human habitats. In Asia, including Japan, pig farms and human habitats are adjacent; therefore, wild rats frequently enter and leave areas of human habitation. In this study, rats carried swine HEV and the HEV from rats was infectious to human A549 cells. The transmission of HEV from pigs to humans has been demonstrated via the consumption of undercooked or uncooked meat. Therefore, high hygienic standards in human habitats and in pig farms are an important issue to prevent the transmission of HEV, and rodent control may be a critical aspect to address the issue.

Conclusions

In this study, 10 of the 56 (17.9%) rats captured around a pig farm were positive for HEV genotype 3, and they were carrying infectious viruses. The contamination risk of HEV via rats needs to be studied in further detail.

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Authors' contributions

YK, SM, SU, SK, YKM, MT, SN, and KH performed the molecular biology and serological examination of the rats. SM, MY, KI, and KH isolated the viruses and helped draft the manuscript. MY, KI, and KH conceived the study, participated in its design and coordination, and drafted the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Protective Role of Human Intravenous Immunoglobulin from Influenza A Virus Infection in Mice

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Abstract: Intravenous immunoglobulin (IVIG) has been manufactured from pooled plasma of 10,000 or more units from healthy donors. Recently, we reported that the IVIG manufactured even before the 2009 influenza pandemic contained antibodies reactive to seasonal H1N1 and pandemic H1N1 2009 (H1N1 pdm) viruses. In this study, we used an animal model to evaluate the efficacy of IVIG against influenza infections. A seasonal influenza H1N1 strain (New Caledonia, A/NC/20/99) and an H1N1 pdm strain (A/Osaka/168/2009) were used. The BALB/c and severe combined immunodeficiency mice (SCID; C.B-17/lcr-scid/scid) were also used. Mice inoculated with A/NC/20/99 or A/Osaka/168/2009 were administrated IVIG and monitored for 3 weeks. The administration of IVIG 48 h before and after inoculation with a mouse-adapted seasonal H1N1 virus, resulted in survival rates of 80 and 88%, respectively. The rate among control mice was 30%. In addition, infectivity in lungs from IVIG-treated mice also decreased significantly. Similar effects of IVIG on the survival rate were obtained with H1N1 pdm. Thus, IVIG was shown to be effective against both viruses in mice.

Keywords: IVIG, Influenza, 2009 Pandemic Influenza, Animal model.

INTRODUCTION

The influenza virus is currently the most important public health concern in the world, especially since the appearance of the 2009 pandemic influenza A/H1N1 (H1N1 pdm) virus. Human intravenous immunoglobulin (IVIG), a product manufactured from plasma derived from more than 10,000 units from healthy donors, most of whom have had natural infections with seasonal influenza viruses as well as vaccinations, could contain a wide variety of antibodies effective for protection against influenza infections.

In Japan, IVIG has not been yet approved for influenza virus infections, although it was recommended for influenza encephalopathy by the *Study Group for Influenza Encephalopathy* [1] and the Japanese Respiratory Society [2].

The mechanism of its effect against influenza is not yet clear. However, Yunoki *et al.* [3] and Hong *et al.* [4] reported that IVIG contains significant titers of hemagglutination-inhibition (HI) and viral neutralization (VN or MN) antibodies against not only seasonal H1N1 but also H1N1 pdm. The cross-reacting antibody against H1N1 pdm seemed to be derived from natural influenza viral infections as well as vaccinations. Here, we examined the efficacy of IVIG against these influenza infections in mice.

MATERIALS AND METHODS

Viruses

A seasonal influenza H1N1 strain (New Caledonia, A/NC/20/99) and an H1N1 pdm strain (A/Osaka/168/2009) were used. Before the experiment, the H1N1 and H1N1 pdm strains were passaged 5 times in BALB/c mice and 4 times in the severe combined immunodeficiency mice (SCID; C.B-17/lcr-scid/scid, CLEA Japan Inc., Japan). The mouse-adopted viruses were named mo-A/NC/20/99 and mo-A/Osaka/168/2009, respectively. To evaluate the adaptation, BALB/c mice (4-week-old males) were infected intranasally with 100 or 1000 focus-forming units (FFUs) of mo-A/NC/20/99 (n=10) or mo-A/Osaka/168/2009 (n=7), then observed for 3 weeks.

Human Intravenous Immunoglobulin (IVIG)

The IVIG (Venoglobulin-IH®; Benesis Corporation, Japan) used was the same lot previously show to contain

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1:160 HI and 1:640 VN titers against seasonal H1N1 (A/NC/20/99) and 1:8 HI and 1:64 VN titers against H1N1 pdm (A/Osaka/168/2009) [3].

Infection with mo-A/NC/20/99

A total of 40 BALB/c mice (4-week-old males) were divided into four groups (n=10 per group). The mice were infected intranasally with mo-A/NC/20/99 at 1000 FFUs per head for the evaluation of IVIG's effect on survival for 3 weeks. To investigate the efficacy of IVIG treatment at 48 h before inoculation (hbi) or at 48 h post inoculation (hpi), each group of mice were intraperitoneally administered with IVIG at 5 mg per head (corresponding to 250 mg/kg) at 48 hbi, and at 48 and 72 hpi with 1000 FFUs per head. As a control, the H1N1-inoculated BALB/c mice were orally administered 100 mg/kg of Oseltamivir (Tamiflu®, Chugai Pharmaceutical Co., Ltd., Japan) for 4 days, and observed for 3 weeks. The dissected lung was fixed with 10% formalin-PB and prepared for tissue sectioning and HE stained for histological examination.

Infection with mo-A/Osaka/168/2009

A total of 14 BALB/c mice were inoculated intranasally with mo-A/Osaka/168/2009 at 1000 FFUs per head. The mice were divided into two groups for the evaluation of IVIG's effect on survival for 2 weeks. To evaluate the antiviral effect of IVIG on the H1N1 pdm virus in SCID mice lacking immunoglobulin and functional T and B cells, SCID mice (8 weeks old, male) were inoculated intranasally with mo-A/Osaka/168/2009 at 1000 FFUs. The mice were intraperitoneally administered IVIG at 5 mg per head (corresponding to 250 mg/kg) at 48 hpi. The untreated control group was administered the solvent of IVIG.

Evaluation of Antiviral Effects

Survival rates were monitored for 3 weeks. The BALB/c mice inoculated with mo-A/NC/20/99 or mo-A/Osaka/168/2009 were monitored. The infected SCID mice treated with or without IVIG were dissected at 3 and 5 days post inoculation (dpi). The lung from infected mice was homogenized with PBS (20% homogenate) and infectivity ($TCID_{50}$) in the lung was determined on days 3 and 5 post administration of IVIG.

We used the commercial statistical analysis software (SPSS 15.0 J, SPSS Japan Inc., Tokyo, Japan) for all statistical analyses in this study. Independent t-test was used for comparisons of averages for two groups between IVIG treatment and untreatment control.

RESULTS

Viral Adaptation to the Mice

The BALB/c mice inoculated with the seasonal H1N1 strain at 100 and 1000 FFUs per head were observed for 3 weeks (data not shown). The survival rate was 30% among the mice infected with 1000 FFUs and 100% among those infected with 100 FFUs. In contrast, the survival rate of BALB/c mice infected with H1N1 pdm at 1000 FFUs was 33%. The histological analysis of lungs from both BALB/c and SCID mice showed hemorrhagic pneumonia. HE-stained sections of dissected lung tissue from BALB/c mice infected with 1000 FFUs of mo-A/NC/20/99 are shown in Fig. (**1**). The titer of virus hereafter was 1000 FFUs per head.

Efficacy Against H1N1 and H1N1 pdm

As shown in Fig. (**2A**), the treatment of BALB/c mice with IVIG at 48 hpi with seasonal H1N1 was highly

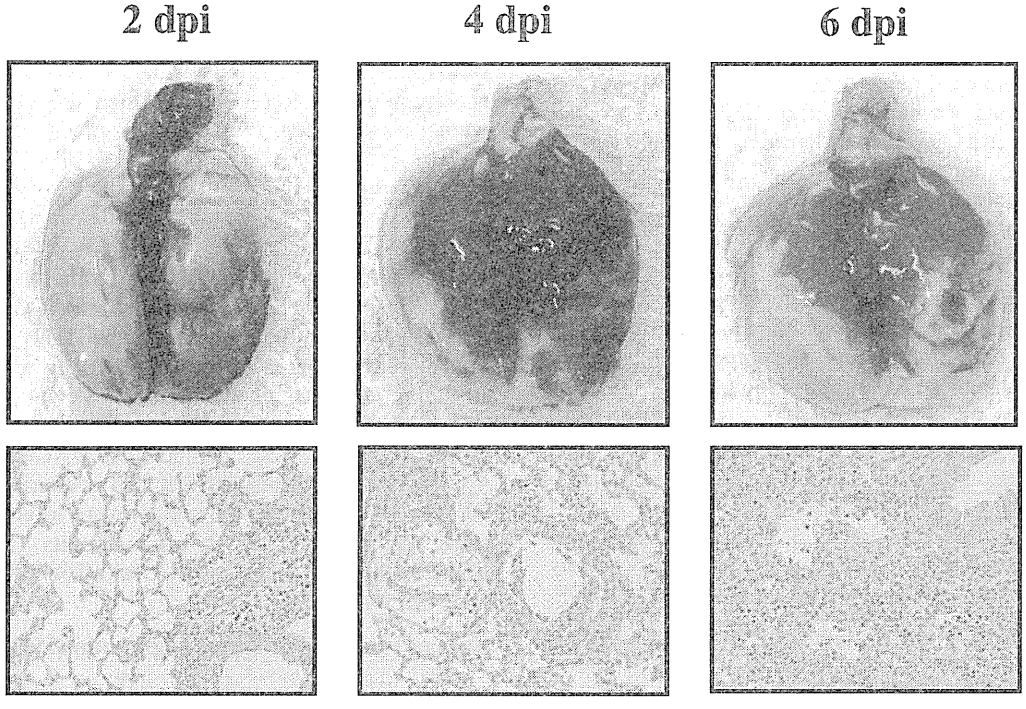

Fig. (1). HE-stained tissue sections of dissected lungs from infected BALB/c mice. Upper: Photos of dissected lungs with hemorrhagic pneumonia in BALB/c mice inoculated with 1000 FFUs of mo-A/NC/20/99 at 2, 4 and 6 dpi. Lower: HE-stained tissue sections of the dissected lungs.

