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The Susceptibility of Cell Lines Used in the Production of Human Biologics to Prion Infection

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THE SUSCEPTIBILITY OF CELL LINES USED IN THE PRODUCTION OF HUMAN BIOLOGICS TO PRION INFECTION

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Abstract

Transmissible spongiform encephalopathies (TSEs) are a group of neurodegenerative human and animal disorders associated with the accumulation of pathogenic or abnormal prion protein (PrP^{Sc}) in the brain. Examples include scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle. Identifying and studying pathogenic prions *in vitro* has been hampered by a limited capacity to detect abnormal prion proteins and incomplete knowledge of cell lines capable of sustaining a prion infection. This study focused on determining the susceptibility of cells commonly used in the manufacturing process of biologics and biotherapeutics to prion infection and on development of an enrichment procedure that used trichloroacetic acid (TCA) precipitation to increase and confirm detection of PrP^{Sc} by Western blot. The newly optimized TCA precipitation protocol demonstrated a two- to four-fold increase in sensitivity compared to other precipitating techniques when used for Western blotting. Work indicated that Sf21, N2a, RmcB, CHO, HFF, HFF-MitC, PBDC, NAMALWA, MRC-5, HEK-293, U937 and Cos-7 cell lines were not susceptible to a PrP^{Sc} infection with BSE as the inoculant strain. N2a cells were susceptible to PrP^{Sc} when a liposome-based delivery system was used. The NIH/3T3 cell line was susceptible to a PrP^{Sc} infection. This work highlighted the need to test a variety of cell lines used in the production of human biologics for their susceptibility to prion infections. Although most of the cell lines tested did not demonstrate a sustained infection, the finding that NIH/3T3 cells were susceptible indicates that cell line origin and endogenous PrP^{Sc} amounts must not be the only factors that decide if a cell lines should be screened or tested.

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List of Abbreviations

APC: Antigen Presenting Cell

Bax: Bcl-2 associated X protein

BSE: Bovine Spongiform Encephalopathy

cAMP: Cyclic Adenosine Monophophate

CHO: Chinese Hamster Ovary

CJD: Creutzfeldt-Jakob Disease

CNS: Central Nervous System

Cos-7: African Green Monkey Fibroblast Cells

Cu^{2+}: Copper Ions

CWD: Chronic Wasting Disease

DC: Dendritic Cell

ENS: Enteric Nervous System

ER: Endoplasmic Reticulum

ERK: Extracellular Signal-Regulated Kinase

fCJD: Familial Creutzfeldt-Jakob Disease

FDC: Follicular Dendritic Cell

FFI: Fatal Familial Insomnia

GABA: Gamma-aminobutyric acid

GPI: Glycosyl-phosphatidylinositol

GSS: Gerstmann-Straussler-Scheinker Syndrome

H: Histidine

HEK-293: Human Embryonic Kidney Cells

HFF: Human Foreskin Fibroblast Cells

Hsp: Heat Shock Protein

iCJD: Iatrogenic Creutzfeldt-Jakob Disease

K: Lysine

LPR/LR: 37 kDa/67kDa Laminin Receptor

LPS: Lipid Polysaccharide

LRP1: Lipoprotein Receptor-Related Protein 1

LRS: Lymphoreticular System

LTM: Long Term Memory

LTP: Long Term Potential

MAP: Mitogen Activate Protein

MRC-5: Human Lung Fibroblast Cells

NAMALWA: Human B-lymphocyte Cells

N2a: Mouse Neuroblastoma Cell Line

NCAM: Neural Cell Adhesion Molecule

NIH/3T3: Mouse Fibroblast Cell Line

NMDA: N-methyl D-aspartate

PBDC: Human Peripheral Blood Dendritic Cells

PKA: Protein Kinase A

PKC: Protein Kinase C

Prnp: Prion gene

PrP: Prion protein

PrP^C: Normal Prion protein

PrP^{Sc}: Abnormal prion protein

PTA: Phosphotungstic acid

RmcB: Mouse hybridoma B lymphocyte Cells

RML: Rocky Mountain Laboratory

ROS: Reactive Oxygen Species

sCJD: Sporadic Creutzfeldt-Jakob Disease

ScN2a: Scrapie-infected Mouse Neuroblastoma Cell Line

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sf21: *Spodoptera frugiperda* Insect Cell Line 21

siRNA: small interfering RNA

STM: Short Term Memory

SWA: Slow wave activity

TCA: Trichloroacetic acid

TSE: Transmissible Spongiform Encephalopathy

U937: Human Histiocytic Lymphoma Cells

UV: Ultra Violet

vCJD: Variant Creutzfeld Jakob Disease

WT: Wild Type

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1.0 Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of genetic, infectious or sporadic neurodegenerative human and animal diseases associated with the accumulation of an abnormal prion protein (PrP^{Sc})(68). Intra-species transmission of TSE was first observed in 1795; however, inter-species transmission was not noted until the 1950's when scrapie in sheep was shown to cause TSE in goats(17,99). TSEs became a public health concern when the development of variant Creutzfeld-Jakob Disease (vCJD), a human TSE, was linked to the human consumption of meat contaminated with bovine PrP^{Sc}(111). These fears brought about the theory that, if PrP^{Sc} from a BSE source were to contaminate human biologics, it could then be transferred to patients during therapeutic treatments, resulting in an epidemic. The production of human biologics often involves a variety of cell lines, reagents and biochemicals before the end product is synthesized. Prusiner *et al.* provided the earliest evidence that a neuronal cell line could maintain a persistent prion infection(18). This observation sparked a debate about the safety of biologics arising from the use of various animal reagents during the manufacturing process. Researchers have since found that prions can also persist in cell lines similar to those used in the production of human biologics. Based on these concerns, the information presented within this thesis examines the susceptibility, to a prion infection, of various cell lines used in the production of human biologics.

Prion Proteins and the Diseases They Cause.

1.1.1 Prion Proteins Prion proteins (PrPs) are cellular proteins that are found abundantly in the neurons and ganglia of the brain and spinal cord(51). These proteins are also found in leukocytes, skeletal muscle and a variety of organs including the heart, lung, intestinal track, spleen, testis and ovaries(10). Two main isoforms of PrP exist: PrP^{C}, a normal cellular protein, and PrP^{Sc}, the disease isoform. PrP^{Sc} has been linked to be the main causative agent for a variety of TSEs. These diseases affect a wide range of species, including humans, and are always fatal. Death is due to extensive neurodegeneration via neuronal loss, vacuolation (spongiosis), reactive astrocytosis and microgliosis(34). The degeneration leads to neuronal impairment through the interference of neurotransmitter systems, synaptic alteration and dendritic atrophy.

Although PrP^{Sc} causes a pathogenic disease, it resists normal methods of decontamination used against bacteria and viruses(100). High temperatures, detergents, huge doses of ultraviolet light and formaldehyde are all ineffective in inactivating prion proteins(100). In contrast to viruses and bacteria, nucleic acids seem to play only a very limited role for PrP^{Sc} replication(100). Studies on nucleic acid involvement as well as mounting evidence that a misfolded protein was the causative agent for TSEs, led to the proposal of the 'protein only hypothesis' by Stanley Prusiner(100). His theory stated that TSEs were caused by a nucleic acid-free proteinaceous infectious molecule that he called a 'prion'(100). This prion could interact with other non-infectious prions and cause a structural conversion, generating new infectious molecules. His hypothesis is generally accepted yet some scientists postulate that slow, atypical viruses known as virinos may be the cause of TSEs(74). To clarify the naming process with prion diseases, PrP^{Sc} acts as an 'umbrella term' that refers to the misfolded protein responsible for the diseases.

1.1.2 Scrapie A disease that affects both sheep and goats, scrapie has been known for the last 200 years(74). Although it has been labelled with a variety of names, records as early as the 1700's have shown that farmers recognized scrapie as a deadly disease that could be transmitted to other animals(100). Sheep are usually infected either during grazing when they feed on scrapie-contaminated pastures or via animal to animal contact(100). Although scrapie symptoms vary greatly among cases, some common signs include itchiness, behavioural changes, coordination difficulties, weight loss, poor wool coat production and tremors(2). The name "scrapie" refers to the tendency of sheep to rub (or scrape) against each other or fences to alleviate this itchiness. Susceptibility to scrapie has been associated with three mutations within the sheep PrP gene: mutations in codons 136, 154 and 171 in various combinations result in a gamut of individual animals which range from highly resistant to very susceptible to scrapie infection(2).

One of the first breakthroughs in studying the disease occurred in 1961 when Richard Chandler infected mice using scrapie obtained from field samples. These studies not only allowed scientists to study scrapie under tightly controlled conditions but were also much less expensive and less time consuming than studies using sheep(100).. From these initial experiments, mice have provided models that have helped in the study of how prions propagate through the host as well as provided a bioassay that is extremely sensitive in detecting infectious prion samples(68).

1.1.3 Bovine Spongiform Encephalopathy (BSE) The transmissible spongiform encephalopathy that affects cows achieved fairly high media coverage under the name of "mad cow disease". First reported in 1987 by Gerald Wells, the changes in bovine brains effected by the disease were identified after infected samples were examined by the Central Veterinary Laboratory(141). Originally, scrapie was linked as the causative agent responsible for the spread of BSE(101). Epidemiological studies pointed to a common source and it was suggest that scrapie-contaminated sheep carcasses used as cow feed were the most likely candidates(101). This has been difficult to prove through experimental studies, however, as direct transmission of scrapie to bovine subjects has not produced any sort of "mad cow" phenotype. Although unproven at this time, scientists are beginning to believe that the BSE outbreak seen in the late 1990's may have actually been caused by recycled BSE-contaminated cattle carcasses that were also sometimes used as cattle feed(101).

Transmission of BSE has been shown to be quite limited horizontally (between bovines) and vertically (between parent and child bovines). Unlike scrapie, BSE does not seem to pass from animal to animal via excreta or secreta or from mother to offspring via maternal transmission(132). The only method identified for transmission seems to be the processing of infected animal products, such as bone meal, that are then used as animal feed(132). Symptoms of the disease can vary but seem to always include at least some of the following: anxious or aggressive behaviour, unusual posture, difficulty with coordination, a decrease in milk production and weight loss(1). These symptoms usually only appear late in the pathology of the disease during the last six months of the animals life(1).

1.1.4 Chronic Wasting Disease (CWD) Affecting deer and elk populations, CWD cases were first identified in the 1980's(144). Scientists had recognized this disease as a culprit that shortened the lifespan of captive mule deer before the 1980's yet knowledge of it being a contagious disease was limited until then(144). Only three species have been found to be naturally susceptible to prion infections: white-tailed deer, mule deer and elk. Subspecies such as the black-tailed deer have also been shown to be experimentally susceptible to prion infections; therefore, it has been hypothesized that all subspecies of the three aforementioned species would be susceptible to infection, were they appropriately exposed to the contagion(144).

The origin of CWD has never been deduced although a few hypotheses exist. The most plausible explanation is that CWD originated from contact with scrapie-infected sheep. Due to the ability of deer or elk PrP^{Sc} to convert sheep PrP^{C} into PrP^{Sc}, it has been hypothesized that this may provide a possibility for CWD to have originated from some sort of interaction with scrapie; however, the fact that deer or elk PrP^{Sc} inoculated into sheep does not produce the same sort of disease nor does it follow the time line of a typical scrapie infection, suggesting another origin(144).

Symptoms of the disease include difficulties in coordination and locomotion, weight loss, behavioural differences, hyper-excitability, altered stature, rough coat composition and a greater risk of sudden death after handling most likely due to lesions caused by CWD in the parasympathetic innervations of the heart(144). The duration of the disease varies dramatically with each case as well as among species(144).

1.1.5 Kuru, Gerstmann-Sträussler-Scheinker(GSS) Syndrome, Fatal Familial Insomnia (FFI) and Creutzfeldt-Jakob Disease (CJD) Kuru, Gerstmann-Sträussler-Scheinker(GSS) Syndrome, Fatal Familial Insomnia (FFI) and Creutzfeldt-Jakob Disease (CJD) are all PrP^{Sc} disease specific to humans. Kuru was first documented in the 1950's when contact from Western societies began with tribes of New Guinea(30). Kuru was shown to be endemic within the Fore groups, a culture living in the Eastern Highlands of New Guinea(30). The term "kuru" actually came from Fore language meaning 'to shiver': a symptom often seen in this fatal neurodegenerative disorder(30). Early research into kuru by a veterinary pathologist indicated that epidemiological, clinical and neuropathological signs resembled those of scrapie(30). Originally, kuru was believed to be a hereditary condition heavily dependent on whether the individual was male or female. The belief that the disease was hereditary stemmed from observations that the male-to-female ratio of individuals with the disease was 1:25(30). Further epidemiological studies demonstrated that the disease was not X-linked but that the real cause for this gender discrepancy was due to the method of cannibalism being practiced(30). Kuru culture dictated that cannibalism occur as a ritual during the funeral rites of relatives. Women typically ate the lesser desired (and much more infectious) materials such as tissues of the CNS while males ate the more preferred (and relatively uninfectious) tissues such as the skeletal muscle(30). Now that cannibalism is no longer practiced with the Fore, cases of kuru have substantially declined since the 1950's(30).

Kuru symptoms include headaches, malaise or cough in the early stages of the disease. From there, difficulties in equilibrium and a decrease in weight occur that is most often noticed first by family members before the patient realizes something is wrong. Within weeks, the individual often becomes extremely unsteady when walking. At this stage, poor arm coordination, eye coordination and articulation appear as well as tremor-like shaking that can be seen when the patient tries to make voluntary movement(30). As the disease progresses, these tremors become more dramatic and uncontrolled(30). In the final stages, infected individuals becomes completely unable to walk or control muscle movement before they often die from starvation or from complications due to static bronchopneumonia or infected pressure sores(30). Interestingly, cognitive abilities are relatively unimpaired until the final stages of the disease. Even at these late stages, it is difficult to judge how damaged cognition is due to the sever impairment in motor and verbal skills that prevents patients from responding to tests(30).

GSS syndrome first became apparent in an Austrian family that suffered from a slowly progressive loss of muscle control mixed with cognitive impairment(30). Similar to kuru, the disease was originally attributed to an autosomal dominant hereditary condition based on the trans-generational pattern seen in the affected family(30). It wasn't until the 1980's that studies finally conclusively demonstrated the same amyloid plaque deposition common to TSEs(81). The disease seems to occur most commonly in individuals exhibiting a mutation to the PrP gene at codon 102(30). Although some transmissibility has been shown in mouse studies, the potential of transmission seems to be very low even in studies where mice express the mutated gene(30).

GSS syndrome symptom onset is typically late in life with individuals showing the initial signs of the disease in their 50-60's(30). Loss of weight, difficulties with coordination, spatial impairment, inability to perform rapid movements, staggered movements, limb weakness and partial paralysis of the lower limbs all commonly affect individuals with GSS(30). Memory impairment is often seen at the beginning of the disease yet may not be present in each case. Learning difficulties, intellectual deterioration and changes in demeanor eventually consume the patient before they die in a bedridden, completely dependent state(30).

FFI originally earned its name from a diagnosis of five members in an Italian family who demonstrated a novel neurodegenerative disorder(30). In 1992, however, studies demonstrated that i) a mutation in the PrP gene was found at codon 178 in each member of the family studied, ii) PrP^{Sc} was obtained in samples from the cortical and sub-cortical regions of the brain and iii) transmissibility of the infectious agent was confirmed via mouse studies(87,128). These observations led to the identification of FFI as a member of the TSE disease family. Degeneration in the brain is focused in the thalami yet the degree of damage in the brain does not necessarily dictate the symptoms that each patient exhibits. As well, a mutation in the PrP gene does not necessarily guarantee an individual will succumb to this disease since other members of the Italian family who had the mutation showed no symptoms as of 2001(30).

Early signs of the disease include sudden weight loss, lethargy and overall tiredness; however, the main symptom of FFI is disruption of the normal sleep-wake cycle(30). Electroencephalographic patterns of sleep become inconsistent, irregular nervous system activity is seen and endocrine functions become abnormal(30). Once sleep patterns have become increasingly interrupted, nocturnal insomnia becomes a hallmark of the disease(30). Hallucinations and motor impairment begin to develop midway through the illness as well as vivid dreams that may be acted upon while the patient is in deep sleep(30). Attention and concentration impairment occurs throughout the disease but typically culminates near the late stages with serious intellectual decline, confusion and disorientation(30). The patient usually then enters into a coma before dying from complications following pneumonia(30).

CJD is the most commonly recognized TSEs by health care practitioners mainly due to the outbreak of variant CJD (vCJD) that is believed to have begun from the consumption of contaminated BSE meat in the UK(111). In reality, more than one form of CJD exists: sporadic CJD (sCJD), familial CJD (fCJD), iatrogenic CJD (iCJD) and variant CJD (vCJD)(111). The different CJD forms are similar in symptoms yet the age of onset as well as the manner with which each disease is transmitted is quite different. First recognized in the 1920's, sCJD was found to be caused by somatic mutations or random conversion of PrP^{C} to PrP^{Sc}(111). Also first discovered in the 1920's, fCJD stems from hereditary genetic mutations within the PrP gene(111). iCJD was first recognized in 1974 when epidemiological studies found that CJD could be passed on through contaminated surgical equipment, blood transfusions or contaminated biologics such as human growth hormone(111). vCJD was first recognized in the early 1990's and is believed to be caused by eating BSE contaminated meat(111). With the exception of vCJD, which is often first seen in young adults, all other forms of the disease typically exhibit themselves late in the adult's lifespan(136).

Early symptoms of most CJDs are difficult to diagnose because of their similarity to other dementias such as Alzheimer's disease. The earliest signs include mood swings, psychological disorders (such as depression and anxiety), issues with memory, social withdrawal, a lack of coordination, insomnia, visual problems, fatigue and uncharacteristic behavior(54). At this point, the disease usually escalates rapidly with patients experiencing blindness, severe dementia, uncontrolled muscle movements, paralysis, speech and swallowing impediments, the inability to control excretory movements and finally entrance into a coma(54).

