Chapter 14
Modeling the Cell Biology of Prions

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Abstract Cell models have been useful for elucidating the function of proteins and/or their role in pathogenesis. Even before the discovery that the prion protein was a normal cellular protein (Oesch et al., Cell 40:735–746, 1985), cell models were developed to investigate prion infection (Rubenstein et al., J Gen Virol 65:2191–2198, 1984). Subsequently, with the discovery of familial forms of human prion diseases (Hsiao et al., Nature 338:342–345, 1989), cell models were developed to investigate the effect of mutations on the metabolism of the prion protein and, in parallel, the normal synthesis and processing of the cellular prion protein. In this chapter, we review the progress made in these two areas to date.

Keywords Cell models · Prion protein · Prions · TSE agent replication · Cellular cultures · Pathogenic mutations

14.1 Cellular Cultures Supporting TSE Agent Replication

Cell cultures represent relevant and useful experimental models to study transmissible spongiform encephalopathies (TSEs) or prion diseases. Our current understanding of the cell biology of both the normal prion protein (PrP^{C}) and the pathogenic isoform (PrP^{Sc}) has utilized infected cell culture models. Cell culture models have also been useful in the development and validation of anti-prion drugs as well as offering an alternative approach to the transmission/infectivity assays historically performed in animal models. Cell culture models have also been used to

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study prion-induced cytopathological changes, which might help to explain the prion disease-associated neuropathogenesis observed in vivo.

Several cell culture models permissive to prion replication are available and some of them allow subpassaging to monitor stable and persistent replication of the infectious agent. The target cell type presumed to be most useful and informative would be cells of neuronal origin from the central nervous system (CNS), since the majority of prion infectivity is found in the CNS and the associated pathology is predominantly neurodegeneration. These include uncloned and cloned mouse neuroblastoma cell lines (N2a, C-1300, N1E-115) (Race et al. 1987; Nishida et al. 2000; Butler et al. 1988; Markovits et al. 1983; Ostlund et al. 2001) and murine GT1 hypothalamic neural cells (Schatzl et al. 1997). The GT1 cells are differentiated gonadotrophin-releasing hormone neurons, and in contrast to some of the neuroblastoma cell lines, they are susceptible to the 139A and 22 L mouse-adapted scrapie strains, as well as prions from familial GSS and sporadic CJD. GT1 cells are particularly useful for studying prion infection-associated cytopathic effects, since they become stably infected in contrast to N2a cells and, therefore, do not require periodic subcloning to maintain an infected culture (Nishida et al. 2000; Schatzl et al. 1997).

In addition, neuronal stem cells isolated from conventional or transgenic mice propagate mouse-adapted prions (Giri et al. 2006; Milhavet et al. 2006). Recently, hippocampal-derived HpL3-4 cells obtained from a PrP^C knockout mouse and transfected with mouse PrP^C were shown to be permissive to the mouse-adapted 22 L scrapie strain (Maas et al. 2007). Finally, cells from the peripheral nervous system, such as MSC80, murine Schwann-like cells, replicate low levels of the Rocky Mountain Laboratories (RML) mouse scrapie strain (Follet et al. 2002). Non-neuronal cell lines can also efficiently propagate prions. Common fibroblast cell lines (Vorberg et al. 2004), a microglial cell line (MG20) established from transgenic mice overexpressing PrP (Iwamaru et al. 2007), and PC12 rat pheochromocytoma cells (Rubenstein et al. 1984, 1991) are susceptible to various murine prion strains. Notably, the mouse-adapted bovine spongiform encephalopathy agent was successfully propagated in MG20 cells. Infection of a skeletal myoblast cell line (C2C12) was recently described (Dlakic et al. 2007), and could be used to investigate the mechanism underlying the prion infection of muscles observed in sheep and cervids.

One well-established feature of the animal bioassay is the species-specificity relationship between the source of the infectious agent and the recipient animal, which dictates both efficiency of infection and latency. In the cell system, both homologous (i.e., species matched) and heterologous (i.e., species mismatched) cell culture model systems have been successfully used. The rationale for using homologous cies-barrier phenomenon observed in animal bioassays in which the efficiency of infection is reduced if there are dissimilar primary amino acid sequences in the PrP of the species from which the prion agent and the host cells were derived. However, cell culture studies have demonstrated that this is not as straightforward as it seems. For example, only a limited number of mouse-adapted scrapie strains

can replicate in murine-derived host cell lines, and rat-derived PC12 cells can only be infected with selected mouse (but not rat)-adapted scrapie strains.

