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Comparison of abnormal isoform of prion protein in prion-infected cell lines and primary cultured neurons by PrP\textsuperscript{Sc}-specific immunostaining

Running title: PrP\textsuperscript{Sc} in immortalized cell lines and primary neurons

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Abbreviations:

Ara-C, cytosine arabinoside; CNs, primary cerebral neurons; DAPI 4',6-Diamidino-2-phenylindole; div, days in vitro; dpi, days post infection; GdnSCN, guanidine isothiocyanate; GFAP, glial fibrillary acidic protein; IFA, immunofluorescence assay; MAP2, microtubule-associated protein 2; N2a, Neuro2a; pAbs; polyclonal antibodies; PK, proteinase K; PrP^C, cellular isoform of prion protein; PrP^Sc, abnormal isoform of prion protein; PrP-res, proteinase K-resistant PrP^Sc; PTA, phosphotungstic acid; RT, room temperature; ScGT1-7-22L, GT1-7 persistently infected with the 22L prion strain; ScN2a-3-22L, N2a-3 persistently infected with the 22L prion strain; ScN2a-3-Ch, N2a-3 persistently infected with the Chandler prion strain
Abstract

We established abnormal isoform of prion protein (PrP\textsuperscript{Sc})-specific double immunostaining using mAb 132 that recognizes aa 119–127 of the PrP molecule and novel PrP\textsuperscript{Sc}-specific mAb 8D5 that recognizes the N-terminal region of the PrP molecule. Using the PrP\textsuperscript{Sc}-specific double immunostaining, we analyzed PrP\textsuperscript{Sc} in immortalized neuronal cell lines and primary cerebral neuronal cultures infected with prions. The PrP\textsuperscript{Sc}-specific double immunostaining showed the existence of PrP\textsuperscript{Sc} positive for both mAbs 132 and 8D5 as well as those positive only for either mAb 132 or mAb 8D5. This indicated that double immunostaining detects a greater number of PrP\textsuperscript{Sc} species than single immunostaining. Double immunostaining revealed cell type-dependent differences in PrP\textsuperscript{Sc} staining patterns.

In the 22L prion strain-infected Neuro2a (N2a)-3 cells, a subclone of N2a neuroblastoma cell line, or GT1-7, a subclone of the GT1 hypothalamic neuronal cell line, granular PrP\textsuperscript{Sc} stains were observed at the perinuclear regions and cytoplasm, whereas unique string-like PrP\textsuperscript{Sc} stains were predominantly observed on the surface of the 22L strain-infected primary cerebral neurons. Only 14% of PrP\textsuperscript{Sc} in the 22L strain-infected N2a-3 cells were positive for mAb 8D5, indicating that most of the PrP\textsuperscript{Sc} in N2a-3 lack the N-terminal portion. In contrast, nearly half PrP\textsuperscript{Sc} detected in the 22L strain-infected primary cerebral neurons were positive for mAb 8D5, suggesting the abundance of full-length PrP\textsuperscript{Sc} that possesses the N-terminal portion of PrP.

These results pose a problem in interpreting the mechanism of prion propagation in different cell types.
Introduction

Transmissible spongiform encephalopathies, also known as prion diseases, are fatal neurodegenerative disorders in humans and animals, which are caused by the infectious agent called prions. The pathological hallmarks of prion diseases include microglial activation, astrogliosis, and accumulation of abnormal isoform of prion protein ($\text{PrP}^\text{Sc}$) in the central nervous system. $\text{PrP}^\text{Sc}$ is generated from a host-encoded cellular isoform of prion protein ($\text{PrP}^\text{C}$) by posttranslational modifications such as conformational transformation (Prusiner, 1998). The generation of $\text{PrP}^\text{Sc}$ is believed to be strongly associated with prion propagation and neurodegeneration in prion diseases (Mallucci et al., 2003). Therefore, the clarification of the cellular mechanism of $\text{PrP}^\text{Sc}$ formation would be useful.

Cell biological studies using immortalized neuronal cell lines such as Neuro2a (N2a) neuroblastoma and GT1 hypothalamic neuronal cells have greatly contributed to the elucidation of the cellular mechanism underlying prion propagation. Earlier studies suggested that $\text{PrP}^\text{Sc}$ is generated from mature $\text{PrP}^\text{C}$ expressed on the cell surface and that the $\text{PrP}^\text{Sc}$ generation occurs at cellular compartments on the endocytotic pathway including the cholesterol-rich membrane microdomain called lipid rafts (Borchelt et al., 1992; Caughey & Raymond, 1991; Naslavsky et al., 1997; Vey et al., 1996). Further cell biological studies within a decade indicated the involvement of endocytic recycling compartments and endocytic recycling pathway in $\text{PrP}^\text{Sc}$ formation (Beranger et al., 2002; Marijanovic et al., 2009; Pimpinelli et al., 2005; Veith et al., 2009; Yamasaki et al., 2012). We recently reported that after the inoculation of prions, newly generated $\text{PrP}^\text{Sc}$ appeared on the cell surface, early endosomes, and late endosomes of N2a cells (Yamasaki et al., 2014a). These cell biological studies suggest the intracellular vesicular compartments to be the major sites for $\text{PrP}^\text{Sc}$
formation, whereas it was reported that PrP<sup>Sc</sup> formation occurs on the cell surface within a minute (Goold et al., 2011). On the other hand, ultrastructural studies using the brains of prion-infected mice showed that PrP<sup>Sc</sup> was frequently detected on the plasma membranes of neuropils but occasionally in the intracellular vesicles (Godsave et al., 2008; Godsave et al., 2013; Jeffrey et al., 1994; Jeffrey et al., 1992). Further immuno-electron microscopic studies using anti-PrP recognizing the N-terminal region of PrP suggested that neuronal plasma membranes such as membrane invaginations and sites of cell-to-cell contact are primary sites for PrP<sup>Sc</sup> formation (Godsave et al., 2013). To resolve the partly contradictory results regarding the site of PrP<sup>Sc</sup> formation between cell culture and ultrastructural studies, primary cultured neurons are considered to be a good ex vivo model. There are only a few reports on prion propagation in primary cultured neurons derived from the cerebellum, striatum, and cerebral cortex of mouse brains (Cronier et al., 2004; Dron et al., 2010; Hannaoui et al., 2013). However, little information is available on the localization of PrP<sup>Sc</sup> in primary neurons infected with prions.