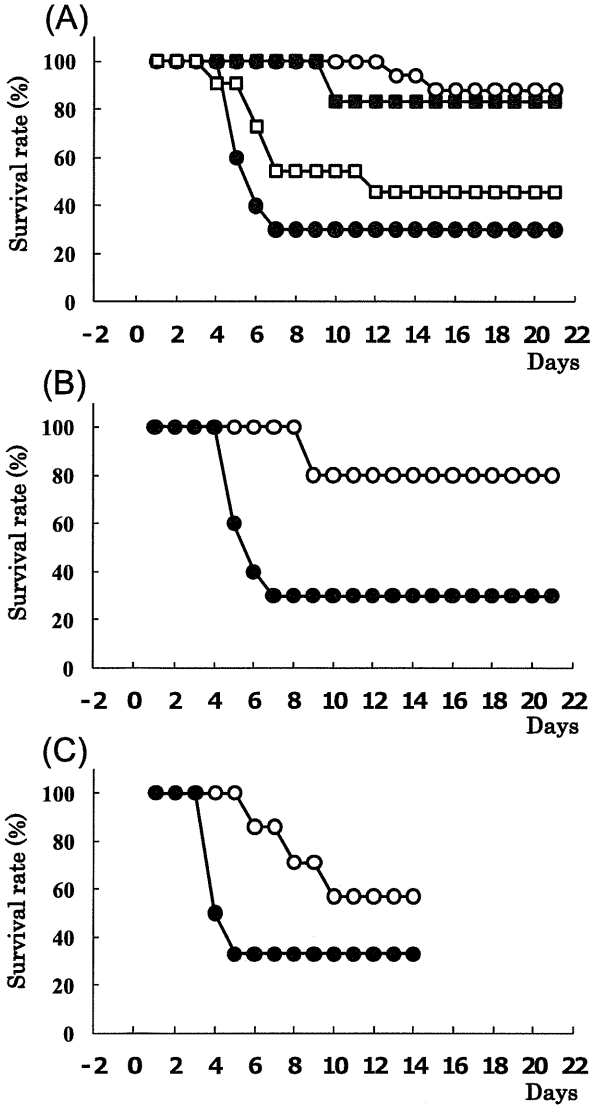

Fig. (2). Efficacy of IVIG against seasonal and pandemic H1N1 infections. **A)** To test the efficacy of IVIG after inoculation with seasonal H1N1, BALB/c mice inoculated with 1000 FFUs of mo-A/NC/20/99 per head were left untreated (closed circle) or else treated with 5 mg of IVIG (open circle) or a total of 2 mg of Oseltamivir (closed square) per head at 48 hpi. In addition, the inoculated mice were treated with 5 mg of IVIG per head at 72 hpi (open square). **B)** To test the efficacy of IVIG against seasonal H1N1 before inoculation of the virus, BALB/c mice were left untreated (closed circle) or treated with 5 mg of IVIG per head (open circle) at 48 hbi with the same dose of H1N1 as above. **C)** To test the efficacy of IVIG against pandemic H1N1 after inoculation of the virus, BALB/c mice inoculated with 1000 FFUs of mo-A/Osaka/168/2009 per head were left untreated (closed circle) or treated with 5 mg of IVIG per head (open circle) at 48 hpi.

effective, since the survival rate was significantly increased to 88%, compared with 30% in the control. Interestingly, when mice were treated with Oseltamivir, a similar survival rate (80%) was obtained. When mice were treated at 72 hpi,

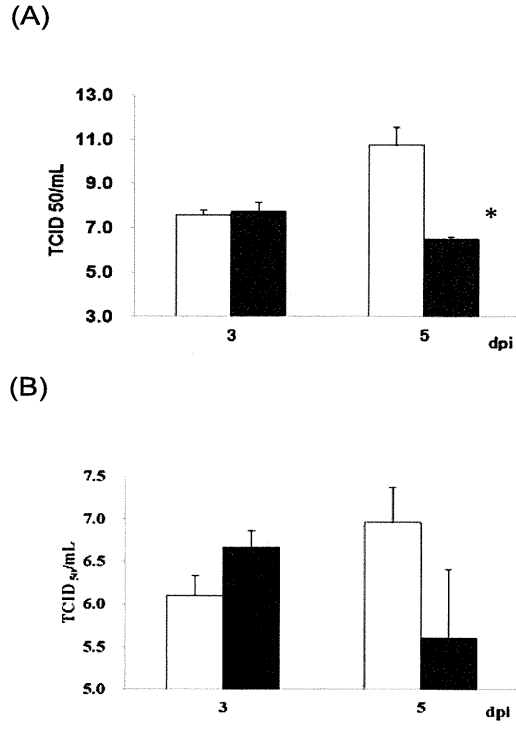

Fig. (3). Effect of IVIG treatment on viral propagation in the infected mouse lung. The amount of infectious virus in the lung from infected (closed bar) and control mice (open bar) at 3 and 5 days post inoculation (dpi) with seasonal H1N1 (**A**) and H1N1 pdm (**B**). The data showed the average and standard deviations of virus titer. Asterisk showed the statistical significance $P<0.05$.

the survival rate greatly decreased to 45.5%, suggesting the delayed treatment reduced by half the survival rate.

We also evaluated the preventive effect of IVIG against seasonal H1N1 infections in BALB/c mice. As shown in Fig. (**2B**), a similar survival rate, 80%, was achieved with the intramuscular administration of IVIG (5 mg/head) at 48 hbi. Interestingly, severe clinical manifestations, such as respiratory symptoms and cyanosis, were not observed in all mice in the pretreated groups, suggesting that pre-treatment with IVIG may prevent the life-threatening influenza virus infection at least in mice. This suggestion was supported by a significant reduction of viral titers in the lungs from BALB/c mice treated with IVIG at 48 hpi: $10^{10.7}$ versus $10^{6.5}$ $TCID_{50}$/ml in control and IVIG-treated mice, respectively (Fig. **3A**).

The treatment of BALB/c mice with IVIG increased the survival rate to 58% at 48 hpi with H1N1 pdm, the survival rate being 33% for control mice (Fig. **2C**). In fact, the experiments using SCID mice for H1N1 pdm revealed that the propagation of this virus also tended to decrease after IVIG treatment (Fig. **3B**).

DISCUSSION

IVIG proved highly effective against influenza viral infections in mice, raising the survival rate from 30% to 88% for seasonal H1N1 infections and from 33% to 58% for H1N1 pdm infections. The treatment's efficacy depended on infection status, and was at least comparable with that

of Oseltamivir treatment at 48 hpi. Interestingly, the administration of IVIG within 48 hpi significantly increased the survival rate of H1N1-infected individuals. This efficacy was also observed in the mice infected with H1N1 pdm. The effect of IVIG differed between the seasonal and pandemic H1N1. The difference in survival rates could be due to different HI and VN titers of the IVIG against the viruses, i.e., 1:160 HI and 1:640 VN titers against seasonal H1N1 (A/NC/20/99) and 1:8 HI and 1:64 VN titers against H1N1 pdm (A/Osaka/168/2009) [3].

Immunoglobulin is believed to be a multi-functional plasma product [5]. There are several options against influenza-related diseases. Generally, IVIG has been used against complications such as influenza encephalopathy and the development of severe pneumonitis whereas Oseltamivir has been used against viral replication. Interestingly, the use of SCID mice revealed that IVIG reduced the viral titer, though only the 1:8 HI and 1:64 VN titers against H1N1 pdm. Thus, IVIG could be an option for treating serious influenza and the complications associated with it. In fact, in several cases, IVIG treatments have proven effective against influenza encephalopathy, severe pneumonitis and flu-related respiratory tract complications [6, 7]. IVIG could be a second-line option for influenza with seasonal H1N1 and H1N1 pdm in patients with hematologic malignancies [8]. In addition, immunoglobulin G_2 deficiency and bacterial co-infections seem to exacerbate H1N1 pdm infections [9-11].