Although the source of the infectious agent is typically homogenized brain tissue originating from infected animals, partially purified preparations of scrapie-associated fibrils or PrP^{Sc} have also been used to achieve a higher-titer inoculum (Race et al. 1987). Cultures are either maintained in a nondividing, neuronal state, or passaged several times, and continually monitored for the disappearance (i.e., dilution) of the initial inoculum and appearance of de novo agent replication. To monitor propagation, cells are harvested at different times after exposure to the source of agent and cell lysates are used in animal bioassays. Alternatively, once it had been demonstrated that there is a close association between PrP^{Sc} and agent replication, the appearance and increase of the proteinase K (PK)-resistant PrP^{Sc} isoforms can be monitored by immunodetection and used as a biomarker of prion agent replication. Cell blotting techniques have been successfully used to detect PrP^{Sc} when only 1% of the cells are infected (Bosque and Prusiner 2000) and a filter retention assay for PrP^{Sc} (Winklhofer et al. 2001), which measures both PK resistance and presence of a detergent-insoluble aggregated state, has also been used. In addition, Vilette et al. have used a post-embedding method able to detect single infected cells (Vilette et al. 2001). This method has the advantage of evaluating the percentage of infected cells present in a particular culture. This is important when one considers the reports that for N2a cells only 1% of the cells were actually infected (Race 1991), although more efficient cell-culture models (Bosque and Prusiner 2000; Nishida et al. 2000) seem to have up to 30% of cells actually accumulating PrP^{Sc}.

The amount of infectivity present in the culture is also an important issue. Recent data on permissible cell lines revealed that cultures have the potential to accumulate as many infectious units per milligram of protein as brain from affected animals (Vilette et al. 2001).

Studying prion propagation in cell culture originally used animal-derived infected cells in which infected cultures were obtained from infected animals. The SMB cell line was established from the brain of a mouse clinically affected by the Chandler scrapie strain (Clarke and Haig 1970a, b). The majority of the initial studies on infecting cells in vitro used murine neuroblastoma cell lines (Race et al. 1987; Butler et al. 1988; Nishida et al. 2000; Markovits et al. 1983; Ostlund et al. 2001; Borchelt et al. 1990). Several investigators have described various biochemical and, at best, only subtle phenotypic differences in scrapie-infected cells. In addition, both increases and decreases in the rates of cell proliferation have been reported in infected cell lines. Unfortunately, it is not clear that the changes described were necessarily only due to the scrapie agent as opposed to clonal differences or to other factors present in the inoculum used to infect the cells. In addition, since the concentration of PrP has been shown to influence infectability, replication, and transmissibility of the prion agent in vivo, using an overexpressing cell line, such as the murine N2a neuroblastoma, allows these cells to be readily infected by the three mouse-adapted scrapie strains, Chandler, 139A, and 22 L (Nishida et al. 2000).

A common feature of susceptible cell lines is that they only support the propagation of TSE strains that have been experimentally adapted to rodents. Recently, Vilette et al. developed a new heterologous model for naturally occurring sheep scrapie. This model was obtained by stable expression of the ovine PrP gene in a rabbit epithelial cell line (RK13) (Vilette et al. 2001). The authors showed that the expression of heterologous PrP in an otherwise refractory system, such as the rabbit system, is sufficient to cross the species barrier ex vivo. The use of gene-edited mouse cell lines with murine prions has now been shown to have the potential to express bank vole and cervid PrP genes following CRISPR–Cas9 editing and lentiviral transduction, allowing for greater insight into chronic wasting disease and cross species comparisons (Walia et al. 2019). Further use of such methods may allow for diverse cellular protein platforms, providing cellular infection models with greater compositional diversity. Still, such models demonstrate the primacy of rodent models, where conventional editing techniques serve to modify such models rather than compete with this standardized modality.

