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SHORT COMMUNICATION

Human Tonsil-Derived Follicular Dendritic-Like Cells are Refractory to Human Prion Infection *in Vitro* and Traffic Disease-Associated Prion Protein to Lysosomes

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The molecular mechanisms involved in human cellular susceptibility to prion infection remain poorly defined. This is due, in part, to the absence of any well characterized and relevant cultured human cells susceptible to infection with human prions, such as those involved in Creutzfeldt-Jakob disease. In variant Creutzfeldt-Jakob disease, prion replication is thought to occur first in the lymphoreticular system and then spread into the brain. We have, therefore, examined the susceptibility of a human tonsil-derived follicular dendritic cell-like cell line (HK) to prion infection. HK cells were found to display a readily detectable, time-dependent increase in cell-associated abnormal prion protein (PrP^{TSE}) when exposed to medium spiked with Creutzfeldt-Jakob disease brain homogenate, resulting in a coarse granular perinuclear PrP^{TSE} staining pattern. Despite their high level of cellular prion protein expression, HK cells failed to support infection, as judged by longer term maintenance of PrP^{TSE} accumulation. Colocalization studies revealed that exposure of HK cells to brain homogenate resulted in increased numbers of detectable lysosomes and that these structures immunostained intensely for PrP^{TSE} after exposure to Creutzfeldt-Jakob disease brain homogenate. Our data suggest that human follicular dendritic-like cells and perhaps other human cell types are able to avoid prion infection by efficient lysosomal degradation of PrP^{TSE}. (*Am J Pathol* 2014, 184: 64–70; <http://dx.doi.org/10.1016/j.ajpath.2013.09.013>)

Variant Creutzfeldt-Jakob disease (vCJD) is a fatal neurodegenerative disease resulting from oral infection with the bovine spongiform encephalopathy (BSE) agent.¹ BSE and vCJD belong to a group of transmissible spongiform encephalopathies (TSE) in which the infectious agent or prion is believed to be a conformationally altered and aggregated form prion protein TSE (PrP^{TSE}) of the host-encoded cellular prion protein (PrP^C), replicated by a templated conformational conversion process that resembles seeded aggregation.² vCJD involves the lymphoreticular system, probably before neuroinvasion and the subsequent appearance of neurological symptoms.^{3,4} Accumulation of PrP^{TSE} in the follicular dendritic cells (FDCs) residing in the germinal centers in the tonsil, spleen, appendix, and lymph node is a consistent feature of vCJD pathology.^{5,6} Exposure of the United Kingdom population to BSE is thought to have been widespread and yet the resultant vCJD epidemic has thus far been

limited to 177 cases [National CJD Research & Surveillance Unit (NCJDRSU), <http://www.cjd.ed.ac.uk/documents/figs.pdf>, last accessed August 30, 2013]. All clinical cases of definite vCJD that have been tested are of the prion protein gene *PRNP* codon 129 *MM* genotype (NCJDRSU, <http://www>.

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This is an independent report and the views expressed in the publication are those of the authors and not necessarily those of the Department of Health, United Kingdom.

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cjd.ed.ac.uk/documents/report20.pdf, last accessed August 30, 2013). However, studies based on PrP^{TSE} immunostaining of routine appendectomy, tonsillectomy, and autopsy tissues indicate that the prevalence of infection in the United Kingdom is higher than the incidence of clinical vCJD would seem to suggest and indicates that all three possible *PRNP* codon 129 genotypes (*MM*, *MV*, *VV*) are susceptible to infection with the vCJD/BSE agent.^{7–11} [Summary results of the second national survey of abnormal prion prevalence in archived appendix specimens are available online (Health Protection Report, <http://www.hpa.org.uk/hpr/archives/2012/news3212.htm#bnrmlprn>, last accessed August 30, 2013)]. The mechanism by which cells interact with prions is only beginning to be defined.¹² Despite a wealth of data showing that cultured cells of a variety of species and phenotypes can propagate animal prions, evidence that cultured human cells have been infected with human prion agents is restricted to a single unconfirmed report from 1995. In this report, Ladogana et al¹³ described infection of the human neuroblastoma SH-SY5Y cell line (*PRNP* codon 129 genotype *MM*) with a brain homogenate from a sporadic CJD patient (also *PRNP* codon 129 genotype *MM*). However, the infection proved to be unstable¹³ and this initial success in infecting human cells with the CJD agent has not been capitalized on. Interestingly, RK13 rabbit kidney epithelial cells expressing human PrP were found to be resistant to prion infection when exposed directly to sporadic CJD prions.¹⁴

