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Recent Advances in Clarifying Prion Protein Functions Using Knockout Mice and Derived Cell Lines

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Abstract: Considerable information on the functions of prion protein (PrP) has been accumulated. One experimental approach is the use of PrP gene-knockout mice and derived cell lines. This approach has contributed to elucidating the functions of cellular prion protein (PrP^C), such as its anti-oxidative and anti-apoptotic roles. This review will introduce the recent advances in prion biology made possible by the availability of these tools.

Keywords: Prion protein, PrP-knockout mice, PrP-deficient cells, apoptosis, oxidative stress.

INTRODUCTION

Transmissible encephalopathies (TSE), also called prion diseases, such as Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), kuru, and fatal familial insomnia (FFI) in humans, and scrapie, bovine spongiform encephalopathy (BSE), and chronic wasting disease (CWD) in animals, are fatal neurodegenerative disorders with pathologies including neuronal cell loss, vacuolation, astrocytosis, and amyloid plaques [1]. The conversion of cellular prion protein (PrP^C) into abnormal isoforms of prion protein (PrP^{Sc}) constitutes a fundamental feature of prion diseases [1]. Prion protein (PrP) contains disulfide links (S–S), Asn-linked glycosylation sites (CHO), a signal peptide sequence (SP), an octapeptide repeat region (OR), a hydrophobic region (HR), and a glycosylphosphatidylinositol (GPI) anchor attached to its C terminus [1]. One approach to studying TSE is the use of PrP gene (*Prnp*)-knockout mice and the derived cell lines, which have contributed to elucidating the functions of PrP^C. Analysis of the phenotype of $Prnp^{-/-}$ mice has contributed to the study of PrP functions [2]. Furthermore, the participation of PrP^C in anti-apoptotic and anti-oxidative functions has been demonstrated using *Prnp*-deficient immortalized hippocampal neuronal cells [3, 4]. From further studies on PrP using $Prnp^{-/-}$ mice and the derived cells, several regions of PrP, such as the OR and HR, have been revealed to be key components for such anti-apoptotic and anti-oxidative functions [5, 6]. The present review will introduce the recent advances in prion biology made possible by the availability of $Prnp^{-/-}$ mice and the derived cell lines.

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STRUCTURE AND FUNDAMENTAL CHARACTERISTICS OF PRP

PrP mRNA is expressed in a wide variety of tissues. Northern blot analysis has shown that the levels of PrP mRNA vary among tissues, with the highest levels found in the brain and placenta, the moderate levels in the testis, heart, and lung, and lower levels found in the spleen and kidney [7, 8]. However, reverse-transcription polymerase chain reaction (RT-PCR) analysis, which has higher sensitivity than Northern blot analysis, detected PrP mRNA in all tissues including the liver, in which PrP mRNA was not detected by Northern blot analysis (Saeki and Onodera, unpublished results). Moudjou *et al*. reported the detailed tissue distribution and quantity of PrP^c in sheep [9]. Generally, 3-5 µg PrP^c/ g tissue is present in brain, whereas the level of PrP^c per g tissue is 100 ng in heart, 100 ng in skeletal muscle, 200 ng in lung, 40 ng in spleen, and 3 ng in liver. Thus, the quantity in those tissues is 20- to 50-fold lower than that in the brain. This suggests that PrP^c plays important roles mainly in the brain, but also in other tissues. *Prnp* is located on chromosome 2 in the mouse, and on chromosome 20 in humans [10]. Human PrP^C contains 253 amino acids [11]. The first 22 amino acids form a signal peptide, which is cleaved after translocation into the endoplasmic reticulum. The last 23 amino acids are removed after the maturation of PrP^C, because a GPI anchor is attached to the serine residue at position 230 [12]. One disulfide bridge and two *N*-linked glycosylation sites are present at positions 179 and 214, and positions 182 and 198, respectively. Therefore, mature PrP^C is anchored on the cell membrane by GPI with zero to two *N*-glycosylations and one disulfide bridge, and is detected as a 33-35 kDa protein by Western blot analysis. In some cases, PrP seems to be hydroxylated [13] or phosphorylated [14, 15]. However, it is unclear whether PrP is subject to these modifications and whether such modifications may modulate PrP^C function. Several regions of PrP have been revealed to be key

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components. Five octapeptide [P(Q/H)GGG(G/-)WGQ] repeats, highly conserved among mammals, can selectively bind copper [16-20]. Zinc, manganese and nickel also bind to PrP, but with lower affinity than does copper [21-23]. The binding of metals occurs *via* histidine residues of the OR. The binding of copper to PrP is thought to be indispensable for the superoxide dismutase (SOD) activity of PrP [24, 25]. In contrast, as the numbers of octapeptide repeats and histidine residues vary among species, the importance of the OR for the normal function of cellular PrP appears to depend on the species [26]. Interestingly, the neuropathological changes observed in prion diseases are similar to those induced in mice by cuprizone, a Cu^{2+}-chelating reagent [27, 28]. Moreover, amino acid deletions and insertions in the OR have been shown to be related to human prion diseases [29-50]. Therefore, the OR and copper appear to play an important role in not only PrP functions but also the pathogenesis of prion diseases.

The HR, which contains the highly conserved sequence motif AGAAAAGA [51, 52], has been shown to be critical for PrP topology in the endoplasmic reticulum membrane [53], normal metabolic cleavage [54] and stress-inducible protein 1 (STI1) binding [55]. A synthetic peptide consisting of residues 106-126 of human PrP activates microglial cells, causing an increase in their oxygen radical production and inducing a neurotoxicity [56, 57]. Moreover, amino acid substitutions of the HR have been shown to be associated with human prion diseases [58-69]. Furthermore, sequence comparison of the amino acid residues between PrP and a PrP homologue, the PrP-like protein Doppel (Dpl), revealed that the OR and HR are apparently absent from Dpl [70, 71], suggesting that this difference may be the major reason for the significant functional divergence between PrP and Dpl.

The identification of PrP-binding proteins will help us to provide insights into the physiological functions of PrP. Many PrP-interacting proteins have been identified by

Table 1. Proteins Interacting with PrP

Protein	Method	References
STI1	Complementary hydropathy	[86]
Tubulin	Cross linking by bis(sulfosuccinimidyl)-suberate	[84]
N-CAM	Cross linking by formaldehyde	[85]
Dystroglycan	Detergent-dependent immunoprecipitation	[81]
nNOS	Detergent-dependent immunoprecipitation	[81]
Grp94	Immunoprecipitation	[33]
Protein disulphide isomerase	Immunoprecipitation	[33]
Calnexin	Immunoprecipitation	[33]
Calreticulin	Immunoprecipitation	[33]
ZAP-70	Immunoprecipitation	[83]
Nrf2	Interaction with PrP23-231-alkaline phosphatase probe	[73]
Aplp1	Interaction with PrP23-231-alkaline phosphatase probe	[73]
F-box protein-6	Interaction with PrP23-231-alkaline phosphatase probe	[73]
NFB42	Interaction with PrP23-231-alkaline phosphatase probe	[73]
PSD-95/SAP-90 associated protein	Interaction with PrP23-231-alkaline phosphatase probe	[73]
Protein tyrosine phosphatase, non-receptor type-21	Interaction with PrP23-231-alkaline phosphatase probe	[73]
Predicted protein KIAA0443	Interaction with PrP23-231-alkaline phosphatase probe	[73]
GFAP	Interaction with radioisotope-labeled PrP27-30	[72]
Hsp60 of Brucella abortus	Pull-down assay	[87]
Bcl-2	Yeast two-hybrid system	[78]
Hsp60	Yeast two-hybrid system	[77]
LRP	Yeast two-hybrid system	[76]
Pint1	Yeast two-hybrid system + immunoprecipitation	[79]
Synapsin I	Yeast two-hybrid system + immunoprecipitation	[79]
Neuronal phosphoprotein Grb2	Yeast two-hybrid system + immunoprecipitation	[79]
Neurotrophin receptor interacting MAGE homolog	Yeast two-hybrid system + *in vitro* binding assay + immunoprecipitation	[80]