1.2 Prion Diseases: A Tragic End

1.2.1 Treatments Unfortunately, no current treatment has been shown to slow or stop the progression of any type of human PrP^{Sc} infection. Typical treatments ensure that the patient is as comfortable as possible by using drugs to inhibit pain and involuntary muscle movements(54). All compounds tested thus far in an effort to slow or stop TSE progression have provided disappointing results. Quinacrine, for example, demonstrated anti-PrP^{Sc} effects *in vitro* yet lacked any effect in animal studies(31). Pentosan polysulphates have been shown to provide promising effects against PrP^{Sc} replication *in vitro* and through hamster *in vivo* studies. Two clinical studies in patients, however, provided much less promise with one reporting no improvement and another reporting mild improvement that could not be directly related to the administration of the drug(41,131,143). Finally, flupirtine has been recorded to help with some of the cognitive defects PrP^{Sc} causes yet it has not been shown to slow the progression of the overall disease nor does it prevent death from PrP^{Sc} diseases(96).

1.3 The Species Barrier Phenomenon

1.3.1 The Species Barrier

The intra-species transmission of prions had been shown as early as 1930 when sheep scrapie was passaged between sheep hosts(11). Inter-species transmission, however, is much more difficult because of a phenomenon known as “the species barrier”. The barrier actually refers to the fact that, when trying to infect a host of another species, not only is it much more difficult to establish an infection but it also takes much longer before clinical signs begin to appear(11). Once an infection has been established within a new species, passage of infected material within that species is no longer subject to the barrier and infectivity rate increases while the length of incubation time decreases(11).

A variety of factors are believed to impact the efficacy of the species barrier. First is the genotype of the animal being infected: specific mutations with the sequence of the PrP gene greatly affect how susceptible a host is to a productive PrP^{Sc} infection. With sheep, for instance, mutations at codons 136, 154 or 171 are needed to establish a prion infection(11).

The original strain used is also important. This was first brought to light when scientists noticed how simple it was to infect a variety of other species with BSE without any phenotypic modification of the sample(11). sCJD or fCJD are also notorious for being able to infect other species. This weak barrier against either BSE or CJD indicates that the species barrier may not entirely depend on sequence but may require some unknown modification/factor that is established when a particular strain is passed through a particular species.

Finally the route of infection also plays a large role in determining how effective the species barrier is. Oral routes often lower the infectivity of a prion strain by a factor of 10^5 while infection via intracranial inoculation is the most efficient(11). As one can imagine, these routes depend heavily on how easy it is for the infectious agent to enter into the host's lymphatic system and then travel to the brain where the greatest concentration of PrP^C exists(11). Intracerebrally-injected PrP^{Sc}, for instance, relies heavily on how effective the particular strain is at infecting tissues at the location of injection(11). For example, experimentally-injected BSE strains are not as effective at infecting lymphatic tissues in comparison with scrapie, CWD and vCJD strains(11).

1.4 The Lifecycle of a Prion Protein

1.4.1 PrP^C Biosynthesis Extra embryonic tissues express the *PrP* gene as early as 6.5 days into embryogenesis while transcripts of the gene in developing brain and spinal cord tissues appear around day 13.5 in the mouse model(79). The mRNA encoding PrP^C is first expressed in embryogenesis both in neuronal and non-neuronal tissues and mRNA levels continue to rise during development. By adulthood, prion proteins are found throughout the central nervous system with the highest expression in neocortical, hippocampal and spinal motor neurons as well as in Purkinje cells(66).

PrP^C in humans originates from the mammalian PrP gene located on chromosome 20(100). The protein is 250 amino acids long and consists of several parts including a N-terminal signal peptide, five proline/glycine rich octapeptide repeats, a highly conserved hydrophobic segment and a C-terminal region that acts as a signal for a glycosyl-phosphatidylinositol (GPI) anchor(51). PrP^C is synthesized in the endoplasmic reticulum before it travels through the Golgi apparatus on its way to the cell membrane. The final protein is membrane bound.

1.4.2 Post-Translational Modifications Several post-translational modifications occur following PrP^C synthesis including removal of the N-terminal signal peptide, addition of asparagine-linked oligosaccharide chains to sites 181 and 197, disulfide bond formation and the addition of the GPI anchor to the C-terminus(42,50,51). What sets the GPI anchor found on PrP^C and PrP^{Sc} apart from other typical GPI anchors is that its core is altered by the addition of sialic acid residues and is resistant to endoglycosidase H treatment(19). In addition to these alterations, multiple cleavage events occur once the prion protein is inserted into the cell membrane. The first cleavage occurs between K_{121} and H_{122}, a highly conserved region containing 16 hydrophobic amino acids(51,147). The C-terminal fragment then undergoes a proteolytic process that separates the GPI anchor from the C-terminal fragment, releasing this fragment into the extracellular millieu(147). Although the physiological significance of these cleavages is poorly understood, the N-terminal fragment may act as a biologically active ligand while the C-terminal cleavage may be a mechanism to remove the neurotoxic region that exists inherently in the C-terminal region of the proteins structure(147).

1.4.3 PrP^C Cellular Localization and Trafficking PrP^C is found mainly on the cell membrane where it is attached by its GPI anchor(51). Other possible forms include cytosolic, type 1 and type 2 transmembrane forms(76). Microscopic immunocytochemistry has shown that PrP^C is concentrated in neuronal structures at synaptic fields such as the olfactory bulb, limbic structures and the striato-nigral complex(51). PrP^C is initially translocated to the endoplasmic reticulum (ER) before it is cleaved into the ER lumen(77). From there, it is sent to the Golgi apparatus and finally to the plasma membrane where it becomes attached, via its GPI anchor(77). Transport between the ER, the Golgi apparatus and the plasma membrane is strongly associated with lipid rafts. These rafts are important in ensuring proper folding and glycosylation of PrP^C(77).

Once the protein has been sent and anchored to the plasma membrane, PrP^C trafficking undergoes a cyclic mechanism. Prion proteins continuously transition between the anchored protein and an unanchored form located within an endosome(51,121). Association of PrP^C with clathrin-coated pits, calveolin and endosomes has been documented by a variety of sources(51,76,77,121). Although the exact reason why PrP^C relies on compartmentalization is not clear, studies have shown that proper folding, cleavage into different forms and protein recycling all seem to depend on various aspects of these transport systems(77).

Further research needs to be made of the endocytic cycle of PrP^C. The recycling pathway mentioned earlier seems to have an important yet poorly understood function. After PrP^C is internalized, large amounts of the protein are sent back to the cell membrane, while a small percentage is sent out to the extracellular matrix. Thus, PrP^C may act as a receptor; however, proof of concept is still lacking(51,77).

1.5 Prion Proteins: PrP^C Proposed Roles

1.5.1 Various Aspects of PrP^C Involvement One of the difficulties in determining the function of PrP^C stems from the lack of any obvious physical abnormalities when PrP knockout mice are generated. No observable differences in development, physical attributes or lifespan seem to occur; however, experimental data suggests that PrP^C plays a role in behaviour, the sleep-wakefulness cycle, memory, synaptic activity and immunomodulation(77).

1.5.2 PrP^C and Behavioural Attributes Although findings are still limited, a clearer picture of the affects PrP^C has on various behaviours is beginning to appear. One study focused on responses to stimuli when wild type (WT) or PrP-null mice were introduced to novel environments. Although PrP-null mice had higher locomoter activity, they lacked hyper-locomotor responses to the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801, a molecule used to stimulate neurons with glutamate receptors. This lack of response suggests that PrP^C may be involved in the glutamatergic system(77). WT mice also seem to demonstrate decreased levels of anxiety when under acute stress indicating a potential for PrP^C to interact in behavioural systems designed to adapt to stress(93).

PrP^C also plays a limited role in the sleep cycle. Studies using PrP-null mice showed that although there is no change in the number of vigilances in comparison with WT mice, sleep fragmentation, cortical temperature, sleep transitions and adaptation to sleep deprivation were all affected(130). PrP-null mice were found to suffer greater amounts of sleep fragmentation and double the amount of short waking episodes, than their PrP^C-expressing counterparts(130). In addition, the increased cortical temperatures of PrP-null mice correlated with the greater number of waking episodes(130). Finally, involvement of PrP^C in the sleep cycle seems to involve entry and the intensity of sleep being exhibited. In one study, slow wave activity (SWA), which is used as a measurement for sleep intensity, was lower in PrP-null mice(130). This indicated that PrP-null mice were not entering and maintaining as deep a sleep as the WT group. All of these observations suggest that PrP^C plays a role in the sleep cycle and in sleep continuity(130).

Memory retention seems to be influenced by PrP^C – a conclusion based on mice studies that have looked at short-term memory (STM), learning capabilities and long-term memory (LTM) loss. Although STM and learning abilities do not seem to be impaired in PrP-null mice, LTM retention is greatly decreased after approximately 8-9 months of age(26). PrP-null mice also demonstrated hippocampal-dependent spatial learning difficulties while non spatial learning remained fully functional(26). Interestingly, these deficits were restored upon neuronal re-expression of PrP^C, indicating that PrP^C has some effect on memory retention(26).

1.5.3 <u>PrP^C and Synaptic Activity</u> The roles that prion proteins play in various behavioural and memory components are suggestive of involvement in different synaptic and neuronal excitement mechanisms. Adding weight to this hypothesis is the localization of PrP^C to both pre and post-synaptic structures(77). Direct evidence of a role in neural communication was first obtained by experiments describing transport of glycosylated PrP^C via axons(107). Furthermore, demonstrations that PrP^C binds copper ions (Cu^{2+}) suggest that PrP^C may buffer Cu^{2+} levels after nerve depolarization, acting as a recycling mechanism and protecting against reactive oxygen species (ROS)(77).

Many of the studies on PrP^C and synaptic activity have looked at long term potentiation (LTP) due to LTPs known role in memory formation(77). One of the significant findings is that PrP-null mice exhibit reduced LTP in the hippocampus. Specifically, when tissue slices from PrP-null mice were examined at physiological temperatures, a lower LTP was seen. As expected, this phenotype was rescued by a transgene encoding PrP^C(29,142). Furthermore, PrP-null mice demonstrated diminished endonucleotidase activity, increased glutamate concentrations at the synaptic cleft and slow after hyperpolarizations(27,43,44,77). All three of these findings are evidence that a lack of PrP^C leads to higher neuron excitability. To further support the role of PrP^C in neuron excitability, researchers found that seizures were easier to trigger in PrP-null mice. This finding suggested that PrP^C may be a key component in preventing seizures *in vivo*(77).

1.5.4 PrP^C and Immunomodulation Data from recent studies suggested PrP^C could be involved in the signalling and activation of various components of the immune response. Studies have shown that PrP^C accumulates at the contact sites between dendritic cells (DCs) and T-cells(5). Experiments preventing DC PrP^C accumulation have shown that the peptide-MHC driven proliferative T-cell response becomes inhibited(5). Not only were DCs affected but macrophages also demonstrated a reduced ability to perform phagocytosis when the GPI form of PrP^C was eliminated. Finally, PrP-null mice showed substantially reduced leukocyte infiltration(5). Taken together these results implicated PrP^C as a modulator of APCs (antigen presenting cells) and of the peripheral inflammatory response(77).

1.5.5 <u>PrP^C and the Cell Life Cycle</u> The evidence that PrP^C production increases over embryonic growth has led to the notion that PrP^C plays a role in development(75,77,79,85). A variety of cellular mechanisms for PrP^C's involvement in the cell cycle have been investigated including cell proliferation, differentiation and apoptosis(77).

With regards to cell proliferation, PrP^C seems to play a significant role in the formation of the brain. Studies in mice have shown that PrP^C positively correlates with the proliferative ability of neurons in the dentate gyrus and the subventricular zone(125). PrP^C seems to increase the sensitivity of cells to growth factors present in the microenvironment. Although support is limited, studies showing that PrP-null mice have to compensate with increased growth factor signal expression does provide some preliminary support to the theory(114,115). This proliferative effect is not the main defining factor in the development of neurons as no morphological defects were noted between mice that had and those that lacked PrP^C expression(125). These results showed that while PrP^C plays a prominent role in cellular proliferation, other factors are able to compensate when it is lacking.

As one would expect, PrP^C plays a large role in neuronal differentiation. The first evidence of this came when cultured, developing hamster neurons were labelled using anti-PrP^C antibodies(77). Antibody signal shifted over the course of neuron development from the axons to the synaptic beds, indicating that PrP^C most likely plays a role in axon growth and guidance in addition to synaptogenesis(112). Growth and guidance of developing neuronal structures are major components of differentiation in neurogenesis(112). Furthermore, based on examination of neuronal growth factors like nestin and MAP-2, a positive correlation seems to exist between the content of PrP^C and the differentiated state of both *in vivo* and *in vitro* neurons(125).

Further proof of a role for PrP^C in neuron differentiation comes from studies where soluble recombinant PrP^C was added to developing neurons. This treatment induced the growth of both dendrite-like outgrowth and a single robust axon like process per neurite(63). It also increased synaptogenesis(63). One important note that the study mentioned was that internalization of PrP^C was not needed to induce neuronal differentiation indicating that PrP^C may act as a ligand on precursor neuronal cells. The molecule or signal complex that may be responsible for receiving PrP^C induced signals has yet to be elucidated(77). This study provided early evidence that soluble PrP^C may not be restricted solely to membrane based roles and that binding of soluble PrP^C may be a catalyst for a variety of cellular pathways such as cell death(77).

The very first evidence that PrP^C played a strong role in controlling cell death arose from studies with hippocampal neurons that did not express PrP^C(77). Unlike their WT counterparts, cells that did not express PrP^C underwent rapid cell death upon serum deprivation. The WT phenotype was quickly restored by transfecting cells with the prion gene (*Prnp*)(67).

Studies looking at apoptosis induced by *Bax* (Bcl-2 associated X protein) demonstrated that cell death was prevented when *Prnp* was co-injected. This protective effect was eliminated when protein trafficking between the Golgi apparatus was blocked yet was restored when a soluble form of PrP^C that lacked its GPI anchor was used(15). These findings are consistent with previous studies that show increased survival with the addition of solubilised PrP^C to cerebellar granule cells and mouse hippocampal neurons(77). The hypothesis that the anti-apoptotic nature of PrP^C is due to its interaction with the *Bax* pathway has been examined in both human and mouse neurons. It was found that PrP^C inhibits the apoptotic conformational change of *Bax*, blocking its ability to induce cytochrome *c* release by the mitochondria(109).

Strong evidence also exists for a role of PrP^C in neuroprotection. Reports that hypoxic-ischemic injury to the brain is diminished in PrP^C mice in comparison with their PrP-null counterparts accompany reports of increased PrP^C expression at the sites of damage (forebrain) in mice(86,138,139). This neuroprotective effect was seen in a gene- or dose-dependant manner where PrP-null mice had the least amount of protection and WT mice had the greatest(86). Overexpression of PrP^C did not seem to enhance protection against brain damage above the WT threshold however improved neurobehavioral signals after cerebral ischemia occurred has been reported(86). The study postulated that the neuroprotection may extend past the prevention of cell death(122).

Some studies claim that protection from apoptosis in cancer cells may be largely attributed to PrP^C(73,77). Samples of gastric adenocarcinomas showed greater expression of PrP^C correlating with the histopathological differentiation and the progression of the tumour(73). The study also noticed that PrP^C seemed to provide protection against cancer cell apoptosis via downregulation of both p53 and *Bax* apoptotic pathways and by an increased production of PrP^C mRNA(73,77). These anti-apoptotic and neuroprotective benefits seem to be more prominent in cancer cell lineages, however, and cannot be assumed to be identical across all cell types. Indeed, in studies that looked at protection conferred by PrP^C in retinal explants of mice, no difference was identified between WT or PrP-null mice(77).

1.5.6 <u>PrP^C and Signal Mediation</u> The role of PrP^C in signal transduction has been established via both direct and indirect evidence(77). PrP^C seems to act as a receptor for a variety of ligands. Different cell signalling pathways have been investigated with regards to the potential role of PrP^C. The studies mainly revolve around the interaction between PrP^C, caveolin and various cell kinases that are believed to cause numerous effects.

Using embryonic carcinoma cells that were able to differentiate into either the serotonergic or catecholaminergic phenotypes, some studies demonstrated that cross-linking of PrP^C to Fyn through caveolin led to the activation of the Fyn non-receptor tyrosine kinase (an activator of the ras pathway which influences cell growth and proliferation). This activation was blocked using antibodies against caveolin-1, suggesting that caveolin-mediated signal transduction is triggered by PrP^C(77). One problem with this theory, however, is that caveolin has been shown to be unable to bind directly to the GPI anchor attached to PrP^C limiting direct interactions between the two proteins(77). This has made it difficult to explain how the interaction between PrP^C and caveolin is occurring. The most likely explanation for this apparent problem is that an intermediate molecule such as a neural cell adhesion molecule (NCAM), a molecule involved in neuron growth, may be the missing link in this transduction pathway(77).

There is direct evidence that PrP^C plays a role in activating the various kinase pathways. Protein kinase A (PKA), for example (which is used in a cAMP dependent manner) has been shown to be mediated by PrP^C(77). The modulation of the PKA pathway is known to confer neuroprotection. This protection was not obtainable in PrP-null mice and was demonstrated to occur when cAMP levels rose in the presence of PrP^C, activating the PKA pathway(22). A study looking at the interaction between PrP^C and the PKA pathway found that neuroprotective effects were abolished in the presence of antibodies that blocked PrP^C. The protective effect was also eliminated when PrP^C binding was blocked, which demonstrated the importance of PrP^C in the activation of the pathway(22).

