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CRBL cells: Establishment, characterization and susceptibility to prion infection

Charles E. Mays^a, Hae-Eun Kang^b, Younghwan Kim^a, Sung Han Shim^c, Ji-Eun Bang^b, Hee-Jong Woo^b, Youl-Hee Cho^c, Jae-Beom Kim^d, and Chongsuk Ryou^{a*}

^aSanders Brown Center on Aging, Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky College of Medicine, Lexington, KY, U. S. A.

^bLaboratory of Immunology, Seoul National University College of Veterinary Medicine, Seoul, Korea

^cDepartment of Medical Genetics, Hanyang University School of Medicine, Seoul, Korea

^dCaliper Life Sciences, Alameda, CA, U. S. A.

Abstract

The cerebellum is involved in complex physiological functions including motor control, sensory perception, cognition, language, and emotion. Humans and animals with prion diseases are characterized clinically by ataxia, postural abnormalities and cognitive decline. Pathology in the cerebellum affected by prions includes spongiform degeneration, neuronal loss, and gliosis. To develop an *in vitro* model system for studying prion biology in cerebellar cells, we established and characterized an immortal cell line (CRBL) isolated from the cerebellum of mice lacking expression of a protein involved in cell cycle arrest. The characteristics of the cells include morphological heterogeneity, rapid proliferation, serum responsiveness during growth, and a change in the number of chromosomes. CRBL cells expressed both neuronal and glial cell markers as well as a considerable level of cellular prion protein, PrP^C. Upon *in vitro* infection, CRBL cells exhibited selective susceptibility to prions isolated from different sources. These cells chronically propagated prions from SMB cells. Strain-specific prion infection in CRBL cells was not due to instability of the cell line, allelic variance, or mutations in PrP gene. Molecular properties of prions derived from SMB cells were maintained in the infected CRBL cells. Our results suggest that the specific interaction between a prion strain and hosts determined the selective susceptibility of CRBL cells, which reflects the conditions *in vivo*. In addition to the future studies revealing cellular and molecular mechanism involved in prion pathogenesis, CRBL cells will contribute to the studies dealing with prion strain properties and host susceptibilities.

Classification terms

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Section

Disease-Related Neuroscience

*correspondence, Name: Chongsuk Ryou, Address: 800 Rose St. HSRB-326, Lexington, KY 40536, Phone: (859) 257 4016, Fax: (859) 257 8382, E-mail: cryou2@email.uky.edu.

Current address of H-E Kang: Sanders Brown Center on Aging, University of Kentucky, Lexington, KY

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Keywords

cerebellum; prion diseases; CRBL cells; prion susceptibility; prion strain

1. INTRODUCTION

The cerebellum has been recognized as the structure involved in motor coordination. It is also known to contribute to non-motor functions such as sensory perception, cognition and emotion (Bastian and Thach, 2002; Ghez and Thach, 2000). The cerebellum contains more neurons than all the other structures of the brain and retains functionally well-defined organization where the networks of neural cells convey millions of bites of information related to cerebellar functions to and from many other regions of the brain and the spinal cord (Bastian and Thach, 2002). This complex network is essential for the roles of the cerebellum in motor, sensory, and cognitive functioning. For this reason, cerebellar lesions typically exhibit deficits during movement execution, difficulties in maintenance of posture and balance, and dysfunctions in eye movement and speaking (Topka and Massaquoi, 2002).

Prion diseases are fatal neurodegenerative disorders caused by the proteinacious pathogen, prions (Prusiner, 1998). Prions are composed of β -sheet rich, disease-associated prion protein (PrP^{Sc}) that underwent conformational transition from α -helix rich cellular prion protein (PrP^C) (Prusiner, 1998). Due to conformational changes, PrP^{Sc} becomes hydrophobic and partially resistant to proteinase K (PK) digestion. Humans and animals with prion diseases exhibit abnormalities in coordination of muscle movement, lack of balance, disturbance of gait, over reactive sensory perception, loss of language, dementia, and irritable demeanor (Collinge, 2001). These diseases accompany pathological lesions in the brain structures including the cerebellum (DeArmond et al., 2004; Ferrer, 2002). Patients with Creutzfeldt-Jakob disease (CJD) frequently exhibit accumulated prion plaques in the cortex, a widespread microvacuolar spongiform change in the molecular layer, neuronal loss in the granular and Purkinje cell layers, and gliosis in astrocytes of the cerebellum (Armstrong et al., 2001a; Armstrong et al., 2002; Armstrong et al., 2001b; Ferrer et al., 2000; Jarius et al., 2003; Schulz-Schaeffer et al., 1996).

PrP^C appears to play an important physiological role for neurons in the cerebellum (Herms et al., 2000; Herms et al., 2001; Katamine et al., 1998; Laine et al., 2001; Legname et al., 2002). The major events leading to pathogenesis also occur in the cerebellum when PrP^C is converted to PrP^{Sc} (Ferrer, 2002). Despite the increasing number of studies, the mechanisms involved in both physiology and pathology of PrP isoforms in the cerebellum is still poorly understood. In order to understand the cellular and molecular mechanisms involved in prion diseases, it is necessary to investigate the roles of the cerebellar cells in a well-characterized *in vitro* model system that mimics the conditions *in vivo*.

The present study describes the establishment and characterization of the immortalized mouse cerebellar cells termed CRBL, and susceptibility of these cells to prions. CRBL cells will be a useful model system for the research involved in prion biology of the cerebellum.