Aplp1: amyloid precursor protein-like protein 1; GFAP : glial fibrillary acidic protein; Grb2: growth factor receptor-bound protein; Grp94: glucose regulated protein; Hsp60: heat shock protein 60kDa; LRP: laminin receptor protein; MAGE: melanoma-associated antigens; N-CAM: neural adhesion molecule; NFB42: neural F-box protein 42kDa; nNOS: neuronal isoform of nitric oxide synthase; Nrf2: NF-E2 related factor 2; Pint1: prion interactor 1; PrP: prion protein; PSD-95: postsynapic density 95kDa; STI1: stress-inducible protein 1; ZAP-70: zeta-associated protein 70.

several methods (Table **1**). Firstly, interaction between glial fibrillary acidic protein (GFAP) and radioisotope-labeled PrP27-30 was discovered [72]. A similar strategy was carried out using alkaline phosphatase-labelled PrP23-231 and revealed that PrP interacts with NF-E2 related factor 2 (Nrf2), amyloid precursor protein 1 (Aplp1), F-box protein-6, neural F-box protein 42 kDa (NFB42), postsynapic density 95 kDa (PSD-95)/ SAP-90 associated protein, protein tyrosine phosphatase non-receptor type-21, and predicted protein KIAA0443 [73]. In addition, laminin [74], 37-kDa/67-kDa laminin receptor [75], 37-kDa laminin receptor precursor [76], heat shock protein 60 kDa (Hsp60) [77], and Bcl-2 [78] were also shown to bind to PrP by experiments in the yeast two-hybrid system. Prion interactor 1 (Pint1) [79], synapsinIb [79], growth factor receptor-bound protein 2 (Grb2) [79], and neurotrophin receptor interacting MAGE (melanoma-associated antigens) homolog (NRAGE) [80] were identified using yeast two-hybrid system, and the PrP-binding activities were further confirmed by co-immunoprecipitation. PrP was co-immunoprecipitated with dystroglycan [81], the neuronal isoform of nitric oxide synthase (nNOS) [81], glucose regulated protein 94 (Grp94) [82], protein disulphide isomerase [82], calnexin [82], and calreticulin [82] and zeta-associated protein-70 (ZAP-70) [83]. Interaction with tubulin [84] and neural adhesion molecule (N-CAM) [85] have also been demonstrated by the cross-linking method. Complementary hydropathy and pull-down assays were able to show the interaction of PrP with stress inducible protein 1 (STI1) [86] and Hsp60 of *brucella abortus* [87], respectively. Several representatives of the proteins described above that interacted with PrP are listed in Table **1**. Most of the interacting proteins are important factors involved in survival, proliferation, differentiation, development and stress response. Interestingly, cross-linking of cell-surface PrP stimulated caveolin-1-dependent interaction with Fyn tyrosine kinase, which is involved in the transduction of many cell signals, such as signals regulating cellular proliferation and survival, in differentiated 1C11 neuronal cells [88]. Therefore, PrP may regulate various types of cell signals *via* Fyn activation. Several recent reports support this idea [83, 89-91]. PrP may act as a conductor to maintain cell integrity and may function especially under emergent situations such as oxidative and apoptotic conditions. However, the biological significance of most of the interactions with PrP remains undefined, and the relationships among the interactions remain obscure. Identification of the interacting proteins has helped us to understand the variations of the molecular participants in the PrP-dependent signal pathway, but can not elucidate the functional significance of the interaction. The results of knockout analysis can aid in elucidating the relationships and interactions.

STUDY OF PRP BY GENE TARGETING METHOD

To study mammalian gene function, gene-targeting and transgenic mouse models are often generated. This strategy has also facilitated the *in vivo* study of PrP function. Six lines of *Prnp* knockout mice, designated Zrch I [92], Zrch II [93], Npu [94], Ngsk [71], Rcm0 [70], and Rikn [95], have been generated (Fig. **1**). All lines of PrP-knockout mice are resistant to infection with prion agents [96-99] and the

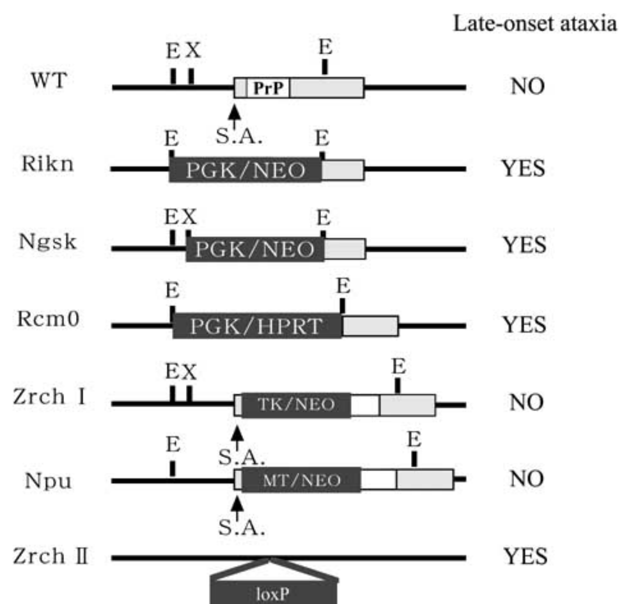

Fig. (1). Knockout constructs of six lines of prion protein gene-knockout mice.