Taraboulos et al. reported that treatment of fixed cells with guanidinium salts prior to incubation with anti-PrP antibodies significantly increases the PrP<sup>Sc</sup> signals in immunocytochemical staining (Taraboulos et al., 1990). This method has been being used for PrP<sup>Sc</sup>-specific detection by immunofluorescence assay (IFA). However, careful adjustment of the threshold level by manipulation of the detector gain or the exposure time is required for the discrimination of PrP<sup>Sc</sup> signals from PrP<sup>C</sup>. One concern to arise is that such manipulations may limit the detailed analysis of PrP<sup>Sc</sup>. We recently reported that mAb 132 recognizing the epitope comprising mouse PrP aa 119–127 distinguishes PrP<sup>Sc</sup> from PrP<sup>C</sup> in prion-infected cells and tissues with a minimum manipulation of threshold setting (Sakai et al., 2013;
Yamasaki et al., 2012). Although the pretreatment with guanidinium salts is still prerequisite, the advantage in threshold setting made it possible to disclose the detailed intracellular localization of PrP\textsuperscript{Sc} in cells persistently infected with prions (Yamasaki et al., 2014\textsuperscript{a}; Yamasaki et al., 2014\textsuperscript{b}; Yamasaki et al., 2012). The use of mAb 132 improved the specificity of PrP\textsuperscript{Sc} detection; however, PrP\textsuperscript{Sc} comprises a heterologous PrP\textsuperscript{Sc} population in the size and rigidity of aggregates. Therefore, whether mAb 132 can detect all of PrP\textsuperscript{Sc} species in cells remains obscure.

Recently, Masujin and his colleagues reported a new PrP\textsuperscript{Sc}-specific antibody, mAb 8D5, which recognizes the N-terminal region comprising mouse PrP aa 31–39 (Masujin et al., 2013). This mAb is expected to react with full-length PrP\textsuperscript{Sc} but not with the N-terminally truncated form of PrP\textsuperscript{Sc} that is generated by endogenous enzymes after PrP\textsuperscript{Sc} formation in the cells (Caughey et al., 1991; Taraboulos et al., 1992). The mAb 8D5 specifically immunoprecipitated Prp\textsuperscript{Sc} from brain homogenates of prion-infected animals, similar to other PrP\textsuperscript{Sc}-specific antibodies reported to date (Curin Serbec et al., 2004; Horiuchi et al., 2009; Korth et al., 1997; Paramithiotis et al., 2003; Ushiki-Kaku et al., 2010). Of relevance is that the mAb 8D5 detected PrP\textsuperscript{Sc} in non-denatured histoblot and the cryosections of scrapie-infected mouse brains. This feature appeared to be useful for the immunocytochemical detection of PrP\textsuperscript{Sc}; thus, we attempted to use mAb 8D5 in combination with mAb 132 for PrP\textsuperscript{Sc}-specific detection.

In the current study, we prepared primary neuronal cultures from mouse cerebra with enrichment of neuronal population by antimitotic treatment and confirmed the prion propagation in the neurons. Then we compared PrP\textsuperscript{Sc} in immortalized cell lines and in primary neuronal cultures using PrP\textsuperscript{Sc}-specific double immunostaining.
Results

Detection of PrP<sup>Sc</sup> in prion-infected immortalized cells by PrP<sup>Sc</sup>-specific staining with mAbs 132 and 8D5

First we examined if mAb 8D5 reacts with PrP<sup>Sc</sup> in prion-infected cells with or without pretreatment of cells with guanidine isothiocyanate (GdnSCN) (Fig. 1a). As expected, mAb 132 detected PrP<sup>Sc</sup> in ScN2a-3-22L cells, which were a subclone of Neuro2a mouse neuroblastoma cell line (N2a-3) persistently infected with the 22L prion strain after pretreatment of cells with 5 M GdnSCN. Although fluorescence intensities were weaker than those by mAb 132, PrP<sup>Sc</sup> signals were detected by mAb 8D5 in ScN2a-3-22L cells with or without GdnSCN pretreatment. Under the same condition of fluorescent image acquisition, mAb 8D5 did not show any specific fluorescent signals in prion-uninfected cells, demonstrating the PrP<sup>Sc</sup>-specific detection.

To confirm the optimum condition of GdnSCN pretreatment for PrP<sup>Sc</sup>-specific double immunostaining with mAbs 132 and 8D5, influence of the pretreatment on the PrP<sup>Sc</sup> detection was analyzed using GT1 hypothalamic neuronal cell line (GT1-7) persistently infected with the 22L prion strain (ScGT1-7-22L) (Supplementary figure 1). The GdnSCN pretreatment did not enhance or degrade the reactivity of mAb 8D5 to PrP<sup>Sc</sup> in ScGT1-7-22L cells so much. In contrast, the reactivity of mAb 132 drastically enhanced after the 5-min GdnSCN pretreatment. However, a longer GdnSCN pretreatment did not affect the reactivity of mAb 132. This result demonstrated that the 10-min pretreatment of cells with 5 M GdnSCN was optimum for the PrP<sup>Sc</sup>-specific double immunostaining with mAbs 132 and 8D5, and thus, in
the following experiments, 10-min GdnSCN pretreatment was performed unless otherwise specified.