The previously mentioned study also indicated that PrP^C activated the extracellular signal-regulated kinase (ERK) pathway(22). Similar to the results mentioned in the preceding paragraph, no ERK detection was obtained in PrP-null retinal cells. Antibody-induced clustering of PrP^C seemed to be the main mechanism in ERK phosphorylation and activation(22). Furthermore, ERK activation was abolished when NADPH oxidase was blocked, indicating that the interaction between PrP^C, NADPH oxidase and reactive oxygen species may be an intermediate step in the activation of the ERK pathway(22,77)

Finally, the other PrP^C related signalling interaction that has been investigated is that of PrP^C and protein kinase C (PKC). The first evidence that PrP^C interacted with this pathway came from studies demonstrating PrP^C controlled mediation of Ca^{2+} influx. Transfecting cells with *Prnp* increased the levels of Ca^{2+} taken in through the plasma membrane but decreased the release of Ca^{2+} from the ER and the mitochondria(16).

These results suggest that PrP^C plays a role in controlling the calcium-dependent PKC-α and PKC-β pathways. Other studies have demonstrated that lectin-induced phosphorylation caused by the calcium dependent PKC-α and PKC-β pathways were decreased in PrP-null mice whereas the calcium independent PKC-δ pathway was unaffected(83). Although these studies provide the basis to support the interaction of PrP^C and the PKC kinase pathway, further evidence will be needed before any direct links can be made(77).

1.6 Prion Proteins: The Dark Side

1.6.1 PrP^{Sc} Biochemistry PrP^{Sc} is the misfolded isomer of PrP^C that undergoes a structural change from an alpha helix-rich form to a prominently beta sheet form. PrP^{Sc} is a hydrophobic protein that contains the exact same amino acid sequence as PrP^C(106). PrP^{Sc} is insoluble in mild detergents, in contrast to PrP^C(106). When purified, PrP^{Sc} forms rods that are visible in electron micrographs(106). These rods, when stained with Congo Red, show the same fluorescence seen in amyloids(88). The protein is approximately 33-35 kDa in size and has the very unusual property of being resistant to proteinase K-induced degredation(106). This is one of the main distinguishing features between PrP^{Sc} and PrP^C as PrP^C is quite susceptible to proteinase K treatement(70,106).

PrP^{Sc} is also different biochemically from PrP^C in that PrP^{Sc} can interact with PrP^C to produce new PrP^{Sc} molecules. These new PrP^{Sc} molecules are able to demonstrate their infectious nature due to their ability to amplify, aggregate and cause disease in a variety of mammals including humans(70).

The actual conversion of PrP^{C} to PrP^{Sc} seems to be a conformational change as opposed to a covalent one(51). The two molecules contain exactly the same amino acid sequence and most likely the same post-translational modifications(51). The increase in the number of beta sheets when the conversion occurs is quite profound going from 3% in PrP^{C} to 43% in PrP^{Sc}(51). Although tertiary structure data on PrP^{Sc} is still quite limited due to the technical difficulties in working with PrP^{Sc}, preliminary evidence suggests that changes take place primarily in the N-terminal region of PrP^{C} when PrP^{Sc} is being generated(51). Some structural data using two dimensional crystalline-like arrays of PrP^{Sc} molecules suggest that PrP^{Sc} has hexagonal symmetry. The N-terminal becomes located on the inside of the protein with helices two and three at the outer side, the glycosyl-groups face the space between the hexagonal units and beta helices may be more prevalent than the beta sheets originally reported by others using spectroscopy(106).

1.6.2 <u>PrP^{Sc} Formation and Conversion</u> It is believed that the formation of PrP^{Sc} occurs via a physical interaction capable of generating new PrP^{Sc} from existing PrP^{C} molecules(51). The first line of evidence came from *in vivo* mice studies where PrP-null mice demonstrated a complete resistance to prion infection(51). Second, studies using transgenics have allowed infections between various species and prion strains. For example, expression of a hamster transgene in mice allows them to become susceptible to hamster prion infections where they normally would be resistant(119). Lastly, *in vitro* conversion system experiments have used purified PrP^{Sc} to generate new PrP^{Sc} from PrP^{C} stocks(38). These experiments required the addition of mammalian RNA, however, indicating that the physical interaction is not directly initiated by PrP^{Sc} on PrP^{C} contact but that single stranded RNA plays a role in inducing the conformational changes needed to alter PrP^{C}'s structure(38).

Some studies have looked at the biochemistry that is considered essential for the conversion of PrP^{Sc} from PrP^{C}(108). Sixty-six amino acids of the N-terminal and thirty-six amino acids that include helix 1 and β-strand 2 of the entire PrP^{Sc} molecule are not required for conversion of PrP^{C}(108). Similarly, N-linked glycosylation is not needed to ensure PrP^{Sc} conversion(126). Sequence conservation within the central region of the protein does play a large role in the conversion effectiveness, with a single mismatched amino acid being capable of preventing the conversion of PrP^{C} to PrP^{Sc}(102). Taken together these results support the idea that for PrP^{Sc} to be formed, the template molecule, PrP^{C}, must be present and that physical interactions of certain parts of the molecule must occur, most likely with the guidance of various RNA molecules.

There are currently several models to explain how PrP^{C} may be altered to its pathogenic isoform, PrP^{Sc}. In one such model, nucleated polymerization, individual monomers of PrP^{C} are attached and stabilized to the ends of pre-existing polymeric PrP^{Sc} structures in a mechanism similar to that seen with actin or tubulin polymerization(51). In a second model, template assistance, the conversion occurs with a template PrP^{Sc} molecule that oligomerizes with PrP^{C} molecules and a putative molecular chaperone, to produce new PrP^{Sc} molecules(51). Which of the two models is correct will require more in-depth understanding of the physical interactions between PrP molecules in the cell.

Of note is the involvement of molecular chaperones in the conversion of PrP. Chaperones influence the folding of proteins during their synthesis and transport. They are often imperative in preventing the misfolding of proteins or preventing aggregation especially during times of stress(51). The first evidence of their involvement in the PrP^C to PrP^{Sc} conversion process was demonstrated when scientists noticed that human PrP^{Sc} propagates poorly in mice transgenically modified with human PrP^C(140). When they modified the human PrP^C to express N- or C-terminals that could interact with chaperones from mice however, conversion of human PrP^C to PrP^{Sc} was much more efficient. As well, some of the most striking evidence of the importance of chaperones comes from studies done on the yeast chaperone Hsp104 and the bacterial chaperone GroEL. These two particular chaperones have been shown to increase the formation of PrP^{Sc} in a cell free system(36). Hsp104 studies originally focused on the formation of PSI+, a protein in yeast that can exist in a prion-like state, however, further studies utilized both GroEL and Hsp104 in the presence of PrP^C to generate PrP^{Sc}(21,36). Although these two proteins provide evidence that chaperones can play a role in converting PrP^C to PrP^{Sc}, they would never normally be found in mammalian cells. Together, these studies indicate that cellular chaperones may need to be in place to ensure formation of PrP^{Sc} in the cell, however, further work will be needed to confirm or refute these findings(49,64). The study of molecular chaperones within mammals will, therefore, be crucial in deciphering prion conversion within the cellular environment.

1.6.3 <u>PrP^{Sc} Cellular Localization and Trafficking</u> Locating the cellular compartment of PrP^{Sc} has been difficult due to the poor immunoreactivity of this protein unless it is treated with denaturants. Unfortunately, such harsh treatment typically destroys the very cells and organelles being examined(51). Immunofluorescence studies of PrP^{Sc} using infected N2a cells suggested that the molecules are intracellular with a high affinity for Golgi, late endosomal and lysosomal markers(51). Immunogold staining indicated that some PrP^{Sc} molecules reside on the surface of the cell(62). Other studies point to specialized lipid rafts as the location of PrP^{Sc} based on results that demonstrate blockage of PrP conversion when cells are depleted of cholesterol or when artificially constructed PrP^{Sc}s that are not encasable in lipid rafts are used(127). Although this evidence provides some insight as to where PrP^{Sc} may reside within infected cells, further studies and more conclusive methods to localize PrP^{Sc} will have to be investigated.

Although PrP^{Sc} shares similar post-translated modifications with PrP^{C}, it seems that the GPI anchor is structurally different. Chemical analysis of PrP^{Sc} has shown that it does have the same GPI anchor found on its isomer yet enzymes that release PrP^{C} from the membrane via its GPI anchor are ineffective in cleaving PrP^{Sc}(51). GPI anchor release is not inhibited because of lumenal sequestration nor is it caused by the anchor being uncleavable by proteases(51). The anchor actually seems to avoid the cleavage one would see in PrP^{C} due to its location. When PrP^{Sc} is bound to the membrane, the GPI anchor becomes inaccessible to enzymes most likely due to the conformation of the protein(51). PrP^{Sc} seems to be structurally similar to PrP^{C} when in the cell yet the dynamics with which it interacts with various cellular components seems to change, probably due to conformational PrP^{Sc} changes.

1.7 Prions: The Ins and Outs

1.7.1 PrP^{Sc} Entry Mechanisms

Few cell lines are easily infected by PrP^{Sc} and the molecular mechanism underlying the susceptibility of cells to PrP^{Sc} infection remains largely unknown. Several cellular receptors have been suggested to mediate the entry of PrP^{Sc} into the targeted cells. Heparan sulphates, for example, appear to serve as cellular receptors. Blocking heparin-based PrP^{C} entry using heparin mimetics limited PrP^{Sc} propagation in Chinese hamster ovary (CHO), hypothalamus GT1-1 and N2a cell lines(55,57,118). The use of heparan sulphate as the main entry receptor may not be universal, however, as studies using heterologous epithelial cells (Rov: a rabbit epithelial cell line) expressing ovine prion protein suggest heparan sulphate is not involved in PrP^{Sc} internalization(97).

The internalization of PrP^{Sc} may occur through multiple routes. The non-integrin 37 kDa/67 kDa laminin receptor (LRP/LR) has been identified as another cell surface receptor for PrP^{Sc}, and is required for PrP^{Sc} propagation in some scrapie-infected cells(46,72). Furthermore, Morel *et al.* reported that bovine PrP^{Sc} is internalized in human Caco-2/TC7 enterocytes via LRP/LR-mediated endocytosis(91). This observation supports the hypothesis that enterocytes, the major cell population of the intestinal epithelium, play a role for the uptake of infectious prion particles during oral infection. Given the wide distribution of laminin receptor as the major glycoprotein of the basement membrane for all cell types, and the varying susceptibility of different cells types to PrP^{Sc} infection, LRP/LR may not be solely responsible for the uptake of PrP^{Sc}. Indeed, mounting evidence suggests that multiple cellular factors or endocytotic routes could be involved(92,97). For example, the presence of PrP^C has been reported to be critical in supporting the propagation of PrP^{Sc}(97,124). Since only a few PrP^C expressing cells can be infected by PrP^{Sc}, PrP^C itself is insufficient to confer permissiveness of cells for infectivity. It was hypothesized that PrP^C could interact with exogenous PrP^{Sc} at the cell surface in the early stage of *de novo* infection of epithelial cells, which might be the first place where PrP^{Sc} multiplication takes place(97). Recent studies propose that the stromal complement receptor CD21/35 could be involved in targeting PrP^{Sc} to follicular dendritic cells (FDCs)(145). It remains unclear as to how PrP^{Sc} replicates in FDCs although B-cells are critical for neuroinvasion of PrP^{Sc} in the mouse model(90). As CD21/35 receptors also exist in B-cells, which appear to express little PrP^C, the requirement for these cells to facilitate accumulation of PrP^{Sc} in the spleen was thought to be related to the way B-cells supply cytokines necessary for the maturation of FDC(65,82,145). Furthermore, other receptors have been speculated to serve as cellular receptors mediating the endocytosis of PrP^C, i.e., stress-inducible protein, neural adhesion molecules and lipoprotein receptor-related protein 1 (LRP1)(113,117,129,146). How exogenous PrP^{Sc} interacts with these receptors has not been as vigorously studied *in vitro* as the normal cellular PrP^C. Collectively, the data documented in the literature suggests that PrP^{Sc} may enter target cells via various routes or PrP^{Sc} entry may be dependant on cell types. The limited knowledge of which cellular receptors mediate PrP^{Sc} entry hamper the understanding of what happens after PrP^{Sc} has entered into the cell. It is unknown what constitutes the most effective environment within cells to support PrP^{Sc} propagation. While results from the studies suggest that the susceptibility of a cell line to PrP^{Sc} infection might be independent of the tissue origin or the level of normal prion proteins, the presence of PrP^{C}, albeit at low levels, appears to be necessary since knock-out of the gene encoding PrP^{C} by small interfering RNA (siRNA) inhibits PrP^{Sc} propagation(35).

1.7.2 <u>PrP^{Sc} Travel Routes</u> The exact method by which PrP^{Sc} spreads throughout the body is not yet fully understood. After pre-oral uptake of a TSE agent, four major components seem to influence the success of prion propagation within the hosts' body. The process begins with accumulation of the prion agent in lymphoid tissues. From there, neuro-invasion occurs as the agents spread to the peripheral nervous system. Third, the pathogen travels and spreads throughout the spinal cord and the brain. And finally, the agent spreads from the central nervous system (CNS) to other peripheral sites such as muscles(9).

In the lymphoid follicles, a variety of cells seem to have a role in the uptake and interaction of PrP^{Sc}. Studies have shown that FDCs, macrophages and epithelial cells all demonstrate uptake of PrP^{Sc}(9). FDCs have been shown in the past to play a large role in the spread of PrP^{Sc} diseases. By blocking the signals used to maintain FDCs in a differentiated state, Mabbott *et al.* demonstrated that PrP^{Sc} infection and spread was completely blocked(78). This protective quality may not be totally reliant on FDCs, however, and may occur due to the interaction they undergo with Payer's patches. Evidence to support this was shown when TNF-$\alpha^{-/-}$ x LT$\alpha^{-/-}$ mouse strain (which lacked Payer's patches) demonstrated resistance to PrP^{Sc} infection(9). As well, mice have shown that once the agent is ingested, PrP^{Sc} accumulates in Payers patches and mesenteric lymph nodes before travelling to the spleen(9). Taken together, the data suggested that

FDCs and Payers patches as well as a variety of other bone marrow derived cells act as a bridge in allowing PrP^{Sc} infection to develop, propagate and then move into the CNS(9).

To sustain any sort of PrP^{Sc} infection, cells need to produce the template molecule PrP^{C}(9). When searching for compartments between the lymphoreticular system (LRS) and the CNS, scientists discovered that the peripheral nerve system was the most likely candidate(9). Although the exact mechanism by which PrP^{Sc} travels between the LRS and the CNS has not been proven yet, theories entertain the idea that cell to cell contact between immune cells and nerves may be the most likely method of transfer(37). Lymphoid organs are known to be innervated via the splanchnic nerve, a part of the sympathetic nervous system and a potential target for the transfer of PrP^{Sc} to the CNS. As well sensory fibres of the vagal nerve are interspersed throughout the gastrointestinal tract. These fibres communicate with activated dendritic cells (DCs) which would fit well with evidence that DCs are key in the transmission of PrP^{Sc}(9). Finally, vagal efferents synapse in the enteric nervous system ganglia have been shown to innervate various targets in the intestinal wall(45). Due to the fact that studies have shown that PrP^{Sc} can undergo transcytosis across nerve endings, the variety of travel methods all provide different targets that may allow for entry of PrP^{Sc} into the CNS from the gastrointestinal tract(9).

Evidence to support the spread of PrP^{Sc} through the CNS has been documented with chronological studies in hamsters infected with scrapie, yet studies with scrapie and BSE in sheep also support these pathways(9). Initially, deposition of PrP^{Sc} occurs in the mesenteric and submucosal enteric nervous system (ENS) ganglia in the small intestine. From there, retrogradual transfer of the agent via autonomic ganglia and efferent fibres of the vagus and splanchic nerves to the dorsal motor nucleus and the intermediolateral grey column in the thoracic spinal cord occurs(9). Travelling along specific neuroanatomical projections within the brain and spinal cord, PrP^{Sc} infections have been shown to spread in an ascending and descending manner(9). Finally, the transmission seems to spread centrifugally to the sensory nodose and the dorsal root ganglia(9,84). Although the general pathways have now been traced, the exact mechanisms and cellular components used in the transportation of PrP^{Sc} are still being discovered in animal models. How exact the travel of PrP^{Sc} from cell to cell through the human body is has yet to be elucidated.

1.8 The Susceptibility of Cell Lines

1.8.1 Cell Culture Systems

Since the 1970's, cells of numerous lineages have been studied for their susceptibility to PrP^{Sc} infections. Clarke and Haig provided the first evidence that PrP^{Sc} could be passaged through cell culture(23). In addition, various cell lines were found to be susceptible to persistent PrP^{Sc} infections as reported by Chesebro's, Pruisner's and other groups over the last 30 years(18,104). A partial list of susceptible cell lines is found in table 1. One of the most studied PrP^{Sc}-infected cell lines is the scrapie-infected mouse neuroblastoma cell line (ScN2a), derived from N2a cells, which was found to be persistently infected with the Rocky Mountain Laboratory (RML) mouse-adapted strain of scrapie(18). Conflicting observations have been reported with respect to the biochemical or phenotypical changes in these PrP^{Sc}-infected cells relative to the parent cell lines. While some researchers have reported changes in catecholamine, serotonin and noradrenaline levels, others have failed to obtain the same results(80,124). Furthermore, some studies have observed morphological changes and increased proliferation in ScN2a cells while others have reported the opposite(13,80,124). Nevertheless, it is of note that most of the cell lines investigated so far display no visible morphological or pathological changes when infected. Although most cell lines are only susceptible to infection by PrP^{Sc} strains adapted from the host of the same species, some cell lines have shown susceptibility to a PrP^{Sc} infection from a different species(105). Given that BSE is believed to be responsible for vCJD infections in humans, the use of non-human cell lines in the production of biologics could potentially result in the transmission of PrP^{Sc}(28). This would most likely occur through a cell line that became infected with BSE PrP^{Sc} and then passed the infectious agent on during production of a human biologic to the end product.