2. RESULTS

2.1. Establishment of the CRBL cells

The normal diploid cells obtained from wild type animals undergo senescence losing their ability to divide when they are cultured *in vitro*. To establish a cell line that mimics the physiological conditions in the cerebellum and is continuously dividing *in vitro*, we obtained cells from the cerebellum of the mice lacking expression of p53 gene. Since the p53 gene product arrests progression of the cell cycle when DNA damage is sensed (Harris and Levine,

2005), the loss of p53 gene expression results in perpetual cellular division and occasionally causes the spontaneous formation of tumors in the animals (Purdie et al., 1994; Tsukada et al., 1993). Thus, our strategy to immortalize the cell relies on the disruption of cell cycle control.

To screen the homozygotes for p53^{-/-}, we genotyped mice using PCR. Use of different sets of primers in two independent PCR distinguished the heterozygotes from the homozygotes for p53^{-/-}. Neo forward and p53 reverse primers generated nearly 800 bp amplicons from genomic DNA of both heterozygotes and homozygotes (Fig. 1, lanes 8–13). However, p53 forward and reverse primers generated amplicons of 650 bp in lengths only from the heterozygotes (Fig. 1, lanes 2, 3, and 4), but not from the homozygotes (Fig. 1, lanes 1, 5, and 6).

Cells isolated from the cerebellum of p53^{-/-} homozygotes were cultured *in vitro* under the condition described in Experimental Procedures. Because the cells are thought to express neomycin acetyltransferase from the cassette in the transgene utilized to disrupt the expression of p53, CRBL cells were tested to confirm whether they survive in the presence of G418. Unlike other established cell lines, the number of CRBL cells under different concentrations of G418 remained unchanged (Fig. 2). The CRBL cells survived even in the high concentrations (750 µg/ml) of G418 where other cell lines do not survive.

2.2. Characterization of the CRBL cells

The CRBL cells grew immortally and were maintained for more than 80 passages. CRBL cells proliferated rapidly during the maintenance of the culture. To examine their growth rate, cells were grown under regular culture conditions comprised of DMEM and 10% fetal bovine serum, and their doubling times were estimated. At passage ten, CRBL cells doubled their numbers once every 15.6 hours, while the cells at the passages sixty and eighty exhibited doubling times of 11.6 and 10.6 hours, respectively (Table 1). The rapid growth of CRBL cells at early passages was accelerated, but stabilized in later passages.

To investigate the serum responsiveness, CRBL cells at passage ten were maintained under different serum concentrations and their growth rates compared. When cultured under reduced serum conditions (5 and 2.5 %), the growth of the CRBL cells were retarded. The doubling time of 15.6 hours at 10 % serum concentration became extended to 17.4 hours at 5 % and 23.5 hours at 2.5 % serum concentrations (Table 1).

Morphological observation revealed that CRBL cells are composed of cells in various sizes, shapes and lineages. Some cells resemble fibroblasts having spindle-like shapes, while others appear as differentiated neurons with the various degrees of dendritic extension. In addition, cells with the typical star-shape of mature astrocytes were observed. CRBL cells grow larger and demonstrate a flattened shape at low density in culture. Such morphology became less obvious after the cell-cell contact in a high density culture (Fig. 3).

Chromosome numbers were counted at metaphase to investigate the aneuploidic state of the cells. Cytogenetic analysis revealed that CRBL cells were maintained as hypotetraploid. The cells had abnormal karyotypes, with the modal chromosome number varying from 66 to 78 (data not shown).

Since CRBL cells appear to be composed of multiple cell types, expression of neuronal and glial cell marker proteins was examined by both Western blotting and flow cytometry. Western blot analysis demonstrated that CRBL cells express both β III-tubulin, a neuronal marker protein, and GFAP, a glial marker protein (Fig. 4). β III-tubulin was abundantly expressed in N2a cells, but not in C6 cells (Fig. 4, Panel A). By contrast, GFAP was expressed in C6 cells, but not in N2a cells (Fig. 4, Panel B). Expression of actin was almost equal in all cell lines. Flow cytometry was utilized to further confirm the Western blotting results and measure the

proportion of the fully differentiated neurons and glial cells within the CRBL cell population. Incubation with anti- β III-tubulin antibody followed by fluorescence measurements demonstrated the separation of β III-tubulin-positive cells in both CRBL and N2a cells, but not in C6 cells (Fig. 5, Panel A). With a comparison to the isotypic controls, the percentages of positive cells were ~ 17 % and ~ 88 % in the CRBL and N2a cells, respectively. Similar analysis with anti-GFAP antibody resulted in GFAP-positive shifts in both CRBL and C6 cells (Fig. 5, Panel B). By comparing to isotypic controls, CRBL and C6 cells included ~ 30 % and ~ 85 % of GFAP-positive cells, respectively.

2.3. PrP^C expression and prion susceptibility of CRBL cells

To investigate if CRBL cells express PrP^C, the cells were analyzed by both Western blotting and flow cytometry. In Western blotting, all three different glycosylated forms of PrP^C were found in CRBL cells. Expression levels of PrP^C in the cells were higher than that in NIH3T3 fibroblasts, while almost comparable to that in N2a cells (Fig. 6, Panel A). N2a cells expressed quite a high level of PrP^C, while RK13 cells, a negative control, showed no expression of PrP^C. However, the level of GAPDH expression in CRBL, N2a, NIH3T3, and RK13 cells was almost identical. Flow cytometry analysis ascertained that CRBL cells express PrP^C. Approximately 62 % of the CRBL cells expressed PrP^C under the experimental condition, while 86 % of N2a cells were PrP^C positive (Fig. 6, Panel B).