Our previous investigations of human embryonic stem cells (hESC) showed extensive and rapid uptake and clearance of PrP^{TSE} present in crude homogenates of bovine and human prion disease brain tissue added directly to culture medium.¹⁴ Uptake and clearance in these hESC did not appear to depend on the species, form of CJD (sporadic or variant), or the *PRNP* codon 129 genotype of the inoculum or the hESC, suggesting involvement of general rather than specific uptake mechanisms.¹⁵ This is consistent with earlier observations made using rodent adapted scrapie prions and rodent cell lines.¹⁶ Considering the involvement of the lymphoreticular system in vCJD, we chose to investigate the mechanisms involved in human cellular susceptibility to prion infection and PrP^{TSE} intracellular fate using the human cell line HK, which is derived from human tonsillectomy tissue and shares features with FDCs.¹⁷ Our results demonstrate that the human FDC-like HK cells, as far as we can determine, are resistant to prion infection *in vitro* and that the endosome/lysosome compartments are the likely sites of PrP^{TSE} intracellular trafficking and degradation.

Materials and Methods

Cell Culture

The HK cell line [a gift from Dr. Yong Sung Choi (Alton Ochsner Medical Foundation, New Orleans, LA)] was isolated from human tonsils and cultivated as previously described.¹⁷ For studies involving confocal microscopy, cells were detached with

0.05% Trypsin-EDTA (Invitrogen, Paisley, UK) and plated onto pre-coated glass chamber slides (Nunc Lab-TekII CC2, Fisher Scientific, Loughborough, UK) 1 day before exposure.

Brain Tissue

Brain tissue from autopsy-proven, well-characterized cases of definite vCJD, iatrogenic CJD (iCJD) associated with human growth hormone therapy, Alzheimer disease (each with consent for use in research), and cattle BSE were selected for the study. The details are summarized in [Supplemental Table S1](#). All human tissues were obtained from the Edinburgh Brain Bank (LREC 2000/4/157) and were handled exclusively in a category 3 biosafety containment facility according to stringent health and safety protocols. The cattle BSE brain tissue was generously provided by the Animal Health Veterinary Laboratory Agency TSE Archive (Weybridge, UK). Detection of PrP^{TSE} in prion disease brain homogenates was confirmed by proteinase K (PK; VWR International, Lutterworth, UK) digestion, and immunoblot analysis as previously described.¹⁸

PRNP Codon 129 Genotyping

HK cells had their *PRNP* codon 129 genotype determined as previously described.¹⁴

Brain Spiked Medium

For all experiments described in this article, brain tissues were homogenized (10% w/v) at 4°C in PBS (Invitrogen) containing 5% glucose (Fisher Scientific) and cleared by centrifugation at $424.16 \times g$ for 5 minutes at 4°C, then disrupted by sonication (Sonicator model 3000; Misonix, Farmingdale, NY) at 300 W for 1 minute, and diluted into culturing medium to a final concentration of 1%. Other preparative methods used in the challenge experiments were as described in [Supplemental Tables S2, S3, S4, S5, S6, S7, and S8](#).

HK Cell Challenges

HK cells were routinely cultured in Corning T25 cell culture flasks (Fisher Scientific) and after reaching 50% to 60% confluence they were exposed to brain-spiked media, which was prepared according to the methodological variations summarized in [Supplemental Tables S2, S3, S4, S5, S6, S7, and S8](#). Every challenge was performed in duplicate and one flask was used for further subcultivation, whereas the other flask was used to prepare a cell lysate for immunoblot analysis.