Infected cell culture models have provided some valuable insights into the biogenesis of PrP^{Sc} in terms of conversion, subcellular localizations, physiopathological consequences, and species-barrier determinants. They have also contributed to the screening and the study of possible therapeutic compounds and to the development of new strategies for the investigation of TSE-specific biomarkers. Studies with infected cell cultures have shown that PrP^{C} and PrP^{Sc} are associated with the cell surface differently, since only the former can be released by phosphatidylinositol-specific phospholipase C treatment of intact infected cells (Caughey et al. 1990; Lehmann and Harris 1996). Analysis of several types of infected cells, including N2a, GT1, and HaB (Schatzl et al. 1997; Taraboulos et al. 1990), made it clear that PrP^{Sc} resides within the cell and accumulates in late endosomes and/or lysosomes (McKinley et al. 1991; Pimpinelli et al. 2005), where amino terminal trimming of PrP^{Sc} may occur (Caughey et al. 1991).

Furthermore, although PrP^{C} is rapidly synthesized and degraded, while the abnormal PrP^{Sc} isoform is relatively stable (Borchelt et al. 1990; Caughey et al. 1989; Nunziante et al. 2003), the infected cells do have the capacity, processing functions, and proteases to degrade PrP^{Sc} (Beringue et al. 2004; Enari et al. 2001; Feraudet et al. 2005; Peretz et al. 2001; Perrier et al. 2004).

The information obtained from the use of infected cell cultures to study events associated with neurodegeneration have been limited. Replication of the prion agent in cultured cells can result in specific alterations in cellular metabolism, some of which can affect cell survival. For instance, infection with several murine prion strains impairs the cellular response of GT1 and N2a cells to oxidative stress (Milhavet et al. 2000), presumably through a decrease in superoxide dismutase activity. It is interesting to note that prion-infected cell lines accumulating infectious titers similar to those in brain tissue do not show any obvious cytopathic effect, with the possible exception of RML-infected GT1 cells that undergo apoptosis inconsistently. The use of primary cultures may lead to a better understanding of the effect of prion agent replication on neuronal death. For example, infection of

primary cultures of neurons and astrocytes by a sheep scrapie agent resulted only in neuronal apoptosis involving JNK-c-Jun signaling (Cronier et al. 2004).

Numerous compounds have been used successfully to inhibit PrP^{Sc} formation in vitro, but the results in vivo have been disappointing. An example is provided by cellular heparan sulfates, which are sulfated linear polysaccharides typically linked to proteins to form heparan sulfate proteoglycans located at the cell surface (Turnbull et al. 2001). A number of studies suggest that heparan sulfates are involved in the biogenesis of PrP^{Sc} possibly by bringing together components involved in the conversion process, such as PrP^{C}, PrP^{Sc}, and other possible cofactors. A variety of sulfated glycans, including pentosan polysulfate (Birkett et al. 2001; Caughey and Raymond 1993), dextran sulfate 500 (Barret et al. 2003; Beringue et al. 2004; Caughey and Raymond 1993), and heparin (Gabizon et al. 1993), are potent inhibitors of PrP^{Sc} accumulation in several cell lines infected with murine prions presumably by competitive inhibition of cellular heparan sulfates for the binding to PrP^{C} (Gabizon et al. 1993).

The use of cell culture models to determine the therapeutic value of compounds in vivo has been disappointing. A large number of compounds have been found to inhibit PrP^{Sc} accumulation in prion-infected cultures, mainly in N2a cells (Kocisko et al. 2003); however, most of them showed no or very limited effects when subsequently tested in infected animals (Trevitt and Collinge 2006). This does not necessarily mean that infected cell models are not adequate to screen for anti-prion drugs, but rather indicates that prion propagation in organisms is a complex biological process. In addition to drugs, passive immunization with anti-PrP antibodies (Abs) has been tested in cell culture models (Enari et al. 2001; Peretz et al. 2001; Perrier et al. 2004; Gilch et al. 2003). These Abs significantly reduced prion agent replication in cell culture by preventing the conversion of PrP^{C} into PrP^{Sc} through blockage of PrP^{C}–PrP^{Sc} binding and/or by stabilizing the PrP^{C} on the cell surface. Although infected animals injected with antibodies did, under certain circumstances, show a modest increase in survival times, it did not reflect the extent demonstrated in cell culture (Sigurdsson et al. 2003; White et al. 2003).