Structures of the constructs used to produce six lines of prion protein gene (*Prnp*)-knockout mice (Rikn, Ngsk, Rcm0, ZrchI, Npu, and ZrchII) are shown. Various *Prnp*-knockout constructs were used for homologous recombination. The structure of the wild-type (WT) *Prnp* exon3 and PrP coding region (open box) is shown at the top. The selection markers are indicated by black boxes. The presence and absence of the exon 3 splicing acceptor (S.A.) are correlated with the development of late-onset ataxia. The selection markers were PGK: mouse phosphoglycerate kinase promoter; NEO: neomycin phosphotransferase; HPRT: mouse hypoxanthine phosphoribosyltransferase; TK: human herpes simplex virus type 1 thymidine kinase promoter; MT: mouse metallothionein promoter; loxP: a 34-bp recombination site from phage P1. The restriction enzyme sites were E: *Eco*RI; X: *Xba*I.

derived neurons lacking PrP^{C} expression cannot be killed by the toxicity of PrP^{Sc} [97, 100] or PrP(106-126) [56, 100-107]. Therefore, the expression of PrP^{C} is essential for the induction of prion diseases, and the conversion of PrP^{C} to PrP^{Sc} is closely associated with the main features of prion diseases. The use of PrP-knockout and PrP-transgenic mice for studies of the pathogenesis of prion diseases is described in detail elsewhere [2]. On the other hand, although some lines (ZrchI and Npu) do not display any anatomical or developmental defects [92], other lines of PrP-null mice (ZrchII, Ngsk, Rcm0, and Rikn) exhibit late-onset ataxia [70, 71, 95]. In the process of attempting to resolve the discrepancies of the phenotypes of PrP-knockout mice, Dpl, which is encoded by *Prnd* located 16 kb downstream of murine *Prnp* was recently discovered [70, 108]. Some lines of PrP-null mice (ZrchII, Ngsk, Rcm0, and Rikn) showed ectopic expression of Dpl, which was completely correlated with the development of late-onset ataxia. Therefore, the ataxic phenotype of some lines of PrP-knockout mice is probably due to ectopic expression of Dpl (derived from *Prnp*/*Prnd* chimeric mRNAs) as a result of the destruction of the splicing acceptor of *Prnp* exon 3 [70, 93, 108]. The ataxic phenotype observed in some murine $Prnp^{-/-}$ lines can be neutralized by crossing the mice with those

Table 2. Abnormality of PrP-Deficient Mice Ectopically Expressing Doppel (Ngsk, Rcm0, ZrchII and Rikn) or not Ectopically Expressing Doppel (ZrchI and Npu)

Abnormality	Strain observed for abnormality	Supporting reports	Opposing reports
Abnormal localization and activity of nNOS	ZrchI	[136]	
Abnormal microglial activation	Ngsk	[193]	
Abnormal myelination	Ngsk, ZrchI	[109]	
Abnormal uptake of glutamate	ZrchI	[146]	
Abnormality of mitochondria	Npu	[147]	[200]
Altered calcium homeostasis	ZrchI	[144, 145]	
Altered circadian rhythms	ZrchI	[110-113]	
Altered cytokine production and proliferation	Rikn, Npu	[149, 150]	
Altered locomotion	ZrchI	[114-116]	
Cellular copper content	Rikn, ZrchI, Npu	[17, 129, 143]	[164]
Decrease of antioxidative enzymes	ZrchI	[138, 139]	[164]
Decrease of SOD activity	Rikn, ZrchI, Npu	[4, 138-141]	[5, 163, 164]
Deficits in spatial learning and memory	Ngsk, ZrchII, ZrchI, Npu	[121, 194, 195]	
Electrophysiological abnormality	ZrchI, Npu	[120-129]	[130, 131]
Higher rate of phagocytosis of apoptotic cells	ZrchI	[148]	
Increasd sensitivity to apoptotic stimuli	Npu, ZrchI	[151, 152]	
Increased oxidative stress markers	Npu	[137]	
Increased proteasome subunit expression and associated enzymatic activities	Npu	[137]	
Increased sensitivity to brain injury	Rikn, Npu	[118, 119]	
Increased sensitivity to copper toxicity	Rikn, ZrchI	[132-135]	
Increased sensitivity to seizure	ZrchI	[117]	
Increased sentitivity to oxidative stress	Rikn, ZrchI, Npu	[56, 132, 135, 138, 140, 152, 196]	
Reduced mitogen response	Npu	[197]	
Reduced PI 3-kinase activity in brain	ZrchI	[142]	
Reduced transfer of copper between neurons and astrocytes	ZrchI	[198]	
Resistance to neurotoxic prion peptide PrP106-126	Ngsk, ZrchI	[56, 100, 101, 103-107, 199]	[102, 201, 202]
Resistance to scrapie infection	Ngsk, Rikn, ZrchI, Npu	[71, 95-99]	
Resistance to PrP^{Sc} neurotoxicity	ZrchI	[97, 100]	

nNOS: neuronal isoform of nitric oxide synthase; PI3: phosphatidylinositol 3; PrP: prion protein ; PrP^{Sc}: abnormal isoform of prion protein; SOD: superoxide dismutase.

overexpressing wild-type mouse PrP [93, 109]. This suggests that PrP has neuroprotective roles and functionally interacts with Dpl, and that the absence of PrP is essential for Dpl neurotoxicity. Interestingly, the neuroprotective functions of PrP require the OR, because the deletion of amino acids 23-88 abrogates the ability of PrP to protect against Dpl toxicity in Ngsk PrP-knockout mice [5]. Furthermore, not only OR but also HR is essential for the neuroprotective functions of PrP in Rikn PrP-deficient cells [6].

Information on the phenotypes of PrP-knockout mice has been accumulated, and include slight abnormalities such as aberrant circadian rhythms [110-113], altered locomotion [114-116], deficits in spatial learning and memory, and high susceptibility to seizure [117] and brain injury [118, 119] in *Prnp*-knockout mice. Electrophysiological abnormality was also reported [120-131], but remains controversial [130-131]. Furthermore, demyelination of the peripheral nervous system in Ngsk and ZrchI PrP-knockout mice is also correlated with PrP expression [109]. High sensitivity to copper toxicity and oxidative stress are observed in primary cerebral and sperm cells from $Prnp^{-/-}$ mice [132-135]. PrP-knockout mice show abnormality of cell signalling molecules such as nNOS [136], oxidative stress markers [137], antioxidative enzymes, including SOD [4, 138-141], proteasome associated molecules [137] and phosphatidyl-inositol 3 (PI3) kinase [142]. Changes in the homeostasis of copper [17, 129, 143], calcium [144, 145], glutamate [146]

were also reported. Abnormality of the mitochondria [147], in which superoxide is produced under oxidative conditions, in the hippocampus and myocardium in PrP-knockout mice may be associated with demyelination and susceptibility to oxidative stress and increased oxidative markers.

Prnp$^{-/-}$ cells also show several abnormalities not only *in vivo* but also *in vitro*. *Prnp*$^{-/-}$ macrophages and microglia show abnormal activation and increased phagocytosis of apoptotic cells [148]. PrP-deficient lymphocytes show reduction of mitogen response, cytokine production and proliferation [149, 150]. Primary cultured *Prnp*$^{-/-}$ neurons demonstrate increased sensitivity to apoptotic insults such as staurosporine, glutamate, AraC, kainic acid and superoxide anion [151, 152]. Representative abnormalities in PrP-knockout mice are listed in Table **2**. Although PrP plays roles in cell survival and is expressed abundantly in the brain, why do PrP-knockout mice not show any striking defects? One possibility is that lack of PrP function may be compensated by other proteins. Another possibility is that PrP may function only in urgent states such as oxidative conditions. It should be noted that SOD1-deficient mice, which lack Cu/Zn SOD expression, can survive to the adult stage [153]. Interestingly, the SOD1-deficient mice show enhanced susceptibility to central nervous system damage caused by axonal injury [154], ischemia [155] and exposure to glutamate analogs [156]. The vulnerability to oxidative stress and the normal phenotype under normal conditions of SOD1-deficeint mice closely resemble those of PrP-knockout mice. These studies support an important role of PrP in antioxidative function and suggest that PrP may regulate Cu/Zn SOD activity. Moreover, a considerable body of evidence shows that PrP binds copper [16-20] and copper-bound PrP itself exhibits SOD activity [24-25]. However, the SOD activity of PrP itself was not reproduced in other studies [157]. On the other hand, PrP-deficient cells are certainly susceptible to oxidative stress and showed decreased SOD activity [3, 4, 6, 132-135, 137-142, 158-162]. Currently, one of the most controversial findings is that PrP was found to appear to be related to Cu/Zn SOD *in vivo* [141], whereas other studies found that PrP did not appear to participate in any modulation of Cu/Zn SOD [5, 163, 164]. The solution to this discrepancy would be of great interest. To resolve the discrepancy, further studies of the mechanisms by which the absence of PrP causes susceptibility to oxidative stress will be important. Furthermore, as PrP appears to play roles under oxidative conditions, further analysis of the relationship between PrP and SOD *in vivo* under oxidative conditions will be necessary.