Immunoblot Analysis

Exposed cells were washed twice with PBS at 4°C, then lysed for 15 minutes on ice with extraction buffer (0.5% NP-40, 0.5% sodium deoxycholate, PBS-T pH 7.4), and collected using a cell scraper into a safe-lock tube. The samples were then digested with proteinase K at a concentration of 50 µg/mL at 37°C for 60 minutes and digestion was terminated with 1

mmol/L Pefablock SC (Roche, West Sussex, UK). Samples were centrifuged at $20817.16 \times g$ for 60 minutes at 4°C and the pellets analyzed by immunoblot as previously described.^{6,18,19}

Immunofluorescence

Immunofluorescence on the cells exposed to 1% (w/v) brain homogenate for specified time periods was performed as previously described.¹⁴ Briefly, cells were extensively washed with PBS, fixed with 4% paraformaldehyde (Sigma-Aldrich, Dorset, UK), and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS. If proteinase K pretreatment was included, then the cells were incubated with 0.3 $\mu\text{g}/\text{mL}$ for 9 minutes at 37°C and digestion was stopped with 1 mmol/L Pefabloc SC for 5 minutes at room temperature. The cells were then washed with PBS and blocked with 3% bovine serum albumin (BSA; Invitrogen) in Dulbecco's phosphate buffered saline (DPBS; Invitrogen) and incubated with anti-prion protein monoclonal antibody 6H4 (Prionics, Schlieren-Zurich, Switzerland) or 8H4.²⁰ In some experiments, the cells were incubated with antibodies to late endosomal/lysosomal-specific proteins Lamp1 or Lamp2b (Abcam, Cambridge, UK) or glial fibrillary acidic protein (GFAP; Dako, Ely, UK). Then the cells were washed and incubated with the fluorescein isothiocyanate secondary antibody, Alexa 488 (Invitrogen) labeling the PrP and Alexa 546 (Invitrogen) revealing late endosomes/lysosomes or GFAP. The nuclei were counterstained with DAPI (Invitrogen). Slides were mounted with Vectashield (Vector Laboratories Ltd., Peterborough, UK) and examined by confocal microscopy.

Confocal Microscopy, Particle-Based Colocalization, and Statistical Data Analysis

Immunostained cultures were examined by laser scanning confocal microscope NIKON Eclipse TE2000-U with a 40×0.75 Plan Fluor DIC M/N2 dry and 60×1.40 Plan Apo VC oil objectives. Images were exported via EZ-C1 software Gold version 3.30 and analyzed using ImageJ version 1.45s (NIH, Bethesda, MD). Z-stacks were acquired with Z-steps of 0.25 μm . Colocalization was assessed using the particle-based approach²¹ using ImageJ plugin JACoP. Manders' colocalization coefficient²² was then calculated and values were plotted on a graph using Microsoft Office Excel (Redmond, WA).

Results

Human FDC-Like HK Cells Are Resistant to Infection with Human Prions *in Vitro*

The HK cells were found to express readily detectable levels of PrP^C as judged by Western blot analysis (Figure 1A). Direct immunoblot comparison showed the PrP^C level to be equivalent to or greater than that found in the normal human

brain reference standard (NHB20/0005) obtained from the National Institute for Biological Standards and Control TSE Resource Centre²³ (data not shown). The *PRNP* codon 129 genotype of the HK cell line used was found to be of VV. Uptake of exogenous PrP^{TSE} was found to be rapid and the amount of HK cell-associated PrP^{TSE} increased steeply with time of exposure to vCJD brain-spiked medium (Figure 1B). Dual immunolabeling for PrP^{TSE} and GFAP (Figure 1C) showed that both proteins accumulated in the cytosol in a partially overlapping pattern. Uptake, therefore, did not appear to be PrP-specific, rather it suggested a nonspecific uptake of complex brain homogenate components. Confocal microscopy Z-stack sections confirmed the intracellular localization of PrP^{TSE} and showed a coarse granular perinuclear appearance of the intracellular PrP^{TSE} (Figure 1D).