The utilization of a cell culture system as a replacement for the expensive and time-consuming animal bioassay has been explored. However, this has been hampered because of low sensitivity due, in part, to the small percentage of cells actually infected (Race et al. 1987). The isolation of N2a subclones with higher permissiveness (Bosque and Prusiner 2000; Enari et al. 2001), along with improved detection of PrP^{Sc}, allowed the development of a quantitative, highly sensitive scrapie cell-based infectivity assay (SCA) for the RML murine prion strain (Klohn et al. 2003). Although the SCA is almost as sensitive as the mouse bioassay while being much less expensive and ten times faster, it is limited in that N2a cells are not permissive to natural strains of the infectious agents.

Further research is still needed for the development of better cell culture models. These models will be important tools to dissect the properties of the prion agents, including their molecular composition, the basis of cell permissiveness, and the identification of the biochemical and molecular mechanisms causing neuronal death. Some interesting studies along these lines have been reported. Weissmann's

group recently demonstrated that the composition of the glycan can affect infection efficiency (Browning et al. 2011). In another study, sialyation of the glycosylphosphatidyl inositol anchor was shown to play a significant role in PrP aggregation, which is associated with neurodegeneration (Bate and Williams 2012).

In addition, there has been progress through the use of unconventional nonmammalian models. Insight within evolutionary distant models has demonstrated the use of *Saccharomyces (S.) cerevisiae* as an effective, affordable screening method within the induction and propagation of prions (Ishikawa 2021). Traditionally, such models utilize [PSI+] and [URE3] yeast prions, leading to the identification of relevant therapeutics, allowing for the potential repurposing of conventional medications, such as the TLR7 agonist imiquimod or the calcium antagonist flunarizine (Oumata et al. 2013; Bamia et al. 2021). The use of these screening modalities has identified the relationship between yeast [PSI+] and the evolutionarily conserved protein folding activity of ribosome, demonstrating the continued value of utilizing nonmammalian cell models to elaborate upon the prion life cycle and potential corresponding treatments (Blondel et al. 2016; Banerjee and Sanyal 2014). Together, this understanding can present as a lower risk, lower cost means to identify homogeneity between eukaryotic prion cell models, granting alternative forms of modeling and treatment through enhanced screening methods.

14.2 Cell Models of Pathogenic Mutations in the Prion Protein

Following the discovery that PrP was a normal cellular protein, pathogenic mutations associated with familial prion diseases were discovered (Hsiao et al. 1989). This provided the opportunity to study the metabolism of the mutant protein in cell culture models with the hope that this might shed light on the conditions that lead to pathogenic conversion of the prion protein. Cell culture models had been used to study the metabolism of PrP in infected cells, which included a detailed study of the synthesis and processing of PrP^C (Caughey et al. 1989). PrP^C is modified in the endoplasmic reticulum by the addition of a glycosylphospatidyl inositol (GPI) anchor and the nonobligatory addition of *N*-linked glycans; there are two N-linked glycosylation sites in the prion protein (Robakis et al. 1986; Locht et al. 1986). The N-linked glycans serve as a major source of heterogeneity in the prion protein (Rudd et al. 1999). As mentioned above, the glycans appear to influence infection (Browning et al. 2011) and may provide the basis for strain determination, as previously conjectured (Rudd et al. 2001).

To study the effect of point mutations on the synthesis and metabolism of PrP^C, a variety of cell models have been established. (The insert mutation in the octapeptide repeat is not included based on the complexity of the clinical phenotype, see Solomon et al. (2010) for a review.) Some of the models use heterologous pairings of cells and homologues of the pathogenic human mutations (Lehmann and Harris

1995, 1996; Ma and Lindquist 2001). An alternative model, in which the human PrP-coding region was placed under the control of a strong promoter in an episomal vector (Petersen et al. 1996) and transfected into a human neuroblastoma cell line (M17), is the only cell model that has been validated by comparison with human tissue. The use of an episomal vector eliminates problems of copy number and integration site effects. In addition, the instantaneous rate of protein synthesis is the same for all constructs, so that modifications that may be concentration dependent, i.e., glycosylation, GPI anchoring, etc., are unaffected. In general, however, similar results have been obtained in all systems.