ESTABLISHMENT OF PRP GENE-DEFICIENT NEURONAL CELL LINES AND PREVENTION OF APOPTOSIS BY PRP

For the detailed analysis of gene function, cell lines are frequently used. This is because primary cultures can be maintained only for a limited period. Moreover, large, pure populations of specific cell types would be very powerful tools. If detailed analysis is necessary, the use of cell lines is the most appropriate approach. As described above, PrP^{C} is highly expresses in neurons; however, primary neuron cultures can be cultured for only several weeks. Furthermore, the effective transfection of genes, which is required for detailed functional analysis, is difficult, especially when using primary culture neurons. If gene transfer to primary cultured neurons is necessary, viral agents would be required for the introduction of inducible genes. As PrP-knockout mice do not show any severe abnormality, it is clear that detailed analysis is needed for the elucidation of PrP functions. Therefore, several cell lines from primary culture neurons have been established to study PrP functions. Neural cell lines are usually established by several strategies, which include cultivation of primary tumor tissue, fusion of primary cells with tumor cells, carcinogen-induced transformation, spontaneous transformation, and gene transfer of oncogenes [165]. Next, we will introduce the recent efforts by which *Prnp*$^{-/-}$ cell lines have been available to study PrP functions [3].

To gain further insights into PrP functions, two distinct systems of cell lines were established by the gene transfer of oncogenes. These cell lines were from Rikn PrP-knockout mice lacking approximately 800 bp of intron 2, as well as the entire ORF, derived from the hippocampal area of the brains of *Prnp*$^{-/-}$ mice and the other *Prnp*$^{+/+}$ mice. Detailed construction of Rikn PrP-knockout mice was described elsewhere [95]. As illustrated in Fig. **2**, primary cultures of hippocampus cells of 14-day-old *Prnp*$^{-/-}$ and *Prnp*$^{+/+}$ mouse embryos were used as a target for infection with a recombinant retrovirus vector containing the simian virus 40 (SV40) T-antigen gene [166]. Under a dissection microscope, hippocampi were aseptically dissected from 5-6 *Prnp*$^{-/-}$ and *Prnp*$^{+/+}$ mouse embryos at embryonic day 14. After dissection, tissues were triturated until small clumps of cells were obtained and then dissociated by incubation at 37°C in Dulbecco's modified Eagle's medium (DMEM, Gibco, Bethesda, MD) with 0.03% trypsin and 0.4 µg/ml DNase I. At the end of the treatment, trypsin activity was stopped by adding 10% volume of DMEM supplemented with 10% fetal calf serum (FCS, Takara, Japan). Approximately 2 X 10^{5} cells from a cell suspension in DMEM:F12 medium supplemented with 5 µg/ml insulin, 50 µg/ml transferrin, 20 nM progesterone, 100 µg putrescence, 20 nM hydrocortisone, 30 nM sodium selenite and 7 ng/ml alpha-tocopherol were seeded on 35-mm dishes coated with 10 mg/ml poly-lysine and incubated at 37°C in 5% CO_2. Twenty-four hours after plating, cells were infected with a recombinant retrovirus carrying SV40 large T antigen in 8 µg/ml polybrene for 4 h [166]. The medium was replaced with fresh medium containing 5% FCS. Non-infected control cells were incubated only with polybrene in the same conditions as above. Nine days after infection, cells were passaged by mechanical dislodging and seeded at 3% confluence in supplemented DMEM:F-12 as described above. The non-infected cells did not survive beyond the first cell passage. After 48 h the cells were subjected to selection in DMEM with 250 µg/ml G418 (Genetecin, Gibco). The resistant cells which formed cell clusters were observed after 6 days and they were isolated, and cloned by a limiting dilution method. The obtained cell lines were expanded in DMEM plus 10% FCS. Three *Prnp*$^{-/-}$ cell lines, HpL2-1, HpL-3-2 and HpL3-4, and 3 *Prnp*$^{+/+}$ cell lines HW8, HW9, HW19, were thereby obtained [3]. The genotypes of the established cell lines were confirmed by PCR analysis with two pairs of primers generating DNA

fragments of the neomycin resistance gene to the 3' noncoding region of the exon 3 of the *Prnp* and that of the open reading frame of *Prnp*. The absence and presence of PrP on the cell surface of *Prnp*$^{-/-}$ and *Prnp*$^{+/+}$ cell lines, respectively, were confirmed by the immunoreactivity of a rabbit anti-PrP polyclonal antibody conjugated with rhodamine [167]. The positive staining appeared most evident at the tip of extended neurites in *Prnp*$^{+/+}$ cell lines.

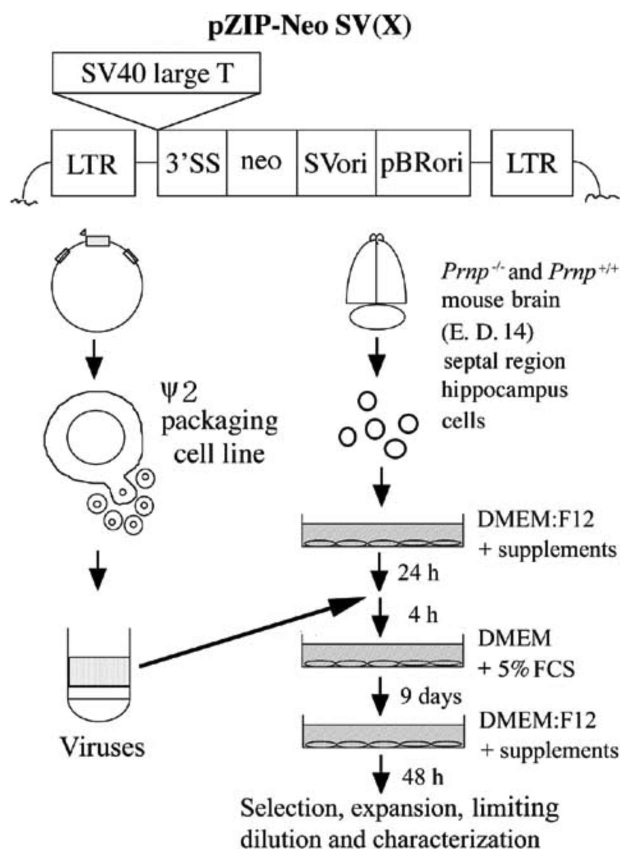

Fig. (2). Establishment of *Prnp*$^{-/-}$ and *Prnp*$^{+/+}$ cell lines.