HK cell cultures were tested for their ability to support a prion infection by exposure to crude homogenates of CJD brain (including vCJD *MM* and iatrogenic CJD of the VV and MV genotypes) and BSE brain (Supplemental Table S1), followed by assessment of cellular PrP^{TSE} by Western blot analysis and immunocytochemistry. A wide variety of inocula preparations and exposure protocols were used based on the previously successful infection of other cell types (Supplemental Tables S2, S3, S4, S5, S6, S7, and S8).^{12,24–34} No conditions were found that established a stable prion infection in HK cells, as judged by the failure of HK cell cultures to maintain PrP^{TSE} levels in the days to weeks after exposure to CJD brain inoculum. An example of an immunoblot of HK cells exposed to iCJD brain inoculum with a *PRNP* codon 129 genotype matching that of the HK cells (VV genotype) and analyzed at specific time points is shown in Figure 1, E and F. This showed increased cell-associated PrP^{TSE} initially, but the signal gradually diminished over time when the cells were grown in fresh (unspiked) medium. After the first passage, the cell-associated PrP^{TSE} could no longer be detected in the cell lysate (Figure 1E). In case cellular infection was a very rare event, requiring time to become established and spread throughout the culture, we continued to culture the cells until they were senescent (>170 days). However, PrP^{TSE} could not be detected in these aged cultures by immunoblot analysis (Figure 1F). Immunocytochemical analysis of the HK cells exposed to iCJD was carried up to the third passage postexposure. No evidence of residual PrP^{TSE} was found (data not shown). If residual (or newly formed) PrP^{TSE} was present in these cultures, it was at levels below those detectable by Western blot analysis and immunocytochemistry. Therefore, despite being cells with a phenotype similar to those susceptible to BSE infection *in vivo*, HK cells appeared resistant to infection *in vitro*, even when there was no species barrier (exposure to vCJD brain homogenate) or when the *PRNP* codon 129 genotype was matched (exposure to iCJD VV2 brain homogenate). Therefore, we decided to investigate the fate of the exogenous PrP^{TSE} taken up by HK cells exposed to the vCJD inoculum.