Table 1. Partial List of Cell Lines Susceptible to PrP^{Sc} Infection

Cell Line Designation	Cell Line	Reference
GT1-7	Hypothalamic neuronal cell line (mouse)	(94,116)
L-fibroblast	Fibroblast cell line (mouse)	(24)
Scn2a	Neuroblastoma cell line (mouse)	(18)
PC12	Pheochromocytoma cell line (rat)	(24)
SMB	Mesodermal cell line (mouse)	(12)
ROV	Kidney epithelial cell line (rabbit) expressing ovine prion protein	(135)
MOV	Neuroglia cell line (mouse) expressing ovine prion protein	(4)
NIH/3T3	Fibroblast cell line (mouse)	(136)
L929	Fibroblast cell line (mouse)	(136)
SN56	Cholinergic septal neuronal cell line (mouse)	(7)
NSC	Fetal neural stem cells (mouse)	(89)
MPC	Adult multipotent progenitor cells (mouse)	(89)
C2C12	Myoblasts (mouse)	(40)
Neurosphere cell line	Central nervous system stem cell (mouse)	(47)
Neurons	Primary cells (mouse)	(32)
Astrocytes	Primary cells (mouse)	(32)
Dendritic Cells	Primary cells (human)	(110)

2.0 Rationale & Statement of Objectives

As discussed in section 1, TSEs caused by PrP^{Sc} result in neurodegenerative diseases that are both difficult to identify and inevitably lethal. Although the mechanism of transmission is not clearly known, data demonstrating that it can be spread between members of the same and of different species have been demonstrated in a number of studies(11,53,68). Transmission of PrP^{Sc} via biologics occurred with a sheep vaccine against mycoplasma that infected various flocks(17). Proof that human biologics could become contaminated and pass on an infection occurred when patients of human growth hormone or gonadotropin treatments became infected with sCJD from PrP^{Sc} contaminated biologics(111). With this in mind, the safety of biologics and biotherapeutics used in current human therapies are constantly debated(39). This concern stems from the fact that a range of animal derived compounds are used in the manufacturing process and could theoretically be contaminated with trace PrP^{Sc} amounts(53,68). These products may include, but are not limited to, blood components for transfusion, tissue transplants, organ transplants, hormones, DNA preparations produced in vitro, vaccines, cytokines and monoclonal antibodies(69).

The work done in this study was proposed to test the validity of some of these safety concerns. **The overall goals** were to test commonly used cell lines in the production of various human biologics for their susceptibility to a PrP^{Sc} infection. This in turn would provide a greater understanding of which substrates needed to be tested and monitored more closely for PrP^{Sc} infectivity.

A variety of human biologics are currently used for therapeutic needs. These biologics are prepared using a number of different cell lines. Thus **the specific goal** of this Master's research was to examine cell lines regulated by Health Canada and used in the production of human biologics for their susceptibility to a PrP^{Sc} infection. It was hypothesized that cell lines expressing PrP^{C} would be more susceptible to a PrP^{Sc} infection than cells that do not produce normal prion protein. In addition, due to the difficulty in isolating small quantities of PrP^{Sc} from cell cultures, determining a more effective means of concentrating PrP^{Sc} was also to be investigated. The **project objectives** were defined below:

- Develop a sensitive PrP^{Sc} identification method and compare it to other commonly used protein concentrating techniques in enriching PrP^{Sc} from *in vitro* samples.
- Test the following cell lines for their susceptibility to BSE infection:
  - *Spodoptera frugiperda* Insect Cell Line 21 (Sf21)
  - Mouse Neuroblastoma Cells (N2a)
  - Mouse Fibroblast Cells (NIH/3T3)
  - Mouse hybridoma B lymphocyte Cells (RmcB)
  - Chinese Hamster Ovary Cells (CHO)
  - Human Foreskin Fibroblast Cells (HFF, HFF-MitC)
  - Human Peripheral Blood Dendritic Cells (PBDC)
  - Human B-lymphocyte Cells (NAMALWA)
  - Human Lung Fibroblast Cells (MRC-5)
  - Human Embryonic Kidney Cells (HEK-293)

- Human Histiocytic Lymphoma Cells (U937)
- African Green Monkey Fibroblast Cells (Cos-7)

- Determine if cell surface interactions play an important part in establishing a persistent PrP^{Sc} infection.

3.0 Materials and Methods

3.1 Cell Culture:

Cell Lines. Mouse neuroblastoma (N2a) and scrapie infected mouse neuroblastoma (ScN2a) cells were obtained from InPro Biotechnology (San Francisco, CA, U.S.A.). Chinese hamster ovary cells (CHO), the insect cell line Sf21, human foreskin fibroblasts (HFF/HFF-MitC), mouse hybridoma (RmcB) cells, mouse fibroblast (NIH/3T3) cells, human B-lymphocyte cells (NAMALWA), human lung fibroblast cells (MRC-5), human embryonic kidney cells (HEK293) and African green monkey fibroblast cells (Cos-7) were obtained from the American Type Cell Collection (ATCC). Human histiocytic lymphoma cells (U937) were a gift from Dr. Karen Copeland (Public Health Agency of Canada). Peripheral blood dendritic cells (PBDC) were purchased from Stem Cell Technologies (Stem Cell Technologies, Vancouver, BC, Canada).

Cell Culture. N2a and Scn2a cells were grown in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L of sodium bicarbonate and supplemented with 10% fetal bovine serum and 0.2% penicillin and streptomycin. CHO cells were grown in F-12K medium supplemented with 10% FBS and 0.2% penicillin and streptomycin. Sf21 cells were grown in Grace's insect cell culture medium supplemented with 10% fetal bovine serum and 0.2% penicillin and streptomycin. HFF and HFF-MitC cells were grown in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5g/L sodium bicarbonate, 4.5g/L glucose and supplemented with

15% fetal bovine serum and 0.2% penicillin and streptomycin. RmcB and U937 cells were grown in RPMI-1640 medium adjusted to contain 1.5g/L sodium bicarbonate and supplemented with 10% fetal bovine serum and 0.2% penicillin and streptomycin. All media as well as the bovine serum albumin were purchased from Gibco (Invitrogen, Carlsbad, CA, U.S.A.). Antibiotics were obtained from Sigma-Aldrich (Sigma-Aldrich, Oakville, ON, Canada).

3.2 Cell Infection:

Inoculation of Cells. Cells were seeded at a density of 1×10^5 cells per well in a 12 well plate and 1 mL of media containing 0.05% (w/v) mouse-adapted bovine spongiform encephalopathy brain homogenate was added to each well. Cells were allowed to grow in the presence of the inoculum for 2-4 days at 37°C with 5% CO_2 before being collected. Adherent cell cultures were washed once with phosphate buffered saline (PBS) and incubated in 0.2 mL of 2.5% Trypsin-EDTA before being collected and centrifuged at 1600 x g for 5 min. Suspension cell cultures were collected, centrifuged at 1600 x g for 5 min, washed once in 1 mL of PBS and then re-centrifuged at 1600 x g for 5 min. Adherent or suspension cells were then split 1:10 and seeded in 6 well plates or plated on plastic cover slips at a density of 1.5×10^5 cells per well in a 24 well plate. Adherent or suspension cells were then allowed to grow to confluency at 37°C with 5% CO_2 and either subjected to a cell blot (see 3.3 Cell blot), collected for Western blot or passaged for further growth.

Pro-ject Inoculation. Pro-ject reagent (a liposome delivery system) was purchased from Pierce Biotechnology (Rockford, IL, U.S.A.). As per the manufacturer protocol, Pro-ject film was suspended in 250 uL of methanol and vortexed for 10-20 seconds at top speed. 10 uL aliquots were distributed into 1.5 mL Eppendorf tubes to evaporate for 4 hours in a laminar flow hood. Aliquots were frozen at -20˚C until further use. Cells were seeded at $3x10^5$ cells per well in a 6 well plate and allowed to grow for 2 days at 37°C with 5% CO_2 before further processing. The protein of interest for delivery (BSE PrP^{Sc} or β-galactosidase) was diluted in PBS to a concentration of 100 ug/mL and added to the aliquot of Pro-ject. The mixture was pipetted up and down 3-5 times and then vortexed for 3-5 seconds at low speed. One aliquot was used per well of a 6 well plate. Serum-free medium was added to the mixture to bring the final volume up to 1 mL. Media was removed and cells were washed once with PBS. The mixture was added to the cells and allowed to incubate at 37˚C with 5% CO_2 for 4 hours. The mixture was removed and 1-2 mL of complete media was added to each well. Cells were then passaged and treated to either the cell blot or with X-gal for β-galactosidase detection.

3.3 Immunoassays:

Cell Preparation. Cell pellets were collected by centrifugation at 1600 x g for 5 min in a microfuge. Pelleted material (approximately $2x10^7$ cells) was incubated with 1 mL of lysis buffer (0.5% sodium deoxycholate, 0.5% Triton X-100, 150 mM NaCl, and Tris HCl [pH 7.5]) for 10 min at 4°C. The cells were then incubated with 5ug/mL (final concentration) of DNase I for 10 min at room temperature and centrifuged for 5 min at

1600 x g to remove cell debris. The resulting supernatant was collected in a new tube and incubated with 20 mM of Protease K (PK) for 1.5 hrs at 37°C with agitation. The reaction was stopped by the addition of 5 mM phenylmethylsulphonyl fluoride (PMSF) for 20 min at room temperature. 100 uL of aliquoted samples were then either processed further or stored at -20°C until further use.

TCA Precipitation. Aliquoted samples were incubated with 100 uL of 0.15% sodium deoxycholate and 900 uL of distilled H_2O and incubated for 10 min at room temperature. Concentrated trichloroacetic acid (TCA) was then added to the mixture to a final concentration of 8.3% (v/v). After incubation for 5 min at room temperature the samples were centrifuged at 16000 x g for 5 min in a microcentrifuge. The supernatant was discarded and the pellets were resuspended in lysis buffer containing 6 M Urea. Samples were then left at room temperature for 2 hours to denature the infectious material before being mixed with equal volumes of 2x sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris HCl) and boiled at 95°C for 5 min. Samples were run through SDS-PAGE for 1 hr at 200 V in a 12% gel and were then subjected to Western blot.

PTA Precipitation. Supernatants were incubated with phosphotungstic acid (PTA) at a final concentration of 0.3%. Samples were left at room temperature for 5 min and then centrifuged at 16000 x g for 30 min. The pellets were resuspended in lysis buffer containing 6 M Urea. Samples were then left at room temperature for 2 hours to denature the infectious material before being mixed with equal volumes of 2x sample buffer and boiled at 95°C for 5 min. Samples were run through SDS-PAGE for 1 hr at 200 V in a 12% gel and were then subjected to Western blot.

Methanol and Ethanol Precipitation. Aliquoted samples were mixed with 4x volumes of either 100% ethanol or methanol and placed at -20°C for at least 30 min. Samples were then centrifuged at 16000 x g for 30 min. The pellets were resuspended in lysis buffer containing 6 M Urea. Samples were then left at room temperature for 2 hours to denature the infectious material before being mixed with equal volumes of 2x sample buffer and boiled at 95°C for 5 min. Samples were run through SDS-PAGE for 1 hr at 200 V in a 12% gel and were then subjected to Western blot.

Western Blotting. Equal amounts of sample were electrophoresed (1 hour, 200 V) onto Pierce pre-cast 12% Bis-Tris buffered sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels using Pierce running buffer (Pierce Biotechnology, Rockford, IL, U.S.A.). Gels were then electroblotted using the Mini Protean 3 transfer system (Bio-Rad, Mississauga, ON, Canada) in Pierce transfer buffer containing 20% (v/v) methanol for 1 h at 90 volts onto polyvinylidene fluoride (PVDF) membrane (Millipore, Etobicoke, ON, Canada). The PVDF membrane was pre-wetted in methanol and then rinsed in water before transfer and immediately incubated in blocking buffer (either 5% (w/v) non-fat blocking grade milk (Bio-Rad, Mississauga, Ontario, Canada) or 5% (v/v) Western blocking solution (Roche, Nutley, NJ, U.S.A.) following transfer in 20 mM Tris-HCL, pH 7.5, 137 mM NaCl, 0.5% Tween-20 (TBST)) for either 1 h at room temperature or overnight at 4°C. The membrane was incubated for 1 h at room temperature with 1 μg/mL of monoclonal antibody 6D11 or 6H4 (Abcam, Cambridge, MA, U.S.A. and Prionics, Schlieren-Zürich, Switzerland), which recognizes a conserved epitope on the murine PrP^C and PrP^{Sc} molecules. After washing 3 times (5 min for each) with TBST, the membrane was incubated with anti-mouse polyclonal serum conjugated with horseradish peroxidase (Bio-Rad, Mississauga, ON, Canada) diluted 1:20000 in blocking buffer. After washing 5 times (5-10 min per wash) with TBST, the membrane was incubated with Millipore chemiluminescent substrate for 5 min, and chemiluminescent signals were visualized by exposing the membrane to Kodak X-ray film (InterScience, Mississauga, Ontario, Canada).

Cell Blotting. The protocol was adapted from a previously published protocol(14). Plastic coverslips were placed in the wells of a 24 well plate. For suspension cell lines, plastic coverslips were incubated in poly-L-lysine for 5 min before being washed with sterile water, dried and placed into the wells of a 24 well plate. Cells were plated at approximately $2x10^5$ cells per well. After 3-4 days of incubation at 37°C with 5% CO_2, the media was removed and the cells were washed once in PBS. The coverslips were removed and placed cell-side up on a sheet of blotting paper. A suitably sized piece of polyvinylidene fluoride (PVDF) membrane was incubated with 100% methanol, washed in distilled water and then soaked in lysis buffer (0.5% sodium deoxycholate, 0.5% Triton X-100, 150 mM NaCl, and Tris HCl [pH 7.5]) before being placed on top of the coverslips. A lysis buffer-soaked sheet of blotting paper was then placed on top of the PVDF membrane. A glass plate was placed on top of this sandwich and weight was applied to the sandwich for 30 sec. The coverslips that adhered to the membrane were carefully removed with forceps. The membrane was then air dried for 1 to 2 h (at this point the membrane was typically stored at -20°C until further use). The blot was then re-wet in lysis buffer before being incubated with 5 ug/mL of Protease K for 1.5 h at 37°C. The blot was washed once in distilled water and then incubated for 20 min in 5 mM phenylmethylsulfonyl fluoride (PMSF) before being immersed in denaturing buffer for 1 hour (5 M Guanidine Thiocyanate, 10 mM Tris HCl [pH 8.0]). Up to this step, the blot was considered potentially infectious and kept within a biosafety cabinet. The blot was then washed twice in distilled water before being incubated in 5% non-fat dry milk for 1 h. The blot was washed 2 times for 5 min in tris buffered saline with 0.5% tween-20 (TBST) and 1 time for 15 min in TBST before being incubated with 1ug/mL of antibody 6D11 or 6H4 for 2 h at room temperature (or overnight at 4°C). The blot was then washed 2 times for 5 min in TBST and 1 time for 15 min in TBST. The blot was incubated in anti-mouse HRP (1:20000) for 1 h at room temperature. The blot was then washed 2 times for 5 min in TBST and 1 time for 15 min in TBST. Detection was performed with the Millipore high sensitivity chemiluminescent substrate as per the manufacturer's instructions for Western blotting. Densitometry was performed using FluorChem software on scanned images of photographic film.

4.0 Results

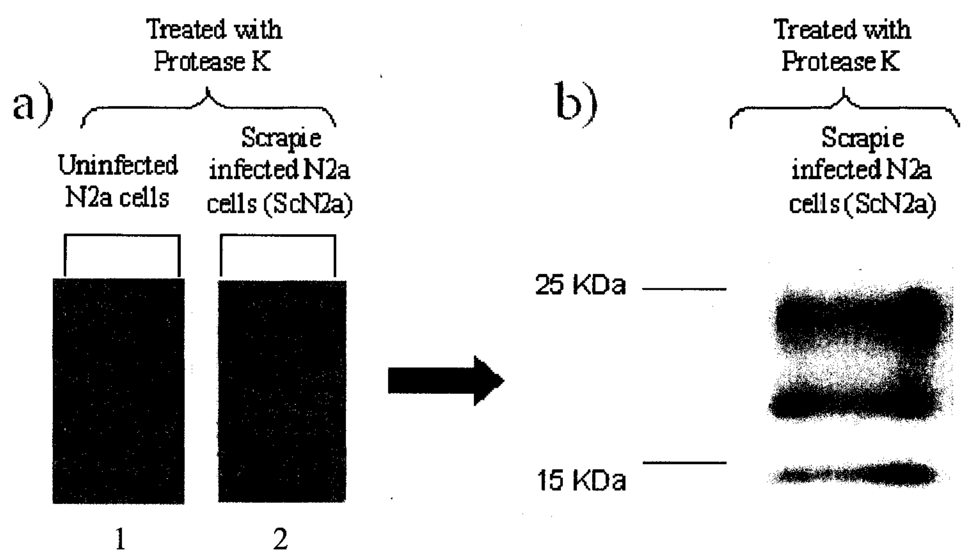
The overall purpose of this project was to examine the susceptibility of cell lines used in the production of human biologics to a PrP^{Sc} infection. One of the earliest steps involved developing a method that would effectively and sensitively allow for visualization of a PrP^{Sc} infection within the cell lines tested.

4.1 <u>The Cell Blot Method:</u> Identification of PrP^{Sc} has always been difficult due to a limited number of cells being permissive to an infection. This inefficiency leads to reduced PrP^{Sc} production *in vitro* and limits identification of the infectious agent. To circumvent this, a variety of techniques have been developed including ELISA, Western blotting, conformation-dependent immunoassays, *in vivo* bioassays, protein misfolding cyclic amplification (PMCA) and cell blotting(68). Due to time and equipment limitations, the cell blot was used because it is rapid, inexpensive and highly sensitive. Approximately 100-fold more sensitive than the commonly used Western blot, the cell blot is an effective technique able to detect infectious prion proteins even when very few cells are infected(14,69).