The retrovirus vector used for cell transformation was derived from pZIP-Neo SV(X) [166]. Simian virus 40 (SV40) large T antigen was transcribed from the long terminal repeat (LTR), while the *neo* gene was translated from a spliced transcript. The virus vector was used for transfection of Ψ2 packaging cells, which are stable cells containing all of the structural retroviral protein genes (*gag*, *pol*, and *env*). The retrovirus particles produced from the transfected cells were used. The hippocampal primary cultures derived from *Prnp*$^{-/-}$ and *Prnp*$^{+/+}$ mice were infected with the above retrovirus, then incubated, selected, expanded, and finally subjected to limiting dilution to obtain cloned cell lines. E.D.: embryonic day; neo: neomycin phosphotransferase; pBRori: pBR origin of replication; SV ori: SV40 origin of replication; 3' ss: 3' splice site.

The *Prnp*$^{+/+}$ and *Prnp*$^{-/-}$ cells were exposed to dibutyl cyclic AMP (dcAMP) at 0.4 – 6 mM, retinoic acid (RA) at 0.1-10 µM, and phorbol 12-myristate 13-acetate (PMA) at 160-1600 nM, for up to 10 days of culture. These compounds all induced morphological changes, i.e., elongation of neurite-like processes, in both *Prnp*$^{+/+}$ and *Prnp*$^{-/-}$ cells. Among the tested compounds, the morphological change was best observed by stimulating the *Prnp*$^{+/+}$ and *Prnp*$^{-/-}$ cells in medium with dcAMP or PMA without FCS (Fig. **3A**,**B**). To further characterize the cell types of the cell lines, RT-PCR was applied to monitor the expression of neurofilament genes. The RT-PCR of RNA samples from cell lines, after treatment with PMA, resulted in the amplification of the predicted size products corresponding to neurofilament (NF)-68K and NF-200K genes. The NF-68K gene was transcribed in all 3 *Prnp*$^{+/+}$ and 3 *Prnp*$^{-/-}$ cell lines with or without PMA treatment. After treatment with PMA, the NF-200K gene was transcribed in all 3 *Prnp*$^{+/+}$ and in only one of the *Prnp*$^{-/-}$ cell lines (HpL2-1). RT-PCR without reverse transcriptase did not show any amplified product. The transcription of GFAP, a glial cell marker, was not detected in *Prnp*$^{-/-}$ or *Prnp*$^{+/+}$ cells. Taken together, the above results suggest that all 6 cell lines used belong to the neuronal precursor cell lineage, although they are variable in their developmental stages.

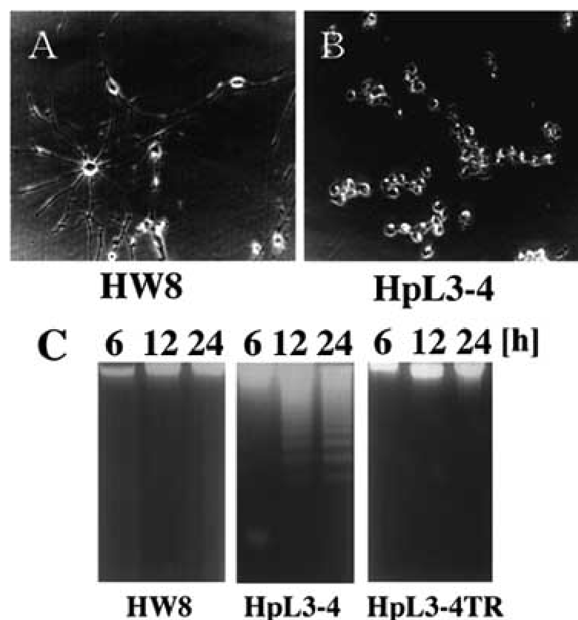

Fig. (3). Neuroprotective role of PrP elucidated by using PrP-deficient neuronal cell lines.

(A-B) Morphological appearance of HW8 *Prnp*$^{+/+}$ cells (A) and HpL3-4 *Prnp*$^{-/-}$ cells (B) cultivated in 1 mM dcAMP-containing, serum-free medium for 1 h. Neurite-like structures were observed in HW8 cells, whereas HpL3-4 cells showed shorter neurite-like extensions. (C) HW8 (*Prnp*$^{+/+}$), HpL3-4 (*Prnp*$^{-/-}$), and HpL3-4TR (*Prnp*$^{-/-}$ cells transfected with PrP-expression vector) cells were subjected to serum deprivation for the indicated times. The fragmented DNA of the cells was analyzed on agarose gels. HpL3-4 cells exhibited fragmented DNA to a higher extent than HW8 and HpL3-4TR cells, suggesting that PrP expression prevents the apoptosis of *Prnp*$^{-/-}$ cells. Similar results were obtained using other PrP-deficient cell lines. Modified from Fig. **1** in Kuwahara *et al*. [3] with permission from Nature Publishing Group.

Next, the characteristics of *Prnp*$^{-/-}$ cells were compared to those of *Prnp*$^{+/+}$ cells to investigate the differences of the characteristics due to the absence of PrP. The most remarkable feature of *Prnp*$^{-/-}$ and compared to *Prnp*$^{+/+}$ cells was observed when the serum was removed from the culture

medium. Withdrawal of serum from the medium caused comparable morphological changes within 1 h in all cell lines whether or not they were treated with dcAMP (Fig. **3A**,**B**). Within 4 days in serum-free culture, *Prnp*$^{-/-}$ cells died. The process started by the rounding up of the cells, cytoplasmic condensation and neurite retraction. In contrast to the 3 *Prnp*$^{-/-}$ cell lines, all *Prnp*$^{+/+}$ cell lines maintained structural integrity under the same culture conditions. DNA fragmentation, a characteristic of apoptosis, was firstly observed at 12 h and peaked at 24 h after serum deprivation in all *Prnp*$^{-/-}$ cells, whether or not they were treated with PMA or dcAMP, whereas it was not detected in any of the *Prnp*$^{+/+}$ cells after serum deprivation (Fig. **3C**). Cultured cells in serum-free medium for 6, 12 or 24 h were ethanol-fixed and stained with propidium iodide before analysis by flow cytometry. A significant peak in the fluorescence low-intensity area (hypodiploid cells), which corresponds to the fraction of apoptotic cells, was detected only in the samples from *Prnp*$^{-/-}$ cells at 6, 12 and 24 h after serum withdrawal (data not shown). To further strengthen the idea that the abnormality observed in *Prnp*$^{-/-}$ cells was due to the absence of PrP, the *Prnp*$^{-/-}$ cell lines (HpL2-1 and HpL3-4) were transfected with PrP expression vector pMAM2-MPR-BSD and the empty vector pMAM2-BSD as a control [3].The pMAM2-MPR-BSD vector carried the ORF of *Prnp* and blasticidin-S-deaminase genes with a SV40 promoter [3]. Resulting cell lines, designated as HpL2-1TR and HpL3-4TR, transfected with pMAM2-MPR-BSD and selected in blasticidin-S (8 µg/ml), were obtained. The HpL2-1TR and HpL3-4TR cells survived under serum-free conditions without any sign of apoptosis (Fig. **3C**). In contrast, HpL2-1 and HpL3-4 cells transfected with pMAM2-BSD lacking *Prnp* ORF showed apoptotic features similar to those of HpL2-1 and HpL3-4 cells under serum-free conditions (data not shown). These results suggest that *Prnp*$^{-/-}$ cells are susceptible to serum deprivation and that apoptotic cell death is suppressed by reintroduction of *Prnp*. This suggests that PrP plays a neuroprotective role. Moreover, as serum-free supernatants of *Prnp*$^{+/+}$ cell cultures could not rescue the *Prnp*$^{-/-}$ cells from apoptosis, molecules secreted from *Prnp*$^{+/+}$ cells appear not to be involved in the neuroprotective mechanism of PrP [3]. In addition to the cell death, *Prnp*$^{-/-}$ cell lines show significantly shorter neurite extension in comparison with *Prnp*$^{+/+}$ cell lines after treatment with PMA, suggesting that PrP is involved not only in survival but also in differentiation [3].