Our study showed a significant effect of IVIG against the influenza virus in an animal model. The effectiveness of IVIG seems to be derived from Japanese blood donors who had received the seasonal influenza vaccines or had a history of natural infections with seasonal influenza viruses. Interestingly, the IVIG lot reactive with H1N1 pdm was detected even before the outbreak of H1N1 pdm [3]. The rate of donors who have antibodies cross-reactive with H1N1 pdm has increased in the population since the H1N1 pdm outbreak and the administration of trivalent inactivated influenza vaccine [12]. This finding suggests that vaccinations against the influenza virus generate cross-reacting antibodies to reassortant H1N1 pdm and may prevent lethal illness. Thus, the circulation of vaccinations may play an important role in producing cross-reactive immunoglobulin against new subtypes of the influenza virus. This study revealed the efficacy of IVIG for the prevention and treatment of both seasonal and pandemic H1N1 infections.

CONFLICT OF INTEREST AND ACKNOWLEDGEMENT

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Review

Cellular Prion Protein: From Physiology to Pathology

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Abstract: The human cellular prion protein (PrP^C) is a glycosylphosphatidylinositol (GPI) anchored membrane glycoprotein with two N-glycosylation sites at residues 181 and 197. This protein migrates in several bands by Western blot analysis (WB). Interestingly, PNGase F treatment of human brain homogenates prior to the WB, which is known to remove the N-glycosylations, unexpectedly gives rise to two dominant bands, which are now known as C-terminal (C1) and N-terminal (N1) fragments. This resembles the β-amyloid precursor protein (APP) in Alzheimer disease (AD), which can be physiologically processed by α-, β-, and γ-secretases. The processing of APP has been extensively studied, while the identity of the cellular proteases involved in the proteolysis of PrP^C and their possible role in prion biology has remained limited and controversial. Nevertheless, there is a strong correlation between the neurotoxicity caused by prion proteins and the blockade of their normal proteolysis. For example, expression of non-cleavable PrP^C mutants in transgenic mice generates neurotoxicity, even in the absence of infectious prions, suggesting that PrP^C proteolysis is physiologically and pathologically important. As many mouse models of prion diseases have recently been developed and the knowledge about the proteases responsible for the PrP^C proteolysis is accumulating, we examine the historical experimental evidence and highlight recent studies that shed new light on this issue.

Keywords: neurodegenerative disease; prion; proteolytic cleavage; C1

1. Introduction

Transmissible spongiform encephalopathies (TSE diseases) or prion diseases are all fatal neurodegenerative conditions that include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, and kuru, Gerstmann-Sträussler-Scheinker syndrome (GSS), sporadic, familial, and variant forms of Creutzfeldt-Jakob disease (CJD) in humans. The central dogma of prion biology is that the normal cellular isoform of prion protein (PrP^C) encoded by the highly conserved single-copy gene *Prnp* [1] is post-translationally refolded into a partially protease resistant and β-sheet-enriched conformation (generally termed PrP^{Sc}) that is infectious [2]. However, neither the mechanism by which PrP^{Sc} causes neuronal dysfunction during prion disease nor the normal function of PrP^C is well defined. Although several functions have been suggested [3,4,5,6,7], none of these has been shown to be associated with prion disease pathogenesis or prion replication.

The neurotoxicity caused by prions is not simply explained by loss of a normal functional activity of PrP^C, since mice lacking PrP^C are healthy and fail to develop prion disease [8,9]. Aguzzi's group investigated whether prion pathology derived from neurotoxicity of PrP^{Sc}, by grafting neural tissue overexpressing PrP^C into the brain of PrP^C-deficient mice which are resistant to prions [10]. The grafted tissues kept producing high levels of PrP^{Sc} and induced localized infectivity after prion-inoculation in those mice, while the surrounding PrP^C-deficient tissues showed no pathological changes and were not significantly damaged by PrP^{Sc}. These results indicate that normal host prion protein is necessary for PrP^{Sc}-induced neurotoxicity.

Additional studies addressed whether neurotoxicity and infectivity are two distinct phenomena. Collinge's group developed double transgenic mice in which PrP^C is conditionally depleted in neurons after 9 weeks of age using the Cre-loxP system [9,11]. After prion inoculation of these mice, they initially suffered from development of clinical disease, but suddenly recovered from the disease and reversed spongiform neuropathology and behavioral abnormalities at around 9 weeks, even when brains of the mice contained high levels of PrP^{Sc}. Furthermore, Chesebro's group established transgenic mice expressing prion protein lacking the glycosylphosphatidylinositol (GPI) membrane anchor to prevent the prion proteins from localizing on the surface of cells [12]. These mice accumulated abnormal protease-resistant PrP^{Sc} in their brains after prion inoculation, and the brains were physically damaged by PrP^{Sc} amyloid plaques, but no clinical signs of disease were observed after 1 to 2 years compared to that seen in the non-Transgenic (Tg) control wild-type mice after 140 to 160 days. Thus, whatever the physiological function of PrP^C, it has become increasingly clear that expression of PrP^C at the cell surface is necessary to mediate the full toxicity induced by PrP^{Sc}.

The uncoupling of neurotoxicity and infectivity is a growing concept in the prion field [13]. Collectively, PrP^{Sc} that is major constituent of prions can propagate using PrP^C as a substrate and then PrP^{Sc} may cause neuronal cell death using membrane bound PrP^C as a detonator. How this occurs is currently unknown, however.

2. Normal cellular prion protein, PrP^C

Three Nobel Prizes have been awarded for seminal work performed in the prion field. Daniel Carleton Gajdusek received the award in 1976 for the discovery of "slow virus" that is now known as prion disease. Stanley B. Prusiner subsequently received the award in 1997 for the discovery of "prion" as the sheep scrapie agent and introduction of the "protein only hypothesis", which stated that misfolded proteins are the infectious agents that cause prion diseases. Finally, Kurt Wüthrich received the award in 2002 for his contributions to the use of NMR to determine three-dimensional structures of proteins and nucleic acids in solution, including unraveling the structure of prion proteins using this technique.

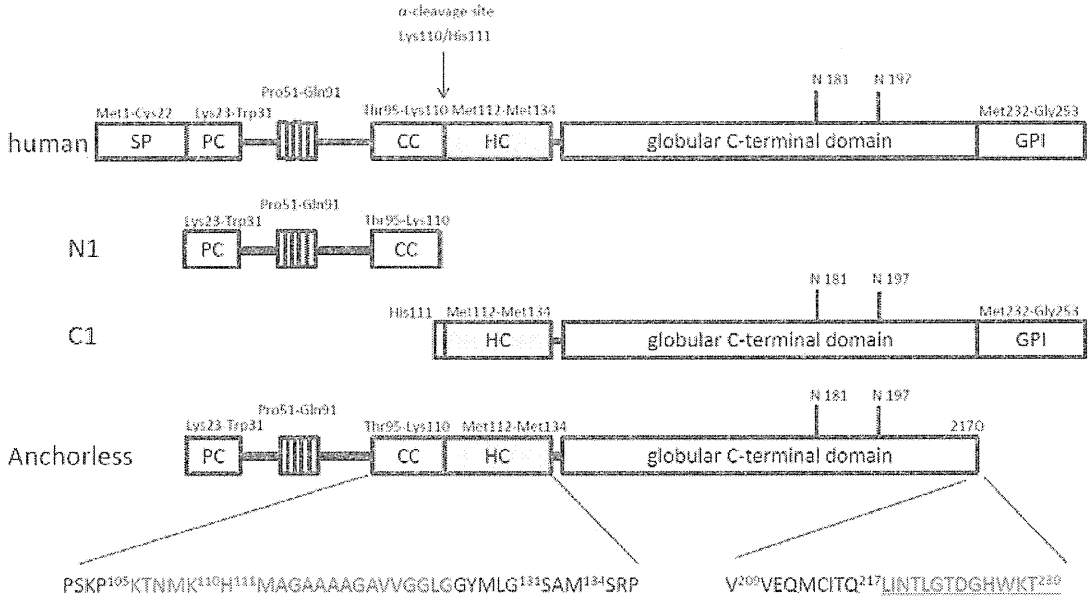
According to the widely accepted "protein-only hypothesis" by Prusiner, proteinase resistant protein, PrP^{Sc}, is infectious. Early studies by Weissmann's group surprisingly demonstrated that PrP^{Sc} is an abnormal isoform of host-encoded cellular prion protein (PrP^C) [1,14]. Since then, many scientific efforts have been performed to address the normal function of PrP^C, but the suggested physiological functions of PrP^C do not completely explain all phenomena seen in prion diseases, and further research is needed. Several of these elements have been discussed in excellent recent reviews [15,16,17]. Here, we briefly elaborate on the processing of PrP^C, involving its normal metabolism.

PrP^C is encoded on the short arm of human chromosome 20 (20q13) as 16 kb single copy PRNP gene and the open leading frame (ORF) is encoded on exon 2, which facilitates the cloning of the ORF by polymerase chain reaction (PCR) directly from genomic DNA. The human prion gene PRNP encodes a 253-residue precursor protein (see Figure 1). The first 22 N-terminal residues are post-translationally removed during transport to the cell surface. The last 23 C-terminal residues are excised after the addition of the glycosyl phosphatidylinositol (GPI) anchor. Thus, mature PrP^C consists of 208 residues. As PrP^C is variably glycosylated at two asparagine residues (N181 and N197), it exists as un-, mono- and di-glycosylated species.

