ASSESSMENT OF RETROVIRUSES AS POTENTIAL VECTORS FOR THE CELL DELIVERY OF PRIONS

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Thesis submitted to the Faculty of Graduate and Postdoctoral Studies of the University of Ottawa in partial fulfillment of the requirements for the degree of Masters of Science

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Dedicated To:

- *My Children Mohammad -Reza and Amir-Reza who were patient and helpful to me during my education specifically those nights that I came home very late.*

- *Nikta, Paul, and Maria my dear friends who always gave me hope and strength for accomplishing my thesis.*

ABSTRACT

Transmissible spongiform encephalopathies (TSEs) or prion diseases are a class of fatal brain disorders better known as Creutzfeldt-Jacob Disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, and chronic wasting disease (CWD) in deer and elk. The infectious agent responsible for these diseases is a misfolded prion protein capable of catalyzing a conformational change in normal cellular prion proteins (PrP^{C}) into aberrant disease-causing structural isoforms (PrP^{Sc}). Although the etiological agent for TSEs has clearly been defined as PrP^{Sc}, there are important gaps in our understanding of how these proteins target and invade brain tissue. It remains to be established how ingested PrP^{Sc} ultimately reach the brain and also to understand why these tissues are particularly targeted, notwithstanding that several other tissues highly express prion proteins. Certain viruses, retroviruses in particular, efficiently hijack host proteins and can carry these proteins with them when they are released from a cell. Several lines of evidence have shown that prions and retroviruses can interact and associate at various stages of the retroviral replication cycle. Of special interest is that most retroviruses can cross the blood-brain barrier and could therefore deliver host-derived proteins to neuronal cells. In view of these observations, this thesis investigates whether retroviruses can act as vectors to capture prions from an infected cell and deliver them to a susceptible target cell.

In this work, I have cloned human and mouse prion cDNAs from PBMCs and the murine cell line NIH 3T3. Either a FLAG epitope tag or the eGFP reporter protein cDNA was inserted into a region of the prion cDNA that is predicted to be amenable to such genetic insertions without affecting protein folding or expression. I then confirmed using both florescent and confocal microscopy and that the recombinant proteins had a similar cell distribution to the endogenous prion protein. Using Western blot analysis, I then showed that endogenous and overexpressed prion proteins can be detected in co-transfected cells producing HIV and murine leukemia virus (MLV) retroviral particles. Finally, I went on to show that prions are also present at high levels in HIV and MLV retroviral particles released from these cells.

This work constitutes the first step in determining whether retroviruses can act as vectors for prion dissemination. Establishing a strong and clear association between retroviruses, pathogenic prions and prion disease would provide the rationale for preventive measures to be taken directly against retroviruses in order to protect humans and animals that have been newly exposed to PrP^{Sc}-infected

products or those who are genetically predisposed to develop prion diseases. Anti-retroviral drugs could also be potentially used to delay disease progression and reduce prion transmission in human and animal tissues. The availability of such a treatment would constitute a significant advancement because there is currently no cure or treatment for prion diseases.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Marc-André Langlois for accepting me as a graduate student in his lab. It was very nice to work in his lab and be a part of his research group. His wisely supervising and continuous support has improved and completed my knowledge and techniques for becoming a good researcher. He has reliably provided advice for my experiments and in writing and I am truly thankful. I am sure his help and support would provide more opportunity in research for me in future.

I would also like to thank my thesis advisory committee, Dr. Ken Dimock and Dr. Alain Stintzi for their advice, efforts, and suggestions on my thesis project.

I would thank to my examiners professors, Dr. Kathryn Wright and Dr. Lionel Filion.

Conclusively, I would like to thank Olga Agah, Tara Read, Kasandra Bélanger, Maria Rosales Gerpe, and Mathieu Savoie for their advice and technical assistance during my study as a graduate student in Dr. Langlois lab.

This work was funded by a CIHR operating grant and OGSST award

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A3G	deoxycytidine deaminase APOBEC3G
AID	Activation-Induced cytidine Deaminase
AIDS	Acquired Immunodeficiency Syndrome
Asn	Asparagine
Bax	pro-apoptotic factor from Bcl-2 gene family
BBB	Brain Blood Barrier
BSE	Bovine Spongiform Encephalopathy
CA	Capsid Antigen
CAEV	Caprine Arthritis Encephalitis virus
CDC	Centers for Disease Control and Prevention
cDNA	complementary Deoxyribonucleic Acid
CJD	Creutzfeldt - Jakob disease
CMV	Cytomegalovirus
CNS	Central Nervous System
COOH-	Carboxyl (C) -terminal
CWD	Chronic Wasting Disease
Cys	Cysteine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
EcoRI	restriction recognition sites, GAATTC

EDTA	Ethylene Diamine Tetra Acetic Acid
EGFP	Enhanced Green Fluorescent Protein
ER	Endoplasmic Reticulum
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FDCs	Follicular Dendritic Cells
FeLV	endogenous Feline Leukemia virus
FFI	Fatal Familial Insomnia
FIV	Feline Immunodeficiency virus
FLAG	epitope tag DYKDDDDK
GALV	Gibbon Ape Leukemia virus
GPI	Glycosyl phosphatidylinositol
GSS	Gerstmann-Sträussler and Scheinker
GTPase	Guanosine triphosphatease
HERV	Human endogenous retroviruses
HEK 293 Tcell	Human embryo kidney cells
hGH	Human growth factor and gonadotropin hormones
HIV-1	Human Immunodeficiency virus type 1
HRP	Horse Radish Peroxidase
ICTV	International Committee on Taxonomy of Viruses
iCJD	iatrogenic CJD
IgG	Immunoglobulin G
IN	Integrase
kDa	Kilo Dalton
KoRV	endogenous retrovirus of Koala bear

LSM	Laser Scanning Microscopy
LTR	Long Terminal Repeats
MA	Matrix Protein
MBM	Meat and Bone Meal
MCS	Multiple Cloning Sites
MLV	Murine Leukemia virus
MoMLV	Moloney Murine Leukemia virus
MVB	Multi-Vesicular Bodies
MMV	Maedi Visna virus
N2a	Neuroblastoma (Neural/Glial) mouse cell line
NC	Nucleocapsid
NH2-	Amino (N)-terminal
NIH 3T3	mouse embryo fibroblast cell line.
OR	Octarepeats Region
ORF	Open Reading Frame
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PI	Protease Inhibitor
PIPLC	Phosphatidylinositol-Specific Phospholipase C
PNS	Peripheral Nervous System
PRNP	Human Prion Protein Gene
prnp	mouse prion protein gene
PrP^{C}	Cellular Prion Protein
PrP^{Sc}	Scrapie (infectious) Prion Protein
PVDF	Polyvinylidene Difluoride Membrane

RIPA	Radioimmunoprecipitation assay (protein lysate buffer)
RNA	Ribonucleic Acid
SAMP8	Senescence-Accelerated Mice
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SFFV	Spleen Focus Forming virus
spCJD	sporadic CJD
TSE	Transmissible Spongiform Encephalopathy
vCJD	variant CJD
VLA	Veterinary Laboratories Agency
VSV-G	Vesicular Stomatitis Virus Glycoprotein.

1.0 INTRODUCTION

1.1 Prions

1.1.1 Prion Diseases, Pathogenesis and Epidemiology

Prion diseases are neurodegenerative disorders caused by cellular prion proteins (PrP^{C}) undergoing a pathological conformational change resulting in infectious prion proteins (PrP^{Sc}). PrP^{Sc} accumulate in the cytoplasm of cells of the central nervous system (CNS) and produce aggregated amyloid rods that are toxic for neurons. Infected cells induce a remodelling of the brain tissue structure, which ultimately resulted in a spongiform appearance of infected human and animal brains. Prion diseases, also known as transmissible spongiform encephalopathies (TSEs) are contagious, untreatable and fatal (Prusiner, 2004).

Physicians first witnessed patients suffering from a strange central nervous system degenerative disease, where the brains of deceased patients displayed a spongiform appearance over 90 years ago; they called it Creutzfeldt-Jakob disease (CJD) (Creutzfeldt, 1989, Jakob, 1921). The early clinical features included headaches, tiredness, sleep or appetite disturbance and depression. The patients then rapidly develop more severe clinical manifestations such as dementia, ataxia, involuntary muscle twitching and speech loss. At the terminal stage, patients loose awareness and responsiveness to their environment, and quickly die thereafter (Colby and Prusiner, 2011a, Colby and Prusiner, 2011b, Prusiner, 2004).

The infectious PrP^{Sc} that causes prion disease is an extremely stable and resilient protein. It is stable when exposed to doses of radiation that could inactivate both viruses and bacteria (Alper, 1985, Bellinger-Kawahara et al., 1987a). The relative insensitivity of PrP^{Sc} to heat and various chemicals also sets it apart from other proteins and especially pathogens (Bellinger-Kawahara et

al., 1987b). It was the research efforts of Stanley B. Prusiner that clarified the characteristics of the infectious agent. His work lead to the identification of prions as being the causative agent of TSEs, which was acknowledged by the Nobel Prize in Physiology or Medicine being awarded to him in 1997 (Bonn and Ault, 1997).

The cellular prion protein is expressed in all mammals, birds and reptiles (Aguzzi and Calella, 2009, Calzolai et al., 2005). There are a variety of human prion diseases including: Kuru, CJD, fatal familial insomnia (FFI), and Gerstmann-Sträussler and Scheinker (GSS) disease. Animal prion diseases are referred to as scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease (CWD) in free-ranging deer such as elk (Colby and Prusiner, 2011a, Prusiner, 2004).

1.1.1.1 Human prion diseases

Roughly 85% of human prion cases are sporadic, whereas less than 1% of cases seem to be the results of an exposure to the infectious PrP^{Sc}. The remainder is caused by the inheritance of a germline mutation in the PRNP gene coding for PrP^{C}. More recent systematic and comprehensive surveys of CJD have determined that the mortality rate is 0.5 to 1.8 cases per million people per year worldwide (Brown et al., 1979, Cousens et al., 1990). Although the transmission of infectious prion proteins to human is rare, considerable attention is still dedicated to CJD (Prusiner, 2004).

Here are some details of the main forms of prion disease in humans:

Kuru is a prion disease that was first identified by European explorers to the highlands of New Guinea in the mid 1950's, where people practiced cannibalism during spiritual ceremonies for immortalization purposes (Alpers, 2008, Gajdusek, 2008, Glasse, 1967).

Heritable prion disease in humans such as GSS, familial CJD and FFI are caused by genetic mutations in the *PRNP* gene, which codes for the cellular prion protein. Beside the familial or genetic form, CJD has three other types: sporadic (sCJD), iatrogenic (iCJD) and variant (vCJD) (Colby and Prusiner, 2011a, Linden et al., 2008, Prusiner, 2004).

Sporadic CJD was first described in 1921 by Jakob, who found similarities to a previous case described by Creutzfeldt a year before. Sporadic CJD occurs in about 50 to 60 cases each year in the UK. It is a late-middle age disease and shows a progressive multifocal neurologic dysfunction, myoclonic, ataxia, involuntary movement, and a terminal state of severe cognitive damage followed by death within a few months of the more severe symptoms (Roos et al., 1973). The cause of sCJD is unknown and all efforts to find a link with a transmittable infection have failed (Aguzzi and Calella, 2009).

Iatrogenic CJD is when the disease is transmitted from one infected person to another. This has occured from corneal grafts, dura matter grafts, contaminated neurosurgical instruments or depth electrodes, and from tainted human growth factors and gonadotropin hormones of human origin (hGH). However, transmission by blood products is very rare. In all these cases, cross-contamination of PrP^{Sc} has been seen specifically when the titer of the infectious prion was very high. Incubation periods for iCJD, from the implantation of the graft to the development of the clinical disease ranged from 1.5 to 18 years, with a mean of approximately 6 years (Brown et al., 2000). The risk of iatrogenic transmission of CJD is reduced significantly when all precautionary guidelines for the transplantation of human organ material have been executed carefully. Guidelines include replacing hormones of human origin with recombinant

hormones, and following modern autoclaving methods (134C° for 18 minutes) are important factors for reducing the incidence of iCJD in humans (Prusiner, 2004).