Since CRBL cells were isolated from the cerebellum, one of the brain structures targeted by prion diseases, the susceptibility of cells to prion infection was assessed by *in vitro* prion infection. CRBL cells were challenged with prion inocula prepared from cells either free of (SMB-PS and N2a) or permanently infected by prions (SMB and ScN2a). In Western blotting, PK-resistant PrP^{Sc} was propagated in the CRBL cells during the period of 4 passages following the inoculation with SMB cell lysate (Fig. 7, Panel A, lane 3). Interestingly, inoculation with prions from ScN2a did not yield PK-resistant PrP^{Sc} in CRBL cells (Fig. 7, Panel A, lane 5). In controls, prions from both ScN2a and SMB cells were able to infect N2a cells, where a high level of PrP^{Sc} was accumulated (Fig. 7, Panel A, lanes 6 and 7). The level of PrP^{Sc} in CRBL cells infected by prions from SMB cells, designated SMBinfCRBL cells, was lower than that in N2a cells infected by the same prions (Fig. 7, Panel A, lanes 3 and 7). As expected, uninfected CRBL cells and those exposed to inocula prepared from SMB-PS and N2a cells maintained their prion-free states (Fig. 7, Panel A, lanes 1, 2 and 4).

To re-examine the inefficient transmission of ScN2a prions to CRBL, N2a and CRBL cells were inoculated with RML prions, which were utilized in the establishment of ScN2a (Butler et al., 1988). When challenged *in vitro* with brain homogenate of uninfected and RML-infected mice, PrP^{Sc} was not accumulated in the CRBL cells (Fig. 7, Panel B, lanes 1 and 2). However, the control N2a cells did propagate PrP^{Sc} when infected with RML-sick mouse brain homogenate, but not with uninfected mouse brain homogenate (Fig. 7, Panel B, lanes 3 and 4). Overall, none of the *in vitro* infections were shown to alter the level of PrP^C (Fig. 7, Panels A and B, upper panels). Our results showed that CRBL cells were selectively susceptible to the prions from SMB cells, but not to the prions from ScN2a cells and RML-infected brains.

To confirm whether this differential susceptibility of CRBL cells represents a transient event in the early stage of *in vitro* prion infection, the cells infected by prions from either SMB or ScN2a cells were further maintained up to 15 extra passages. During this period, the low levels of PrP^{Sc} in the early passage (Fig. 7, Panel A, lane 3) increased, and remained steady in SMBinfCRBL cells (Fig. 7, Panel C, lanes 2 and 3). This indicates that CRBL cells are permissible for the robust replication of SMB prions and persistently infected. However, CRBL cells infected by ScN2a prions lacked PK-resistant PrP^{Sc} after the extended cell culture (Fig. 7, Panel C, lanes 5 and 6), suggesting the cells were devoid of ScN2a prion replication.

CRBL cells infected by SMB and ScN2a prions were further analyzed to determine whether differential susceptibility was influenced by a shift of cell population from multiple to a single cell type of either neurons or glial cells. Expression of both β III-tubulin and GFAP in CRBL cells infected by either SMB or ScN2a prions remained consistent with the levels prior to inoculation (Fig. 7, Panel D). Therefore, this indicates that selective susceptibility of CRBL cells to different prion strains was not due to the instability of CRBL cells.

In order to examine if such selective prion susceptibility of CRBL cells was caused by variance of Prnp alleles and unexpected mutations occurred in PrP gene during the cell line establishment and maintenance, DNA sequences of the PrP ORF from CRBL cells in various passages (passages 10, 60 and 80) were compared to those from N2a, ScN2a, SMB, and SMB-PS. Determination of DNA sequences revealed that PrP ORFs from these cell lines were identical to each other and no mutation was found (data not shown). Moreover, DNA sequences of the PrP ORF from the cell lines corresponded to the mouse Prnp^{a/a} short incubation time gene (GenBank accession number: [U29186](#); data not shown). This supports the idea that selective susceptibility of CRBL cells to SMB prions was not due to the allelic variance or mutations in PrP gene. Rather, our data implicates that a specific host-agent interaction caused selective prion susceptibility of CRBL cells. The mechanism underneath the host-prion interaction is unclear and requires further investigation.

2.4. Molecular properties of PrP^{Sc} and PrP processing events in SMBinfCRBL cells

Since CRBL cells were shown to propagate prions of SMB cells, it was hypothesized that molecular aspects of strain properties remained unaltered between SMB and SMBinfCRBL cells. The specific glycosylation banding pattern of PrP^{Sc}, a molecular signature associated with different prion strains, was examined. In a thorough comparison of PK-resistant PrP^{Sc} bands from multiple Western blots, the ratio of differentially glycosylated PrP^{Sc} from both cells was found unchanged (Fig. 8, Panel A). Furthermore, deglycosylation of PK-resistant PrP^{Sc} revealed that the size of C2, a 21 kDa C-terminal core (90–230) of PrP^{Sc}, was not different in both cells (Fig. 8, Panel B, lanes 2, 4, 6, and 8). This indicates that conformation of PrP^{Sc} found in both cells was identical. Our results suggest that the strain properties of SMB prions were retained after transmission to CRBL cells. Nevertheless, examination on incubation periods, and PrP^{Sc} distribution in the local brain regions in animals will provide a further understanding of the prion strain maintained in SMB and SMBinfCRBL cells.