Fig. 1(b) shows double immunofluorescent staining of PrP\textsuperscript{Sc} in ScN2a-3-22L cells using mAbs 132 and 8D5. In ScN2a-3-22L cells, mAb 132 detected bright granular PrP\textsuperscript{Sc} signals at perinuclear regions as previously described (Yamasaki et al., 2012). In contrast, mAb 8D5 showed relatively faint granular PrP\textsuperscript{Sc} signals in ScN2a-3-22L cells (Fig. 1b, arrowheads). Although some granular PrP\textsuperscript{Sc} signals detected by mAb 132 merged with those by mAb 8D5 (Fig. 1b, arrow), the majority of granular PrP\textsuperscript{Sc} stains by mAb 132 did not appear to be co-localized with those by mAb 8D5. The results of the detailed colocalization analysis are described later (see Fig. 6).

The same double immunofluorescent staining was carried out on ScGT1-7-22L cells. MAb 132 showed perinuclear granular stains of PrP\textsuperscript{Sc}, however, the granular signals appeared to be scattered to cytoplasm compared with ScN2a-3-22L cells (Fig. 1c). MAb 8D5 appeared to detect greater number of PrP\textsuperscript{Sc} granules in ScGT1-7-22L cells than in ScN2a-3-22L cells. PrP\textsuperscript{Sc} granular stains detected by mAb 8D5 were scattered to the cytoplasm and some granules with intense fluorescence were observed at the peripheral region of cytoplasm, possibly proximate the plasma membranes (Fig. 1c, arrowheads). PrP\textsuperscript{Sc} detected by mAbs 132 and 8D5 was partly but not extensively merged (Fig. 1c, arrows).

**Neuron-enriched primary cultures from mouse cerebra**

Cells isolated from the cerebra of mouse embryos were abundant in neurons and contained a few glial cells (Fig. 2b, e.g., 7 days in vitro [div], cytosine arabinoside [Ara-C] [-]). However, in the absence of Ara-C, glial fibrillary acidic protein (GFAP)-positive
astrocytes readily increased by 14 div (Fig. 2b). Ara-C treatment at 0.25 µM from 4 to 7 div and following treatment at 0.125 µM from 7 to 11 div successfully suppressed the appearance of GFAP-positive astrocytes up to 28 div; only a few astrocytes were found in Ara-C-treated cultures until 28 div. The result of GFAP expression in immunoblot analysis also demonstrated the successful reduction of astrocytes (Fig. 2c). A neuron-specific protein, βIII-tubulin, was detected from primary neuronal cultures in the presence or absence of Ara-C by immunoblot analysis (Fig. 2c). Lower levels of GFAP but higher levels of βIII-tubulin in Ara-C-treated primary neuronal cultures at each time point also indicated that the Ara-C treatment by the indicated schedule securely resulted in the enrichment of neurons in the primary neuronal cultures. We designate this culture as primary cerebral neurons (CNs) in the description below.

PrP^sc generation in CNs

The CNs at 7 div were exposed to microsomes as described in the section of Materials and Methods. Four days after the exposure, the medium was replaced with fresh, Ara-C-free Neuronal Medium to remove inocula (Fig. 2a). The CNs at 0, 7, 14, 21, 28, and 35 days post infection (dpi) were subjected to immunoblot analysis for proteinase K (PK)-resistant PrP^sc (PrP-res) detection (Fig. 3a). PrP-res was detected in cells exposed to three different prion strains from at least 7 dpi. In CNs infected with 22L or Chandler strain, PrP-res levels increased up to 21 dpi, demonstrating prion propagation. In Obihiro strain-infected CNs, the PrP-res level was lower than CNs infected with other two prion strains. No PrP-res was detected in mock-infected CNs. Figure 3(b) shows PrP^sc-specific immunostaining using mAb 132. PrP^sc signals were observed around cell bodies and neurites in prion-infected CNs from
7 dpi but not in mock-infected CNs. The PrP\textsuperscript{Sc} stains per cell appeared to gradually increase up to 21 dpi, and most CNs were positive for PrP\textsuperscript{Sc} at 14 dpi (data not shown). The granular PrP\textsuperscript{Sc} stains at perinuclear regions, as observed in ScN2a-3-22L cells and N2a-3 cells persistently infected with the Chandler prion strain (ScN2a-3-Ch), were scarcely observed in prion-infected CNs. However, string-like stains around the edges of cell bodies and neurites were evident during the later stage of infection (Fig. 3b, arrows).

**PrP\textsuperscript{Sc}-specific double immunostaining of prion-infected CNs using mAbs 132 and 8D5**

To characterize the string-like PrP\textsuperscript{Sc} stains in prion-infected CNs that were detected by mAb 132 (Fig. 3b), PrP\textsuperscript{Sc}-specific double immunostaining was performed with mAbs 132 and 8D5. In 22L or Chandler strain-infected CNs (at 14 dpi), string-like PrP\textsuperscript{Sc} stains (Fig. 4, closed arrows) were detected by both mAbs 132 and 8D5, and they were partly merged (Fig. 4, arrowheads). In addition to string-like PrP\textsuperscript{Sc} stains, granular PrP\textsuperscript{Sc} stains single positive for mAb 132 were observed at neurites but hardly at somas (Fig. 4, open arrows). The string-like PrP\textsuperscript{Sc} stains were evident but less obvious in Obihiro strain-infected CNs compared with 22L or Chandler strain-infected CNs.

Since PrP\textsuperscript{Sc} stains in prion-infected CNs appeared at the plasma membranes or extracellular space, living CNs were incubated with Alexa Fluor 488-conjugated mAb 8D5 and subjected to microscopic examination to confirm whether the string-like PrP\textsuperscript{Sc} is present on the cell surface (Fig. 5). The CNs infected with 22L, Chandler, or Obihiro strains at 14 dpi were incubated with mAb 8D5 and were directly subjected to microscopic observation without fixation, permeabilization or denaturation. String-like and granular-like stains of PrP\textsuperscript{Sc} similar to those in fixed cells (Fig. 4) were observed in CNs infected with prions,
demonstrating the presence of PrP<sub>Sc</sub> on the cell surface (Fig. 5). The result also indicated that certain population of PrP<sub>Sc</sub> on the cell surface of CNs possesses the N-terminal region that is recognized with mAb 8D5. PrP<sub>Sc</sub> stains were also clearly detected on the surface of ScGT1-7-22L cells but not obvious on the surface of ScN2a3-22L cells (Supplementary figure 2).