By using the cell blot on a cell line constitutively infected with PrP^{Sc} (Scn2a cells), the effectiveness of the technique in demonstrating PrP^{Sc} infections is shown in figure 1a where the coloured circles outline the coverslips that cells were grown on before being transferred and subjected to the cell blot protocol.

In figure 1a, a clear distinction is made between cells that are not-infected (lane 1) and those that are infected (lane 2) with PrP^{Sc}. Infected cells retain a signal after protease

Figure 1: Positive Control Cell Line ScN2a Demonstrating A Sustained PrP^{Sc} Infection. N2a cells and ScN2a cells were purchased from InPro Biotechnologies before being grown on plastic coverslips for 2-4 days in a 24 well plate until a relatively confluent monolayer was achieved. A) Cell blot of N2a and ScN2a cells after protease K treatment. Lane 1: N2a cells expressing normal prion protein (PrP^{C}) were treated with protease K, which cleaves PrP^{C}, eliminating all PrP chemiluminescence. Lane 2: ScN2a cells, which express abnormal prion protein (PrP^{Sc}), were also treated with protease K; however, protease K does not fully cleave PrP^{Sc} so samples exhibit chemiluminescence. This technique can therefore distinguish normal from abnormal prions in cell lines. B) Western blot of the various glycosylated (di-, mono- and unglycosylated) forms of PrP^{Sc} after protease K treatment. ScN2a samples were submitted to SDS-PAGE and transferred overnight to PVDF membrane. Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being treated with HRP-labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate.



Scrapie Infected Cells Demonstrating the Efficacy and Sensitivity of the Cell Blot. A Western Blot Confirming the Various Glycosylated Forms of PrP^{Sc}.

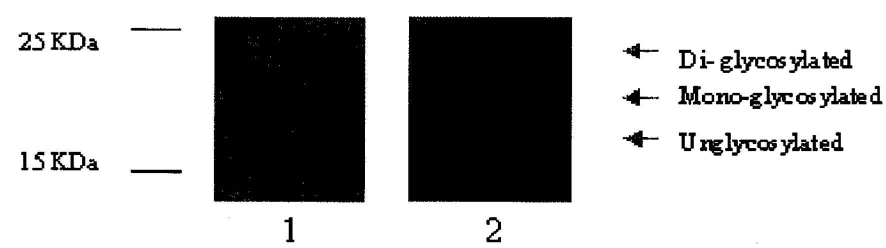
K treatment while uninfected cells do not. The cell blot in particular is able to demonstrate signal for individual cells or groups of cells that are exhibiting a PrP^{Sc} infection. Demonstrating that the cell blot is a sensitive method to identify PrP^{Sc} in Scn2a cells, the next step involved confirming the various glycosylated forms of PrP^{Sc} via Western blot. Figure 1b confirms the presence of PrP^{Sc} indicating the three classic forms including di(top), mono(middle) and unglycosylated(bottom) forms.

4.2 TCA Precipitation: Having shown that the cell blot is effective at identifying cells infected with PrP^{Sc}, a Western blot was used to confirm these results. Obtaining signal via the Western blot however was originally impeded by low quantities of PrP^{Sc} within the samples. To enrich PrP^{Sc} cell lysates, TCA precipitation was used. At first, samples were examined with or without TCA. In figure 2a, a Western blot demonstrates a stronger signal is obtained when TCA is used to precipitate samples in comparison with samples that are not precipitated. When quantified via densitometry of the bands in figure 2b, TCA precipitation results in an almost three-fold increase in its efficacy at precipitating PrP^{Sc} from cell samples. To test how dilute samples could be before TCA no longer captures enough PrP^{Sc} to detect via Western blot, 10-fold dilutions of cell lysates were treated to TCA precipitation. In figure 3, TCA is still able to precipitate PrP^{Sc} while the non-precipitated sample no longer generates detectable signal.

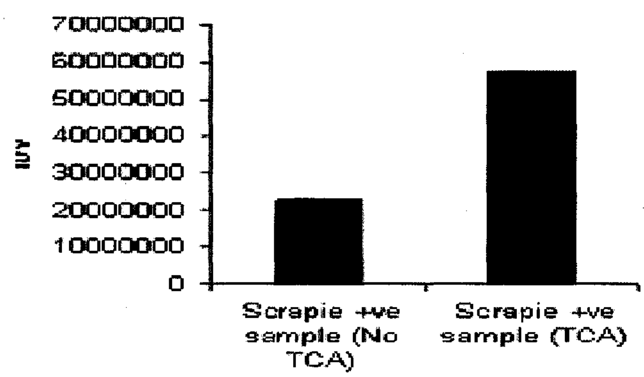
The effectiveness of TCA precipitation was then compared to other reported PrP^{Sc} precipitation techniques. Ethanol, methanol, phosphotungstic acid (PTA) and phenol precipitation are all commonly used precipitation techniques that have been documented

Figure 2: TCA Precipitation Increases the Sensitivity of Western Blot by Approximately Three-Fold Compared to Untreated Controls. A Western blot of ScN2a lysate samples with and without TCA precipitation was performed. Lane 1: ScN2a cell samples were not treated with TCA. Lane 2: ScN2a cell samples were treated with TCA. Densitometry was used to quantify immunofluorescent signals obtained. Cells were lysed and incubated with TCA (8.3%, final concentration) before being centrifuged at 13000 x g for 5 mis and then resuspended in sample buffer for SDS-PAGE. Samples were transferred overnight to PVDF membrane. Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being incubated with HRP labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate. IDV: Integrated densitometric values.

a)

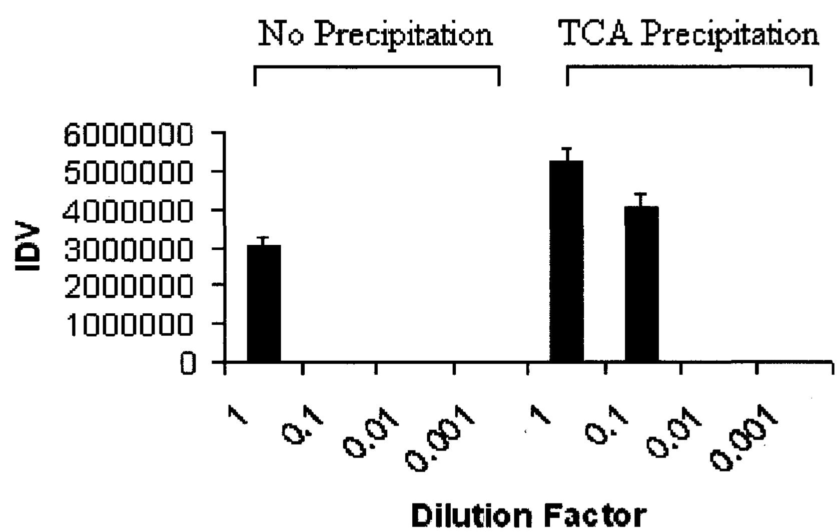


b)



Comparison of No Precipitation Technique to TCA Precipitation of PrP^{Sc} from ScN2a Lysates.

Figure 3: TCA Precipitation Increases the Detection Limit of a Western Blot by 10-fold. ScN2a lysates were diluted 10-, 100- and 1000-fold in lysis buffer. Samples were then treated with TCA as described in Methods. Briefly, cells were lysed and incubated with TCA (8.3%, final concentration) before being centrifuged at 13000 x g for 5 min and then resuspended in sample buffer for SDS-PAGE. Samples were transferred overnight to PVDF membrane. Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being treated with HRP labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate. IDV: Integrated densitometric values.



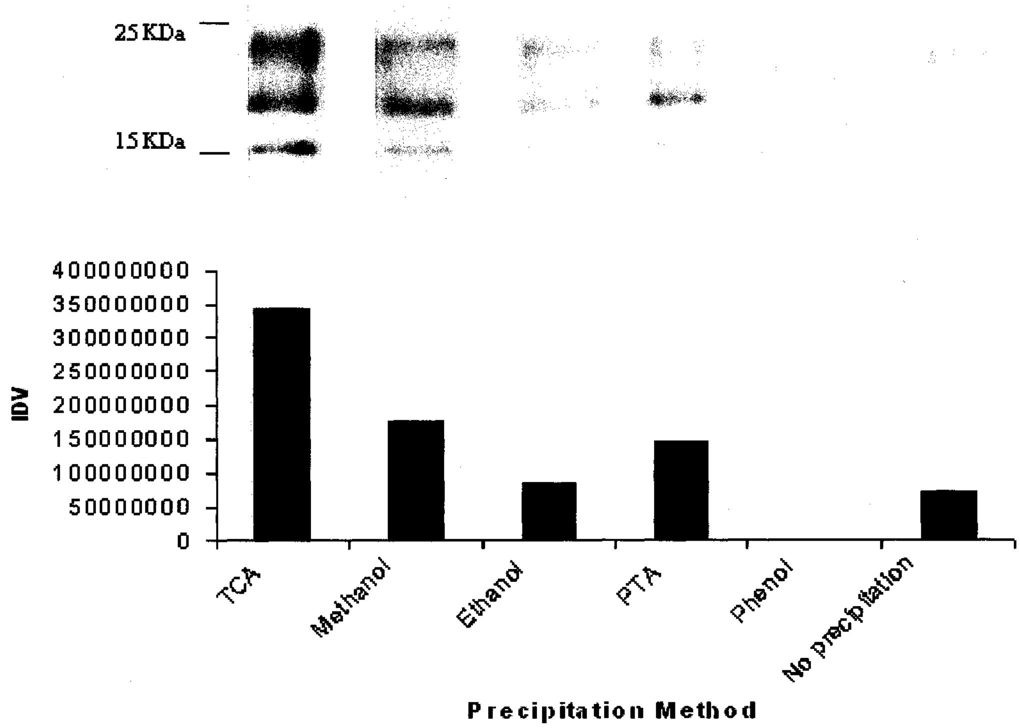
TCA Precipitation is Able to Detect PrP^{Sc} in Samples Diluted 10-fold.

in the past(14,59,68). Using Scn2a cells, lysates were processed using the different precipitating agents, treated with protease K to eliminate endogenous PrP^{C}, and then submitted to Western blot before being quantified by densitometry. As can be seen in figure 4, TCA was once again the most effective precipitating agent increasing the amount of PrP^{Sc} isolated by a factor of two to four versus ethanol, methanol, PTA or no precipitation. TCA was also much more effective than phenol precipitation where not enough PrP^{Sc} was recovered using phenol as a precipitant to produce a signal detectable by Western blot.

4.3 <u>Testing Various Cell Lines for PrP^{Sc} Susceptibility:</u> Having tested an effective, sensitive technique (the cell blot) to identify PrP^{Sc} in cell samples after treatment with infectious prions and having established an enrichment technique to confirm any infections via Western blotting, analysis of the cell lines listed in section 2.0 for PrP^{Sc} susceptibility was conducted. As a control for all cell lines tested, ScN2a cells were used to provide an example of the chemiluminescent signal that can be obtained from infected cells before and after protease K treatment. The signal represents PrP^{C} and PrP^{Sc} before protease K treatment and PrP^{Sc} that has resisted protease K degredation after treatment respectively.

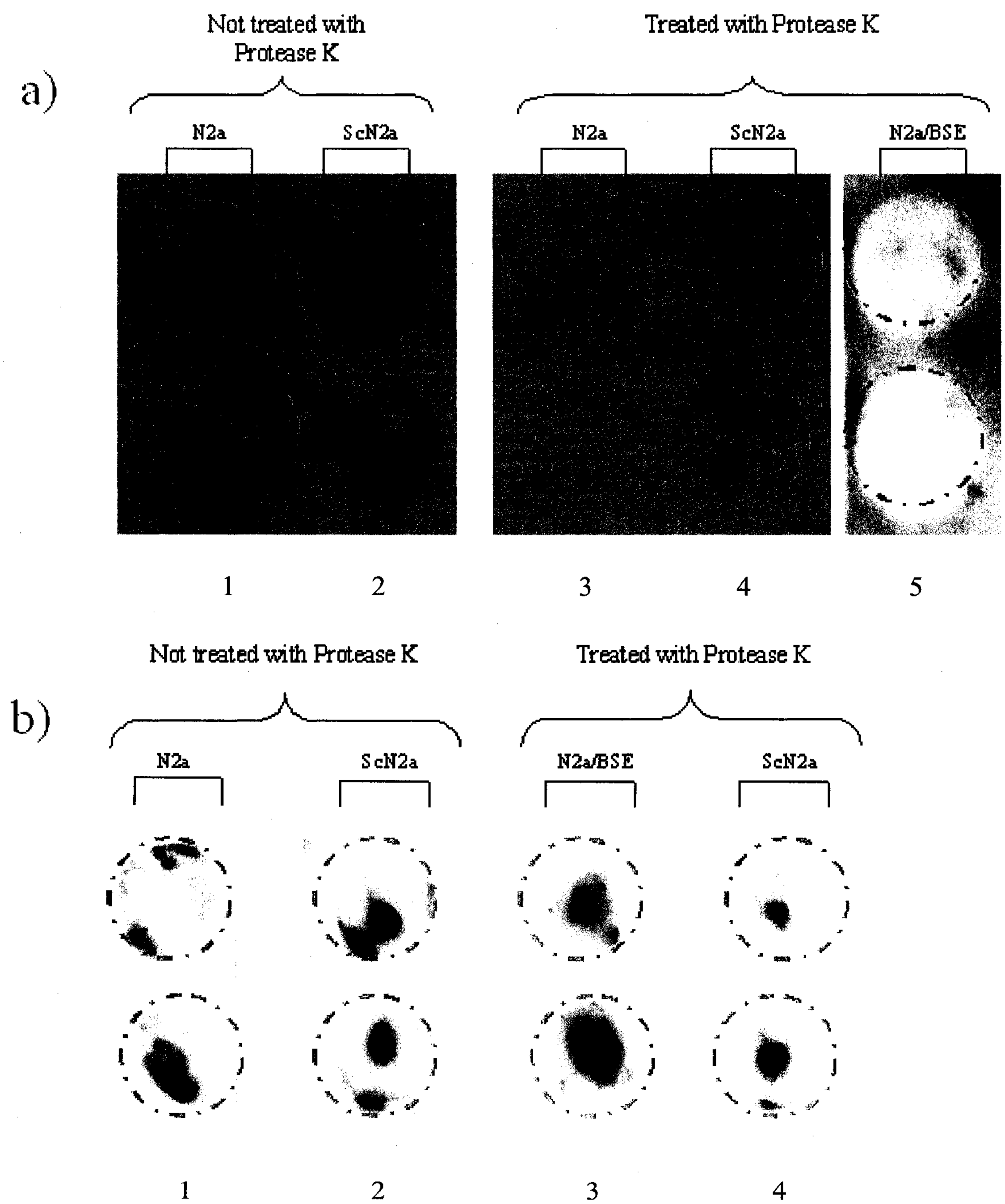
4.3.1 <u>N2a:</u> Mouse neuroblastoma cells were one of the first cell lines to propagate a PrP^{Sc} infection after Clarke and Haig demonstrated that N2a cells are susceptible to the Rocky Mountain Laboratory (RML) strain of scrapie(23). Although not necessarily used in the production of biologics, N2a cells provide a cell line that retains scrapie strain PrP^{Sc} infections for multiple passages. These cells are commonly

Figure 4: A Comparison of Various Techniques for Precipitation of PrP^{Sc}. Samples were treated with different precipitation methods before being run through SDS-PAGE and Western blot analysis with primary antibody 6H4 (1:8000) and anti-mouse HRP conjugated secondary antibody (1:40000). Visualization was performed with the Millipore chemiluminescent substrate. The bottom panel shows densitometry results for methanol, ethanol, phosphotungstic acid (PTA), phenol and trichloroacetic acid (TCA) precipitation. DV: Integrated densitometric values.



TCA Precipitation Demonstrates Increase Sensitivity in Comparison with Other Enrichment Techniques.

Figure 5: N2a Cell Blot Demonstrating No Susceptibility to BSE PrP^{Sc} Infection. N2a cells were inoculated with media containing BSE PrP^{Sc} and allowed to grow for 2-4 days until confluency was reached. A) Lane 1 and 2 demonstrate endogenous PrP^{C} and PrP^{Sc} produced by N2a and ScN2a cell lines reacting with monoclonal antibody 6D11. After protease K treatment however, PrP^{C} is cleaved (lane 3) while PrP^{Sc} (lane 4) resists degradation and retains signal when treated with 6D11. N2a cells (lane 5) treated with BSE PrP^{Sc} do not retain any signal when our inoculation methods are used, indicating that no sustained infection was obtained. ScN2a cells (lane 4) act as a positive control and retain signal. B) N2a cells retain a BSE PrP^{Sc} for at least 3 passages when treated with a liposome delivery system. Lanes 1 and 2 show that both N2a and ScN2a cells express PrP^{C}. After treatment with protease K, lanes 3 (that contains a cell blot of cells treated with BSE PrP^{Sc} via liposome delivery) and lane 4 shows remaining BSE and scrapie PrP^{Sc}, respectively. Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being treated with HRP labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate.