Variant CJD was discovered in the UK in 1996 (Will et al., 1996) and was associated with the emergence of BSE in cattle at the time. Many studies identified a strong link between BSE and vCJD according to epidemiology and neuropathological features (Bruce et al., 1997, Hill et al., 1997, Scott et al., 1999). The vCJD cases had a mean onset of 29 years in comparison to 66 years in sCJD, and a duration of about 14 months compared to a maximum of 4.5 months in sCJD (Will et al., 1996). Neuropathology experiments in macaque monkeys inoculated with BSE have identified this animal's susceptibility to vCJD. The prion protein subtype that caused disease in the monkey brain was similar to that in BSE but had a shorter incubation period (Lasmezas et al., 1996). The transmission of BSE to other animals and humans can occur via food by eating infectious prion proteins. There are several studies that confirmed the possibility of oral transmission to different animals including, mice (Bruce et al., 1997), lemurs (Bons et al., 1999), minks (Robinson et al., 1994) and sheep and goats (Foster et al., 1994). The transmission of BSE to the human population involves the crossing of species barrier, which could explain the longer incubation period compared to intra-species transmission of vCJD or iCJD. According to epidemiological studies, human exposure to BSE may have begun approximately in 1983 (Prusiner, 2004, Spencer et al., 2002).

1.1.1.2 Animal Prion diseases

Scrapie was first reported in England in 1732 and in Germany in 1759. In 1962, one researcher claimed that host genes and natural mutations were responsible for developing scrapie and proposed that it is a genetic disease in sheep and could be eradicated by proper breeding methods (Parry, 1962, Wood and Done, 1992, Wood et al., 1992) In the following

decades, the infectious prion protein (PrP^{Sc}) has been identified as the cause of the disease. It seems that sheep, goats, and moufflon are at risk for natural scrapie disease that occurs during their breeding age (Wood et al., 1992). The clinical signs of disease begin with mildly impaired social behaviour such as unusual nervousness and restlessness. The illness can last from 2 weeks to 6 months. In the late stage of the disease, clinical signs get worse and the affected animal will scratch against the fence posts and bite the affected area. The brain of infected sheep showed patterns of vacuolation in the following areas: medulla, pons, cerebellum, substantia nigra, mesencephalon, hypothalamus, thalamus, septal area, corpus stratum, and neocortex (Wood and Done, 1992).

Bovine Spongiform Encephalopathy (BSE) was identified in 1986 in the brain of three cows with unknown neurological disease at the Veterinary Laboratories Agency (VLA), Weybridge, UK. These dairy cows displayed neurohistological changes in the brain that were very similar to scrapie in sheep. The disease was therefore called Bovine spongiform encephalopathy (BSE). The infected cattle had altered behaviours such as apprehensive and aggressive responses, ataxia, and dysesthesia (dislike to be touched). The disease progression occurred over 1 to 6 months (Wells et al., 1987). In 1987, a major epidemic of BSE started in Great Britain and continued to increase every year up until 1993 when 1000 new cases per week of BSE were reported in UK (Aguzzi et al., 2008, CDC, Centers for Disease Control and Prevention, 2011). This new outbreak of prion disease was widely studied to understand how the disease raised up almost simultaneously in the entire of Great Britain (Wilesmith et al., 1988). In 1991, Wilesmith suggested that one common source for all 200 cow cases was the use of dietary protein supplement, meat and bone meal (MBM) that was regularly fed to dairy cattle from the first weeks of life. Despite the inactivation procedures in MBM production, those

cows were fed MBM contaminated with PrP^{Sc} and were shown to develop BSE (Wilesmith and Wells, 1991). The age of onset in the first wave of BSE cases was 3-5 years old, which matched the incubation time for prion disease assuming the exposure to infectious materials began shortly after birth. Another important fact is that scrapie is endemic in the UK and the high population of sheep and cattle proportionately increased the chance of prion-contaminated MBM feed for dairy cattle. Governmental actions banned the addition of any livestock raw material in protein-supplemented food for cattle and birds. By applying proper changes in the MBM procedures, dramatic reductions of BSE cases resulted every year. In October 2002, it reached its lowest incidence of 0.4 % cases since the onset of the epidemic. The waning of BSE directly correlates with the MBM feed ban (Nathanson et al., 1993, Prusiner, 2004). Several studies have also shown a strong correlation between the incidence of BSE and vCJD (Bruce et al., 1997, Hill et al., 1997, Scott et al., 1999). When BSE causes plummeted, so did the diagnosis of new cases of vCJD in humans (Sanchez-Juan et al., 2007).

Chronic Wasting disease (CWD) is a prion disease that has been diagnosed in white-tailed deer, free-ranging mule deer and Rocky Mountain elk living within 12 states of the USA and 2 Canadian provinces (Sigurdson and Aguzzi, 2007). The first case of CWD recognised as spongiform encephalopathy was documented in 1977 in a mule deer (Spraker et al., 1997, Williams and Young, 1992). The similarity between the glycoform patterns of CWD prion and scrapie prion proteins suggests that CWD may have arisen from a zoonotic transmission of scrapie disease (Race et al., 2002). Since the infectious prion protein can be transmitted to humans by eating contaminated meat, then CWD disease could potentially be transferred to humans.

1.2 Molecular Biology of Prion

1.2.1 The Cellular Prion Gene

The human prion protein (*PRNP*) gene is located on the short arm of human chromosome 20 and the orthologous mouse prion protein gene is located on chromosome 2 (Sparkes et al., 1986). The PrP^C protein sequence is highly conserved in mammals and has an amino acid similarity ranging from 92.9-99.6% (Damberger et al., 2011, Schatzl et al., 1997).

1.2.1.1 The molecular structure of PrP^C

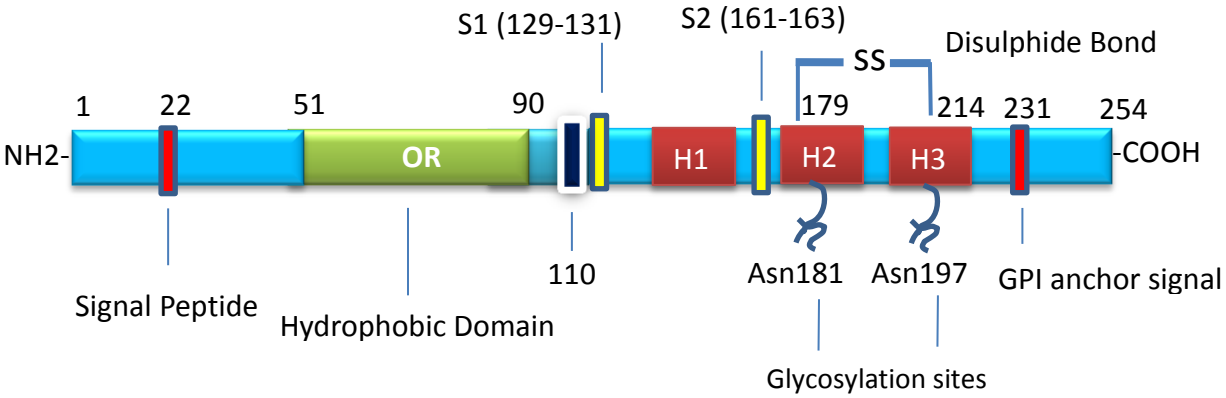
The cellular prion protein has 254 amino acids (aa.) with several distinct domains including an amino-terminal signal peptide (NH2-), a series of eight peptide repeats (PHGGGWGQ or octarepeats (OR), that are in a central hydrophobic domain and highly conserved), and finally a carboxyl-terminal hydrophobic region (COOH-) which has a signal for the addition of a glycosylphosphatidylinositol (GPI) anchor (Fig1.1). PrP^C undergoes post-translational modifications such as an amino-terminal cleavage at the signal peptide domain that allows the protein to be transported to the endoplasmic reticulum (ER) and then to the Golgi apparatus to become glycosylated. N-linked oligosaccharide chains are added in the ER and are sensitive to digestion by endoglycosidase H (Caughey et al., 1989a). These oligosaccharide chains will further be modified in the Golgi, resulting in complex structures that have sialic acids and are resistant to endoglycosidase H. Additional post-translational processing occurs at the carboxyl-terminal hydrophobic segment (GPI signal peptide), where a GPI molecule is added and anchors the protein into the membrane bilayers. PrP^C can be released from the cell surface by treating the cell with the bacterial enzyme phosphatidylinositol-specific phospholipase C (PIPLC) which can specifically break the GPI anchor (Caughey et al., 1989a, Stahl et al., 1992).

The secondary structure of human PrP^C consists of a globular domain with three- α helix (A or H1, B or H2 and C or H3) and a two-stranded antiparallel β sheets (S1 and S2). There is a disulphide bond between cysteine (Cys) 179 of the second helix (H2) and Cys 214 of the third helix (H3) which is important for the overall structure of the prion protein. Glycosylated sites in human PrP^C are on H2 and H3 at Asparagine (Asn) 181 and Asn 197 respectively, and in mouse PrP^C they are on Asn 180 and Asn 196 (Haraguchi et al., 1989). Human PrP^C has three different glycosylated isoforms, un-glycosylated PrP^C (U ~ 21 kDa), the immature glycosylated (I ~27 kDa) and the mature highly glycosylated isoform (H~33 kDa). Moreover, these glycosylated isoforms are sensitive to N-glycosidase F and they are insensitive to endoglycosidase H. Prion protein extracts treated with N-glycosidase F show only a single band representing the un-glycosylated 21 kDa isoform (Capellari et al., 1999, De Keukeleire et al., 2007).

The highly conserved octarepeats region (OR) is the principal metal-binding site that has high affinity for copper, followed by nickel, zinc, and manganese (Jackson et al., 2001). In recent studies, iron was shown to interact with the OR and with histidine residues at amino acid positions 96 and 111. This interaction between iron and prions is dependent on the conformation of PrP^C rather than the specific amino acid sequences involved in the binding. It is speculated that PrP^C may be important for copper and iron uptake (Singh et al., 2010) (Fig.1.1).

Figure 1.1 Structures of the cellular prion protein and its posttranslational modifications

The H1, H2 and H3 are: α-helix 1, α-helix.2 and α-helix 3, S1 and S2 are two βsheets as structure positions, octarepeats region (OR), S-S, disulphide bond and Asn for asparagine amino acid is glycosylation site of the prion molecule (Adapted from: Prion Biology and Diseases, Stanley B. Prusiner, 2004)


S1 (129-131)
S2 (161-163)
Disulphide Bond
SS
1
22
51
90
179
214
231
254
NH2-
OR
H1
H2
H3
-COOH
110
Asn181
Asn197
GPI anchor signal
Signal Peptide
Hydrophobic Domain
Glycosylation sites

1.2.1.2 Conformational conversion of PrP^C

It is the abnormal folding and conformational changes in the PrP^C structure that generate the infectious prion protein scrapie (PrP^{Sc}). This isomer of the prion protein has a different secondary and tertiary structure from the cellular prion protein but has an identical primary sequence. Fourier-transform infrared spectroscopy revealed that PrP^C had 43% α-helix and almost no- β-sheets (3%), while PrP^{Sc} is comprised of 30% α-helixes and 43% β-sheets (Pan et al., 1993) (Fig 1.2). The PrP^{Sc} isoform is detergent-insoluble and extremely resistant to Proteinase K (50μg/μl at 37 ^oC for 30 minutes), with a truncated polypeptide PrP^{Sc} of ~ 142 amino acid remaining after digestion. This specific segment of the molecule has 27-30 kDa and is referred to as PrP^{27-30}. The PrP^C isoform, however, is detergent-soluble and will digest completely with Proteinase K. The conversion mechanism of PrP^C to PrP^{Sc} is unknown, but it appears that PrP^C must bind to an infectious PrP^{Sc} and possibly another ancillary protein to form an intermediate complex to produce a nascent PrP^{Sc} molecule (Meier et al., 2003) Propagation of the new infectious prion molecules induce the formation of febrile strands or amyloid rods in neuronal tissues. The aggregation mechanism of amyloid plaques and the cause of their neurotoxicity is still a mystery (Aguzzi and Calella, 2009).