Colocalization of PrP<sub>Sc</sub> detected by mAbs 132 and 8D5 in immortalized cells and CNs

PrP<sub>Sc</sub>-specific double staining revealed differences in immunoreactivity of PrP<sub>Sc</sub> between immortalized cell lines and CNs; PrP<sub>Sc</sub> positive for mAb 8D5 was more apparent in CNs infected with the 22L strain than in ScN2a-3-22L cells. To assess the differences in detail, quantitative analysis of PrP<sub>Sc</sub> populations was performed based on the reactivity with mAbs 132 and 8D5 (Fig. 6). In ScN2a-3-22L cells, only 6.6 % of the total PrP<sub>Sc</sub> signals in cells were positive for both mAbs (Fig. 6b). In contrast, the proportion of double-positive PrP<sub>Sc</sub> appeared to be higher in ScGT1-7-22L cells and prion-infected CNs than in ScN2a-3-22L cells; 18.9% in ScGT1-7-22L cells and 13.5%–24.9% in CNs infected with three different prion strains. In ScN2a-3-22L cells, 92.3% of PrP<sub>Sc</sub> were positive for mAb 132 (Fig. 6c, red and yellow bars), whereas only 14.3% were positive for mAb 8D5 (Fig. 6c, green and yellow bars). The proportion of PrP<sub>Sc</sub> population detected by mAb 132 in CNs infected with the 22L prion strain (78.9%) was significantly lower than that in ScN2a-3-22L cells (Fig. 6c, p<0.05). Conversely, nearly half of PrP<sub>Sc</sub> was positive for mAb 8D5 in CNs infected with 22L prion strain (46.0%), which was significantly higher than that in ScN2a-3-22L cells (Fig. 6c, p<0.01). Although no difference was observed in the proportion of mAb 132-positive
PrP<sup>Sc</sup> between ScN2a-3-22L and ScGT1-7-22L cells (88.8%) or between ScGT1-7-22L and CNs infected with the 22L prion strain, the proportion of mAb 8D5-positive PrP<sup>Sc</sup> in ScGT1-7-22L cells (30.2%) was significantly higher than in ScN2a-3-22L cells, but lower than in CNs infected with 22L prion strain (Fig. 6c, p< 0.05).

**Biochemical properties of PrP molecules in cells**

PrP<sup>Sc</sup>-specific immunofluorescence staining revealed differences in staining pattern of PrP<sup>Sc</sup> among cell types, particularly between ScN2a-3-22L cells and ScGT1-7-22L cells or CNs infected with the 22L strain. To address the differences, we performed immunoblotting to detect total PrP (both PK-sensitive and PK-resistant PrP) without PK treatment (Fig. 7a). The level of the full length PrP was apparently lower in ScN2a-3-22L cells than in N2a-3 cells. In contrast, GT1-7 and ScGT1-7-22L cells, and CNs and CNs infected with the 22L strain had comparable amount of full length PrP. These results suggest that ScN2a-3-22L cells have a higher N-terminal processing activity and that the cells produce the N-terminal truncated PrP<sup>Sc</sup> that lacks the epitope for mAb 8D5. This idea is consistent with the results of PrP<sup>Sc</sup>-specific immunofluorescence staining: PrP<sup>Sc</sup> stains positive for mAb 132 were more evident than those positive for mAb 8D5 in ScN2a3-22L cells (Fig. 1a). By contrast, mAb 8D5 seemed to detect PrP<sup>Sc</sup> efficiently in ScGT1-7-22L cells (Fig. 1d) and CNs infected with the 22L strain (Figs 4 and 5).

To analyze PrP<sup>Sc</sup> recognized by mAb 8D5, PrP<sup>Sc</sup> was immunoprecipitated from GT1-7 cells with mAb 8D5 and then subjected to immunoblotting with mAb 110, which recognizes the epitope in the octapeptide repeat region (Fig. 7b). PrP bands were detected only in the precipitates from ScGT1-7-22L cells, demonstrating the PrP<sup>Sc</sup>-specificity of
immunoprecipitation. The mAb 110 detected broad bands around 36 and 30 kDa as well as bands lower than 25 kDa. The 36 kDa broad band corresponds to full length PrP\(^{Sc}\), indicating the existence of full length PrP\(^{Sc}\) on the cell surface. Regarding broad bands around 30 kDa and band with approx. 25 kDa, it is difficult to conclude whether these bands represent mono- or non-glycosylated full length PrP\(^{Sc}\) or di- or mono-glycosylated N-terminal truncated PrP\(^{Sc}\) that were co-precipitated with full length PrP\(^{Sc}\) during the immunoprecipitaion. However, no immunoreactive bands were observed below 15 kDa, even around the running front (data not shown). This suggests that PrP molecule possessing the extreme N-terminus but lacking the C-terminal core region will be negligible even if it exists.