When Wilfried W. de Jong's group compared PrP coding sequences in 26 mammalian species [18], they noted that a hydrophobic region in the middle of PrP^C is perfectly conserved, suggesting that the region is functionally important for PrP^C. This hydrophobic core (HC) region is immediately adjacent to the putative cleavage site of PrP^C, which is enriched in charged residues (Figure 1), designated the charged cluster (CC) region. The putative cleavage site has been commonly referred to as the α-cleavage site.

Until more recently, the existence of the truncated form of PrP^C was not widely recognized by many prion biologists, since western blot analysis for brain homogenates or cell lysates showed several bands, which were attributed to differential glycosylation.

Figure 1. Diagrammatic representation of protein structure of normal cellular prion proteins. Based on the knowledge of physiological processing of prion protein (PrP^C), it is likely that mature PrP^C (residues from 23 to 253), N-terminal (N1) (23 to 110), C-terminal (C1) (111 to 253), and anchorless PrP (23 to 230) may exist in normal brains of humans. Anchorless PrP seems to be unglycosylated and non-cleavable at putative α-cleavage site. The minimum sequence that causes spontaneous neurodegeneration when deleted in mice is shown in red. The additional open leading frame for anchorless PrP is underlined. SP, signal peptide; PC, positively charged region; CC, charged cluster region; HC, hydrophobic core region.



The discovery of the truncated form of PrP^C can be traced back to the early 1990s, when Prusiner's group characterized PrP^C from Syrian hamster brain [19]. They found two PrP^C forms designated PrP^C-I and PrP^C-II which appeared to be generated from PrP^C-I by limited proteolysis of the N-terminus, as confirmed by epitope mapping with different antibodies. Spontaneous degradation during the purification procedure was ruled out, since various protease inhibitors failed to alter the detection of the two protein forms. The same group further characterized PrP^C-II and concluded that PrP^C-II appears to lack the putative α-helical domain (codons 109-122), which is likely to be necessary for PrP^{Sc} formation. They also identified that chymotrypsin-like activity is a candidate mechanism by which a ~9 KDa fragment is proteolytically excised from the N-terminus of PrP^C [20].

In 1995, Gambetti's group examined PrP^C metabolism in more detail. The group characterized the PrP forms present in normal and pathological human brains and in neuroblastoma cells [21]. They demonstrated that a COOH-terminal fragment of PrP^C, which was named "C1", is found in normal and CJD brains, as well as in human neuroblastoma cells. C1 turned out to have alternative N-termini starting at either His-111 or Met-112 using epitope mapping and radiosequencing. Thus, it seems that C1 contains at least the HC region (amino acids 112-133). Interestingly, both PrP^C and C1 seem to be anchored to the cell membrane, since both are mostly recovered in the membrane fraction and are released after treatment with phosphatidylinositol-specific phospholipase C (PIPLC) [21]. These data provide supporting evidence that C1 is not a degradation product generated during experimental procedures, as suggested by others [14,19].

In 1993, Harris's group investigated chicken brain and murine neuroblastoma cells transfected with the chicken homologue of PrP^C [22]. Chicken PrP^C was demonstrated to undergo two cleavages as part of its normal metabolism. One product is a secreted form of full-length PrP^C, which is released from the membrane when the GPI-anchor is cleaved. Importantly, they also found a second truncated form that accounts for most of the surface-anchored molecules in transfected murine cells, indicating that the machinery to generate C1 is highly conserved between species. The N-terminal cleavage fragment, termed N1, was found in medium from neuroblastoma cell cultures, as well as in chicken cerebrospinal fluid. We have also detected N1 fragments in the cell lysates of murine neuroblastoma N2a cells, although N1 is unstable even when stored at -80 ˚C in the presence of proteinase inhibitors, resulting in analysis having to be performed immediately to avoid degradation (unpublished data). Collectively, PrP^C appears to have two physiological forms on cell surfaces; one is full-length and the other is the truncated C1 form, and both may have functions (Figure 1). Whether the secreted forms (GPI-cleaved PrP or N1) have physiological functions will require more detailed investigation in the future.

Interestingly, physiological anchorless prion protein may exist in humans due to alternative splicing of exon 2 of the PrP gene, which eliminates the GPI-anchor [23] (see Figure 1). Moreover, analysis of the transcriptome derived from zebrafish embryo mRNA present at 5.3 hours postfertilization (hpf) indicates splice variants of PrP-2 which lacks GPI-anchor [24]. Since the anchorless form is unglycosylated and unprocessed, the full-length anchorless PrP^C may have physiological function in the absence of N-linked glycosylation and without α-cleavage [23]. Transgenic mice expressing artificial GPI-anchorless PrP^C protein also showed that the exogenous anchorless PrP^C is unglycosylated, reside in the cytosol, and is secreted [12]. However, the glycosylation status is controversial, since GPI-anchorless PrP^C is fully glycosylated in different cell types [25]. Collectively, there are at least four forms of normal cellular prion protein; full-length GPI-anchored, N1, C1, and GPI-anchorless (Figure 1). None of the physiological functions of all these prion proteins has so far been clarified.

3. Transgenic mouse models

Prion diseases were originally thought to be caused by a kind of slow-virus, as suggested by Gajdusek. Subsequently, the nature of materials that cause prion diseases was hypothesized by Prusiner to be the PrP^{Sc} protein, but not a virus. However, a new paradigm has appeared since normal cellular PrP^C was found and examined. In 1998, Weissmann's group produced several truncated forms of PrP^C to identify the regions necessary for its pathogenic conversion, since PrP^C-deficient mice are resistant to scrapie which is induced by mouse-adapted sheep prions [26]. Unexpectedly, they found that PrP lacking residues 32-121 or 32-134 caused severe ataxia and neuronal death limited to the granular layer of the cerebellum as early as 1-3 months after birth (Figure 2). Importantly, attempts to transmit disease with brains of these spontaneously ill mice failed, no PrP aggregation was found, and the introduction of a single copy of the wild-type *Prnp* allele (PrP^C) rescued the phenotypes. Thus, PrP lacking residues 32-121 or 32-134 (ΔF) causes neuronal dysfunction irrespective of "infectious prions". Before this report, in 1990 Prusiner's group produced transgenic mice that have a leucine substitution at codon 101 (corresponding to P102L in human), which is the genetic basis of the human inherited prion disease, Gerstmann-Sträussler-Scheinker syndrome [27]. The mice also spontaneously

developed prion diseases, showing spongiform degeneration, astrogliosis, amyloid plaques, and infectivity to other mice [28,29]. Based upon these reports, we have categorized the transgenic mouse models in two groups; one with the non-infectious "ΔF phenotype", the other with the infectious "PrP^{Sc} phenotype" (Table 1. Due to limited information some transgenic mice are excluded from these categories.

Weissmann's group produced mice expressing PrP with several amino-proximal deletions in addition to ΔF (32-134) [26]. Some of these were normal, while others suffered from spontaneous neurological diseases which were non-infectious and did not generate protein aggregates. Interestingly, there was a strong correlation between the neurotoxicity caused by the truncated forms of PrP and the blockade of their proteolysis (see Figure 2). Wild-type PrP^{C} (non-toxic), exogenous PrP^{C} (non-toxic) (i.e. *Tga*20), ΔB(32-80)(non-toxic), ΔC(32-93)(non-toxic), and ΔD(32-106)(non-toxic) all showed the appearance of C1 by western blot analysis and no cerebellar syndrome (Figure 2). On the other hand, ΔE(32-121) (toxic) and ΔF(32-134) (toxic) did not show detectable C1 and caused neurodegenerative symptoms, (however in the publication of the ΔF mice it is difficult to judge C1 due to their similar size of ΔF(32-134) and C1). The absence of C1 in ΔF(32-134) is also examined in an *in vitro* assay using the HpL 3-4 *Prnp*-deficient cell line [30], indicating that ΔF is slightly larger than C1. The different phenotypes between the former (non-toxic) and the latter (toxic) are not simply explained by aberrant cellular localization of the truncated forms or by distinct expression levels, since all forms were shown to be expressed on the cell surface and the levels of transgene expression were comparable among the mice [26].

Figure 2. Schematic of PrP constructs and comparison of α-cleavage. All constructs shown here are derived from murine PrP^{C}. The constructs were inserted into half genomic expression vectors, containing the promoter, 5'intronic, and 3'untranslated sequences of the murine prion protein gene [31,32]. All mice are $Prnp^{0/0}$ background. Spontaneous neurodegeneration (Yes), C1 generation (Yes) are shown, according to the publications. *The sizes of C1 and exogenous PrP are similar so that it might be difficult to judge.