Interestingly, deglycosylation of PrP revealed the unique state of proteolyzed PrP in SMBinfCRBL cells. The level of C2 fragments generated natively by the cellular machinery in SMBinfCRBL cells was found to be lower than that in SMB cells (Fig. 8, Panel B, lanes 1, 3, 5, and 7). However, the level of C1, the 17 kDa C-terminal core (111–230) generated from PrP^C, was presented equally from both cell lines (Fig. 8, Panel B, lanes 5 and 7). The low level of native C2 fragment (Fig. 8, Panel B, lanes 3 and 7) correlated with that of C2 fragments generated after PK digestion (Fig. 8, Panel B, lanes 4, and 8) and PrP^{Sc} accumulated in SMBinfCRBL cells (Fig. 7, Panel A and C).

3. DISCUSSION

Establishment and characterization of the immortalized cells from the cerebellum of mice have made it possible to investigate the susceptibility of cerebellar cells to prions. Since the cerebellum is largely affected by prions and PrP^C appears to be involved in the physiology of the cerebellar cells, the importance of studying cellular and molecular events in the cerebellum under the state of prion infection has become greater. Difficulties with *in vivo* models and limitations in the current *ex vivo* models necessitate a new model system, which circumvents obstacles frequently associated with primary cell culture and recapitulates the conditions *in vivo*.

CRBL cells have the capacity to bypass cellular senescence, which is frequently observed during the cultivation of primary cells. In this study, viral oncogenes were not introduced to establish the cells proliferating immortally. Instead, CRBL cells were isolated from mice in which tumor suppressor gene p53 was inactivated. Lack of functional p53 in the cells causes cell cycle arrest, gene amplification, and loss of growth control resulting in immortal proliferation, and increased spontaneous tumorigenesis in animals (Donehower et al., 1992; Purdie et al., 1994; Tsukada et al., 1993). The properties of CRBL cells, revealed in this study, support the features associated with inactivation of p53. CRBL cells immortally survive > 80 passages without losing the ability of constant proliferation.

A majority of CRBL cells were found to have hypotetraploidy in both early and later passages. However, diploid was not common in CRBL cells in any passage of the culture. An earlier study demonstrated that *ex vivo* cell culture from p53-deficient mice were predominantly diploid in early passages, but gradually became hypertetraploid over several passages (Tsukada et al., 1993). Interestingly, CRBL cells divided about 4 times faster than the explanted cells from p53-deficient mice. The explanted cells made about 30 cumulative doublings over a 2 month period (Tsukada et al., 1993). The calculated doubling time of CRBL cells was around 12 hours in our studies. These differences might be caused by different culture conditions, tissues where the cells were explanted, and age of animal where cells were obtained. The aneuploid conditions correlate to a high rate of cell division in CRBL cells. Although immortalization and aneuploidy are prerequisites for transformation, the ability of CRBL cells to be transformed requires to be tested in animals if they induce tumors after transplantation.

Our newly established CRBL cell line more closely represents the environment found *in vivo*. Most prion-susceptible cell lines, previously established, represent a single lineage of cells (Solassol et al., 2003). Such cell lines have not provided the appropriate conditions to study the cellular interactions between different cell types, *in vitro*, under the prion-infected state. As suggested from our morphological observation and marker protein expression studies, it is likely that CRBL cells are composed of several different cell types including neurons and glial cells. Moreover, cells at various stages of differentiation such as stem cells and fully differentiated cells are thought to be included in the CRBL cell population. Future studies dealing with the sub-types composing CRBL cells and their interaction during prion pathogenesis remain to be conducted.

Adequate *in vitro* models for studying the cellular and molecular events in prion biology must meet the requirement of PrP^C expression and competency for prion infection. Our studies with CRBL cells showed that a considerable portion of the cell population abundantly expressed PrP^C and were susceptible to prions. Interestingly, the accumulated PrP^{Sc} level in SMBinfCRBL cells was lower than that in the neuronal lineage N2a cells when both cell lines were infected by the same SMB prions. Even after chronic infection with SMB prions, SMBinfCRBL cells maintained less PrP^{Sc} than SMB cells. It can be postulated that the ability of CRBL cells to replicate the limited level of PrP^{Sc} results in persistent, but low PrP^{Sc} accumulation. It is possible that not all CRBL cells are able to support the PrP^{Sc} propagation. Presumably, PrP^C expressing neuronal cells within CRBL cells are considered to be the cell population responsible for maintaining PrP^{Sc} propagation although a neuronal subclone of CRBL cells has not been identified to propagate prions independently of interaction with other cell types. It is likely that CRBL cells resemble the cellular and tissue conditions of prion-infected host brains.

Our results showed that generation of native C2 fragments was extremely low in SMBinfCRBL cells, but abundant in SMB cells. This implicates that cellular machinery involved in PrP^{Sc} processing is regulated differently in both cell lines. SMBinfCRBL cells may be a useful model to study PrP processing events because the interaction between multiple cell types within the

CRBL cells can be responsible for the orchestrated control of enzymes and inhibitors involved in PrP processing.