**Discussion**

The mAb 8D5 recognizes the epitope composed of the extreme N-terminus of PrP (aa 31–39) (Masujin et al., 2013). It is well known that the N-terminus of PrP is partly digested by cellular events (Caughey et al., 1991; Chen et al., 1995). MAb 132 recognizes the epitope composed of aa 119–127 of PrP that remains in the C-terminal core fragment of PrP\(^{Sc}\), possibly analogous to the C2 fragment (Chen et al., 1995). Thus, PrP\(^{Sc}\)-specific double staining using the mAbs is expected to provide more precise information on intracellular localization of PrP\(^{Sc}\). Indeed, PrP\(^{Sc}\) stains with mAb 132 were not always merged with those by mAb 8D5, indicating that the double staining detected greater number of PrP\(^{Sc}\) molecules than single-staining (Figs. 1, 4, and 6). The mAb 132-positive but mAb 8D5-negative PrP\(^{Sc}\) signals may represent the N-terminally truncated PrP\(^{Sc}\) molecules. Unexpectedly, however, certain PrP\(^{Sc}\) was mAb 8D5 positive but mAb 132 negative in the double staining. The
reactivity implies the existence of PrP\textsuperscript{Sc} composed only of the N-terminal portion of the PrP molecule; however, this is unlikely. The N-terminal portion of PrP\textsuperscript{Sc} is easily digested by PK treatment, indicating that the N-terminal portion locates the accessible surface of PrP\textsuperscript{Sc} oligomers/aggregates. Thus, anti-PrP antibodies may be able to more readily access the N-terminal portion than the C-terminal portion of PrP\textsuperscript{Sc}. The antibody molecule is nearly 5-fold larger than a single PrP molecule; therefore, once mAb 8D5 binds to PrP\textsuperscript{Sc}, it may disturb the binding of mAb 132 to the epitope even on the neighboring PrP molecule of PrP\textsuperscript{Sc} oligomers by steric hindrance. Indeed, ScN2a-3-22L cells were stained with Alexa Fluor 488-labeled mAb 132 and Alexa Flour 647-labeled mAb 132 simultaneously, 65.7 ± 2.3 % of Alexa 488-labeled PrP\textsuperscript{Sc} stains were also stained with Alexa Flour-647. These factors will affect the interpretation of the results; nevertheless, PrP\textsuperscript{Sc}-specific multiple staining using two or more antibodies, as shown here, is one of the effective approaches for precise understanding of intracellular localization of PrP\textsuperscript{Sc} and the mechanism for prion propagation.

Although PrP\textsuperscript{Sc} has been reported to be detected on the cell surface of prion-infected immortalized neuronal cells (Goold et al., 2011; Rouvinski et al., 2014; Yamasaki et al., 2012), cell biological studies using immortalized cell lines strongly suggested that the PrP\textsuperscript{Sc} formation takes place at cellular compartments along with the endocytic recycling pathway such as the endocytic recycling compartments, or those along with the endo-lysosomal pathway such as multivesicular bodies (Beranger et al., 2002; Borchelt et al., 1992; Marijanovic et al., 2009; Pimpinelli et al., 2005; Veith et al., 2009; Yamasaki et al., 2014a; Yamasaki et al., 2012). In contrast, ultrastructural analyses using brains of prion-infected mice and hamsters frequently identified PrP\textsuperscript{Sc} at the plasma membranes and less frequently at synapses and compartments of the endo-lysosomal system (Arnold et al., 1995; Fournier et al.,
2000; Godsave et al., 2008). Recently, Godsave et al. reported that in RML strain-infected mouse brains, the majority of PrP^Sc clusters were detected at the plasma membranes, including membrane invaginations and the site of cell-to-cell contact, whereas in the other subcellular regions such as synapses and intracellular vesicles, PrP^Sc clusters were detected only occasionally by immuno-electron microscopic examination using an antibody that recognizes the N-terminal region of PrP (Godsave et al., 2013). The authors believed that PrP^Sc detected by the anti-PrP N-terminus mAb Saf32 included nascent full-length PrP^Sc, as the N-terminal region tends to be processed after generation of PrP^Sc (Caughey et al., 1991; Taraboulos et al., 1992). Considered collectively, ultrastructural studies suggest the plasma membranes as the primary site for PrP^Sc formation. In the current study, we found that morphology and cellular localization of PrP^Sc differ in immortalized cell lines and CNs: in N2a-3 and GT1-7 cells infected with 22L prion strain (Fig. 1b and c), PrP^Sc was mainly detected as granule-like stains at the perinuclear regions, as previously reported (Schatzl et al., 1997; Yamasaki et al., 2012), whereas in CNs infected with the 22L- or Chandler strain, PrP^Sc was detected as unique string-like stains, possibly on the cell surface; however, a few were detected in the cytoplasm (Figs. 4 and 5). Rouvinski et al. recently reported that in addition to granule-like PrP^Sc stains in the cytoplasm, strings-like PrP^Sc stains were detected on the surface of 22L or RML prion strain-infected GT1 cells. The string-like PrP^Sc was detected by anti-PrP antibodies recognizing the N-terminal part of PrP and was demonstrated as glycosylphosphatidylinositol-anchored PrP^Sc amyloids (Rouvinski et al., 2014). However, compared with the results by Rouvinski et al, string-like PrP^Sc stains were predominantly observed on the surface of CNs; however, a few intracellular granule-like PrP^Sc stains were observed in the CNs (Fig. 4). Taken together, the differences in PrP^Sc stains suggest that the
mechanism of PrPSc formation in immortalized cell lines may not be exactly the same as that in CNs. Interestingly, Rouvinski et al. also detected string-like PrPSc stains in brains of scrapie-infected mice (Rouvinski et al., 2014). Furthermore, filamentous depositions of PrPSc were frequently observed in the white matter of patients with inherited prion diseases (Reiniger et al., 2013) and in the neuropils of the 22L prion strain-infected cerebellar slices (Wolf et al., 2015). In addition, it was reported that PrPSc accumulates predominantly as unprocessed forms in brain tissues and in the primary cultured cerebellar granule neurons (Dron et al., 2010). Since PrPSc detected by mAb 8D5 is expected to be a full-length PrPSc, therefore, the morphological, and locational as well as biochemical similarities of PrPSc stains in prion-infected CNs and brains of patients or animals affected by prion diseases suggest that the mechanism of PrPSc formation in CNs is, at least to some extent, similar to that in neurons in CNS.