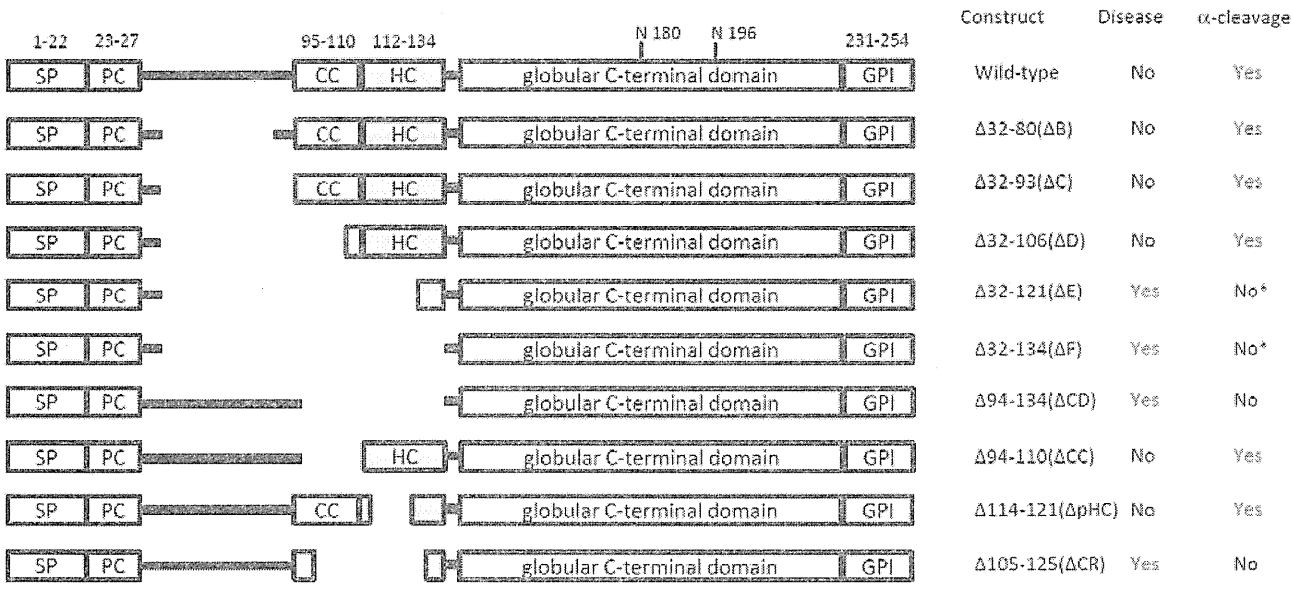


Table 1. Transgenic mouse models with mutant prion proteins.

Table 1. Transgenic mouse models with mutant prion proteins

Type	Designation	Human diseases	Copy number	Expression	$Prnp^{+/+}$	$Prnp^{0/0}$	Onset	PrP^{Sc}	Transmission	C1	Refferences
	MoPrP-P101L	Familial: GSSP102L	60 copies	8-fold	prion disease	[5]Accelerated	150-300 days	[3]PrPSc-like	Yes; P101L mice	N.S.	Hsiao et al. 1990 Science 250: 1587-1590. Hsiao et al. 1994 PNAS 91: 9126-9130. Telling et al. 1996 Genes Dev 10: 1736-1750.
[1]PrP^{Sc}-type	MoPrP(3F4)-PG14	Familial:Octarepeat	28 copies	4-fold	prion disease	[5]Accelerated	100-200 days	[3]PrPSc-like	no	N.S.	Chiesa et al. 1998 Neuron 21: 1339-1351. Chiesa et al. 2003 J Virol 77: 7611-7622.
	MoPrP-D177N/L108M/V111M	Familial: CJD(FFI)D178N	2 copies (knock-in)	N.D.	N.D.	prion disease	16 months	Yes	Yes	N.S.	Jackson et al. 2009 Neuron 63: 438-450.
	MoPrP-D177N/V128	Familial CJD(FFI)D178NV129	22 copies	1-fold	[5]Accelerated	prion disease	72 days	Yes	N.S.	N.S.	Dossena et al. 2008 Neuron 60:598-609.
	MoPrP-A116V/V128	Familial: GSSA117V	N.S.	6-fold	N.S.	Neurodegeneration	140-170 days	Yes	N.S.	C1	Yang et al. 2009 J Neuroscience 29: 10072.
	MoPrP(Δ GPI)	Familial: GSS	N.S.	1.7-fold	[5]Accelerated	prion disease	597 days	Yes	Yes	N.S.	Stohr et al. 2011 PNAS 108: 21223-21228.
	MoPrP-Δ F(Δ 32-134)	Artificial	20 copies	2-fold	[5]rescued	Neurodegeneration	30-90 days	no	no	[4]no C1	Shmerling et al. 1998 Cell 93: 203-214.
	MoPrP-Δ CD(Δ 94-134)	Artificial	1 - 5 copies	0.8-fold	[5]rescued	Neurodegeneration	10-25 days	no	no	[4]no C1	Baumann et al. 2007 EMBO J 26:538-547.
	MoPrP-Δ pHC(Δ 114-121)	Artificial	1 - 5 copies	0.8-fold	N.S.	normal	no	no	no	reduced C1	Baumann et al. 2007 EMBO J 26:538-547.
	MoPrP-Δ CC(Δ 94-110)	Artificial	N.S.	N.S.	N.S.	normal	no	no	no	reduced C1	Bremer et al. 2010 Nat Neurosci 13: 310-320.
[2]Δ F-type	MoPrP-Δ CR(Δ 105-125)	Artificial	N.S.	1-fold	[5]rescued	Neurodegeneration	6 days	no	no	[4]no C1	Li et al. 2007 EMBO J 26: 548-558.
	Mo-CD_Dpl(PrP90-133@Dpl65-66)	Artificial	126 copies	1-fold	N.S.	normal	no	no	no	C1	Baumann et al. 2009 PLoS One 4: e6707.
	Mo-PrP_Dpl(PrP1-133@Dpl66)	Artificial	180-120 copies	0.4-fold	N.S.	normal	no	no	no	[4]no C1	Baumann et al. 2009 PLoS One 4: e6707.
	MoPrP-Δ 23-134a	Artificial	N.S.	2-fold	N.S.	normal	no	no	no	-	Westergard et al. 2011 J Neuroscience 28: 14005-14017.
	MoPrP-Tg(C1) (Δ 23-111)	Artificial	N.S.	7-fold	N.S.	normal	no	no	no	-	Westergard et al. 2011 JBC 286: 44234-44242.
	Sha-T183A/T199A	Aritificial	N.S.	4-fold	N.D.	normal	no	no	no	N.S.	DeArmond et al. 1997 Neuron 19: 1337-1348.
	MoPrP-P101L	Familial: GSSP102L	2 copies (knock-in)	N.D.	normal	normal	no	no	no	N.S.	Manson et al. 1999 EMBO 18: 6855-6864.
	MoPrP-L9R/3AV(A112A114A117→V)	Artificial	N.S.	0.5-fold	[5]Accelerated	Neurodegeneration	21-389 days	no	no	N.S.	Stewart et al. 2005 J Neuroscience 25: 3469-3477.
	MHM2-(Δ 23-88&Δ 177-200)	Artificial	N.S.	0.5-fold	N.S.	Neurodegeneration	46-227 days	[3]PrPSc-like	N.S.	N.S.	Muramoto et al. 1997 Nature Med. 3: 750-755.
	MHM2-(Δ 23-88&Δ 201-217)	Artificial	N.S.	2-fold	N.S.	Neurodegeneration	67-165 days	[3]PrPSc-like	N.S.	N.S.	Muramoto et al. 1997 Nature Med. 3: 750-755.
Others	MoPrP-E199K	Familial: CJDE200K	N.S.	8-fold	normal	N.S.	no	no	no	N.S.	Telling et al. 1996 Genes Dev 10: 1736-1750.
	MH2M-AV3(A113A115A118→V)	Artificial	N.S.	4-fold	N.D.	Neurodegeneration	60 days	no	N.D.	N.S.	Hegde et al. 1998 Science 279:827-834.
	Sha-A117V	Familial: GSSA117V	N.S.	4-fold	N.D.	Neurodegeneration	572 days	no	no	N.S.	Hegde et al. 1999 Nature402: 822-826.
	Sha-N108I	Familial: CJDN108I	N.S.	4-fold	N.D.	Neurodegeneration	312 days	no	no	N.S.	Hegde et al. 1999 Nature402: 822-826.
	Sha-KH→II	Artificial	N.S.	4-fold	N.D.	Neurodegeneration	58 days	no	no	N.S.	Hegde et al. 1998 Science 279:827-834. Hegde et al. 1999 Nature402: 822-826.
	Sha-Δ STE(Δ 104-113)	Artificial	N.S.	4-fold	N.S.	normal	no	no	no	N.S.	Hegde et al. 1999 Nature402: 822-826.

[1]PrP^{Sc}-type: Neurodegeneration occurs even in the presence of PrP^{C}., meaning that PrP^{C} does not rescue it.

[2]Δ F-type: Neurodegenerative phenotypes are rescued by the introductio of PrP^{C}.

[3]Amyloid plaque, Insoluble PrP, or PrP deposition are seen in the ill mice, but they are largely proteinase K sensitive.

[4]no C1 means that it is under detection level by western blot analysis.

[5]Neurdegeneration is accelerated or resuced in the presence or absence of PrP^{C}.

MoPrP, mouse prion protein; Sha, syrian hamster prion protein; MHM2, a chimeric mouse-Syrian hamster prion protein with L108M/V111M; MH2M, a mouse-hamster chimera in which residues 94 to 188 are from hamster.