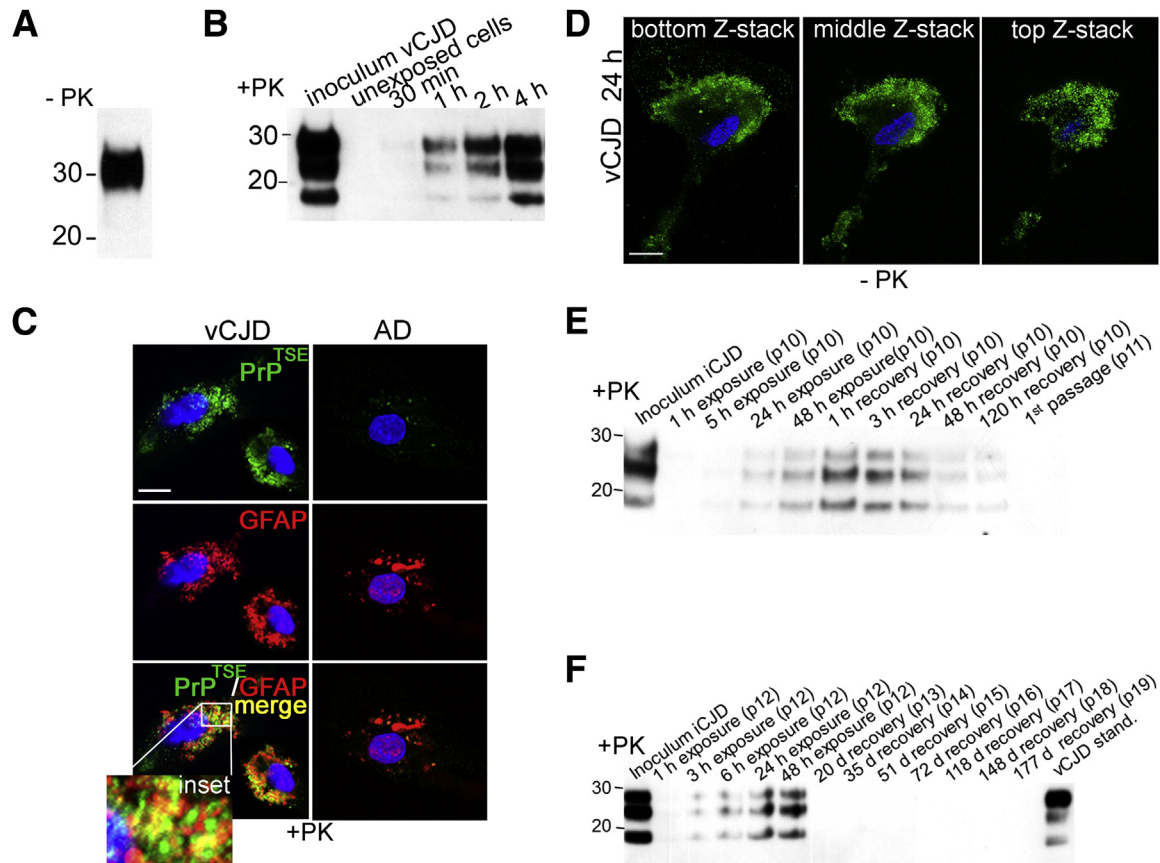


Figure 1 **A:** PrP^C in HK cells was analyzed by immunoblot analysis using anti-PrP mAb 3F4. **B:** The time-dependent uptake of PrP^{TSE} (mAb 6H4) by HK cells exposed to vCJD brain homogenate. **C:** Uptake of PrP^{TSE} (mAb 6H4, green) and GFAP (red) by HK cells demonstrated by immunofluorescence of cells exposed to vCJD and Alzheimer disease (AD) brain homogenate for 48 hours. Nuclei were counterstained with DAPI (blue). **D:** Immunofluorescence analysis of the intracellular accumulation of the exogenous PrP^{TSE} in HK cells exposed to vCJD for 24 hours by Z-stack analysis (optical sectioning), allowing visualization of different levels of the immunostained cell with the bottom Z-stack, middle Z-stack, and top Z-stack. PrP mAb 8H4 (green). Nuclei were counterstained with DAPI (blue). **E:** Immunoblot analysis of the uptake and clearance of PrP^{TSE} from HK cells exposed to iCJD brain-spiked medium (inoculum iCJD) for indicated times, followed by indicated recovery times (up to 120 hours). Subsequently the cells were split at a ratio of 1:2 and grown until confluence at which time point they were also analyzed. The anti-PrP mAb used was 6H4. **F:** Immunoblot analysis showing the uptake and clearance of PrP^{TSE} from HK cells and their failure to support prion propagation. Cells were exposed to iCJD₁ brain-spiked medium for 48 hours and then cultured in fresh medium and passaged until senescence (177 days post challenge, at passage 19). Samples were taken for immunoblot analysis at the stated number of hours (h) of exposure and at subsequent stated days (d) postexposure. The passage number (p) is also given. The anti-PrP mAb used was 6H4. The molecular mass in the immunoblots (**A**, **B**, **E**, and **F**) is marked in kDa (**left**) on each blot. Scale bars: 20 μ m (**C** and **D**).