In the previous reports using prion-infected primary cultured neurons, antimitotics were used throughout the culture period to suppress the growth of astrocytes (Carimalo et al., 2005; Cronier et al., 2007; Hannaoui et al., 2013), and some of them confirmed the neuronal predominance with astrocytes at 4%–5% of the cellular composition at an early stage (Cronier et al., 2004). However, antimitotics will be harmful to neurons depending on concentrations and the period of treatment. In the current study, we showed that temporary treatment of Ara-C successfully reduced astrocyte proliferation and enabled us to keep CNs up to 4 weeks after prion inoculation (Fig. 2). The CNs supported prion propagation with various prion strains including Obihiro strain that is not propagated well in N2a-3 and GT1-7 cells (Uryu et al., 2007). Difference in PrPSc stains in CNs infected with Obihiro strain (Fig. 4) may be partly due to a biochemical difference of PrPSc among prion strains. Furthermore,
PrP<sup>Sc</sup>-specific double staining using mAbs 132 and 8D5 suggested that CNs share the same mechanism for prion propagation with neurons in central nervous system. The presence of PrP<sup>Sc</sup> at neurites in the CNs is of interest, because neurodegeneration in prion diseases is reported to be initiated at axonal terminals (Gray et al., 2009), dendrites (Fuhrmann et al., 2007) or synapses (Jeffrey et al., 2000) rather than at the soma of neurons. Thus, neuron-enriched primary cultures used in the current study will be an invaluable ex vivo model for analyzing the mechanism of neurodegeneration caused by prion infection.

**Materials and methods**

**Antibodies**

MAbs 31C6, 132, and 8D5 that recognize epitopes consisting of mouse PrP aa 143–149, 119–127 (Kim et al., 2004), and 31–39 (Masujin et al., 2013), respectively, were used. MAb 110 that recognizes the epitope in the octapeptide repeat of PrP, PHGGGWG, at aa 59–65 and 83–89 was also used (Kim et al., 2004). For direct immunofluorescent staining of PrP<sup>Sc</sup>, mAbs 132 and 8D5 were conjugated with Alexa Fluor 647 and 488, respectively, using Alexa Fluor Carboxylic Acid Succinimidyl Ester mixed isomers (Life Technologies). The following antibodies were also used for IFA or immunoblot analysis: anti-microtubule-associated protein 2 (MAP2) chicken polyclonal antibodies (pAbs) (Abcam, ab5392), anti-GFAP rabbit pAbs (Dako, 0334), and anti-βIII-tubulin rabbit pAbs (Abcam, ab18207). MAb P2-284 against feline panleukopenia virus was used as an isotype control antibody (Horiuchi et al., 1997).
Cell lines

A subclone of the Neuro2a mouse neuroblastoma cell line, N2a-3 (Uryu et al., 2007), and a subclone of the GT1 hypothalamic neuronal cell line, GT1-7 (Schatzl et al., 1997), were used. ScN2a-3-22L (Nakamitsu et al., 2010), ScN2a-3-Ch (Uryu et al., 2007), and GT1-7 persistently infected with the 22L prion strain (ScGT1-7-22L) (Yamasaki et al., 2012) were used as prion-infected cells. Those cells were cultured as described previously (Uryu et al., 2007; Yamasaki et al., 2012) and used at passage history between 5 and 30.

Primary neuronal cell culture

Chamber covers of 8-well configurations (Matsunami), 96-well µ-plates (ibidi), and 24-well plastic culture plates were coated with 20 µg poly-L-lysine (Sigma) ml⁻¹ in PBS and settled overnight at room temperature (RT). After washing twice with sterilized de-ionized water, Neurobasal Medium (Life Technologies) containing 1 × B-27 Supplement (Life Technologies) and 6 mM GlutaMAX (Life Technologies) (hereafter Neuronal Medium) was added into each well and the plates were kept at 37 °C in 5% CO₂ atmosphere until use.

Primary neuronal cell cultures were prepared from ICR mouse embryos of embryonic day 14 (pregnant mice were purchased from Japan Clea Inc.). The experimental procedures have been approved by the Institutional Animal Care and Use Committee at Hokkaido University (No. 13-0141). The diencephalon, mesencephalon, medulla oblongata, and cerebellum were removed from embryonic brains, and the olfactory bulb, hippocampi, and meninges were resected under a stereo microscope. The remaining tissues including cerebral cortices were digested using the Neural Tissue Dissociation Kit (P) (Miltenyi Biotec) according to the manufacturer’s instructions but modified by enzymatic reactions being
performed on ice. Tissues were dispersed by gentle pipetting and filtrated through cell strainer with 100 µm nylon mesh (Corning). The cells were collected by centrifugation at 300 × g for 5 min at 4 °C. All procedures described above were performed using ice-cold Hank’s Balanced Salt Solution (Sigma) containing 100 µM HEPES (Life Technologies) and 10 µM sodium pyruvate (Life Technologies). Cells were resuspended in the Neuronal Medium and were plated at a density of 1.0 × 10^5 cells on poly-L-lysine pre-coated glass or plastic plates cm^2. Half of the culture medium was replaced with fresh Neuronal Medium every week. To inhibit astrocyte proliferation, Ara-C (Sigma) was added at a final concentration of 0.25 µM to cultures at 4 div. The Ara-C concentration was reduced by half accompanying the prion infection at 7 div, following which cultures were maintained in Ara-C free Neuronal Medium after 11 div (see Fig.2a).

Inocula containing prions were prepared from brains of mice infected with the 22L, Chandler, or Obihiro prion strains (all are mouse-adapted scrapie prions) at the terminal stage of the disease, and brains of age-matched, uninfected ICR mice were used as a control. The brains were homogenized in sterile PBS at a concentration of 10% w/v. The homogenates were sonicated for 3.5 min and centrifuged at 3,000 × g for 10 min at 4 °C. The resulting supernatant was centrifuged at 100,000 × g for 60 min at 4 °C, and the pellet containing microsomes was suspended in sterile PBS. The amount of PrP-res in the material was quantified by immunoblot analysis using purified PrP-res as a standard (Yamasaki et al., 2014a). Primary neuronal cell cultures at 7 div were exposed to the inocula equivalent to 5 ng of PrP-res per 1.0 × 10^5 cells by replacement of half of medium in each well. The medium was completely replaced by fresh Neuronal Medium at 4 dpi that corresponds to 11 div (see Fig. 2a).
Immunoblot analysis