N.S.: not shown, N.D.: not determined.

Aguzzi's group developed transgenic mice, expressing PrP variants lacking residues 94-134(ΔCD) or 114-121(ΔpHC) (Figure 2) [33]. These deletion mutants are quite interesting because 94-134 encompasses the CC, α-cleavage and HC sites, and 114-121 includes the N-terminal palindrome sequence (AGAAAAGA), which is highly conserved among species and is thought to be important for the conversion into PrP^{Sc} [34,35]. These transgenic mice showed distinct phenotypes on a $Prnp^{0/0}$ background. ΔCD (94-134) mice spontaneously developed neurodegenerative diseases, while ΔpHC (114-121) mice did not [33]. The striking difference compared to ΔF (32-134) mice is that ΔCD (94-134) mice show more severe neurodegeneration, since the phenotypes were observed even on a $Prnp^{+/+}$ background. To overcome the neurotoxicity caused by ΔCD (94-134), either low expression of the exogenous ΔCD (94-134) or the introduction of exogenous high PrP^{C} expression, such as in *Tga*20 mice, are required [33]. Upon PNGase-F treatment of the brain homogenates from those mice, PrP^{C} displayed a significant amount of C1, whereas C1 formation was reduced in ΔpHC (114-121) and absent from ΔCD (94-134) [33], supporting the model dictating that blockade of α-cleavage of PrP^{C} makes it toxic. Similar results were also obtained in Tg(ΔCR) mice by Harris's group where PrP^{C} was deleted between residues 105 and 125, which include the CC and HC regions (105-125). This deletion appears to be the minimum mutation required to block α-cleavage of PrP^{C} *in vivo* using transgenic mice. Interestingly, accumulating results have shown that PrP peptide 106-126 is highly toxic in both murine and human neurons. However, further investigation is required to clarify the relation between PrP 106-126 and α-cleavage phenomenon. The same group also showed that ΔCR (105-125) is expressed on the surface of neurons, but C1 generation is undetectable by western blot analysis of brain homogenate (Figure 2) [36].

All of the above mice had spontaneous neurodegenerative dysfunction and importantly the dysfunction can be rescued by normal PrP^{C} in a dose dependent manner of PrP^{C} expression. In contrast, other transgenic mice including MoPrP-P101L, MoPrP-PG14, MoPrP-D177N and MoPrP-A116V showed spontaneous neurodegeneration, but the phenotypes were not rescued by PrP^{C} expression, which instead exacerbated the phenotypes (Table 1) [27,28,29,37,38,39,40,41]. Most strikingly, these mice appear to spontaneously generate PrP^{Sc} or amyloid plaque. In addition, their disease is often transmissible if "species barrier" is considered. Thus, these transgenic mice are likely a valuable model for prion disease. Although the generation of C1 was not assessed in these mice, α-cleavage of these mutant PrPs would occur fully or partially, since an *in vitro* system by Oliveira-Martins et al. showed that α-cleavage is unexpectedly tolerant to mutations unless a large portion of PrP^{C} is deleted [30]. However, it still remains to be determined how "PrP^{Sc}-type" could be explained by the "α-cleavage phenomenon".

In 1995 Gambetti's group described that an additional fragment longer than C1, designated C2, is present in substantial amounts in CJD brains, but not in normal controls [21]. Thus, C1 and C2 seem to be completely different in terms of physiological function, because C2 appears to be pathogenic. Interestingly, C2 seems to have the 3F4 epitope, meaning that C2 contains at least the residues between 90 and 104, which is upstream of the α-cleavage site (110 or 111). Thus, C2 is the fragment of PrP^{C} that was not cleaved at the α-cleavage site.

In 2006, Telling's group produced transgenic mice, expressing GFP-tagged murine PrP at codon 22 [42]. In addition to the study of the GFP-PrP^{C}, they tested a murine adopted scrapie (RML) infection. Interestingly, they used both PNGase F and Proteinase K to detect PrP^{Sc}. Normal mice showed two

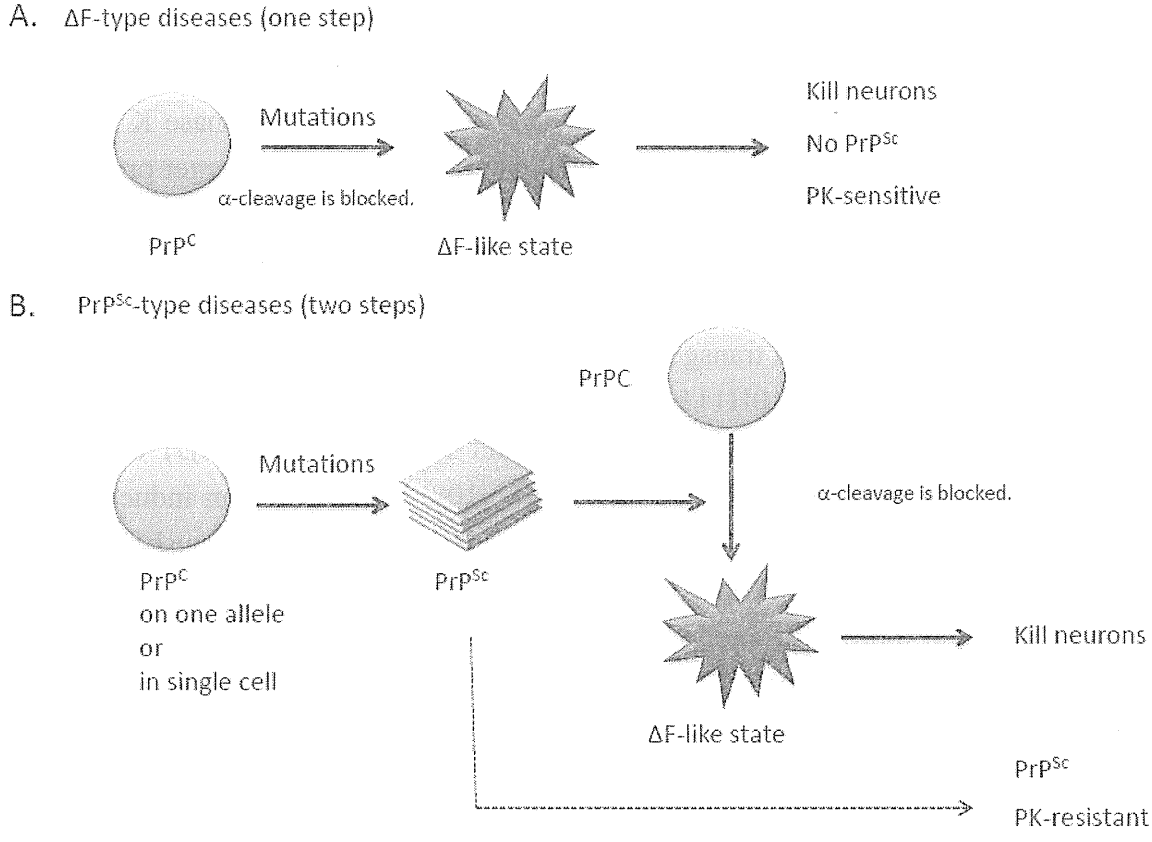
bands on WB, PrP^C and C1, and both bands were completely digested by Proteinase K treatment at 20 μg/ml. On the other hand, normal mice infected with RML showed three bands, PrP^C, C1 and C2. The data demonstrates that after Proteinase K treatment at 20 μg/mL C2 is retained, but not PrP^C and C1, suggesting that C2 is the PrP^{Sc} [43].

As previously described, Prusiner's group produced Tg mice overexpressing MoPrP-P101L, which spontaneously developed central nervous system dysfunction and transmissible, even though Proteinase K resistant PrP^{Sc} was not detected. In 2005, Telling's group revisited this issue [44]. They used the cold PK technique (Proteinase K treatment on ice), PNGase F, and PrP^{Sc}-specific antibody (15B3) to show the appearance of a proteinase resistant form of MoPrP-P101L with molecular weights corresponding to C2. Thus, PNGase F can be a powerful tool to detect proteinase K sensitive PrP^{Sc}, because contrary to Proteinase K treatment, which can completely digest the target protein depending on incubation time and concentration, the experimental impact of varying PNGase F treatment conditions is minimal. It will be interesting to test for the presence of such PrP^{Sc} using a similar technique in the MoPrP-PG14, MoPrP-D177N, and MoPrP-A116V transgenic mice.

In 1998, Lingappa's group reported transgenic mice expressing several mutant PrP, including Δ STE(104-113), G123P, KH→II(K110H111 to I110I111 substitutions), AV3(V113V115V118 to A113A115A118 substitutions), A117V (human GSS) and N108I (human CJD) on hamster PrP^C [45,46]. Among these mice, KH→II, A117V, AV3, and N108I PrP expression induced spontaneous neurodegeneration, but importantly, PrP^{Sc} was not detected in these mice. Previously, the same group studied PrP translocation at the endoplasmic reticular (ER) membrane and revealed unusual features in its biogenesis. They found more than one topologic form of full-length PrP^C synthesized in cell-free translation systems, including at least secPrP, NtmPrP, and CtmPrP [45]. Interestingly, they also showed that favored synthesis in the CtmPrP form strongly correlates with neurodegenerative phenotypes such that mice expressing KH→II (toxic), A117V (toxic), and AV3 (toxic) have relatively more CtmPrP in their brains. They concluded that expression of CtmPrP produces neurodegenerative changes. It remains to be determined whether the neurodegenerative phenotypes by KH→II, A117V, and AV3 mice are rescued by PrP^C or whether C1 is generated in these mice.