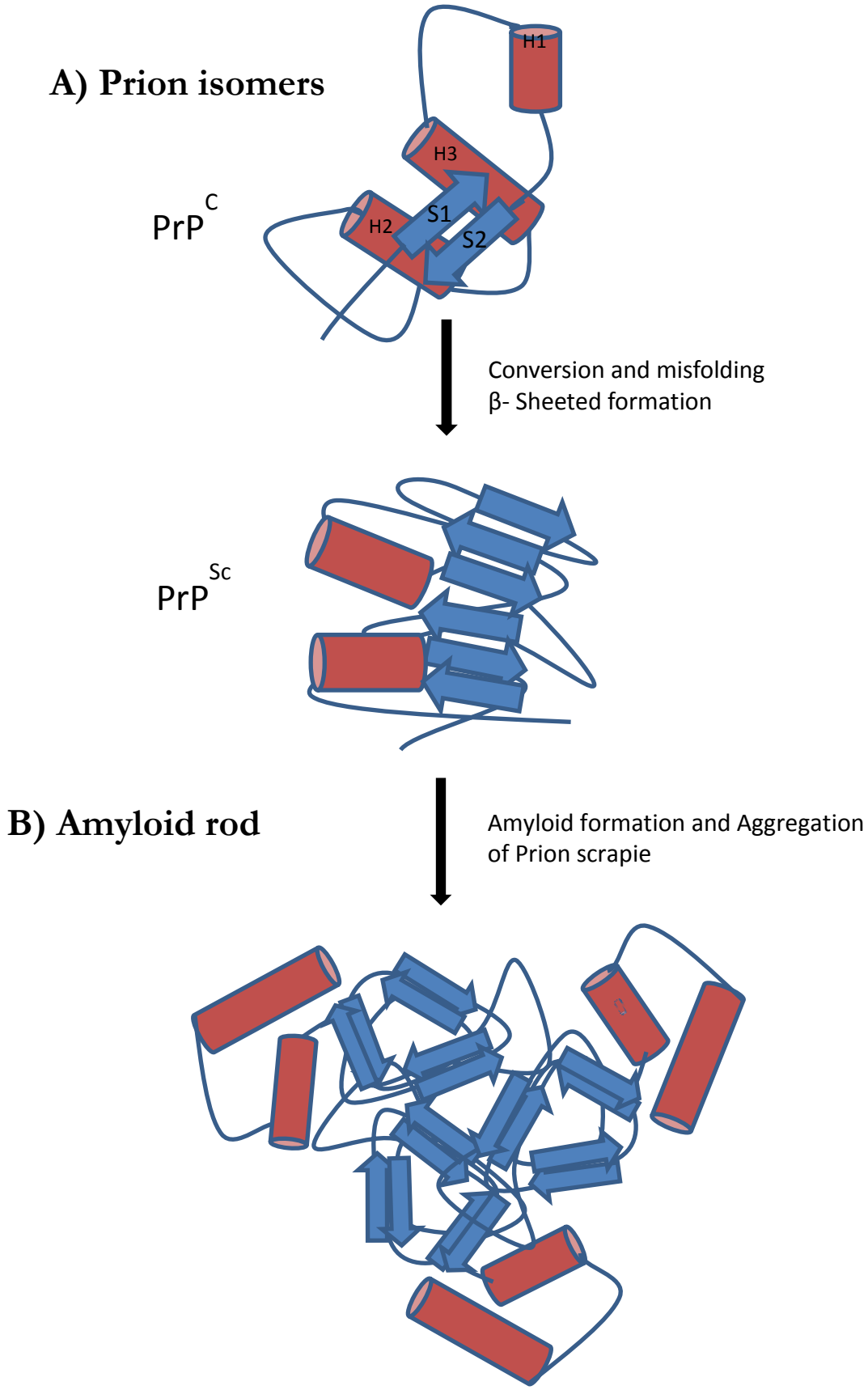
Many studies have shown that PrP^C is essential for prion replication and neurodegenerative disease. Knockout mice for the prion gene are resistant to prion disease and PrP^C expression is necessary for TSE to develop in the infected host (Bueler et al., 1993). Inoculation of PrP^{Sc} alone in the PrP^C knockout mice was unable to induce prion disease and did not induce any pathological changes (Brandner et al., 1996). Also, transgenic mice expressing only a secreted form of PrP^C lacking the GPI anchor did not develop clinical prion disease, although

inoculation of PrP^{C} into the brain of transgenic mice infected with PrP^{Sc} could induce the formation of amyloid plaques(Chesebro et al., 2005).

In a recent study, the replication site of PrP^{Sc} in the gut of wild type and prion-deficient mice (prnp-/-) after feeding PrP^{Sc}, was studied by using high-resolution immunofluorescence and cryo-immunogold electron microscopy (Kujala et al., 2011). The trafficking of PrP^{Sc} toward Peyer's patches was transiently detectable at day 1 post-feeding, within the large multi-vesicular endosomes of enterocytes in the follicle-associated epithelium (FAE). PrP^{Sc} was also detected in M cells, although at much lower levels. Later, at day 7–21 post feeding, increased PrP^{Sc} labeling was observed only in the plasma membranes of follicular dendritic cells (FDCs) in germinal centers of Peyer's patches from wild-type mice, identifying FDCs as the first sites of PrP conversion and replication in mice (Kujala et al., 2011). This result contradicts previous studies that have shown M cells to be a major entry site for prion proteins via the gut and FDCs as unlikely carriers of PrP^{Sc} to the peripheral nervous system (PNS) (Foster and Macpherson, 2010, Heppner et al., 2001, Raymond and Mabbott, 2007, Raymond et al., 2007).

Figure 1.2 Conformational changes of PrP^{C} into PrP^{Sc}

A) Conversion and misfolding of PrP^{C} into PrP^{Sc}, B) Amyloid formation and aggregation of prion Amyloid rod formation (Adapted from: Prion Biology and Diseases book, Stanley B. Prusiner, 2004)


A) Prion isomers
PrP C
H1
H3
S1
S2
H2
Conversion and misfolding
β- Sheeted formation
PrP Sc
B) Amyloid rod
Amyloid formation and Aggregation
of Prion scrapie

1.2.1.3 The Trafficking and Function of PrP^C in the cell

The cellular prion protein is highly expressed in the brain, but has also been identified in other non-neuronal tissues like lymphoid cells, lung, heart, kidney, gastrointestinal tract, muscle, and mammary glands. Although PrP^C is a cell membrane-associated glycoprotein and is glycosylated in the ER and again in the Golgi on its way to the cell surface, apparently the protein will not remain on the surface of the cell and will essentially cycle between the plasma membrane and the cytoplasm. This occurs in endocytic compartments via clathrin-coated vesicles that are dynamin-I endocytosis-dependent (Laine et al., 2001, Linden et al., 2008, Madore et al., 1999, Shyng et al., 1994).

After the protein has reached the cell membrane, it goes through several physiological changes such as cleavage near residue 110, an extremely conserved hydrophobic domain (Fig 1.1) (Caughey et al., 1989a, Caughey et al., 1989b, Chen et al., 1995, Harris et al., 1993, Prusiner, 2004). The proteolytic cleavage occurs in the presence of metallo-proteases (Vincent et al., 2001). During each cycle, 1–5% of the molecules will be cleaved at amino acid 110. This cleavage can be inhibited with lysosomotropic amines, leupeptin and brefeldin A, and is consistent with what is happening in an endocytic compartment. In addition, kinetic analysis using fluorescently labeled antibodies showed that PrP^C molecules will take about 6o minutes to travel from the cell surface to the endocytic compartment (Harris, 2003, Shyng et al., 1993).Gold labeling electro microscopic analysis has also identified that prions are located in endocytic compartments, coated with clathrin, between Golgi and the cell surface (Laine et al., 2001, Madore et al., 1999, Shyng et al., 1994). Since the prion protein has a GPI anchor, it seems that PrP^C associates with lipid rafts first in the secretory pathway and then, at the cell surface, PrP^C leaves the rafts to be internalized via clathrin vesicles (Linden et al., 2008).

The function of the cellular prion protein in neurons is poorly understood, as well as the understanding of how the transformation of PrP^{C} into PrP^{Sc} leads to neurodegeneration. The endocytic recycling pathways of PrP^{C} suggests a role for the prion protein in cellular uptake, possibly that of copper and iron (Brown and Harris, 2003, Singh et al., 2010, Zomosa-Signoret et al., 2008). A study showed that increasing copper levels in the brain and blood of mice influences the rate of progression of prion disease; therefore using copper chelation therapy was advised for delaying the disease (Sigurdsson et al., 2003). That interaction of PrP^{C} with certain metals may induce conformational changes in PrP^{C} is a hypothesis that was advanced to attempt to explain prion disease pathogenesis (Basu et al., 2007, Deleault et al., 2007).

In another study on a *PRNP* gene carrying pathogenic predisposing mutations, it was shown that these prion proteins misfolded very soon after their synthesis, before exiting the ER and trafficking to the Golgi (Harris, 2003, Stewart and Harris, 2003). The transportation of the abnormal protein was also slower than wild-type molecules. However, neither mutant nor wild-type PrP translocate or are degraded by proteasomal enzymes in the ER. These observations could explain the accumulation of misfolded PrP^{Sc} in the ER lumen, rather than in the cytosol, which occurs in pathogenic events in a subset of familial prion diseases (Harris, 2003, Stewart and Harris, 2003). The spreading mechanism of infectious prion proteins from one cell to another has not been well documented. In recent *in vitro* studies, cell-to-cell contact was required for transmission of prion molecules (Kanu et al., 2002, Schatzl et al., 1997). In other studies, both infectious and cellular prion proteins have been found in extracellular medium of scrapie infected cells in association with exosomes and it seems that prion proteins are "riding exosomes bubbles" for travelling from one cell to another (Fevrier et al., 2005, Paquet et al., 2004, Porto-Carreiro et al., 2005).

1.3 Retroviruses

1.3.1 Description and Molecular Biology

Retroviruses are enveloped viruses from the retroviridae family and are 80–100 nm in diameter. They encode a reverse transcriptase (RT) that produces double-stranded proviral DNA (dsDNA) from their viral RNA genome. This dsDNA can then integrate into the genome of the host cell to allow for viral replication. The viral RNA genome of retroviruses is 7-12 kb in size, linear, single-stranded, non-segmented, and has a positive polarity. There are two copies of the viral RNA genome in the icosahedral capsid, which are covered and stabilized by nucleocapsid (NC) proteins. The shape of the capsid is specific to each genera of the retroviridae family. Several copies of integrase (IN) are found within the capsid, allowing for the integration of the proviral DNA into the cellular genome, along with RT molecules required for reverse transcription of the genomic RNA (Fig 1.3).

The viral genomic RNA has a 5' terminus methylated nucleotide cap and a poly (A) tract at the 3' terminus, much like mRNA. At the two termini of the genome, there are noncoding sequences with redundancy repeats including two direct repeats (R), a U5 (5'unique) at the 5' end, and a U3 (3'unique) at the 3' end. These sequences in the RNA genome are the precursors of the long terminal repeat regions (LTR) at the 5' and 3' ends of the retroviral DNA. The LTR contains regulatory sequences for both the promotion and termination of viral RNA transcripts.

All retroviruses harbor three major coding domains for viral proteins: *gag*, the structural proteins that make up the matrix (MA), the capsid (CA), and the nucleocapsid protein (NC) structure proteins; *pol*, which codes for the reverse transcriptase, integrase and RNase H; and

env, which encodes the viral envelope protein constituted of both a surface (SU) and transmembrane (TM) domain. There is also a small coding domain present in all retroviruses between the *gag* and *pol* gens called *pro*, which codes for the viral protease (Fig 1.4) (Coffin et al., 1997, Kinpe, 2007). The genome of retroviruses is generically classified in two major groups; simple and complex according to the number of open reading frames (ORF) and alternative mRNA splicing variants. Most of the simple retroviruses transcribe only one or two spliced mRNA; as opposed to multiple spliced mRNAs for the complex retroviruses (Coffin et al., 1997) (Fig 1.4).

Retroviruses are classified into seven groups according to their evolutionary and genetic similarities (Table 1.1). Five of these groups characterize retroviruses with oncogenic potential (oncoviruses), and the other two groups are the lentiviruses and the spumaviruses. All oncogenic members except the human T-cell leukemia virus–bovine leukemia virus (HTLV-BLV), are simple retroviruses and the lentiviruses (e.g., HIV), spumaviruses, HTLV and BLV are complex (Coffin et al., 1997).