Neurons cultured on 24-well plastic plates were washed with PBS and treated with 200 µl of lysis buffer (0.5% TritonX-100, 0.5% sodium deoxycholate, 150 mM NaCl, 5 mM EDTA, and 10 mM Tris-HCl [pH 7.5]) for 30 min at 4 °C. The cells were then lysed by one cycle of freeze-thaw and subsequent pipetting. The lysate was clarified by centrifugation at 2,000 × g for 5 min at 4 °C. Protein concentration of the lysates was measured using a DC protein assay kit (Bio-Rad) and adjusted to 0.3 mg ml⁻¹. For detection of PrP-res, PK were added to the lysates at a final concentration of 4% to the amount of total protein and incubated at 37 °C for 20 min. PK digestion was terminated by the addition of Pefabloc (Roche) to 1 mM, and the lysates were then treated with 50 µg DNase I ml⁻¹ at RT for 15 min. Precipitation of PrP-res by phosphotungstic acid (PTA) and following SDS-PAGE, Western transfer, and chemiluminescence detection were carried out as described elsewhere (Shindoh et al., 2009; Uryu et al., 2007). For detection of GFAP and βIII-tubulin, the procedures from PK digestion to PTA precipitation were omitted, and 2 µg of total protein was loaded onto each lane.

Immunoprecipitation

GT1-7 cells grown on 10 cm dish at approximately 80% confluence were used. After washing cells gently with cold PBS, cells were incubated with 15 µg of mAb at 4 °C for 1 h. After washing the cells three times, cells were collected by cell scraper and lysed with PBS containing 1% Zwittergent 3-14 (Calbiochem) and 1× cOmplete Protease Inhibitor Cocktail (Roche). The cell lysates were clarified by centrifugation at 500 × g for 10 min, the resulting supernatants (50 µg protein equivalent) were mixed with Dynabeads Protein G (Milteny) that
were blocked with 1% I-BLOCK (Applied Biosystems) and 20% N-101 (NOF Cooperation) and pre-incubated with anti-mouse IgG Fc-specific goat IgG. Mixtures were incubated at 4 °C for 90 min with constant rotation and then the beads were washed five times with PBS containing 1% Tween 20 using magnetic separator. Immunoprecipitates were eluted with the SDS sample buffer by boiling for 10 min and subjected to immunoblotting. Blots were probed with mAb 110 labeled with horseradish peroxidase (Horiuchi et al., 2009).

**IFA**

Primary neurons grown on 8-well chamber covers or 96-well μ-plates, and N2a-3 or GT1-7 cells grown on 8-well Lab-Tek II chambered coverglass (Nunc) were fixed with PBS containing 4% paraformaldehyde and 4% sucrose at RT for 10 min. Subsequently, procedures for IFA were carried out as described previously (Yamasaki et al., 2014a; Yamasaki et al., 2014b). In some cases, mAbs conjugated with Alexa Fluor fluorescence dye were used for the direct immunostaining of PrP Sc.

For the detection of PrP Sc on the cell surface, cells were gently rinsed with prewarmed sterile PBS and incubated with the Neuronal Medium containing 1 μg Alexa Fluor 488-conjugated mAb 8D5 ml⁻¹ at 37 °C for 1 h. The cells were gently rinsed and covered with PBS for the microscopic examination.

**Colocalization analysis**

3D fluorescent images were constructed from the Z-stack images and surfaces. The 3D bodies that were positive for each antibody, were identified by Imaris software version 7.6.1 (Bitplane). The minimum unit of the surfaces, termed voxel(s), which was positive for each
antibody, was counted for colocalization statistics.

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anti-prion mechanism of four different anti-prion compounds, anti-PrP monoclonal antibody 44B1, pentosan polysulfate, chlorpromazine, and U18666A, in prion-infected mouse neuroblastoma cells. *PLoS One* 9, e106516.

**Figure legends**

**Figure 1. PrPSc-specific staining in immortalized cell lines persistently infected with 22L prion strain.** (a) Detection of PrPSc in ScN2a-3-22L with [Gdn(+)] or without GdnSCN-pretreatment [Gdn(−)]. Alexa Fluor 647-conjugated mAb 132 (red) and Alexa Fluor 488-conjugated mAb 8D5 (green) were used for direct detection of PrPSc. Nuclei were counterstained with 4',6-Diamidino-2-phenylindole (DAPI) (blue). (b and c) Double staining of PrPSc using mAbs 132 and 8D5. PrPSc in GdnSCN pretreated ScN2a-3-22L cells (b) and ScGT1-7-22L cells (c) was simultaneously detected by Alexa Fluor-labeled mAbs 132 (red) and 8D5 (green). Magnified images of the boxed areas in the leftmost panels are shown in the three panels on the right. Arrowheads show representative granular stains with mAb 8D5 near the edges of cells. Arrows indicate representative overlapping stains with mAbs 132 and 8D5. Scale bars: (a and c) 10 µm, (b) 5 µm.

**Figure 2. Purity of primary neuronal culture from mouse cerebra.** (a) The scheme for the Ara-C treatment. Cells were treated with 0.25 and 0.125 µM of Ara-C from 4 to 6 and 7 to 10 div, respectively, and Ara-C was completely removed at 11 div, corresponding to 4 dpi. (b) Visualization of neurons and activated astrocytes in primary neuronal cultures. Mock-infected cultures at 7, 14, and 28 div were stained with MAP2 (gray), GFAP (red), and DAPI (blue). Scale bars: 50 µm. (c) Kinetics of the expression of GFAP and β-III tubulin.

**Figure 3. Generation of PrPSc in CNs.** (a) Kinetics of PrP-res generation in CNs. PK-treated cell lysates were subjected to SDS-PAGE and following immunoblot using anti-PrP antibody
mAb 31C6. Purified recombinant PrP (rPrP) (5 ng lane⁻¹) was used as a standard for the quantification. The sample at 0 dpi was harvested before the exposure to prions. Figures show representative immunoblot images of PrP-res. The graph on the right shows quantitative results (means and standard deviations of triplicate experiments, except 22L or Chandler strain-infected CNs at 35 dpi, which are shown as a single datum). Values indicate the total amount of PrP-res in each well. (b) PrPSc in CNs. PrPSc in prion-infected CNs was detected by PrPSc-specific immunostaining using mAb 132 (green) at 7, 14, and 28 dpi. Arrows indicate string-like PrPSc stains. Neuronal cell bodies and dendrites were identified by the staining of MAP2 (gray), and nuclei were counterstained with DAPI (blue). Scale bars: 10 µm.