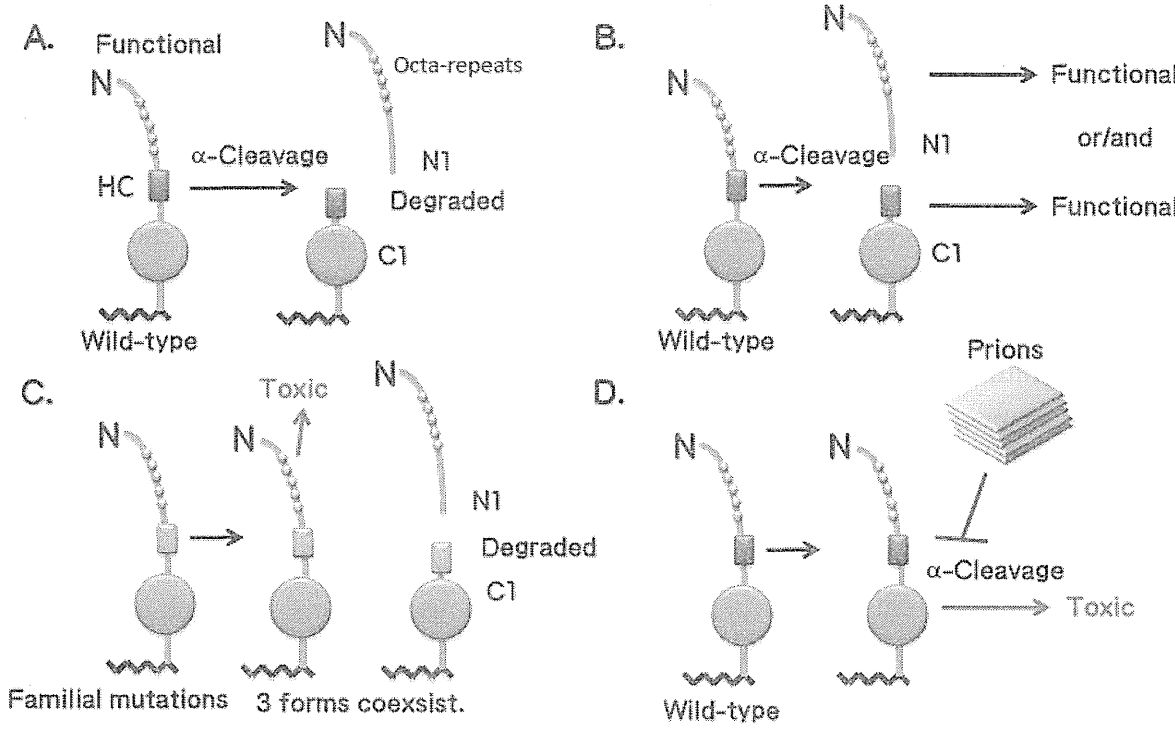
Mice expressing the "ΔF type" PrP have so far not been shown to generate a relevant C2 fragment. On the contrary, it is possible that all mice with the "PrP^{Sc} type" may show a prominent C2. The finding that PrP^{Sc} might be the C2 is interesting, because PrP^{Sc} is not cleaved at the α-cleavage site. Therefore, expression of "PrP^{Sc} type" mutants would also affect α-cleavage because certain mutations of PrP^C result in the generation of PrP^{Sc} that is resistant to α-cleavage or PrP^{Sc} that blocks α-cleavage of PrP^C through interactions with each other (see Figure 3).

Figure 3. (A) When PrP^C is not cleaved by deletion or mutation, the PrPs elicit neurotoxicity. (B) When the α-cleavage of PrP^C is blocked, the unstable PrPs tend to spontaneously aggregate to generate PrP^{Sc}. The generated PrP^{Sc} then affect the α-cleavage of neighboring PrP^C to elicit neurotoxicity or the conversion process from PrP^C to PrP^{Sc} by itself transduces a toxic signal. A mutation or deletion may occur on single allele or in single cell.



Is the reduction of C1 or inefficient α-cleavage of PrP toxic? One hint to answer this question can be deduced from the experiments performed by Aguzzi's group [33]. As discussed above, C1 formation was reduced in mice expressing ΔpHC (114-121) and absent from when ΔCD (32-134) is expressed (Figure 2). It might be possible that the presence of ΔpHC still retains un-cut PrP^C, which was supposed to be cleaved at the correct position and time. Therefore, presence of ΔpHC may retain some residual toxicity, which cannot be observed in the short life span of a mouse. Accordingly, the introduction of ΔpHC into ΔCD mice enhanced the phenotypes [33], suggesting that ΔpHC induces residual toxicity. In addition, the introduction of ΔpHC into ΔF mice partially rescued the ΔF phenotypes [33], indicating that ΔpHC also retains a function of PrP^C, since it is partially cleaved. These findings support a mechanistic model that cleaved and un-cleavable PrPs exist simultaneously with ΔpHC; one is non-toxic and able to rescue ΔF phenotypes and the other is toxic and enhances ΔCD neurotoxicity (Figure 4C).

Figure 4. (A) Full-length PrP^C has a function so that it is immediately cleaved at α-cleavage site after activation. (B) Either N1 or C1 is an active form so that it is cleaved at α-cleavage site to activate it. (C) PrP^C with mutations is not completely cleaved so that the uncleavable PrP show some residual toxicity. (D) Spontaneously generated PrP^{Sc} on one gene or derived from single cell blocks the α-cleavage of the remaining PrP^C, resulting in neurodegeneration. Infectious Prions also blocks the α-cleavage of host PrP^C.



What is the nature of neurotoxicity caused by expression of PrPs of the "ΔF type"? Although the endogenous physiological C1 does not contain residues 1 to 110 or 111, Harris's group pointed out that the artificial PrP constructs, such as Δ32-134, Δ94-134, and Δ105-125, retain the N-terminal 9 amino acids (residues 23-31, KKRPKPGGW) (Figure 2). Therefore, they engineered transgenic mice expressing a PrP mutant with 23-134 deleted (Δ23-134) [47]. According to their report, these mice surprisingly did not display clinical symptoms or neuropathology, in contrast to mice expressing Δ32-134, Δ94-134, or Δ105-125 [26,33,48]. Importantly, Δ23-134 (non-toxic), Δ32-134(toxic), Δ94-134(toxic), andΔ105-125(toxic) PrPs are expressed on the surface of cells, but did not seem to be cleaved to generate C1, thereby rejecting the notion that the blockade of α-cleavage correlates with neurotoxicity by PrP mutants. In addition, neither Δ23-134 (non-toxic) nor Δ32-134 (toxic) PrPs were efficiently endocytosed, while Δ105-125 (toxic) PrP was efficiently endocytosed in an *in vitro* cell culture system, indicating that the lack of toxicity of the Δ23-134 mutant cannot be attributed to its impaired endocytosis. This report suggests that the toxicity caused by PrP mutants that cannot generate C1 relates to presence of the nine amino acids (KKRPKPGGW). Similar evidence was previously shown by Prusiner's group, where a Δ23-88 mutant PrP was introduced into *Prnp*-deficient mice [49]. Their transgenic mice seemed to be normal, but the 23-88 mutant PrP does not seem to be functional, because it failed to rescue the mice from the Purkinje cell death in Ngsk $Prnp^{0/0}$ mice [50]. Thus, both Δ23-134 (un-cleavable) and Δ23-88 (cleavable) mutant PrPs might be non-functional.

As discussed in the Introduction, both function and toxicity require prion protein localization at the plasma membrane. Consistent with this requirement, PrPΔCDs lacking residues 94-134 or the GPI-anchor were shown to be non-toxic [33]. In addition, expression of GPI-anchorless PrP did not rescue Mo-PrPΔF(Δ23-134), although it did show partial residual rescue [51]. Recently, Prusiner's group produced Tg(PrP,ΔGPI)-expressing mice on a *Prnp*$^{0/0}$ background [52]. Mice engineered with low expression of the exogenous ΔGPI mutant remained healthy, as previously reported [12]. In contrast, 1.7-fold higher expression of the ΔGPI mutant compared with PrP^C expression levels of wild-type mice resulted in late-onset, spontaneous neurologic illness accompanied by amyloid plaques. In addition, the disease was transmissible. Thus, Tg(PrP,ΔGPI) mice can be categorized into the "PrP^{Sc} type". Because anchorless PrPs are not cleaved, it might be possible that the un-cut PrP tends to spontaneously aggregate (Figure 3B).

In conclusion, there have been at least two major types of transgenic mouse models produced to date. The first type directly produce PrP^{Sc} derived from exogenous mutant PrP transgenes so that the mice suffer from prion disease and the disease can be transmissible to other mice. Thus, endogenous PrP^C expression rather enhances the disease in these mice. The second type has nothing to do with prion disease in terms of PrP^{Sc} and transmissibility. These mice show neurodegenerative phenotypes in the absence of PrP^C. Thus, the introduction of PrP^C rescues the phenotypes of these mice. In these mice, the exogenous artificial PrP mutants are extremely resistant to "α-cleavage", while PrP^C is mainly cut into two fragments, N1 and C1.