Recently, several groups independently confirmed the neuroprotective function of PrP using the above-mentioned PrP-deficient cell lines or other cell lines. Kim *et al*. reproduced our results using HpL3-4 (*Prnp*$^{-/-}$) cells, and further found greater changes of the calcium ion level, transmembrane potential and cytochrome c level in the mitochondria of HpL3-4 cells than in those of *Prnp*-reintroduced HpL3-4 cells after serum deprivation [158]. Mange *et al*. also confirmed that cell viability was increased by the reintroduction of *Prnp* in HpL3-4 cells [159]. Recently, Vassallo *et al*. investigated the signal cascade of apoptosis induced by serum deprivation and 3-morpholinosydnonimine (SIN-1), and showed that recruitment of PI 3-kinase by PrP^{C} contributes to cellular survival under conditions of oxidative stress imposed by SIN-1 or serum deprivation in HpL3-4 cells [142]. The apoptosis cascade and oxidative metabolism were investigated in HpL3-4 cells after serum deprivation [4, 168]. The data revealed the decreased expression of anti-apoptotic proteins Bcl-2 and Bcl-x_L in HpL3-4 cells during the apoptosis induced by serum deprivation and the suppression of the apoptosis by overexpression of Bcl-2 and Bcl-x_L, suggesting that cell death in this model system is apoptotic, and Bcl-2 and Bcl-x_L play important roles in the apoptosis of HpL3-4 cells [168]. Furthermore, a more marked decrease of intracellular copper concentration, greater increases of intracellular superoxide anion and caspase-3/9 activation, and smaller increases of intracellular hydrogen peroxide after serum deprivation was observed in HpL3-4 cells compared to *Prnp*-reintroduced HpL3-4 cells

Table 3. PrP Gene-Deficient Cell Lines and the Characteristics

Name	Method of production of cell lines	Cell type	Mouse origin	Main characteristic compared to PrP-expressing cells	Reference
HpL2-1, HpL3-2, HpL3-4	Retrovirus-mediated method using SV40 large T antigen expressing vector	Neuronal-precursor cells (expessing NF-68K)	Rikn (C57BL)	Susceptible to serum deprivation	[3]
Zpl2-1, Zpl2-4, Zpl3-4	Lipofection of SV40 large T antigen expressing vector	Neuronal cells (expressing NeuN)	ZrchI (ICR)	Higher proliferation rate	[171]
SFK-B, SFK-C	Continuous culture of abdominal skin explants	Skin fibroblast cells	Ngsk (C57BL)	Decreased expression of Ras and Rac related proteins	[172, 173]
F14	Fusion of PrP-knockout cerebellar cells and mouse neuroblastoma cells	Neuronal cells (expressing NeuN, MAP-2 and synaptophysin)	Npu (129/Ola)	Similar distribution to GFP-PrP fusion protein	[174]
NpL2	Retrovirus-mediated method by SV40 large T antigen-expressing vector	Neuronal cells (expressing NF-L, NF-M, NF-H and MAP-2)	ZrchI (C57BL)	Susceptible to serum deprivation	Unpublished results (Nishimura T. Sakudo A. and Onodera T)
GpL1	Retrovirus-mediated method by SV40 large T antigen-expressing vector	Glial cells (expressing GFAP)	ZrchI (C57BL)	Susceptible to serum deprivation	Unpublished results (Nishimura T. Sakudo A. and Onodera T)

GFAP: glial fibrillary acidic protein; GFP: green fluorescent protein; NeuN: nuclear marker for mature neurons; NF: neurofilament; MAP-2: microtubule-associated protein 2; PrP: prion protein; SV40: simian virus 40.

Table 4. Abnormality of PrP Gene-Deficient Cell Lines

Name	Main characteristic compared to PrP expressing cells	Reference
HpL2-1, HpL3-2, HpL3-4	Higher rate of apoptosis by serum deprivation	[3, 142, 158]
HpL3-4	Decrease of intracellular copper concentration after serum deprivation	[143]
HpL3-4	Decrease of cellular SOD activity	[4]
HpL3-4	Higher increase of intracellular superoxide anion after serum deprivation	[4]
HpL3-4	Lower increase of intracellular hydrogen peroxide after serum deprivation	[4]
HpL3-4	Higher increase of caspase-3/9 activation after serum deprivation	[4]
HpL3-4	Higher sensitivity to poliovirus infection	[170]
HpL3-4	Higher coxsackievirus B3 production and apoptosis rate	[169]
HpL3-4	Alteration of Ca^{2+} concentration in mitochondria after serum deprivation	[158]
HpL3-4	Alteration of transmembrane potentials in mitochondria after serum deprivation	[158]
HpL3-4	Alteration of cytochrome c level in mitochondria after serum deprivation	[158]
HpL3-4	Higher rate of apoptosis by SIN-1	[142]
HpL3-4	Reduced PI 3-kinase activity	[142]
HpL3-4	Decrease of cell viability	[159]
HpL3-4	Shorter neurite extension after differentiation	[3]
SFK-B, SFK-C	Decreased expression of Ras and Rac related proteins	[172, 173]
Zpl2-1, Zpl2-4, Zpl3-4	Higher proliferation rate	[171]
F14	Similar distribution to GFP-PrP fusion protein	[174]
NpL2	Susceptible to serum deprivation	Unpublished results (Nishimura T., Sakudo A. and Onodera T)
GpL1	Susceptible to serum deprivation	Unpublished results (Nishimura T., Sakudo A. and Onodera T)

GFP: green fluorecent protein; PI3: phosphatidylinositol 3; PrP: prion protein; SIN-1: 3-morpholinosydnonimine; SOD: superoxide dismutase.