Figure 1.3 Schematic cross section of a typical retrovirus particle

The virus envelope contains a phospholipid bilayer derived from the cell and contains two major glycoprotein encoded by the *env* gene, surface Protein (SU) and trans-membrane protein (TM) that are inserted. SU and TM components are linked together by disulfide bonds. Matrix protein (MA) is located under the envelope, PR is viral protease and CA is capsid protein. Inside the capsid two copy of virus RNA genome is covered with nucleocapsid proteins (NC), which is made from C-terminal of *gag* poly protein and numbers of viral Integrase (IN) and reverse transcriptase (RT) enzymes. (Adapted from: Retroviruses, Coffin et al., 1997)

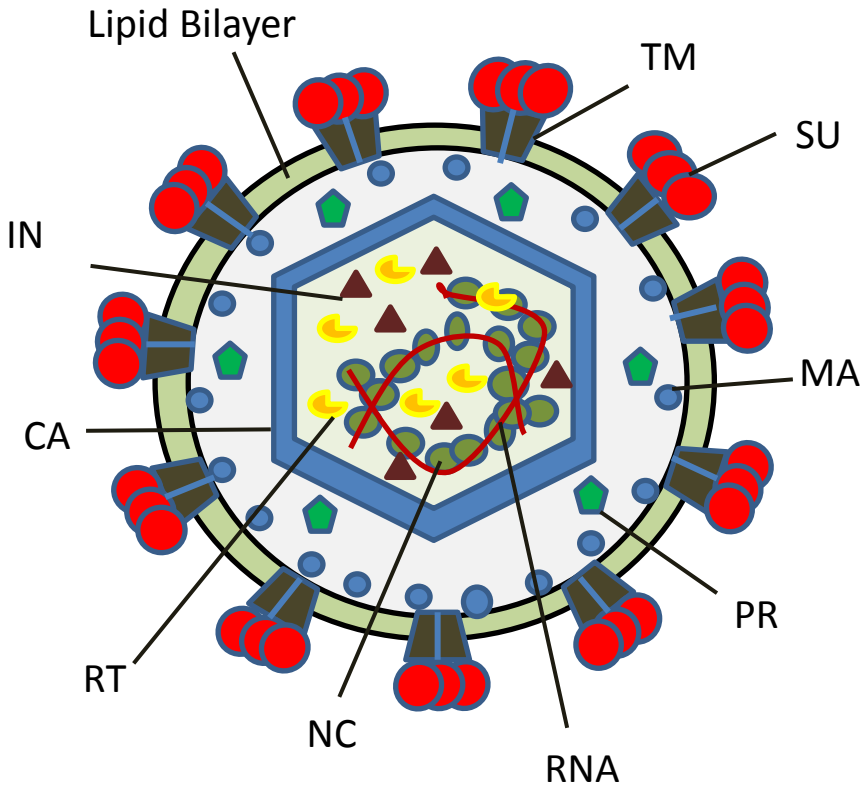
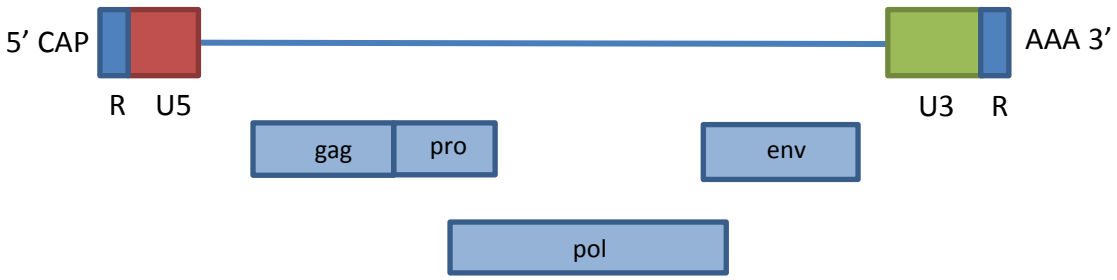

Lipid Bilayer
TM
SU
IN
MA
CA
PR
RT
NC
RNA

Figure 1.4 Genome structure of simple and complex retrovirus

A) A simple retrovirus genome ALV contains, *gag, pro, pol* and *env*. Different reading frames are indicated by vertical displacement of the coding region. The *pro* gene is encoded in the *gag* reading frame. At the 5'end Cap structure there are repeat sequences (R), a non-coding sequence U5 and at the 3'end lays a noncoding sequence U3 and repeat sequences (R). B) A complex retrovirus genome HTLV contains the genetic data of two regulatory proteins, Tax and Rex which have the same open reading frame (ORF) as the *env* protein, but they are produced by different splicing. The *gag, pro* and *pol* are encoding from another ORF. (Adapted from: Retroviruses, Coffin et al. 1997)

A) ALV (Simple)



B) HTLV (Complex)

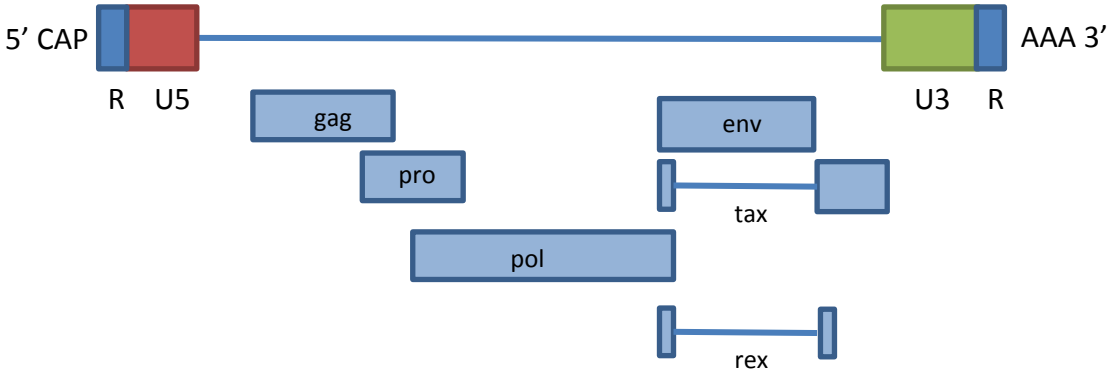


Table 1.1 Retroviruses classification according to the international committee on taxonomy of viruses

Family	Genus	Type Species	Genome	Hosts
Retroviridae	Alpharetrovirus	Avian leukosis virus (ALV)	Simple	Vertebrates
	Betaretrovirus	Mouse mammary tumor virus (MMTV)	Simple	Vertebrates
	Gammaretrovirus	Murine leukernia virus (MLV)	Simple	Vertebrates
	Deltaretrovirus	Bovine leukemia virus (BLV) and Human–T cell leukemia virus (HTLV-I)	Complex	Vertebrates
	Epsilonretrovirus	Walley dermal sarcoma virus (WDSV)	Complex	Vertebrates
	Lentivirus	Human immunodeficiency virus 1 (HIV-1)	Complex	Vertebrates
	Spumavirus	Simian foamy virus (SFV) and chimpanzee foamy virus (CFV)	Complex	Vertebrates

(NCBI website, Taxonomy browser (http://www.ncbi.nlm.nih.gov)

1.3.2 Life Cycle of Retroviruses

The life cycle of retroviruses starts with attachment of the surface envelope glycoproteins to specific plasma membrane receptors on the host cell. Retroviruses can enter the target cell by three possible pathways. The first is the canonical or direct fusion with the plasma membrane after attachment to the viral receptor on the surface of the cell. The second pathway is through clathrin-dependent endocytic internalization followed by intercellular compartment membrane fusion. By this pathway, the viral capsid either enters the cytosol or is degraded in the lysosome. The third possible method is the engagement of a cell receptor and co-receptor by the viral glycoproteins, whereby this interaction increases viral and endosomal membrane fusion in a dynamin-dependant mechanism. Dynamin is a large GTPase that binds the membranes of endocytic vesicles. In the case of co-receptor inhibition, viral particles could be degraded in the lysosome or may be recycled back to the extracellular medium as a reusable infectious particle and potentially initiate a new productive infection (Fig 1.5) (Miyauchi et al., 2009, Permanyer et al., 2010).

Following virus entry into the cytoplasm, the viral RNA genome is enclosed in a core complex of nucleocapsid proteins where the viral RT will reverse-transcribe it into dsDNA. Reverse-transcription into DNA involves two 'jumps' of the reverse transcription complex from the 5' terminus to the 3' terminus of the template molecule. The result of these 'jumps' is a duplication of sequences located at the 5' and 3' ends of the viral RNA. These sequences will fuse to initially form a circle, creating the long terminal repeats (LTRs) that consist of the non-coding sequences U3-R-U5. Once the reverse transcription process is complete and the full-length proviral DNA is produced, the viral integrase will promote and catalyze its insertion into the host genome.

Figure 1.5 Putative retroviruses entry pathways

1) Cell surface internalization: viral attachment to the receptor and coreceptor at the cell surface facilitates fusion between plasma membrane and virus envelop. 2) Endocytic internalization: viral attachment to the receptor and coreceptor can direct viral particle to the lysosome vesicles or enhance endosomal intracellular membrane fusion.3) Co-receptor independent transfer, which may end in lysosomal compartment or be directed to the cell surface for release where active virus recycling may take place (Permanyer et al., 2010). (Adapted from: Permanyer et al. 2010)

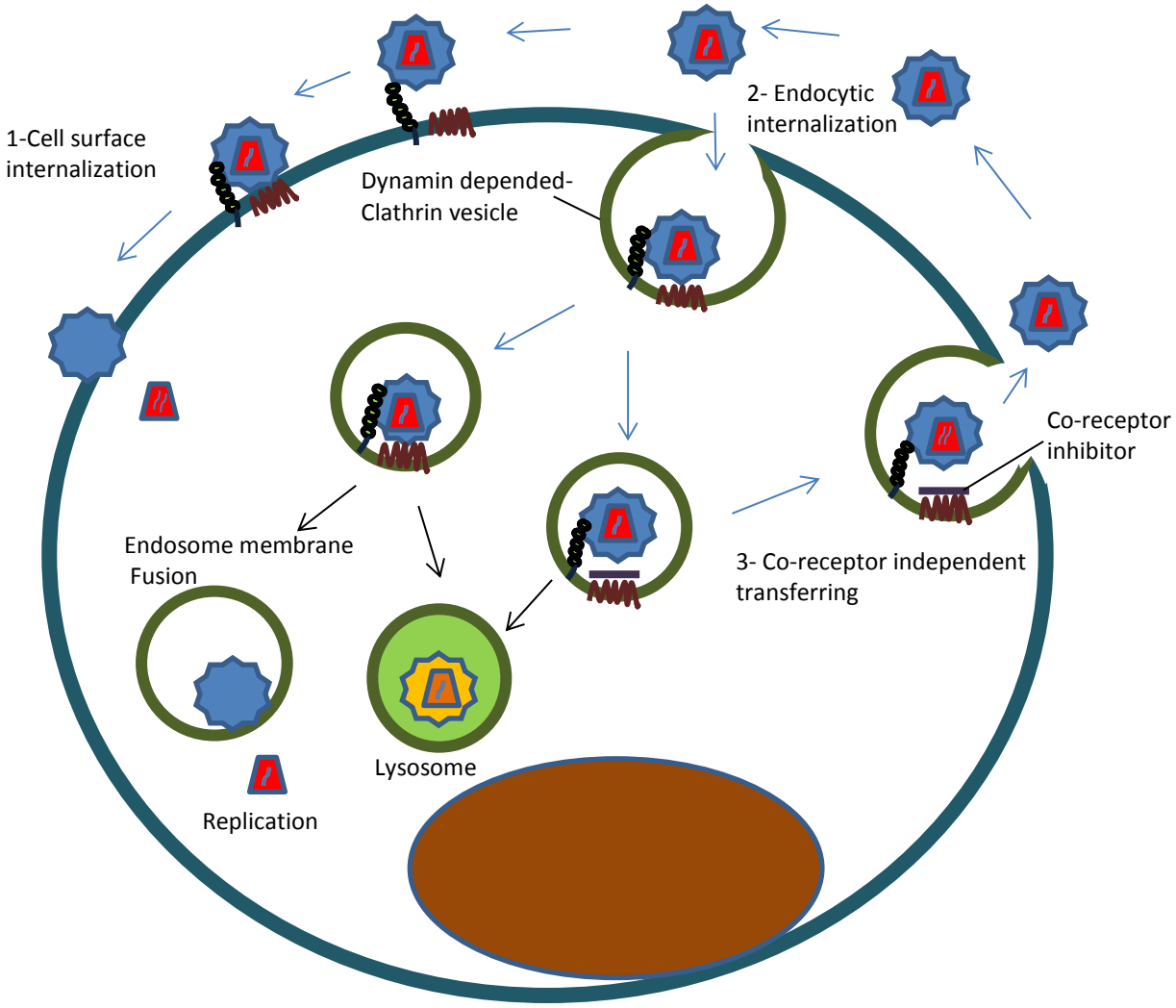
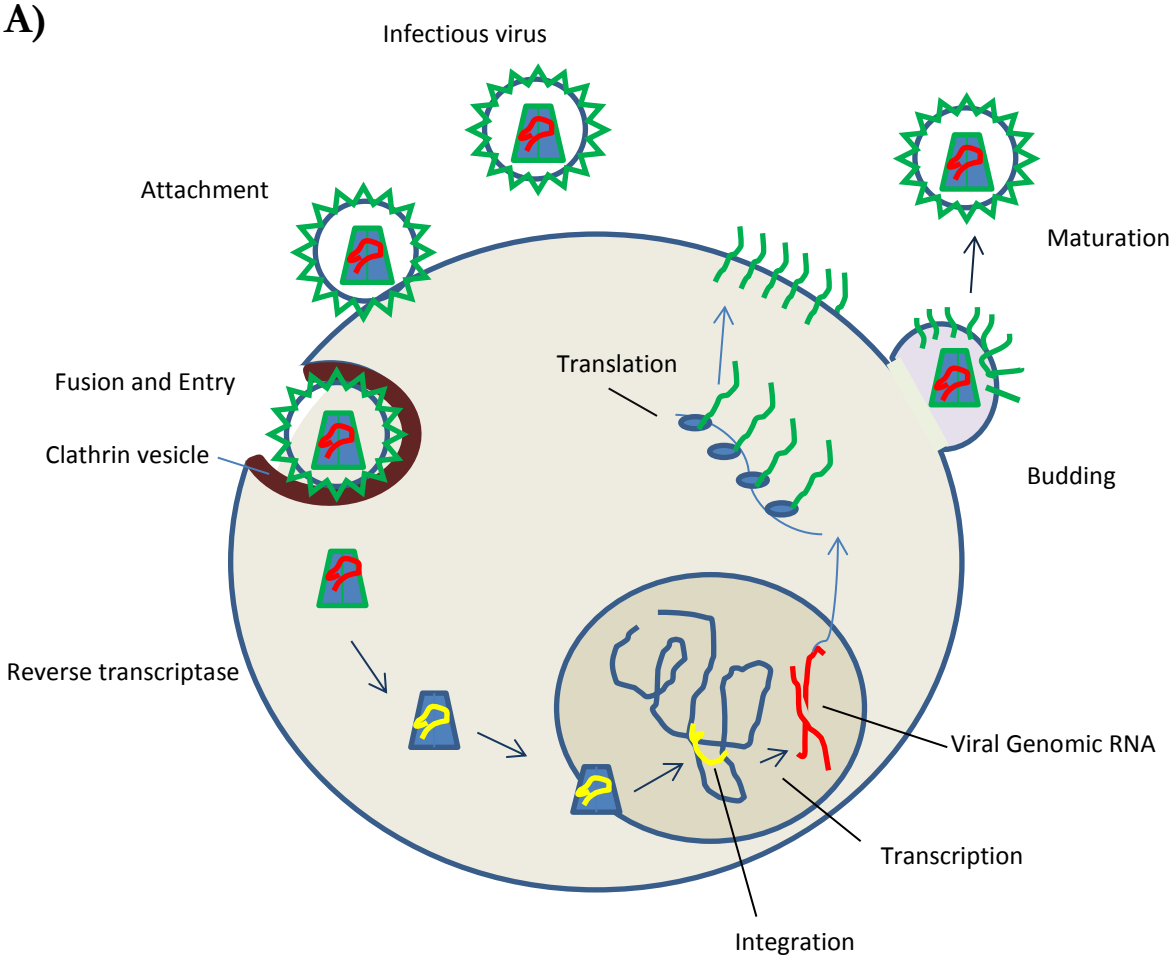

