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# hTERT-immortalized ovine microglia propagate natural scrapie isolates

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ABSTRACT

*Ex vivo* propagation of natural prion isolates (*i.e.*, propagated solely in the natural host) is crucial for the characterization and study of transmissible spongiform encephalopathies (TSEs). Several well-established, prion-permissive cell culture systems are available; however, only a few cell lines are permissive to natural prion isolates and these cells are not pathophysiologically relevant (*e.g.*, renal epithelium and fibroblast-like cells). Therefore, a pathophysiologically relevant cell line derived from a natural TSE host could be used for propagation of natural prion isolates. In this study, ovine brain macrophages (microglia) were immortalized by transfection with the human telomerase reverse transcriptase (hTERT) gene to identify cell lines (hTERT-microglia) permissive to natural scrapie prion isolates. Following transfection, hTERT-microglia were passaged up to 100 times and their lifespan was significantly longer compared to parental cells (Fisher's exact test, $P < 0.001$). Multiple sublines were permissive to cell culture-adapted prions; two sublines were also permissive to natural scrapie isolates (*i.e.*, derived from brain homogenates of sheep infected with scrapie). Prion infectivity and partial protease resistance of the prion protein were maintained in hTERT-microglia. Comparisons between scrapie-permissive and non-permissive hTERT-microglia sublines revealed that overall quantity of the normal cellular prion protein was not associated with prion permissiveness. The use of hTERT-microglia in future TSE studies may be more germane to the characterization of the cellular and subcellular pathophysiology of natural scrapie prion isolates and to investigate host-specific factors involved in prion replication.


## 1. Introduction

Prions are infectious proteins causing transmissible spongiform encephalopathies (TSEs), a group of lethal neurodegenerative diseases that affect humans and several animal species. Such diseases include Creutzfeldt–Jakob disease in humans, scrapie in sheep and goats, chronic wasting disease in cervids, and bovine spongiform encephalopathy in cattle. Prions are primarily, if not solely, composed of a partially protease-resistant form of the prion protein ($PrP^{res}$). According to the protein-only hypothesis, $PrP^{res}$ catalyzes the conversion of the cellular prion protein ($PrP^{C}$) into β-sheet rich $PrP^{res}$, which imparts decreased solubility in detergents and partial resistance to cleavage by proteinase K (PK) (Prusiner, 1998). Progressive prion accumulation, neuronal degeneration, and astrogliosis are the pathological hallmarks of TSEs (Prusiner, 1998).

Substantial knowledge regarding the pathogenesis of TSEs has been gained from *in vivo* and *ex vivo* models of prion infection (Bosque and Prusiner, 2000; Vilette, 2008). Nevertheless, there are no effective treatments for TSEs and the mechanisms of prion replication and intercellular transmission remain poorly understood. The animal bioassay is considered the gold-standard technique for characterization of neuropathology and quantification of prion infectivity; however, prion-permissive cell culture systems offer a quicker, more cost-effective, and more ethically acceptable alternative for the quantification and study of prion

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infectivity (Arellano-Anaya et al., 2011; Boerner et al., 2013; Bosque and Prusiner, 2000; Klohn et al., 2003) and identification of prion isolates and strains (Mahal et al., 2007; Bian et al., 2010). Most of the available cell culture systems, however, either derive from species without natural TSEs (*e.g.*, rodent-derived cells (Mahal et al., 2007)) or consist of genetically modified cells that are not relevant to the pathogenesis of TSEs (*e.g.*, renal epithelial cells) and overexpress allospecific $PrP^C$ (Vilette, 2008; Bian et al., 2010). Furthermore, most of these cell culture systems are robustly permissive only to cell culture-adapted (Vilette, 2008) and rodent-adapted prion isolates (Archer et al., 2004; Cronier et al., 2004), whereas they have limited or no permissiveness to natural prion isolates (*i.e.*, derived directly from infected tissues of the natural host). For instance, ovinized (ovine $PrP^C$) RK13 (Rov) cells and mouse glial cells were only permissive to seven out of thirty natural scrapie isolates (Neale et al., 2010).

Even with these constraints, prion-permissive cell culture systems have been used to screen for anti-prion compounds (Kocisko et al., 2005). Significant challenges remain, however, given the inconsistency of results sometimes produced when different cell lines and prion isolates are compared (Kocisko et al., 2005), and the fact that all candidate compounds have failed when tested in animal disease models. Alternatively, the use of a pathophysiologically relevant cell type derived from a natural TSE host is intuitively expected to be a better candidate for *ex vivo* propagation of natural prion isolates, and is more likely to produce results that reliably translate back to the natural host species (Kocisko et al., 2005; Stanton et al., 2012). Neurons are undoubtedly the most pathophysiologically relevant cell type in TSEs; however, current prion-permissive cell cultures are limited to rodent species (Vilette, 2008). Another group of pathophysiologically relevant cells are myeloid-derived monocytic cells, which contribute to the pathogenesis of TSEs inside and outside of the central nervous system (Wathne and Mabbott, 2012). Thus, the development of a myeloid-derived cell line (including microglia) permissive to natural scrapie isolates could offer an alternative to characterize the molecular mechanisms associated with transport (Huang et al., 2002), accumulation (Baker et al., 2002), and degradation (Luhr et al., 2004) of prions. Additionally, depletion of microglia in brain slices leads to an increase in prion replication (Falsig et al., 2008), suggesting an important role of microglia in prion clearance. Thus, the identification of prion-permissive and prion-resistant microglia would allow comparative analysis that could determine the mechanisms of prion neutralization or clearance and provide a system to complement and validate findings from other species and cell types.