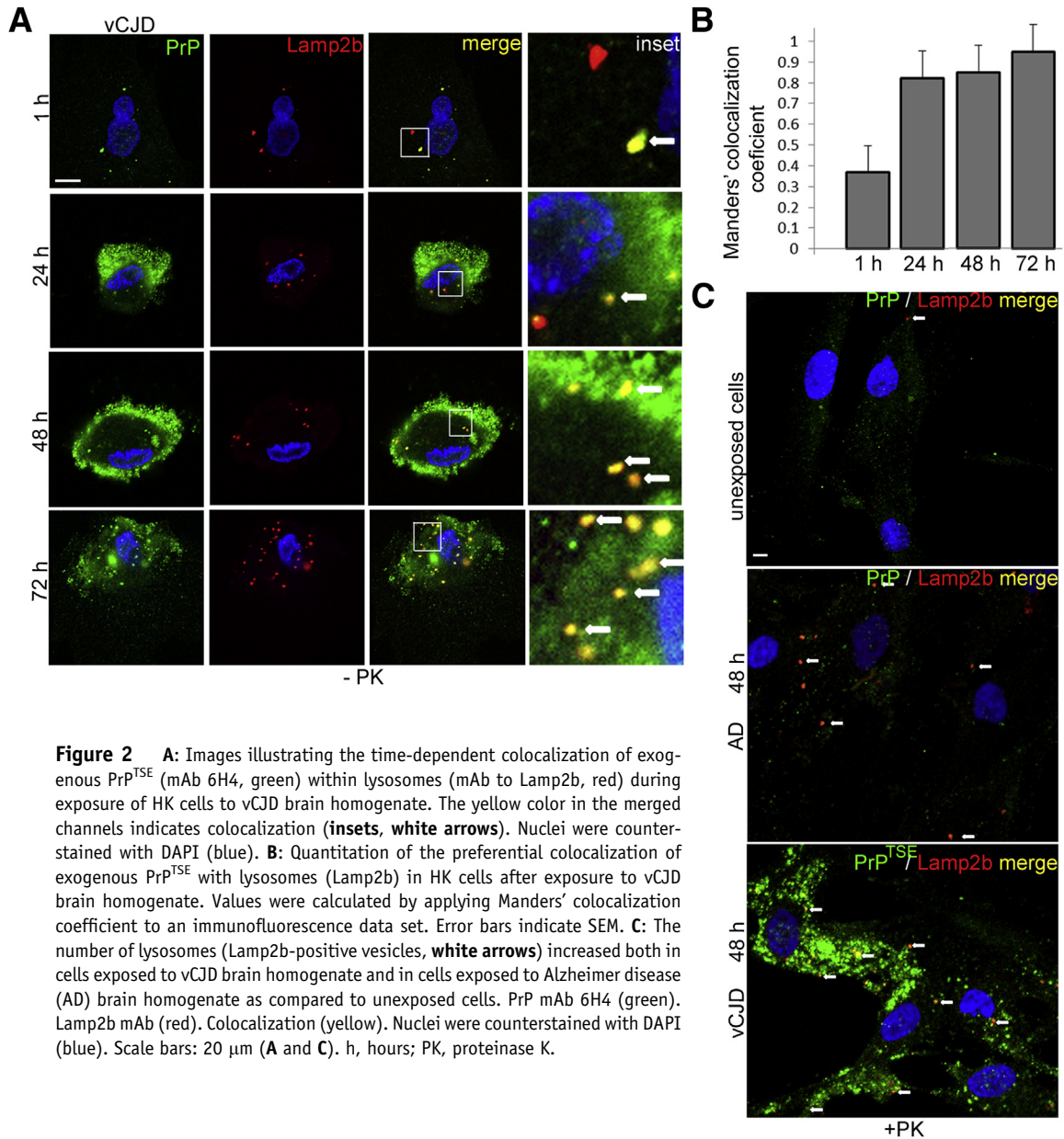
Late Endosomes/Lysosomes Are a Destination of Exogenous PrP^{TSE} in HK Cells

The intracellular fate and the subcellular site of the endocytosed exogenous PrP^{TSE} was investigated after vCJD brain homogenate exposure using immunohistochemistry to colocalize PrP^{TSE} with a range of markers of different cellular compartments including endoplasmic reticulum, golgi apparatus, early endosome, and recycling endosomes. Only in the case of the late endosomal/lysosomal compartment was there clear evidence of colocalization found (Figure 2A). The cells were immunolabeled for PrP [monoclonal antibody (mAb) 6H4] and lysosomes (mAb against Lamp2b). A time-dependent increase of cell-associated PrP^{TSE} was clearly observed and an apparent colocalization was seen in the merged channels. Almost all visible Lamp2b-labeled structures were positive for exogenous PrP^{TSE} at 48 and 72 hours postexposure. The observation was confirmed

using an antibody against Lamp1 (data not shown). However, the PrP^{TSE} localizing with markers of lysosomes only represents a small proportion of detectable intracellular PrP^{TSE}. The exogenous PrP^{TSE} colocalization with lysosomes was assessed statistically using a particle-based colocalization method (Figure 2B). The graph confirms an increasing trend of PrP^{TSE} positive lysosomes with time of exposure. The data are representative of seven independent, but nonidentical experiments. We also found that the number of Lamp2b-positive structures within the cell increased with time of exposure and that this was a general response to exposure to brain homogenate, occurring after exposure to Alzheimer disease, as well as vCJD brain homogenate (Figure 2C).