1-Cell surface internalization
2- Endocytic internalization
Dynamin depended-Clathrin vesicle
Co-receptor inhibitor
3- Co-receptor independent transferring
Endosome membrane Fusion
Lysosome
Replication

Figure 1.6 Schematic picture of retrovirus replication

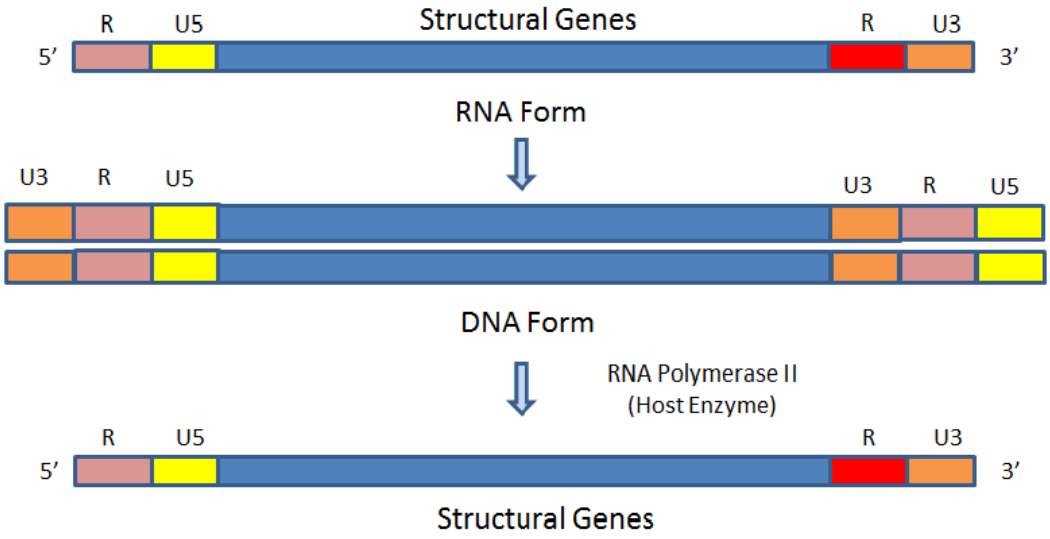
A) Life cycle of a replication of retrovirus in an infected cell, from attachment proceding to budding and maturation of new viruses.

B) Structures of retrovirus genome in different stages of replication: positive strand RNA, provirus DNA, and new genomic RNA

A)


Infectious virus
Attachment
Maturation
Fusion and Entry
Translation
Clathrin vesicle
Budding
Reverse transcriptase
Viral Genomic RNA
Transcription
Integration

B)


R
U5
Structural Genes
R
U3
5'
3'
RNA Form
U3
R
U5
U3
R
U5
DNA Form
RNA Polymerase II
(Host Enzyme)
R
U5
R
U3
5'
3'
Structural Genes

During their exit from the host cell, retroviruses acquire their phospholipid bilayer envelope from the cell's plasma membrane, where the viral envelope glycoproteins accumulate. This stage of the viral lifecycle is referred to as budding (Fig 1.6) (Coffin et al., 1997). The new viral progenies released from the cells are then able to infect other cells and propagate the infection.

There are numerous studies that show enveloped viruses are able to capture some of the host proteins before they bud from the cellular membrane (Cantin et al., 2005). A variety of host cytidine deaminases such as APOBEC3G, APOBEC3F, and APOBEC3C can be incorporated into budding virions of retroviruses like HIV-1, MLV and SIV (Bogerd et al., 2004, Cantin et al., 2005, Kao et al., 2003, Langlois et al., 2005). Clathrin, a vesicular transport protein; Thy-1, a GPI anchor protein similar to prion; and actin and β-Tubulin, cytoskeleton proteins are some examples of host molecules that are incorporated into retroviruses (Cantin et al., 2005, Nermut et al., 1999, Wang et al., 2003).

1.3.3 Endogenous and Exogenous Retroviruses

Retroviruses can also be divided into two groups as either endogenous or exogenous retroviruses according to their mode of transmission. Exogenous retroviruses are retroviruses that transmit horizontally by infecting the somatic cells of new hosts (Table1.1), but endogenous retroviruses (ERVs) have been integrated into the germline for millions of years and have been transmitted vertically to offspring as an integral part of the host's genome. For example, MLV (murine leukemia virus) entered the germ line of mice approximately 1.5 million years ago and its copy number ranges from 25 to 70 depending on the mouse strain (Maksakova et al., 2006, Miyazawa, 2010). ERVs are present in the genome of all vertebrates

and originate from the infection of exogenous retroviruses. Co-evolution of ERVs with their host's genome has provided a positive contribution to genome plasticity (flexibility or adaptability) that confers protection against the infection by related pathogenic exogenous retroviruses. Therefore, some ERVs have been positively selected and maintained in the host genome throughout evolution (Black et al., 2010, Gifford and Tristem, 2003). ERVs make up approximately 5%-8% of the human genome (Lander et al., 2001), additionally murine endogenous retroviruses together with LTR retrotransposons comprise almost 10% of the mouse genome (Mouse Genome Sequencing Consortium et al., 2002). Most of these ERVs are functionally defective, since they encode partial protein sequences, or their genomes have accumulated stop codon mutations. However, a small number appear to be expressed as full-length viral mRNAs (Gifford and Tristem, 2003). It is also possible that defective ERVs that have intact ORFs can encode proteins. For example, the endogenous feline leukemia virus (FeLV) expresses the *env* protein that can block the exogenous FeLV subgroup B (Katzourakis and Gifford, 2010, McDougall et al., 1994).

Almost 26 lineages of human endogenous retroviruses (HERVs) have now been identified and are classified into at least three classes: I, II and III according to their phylogenetic relationship (Gifford and Tristem, 2003). Type I ERVs are similar to their homologous exogenous gammaretrovirus and epsilonretrovirus counterparts. Type II and III ERVs are most closely related to alpharetroviruses, betaretroviruses, and spumaviruses, respectively (Miyazawa, 2010). Lentiviruses and deltaviruses are the only retroviral genera without any endogenous counterparts, and therefore are fully exogenous retroviruses (Gifford and Tristem, 2003).

There are four classes of endogenous murine leukemia viruses (MLV): ecotropic viruses predominantly infect mouse cells; xenotropic viruses are able to infect cells from species other

than mouse; amphotropic viruses infect many species of animal cells; polytropic viruses are recombinant viruses that replicate in mouse as well as non-mouse cells (Weiss, 2010). Normally ERVs are not pathogenic in their original host; however, some studies have shown that they can induce disease such as lymphoma, as for example in AKR mice (Reus et al., 2001). Another example is the exogenous gibbon ape leukemia virus (GALV) that causes lymphoma in gibbon apes, and has 78% similarity to the endogenous koala retrovirus (KoRV). KoRV is linked to a neoplastic and immune suppression disease in koalas and seems to be a very active ERV since it still shows many of the features of an exogenous virus including the capacity to produce infectious virus particles and variable proviral sequences. Sometimes interactions between an infectious exogenous retrovirus with an endogenous retrovirus may produce new pathogenic recombinants in the same host or in a new host (Gifford and Tristem, 2003). In the event that retroviral envelope glycoproteins are expressed at the cell surface, newly-generated retroviral particles could potentially go on to infect a new range of target cells and/or hosts (Khan, 1984, Sheets et al., 1993).

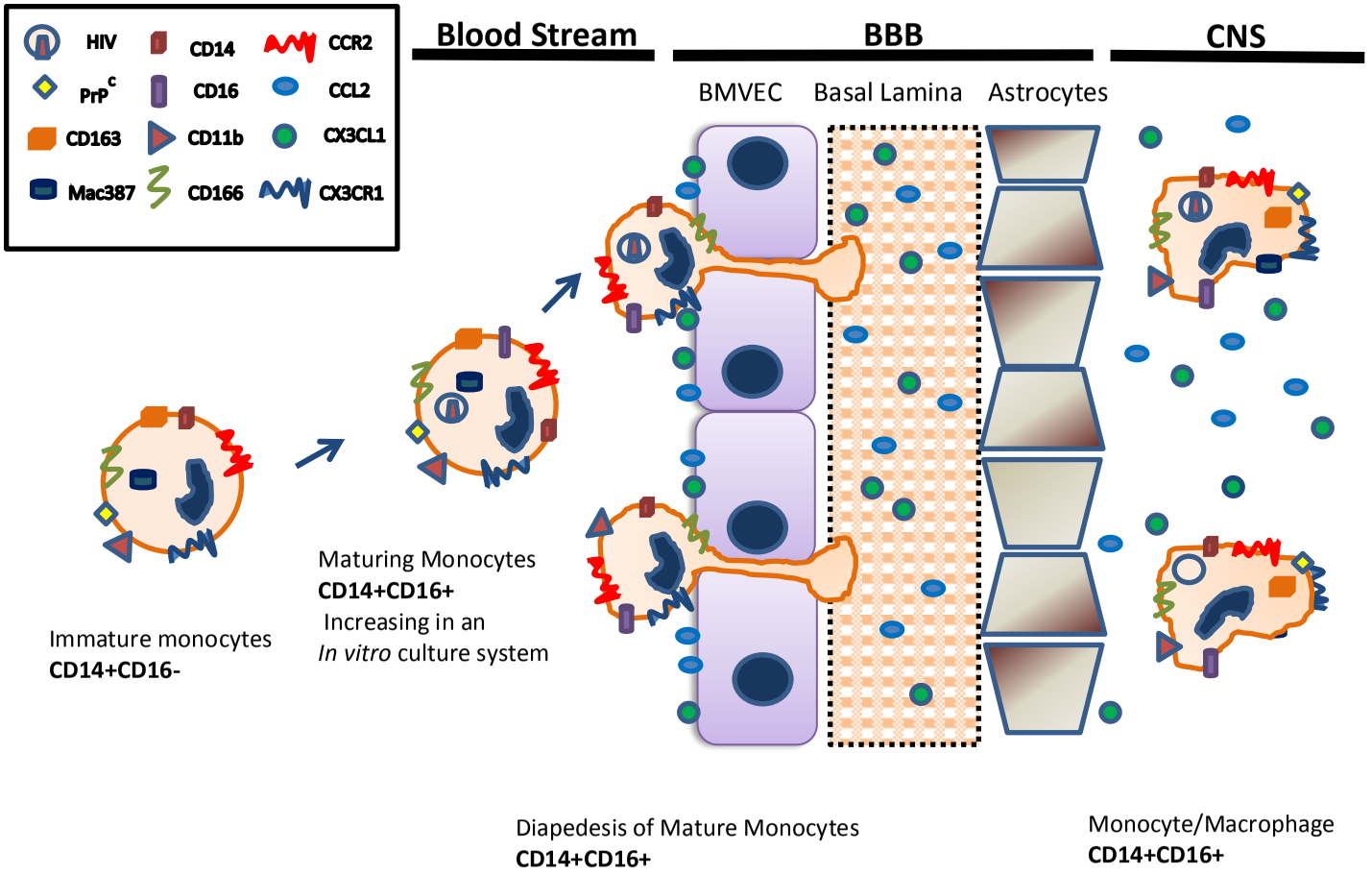
1.3.4 Retroviruses and the Central Nervous System