**Figure 4. PrPSc-specific double staining of prion-infected CNs.** CNs infected with 22L, Chandler, or Obihiro prion strain were fixed and pretreated with GdnSCN and stained simultaneously with Alexa Fluor 647-conjugated mAb 132 (red) and Alexa Fluor 488-conjugated mAb 8D5 (green) at 14 dpi. Neurons were visualized with anti-MAP2 antibody (gray), and nuclei were stained with DAPI (blue). Boxed areas in merged images are enlarged in the corresponding rightmost columns. Closed arrows indicate string-like stains of PrPSc by mAbs 132 and 8D5, and open arrows indicate granular stains of PrPSc. Arrowheads show representative overlapping stains with mAbs 132 and 8D5. Scale bars: 10 µm.

**Figure 5. Detection of PrPSc on the cell surface of living CNs by mAb 8D5.** CNs at 14 dpi were incubated with Alexa Fluor 488-conjugated mAb 8D5 at 37 °C for 1 h without fixation, permeabilization, or denaturation. Upper panels show the signals detected by mAb 8D5
(green), and lower panels show the merged images with a differential interference contrast image (DIC). Scale bars: 20 µm

**Figure 6. Colocalization analysis of PrP$^{\text{Sc}}$ in cells.** (a) A scheme for the colocalization analysis. First, 3D-fluorescent images were constructed from Z-stack images, and fluorescence-positive 3D-regions, “surface”, were created by IMARIS software. Next, PrP$^{\text{Sc}}$-positive surfaces were broken down into voxel(s), the minimum unit of 3D-images, for following quantification. Voxel(s) positive for mAb 132, mAb 8D5, or both mAbs were counted, and the proportion to the total voxels positive for PrP$^{\text{Sc}}$ detected in cells was calculated by dividing the voxel number of each population by that of total PrP$^{\text{Sc}}$. (b) Proportions of PrP$^{\text{Sc}}$ populations with different immunoreactivity. Each bar shows the percentage of PrP$^{\text{Sc}}$ detected by only mAb 132 (red) or 8D5 (green), or both mAbs (yellow) in cells. The numbers in yellow bar indicate the percentage of PrP$^{\text{Sc}}$ positive for both mAbs. Cell types used for this analysis are shown at the bottom: ScN2a-22L and ScGT1-7-22L cells as immortalized cell lines; CNs infected with 22L (CN-22L), Chandler (CN-Ch), or Obihiro (CN-Obi) strain (at 14 dpi). (c) Proportions of PrP$^{\text{Sc}}$ detected by mAb 132 or mAb 8D5 in three cell types. The proportions of mAb 132-positive or mAb 8D5-positive PrP$^{\text{Sc}}$ to total PrP$^{\text{Sc}}$ in the corresponding cells were picked out from (b). Graphs represent the means and standard deviations of 9 wells from 3 independent experiments for N2a-3 and GT1-7 cells, and those of 6 wells from 2 independent experiments for CNs. Z-stack images were acquired from three to four view fields per well, which contained 10 cells for N2a-3, 7 cells for GT1-7, or 4 cells for CNs. Statistical analysis was done by one-way ANOVA, and *post hoc*
comparisons were carried out using Tukey-Kramer multiple comparisons test. *, \( p<0.05; \) ** \( p<0.01\).

**Figure 7. Biochemical properties of PrP molecules in cells.**

(a) Total PrP molecules in cells. PK-untreated cell lysates from N2a-3, GT1-7 and primary cerebral neurons (CNs, at 21 dpi) were subjected to immunoblotting using mAbs 110 and 31C6. Lysate equivalent to 3 \( \mu \)g total protein was loaded onto each lane. Arrows indicate full length PrP. Un, uninfected; Inf, infected with 22L prion strain; rPrP, recombinant PrP as a marker. (b) Immunoprecipitation of PrP\(^{Sc}\) with mAb 8D5. Immunoprecipitates from GT1-7 (GT1) or ScGT-17-22L (ScGT1) cells with mAb 8D5 or negative control mAb (NC) were subjected to immunoblotting using mAb 110 that recognizes the epitope in the octapeptide repeat at aa 59–65 and 83–89.
Fig. 1 Tanaka et al.
Fig. 2 Tanaka et al.

(a) Ara-C treatment (µM)

Plating (div) 0 0.25 0.125
Exposure to prions

(dpi) 0 4 7 11

(b) Ara-C (-) Ara-C (+)

7 div 14 div 21 div 28 div

(c) Ara-C (-) Ara-C (+)

GFAP 50 kD

β III-tubulin 50 kD

Ara-C (-) 7 14 21 28 35 42 (div)
Ara-C (+) 7 14 21 28 35 42 (div)
Fig. 3 Tanaka et al.
Fig. 5 Tanaka et al.
(a) Z-stack images → 3D-Construction & Quantification

<table>
<thead>
<tr>
<th>Total (T)</th>
<th>mAb 132 (+)</th>
<th>Co-localized</th>
<th>mAb 8D5 (+)</th>
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<td>X/T %</td>
<td>Y/T %</td>
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(b) Detected by:
- Only mAb 132
- Only mAb 8D5
- Both mAbs

(c) Detected by:
- Only mAb 132
- Only mAb 8D5
- Both mAbs

Proportion of PrPSc population:
- ScN2a-3
- ScGT1-7
- CNs
- CNs-Ch
- CNs-Obi

14 dpi

Infected with 22L strain

Fig. 6 Tanaka et al.
Fig. 7 Tanaka et al.