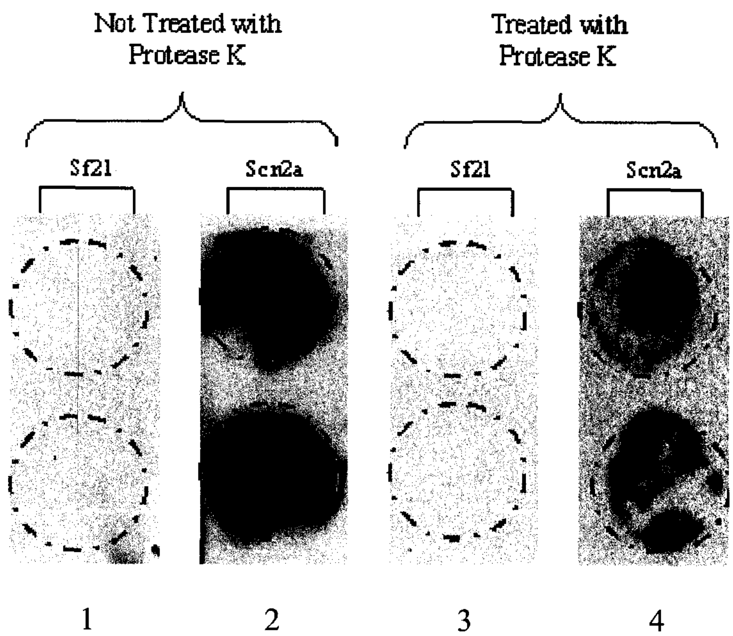


N2a Cells Infected or not Infected with Scrapie (ScN2a) or BSE and Either Treated with Protease K or Not Treated with Protease K.

referred to as ScN2a once they have been infected with RML and are used consistently in this study as a positive control cell line to demonstrate what a PrP^{Sc} cell line looks like after protease K treatment. As seen in figure 5a, one can see that N2a cells naturally produce PrP^{C} (lane 1) and are indistinguishable from PrP^{Sc} (lane 2) samples. After protease K treatment PrP^{C} is cleaved (lane 3) while PrP^{Sc} remains intact (lane 4). This leads to a complete loss of signal in the N2a-protease K-treated lane (lane 3) while remaining PrP^{Sc} in the ScN2a protease K-treated lane (lane 4) retains its signal. When tested for their susceptibility to BSE infection, (lane 5), the cells do not seem to retain an infection after incubation with BSE inoculum *in vitro*. An infection is re-established, however, once a liposome-based delivery system is used to insert the BSE proteins directly into the cells (figure 5b lane 3). This will be discussed further in section 5.0.

4.3.2 Sf21: An insect cell line adapted from the Fall Army Worm *Spodoptera frugiperda*, Sf21's are used in the production of recombinant proteins and vaccines for human biological use(98). Known for their ability to generate high yields of structurally native proteins *in vitro*, Sf21's typically express proteins using baculovirus vectors that encode various recombinant human cDNA's(98). Due to their use in the production of human biologics, Sf21 cells were cultivated with BSE in 6-well plates and were then investigated for their ability to sustain a prion infection. As shown in figure 6, Sf21 cells do not produce detectable amounts of PrP^{C} naturally (lane 1). Although ScN2a cells produced enough PrP^{Sc} to elicit a chemiluminescent response after protease K treatment (lane 4) acting as a control, Sf21 cells showed no chemiluminescent signal and hence, no infection after incubation with BSE (lane 3).

Figure 6: Sf21 Cell Blot Demonstrating No Susceptibility to BSE PrP^{Sc} Infection. Sf21 cells were inoculated with media containing BSE PrP^{Sc} and allowed to grow for 2-4 days until confluency was reached. Cells were then exposed to the cell blot protocol described in the Methods. Sf21 cells do not retain a PrP^{Sc} infection after exposure to BSE PrP^{Sc}. Lane 1 demonstrates the absence of any normal endogenous PrP^{C}. After protease K treatment, PrP^{C} is cleaved while PrP^{Sc} resists degradation and retains signal when treated with 6D11. Sf21 cells (lane 3) treated with BSE PrP^{Sc} do not retain any signal indicating that no sustained infection was obtained. ScN2a cells (lane 4) act as a positive control and retain signal. Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being treated with HRP labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate.



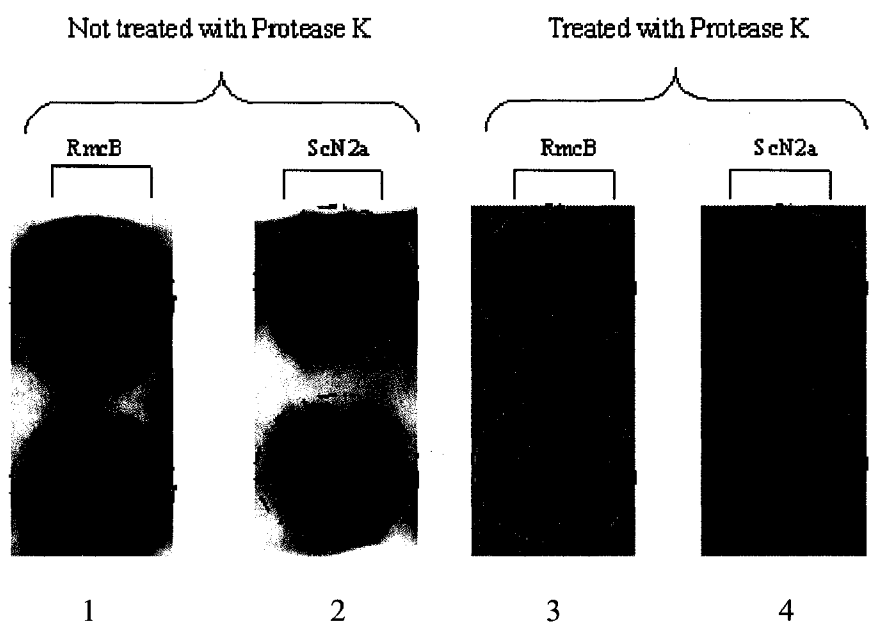
Sf21 Cells With or Without Protease K Treatment After BSE Inoculation.

4.3.3 RmcB: Originally developed in 1985 by Crowell *et al.*, this mouse hybridoma cell line was formed by fusing spleen cells with myelomas(33). Often used in the production of monoclonal antibodies, RmcB cells are involved in the research and manufacturing of human biologics. Originating from blood cell precursors, RmcB cells naturally produce PrP^C in fairly large quantities as seen in figure 7 (lane 1). Although large amounts of template PrP^C are present, after treatment with protease K, no signal is retained in the BSE treated lane (lane 3). This indicates that RmcB cells are not susceptible to BSE infection under these infection conditions.

4.3.4 CHO: Chinese hamster ovary cells were originally developed by scientists to compensate for the low levels of some biologically active molecules that were being obtained from bacterial protein production systems. Now, they are used in the production of a variety of recombinant proteins that are applied in human therapeutics(95). As seen in figure 8, CHO cells produce PrP^C naturally (lane 1), whereas no signal can be seen after BSE inoculation and protease K treatment (lane 3) indicating that no sustainable infection occurs in CHO cells using the infection methods found in this study.

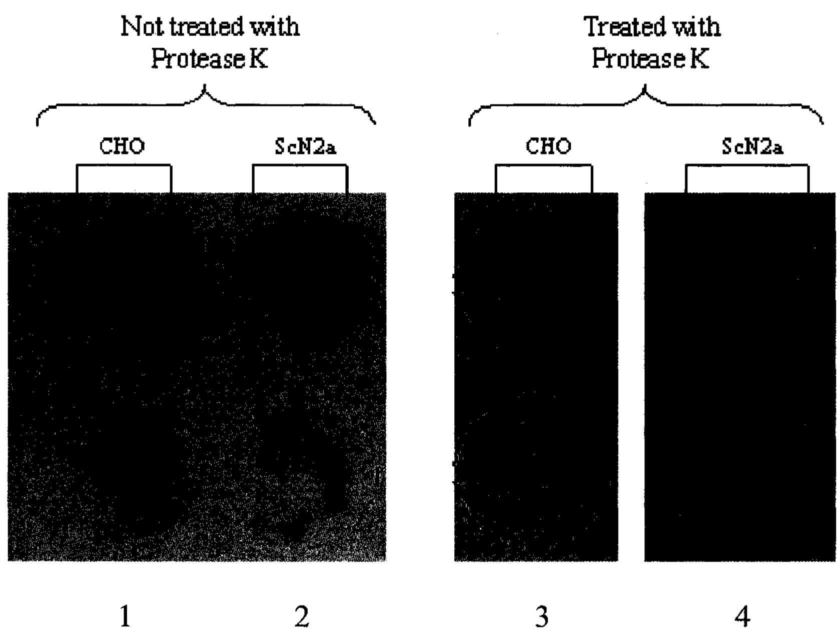
4.3.5 HFF/HFF-MitC: Human foreskin fibroblasts have a large role in the ever-emerging research designed around using stem cells for human therapeutics(134). These cells act as feeder cell layers that provide the required molecules to maintain a pleuripotent state in stem cell populations. HFF/HFF-MitC's could theoretically be a direct contact between PrP^{Sc} molecules and stem cells before being used in patient therapies. The main difference between the two HFF cell lines investigated in this study is that HFF-MitC cells were treated with Mitomycin C at passage 13, eliminating their

Figure 7: RmcB Cell Blot Demonstrating No Susceptibility to BSE PrP^{Sc} Infection. RmcB cells were inoculated with media containing BSE PrP^{Sc} and allowed to grow for 2-4 days until confluency was reached. Cells were then exposed to the cell blot protocol described in the Methods. RmcB cells do not retain a PrP^{Sc} infection after exposure to BSE PrP^{Sc}. Lane 1 demonstrates normal endogenous PrP^{C}. After protease K treatment, PrP^{C} is cleaved while PrP^{Sc} resists degradation and retains signal when treated with 6D11. RmcB cells (lane 3) treated with BSE PrP^{Sc} do not retain any signal indicating that no sustained infection was obtained. ScN2a cells (lane 4) act as a positive control and retain signal. Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being treated with HRP-labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate.



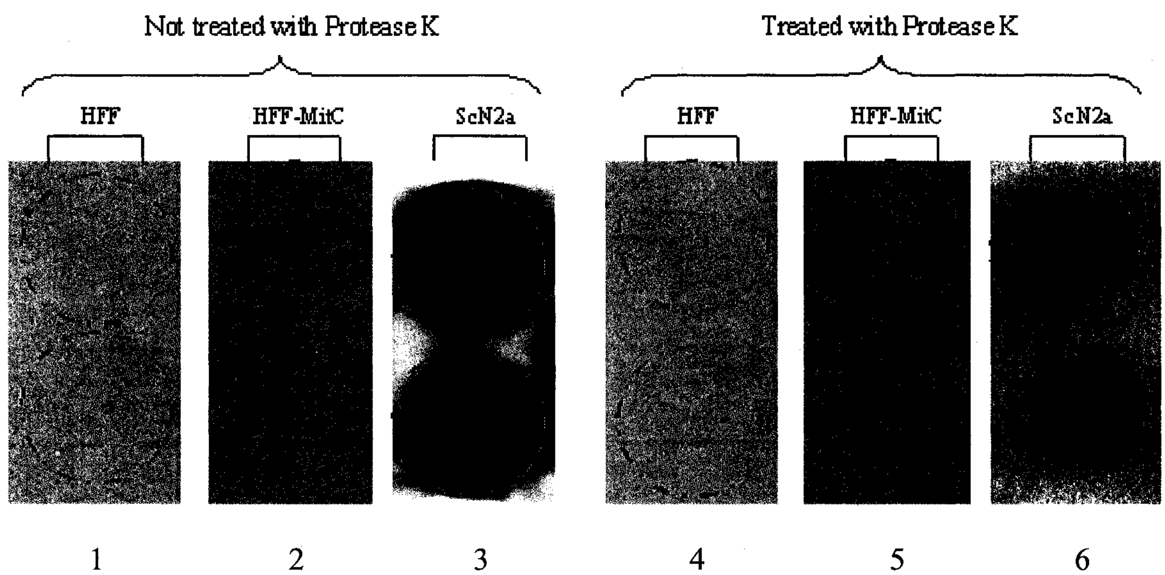
RmcB Cells With or Without Protease K Treatment After BSE Inoculation.

Figure 8: CHO Cell Blot Demonstrating No Susceptibility to BSE PrP^{Sc} Infection. CHO cells were inoculated with media containing BSE PrP^{Sc} and allowed to grow for 2-4 days until confluency was reached. Cells were then exposed to the cell blot protocol described in the Methods. CHO cells do not retain a PrP^{Sc} infection after exposure to BSE PrP^{Sc}. Lane 1 demonstrates normal endogenous PrP^{C}. After protease K treatment, PrP^{C} is cleaved while PrP^{Sc} resists degradation and retains signal when treated with 6D11. CHO cells (lane 3) treated with BSE PrP^{Sc} do not retain any signal indicating that no sustained infection was obtained. ScN2a cells (lane 4) act as a positive control and retain signal. Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being treated with HRP-labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate.



CHO Cells With or Without Protease K Treatment After BSE Inoculation.

Figure 9: HFF/HFF-MitC Cell Blot Demonstrating No Susceptibility to BSE PrP^{Sc} Infection. HFF or HFF-MitC cells were inoculated with media containing BSE PrP^{Sc} and allowed to grow for 2-4 days until confluency was reached. Cells were then exposed to the cell blot protocol described in the Methods. HFF/HFF-MitC cells do not retain a PrP^{Sc} infection after exposure to BSE PrP^{Sc}. Lane 1 demonstrates the absence of normal endogenous PrP^{C}. After protease K treatment, PrP^{C} is cleaved while PrP^{Sc} resists degradation and retains signal when treated with 6D11. HFF/HFF-MitC cells (lane 3) treated with BSE PrP^{Sc} do not retain any signal indicating that no sustained infection was obtained. ScN2a cells (lane 4) act as a positive control and retain signal. Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being treated with HRP-labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate.



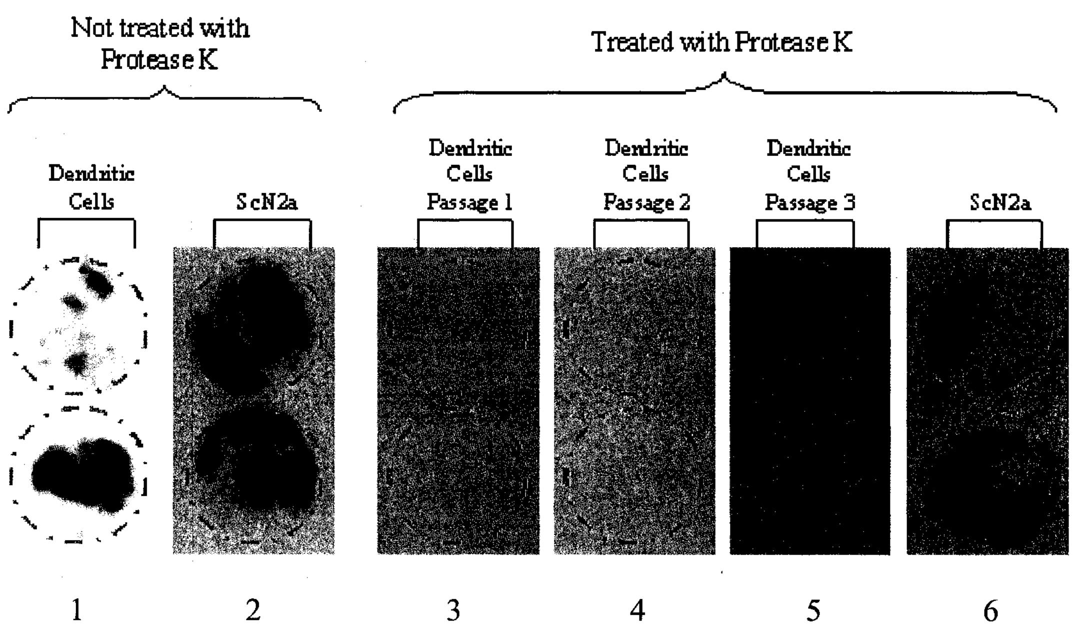
HFF and HFF-MitC With or Without Protease K Treatment After BSE Inoculation.

ability to replicate. Although some fibroblast cells do produce observable amounts of PrP^C, HFF's do not seem to produce any observable levels (figure 9, lane 1 and 2). As can be seen in figure 9, HFF (lane 4) and HFF-MitC (lane 5) cells do not retain any sort of BSE infection after inoculation with the agent and treatment with protease K.

4.3.6 PBDC: Human peripheral blood dendritic cells are well recognized for their ability to act as potent antigen presenting cells(8). They are typically used as either vaccine carriers or in immunotherapies. Their putative involvement in the transport of PrP^{Sc} causes grave concerns that should these cells become infected during manipulation/human biologic production, the implication could lead to an infection of the patient(68). Due to the fact that dendritic cells are not immortalized, passaging these cells becomes a difficult problem that had to be surpassed before their susceptibility could be tested. To circumvent this issue, samples of cells inoculated with BSE were lysed and then used to inoculate a fresh lot of PBDC's. As shown in figure 10, PBDC's do produce small amounts of PrP^C naturally (lane 1). These cells were inoculated with BSE before half were subjected to a cell blot or collected, lysed and applied to the media of a new batch of PBDC's. This procedure was done three times in total and cell blots from each 'passage' were compared for any infection. Figure 10 shows in lanes 3, 4 and 5 that no infection was obtained after three passages.

4.3.7 NAMALWA: Of human origin, these immortalized B-lymphocytes were originally obtained from a young girl named Namalwa who suffered from Burkitt's lymphoma(6). The use of these cells was strongly debated when proposed for use in human biologic production due to concern that transferring contaminating oncogenic

Figure 10: PBDC Cell Blot Demonstrating No Susceptibility to BSE PrP^{Sc} Infection. PBDC's were inoculated with media containing BSE PrP^{Sc} and allowed to grow for 2-4 days until confluency was reached. Cells were then exposed to the cell blot protocol described in the Methods. PBDC's do not retain a PrP^{Sc} infection after exposure to BSE PrP^{Sc}. Lane 1 demonstrates normal endogenous PrP^{C}. After protease K treatment, PrP^{C} is cleaved while PrP^{Sc} resists degradation and retains signal when treated with 6D11. PBDC's in lane 3, 4 and 5 treated with BSE PrP^{Sc} do not retain any signal indicating that no sustained infection was obtained. ScN2a cells (lane 4) act as a positive control and retain signal. Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being treated with HRP-labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate.