HIV is a global health issue with more than 34 million people infected worldwide. Clinical studies have demonstrated that AIDS patients often develop cognitive disorders, which can change from distractibility and delirium to reduced memory and dementia (Faulstich, 1986, Gabuzda and Hirsch, 1987, Mangos et al., 1989, Navia et al., 1986a, Snider et al., 1983). Brain autopsies of AIDS patients showed cortical atrophy, invasion of macrophages and giant cell formation (Budka, 1989, Gelman, 1993, Navia et al., 1986b); as well as damage to neurons in the frontal, temporal, and parietal regions of the brain (Ketzler et al., 1990, Navia et al., 1986b, Wiley et al., 1991). This neuronal pathology is likely caused by the HIV virus itself; therefore

many researchers have focused on understanding how a retrovirus can cause neuronal and synaptic loss in the central nervous system (CNS), and if HIV can pass through the brain blood barrier (BBB) (Buckner et al., 2011, Giulian et al., 1996). New studies clarified that HIV-associated neurocognitive disorders (HAND) are increasing among HIV-infected patients who are receiving antiretroviral therapy (Cysique et al., 2009, Sacktor et al., 2002). A recent study identified that HIV can enter the CNS within two weeks of infection, and regardless of antiretroviral therapy, establish HAND in 40-60% of HIV-positive patients (Williams et al., 2012). One study showed that $CD14^{+}CD16^{+}$ monocytes represent a mature population that is able to transmigrate across the blood brain barrier (BBB) into the CNS parenchyma and subsequently accumulate in the brain. Monocytes have long been considered to be resistant to HIV infection, but recent studies determined that these mature monocytes are susceptible to HIV infection and can produce high titres of HIV. Mature monocytes increase in the blood of HIV infected patients due to CCL2 (perhaps CX3CL1) chemotactic signals. These monocytes have an increased number of junctional proteins on their cell surface that could improve the interaction between mature monocytes and brain microvascular endothelial cells (BMVECs) of BBB. This process could facilitate the transmigration of monocytes across the BBB (Fig1.7). Because these cells are able to access the CNS, they could establish neuro-inflammation in the brain as a result of HIV infection (Buckner et al., 2011, Williams et al., 2012).

Figure 1.7 Schematic representation of monocytes maturation and transmigration across the BBB into the CNS

Most circulating peripheral blood monocytes have $CD14^+$ while a small ratio also express $CD16^+$, which represents 5-10% of monocytes in the blood. In this nonadherent culture system model for $CD14^+$, during the maturation process the number of $CD14^+CD16^+$ will increase as well as other junction proteins including CD11b, CD163, PrP^C, Mac387, CD166, CCR2, and CX_3CR1. The mature monocyte ($CD14^+CD16^+$) is susceptible to HIV infection and are able to transmigrate across the blood brain barrier (BBB) in to the centre nervous system (CNS) in the response to specific chemokine gradients of CCL2 and CX3CL1. Diapedesis of the monocytes allow the passage through the brain microvascular endothelial cells (BMVEC). (Adapted from: Williams et al., 2012)


HIV
PrP^C
CD163
Mac387
CD14
CD16
CD11b
CD166
CCR2
CCL2
CX3CL1
CX3CR1
Blood Stream
BBB
CNS
BMVEC
Basal Lamina
Astrocytes
Immature monocytes
CD14+CD16-
Maturing Monocytes
CD14+CD16+
Increasing in an
In vitro culture system
Diapedesis of Mature Monocytes
CD14+CD16+
Monocyte/Macrophage
CD14+CD16+

1.4 The Relationship between Prion Disease and Retroviruses

Prion proteins have a GPI anchor that allows for their insertion into the phospholipid bilayer of cell membranes and cycle between the cell surface and endosomal compartments (Linden et al., 2008, Peters et al., 2003). There are several studies that have described the association of prion proteins and exosomes in extra cellular mediums of PrP^{Sc}-infected cell cultures. In these studies, prions are believed to have been taken-up by exosomes and were detected in the supernatants of cells (Fevrier et al., 2004, Fevrier et al., 2005, Paquet et al., 2004). Retroviruses and exosomes share many similarities in size, density, lipid composition, cellular protein content, site of assembly and release, and in their ability to activate immune cells (Cantin et al., 2008, Pelchen-Matthews et al., 2004).

Furthermore, retrovirus assembly happens in a multi-step fashion involving the viral *gag* polyprotein, genomic viral RNA and the cellular membrane where the viral envelope glycoproteins are anchored (Cimarelli and Darlix, 2002). Many studies have determined that the composition of proteins and lipids in the viral envelopes are dependent on the site of virus assembly and the host endosomal membrane proteins. These host proteins can then be hijacked by enveloped viruses during the budding and release from infected cells. For example, GPI-anchored proteins such as Thy1, CD59 or CD55 are cellular bilayer membrane glycoproteins and regularly found in lipid rafts therefore they are more susceptible to incorporation into enveloped viruses particularly retroviruses (Cantin et al.,2005, Leblanc et al., 2006). Beside GPI-anchored proteins, trans-membrane molecules such as MHC class I and class II, and ICAM, are also taken-up into nascent retrovirus particles (Leblanc et al., 2006, Nguyen and Hildreth, 2000, Ott, 1997, Pelchen-Matthews et al., 2004, Raposo et al., 2002).

Since the prion protein is a GPI-anchored glycoprotein, and retroviruses assembly co-localized in the same intracellular endosomal vesicles such as multi vesicular bodies (MVB), they have direct opportunity to interact with each other and prions can incorporate into the retroviruses (Ashok and Hegde, 2006, Leblanc et al., 2006).

Western blot analysis using an anti-prion antibody have shown that infection by the Moloney murine leukemia virus (MoMLV) enhances the release of PrP^{C} and PrP^{Sc} in association with retroviral particles and exosomes in the supernatants of infected NIH3T3 cells (Leblanc et al. 2006). Similar results have been obtained when HIV-1 was used (Leblanc et al. 2006).

In addition, the potential interaction between the retroviral Env glycoprotein and the Gag capsid protein (CA) with prions has been investigated in several studies. Prion proteins co-fractionate with the retroviral Env and Gag proteins in Optiprep gradient assays (Kolesnikova et al., 2004). Gag-pol polyprotein production also enhanced prion release from the cell, whereas Env expression had no effect (Leblanc et al., 2006). Furthermore, scrapie infectivity has been shown to be associated with both MoMLV virions and exosomes in the extracellular media (Leblanc et al., 2006).

In another study, mouse neuroblastoma cells (N2a) infected with PrP^{C} or PrP^{Sc} displayed an increased release of prion proteins in the cell culture medium in association with exosomes and /or virus-like particles (VLPs). Evidence has been brought forth showing that endogenous retroviruses can produce enveloped VLPs that associate with prions in the cell culture medium (Alais et al., 2008). In a similar manner, the release and accumulations of PrP^{Sc} in the extracellular medium of infected sheep microglial cells were found to be increased two fold when co-infected with the small-ruminant lentivirus, caprine arthritis encephalitis virus

(CAEV)-Cork strain (Stanton et al., 2008). Likewise, Ligios et al. found that the presence of PrP^{Sc} in mammary glands of sheep diagnosed with scrapie and mastitis clearly correlated with seropositivity of the maedi visna virus (MVV) (Ligios et al., 2005). Since MVV and CAEV are generally endemic in sheep and goats, prion infectivity could be radically enhanced upon retroviral infection if an association existed (Leblanc et al., 2006)

There are also several more associations that have been made between retroviruses and prions *in vitro*. A functional interaction of cellular prion proteins with the nucleocapsid protein (NC) and genomic viral RNA of feline immunodeficiency virus (FIV) has also been observed (Moscardini et al., 2002). The retroviral NC protein is a major nucleic acid-binding protein and is required for packaging the dimeric RNA genome in the viral capsid (Darlix et al., 2000). Some studies have shown that cellular prion proteins are very similar to the NC protein of retroviruses in that they can bind nucleic acids such as viral genomic RNA (Darlix et al., 2000, Gabus et al., 2001a, Gabus et al., 2001b, Moscardini et al., 2002).

Several *in vivo* studies have also established correlations between prions and retroviruses. One study has unravelled a link between scrapie infection and the replication of ecotropic, xenotropic and polytropic MLVs in the brain and spinal cord of senescence-accelerated mice (SAMP8) (Lee et al., 2006). In another *in vivo* study, endogenous MLV titers were reported to be increased in the brains of SAMP8 and AKR mice when these were inoculated with scrapie proteins (Carp et al., 2000). Finally, because the finding that ERV expression is increased in prion disease is so robust, one research group has recently assessed whether RNA transcript levels of human endogenous retroviruses (HERV) in cerebrospinal fluids of patients with CJD could be considered as a marker for sCJD diagnosis (Jeong et al., 2010).

Altogether, these data convincingly show that there is a strong relationship between prions and retroviruses in tissue culture and in animal models. It is currently believed that prions may be released from retrovirus-infected cells as part of the viral envelope during the budding of the retrovirus from the cell membrane and/or can they could bind directly to the NC protein and be encapsidated into the viral capsid of nascent retroviral progeny. Experimental and clinical data have also shown that retroviruses can cross and disrupt the BBB. On the other hand, prions released in the blood stream are not capable on their own of breaching the tight BBB junction and entering the brain. Finally, natural associations have been established between prion disease and co-infection by endemic retroviruses in sheep and goats.

Although it may appear to some that there is a causative association between prion disease and retroviruses, there is, however, an important piece of the puzzle that is missing. To formally prove that retroviruses, either endogenous or exogenous, could act as vectors for the dissemination of prion disease, it must be shown that retroviruses can acquire prions from PrP^{Sc}-infected cells, transport and deliver them into susceptible target cells in the brain of the host, and ultimately cause disease in the experimental animal.

2.0 HYPOTHESIS

Prion proteins can be taken-up as cargo within nascent retroviral particles, and therefore may be transported and delivered into susceptible target cells by retroviruses.

2.1 Objectives

- Generate human and mouse cellular prion protein expression constructs with or without an internal Flag epitope tag or an eGFP reporter gene.

- Develop an *in vitro* assay system to analyze the uptake of overexpressed and endogenous cellular prion proteins by human and mouse retroviruses.

- Establish fluorescent microscopy techniques and assays to track the transfer of fluorescent prion proteins from transfected producer cells to retroviruses and subsequently to target cells.

- Determine whether prion proteins that are hijacked by retroviruses can be transferred to the cytoplasm of susceptible target cells.