Another intriguing aspect of our study is the selective susceptibility of CRBL cells to prions obtained from different cell lines. Prions from SMB, but not from ScN2a cells were able to infect CRBL cells. Efficient transmission of prions may depend on either the genotype of the host or the prion strain (Bruce et al., 1976). Although CRBL cells maintain a complex genetic background as a result of using numerous mouse strains to generate the transgenic mice from which CRBL cells were isolated (Kim et al., 2007), all mouse strains utilized retain common Prnp^{a/a} alleles responsible for short incubation periods upon prion infection (Carlson et al., 1986; Westaway et al., 1987). Furthermore, our DNA sequencing data showed that CRBL cells included Prnp^{a/a} gene with no mutation. Thus, our results indicate that the selective susceptibility of CRBL cells may be attributed to prion strain specificity in infecting host cells.

Maintenance of strain properties is an important parameter to determine the strain-specific transmission of prions. Although it remains to be confirmed using animal systems, the similarities in molecular aspects of PrP^{Sc} support that the strain properties of SMB prions were maintained in SMBinfCRBL cells. Furthermore, the observation that CRBL cells are more susceptible to prions from SMB cells than those from ScN2a cells may reflect prion strain tropism to a certain region of the brain. Regional distribution of neuropathology is a key feature that has commonly been used to define the prion strain because strain-specific replication has been shown to occur in certain subpopulations of brain cells resulting in a distinct lesion profile (Bruce et al., 1989; Hecker et al., 1992; Ridley and Baker, 1996). SMB and ScN2a prions were derived from 139A and RML prions, respectively (Birkett et al., 2001; Bosque and Prusiner, 2000). Inoculation of mice with 139A prions resulted in intense vacuolation in the hippocampus and white matter of the cerebellum, while similar studies with RML prions revealed vacuolation limited to the neocortex of the cerebrum (Carp et al., 1998; Tremblay et al., 2004). The pathological targeting to the cerebellum by 139A, but not by RML prions correlates to selective susceptibility of CRBL cells to prions from SMB cells, but not from ScN2a cells or RML-infected brain homogenate. In a different scenario, it is possible that the parental Chandler prions diverged into SMB- and ScN2a-specific prions that may have evolved to replicate in mesodermal and neuronal specific cell types, respectively, during the complex adaptation processes although both 139A and RML strains commonly originated from Chandler isolates (Butler et al., 1988; Chandler, 1961; Clarke and Haig, 1970).

In conclusion, our newly established CRBL cells will be a valuable tool to understand the mechanisms of physiological process involved in PrP^C and neuropathological events caused by prion infection as a consequence of cellular interaction among cerebellar cells. Additionally, CRBL cells represent a new cell culture model which is useful in evaluating host response to different prion strains and studying the mechanism of host-prion interactions.

4. EXPERIMENTAL PROCEDURES

4.1. Mice and genotyping

Mice lacking expression of the p53 gene and genotyping procedures are described elsewhere (Kim et al., 2007). Although the functional assessment of focal adhesion kinase (FAK) in both the establishment of CRBL cells and the study of their susceptibility to prions is beyond the scope of our present studies, FAK floxed p53 null (p53^{-/-}) mice were utilized in this study. The animals used in this study have been acquired and cared for in accordance with the guidelines published in the NIH *Guide for the Care and Use of Laboratory Animals*.

4.2. Preparation of cells from the cerebellum

The cerebella were extracted from three-month old mice. After the meningeal layer was removed, the cerebellum was dissected into small pieces under sterile conditions and dissociated by trypsin treatment (0.25%, Invitrogen, Carlsbad, CA) for 15 min. Followed by trypsin deactivation with serum, brief straining (nylon mesh, 100 μ m, Falcon, BD Biosciences, San Jose, CA), and centrifugation for 5 min at 1000 $\times$ g, the cells were plated in 24 well culture plates (BD Biosciences).

4.3. Cell Culture

The newly established CRBL cells and all other cell lines used in this study were maintained in Dulbecco's Modified Eagle Medium (DMEM, high glucose) containing 10% fetal bovine serum (FBS), 1% glutamax, and 1% streptomycin/penicillin (Invitrogen) with 5 % CO₂ and humidity. Depending on experiments, $\sim 1 - 20 \times 10^4$ cells were plated in the proper culture dishes or plates (Corning, Lowell, MA). The following established cell lines were utilized for the study: N2a (mouse neuroblastoma, CCL-131, ATCC) (Klebe and Ruddle, 1969), ScN2a (scrapie infected N2a) (Butler et al., 1988), SMB (scrapie infected Swiss mouse neural) (Clarke and Haig, 1970), SMB-PS (SMB cured) (Birkett et al., 2001), C6 (rat glioma, CCL-107, ATCC) (Benda et al., 1968), NIH3T3 (mouse fibroblast, CRL-1658, ATCC) (Jainchill et al., 1969), and RK13 (rabbit kidney cells, CCL-37, ATCC) (Beale et al., 1963). To induce a high level expression of GFAP, C6 cells were treated with 1 mM N6, 2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt (Bt₂AMP, Sigma, St. Louis, MO) for 3 days as described earlier (Takanaga et al., 2004). The Bt₂AMP treated C6 cells were used in Western blotting and flow cytometry analyses. For the studies of cell growth in response to serum concentrations, the CRBL cells were maintained in DMEM with 5 % and 2.5 % FBS. For the studies of neo cassette expression, the cells were maintained in DMEM containing 10 % FBS and 0–750 μ g/ml Geneticin sulfate (G418, Invitrogen). The cell morphology was estimated and photographed under a phase contrast microscope.