Detailed studies examining the metabolic defects associated with expression of mutant PrP, PrP^M, suggest that the effects of the mutations fall into two general categories (see Fig. 14.1). The first category includes mutations around the normal amino terminal cleavage site at residue 111/112, which includes those at codons 102, 105, and 117. In the human neuroblastoma cell models, these mutations do not appear to affect overall metabolism, but seem to cause an altered cleavage of PrP (Mishra et al. 2002) (RBP, unpublished). The truncated fragments are generally associated with Gerstmann–Straussler–Scheinker syndrome, a familiar form of prion disease that presents with prion plaques. Novel PrP fragments have been found in cells expressing the F198S, E200K, and Q217R mutations, indicating an alteration in protein processing (Zaidi et al. 2005; Capellari et al. 2000a; Singh et al. 1997).

The second category of mutations is clustered in and around the site of post-translational modifications that include nonobligatory addition of two *N*-linked glycans and the formation of a disulfide bridge. These mutations include those at codons D178N (Petersen et al. 1996), T183A (Capellari et al. 2000b), F198S (Zaidi et al. 2005), E200K (Capellari et al. 2000a), and Q217R (Singh et al. 1997). In this category, the mutations shared four common alterations in metabolism or processing: (1) PrP^M is unstable and degraded. This is particularly evident in the unglycosylated form of the PrP^M, which is virtually absent in some of these mutants. In addition, treatment with tunicamycin, which inhibits glycosylation, results in the rapid degradation of PrP^M compared to PrP^C in the secretory pathway. These results

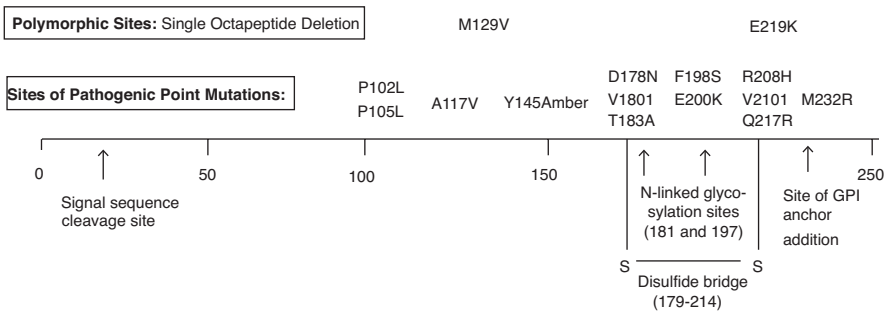

Fig. 14.1 Linear map of the prion protein showing a limited number of the pathogenic mutations in the human PrP as well as the sites of major post-translational modifications

support the role of *N*-linked glycans in facilitating protein folding. An early study suggested that loss of the first glycosylation site blocks transport of the mutant protein (Lehmann and Harris 1997); however, it was later established that the T183A mutation results in a structural change in the protein (Capellari et al. 2000b). (2) As a result of the decreased stability of the PrP^{M}, less PrP^{M} is found on the surface of cells expressing PrP^{M} and the ratio of glycoforms found at the cell surface parallels that found in the cells with marked underrepresentation of the unglycosylated form. Approximately 90% of PrP^{C} reaches the cell membrane, indicating that 10% of the normal protein fails to fold properly (Cohen and Taraboulos 2003). (3) A greater proportion of PrP^{M} partitions in a detergent insoluble fraction, indicating that PrP^{M} is aggregated in cells. (4) Most of these defects can be alleviated, in part, by incubating the cells at reduced temperature, 24 °C, suggesting that the processing defects arise due to misfolding of the PrP^{M}; misfolding has been shown to be partially corrected by reduced temperature (Singh et al. 1997). It is interesting to note that biophysical studies using recombinant PrP indicated that, with the exception of mutations at codons 183 and 198, these mutations do not appear to affect the physical properties of PrP^{M} versus PrP^{C} (Liemann and Glockshuber 1999).