3.0 MATERIALS AND METHODS

3.1 DNA Constructs

3.1.1 RNA Extraction and cDNA Synthesis

Total cellular RNA was extracted using TRIzol® (Invitrogen, USA) according to the manufacturer's specifications. A ratio of 9:1 of TRIzol to cell lysate was used for the RNA extraction. One microgram of total RNA was used in the reverse transcriptase polymerase chain reaction (RT-PCR) to produce cDNA by using either random primer hexamers or oligo dT primers. The ImProm-II™ Reverse Transcription System kit was used for this preparation (Promega, Canada). The RT-PCR program was as follows: 5 minutes at 70 ^{o}C (RNA secondary structures are denatured), then add master mix. Reverse transcription of mRNA into cDNA was performed for 60 minutes at 42 ^{o}C. cDNA samples were stored at (-20^{o}C) for future work.

3.1.2 DNA Polymerase Chain Reaction (PCR)

For the amplification of the prion cDNA, specific primers for human and mouse prion were designed separately by using Genbank on NCBI (National Center for Biotechnology Information). Additional DNA sequences such as restriction endonuclease (RE) sites, FLAG epitope tag and eGFP reporter sequences have been designed in the cloning primers for generating human and mouse prion protein constructs. The designed specific primers are as follows:

Table 3.1 The cloning primer sequences for constructing the various human and mouse prion expression vectors

Forward Human Prion Primers:	
Fwd HP1-BglII	5'ACTGACTAGATCTATGGCGAACCTTGGCT3'
Fwd HP2-EcoRI	5'TCTCAGGAATTCGCCTATTACCAGAGAG3'
Fwd HP2-EcoRI-FLAG	5'TCTCAGGAATTCATGGACTACAAGGACGATGATGACCCGGCCTATTACCAGAGAG3'
Reverse Human Prion Primers:	
Rev HP1-EcoRI	5'ATAGGCGAATTCCTGAGATTCCCTCTCG3'
Rev HP2-HpaI	5'GTCTGTATGTTAACTCATCCCACTATCAGGAAGAT3'
RevHP2-EcoRI-FLAG	5'TAGTCCATGAATTCCTGAGATTCCCTCTC3'
Forward Mouse Prion Primers:	
Fwd MP1-BglII	5'TCAGATCTCGAGATGGCGAACCTTGGCTACTGGC3'
Fwd MP2-EcoRI	5'GACTGCGAATTCGTCAATATCACCATC 3'
Fwd MP2-EcoRI-FLAG	5'GACTGCGAATTCATGGACTACAAGGACGATGATGACAAGGTCAATATCAC3'
Reverse Mouse Prion Primers:	
Rev MP1-EcoRI	5'TGACGAATTCGCAGTCGTGCACGAA3'
Rev MP2-HpaI	5'ATGGTTAACTTATCCCACGATCAGG3'
Internal eGFP cloning vector:	
Fwd eGFP-EcoRI	5'ATCTCAGGAATTCATGGTGAGCAAGGGC3'
Rev (eGFP-EcoRI)	5'ATAGGCGAATTCCTTGTACAGCTCGTCC3'

All primers were ordered through (Sigma-Alderich, Canada). PCR was performed using PrimeSTAR® HS DNA Polymerase (2.5u/μl), 5X PrimeSTAR® GC buffer (Mg^{+2}plus), dNTP mix (25mM each) (from Takara Bio INC, Genetic Engineering Research), specific forward and reverse primers (10μM) and DNA template (<200ng). PCR was performed as follows: 94°C-40s, (94°C-10s, 55°C-10s, 72°C-60s) x 40 cycles, 72°C-10min. The correct band size was confirmed by 1% agarose gel electrophoresis and samples were gel purified using the Wizard SV gel and PCR clean-up system kit (Promega, Madison, Wisconsin, USA).

3.2 Generation of Prion Expression Vectors

All of the prion expression vectors have an internal EcoRI endonuclease restriction site created by PCR. This restriction site was used to insert a FLAG epitope tag or the cDNA of the eGFP reporter protein.

The reason for making internally tagged prion expression vectors is that there is post-translational cleavage of the prion protein in both its N-terminus and C-terminus. Human peripheral blood mononuclear cells (PBMC) isolated by Ficoll-preparation (Glinski et al., 1976) were used as a source of mRNA for cloning the human prion cDNA, and total RNA from the mouse cell line NIH 3T3 was used for cloning the mouse prion cDNA. The fragment of human prion cDNA between restriction sites BglII and EcoRI is referred to as HP1 and the fragment between EcoRI and HpaI is named HP2 (see Figures in section 3.2.1). The same concept was used for the mouse prion cDNA, where the first segment of the prion molecule from BglII to HpaI was named MP1, and second domain after EcoRI is MP2. The first plasmid construct was made by cloning the PCR fragments in the first part of the prion gene (HP1 or MP1) into the peGFP-N1 plasmid. This new construct was then used for cloning the second

part of the prion gene (HP2 or MP2), which has the EcoRI RE site at the 5' end of the cDNA and an HpaI RE site after the stop codon at the 3' end. The PCR inserts and expression vector were digested using EcoRI and HpaI. The digested vector was dephosphorylated with Antarctic Phosphatase (5u/μl) (BioLabs Inc, New England) to avoid re-ligation of cleaved ends. T4 DNA Ligase (New England BioLabs Inc.) was used to ligate the cassette into the dephosphorylated and purified vectors. The ligation was performed at 16°C overnight. DH5α competent E.coli cells (100μl) were transformed with 10μl of the ligation mixture. The transformed cells were streaked onto LB plates containing the appropriate antibiotic for selection. The next day, colonies were picked and grown overnight in 1.5 ml of LB media with antibiotics, allowing for the selected bacteria to increase exponentially. The vector (peGFP-N1) contains a CMV promoter and a resistance gene for the antibiotic kanamycin. Roughly 16 to 24 colonies were picked and plasmid mini preparations (mini-prep) were performed. Screening for successful clones was performed by digestion of the purified DNA plasmids with appropriate RE(s). The samples were then separated by gel electrophoresis (1% agarose gel), and screened for the correct fragment size. Successful mini-preps were then purified using phenol-chloroform extraction and sent for sequencing.

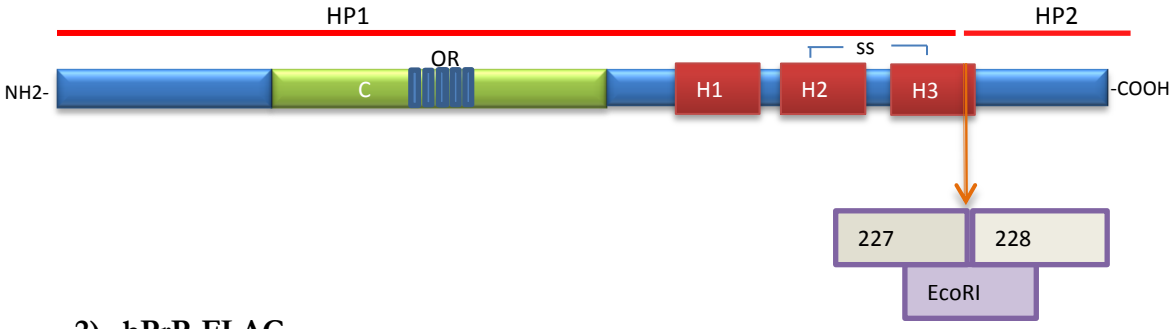
3.2.1 Human Prion Protein Expression Constructs

Three expression vectors for the human prion protein were made. One without an epitope tag or reporter gene called hPrP-EcoRI, one with a FLAG epitope tag called PrP-FLAG and one containing the eGFP reporter protein (PrP-eGFP). The two latter expression plasmids have their transgene inserted between amino acid 227 and 228 (C-terminus of helix 3). The structure of these protein recombinants are shown in figure 3.1. The plasmid backbone is peGFP-N1 (Clontech), which has a strong CMV promoter upstream of a multiple cloning site (MCS).

Figure 3.1 Schematic structures of the predicted human prion protein expression constructs.

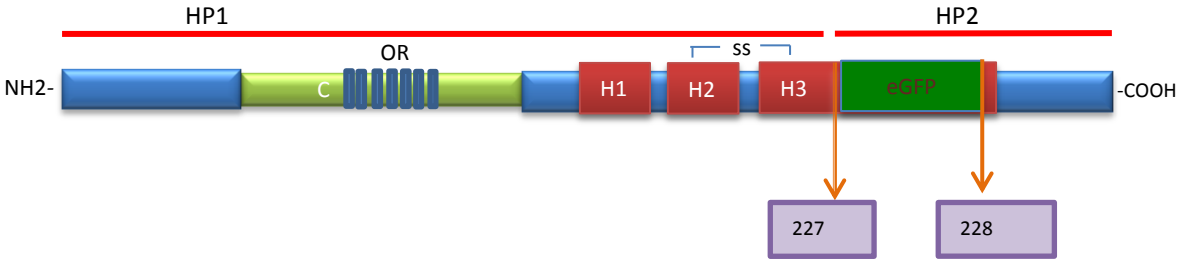
HP1 is the name of the section between N-terminal and amino acid(a.a) 227, HP2 is the name of the section between amino acid 228 and C-terminal of the construct protein. S-S is the disulphide band between H2 (α helix2) and H3(α helix3). OR is octarepeat region, and EcoRI is endonuclease restiction enzyme (ER) site inserted between a.a 227 and 228.

1) **hPrP-EcoRI**



2) **hPrP-FLAG**

3) **hPrP-eGFP**



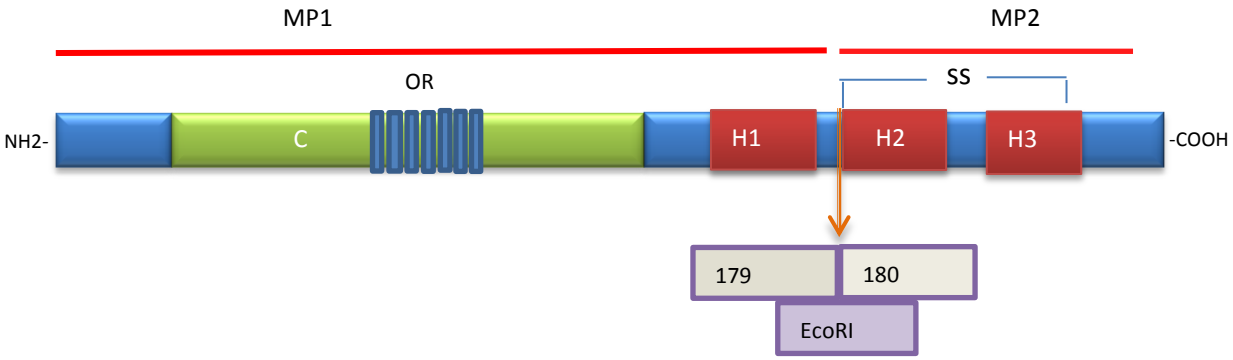
3.2.2 Mouse Prion Protein Expression Constructs

The mouse prion protein expression vectors have been made for future use and have not been used at this stage of the research project. The source for cloning the mouse prion protein cDNA was the mouse embryonic fibroblast NIH 3T3 cell line. Both halves of the cDNA cassette were generated as described in the previous section, and were inserted in the peGFP-N1 expression vector. Two mouse prion protein expression vectors that have the EcoRI site with or without the FLAG tag epitope between amino acids 179 and 180, which is before α helix, 2(H2) were generated. It is predicted that the transgenes in this location will not affect the structure of the helix. Sequence analysis confirmed the expression constructs of the mPrP cloning vectors (Fig 3.2).

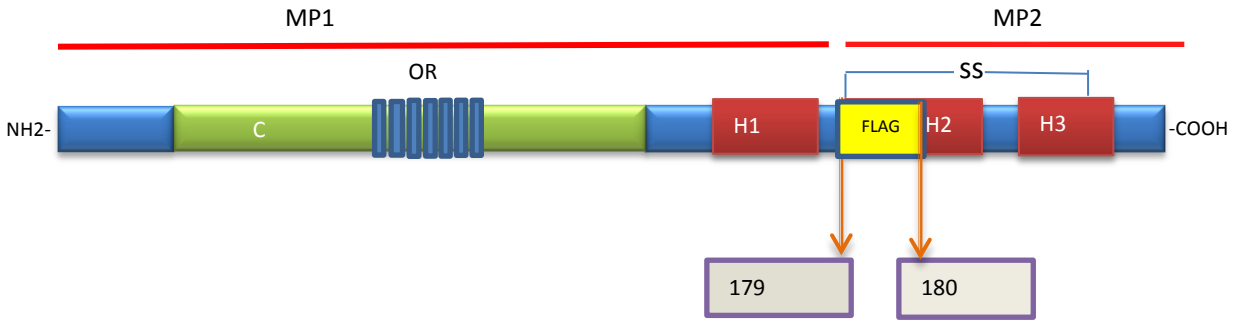
Figure 3.2 Schematic structures of the predicted mouse prion protein expression constructs.

