc) following transient transfection with the pBI-EGFP vector (Clontech) containing an enhanced green fluorescent protein (EGFP) gene under the control of a TRE element. Twenty selected clones were incubated for 2 days with 50 μg/ml Tc and the regulation was monitored by fluorescence microscopy. One of the resulting cell clones was finally selected. This new, $PrP^{0/0}$ and Tc-controlled, cell line will be referred to as LHABT. LHABT cells were grown on a 10 cm cell culture dish. After reaching 80% confluence the medium was removed and the cells were