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Replication of prions in differentiated muscle cells

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We have demonstrated that prions accumulate to high levels in non-proliferative C2C12 myotubes. C2C12 cells replicate as myoblasts but can be differentiated into myotubes. Earlier studies indicated that C2C12 myoblasts are not competent for prion replication.[1] We confirmed that observation and demonstrated, for the first time, that while replicative myoblasts do not accumulate PrP^{Sc}, differentiated post-mitotic myotube cultures replicate prions robustly. Here we extend our observations and describe the implication and utility of this system for replicating prions.

Keywords: prion, prion replication, muscle, C2C12, myotube, PrP, Prnp, differentiation, scrapie

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Although a number of cell lines have been developed or identified that can replicate prions, most cell lines are not permissive to prion accumulation despite demonstrable prion interaction and uptake. Why are some cells permissive and others resistant to prion accumulation? One factor as suggested by Ghaemmaghami et al.[1] is that cellular division rate competes with prion replication rate. The balance between the two is the steady-state level of PrP^{Sc}. In addition to prion replication permissivity, some cell lines also discriminate in their ability to replicate different prion strains. Given that the host cell is apparently competent for replication of one strain of prion, but resistant to others, a reasonable generic explanation may lie in the difference of replication rate between the two strains, the "faster" strain able to out replicate cell division and the slower strain lost by the dilutive effect of cell proliferation. In our recent manuscript[2] we show that C2C12 myotubes can replicate mouse prions to high levels. This system takes advantage of the fact that myotubes are differentiated non-proliferative cells, removing the dilutive effect of cell replication from prion amplification. The result is robust accumulation of PrP^{Sc} and infectivity.

C2C12 cells are grown as proliferative myoblasts and as long as they do not reach confluence, cells can be passaged and expanded indefinitely. Upon reaching confluence, however, myoblasts begin to differentiate, fusing into elongated, multinucleated, and occasionally contractile fibers. This differentiation is stimulated by withdrawal of growth factors and involves the coordinated expression of many muscle specific genes. Our studies were not the first to examine C2C12 cells for the ability to replicate prions. Dlakic et al.[3] examined prion replication in C2C12 myoblasts using co-infection with N2a neuroblastoma cells. Co-culture was necessary to maintain prion infectivity as myoblasts were not capable of replicating prions. Ultimately, Dlakic et al., were able to clear the co-culture of N2a cells by antibiotic selection against sensitive N2a cells. Clearly, however, muscle is competent for prion replication and accumulates substantial titers in vivo.[4]

In our approach, by first differentiating confluent cultures of myoblasts into multi-nucleated myotubes, the cell line becomes infectable through simple addition of infectious brain homogenates. We routinely obtain infected cell layers by incubation with 0.1% (w/v) whole brain homogenate (equivalent to approximately 1×10^6 LD_{50}/mL of infectious prions). Although further dilution of the infectious agent is possible, below a multiplicity of infection of one, infections

are not detectable by western blotting. We think this is indicative of limited cell-to-cell spreading of the infectious prions in this system. At appropriate MOI values, however, myotube cell layers accumulate significant infectivity. A further advantage of murine myotube cultures is that the input PrP^{Sc} can be distinguished from de novo replicated PrP^{Sc} as the glycosylation pattern of PrP is different in myotubes than PrP from the central nervous system. Mouse brain PrP^{Sc} is predominantly mono-glycosylated while PrP^{Sc} generated in the myotubes is predominantly di-glycosylated. The demonstration of robust replication in differentiated C2C12 myotubes compares favorably with the observed infectivity present in PC12 cells[5] and neurospheres[6] which are also differentiated. We conclude that the heightened level of prion accumulation is a general feature of non-dividing cells.

Prion infection of most susceptible cell culture lines results in persistently infected cells. These cells can then be compared with parental uninfected lines or, alternatively, persistently infected cells can be cured by pentosan polysulfate (PPS) treatment.[7] C2C12 myotubes are, however, infected de novo in each experiment. As myotube layers are seeded as myoblasts and subsequently differentiated prior to infection, exquisitely matched control cell layers can be prepared. Myotubes are post-mitotic and the cell layers exhibit minimal drift with regard to their fundamental properties. Finally, and quite distinctly from proliferating cells, C2C12 myotubes replicate, accumulate and degrade PrP^{Sc} in an equilibrium without the influence of cell proliferation and associated technical variance due to splitting cells.