4.4. Cell counting and calculation of doubling time

At given time points during experiments, cells were collected, stained with trypan blue solution (0.4 %, Sigma) and counted in hemocytometer chambers. Only live cells excluding the dye staining were counted. The growth of the cells was monitored by estimating doubling time. Doubling time (T_d) was calculated using the following formula: $T_d = \log 2 \times [(T_2 - T_1) / (\log N_2 - \log N_1)]$, where $T_2 - T_1$ indicates the length of time between two measurements and N_1 and N_2 denote the numbers of cells at two points of measurement.

4.5. Cytogenetic studies

Chromosome analysis of CRBL cells was performed with conventional G-banding techniques (Pathak, 1976). The exponentially growing CRBL cells were sequentially incubated with 0.1 μ g/ml N-deacetyl-N-methylcolchicine (Sigma) and 0.25 % sodium citrate/60 mM potassium chloride solutions. After fixation with cold glacial acetic acid:methanol (1:3 v/v), the cells were smeared and stained with Giemsa stains (Sigma). Chromosome numbers were counted in each of the multiple spreads.

4.6. Immunoblotting

Lysates were prepared from confluent cell cultures with lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.5 % Nonidet-P 40 and 0.5 % sodium deoxycholate). After quantification of the cell lysates by BCA protein assay (Pierce, Rockford, IL), an equal amount of each cell lysate (30 μ g) was separated on a 12% Tris-Glycine SDS-PAGE gel and transferred onto a PVDF membrane (Immobilon-FL Transfer Membrane, Millipore, Billerica, MA). After blocking with 5 % non-fat Carnation milk, the membrane was incubated sequentially with primary and

secondary antibodies. Monoclonal primary antibodies utilized for these studies include anti- β III-tubulin (TuJ-1, R&D Systems, Minneapolis, MN), anti-GFAP (GA5, Sigma), anti-actin (ACTN05, Neomarker, Fremont, CA), and anti-GAPDH (6C5, Ambion, Austin, TX). Anti-PrP Fab fragments D13 and D18 (Inpro, South San Francisco, CA) were used for detection of PrP. D18 was used only for the detection of C1 fragments. The peroxidase conjugated secondary antibodies were goat anti-mouse IgG (Pierce) and goat anti-human Fab (Pierce). Western blots were developed using ECL PlusTM Detection Reagents (Amersham Biosciences, Piscataway, NJ) and visualized after scanning in Fuji Film FLA 5000 image reader (Fuji Film, Edison, NJ).

4.7. Flow cytometry

The cells for flow cytometry analysis were prepared from cell culture by trypsinizing, repeated washing and passing through 70 μ m cell strainer (Falcon). Approximately 1×10^6 cells were fixed with 2% paraformaldehyde (Fisher, Pittsburgh, PA) for 30 min on ice and incubated with phosphate buffered saline (PBS) containing 0.2% saponin for 15 min at 37°C. After blocking with antibody binding buffer (PBS containing 3% FBS and 0.05% NaN₃) for 30 min on ice, cells were incubated with anti- β III-tubulin (TuJ-1, R&D Systems) or anti-GFAP (GF12-24, Chemicon, Temecula, CA) antibodies. Purified mouse IgG2a (Chemicon) was used as an isotypic control for both monoclonal primary antibodies. Cells were further incubated with FITC-conjugated goat anti-mouse IgG (Jackson Immuno Research, West Grove, PA) diluted 1:200 in antibody binding buffer. For PrP^C detection, anti-PrP Fab fragment D13 (Inpro) and FITC-conjugated goat antihuman IgG (1:500 dilution, Sigma) were used. Then, cells were washed and resuspended in antibody binding buffer prior to flow cytometry analyses (FACSCalibur, BD Biosciences).

4.8. Prion infection *in vitro*

Inocula from ScN2a, N2a, SMB, and SMB-PS cells were prepared by the method previously described (Bosque and Prusiner, 2000). Briefly, confluent cells were harvested in 1 ml of PBS and lysed by repeated cycles of freezing and thawing. These lysates were then serially passed multiple times through smaller hypodermic needles, from 16 to 26 gauges. Brain homogenates (10% w/v) from RML scrapie-infected and uninfected CD-1 mice were prepared in sterile PBS by the same manner. *In vitro* prion inoculation was conducted as described elsewhere (Bosque and Prusiner, 2000) with minor modifications. CRBL and N2a cells were plated at a density of 4×10^3 cells/well in a 6-well plate and cultured in 3 ml culture media with 100 μ l of inoculum for 7 days. If the culture became confluent within the inoculation period, it was split at a 1:10 dilution into a new well and supplied with new media and inoculum. After inoculation period, the cells were maintained 4 more passages in the inoculum free culture media. It is known that all prions provided by the inoculum were washed away by dilution during the first 4 passages, leaving no detectable PrP^{Sc} in the culture (Bosque and Prusiner, 2000). When necessary, CRBL cells inoculated with prions of SMB and ScN2a cells were cultured up to 15 extra passages. For the detection of PrP^{Sc}, ~1 mg of each cell lysate was treated with 100 μ g/ml PK (Invitrogen) for 1h at 37°C. Following PK inactivation by adding PMSF to the final concentration of 2 mM, the lysates were ultracentrifuged at $100,000 \times g$ for 1h at 4°C in a bench top ultracentrifuge (TLX-50, Beckman, Fullerton, CA). PK-resistant PrP^{Sc} was detected by Western blotting analysis.