We have previously demonstrated that primary ovine microglia (homozygous for the *PRNP* $V_{136}R_{154}Q_{171}$ allele) are permissive to cell culture-adapted scrapie prions (Stanton et al., 2008). Unfortunately, these primary cells have a short lifespan and permissibility to natural scrapie isolates has not been demonstrated. Previous attempts to immortalize these cells using the SV40 large T antigen have resulted in immortalized microglia, but with decreased permissiveness to cell culture-adapted prions, as compared to primary microglia (Stanton et al., 2008). This is presumably due to an increased rate in cell division (Ghaemmaghami et al., 2007); a direct effect of oncogene-based immortalization. In contrast, immortalization of primary cells can also be achieved using human telomerase reverse transcriptase (hTERT), which uniquely prevents cellular senescence without increasing the rate of cell division (Roy et al., 2007; Verdun and Karlseder, 2007). Therefore, in this study, primary ovine microglia were immortalized using hTERT and subsequently tested for permissiveness to natural prion infection using different scrapie isolates. Different strains of prions have been characterized by their incubation time (Hecker et al., 1992), brain lesion profile (Fraser and Dickinson, 1968), and pattern of $PrP^{Sc}$ glycosylation (Xanthopoulos et al., 2009). Given the lack of robust characterization of scrapie strains in the United States, in this manuscript, the term "scrapie isolate" is preferred and refers to sheep-passaged $PrP^{Sc}$ deriving from animals infected with classical scrapie.

Herein, a pathophysiologically relevant cell culture system for the study of TSEs is described, as is a method of immortalization that may be particularly useful for the development of other TSE cell culture systems (*e.g.*, human cell cultures).

## 2. Materials and methods

### 2.1. hTERT transfection of ovine microglia

Cryogenically stored parental microglia (previously obtained from a near-term Suffolk sheep (Stanton et al., 2008)) were thawed and maintained in Opti-MEM medium supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals) 2 mM L-glutamine, 10 IU of penicillin, and 10 mg/ml streptomycin (OMEM). At passages 7 and 8 of primary cell lifespan, approximately $1.5 \times 10^5$ cells were plated into 12-mm diameter wells and transfected with pBABE-puro-hTERT plasmid (1.6 μg of plasmid DNA, Addgene plasmid 1771) (Counter, 1998) using Lipofectamine 2000 per manufacturer's instructions (Invitrogen). To control for effects of transfection on prion permissibility, primary microglia were mock transfected (*i.e.*, no plasmid) or transfected with the pBABE-puro plasmid (Addgene plasmid 1764) (Morgenstern and Hartmut, 1990). Sublines are defined by individual transfection reactions. Fifteen sublines were created with pBABE-puro-hTERT (hTERT-microglia), seven were created with pBABE-puro (puro-microglia), and three were created by mock transfection. Ten hTERT-microglia and five puro-microglia sublines were selected with 1 μg/ml puromycin (concentration determined by kill-curve analysis of untransfected ovine microglia) 48 h after cell expansion, whereas the other transfected sublines did not undergo puromycin selection. After selection, all transfected sublines were split 1/5 every 4 days using new 25-$cm^2$ culture flasks. Under such conditions, primary ovine microglia typically reach senescence in 20–22 passages (unpublished data). Thus, for the purposes of this experiment, we attempted to passage all sublines at least 30 times. A Fisher's exact test was used to determine the statistical significance of the number of cell lines surviving to passage 30.

### 2.2. Characterization of hTERT-microglia and cloning

Following expansion and subcultivation, RNA was collected from transfected sublines using the RNeasy Mini kit (QIAGEN) following manufacturer's instructions. One hundred micrograms of total RNA from each subline were reverse-transcribed in a 10 μl reaction using the Superscript™ first-strand synthesis system kit (Invitrogen) following manufacturer's instructions. The DreamTaq Green PCR Master Mix (Thermo Scientific) was used for PCR reactions. A segment of hTERT was amplified using the WH33 (5′-GTGGTGGAATTCTAGATTTGCAGGTGAACAGCCTC-3′) and AS1310 (5′-GACACACATTCCACAGGTCG-3′) primers (Masutomi et al., 2003) with an expected amplicon of ~460 bp. A 156 bp segment of the puromycin resistance gene was amplified with the primers puroFW (5′-CGCCACATCGAGCGGGTCAC-3′) and puroRV (5′-GGCCGATCTCGGCGAACACC-3′). Plasmid DNA and water were used as template to control PCR reactions. Telomerase activity was measured using TeloTAGGG Telomerase PCR ELISA (Roche) following manufacturer's instructions. Negative controls were created by treating cell lysates with 1 μg of RNase for 20 min at 37 °C. Absorbance was measured at density of 450 nm with a reference wavelength of 690 nm. Immunocytochemistry using the monoclonal antibody MM61A was used to test for CD14 expression (a