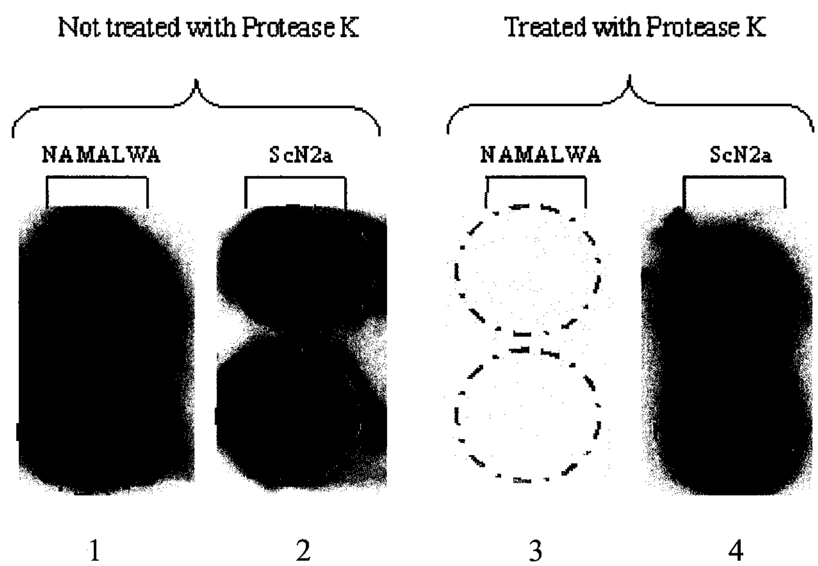
PBDC's With or Without Protease K Treatment After BSE Inoculation.

DNA could lead to tumor growth in the host(6). By the mid 1980's purification techniques combined with studies led by Petricciani *et al.* helped to alleviate these fears and led to the use of NAMALWA cells in the production of interferon for human application(6). NAMALWA cells do produce large amounts of PrP^{C} (figure 11, lane 1) indicating that they do provide the template to sustain a PrP^{Sc} infection. Once inoculation and passaging has occurred, however, no signal from PrP^{Sc} can be seen after protease K treatment (lane 3), indicating that NAMALWA cells are not susceptible to a BSE PrP^{Sc} infection via the techniques described in this study.

4.3.8 <u>MRC-5:</u> Developed in 1966 in the United Kingdom, the Medical Research Council strain 5 was derived from an aborted 14 week old male fetus(61). These lung fibroblast cells have been used to produce viral vaccines for use in human therapies due to their susceptibility to a range of human viral infections(61). This involvement in human biologics puts the patient at risk if an infection with PrP^{Sc} is possible. As can be seen in figure 12, however, even though MRC-5 cells do produce PrP^{C} naturally (lane 1), after inoculation with BSE, infection (lane 3) is not maintained using the infection protocol from this thesis.

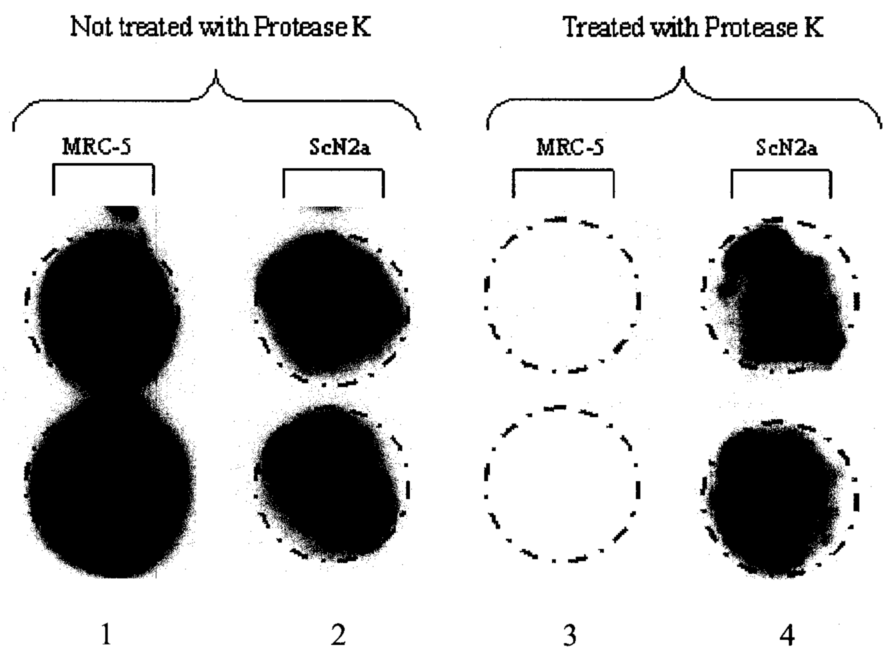
4.3.9 <u>HEK-293:</u> Created by adding adenovirus 5 DNA to human embryonic kidney cell cultures, the HEK-293 cell line was used to demonstrate one of the fundamental principles of molecular biology. Graham *et al.* showed that DNA could be transfected directly into mammalian cells and then expressed(48). These cells have since been used for human biologics where they were employed in vaccines production, adenoviral production (which is used in a host of different treatments) and gene therapy(120). In figure 13, one can see that HEK-293 cells produce PrP^{C} (lane 1)

Figure 11: NAMALWA Cell Blot Demonstrating No Susceptibility to BSE PrP^{Sc} Infection. NAMALWA cells were inoculated with media containing BSE PrP^{Sc} and allowed to grow for 2-4 days until confluency was reached. Cells were then exposed to the cell blot protocol described in the Methods. NAMALWA cells do not retain a PrP^{Sc} infection after exposure to BSE PrP^{Sc}. Lane 1 demonstrates normal endogenous PrP^{C}. After protease K treatment, PrP^{C} is cleaved while PrP^{Sc} resists degradation and retains signal when treated with 6D11. NAMALWA cells (lane 3) treated with BSE PrP^{Sc} do not retain any signal indicating that no sustained infection was obtained. ScN2a cells (lane 4) act as a positive control and retain signal. Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being treated with HRP-labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate.



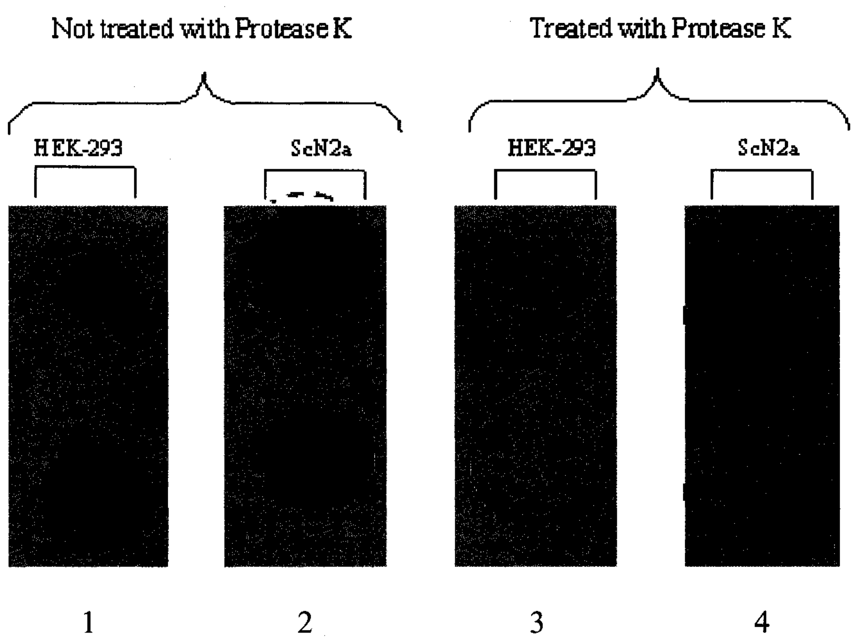
NAMALWA Cells With or Without Protease K Treatment After BSE Inoculation.

Figure 12: MRC-5 Cell Blot Demonstrating No Susceptibility to BSE PrP^{Sc} Infection. MRC-5 cells were inoculated with media containing BSE PrP^{Sc} and allowed to grow for 2-4 days until confluency was reached. Cells were then exposed to the cell blot protocol described in the Methods. MRC-5 cells do not retain a PrP^{Sc} infection after exposure to BSE PrP^{Sc}. Lane 1 demonstrates normal endogenous PrP^{C}. After protease K treatment, PrP^{C} is cleaved while PrP^{Sc} resists degradation and retains signal when treated with 6D11. MRC-5 cells (lane 3) treated with BSE PrP^{Sc} do not retain any signal indicating that no sustained infection was obtained. ScN2a cells (lane 4) act as a positive control and retain signal. Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being treated with HRP-labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate.



MRC-5 Cells With or Without Protease K Treatment After BSE Inoculation.

Figure 13: HEK-293 Cell Blot Demonstrating No Susceptibility to BSE PrP^{Sc} Infection. HEK-293 cells were inoculated with media containing BSE PrP^{Sc} and allowed to grow for 2-4 days until confluency was reached. Cells were then exposed to the cell blot protocol described in the Methods. HEK-293 cells do not retain a PrP^{Sc} infection after exposure to BSE PrP^{Sc}. Lane 1 demonstrates normal endogenous PrP^{C}. After protease K treatment, PrP^{C} is cleaved while PrP^{Sc} resists degradation and retains signal when treated with 6D11. HEK-293 cells (lane 3) treated with BSE PrP^{Sc} do not retain any signal indicating that no sustained infection was obtained. ScN2a cells (lane 4) act as a positive control and retain signal. Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being treated with HRP-labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate.



HEK293 Cells With or Without Protease K Treatment After BSE Inoculation.

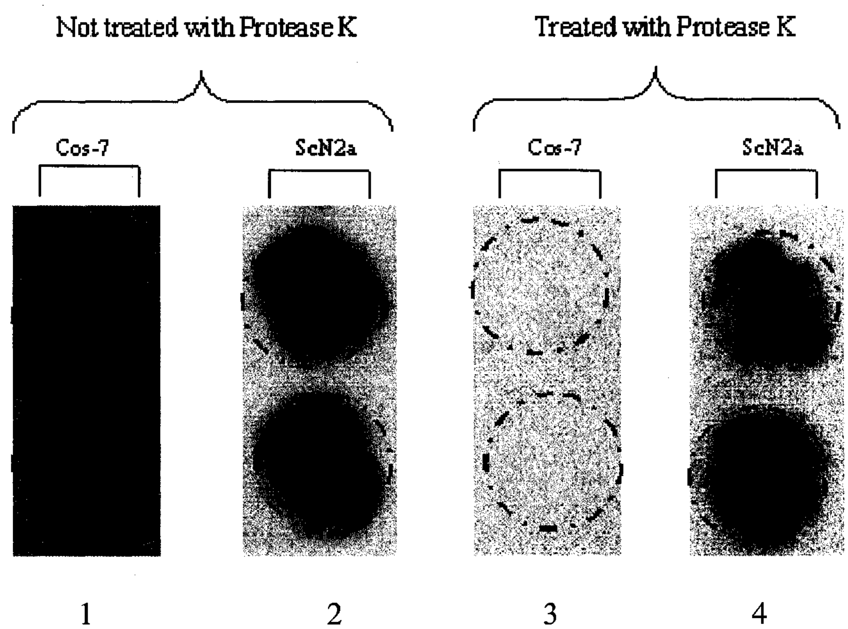
however after attempts at infection with BSE inoculum, no sustained infection was produced (lane 3).

4.3.10 Cos-7: Originally obtained from the kidney of the African green monkey by Yakov Gluzman, this cell line was generated by transformation of the CV-1 line with a defective mutant of the SV40 virus. This strain codes for the wild-type virus T-antigen. Although the use of these cells has been heavily contested over the last decade due to concerns that the T-antigen they produce may cause certain types of cancers in humans, they are still employed in the production of a variety of components used in cell culture and differentiation(20,25). As seen in figure 14, these cells produce PrP^C at observable levels (lane 1) but they do not show susceptibility to BSE infection when incubated with the inoculating agent (lane 3).

4.3.11 U937: Derived from a diffuse human histiocytic lymphoma, the U937 cell line is used as an *in vitro* model of monocyte/macrophage differentiation, anti-tumour product testing and various tumorigenicity factors. With regards to human biologics, U937 cells have been demonstrated to be successful producers of interleukins, like interleukin 1, which can be used in a variety of applications(3). Similar to RmcB cells in their high production capacity of PrP^C, U937 cells produce a strong signal before protease K treatment (figure 15, lane 1). This signal is eliminated after BSE inoculation and degradation with protease K (figure 15, lane 3) demonstrating that these cells are not susceptible to a BSE PrP^{Sc} infection.

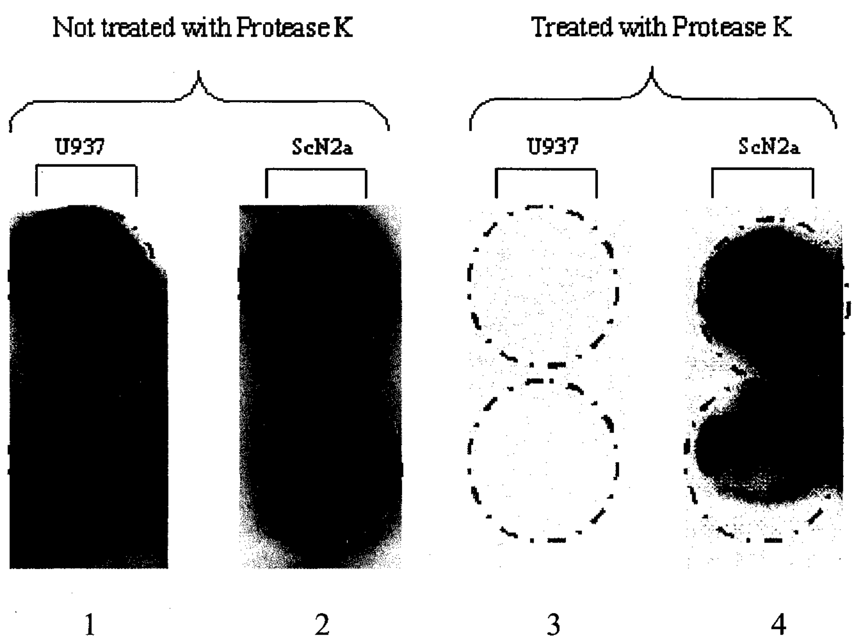
4.3.12 NIH/3T3: Established in 1962 by Todaro and Green at New York University, this Swiss mouse cell line has become the standard fibroblast cell line. The "3T3" name of the cell line refers to the protocol that Todaro and Green developed

Figure 14: Cos-7 Cell Blot Demonstrating No Susceptibility to BSE PrP^{Sc} Infection. Cos-7 cells were inoculated with media containing BSE PrP^{Sc} and allowed to grow for 2-4 days until confluency was reached. Cells were then exposed to the cell blot protocol described in the Methods. Cos-7 cells do not retain a PrP^{Sc} infection after exposure to BSE PrP^{Sc}. Lane 1 demonstrates normal endogenous PrP^{C}. After protease K treatment, PrP^{C} is cleaved while PrP^{Sc} resists degradation and retains signal when treated with 6D11. Cos-7 cells (lane 3) treated with BSE PrP^{Sc} do not retain any signal indicating that no sustained infection was obtained. ScN2a cells (lane 4) act as a positive control and retain signal. Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being treated with HRP-labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate.



Cos-7 Cells With or Without Protease K Treatment After BSE Inoculation.

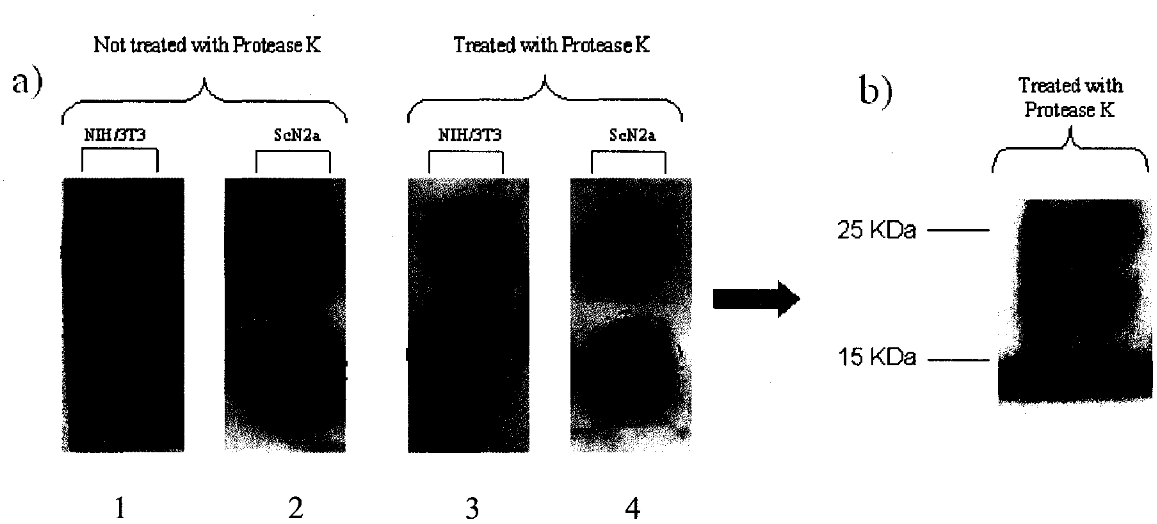
Figure 15: U937 Cell Blot Demonstrating No Susceptibility to BSE PrP^{Sc} Infection. U937 cells were inoculated with media containing BSE PrP^{Sc} and allowed to grow for 2-4 days until confluency was reached. Cells were then exposed to the cell blot protocol described in the Methods. U937 cells do not retain a PrP^{Sc} infection after exposure to BSE PrP^{Sc}. Lane 1 demonstrates normal endogenous PrP^{C}. After protease K treatment, PrP^{C} is cleaved while PrP^{Sc} resists degradation and retains signal when treated with 6D11. U937 cells (lane 3) treated with BSE PrP^{Sc} do not retain any signal indicating that no sustained infection was obtained. ScN2a cells (lane 4) act as a positive control and retain signal. Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being treated with HRP-labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate.