4.9. Analysis of PrP sequences

N2a, ScN2a, SMB, SMB-PS cells and CRBL at passage 10, 60 and 80 were grown by the method described above. Genomic DNA was isolated from these cells using Puregene DNA purification kit (Qiagen). The coding region for the full length mouse PrP was amplified by PCR using genomic DNA and PrP gene specific primers (forward primer: 5'-

cgaattcatggaaccttgcta-3' and reverse primer: 5'-ccgcccgcgtcatccacgatca-3') under the following condition: 95°C for 3 min; 40 cycles of 95°C for 30 sec/61°C for 30 sec/72°C for 1 min; 72°C for 3 min. DNA sequences were determined by the fluorescent dye-terminator sequencing method in ABI Prism™ 3730x DNA sequencers (SeqWright DNA Technology Service, Houston, TX). The sequence data was analyzed using BLAST (National Center for Biotechnology Information).

4.10. Quantification of PrP^{Sc} glycosylation states

Density of di-, mono-, and un-glycosylated bands of PK-resistant PrP^{Sc} in multiple Western blots was measured using the UVP Doc-It Densitometry System (UVP, Upland, CA). Relative ratio of these bands was obtained from the densitometry of 6 (SMB cells) and 7 (SMBinfCRBL cells) independent blots of PrP^{Sc}.

4.11. Deglycosylation of PrP

Cell lysate with or without PK digestion was incubated in 1x denaturing buffer (0.5 % SDS, 40 mM DTT) for 3 min at 100°C. Denatured cell lysate were further incubated with ~ 1000 units of Peptide: N-Glycosidase F (PNGase F, NEB, Ipswich, MA) in 50 mM sodium phosphate buffer, pH7.5, supplemented with 1 % Nonidet-P 40 for 3 h at 37°C. Deglycosylated PrP bands were analyzed by Western blotting.

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Abbreviations

PrP, prion protein
 PrP^C, cellular prion protein
 PrP^{Sc}, scrapie prion protein
 CJD, Creutzfeld-Jakob disease
 p53^{-/-}, p53 null
 CRBL, a cell line established from the cerebellum
 N2a, Neuro2a
 ScN2a, scrapie-infected N2a
 SMB, scrapie-infected mouse brain cells
 SMB-PS, SMB cells cured by pentosan sulfate
 PK, proteinase K
 Bt₂AMP, N6, 2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate
 PMSF, phenylmethylsulphonyl fluoride
 GFAP, glial fibrillary acidic protein
 GAPDH, glyceraldehyde-3-phosphate dehydrogenase
 ORF, open reading frame

Fig. 1. Screening of p53 null mice

p53 null homozygote (lanes 1, 5 and 6) and heterozygote (lanes 2, 3, and 4) mice were screened by PCR. Genomic DNA from mouse tail snips were purified by gDNA isolation kit (Qiagen) after PK and RNase treatment. The p53- and neo-specific amplicons were generated by the combination of different forward primers (p53 forward primer: 5'- gacaagtatgcatcataca -3' and neo forward primer: 5'- gaacctgcgtgcaatccatct -3') and the common reverse primer (5'- ctctcaacatcctggggcag -3'). The PCR condition for p53-specific amplicon was 94°C for 2 min; 40 cycles of 94°C for 1 min/60°C for 2 min/72°C for 3 min; 72°C for 15 min. The PCR condition for neo-specific amplicon was 94°C for 3 min; 35 cycles of 94°C for 45 sec/61°C for 25 sec/72°C for 30 sec; 72°C for 15 min. The amplicons were separated on the 0.8% agarose gel. Lanes 1–6: amplicons from p53 forward and p53 reverse primers, Lane 7: 1Kb plus DNA marker (Invitrogen), Lanes 8–13: amplicon from neo forward and p53 reverse primers.

Fig 2. Resistance of CRBL cells against G418 treatment

The cells were seeded at 1×10^4 cells/well, cultured with 0 – 750 $\mu\text{g/ml}$ G418 for 7 days, and surviving cells were counted after trypan blue staining. The numbers of live cells were normalized with the cell numbers obtained from the untreated cells, which differ from $\sim 2 - 9 \times 10^5$ cells depending on cell types. CRBL (squares, solid line); 2.0×10^5 cells, N2a (triangles, dashed line); 5.6×10^5 cells, ScN2a (diamonds, double-dot line); 6.5×10^5 cells, SMB (circle, single-dot line); 8.7×10^5 cells. The viability of CRBL was not significantly affected by treatment with G418.

Fig 3. Photomicrographs of CRBL cells

The cells (1×10^4 cells) were plated and cultured in 100-mm culture dish as described in Experimental Procedures. The images were obtained by phase contrast microscope (CKX41, Olympus) after 48 (A) and 96 hrs (B) of cultivation. Magnifications were 200x (A) and 100x (B).

Fig 4. Western blot analysis of β III-tubulin and GFAP expression in CRBL cells

Expression of β -III tubulin (A) and GFAP (B) in CRBL cells was examined by Western blotting using anti- β -III tubulin and anti-GFAP antibodies. Molecular weights of both proteins coincide at ~ 50 kDa. β -actin (42 kDa) was used as a reference protein to ensure the equal amounts of each cell lysate were analyzed in both panels. Lane 1: CRBL cells, Lane 2: N2a cells, Lane 3: C6 cells treated with Bt₂cAMP.