How could the divergent phenotypes of these two mouse models be explained by a unified hypothesis? One hypothetical model to explain this dichotomy is illustrated in Figure 3. We propose that when PrP^C is not cleaved at all by deletion or mutation, the PrPs elicit neurotoxicity (ΔF type) or the PrPs spontaneously aggregate to generate PrP^{Sc} (PrP^{Sc} type), depending upon the mutations involved. When PrP^C is partially cleaved by mutation, three forms should exist simultaneously: un-cut PrP, N1, and C1 (i.e. ΔpHC). Thus, un-cut PrP may induce mild neurotoxicity that cannot be observed under normal conditions. We further propose that neurotoxicity by the un-cut PrP is evident in the presence of other mutant PrPs, since double transgenic mice with ΔpHC (non-toxic) and ΔCD (toxic) enhanced the neurodegenerative phenotypes of ΔCD mice [33]. On the other hand, expression of ΔpHC can partially rescue the neurotoxicity induced by ΔF (toxic). It might be possible that N1 or C1 derived from ΔpHC rescues the neurotoxicity induced by ΔF. Furthermore, we predict that ΔCD expression cannot be rescued by ΔpHC because ΔCD possesses a long N-terminal domain, which might block the functions of N1, C1, or full-length PrP^C.

4. Doppel and Shadoo

4.1. Doppel

In order to address the function of PrP^C, several PrP^C-deficient mouse lines were developed by many laboratories, including Zrch I, Rcm0, Npu/Edbg, Ngsk, Rikn, and Zrch II [8,53,54,55,56,57,58]. Zrch I and Npu mice developed and reproduced normally, while Rcm0, Ngsk, Rikn and Zrch II mice exhibited severe ataxia and Purkinje cell loss in later life, as well as demyelination of peripheral nerves. Spontaneous cerebellar neurodegeneration seen in these mice led to the discovery of doppel

(Dpl), a protein structurally related to PrP^C [54]. The mouse gene *Prnd* (Dpl) is located 16 kilobases downstream of *Prnp* (PrP^C) and encodes a 179-amino acid protein. Dpl is absent from the brain and is highly expressed in testis under normal physiological condition. In accordance with its expression, Dpl-deficient mice showed a male infertility phenotype [59,60]. The deletions designed in Rcm0, Ngsk, and Zrch II mutants resulted in the generation of high levels of Dpl expression in the brain by an unusual mechanism involving exon-skipping and intergenic splicing. Interestingly, the introduction of a single wild-type PrP allele fully rescued the deleterious phenotype caused by the ectopic expression of Dpl in the brain [58], indicating that Dpl has a functional overlap with PrP^C. Dpl shows structural homology with the C-terminal globular domain of PrP^C, but lacks the N-terminal octarepeats and the hydrophobic core (HC) [61], suggesting that Dpl is not cleaved at the same position as PrP^C. As expected, murine Dpl appears as a single band of ~17 KDa after PNGase F treatment [62], although human Dpl from testis additionally possesses an O-bound oligosaccharide [63,64]. Interestingly, Aguzzi's group produced mice expressing fusion proteins in which the CC and HC regions of PrP^C (residues 90-133) (cleavable) were inserted into the middle of Dpl (between 65 and 66), and the chimeric CD-Dpl proteins are cleaved into two fragments [65]. These mice did not show the neurodegeneration phenotype seen by ectopic Dpl expression in several PrP^C-deficient mice (i.e. Rcm0, Ngsk, Rikn and Zrch II), although these fusion proteins are artificial so their functional integrity is unclear. However, the CD-Dpl expression in either PrP-ΔF (Δ32-134) or PrP-ΔCD (Δ94-134) mice partially rescued the severe neurodegeneration caused by expression ΔF or ΔCD [65], indicating that the CD-Dpl proteins are at least partially functional. The same group also produced mice expressing the PrP-Dpl proteins in which the N-terminal domain of PrP (1-133) is fused to the C-terminal domain of Dpl (66-179). These PrP-Dpl fusion proteins are not toxic in the PrP^C-deficient mice, even in the absence of C1-like molecules as shown by western blot analysis, implying no correlation between toxicity and lack of C1. It is possible that the PrP-Dpl fusion did not cause neurodegeneration because it is non-functional, but this does not seem to be the case as it partially, albeit weakly, rescued the ΔF and ΔCD phenotypes [65]. One possible explanation for the rescue of the ΔF and ΔCD phenotypes by PrP-Dpl is that the fusion protein still retains capacity to generate a residual level of C1 fragment that is below detection level by normal western blot analysis, since weak C1 expression was shown to partially rescue theΔF phenotypes in ΔpHC and ΔF double transgenic mice [33]. In addition to the α-cleavage phenomenon, one could conclude from these studies that the N-terminal 9 amino acids (23-31) and residues 90 to 133, which include the CC, α-cleavage site, and HC domains, but not the octarepeats, are sufficient to rescue the toxicity caused byΔF, ΔCD, and Dpl.

4.2. Shadoo

Database analysis by Gready's group identified a novel gene named SPRN (Shadoo), shadow of prion protein (Sho) [66]. Dpl resembles the α-helical C-terminal half of PrP^C, while Sho is reminiscent of the flexible N-terminal half, although Sho is extracellular and GPI-anchored. The SPRN gene was also found in zebrafish, Fugu, rat, mouse, cow and human [67] and was shown to have an overlapping pattern of expression in the central nervous system, similar to that of PrP^C [68,69]. In particular, the similarity between PrP and Sho is striking within the putative HC region, suggesting that Sho, but not Dpl, undergoes α-cleavage.

As anticipated, Westaway's group have shown that Sho is indeed cleaved at the putative α-cleavage site [68], although the sequence of the Sho-C1 has not yet been determined to identify the exact cleavage site. If Sho is cleaved to generate a "C1-like molecule", it would be interesting to test whether expression of an "un-cleavable Sho" mutant can cause neuronal cell death like expression of the ΔF PrP mutant in the absence of wild-type Sho or PrP^C. Obviously, Sho does not seem to compensate for the lack of PrP^C, since ectopic expression of Dpl causes neuronal dysfunction in the absence of PrP^C, but Sho should still be present, although Sho expression was not tested in the Rcm0, Ngsk, and Zrch II mice.

Collectively, the mammalian prion protein family consists of three members: ubiquitously expressed PrP^C, testis-localized (although not exclusively) Dpl, and neuron-localized (although not exclusively) Sho. Since PrP^C null mice develop normally and are completely resistant to prion infections, another molecule has been suggested to compensate the function of PrP^C. Sho is a good candidate for such compensation, but this does not seem to be the case. A recent publication by Westaway's group has reported the analysis of mice deficient in both Sho and PrP^C, and these mice were fertile and viable for up to 690 days of age [70]. These results directly contradict the hypothetical redundancy between PrP^C and Sho. In sharp contrast, however, Laude and Vilotte's group [69] previously reported that *Sprn* knockdown in PrP^C deficient embryos using a lentiviral system resulted in embryonic lethality. The explanation for this discrepancy needs further investigation, but may be due to the different experimental strategies used.

5. The cellular compartment in which α-cleavage occurs

Prion infection into animals and humans results in the conversion of PrP^C into scrapie prion protein (PrP^{Sc}) [71]. The conversion of PrP^C to PrP^{Sc} is clearly a posttranslational process [14]. One study proposed that the conversion process occurs intracellularly between the Golgi and the cell surface, since PrP^{Sc} was not released by phosphatidylinositol-specific phospholipase C (PIPLC) in the scrapie-infected murine neuroblastoma cell line, N2a [72]. In support of this mechanism, PrP^{Sc} was shown to be co-localized with the Golgi marker in murine N2a and Syrian hamster-derived HaB cells [73]. In contrast, Caughey's group reported in 1991 that the precursor of PrP^{Sc} was eliminated from intact cells by treatments with PIPLC and trypsin, suggesting that the conversion of PrP^C to PrP^{Sc} occurs after the precursor reaches the cell surface in rat PC12 cells [74]. Collectively, it seems that the conversion occurs between the cell surface and the Golgi compartment and further study is warranted.

N2a is the most frequently and broadly used cell model for *in vitro* study of prion infection, although it is derived from murine cells, thereby limiting its use [75]. Many laboratories have reproducibly demonstrated prion infection into N2a cells, presumably due to a unique cellular attribute. The mechanism of PrP^C endocytosis has been studied frequently using N2a cells [76]. In 1993, Harris's group used N2a cells to demonstrate that PrP^C cycles between the cell surface and an endocytic compartment [77]. In 2004, Kaneko's group expressed fusion proteins of PrP with either EGFP or DsRed in N2a cells to show that both endogenous PrP^C and the fluorescent fusion proteins are localized at both the cell surface and the perinuclear compartment (PNC), part of which seems to be the Golgi [78]. Hachiya's report also indicated that C1 is predominantly seen at the cell surface, while full-length PrP^C resides inside the cell, suggesting that full-length PrP^C can be recycled. The