In addition to the common changes resulting from the mutations, some of the mutants exhibited their own specific alterations. First, the Q217R mutation results in the production of a 32-kDa PrP lacking the GPI anchor, which attaches PrP to the cell surface (Singh et al. 1997). The F198S mutation results in the most profound reduction in the unglycosylated form of PrP^{M} (Zaidi et al. 2005). This arises for two reasons. First, the unglycosylated form is unstable, and second, the mutation replaces the phenylalanine residue, which is in the middle of the second glycosylation site, with a serine residue that is known to produce a more efficient glycosylation site. The F198S mutation also established that while the protein can achieve a normal conformation when expressed in a cell, after denaturation, it fails to refold into a native conformation (Zaidi et al. 2005). The E200K mutation, which is just beyond the second N-linked glycosylation site, results in the delayed maturation of PrP^{M} and the production of an abnormally modified glycan that is observed by its abnormal migration in SDS gels (Capellari et al. 2000a). Finally, comparison of the D178N/129 M mutation (FFI) and D178N/129 V mutation (CJD^{178}) did not exhibit specific differences, although the reduction of the unglycosylated form was more pronounced in the FFI expressing cells (Petersen et al. 1996). It is interesting to note that the prion disease referred to as sporadic fatal insomnia is also linked to codon 129 methionine, indicating that methionine may be required for the initiation of the disease process in the thalamus (Parchi et al. 1999).

Although some of the mutations studied resulted in general and specific changes in the metabolism of PrP^{M}, such as aggregation, none of the transfected human neuroblastoma lines produced bona fide protease-resistant PrP (PrP^{Sc}), as assessed by the gel migration pattern or infectivity. In fact, using an antibody that recognizes the carboxyl terminal region of PrP, wild-type PrP is as resistant to proteinase K treatment as PrP^{M} (Capellari et al. 2000a). The carboxyl terminal region of PrP is inherently resistant to protease digestion, and mutations in the region of post-translational modification appear to extend the tertiary structure through residues 90–112 that are typically unstructured. Thus, the weak protease resistance that has been associated

with PrP^{M} expressed in cell culture probably reflects a conformational change of the protein. In retrospect, it is not surprising that the cell culture models expressing the mutant PrP failed to produce PrP^{Sc}. The inherited human prion diseases are age-related diseases, so while the mutations may be necessary for the development of disease, they are clearly not sufficient. This suggests that some age-related deficit in the cellular repair/defense mechanisms is required to enable the initiation of the disease process.

While the cells expressing the mutant PrP grow normally, inhibition of the proteasomal degradation has been observed to result in neuronal cytotoxicity. The first observation of PrP^{M} accumulation was in cells expressing the nonsense mutation at codon 145, Y145Stop (Zanusso et al. 1999), in which the mutant protein accumulated in the cell after inhibition of the proteasome with lactacystin. Subsequent studies demonstrated that the codon 177 murine homologue of the human D178N mutation also accumulated in cells, even in the absence of proteasome inhibition (Ma and Lindquist 2001), and that this resulted in neurotoxicity (Ma et al. 2002).

In addition to conventional cell models, cerebral organoid (CO) creation has appeared as a frontier within prion modeling (reviewed in Walters and Haigh 2022). Utilizing induced pluripotent stem cell technology, complex, three-dimensional organoids now illustrate prion pathology, metabolism, and pharmacology within neuronal lineage cells (Groveman et al. 2021). Through their ability to model structures directly mimicking functional neuroanatomy, CO demonstrate the potential for personalized drug screening within familial prion diseases, offering increased therapeutic relevance within such schemes (Pineau and Sim 2021). Yet, despite their conservation of functional–structural relationships, these models face challenges seen within conventional cell culture models, such as the inability of mutations to induce a disease state independent of relevant aging processes or genetic modifiers. Such limitations were demonstrated by the insufficiency of CO generated from E200K asymptomatic donors to present disease isoforms 12 months post-differentiation (Foliaki et al. 2020). Furthermore, issues with diffusion and the lack of non-neuronal lineage cells present challenges in accurately representing human anatomical composition, where further developments, such as 3D-printed vasculature and regional specification, may alleviate current compositional concerns (Groveman et al. 2021). Collectively, these notions demonstrate the therapeutic potential seen in the correspondence between CO models and human tissue while still acknowledging the reality that mutations independent of aging or genetic determinants fail to reproduce the clinical entity demonstrating the need to further refine CO models.

14.3 Conclusion

Cell models have been invaluable for studying the infectious process at a cellular level. The infected cell model is currently facilitating studies that will help clarify the origin of prion strains. The cell models of the pathogenic prion mutations

indicate that the mutations are not sufficient to produce the disease-associated form of PrP, but show the potential for chronic stress in the secretory pathway that may facilitate the disease process.

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