[4]. HpL3-4 cells also showed a decrease of cellular SOD activity compared to HpL3-4 cells expressing PrP [4]. Higher coxsackievirus B3 and poliovirus production in HpL3-4 cells showed that PrP may be involved in not only the inhibition of virus replication but also anti-apoptotic functions against virus-induced apoptosis [169, 170]. Kim *et al*. recently established neuronal cell lines from hippocampal neurons of ZrchI PrP-deficient mice by lipofection of SV40 large T antigen-expressing vector and found higher proliferation of neuronal cell lines from PrP-deficient mice (Zpl) than of those from wild-type mice (ZW) [171]. Zpl cells showed a higher proliferation rate than ZW cells [171]. SFK-B and SFK-C skin fibroblast cell lines derived from continuous cultures of abdominal skin explants of Ngsk PrP-deficient mice showed decreased expression of Ras- and Rac-related proteins compared to wild-type skin fibroblast cell lines [172, 173]. Using fusion of PrP-knockout cerebellar cells and mouse neuroblastoma cells, an F14 neuronal cell line was obtained for use in investigating the effect of PrP mutations on PrP membrane orientation, although the characteristics of PrP-expressing cells and PrP-deficient cells have not been compared [174]. Taken together, the availability of *Prnp*$^{-/-}$ cell lines enabled the performance of broad variety of studies of PrP. Thus far, the study of *Prnp*$^{-/-}$ cell lines has revealed that PrP plays important roles in differentiation, proliferation and cell survival such as anti-apoptosis and anti-oxidative roles *via* regulating a variety of cell signal cascades. The results obtained from the study of *Prnp*$^{-/-}$ cell lines are consistent with the results gained in other studies using primary cultures and other cell lines, which also showed the involvement of PrP in neuroprotection and differentiation [175, 176].

MECHANISMS BY WHICH PRP PREVENTS APOPTOSIS IN PRP-DEFICIENT CELL LINES

Recent studies have provided clear evidence for the neuroprotective role of PrP^C against apoptosis induced by serum deprivation in an immortalized *Prnp*-deficient neuronal cell line, but the mechanisms remain unclear. Accumulating evidence indicates that PrP^C acts as a regulator of SOD activity, which is at least in part involved in the neuroprotective roles of PrP. Cellular prion protein PrP^C contains two domains evolutionarily conserved among mammals; viz., the OR (amino acid residue 51–90) and the HR (amino acid residue 112–145). Therefore, to further investigate the mechanisms by which PrP^C prevents apoptosis, the apoptosis and SOD activity of *Prnp*$^{-/-}$ cells were compared with those of *Prnp*$^{-/-}$ cells expressing the wild-type PrP^C or PrP^C lacking the OR and HR under serum-free conditions.

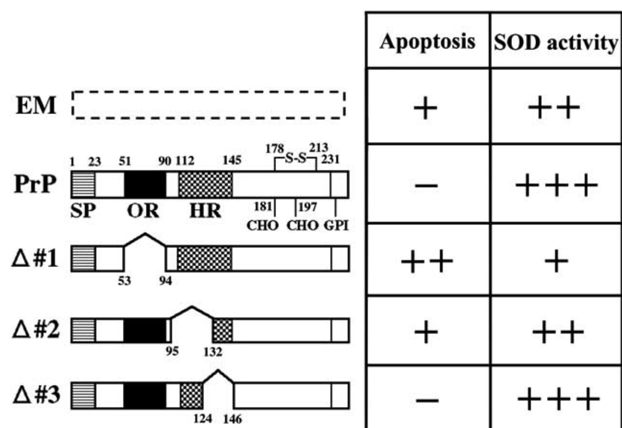

Fig. (4). Schematic representations of PrP deletion mutants.

Schematic representations of deletion mutants of mouse PrP are shown on the left. Mutants of mouse PrP [PrP: wild-type PrP; Δ#1: PrP(Δ53-94, Q52H); Δ#2: PrP(Δ95-132); Δ#3: PrP(Δ124-146)] were prepared using restriction digestion/ ligation. Schematic locations of the deletions as compared with the wild-type protein are shown by a space within the bar next to the indicated protein. Numbers refer to the amino acid residues in the mouse PrP sequence. The disulfides (S-S), two Asn-linked glycosylation sites (CHO), signal peptide sequence (SP), octapeptide repeat region (OR) and hydrophobic region (HR) are shown. PrP has a glycosylphosphatidylinositol (GPI) anchor attached to its C terminus. HpL3-4 cells expressing wild-type PrP (PrP), PrP(Δ53-94, Q52H) (Δ#1), PrP(Δ95-132) (Δ#2), PrP(Δ124-146) (Δ#3) or the empty vector per se (EM) were serum-deprived. The rate of apoptosis of the cells after serum deprivation for 24 h, when maximum apoptosis occurs, and the superoxide dismutase (SOD) activity of the cells after serum deprivation for 6 h, when reactive oxygen species start to be generated, are shown on the right. The number of plusses (+) indicates the degree of apoptosis or SOD activity. Minus (-) indicates a lesser degree. Modified from Fig. **4** in Sakudo *et al*. [179] with permission from Elsevier.

To investigate the roles of the OR and HR in the anti-apoptotic function of PrP, several deletions were made within these regions of mouse PrP (Fig. **4**). The anti-apoptotic function of three PrP deletion constructs and the control constructs was tested following stable transfection into *Prnp*-deficient neuronal cells HpL3-4 [HpL3-4 cells expressing wild-type PrP (PrP: HpL3-4-PrP), PrP(Δ53-94, Q52H) (Δ#1: HpL3-4-Δ#1), PrP(Δ95-132) (Δ#2: HpL3-4-Δ#2), and PrP(Δ124-146) (Δ#3: HpL3-4-Δ#3) or the empty vector per se (EM: HpL3-4-EM)]. The cell clones of the deletion mutants with levels of expression similar to those of clones expressing the full-length protein were checked by Western blotting using either anti-PrP SAF32 or SAF83 and cell surface staining with SAF53 and SAF61 using flow cytometry [6]. In the absence of serum, HpL3-4 cells undergo cell death with features of apoptosis, and $Prnp^{-/-}$ cells transfected with *Prnp* are resistant to serum deprivation [3, 4, 6]. The lactate dehydrogenase (LDH) activity assay showed that the control transfectants (HpL3-4-EM) underwent cell death rapidly upon serum deprivation, while the full-length PrP-transfected cells were significantly protected (Fig. **4**). In contrast, the cells expressing OR-deficient PrP (HpL3-4-Δ#1) showed enhancement of cell death compared with HpL3-4-EM cells. Interestingly, the cells expressing the N-terminal half of HR-deficient PrP (HpL3-4-Δ#2) underwent cell death at equivalent levels to HpL3-4-EM cells (Fig. **4**). In contrast, the cells expressing the C-terminal half of HR-deficient PrP (HpL3-4-Δ#3) underwent cell death at equivalent levels to HpL3-4-PrP cells (Fig. **4**). Comparative differences of apoptotic cells in each deletion mutant were also confirmed by apoptosis assays including the DNA ladder analysis, measurement of the histone-associated DNA fragments (mono- and oligonucleosomes) in cytosolic fractions of the cells with ELISA and nuclear morphological analysis using 4',6-diamido-2-phenylindole hydrochloride (DAPI) staining [6]. To further examine whether the susceptibility of each deletion mutant to serum deprivation was due to the activity of SOD, the SOD activity in the presence and absence of serum was analyzed in each deletion mutant. HpL3-4-Δ#1 cells showed significantly lower activity of SOD than HpL3-4-EM cells, whereas HpL3-4-PrP and HpL3-4-Δ#3 cells showed significantly higher activity of SOD than HpL3-4-EM cells under serum deprivation for 6 h (Fig. **4**). In contrast, HpL3-4-Δ#2 cells showed SOD activity comparable with that of HpL3-4-EM cells (Fig. **4**). Therefore, the data suggest that the anti-apoptotic function of PrP^C can be regulated by not only the OR but also the N-terminal half of HR, and this quantitative difference in the anti-apoptotic function of PrP deletion mutants is correlated with the level of SOD activity in transfectants expressing PrP deletion mutants (Fig. **4**). The data are consistent with the notion that both the OR and N-terminal half of HR are necessary for the activation of cellular SOD. The OR and HR of PrP are well conserved among mammals [51, 177]. The high evolutionary conservation of these regions is consistent with the notion that these regions are important domains for the function of PrP. Our ongoing studies have shown that HpL3-4 cells expressing Dpl, which structurally resembles an N-terminally truncated PrP, fused to PrP(1-124) with the OR and N-terminal half of HR of PrP shows anti-apoptotic function, whereas Dpl fused to PrP(1-95) with OR does not rescue cells from apoptotic cell death induced by serum deprivation (Lee DC, Sakudo A, and Onodera T, unpublished results). These results indicated that a fusion protein containing the OR of PrP was not protective, whereas extending the fusion to the N-terminal half of HR provided protection, also supporting the notion that both the OR and N-terminal half of HR are important domains for the PrP function.