Fig 5. Flow cytometry analysis of β III-tubulin and GFAP expression in CRBL cells

Expression of β III-tubulin (A) and GFAP (B) in CRBL cells was quantified by flow cytometry. After incubation with anti- β III-tubulin and anti-GFAP antibodies, CRBL cells were further incubated with secondary antibody conjugated to FITC prior to the measurement. N2a cells were used as a positive control for β III-tubulin expression and a negative control for GFAP expression. C6 glial cells treated with Bt_2cAMP were used as a positive control for GFAP expression and a negative control for β III-tubulin expression. A purified naïve mouse IgG2a was used to define the background. The percentage of positive cells is indicated in the lower right-hand corner of each graph.

Fig 6. Expression of PrP^C in CRBL cells

(A) Western blotting analysis. The cells known to differentially express PrP^C were used as controls. PrP^C is expressed at a high level in N2a, a medium level in NIH3T3 and none in RK13 cells. Lane 1: CRBL cells, Lane 2: N2a cells, Lane 3: NIH3T3 cells, Lane 4: RK13 cells. GAPDH (36 kDa) was used as a reference protein to ensure the equal amounts of each sample were analyzed. (B) Measurement of PrP^C-positive population using flow cytometry analysis. The percentage of CRBL cell (filled) population expressing PrP^C was compared to that of N2a cells (open). The background was accounted for using the secondary antibody alone. Anti-PrP antibody, D13 (InPro) was used for both Western blotting and flow cytometry analysis.

Fig 7. PrP^{Sc} accumulation in CRBL cells infected by prions

PrP^C and PrP^{Sc} were detected after *in vitro* infection with inoculum prepared from cells (A and C) and mouse brains (B). Both isoforms of PrP were detected before PK treatment (-PK, upper panels). PrP^{Sc} was detected after PK treatment (+PK, lower panels). Anti-PrP antibody, D13 (InPro) was used for Western blotting. (A). The infected cells at the 5th passage post-inoculation were analyzed. Lane 1: uninfected CRBL cells, Lane 2: CRBL cells infected by SMB-PS inoculum, Lane 3: CRBL cells infected by SMB inoculums (SMBinfCRBL), Lane 4: CRBL cells infected by N2a inoculum, Lane 5: CRBL cells infected by ScN2a inoculum, Lane 6: N2a cells infected by ScN2a inoculum, Lane 7: N2a cells infected by SMB inoculum, Lane 8: uninfected N2a cells, Lane 9: ScN2a cells. (B). The infected cells at the 5th passage post-inoculation were analyzed. Lane 1: CRBL cells infected by brain homogenate of uninfected mice, Lane 2: CRBL cells infected by brain homogenate of scrapie (RML)-sick

mice, Lane 3: N2a cells infected by brain homogenate from uninfected mice, Lane 4: N2a cells infected by brain homogenate from scrapie (RML)-sick mice, Lane 5: N2a cells, Lane 6: ScN2a cells. (C). The infected cells were cultured extra 15 passages post-inoculation since the initial confirmation of PrP^{Sc} propagation shown in Panel A. Lane 1: SMB cells, Lane 2: SMBinfCRBL cells, passage 8, Lane 3: SMBinfCRBL cells, passage 15, Lane 4: ScN2a cells, Lane 5: CRBL cells infected by ScN2a inoculums, passage 7, Lane 6: CRBL cells infected by ScN2a inoculums, passage 15. (D) Expression of β III-tubulin (upper panel) and GFAP (lower paner) in SMBinfCRBL cells. Lane 1 and 4: uninfected CRBL cells, Lane 2: SMBinfCRBL cells, passage 8, Lane 3: SMBinfCRBL cells, passage 15, Lane 5: CRBL cells infected by ScN2a inoculums, passage 7, Lane 6: CRBL cells infected by ScN2a inoculums, passage 10.

Fig 8. Molecular properties of PrP^{Sc} in SMBinfCRBL cells

(A) Glycosylation states of PrP^{Sc} in SMB (filled) and SMBinfCRBL (open) cells were analyzed from multiple (n=6, SMB cells; n=7, SMBinfCRBL cells) Western blots obtained and archived in our laboratory. The percent ratio of di- (D), mono- (M), and unglycosylated (U) PrP^{Sc} bands was compared. (B) Deglycosylation of PrP in SMB (lanes 1, 2, 5, and 6) and SMBinfCRBL (lanes 3, 4, 7, and 8) cells. The proteolytic processing of PrP was monitored by detecting C1 and C2 PrP fragments using D13 (lanes 1–4) and D18 (lanes 5–8) antibodies. SMB (30 µg) and SMBinfCRBL (60 µg) cell lysates were treated with PNGase F without PK-treatment (lanes 1, 3, 5, and 7) to exhibit native PrP processing, while SMB (300 µg) and SMBinfCRBL (1 mg) cell lysates were deglycosylated after PK digestion (lanes 2, 4, 6, and 8) to assess the conformation of PrP^{Sc}. F: full-length PrP, C1: a 17 kDa fragment associated with PrP^C, C2: a 21 kDa fragment associated with of PrP^{Sc}.

Table 1

Doubling time of CRBL cells.

% fetal bovine serum	p10	Passages p60	p80
10%	15.6 ± 2.5 hr	11.6 hr	10.6 ± 0.7 hr
5%	17.4 ± 3.1 hr	n.d.	n.d.
2.5%	23.5 hr	n.d.	n.d.

n.d.: not determined. Doubling times presented without standard deviation were obtained from a single measurement.