We conclude that C2C12 myotubes provide a significantly better cell culture model based upon multiple factors. C2C12 cells are widely available and distributed by ATCC. From the box, they can be expanded and utilized immediately in assays of mouse prion replication. They are stable both genetically and phenotypically. In addition, C2C12 myotubes accumulate significant infectivity. In our hands, the level of PrP^{Sc} is substantially greater (>10×) than observed in standard N2a or SMB type cells. Although there have been improvements in prion replication within N2a derived cultures, these successes are limited to a subset of highly susceptible N2a subclones, selected from background for their prion replication proclivity. Many other N2a subclones do not replicate prions or replicate them stably. Highly selected lines are, in many ways, different (morphology, chromosome number, PrP^{C} expression, division rate, etc.), from parental N2a lines as distributed by ATCC and as such most investigators give them new names (e.g., PK1, R33) to distinguish them.

One enigma of prion diseases is the mechanism by which PrP^{Sc} replication or accumulation leads to neurodegeneration and neuroinflammation. In animals, the impact is clear, prion diseases are always fatal. In cell culture systems, however, the toxicity of prion replication is absent or muted, presumably because cells lack the requisite toxicity interactors. Similarly, peripheral tissues are not affected by prions despite accumulation of significant infectivity. The generation of high levels of PrP^{Sc} and prion infectivity in myotube cultures and ability to generate replicate platings of uninfected myotubes, allowed us to contrast the gene expression profile of prion infection in differentiated cells. Surprisingly, we found almost no toxic impact of prion replication in myotubes. Infected myotube cultures looked analogous to control cells throughout the experiment. The gene expression data confirm this and the data are exceedingly tight ($R^2 = 0.998$). Even relaxing the false discovery rate cut off to an 85% confidence interval did not reveal significant gene expression changes in response to prion infection. Despite this, we asked whether any prion specific signature existed by comparing those genes which were most increased (1.3–1.6-fold) in prion infected C2C12 myotubes brains with gene expression profiles from prion infected mice.[8] Shockingly 4 genes whose expression was increased (not statistically significantly) in C2C12 myotubes were also upregulated in RML infected mouse brains. These genes are associated with neuroinflammation and interferon responses, a potentially significant finding considering the muscle origin of the cells profiled. Whether this has any deeper meaning in prion neuropathology remains unclear.

C2C12 myotubes were infectable with 3 different strains of prions (RML > Me7 > 22L) albeit with differential accumulation of PrP^{Sc}. The basis of the differential accumulation of PrP^{Sc} must be a prion strain-specific property as all the myotube cultures were equivalent. C2C12 myotubes also failed to replicate or accumulate HY or CWD prions in accordance with the significant molecular species barrier between mouse PrP^{c} and hamster and deer prions.

One obvious application of cell based assays for prion replication is screening compounds for inhibition of PrP^{Sc} replication. To explore the utility of C2C12 myotubes as a platform for prion inhibition, we used pentosan polysulfate (PPS), a compound with well defined anti-prion activity,[9] to "cure" infected myotube cultures. Control cultures without PPS replicated prions as expected. In this application, cells were infected and PPS treatment started 2 d post exposure. PPS was able to inhibit de novo prion replication as indicated by the lack of C2C12 type di-glycosylated PrP^{Sc}. Intriguingly, however, PPS treatment increased the persistence of input PrP^{Sc} dramatically. This approach envisions the screening of compound libraries without the confounding technical and biological effects of cell division, more closely approximating prion replication in post-mitotic neurons, albeit without induced cytotoxicity.

While robust accumulation of PrP^{Sc} is seen within the 15 d of a routine RML experiment, maintaining myotube cultures for longer periods of time would perhaps allow "slower" strains of prions to accumulate more robustly e.g., 22L. Adapting the myotube approach toward expression of prion proteins from other species will allow a multitude of studies including in vitro cross-species transmissions. In summary, C2C12 myotube are useful for replicating mouse prions. This cell based assay is inexpensive and simple to execute and interpret.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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