Several potential mediators of PrP^C signals have been reported recently. Copper specifically binds the OR of PrP^C [16-20] and enhances the endocytosis of PrP^C [178]. A PrP^C-binding molecule, STI1, binds with amino acid residues 113-128 located in the N-terminal half of the HR of PrP^C [55]. Therefore, additional studies were performed in order to determine how STI1 and copper might contribute to PrP^C-dependent anti-oxidative signaling. To investigate whether the STI1 is important for the biological activities displayed by PrP^C, the effect of the inhibitory peptides against PrP^C-STI1 on $Prnp^{-/-}$ cells was compared to that on PrP^C-expressing $Prnp^{-/-}$ cells under serum-free conditions. The inhibitory peptides are toxic to PrP^C-expressing cells due to inhibiting the SOD activity, although such is not the case for

Prnp$^{-/-}$ cells [179]. Furthermore, immunoprecipitation indicated that STI1 interacted with PrP^{C} in PrP^{C}-expressing cells [179]. The cellular copper concentration was decreased in *Prnp*$^{-/-}$ cells, but not in PrP-expressing cells under oxidative conditions [143]. Therefore, it is proposed that STI1 and copper might be involved in PrP^{C}-dependent SOD activation that can inhibit apoptosis *via* the OR and N-terminal half of PrP. Considering the data reported in this review and obtained in our work using *Prnp*$^{-/-}$ mice and the derived cell lines, it is further proposed that the susceptibility of *Prnp*$^{-/-}$ cells to cell death is probably due to reduced SOD activity, induced at least in part by the absence of PrP^{C}-STI1 signals and the abnormality of copper homeostasis, suggesting that PrP plays antioxidative roles and functions only in urgent states such as oxidative conditions.

CONCLUSIONS AND PERSPECTIVES

In this review, the recent advances in prion biology made possible by the availability of *Prnp*$^{-/-}$ mice and the derived cell lines were described. Since PrP is expressed in not only neuronal cells but also non-neuronal cells, further studies using non-neuronal *Prnp*$^{-/-}$ cell lines will be necessary. Our current focus is studies using the GpL1 glial cell line derived from ZrchI PrP-deficient mice (T. Nishimura, A Sakudo and T. Onodera, unpublished results). PrP is expressed at a variety of levels in different regions of the brains of animals. For example, in the hamster brain, PrP is most abundant in the stratum radiatum and stratum oriens of the CA1 region of the hippocampus, and is virtually absent from the granule layer of the dentate gyrus and the pyramidal cell layer [180, 181]. Therefore, the establishment of *Prnp*$^{-/-}$ cell lines derived from various brain regions of *Prnp*$^{-/-}$ mice is also necessary. Further establishment of PrP-deficient cell lines derived from various cell types and origins in various brain regions and also in other organs would facilitate detailed analysis of PrP functions.

With the development of proteomics, analysis of the information obtained from the application of *Prnp*$^{-/-}$ mice and the derived cell lines by proteomics will be increasing. For example, using these materials to compare their characteristics with those of PrP-expressing counterparts by proteomics, we can easily and rapidly obtain outlines of the effects of PrP-deficiency and assess fundamental differences caused by PrP-deficiency. Therefore, *Prnp*$^{-/-}$ mice and the derived cell lines coupled with proteomics would provide a powerful tools for exploratory analysis of PrP deficiency. The accumulated knowledge using this system will be of great help for understanding PrP functions.

Prnp$^{-/-}$ mice and the derived cell lines will also contribute to the production of materials for studies of prions. For example, by immunizing *Prnp*$^{-/-}$ mice with recombinant murine PrP [182], human recombinant PrP folded into α or β [183], purified PrP^{C} and PrP^{Sc} [99, 184], scrapie-infected mouse neuroblastoma cells [185], DNA plasmids encoding *Prnp* [186, 187], and PrP-displaying retrovirus particles [188], monoclonal and polyclonal antibodies have been produced. By immunizing *Prnp*$^{-/-}$ mice with the derived *Prnp*$^{-/-}$ cell lines transfected with *Prnp* or mutated *Prnp*, specific antibodies to wild-type or mutated PrP may easily be obtained. Furthermore, by using prion-infected *Prnp*$^{-/-}$ cells transfected with *Prnp* as immmunogen, specific antibodies to PrP^{Sc} may be also produced. These newly generated PrP^{C} and PrP^{Sc} antibodies will be useful for exploring the biology of PrP^{C} and establishing the diagnosis of prion diseases in both humans and animals.

Finally, we emphasize that the elucidation of PrP^{C} function is also important in terms of clarifying the etiology of prion diseases. This is true because PrP^{Sc} is derived from PrP^{C} before being accumulated, when animals are infected with prions. The conversion of PrP^{C} to PrP^{Sc} leads to PrP^{C} deficiency [95], suggesting that not only the gain-of-function of PrP^{Sc} but also the loss-of-function of PrP^{C} contributes etiologically to the induction of prion diseases. Therefore, the study of PrP^{C} functions will become more and more important. Moreover, PrP^{C} expression is required for prion infection [96-99], while interference of endogenous PrP with the pathogenicity of TSE agents from other species has been reported [189-192]. Hence, *Prnp*$^{-/-}$ mice and the derived cell lines lacking endogenous PrP will serve to analyze the domain of PrP^{C} required for prion pathogenicity by the transfection of various deletion mutants of PrP following prion infection without interference by the endogenous PrP. The discoveries made from approaches using *Prnp*$^{-/-}$ mice and the derived cell lines should provide valuable new insights into the roles of PrP^{C} and the etiology of prion diseases.

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