Discussion

The known involvement of FDCs in vCJD and the high levels of PrP^C expressed by FDC-like HK cells suggested



that these cells might be susceptible to prion infection and competent to propagate the infection *in vitro*. We tested this hypothesis directly by challenging HK cells with prion disease brain homogenates. Despite using a wide variety of inocula types (BSE, vCJD, and iCJD) and different preparation and exposure protocols in our attempts to infect HK cells with prions, no condition could be found in which cell-associated PrP^{TSE} was maintained, even when the *PRNP* codon 129 genotype of the cells and the inoculum was matched and without species barrier (iCJD VV). This led us to conclude that HK cells are either not competent to support prion replication or that HK cells, similar to hESC, have mechanisms that allow for the uptake, but also efficient mechanisms to effect clearance of exogenous PrP^{TSE}.

Dual immunolabeling for PrP^{TSE} and GFAP indicated that the uptake of material from brain homogenate is likely via a

general rather than a PrP-specific uptake mechanism. Judging by the increase and subsequent loss of cell-associated PrP^{TSE} from the cultures exposed to prion disease brain-spiked medium, it appears that PrP^{TSE} is most likely actively internalized and then either degraded or excreted by the cells. The intracellular fate of the endocytosed exogenous PrP^{TSE} was investigated after exposure to vCJD brain homogenate and this showed that exogenous PrP^{TSE} was being trafficked to the late endosomal/lysosomal compartment. The combination of i) an increased number of lysosomes in these cells in response to exposure to brain homogenate, ii) a proportion of PrP^{TSE} localizing to this compartment, and iii) the subsequent loss of PrP^{TSE} from these cells is strongly suggestive of lysosomal degradation of PrP^{TSE}. Definitive demonstration of a role for lysosomal degradation of PrP^{TSE} would require inhibition of lysosomal function, nor on the basis of this current data can we

exclude the involvement of other systems in processing PrP^{TSE}, however, the data we show here are entirely consistent with previous studies implicating lysosomal proteases in PrP^{TSE} degradation in rodent cells.^{35–41} Our observation is also in agreement with a very recent report showing that in rodent cell cultures with a stimulated autophagic pathway, fluorescently labeled PrP^{TSE} is not processed via autophagy, but rather it is directly targeted for degradation to lysosomes.⁴²

In summary, to our knowledge this is the first report of the fate of PrP^{TSE} from a naturally infectious human inoculum in a physiologically relevant human cell line. Internalization of misfolded PrP and its subsequent degradation in the lysosomal compartment might function as a self-protective cellular mechanism, serving to eliminate non-native, presumably dysfunctional, and potentially dangerous PrP conformers, whether generated endogenously or acquired through exposure to exogenous prion infectivity. The efficient degradation of exogenous PrP^{TSE} shown here appears to be at variance with the hypothesis previously advanced that the late endosome/lysosome compartment is the site of PrP^C to PrP^{TSE} conversion and that lysosomes function as the bioreactor of PrP^{TSE} formation during scrapie pathogenesis.^{43,44} The efficient uptake and clearance of PrP^{TSE} by HK cells after acute exposure *in vitro* also contrasts markedly with the accumulation of PrP^{TSE} in FDCs in vCJD patients. These differences might be attributed to the postmitotic status of FDC *in situ* or they may be taken to indicate that HK cells share some, but not all phenotypic traits of FDCs. The derivation of FDCs, neurons, and glia from human embryonic or human-induced pluripotent cell lines of defined *PRNP* codon 129 genotype will allow the investigation of the balance of prion uptake, replication, and/or degradation in cells more closely resembling their *in vivo* counterparts.

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Z.K. performed all experiments, collected and analyzed data, and generated the figures, and the manuscript was written by M.W.H. and Z.K. with the involvement and approval of P.DeS., J.M., and J.W.I.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.09.013>.

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