U937 Cells With or Without Protease K Treatment After BSE Inoculation.

when cultivating these cells *in vitro*. Every three days (the first "3"), transfer of (the "T") $3x10^5$ cells (the second "3") per 20-cm^2 plate was performed. Typically used as feeder cells for the development of stem cells into keratinocytes, these cells have been shown in the past to be susceptible to scrapie PrP^{Sc} but have yet to be infected with a BSE-PrP^{Sc} infection. Shown in figure 15a, NIH/3T3 cells express PrP^{C} (lane 1) and demonstrate the production of PrP^{Sc} after BSE inoculation (lane 3). Cells are shown at passage 10 and in figure 15b demonstrate the various forms of glycosylated PrP^{Sc} via Western blot confirming that an infection was sustained. This indicated that NIH/3T3 cells were susceptible to a BSE infection *in vitro*.

Figure 16: NIH/3T3 Cell Blot Demonstrating Susceptibility to BSE PrP^{Sc} Infection. NIH/3T3 cells were inoculated with media containing BSE PrP^{Sc} and allowed to grow for 2-4 days until confluency was reached. A) Cells were then exposed to the cell blot protocol described in the Methods. NIH/3T3 cells do not retain a PrP^{Sc} infection after exposure to BSE PrP^{Sc}. Lane 1 demonstrates normal endogenous PrP^{C}. After protease K treatment, PrP^{C} is cleaved while PrP^{Sc} resists degradation and retains signal when treated with 6D11. NIH/3T3 cells (lane 3) treated with BSE PrP^{Sc} retain a signal indicating that a sustained infection was obtained. ScN2a cells (lane 4) act as a positive control and retain signal. B) Western blot confirming infection of NIH/3T3 cells with the three glycosylated forms of PrP^{Sc}: diglycosylated (top), monoglycosylated (middle) and unglycosylated (bottom). Blots were incubated with monoclonal antibody 6D11 (1 ug/mL) for 2 hours at room temperature before being treated with HRP-labelled secondary antibody (1:25000). Membranes were visualized using Millipore chemiluminescent substrate.



NIH/3T3 Cells With or Without Protease K Treatment Demonstrating a Persistent BSE Infection.

5.0 Discussion

The objective of this thesis was to determine the susceptibility of various cell lines used in the production of human biologics to a prion infection. The concern about prion contamination stemmed from the fact that i) cell lines often come in contact with a variety of agents derived from animal products and ii) some cell lines have been shown to be susceptible and capable of sustaining a prion infection using the methods employed in this thesis(14,68,136). Infection of a cell line could lead to the production of contaminated biologics and to the infection of the general (human) population. The cell blot method was used in preliminary examination of each cell line due to its high sensitivity, its low cost and its relative ease of use. Although less sensitive, the Western blot was used to confirm any results obtained from other immunoassays when working with PrP^{Sc} due to its identification of the various glycosylated forms of PrP^{Sc}(14). TCA precipitation has been used by our group in the past to enrich proteins and was optimized to confirm any positive cell blot results because of its efficiency in concentrating PrP^{Sc} and because it is inexpensive and rapid.

Early work began around designing a method to concentrate the amount of PrP^{Sc} obtained from samples of a control cell line: scrapie-infected N2a (ScN2a) cells (figure 1). These cells were used due to their consistent PrP^{Sc} infection that could be maintained through multiple passages. While cell blots demonstrated obvious differences between ScN2a and N2a cells after protease K treatment (figure 1a), preliminary Western blots provided only a weak signal for the three forms of PrP^{Sc} including the un-, mono- and di-glycosylated variations (figure 2a, sample with no TCA). For this reason, protein concentration techniques were employed to enrich the samples. TCA precipitation, commonly used in our lab to concentrate proteins, was tested to determine its efficiency in enriching PrP^{Sc} from scrapie-infected samples. TCA precipitation increased the signal obtained from PrP^{Sc} samples (figure 2a) and, when quantified via densitometry, provided a three-fold increase in sensitivity of the Western blot method. In addition, TCA precipitation was able to recover PrP^{Sc} from samples diluted 10-fold whereas non-precipitated samples demonstrated no signal (figure 3). Having seen TCA precipitation's efficiency in concentrating PrP^{Sc} from ScN2a cell samples, this method was compared to a variety of other prion precipitating techniques that had been reported in the literature(13,59,70). Based on densitometric values, TCA precipitation was at least two to four-fold more effective in precipitating PrP^{Sc} from cell samples than ethanol, methanol, PTA or phenol precipitation (figure 4). The molecular basis for TCA precipitation seems to involve the three chloro groups found on the molecule. Studies that investigated the mechanism found that stronger acids, or acids that were molecularly similar (such as trifluoroacetic acid) but that lacked the chlorine groups were much less effective in precipitating proteins(123). The identification of TCA precipitation as a prion concentration technique was significant for several reasons including i) it demonstrated TCA is more sensitive in precipitating PrP^{Sc} than a variety of other techniques, ii) it enables all manipulation of the samples to occur within a biosafety cabinet allowing for safer methods when concentrating and iii) it could theoretically provide a new tool to investigate the interactions between prion proteins and chlorinated acids.

After establishing TCA as a powerful technique that could be used to help in confirming our results, cell blots of various cell lines used in the production of human biologics began. The selection of these cells is heavily influenced by their relevance to the manufacture of biologics and biotherapeutics regulated by Health Canada. Cells that produced no PrP^{C} such as the Sf21 (figure 6), HFF (figure 9) and HFF-MitC (figure 9) cell lines did not retain a detectable infection. This is most likely due to the lack of template PrP^{C} material that is believed to be a requisite component in establishing and maintaining a PrP^{Sc} infection(97). This requirement has been best shown in a study involving cells that were transfected to over-express PrP^{C}. The study demonstrated that infection of these cells was prevented if no PrP^{C} was expressed at the time of infection(97). On top of this, Paquet *et al.* also showed that restoration of PrP^{C} after inoculation of PrP^{Sc} did not produce infectivity, indicating that production of PrP^{C} needs to be present early during infection and that initial conversion events may occur at the cell surface before internalization of PrP^{Sc} occurs(97). Paquet's study highlights the reasons Sf21, HFF and HFF-MitC cells were not susceptible to a PrP^{Sc} infection. A lack of any template PrP^{C} hinders further PrP^{Sc} production, limiting propagation of the infectious agent even if PrP^{Sc} is able to enter into the cell. This would indicate that cells that do not express PrP^{C} are most likely not candidates for sustaining a PrP^{Sc} infection and that screening would most likely not be needed within these cell lines before they were used for human biologic production.

Some of the other cell lines examined did produce PrP^{C} naturally in varying quantities including N2a cells (figure 5a), RmcB cells (figure 7), CHO cells (figure 8), PBDCs (figure 10), NAMALWA cells (figure 11), MRC-5 cells (figure 12), HEK-293 cells (figure 13), Cos-7 cells (figure 14), U937 cells (figure 15) and NIH/3T3 cells (figure 16). These cells are from a variety of origins including mice, hamsters, primates and humans. Attempts to infect each cell line produced only one viably infected cell line via BSE PrP^{Sc} using our infection methods: the NIH/3T3 cell line (figure 15).

N2a, CHO, RmcB, PBDC, NAMALWA, MRC-5, HEK-293, Cos-7 and U937 cells demonstrated no persistent infection after at least three passages via the infection protocol used throughout this study. The absence of persistent infection has several possible explanations. The first is that entry of the PrP^{Sc} molecules may be required to establish an infection. Previous studies have shown that a variety of entry mechanisms are required to induce a prion infection; including, heparan sulphate receptors, the 37/67 kDa laminin receptor and CD 21/35(68,97). N2a, CHO and Cos-7 cells have been shown to express the 37/67 kDa laminin receptor(46,60,72). PBDC, NAMALWA, MRC-5, HEK-293 and U937 cells were reported to have heparan sulfate receptors(52,58,71,133,137). RmcB cells are not documented to contain any of the proposed receptors. Although most of the cell lines do express one of the cell receptors linked to the internalization of PrP^{Sc}, one study has shown that it may be the interaction of a combination of various receptors that allow for efficient internalization(60). A lack of any of the receptors could prevent steps in the cell surface interactions required to internalize exogenous PrP^{Sc} that could, in turn, prevent further conversion of PrP^{C} in these particular cell lines.

The species barrier may be another reason why some cells were unable to sustain an infection. As discussed in section 1.6.1, genotype, strain variations and inoculation methods can greatly influence the efficiency of PrP^{Sc} converting PrP^{C}. Due to the heterogeneity of the cell populations used in this study, the genotype of each cell may have varied, leading to different susceptibilities to PrP^{Sc} infection. Different susceptibilities in heterogeneous cell lines is in fact a common situation with PrP^{Sc} infected cell lines. This variability can also be a strong barrier to persistent infections as only small numbers of cells may become infected and these infected cells may eventually be eliminated during the passaging process(14).

Strain variations inhibit transmission of PrP^{Sc} infections between species *in vivo*. This interference also applies *in vitro* when adapting cell lines to infection from a strain not of the same species. Rabbit epithelial (Rov) cells provide an obvious example of how manipulating the strain of PrP^{C} being produced by the cells must be accomplished before any sort of infection can occur(135). This cell line was not susceptible to scrapie PrP^{Sc} infection unless normal sheep PrP^{C} was transgenically expressed *in vitro*. Following sheep PrP^{C} production, these cells maintained a scrapie PrP^{Sc} infection from passage to passage(135). One argument that the authors claim is that the overexpression of endogenous PrP may be what limits infections from crossing the species barrier. As seen in cell-free conversion systems, endogenous PrP tends to prevent a different species' PrP^{Sc} from converting PrP^{C} due to heterologous binding between the PrP of two species that limits further conversion(56). Over-expression of the altered species PrP^{C} has been shown to bypass this limitation and speed up incubation times(103). This observation supports the idea that due to the high ratio of sheep PrP^{C} to rabbit PrP^{C} within the Rov cell line, heterologous binding was not sufficient to prevent enough PrP^{Sc} from continuing to convert PrP^{C}, effectively bypassing the species barrier(135). The lack of bovine PrP^{C} expression within any of the cell lines investigated may have prevented further infection by bovine PrP^{Sc}. Although transgenic over-expression of bovine PrP^{C} would only occur under experimental conditions, it does lead one to wonder if manipulation of the mentioned cell lines, via transfection, small molecule treatments, etc. could cause the cells to become permissive to infection *in vitro*(69). With all the potential reasons listed as to why the previously mentioned cell lines would not be susceptible to a PrP^{Sc} infection and the data in this thesis demonstrating a lack of any sustained infection in many of the cell lines, one would conclude that the chances are very low for N2a, RmcB, CHO, PBDC, NAMALWA, MRC-5, HEK-293, Cos-7 and U937 cells becoming infected persistently with the infection then being incorporated in the final human biologic product.

Evidence that entry may be the main inhibitory factor in establishing a persistent infection can be seen in figure 5b, where a liposome-based delivery system was used to introduce PrP^{Sc} directly into N2a cells. Upon delivery, a persistent infection could be maintained. Due to the fact that liposomal delivery bypasses a variety of the cell surface interactions mentioned in this thesis, the delivery system highlights the importance of particular cellular receptors or structural PrP similarities of PrP^{C} and PrP^{Sc} being major requirements to propagate a PrP^{Sc} infection. PrP^{Sc} may infect cells by converting PrP^{C} on the cell surface first, with or without being taken into the cell. Or, it could be that conversion within the cell must occur before PrP^{Sc} is recycled to the cell surface where further infection is propagated and shared between cells. However, before a persistent infection can be developed it would most likely be dependent on a few factors. Interactions between endogenous and exogenous PrP may depend on the conformational compatibility between the normal prions on the cell surface and the abnormal prions initiating the infection. Cell surface receptors may be needed to allow for entry before further interaction can occur between PrP^{C} and PrP^{Sc}, as discussed in previous paragraphs. Finally, factors that have yet to be defined may need to be included to allow for infection of cells that do not express the PrP^{C} of the infectious strain. The speculation that a liposome delivery system bypasses cell surface interactions indicates that other factors that have yet to be defined in the literature need to be elucidated prior to the full understanding of PrP^{Sc} entry mechanisms. It also highlights the use of liposome delivery as a new tool to establish infections in cell lines that may not have produced detectable infections previously and introduces a new mechanism that may need to be investigated for further understanding of how PrP^{Sc} infections become established.

Of all the cell lines examined, only one cell line was particularly susceptible to a BSE PrP^{Sc} infection by the infection protocol described in the Methods. The NIH/3T3 cell line maintained evidence of a prion infection for at least 10 passages following the initial exposure to PrP^{Sc}. Although the use of BSE PrP^{Sc} to establish the infection involves a strain of PrP^{Sc} that has never been used to infect these fibroblasts, NIH/3T3 cells have been shown in the past to be susceptible to PrP^{Sc} infections using scrapie as the inoculant(136). Thus, the susceptibility of a particular cell line is not dependent on the cell origin nor is it required that high levels of endogenous PrP^{C} be produced(136). Vorberg's study supports that conclusion as NIH/3T3 cells sustained a BSE infection even though the cell line is not of neuronal origin nor does it produce high levels of PrP^{C}. The finding that NIH/3T3 cells are susceptible to BSE PrP^{Sc} infection supports not only the notion that i) the infection of cell lines has more to do with the prion strains involved and the cell receptors present on the surface than it does with the type of cell used or the level of PrP expressed but also ii) cell line susceptibility needs to be examined on a case by case basis as a cell line's susceptibility to infection cannot be based solely on its origin or PrP expression level.

The main conclusions of this study are supported by previous studies that demonstrate the difficulties in infecting cell lines using PrP^{Sc}(72). N2a, Sf21, HFF, HFF-MitC, RmcB, CHO, PBDC, NAMALWA, MRC-5, HEK-293, Cos-7 and U937 cells did not show any susceptibility to a BSE PrP^{Sc} infection using the inoculation methods described in this thesis. NIH/3T3 cells were susceptible to a sustained BSE PrP^{Sc} infection via the infection protocol as confirmed by western blot using TCA precipitation to better concentrate and visualize PrP^{Sc} molecules. N2a cells were susceptible to a BSE PrP^{Sc} infection when treated with a liposome delivery system and confirmed via TCA precipitation. Although most of the cell lines examined are not susceptible to a prion infection, the evidence that NIH/3T3 cells are susceptible to a BSE PrP^{Sc} infection provides a strong indication that the use of specific prion-susceptible cell lines may pose a threat to the safety of human biologics and biotherapeutics. Of note is the susceptibility of fibroblast cells to PrP^{Sc} infection since these cells have been used as feeder cell layers for keratinocyte cultures in cutaneous gene-therapy applications(136). The data presented in this thesis support the idea that routine monitoring, especially of cell lines that do show susceptibility to prion infections, should be undertaken before using any cell line in the production of human biologics.

While a variety of cellular receptors have been implicated in the mediation of the endocytosis of PrP^{Sc} or PrP^{C}, additional cellular factors remain elusive. The identified receptors often allow entry of a plethora of other substances or exist in all cells whether these cells are susceptible to prion infection or not. Therefore, differences in receptors alone cannot explain the discrepancy in susceptibility to PrP^{Sc} infections seen in the various cell types. Furthermore, it is still unclear as to whether *ex vivo* treatments of cells with various cytokines or stimuli in exploratory cell or gene therapies might make these cells more susceptible to a prion infection, given that cytokines could affect PrP^{Sc} accumulation and that the susceptibility of the cells to prion infection might be independent of tissue origin and the level of endogenous prion protein expression(28,65,82,145). To answer these questions, more in-depth studies will be needed, especially at a time when the number and the diversity of cell substrates used for the manufacture of biologics and biotherapeutics or as gene/cell therapies will only increase in the years to come.

6.0 Conclusions

The overall goal of this project was to test the susceptibility of various cell lines used in the production of human biologics to a prion infection. The main hypothesis of this project was that multiple cell lines, especially those that produce PrP^C endogenously, could be susceptible to an infection. Experiments demonstrated that this was not the case and that most cell lines, with the notable exception of NIH/3T3 cells, were not susceptible to a PrP^{Sc} infection using the infection method established in this work. Although not directly part of the main hypothesis, this study also highlighted the efficacy of using TCA precipitation to concentrate PrP^{Sc}, as TCA precipitation was two- to four-fold more effective at precipitating PrP^{Sc} in comparison with other precipitating techniques.

Early work demonstrated that the ScN2a cell line was capable of maintaining a PrP^{Sc} infection when N2a cells were infected with scrapie PrP^{Sc} and then passaged multiple times. The cell blot technique was sensitive enough to visualize this sustained infection yet confirmation by Western blot was originally not providing a very strong signal via chemiluminescence. This was compensated for by using TCA precipitation to concentrate cell samples before performing the Western blot protocol.

Testing of cell lines for their receptiveness to prion infection demonstrated that N2a, Sf21, CHO, HFF, HFF-MitC, RmcB, PBDC, NAMALWA, MRC-5, HEK-293, Cos-7 and U937 cells were not susceptible. Interestingly, N2a cells became subject to an infection once a liposome delivery system was used to deliver the BSE PrP^{Sc} indicating that cell surface interactions may be an important part of the infection process.

This thesis also describes the first evidence that NIH/3T3 cells are susceptible to a BSE PrP^{Sc} infection *in vitro*. In previous studies, NIH/3T3 cells have shown susceptibility to scrapie PrP^{Sc}, and the finding that a BSE strain could be propagated in these fibroblast cells as opposed to neuronal N2a's (which originate from the brain and express high amounts of PrP^{C}) indicated that all cell lines need to be tested regardless of their heritage. The research presented in this thesis supports the concept that some cell lines may sustain a BSE PrP^{Sc} infection *in vitro* and that theoretically this prion infection could be maintained throughout the manufacturing process. This highlights the concern that prior to cell lines being used for human biologic production, testing of PrP^{Sc} should be employed to ensure no infection is present.

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