MP1 is the name of the section between N-terminal and amino acid (a.a) 179, MP2 is the name of the section between amino acid 180 and C-terminal of the construct protein. S-S is the disulphide band between H2 (α helix2) and H3 (α helix3). OR is octarepeat region, and EcoRI is endonuclease restiction enzyme (ER) site inserted between a.a 179 and 180.

1) **mPrP-EcoRI cloning vector**



2) **mPrP-EcoRI-FLAG tag cloning vector**



3.3 Retroviral Expression Vectors and Controls

Moloney-GFP (also known as pMOV-eGFP) is a replicative Moloney MLV virus which has the eGFP reporter gene inserted in the proline rich region of Env and is expressed as a fusion protein (Sliva et al., 2004). The single-cycle pseudoviruses, MLV (HcRed), MLV (eGFP), and HIV (expressing the eGFP reporter protein (HIV (eGFP)) were generated by a multi-plasmid expression system. Viruses were produced by co-transfecting a shuttle vector for MLV (M5P) and HIV (CSGW), a Gag-Pol expression vector (GP), and a plasmid encoding the viral envelope glycoprotaein, in this case the vesicular stomatitis virus G glycoprotein (VSV-G). HIV (eGFP) expresses eGFP under the control of an internal SFFV (Spleen focus forming virus) 5' LTR promoter, while in MLV the eGFP or HcRed genes are expressed from the native 5' LTR promoter and also from a SV40 promoter located upstream of the viral LTR on the plasmid DNA (Langlois et al., 2005). The SV40 promoter allows for increased yields of proviral RNA being produced in transfected cells. Two plasmids (peGFP-C3 and peGFP-N1) were used as transfection and expression controls for eGFP. Other expression vectors that have been used as positive controls for eGFP and FLAG domains in this study are APOBEC3G-eGFP (A3G-eGFP), APOBEC3G-FLAG (A3G-FLAG), activation-induced cytidine deaminase (AID)-eGFP and AID-FLAG. These were previously generated by our lab.

3.4 Cell Culture and Protein Expression

Human embryonic kidney epithelium cells (293T), NIH Swiss mouse embryonic fibroblast (NIH 3T3) and mouse neuroblastoma cells (N2a) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (HyClone; high Glucose (4,500 mg/L), L-glutamine (4.0 mM) with sodium pyruvate, (Thermo Fischer Scientific, Waltham, Massachusetts, USA) with or without phenol

red. This media was supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, Missouri) and 100 U/ml Penicillin and 100-μg /ml streptomycin (Multicell, Wisent Inc., Canada) and propagated at 37 ºC in a 5% CO2 incubator. Polystyrene (25 and 75 cm^2 Corning®, USA) flasks were used for growing the cells and 6-well or 12- well cell culture plates were used for transfection and infection assays (Corning®, Costar®, USA).

3.5 Transfection Assays

N2a and 293T cell lines were used to overexpress and to produce infectious viral particles (non-replicative HIV-eGFP and MLV). Cells were seeded in 6-well plates at 2.0 x 10^5 cells/well, and grown overnight. Transfections were performed when cell confluence was almost 30% to 40%. The growth medium was changed prior to transfection. For transfection of the hPrP constructs, 300ng of plasmid DNA was used. For pseudoviruses HIV (eGFP), MLV (HcRed) or MLV (eGFP), a total of 800ng of plasmid DNA was used as follows: *i)* MLV (eGFP) virus: 400ng of MLV (HcRed) or MLV (eGFP), 280ng of Gag-Pol expression vector (GP) and 120ng of VSVG expression vector were co-transfected (with a ratio of 10:7:3 respectively); *ii*) HIV (eGFP): 400ng of the Gag-Pol expression vector p8.2 (Gag-Pol and accessory genes except *env*), 280ng of packaging vector (LTR eGFP packaging vector) and 120ng of plasmid coding for VSV-G were used to produce a HIV(eGFP) pseudovirus (with a plasmid ratio of 10:7:3 respectively).

For the co-transfection of retroviral plasmids with prion expression vectors, a total of 1100ng of plasmid DNA was used. Positive controls for eGFP (e.g. plasmid peGFP-N1 or peGFP-C3) were performed using 300ng. For the co-transfection of A3G-eGFP or A3G-FLAG with MLV or HIV expression vectors, 300ng of A3G were used with a combined total of 800ng for the

plasmid vectors for the retroviral constructs. The transfection reagent Genejuice (Novagen-EMD4 Biosciences, Darmstadt, Germany) was used with a ratio of 3:1 (3μl Genejuice: 1 μg DNA plasmid) in a final volume of 2ml/well. After transfection, 293T cells were incubated for 72 hours in order to produce optimal amounts of protein and infectious viral particles for the subsequent assays. After 24 hours post-transfection, fluorescence in transfected cells was analyzed using a fluorescence microscope with emission peaks expected at 509nm for eGFP and at 637 nm for HcRed.

3.6 Infection Assays

The day before infection with the viruses, 2.0 x 10^5 (NIH 3T3 or 293T) cells were seeded per well in a 6-well plate. After 24 hours, the cells were 30-40% confluent, which is ideal for the infection assays. After 72 hours post-transfection, supernatants containing infectious viral particles were harvested and centrifuged for 5 min at 2000rpm. The supernatant was then filtered through a 0.22 μm cartridge filter to remove cells and debris from the extracellular medium.

For viral infectivity assays, the monolayer of NIH 3T3 or 293T cells was then infected using 0.3 ml of the virus-containing supernatant in the presence of 8μg/ml of polybrene. Spinoculation was performed at 2000 rpm for 1 hour, allowing for an increase in viral attachment and therefore infection efficiency. Infected cells were overlaid with a total of 3 ml of complete DMEM, and incubated for 24 hours at 37ºC. For incubations longer than 24 hours, overlay medium was removed 24 hours post-infection and 3ml of fresh complete DMEM medium was added to each well to maintain ideal cell culture conditions.

3.7 Flow cytometry Assays

Fluorescent proteins expression post-transfection or infection were analyzed by Fluorescence-Activated Cell Sorting (FACS) on a CyAn ™ ADP analyzer (Beckman Coulter Inc., Brea, California, USA), which has three lasers and 9 colour detectors.

In flow cytometry, a laser is focused on the flow cell where the cells are directed to pass through its center in sheath liquid. This instrument can provide the relative size of the cells, or forward scatter, using the blue laser (488 nm). Beside the size, the granularity of the cells can also be assessed as a measure of deflected light off internal structures or granules in the cell, better known as side scatter. Fluorophores bound to cells can absorb the laser energy and emit fluorescence. There are filters that separate the fluorescence emitted by cells and can direct them to individual photomultiplier tubes for measurement. In this study, the eGFP reporter protein of plasmids peGFP-N1 or hPrP-eGFP was excited by a laser with a wave length of 470nm and the emitted peak fluorescence wavelength was 509 nm. This emitted light was directed to the FL1 detector by first passing through a 530/40 nm band pass filter. HcRed has an excitation wavelength of 590 nm and emits at 637 nm. The emitted light spectrum can be analysed by detector 3 (FL3) using a band pass filter of 613/20.

Flow cytometry assays were used to determine transfection and infection efficiencies. For these assays, cells at defined times following transfection or infection were washed with PBS, pH 7.4 and then detached with Trypsin for few minutes (for NIH 3T3 cells). Adding about 0.5ml fresh media (DMEM, FCS 10%) in each well inactivates trypsin. After detaching, media was removed by pelleting the cells for 5 min centrifuge at 2000 rpm. Cells were then re-suspended in EDTA-PBS pH 7.4 and analyzed.

3.8 Virus Titration

After 72 hours post-transfection of $2x10^5$ 293T cells with MLV (eGFP) expression plasmids, the supernatant was removed and transferred to a round-bottom polystyrene tube. To remove floating cells and debris, the supernatant was centrifuged for 5 minutes at 2000 rpm and then filtered through a 0.22 μm cartridge filter (MILLEX-GV, Millipore, Ireland LTD). Various dilutions of virus-containing supernatants were then use to infect NIH 3T3 target cells. Cell infections were monitored 24 hours later by flow cytometry. The virus titer was calculated by he number of transducing particles per ml for supernatant.

3.9 Western Blot Analysis

3.9.1 Preparation of Cells and Viral Particles

For determining protein expression in transfected or co-transfected cells, Western blots were used. This assay starts with seeding 2.0 x 10^5 293T producer cells per well in a 6-well plate for 24 hours. When the cells were 30-40% confluent, transfection or co-transfection of the expression vectors was performed as previously mentioned. A positive control vector (peGFP-C3 or peGFP-N1), that expressed the green fluorescent protein, allowed verification of the transfection efficiency using an inverted fluorescence microscope. Virus and cell lysate preparations were performed 72 hours post co-transfection to ensure maximum protein expression.

3.9.2 Viral Encapsidation Assay

The cell culture medium was removed 72 hours post-transfection, centrifuged and filtered using a 0.22 μm syringe filter to prevent carryover of producer cells into the viral particle supernatant.

The viral particles were then concentrated by ultracentrifugation for 1 hour at 175,000 g (75,000 rpm) at 4°C in TL-100.3 rotor (Beckman, USA). RIPA lysis buffer (75 µl) with 1X protease inhibitor (PI) was then added to the viral pellet. RIPA buffer was comprised of 50 mM Tris-HCl (pH 8.0), 150mM NaCl, 1%NP40 (Igepal), 0.2%, sodium dodecyl sulphate (SDS), 0.5%, Na-Deoxycholate, 1mM EDTA, and freshly supplemented with 1X protease inhibitor (PI) cocktail (Roche, Mannheim, Germany).

3.9.3 Preparation of Cell lysates and Protein Extraction

Transfected or co-transfected cells in 6-well plates were used for cell lysate preparation. Sterile cold PBS, pH 7.4, was used to wash the cells prior to detaching them with 1X trypsin and DMEM was then added to inactive the trypsin. The detached cells were collected in 3ml sterile tubes and centrifuged for 5 minutes at 2000rpm, after which the media was removed. Cells were washed once with PBS and transferred to 1.5ml microtubes for lysis.

Cell pellets were lysed by adding 600µl cold RIPA lysis buffer for 15 minutes on ice. Lysates were then centrifuged at 13,000 rpm at 4°C for 5min to pellet the cell nuclei and debris. Next, 75µl of the cell or viral lysate were mixed with 25µl of 5X protein loading Laemmli buffer (0.5M Tris pH 6.8, glycerol, 20% SDS, bromophenol blue, and dH_2O) freshly supplemented with 5% ß-mercaptoethanol. The mixture was then boiled at 100 °C for 5min to denature the proteins and prepare them for western blotting. Denatured lysates were stored at -20 °C, whereas non-denatured protein extractions were kept at -80°C.

3.9.4 Polyacrylamide Gel Electrophoresis (PAGE) Assay

To separate the denatured proteins according to their molecular weights, polyacrylamide gel electrophoresis (PAGE) was performed. The resolving gel buffer (1.5 M Tris-HCl, pH 8.8) was used to make a 10% resolving polyacrylamide gel by adding 30% Bis-Acrylamide and 10% SDS. 10% APS and TEMED were added as catalyzers to start the polymerization of the resolving gel. The samples were loaded onto the stacking gel which was made with stacking buffer (0.5M Tris-HCl, pH 6.8) with 30% Bis-Acrylamide and 10% SDS. Again, 10% APS and TEMED was added before pouring the gel on top of the resolving gel to make a 4% stacking polyacrylamide gel. Protein electrophoresis was carried out at 175 V in 1X running buffer for about 50 minutes.

Protein Detection by Western blot

The separated proteins on the gel were then electroblotted to 0.45μm PVDF (Polyvinylidene fluoride) membranes (Amersham, Pharmacia Biotech, USA) at 0.35 Amps constant for 1 hour. The membrane was then blocked with 5% skim milk in 1X PBS-T (0. 1% Tween 20) (Acros, New Jersey, USA) for 1 hour at room temperature, or overnight at 4ºC on a platform shaker. Membranes were probed first with primary monoclonal antibody (e.g. anti-human prion protein antibody (3F4) or anti-eGFP) diluted in 5% skimmed milk for 1 hour, or overnight at 4ºC. After staining with the primary monoclonal antibody, the membranes were washed three times in 1X PBS-T for 10 minutes to remove excess antibodies that are attached non-specifically to the membrane. The membranes were then stained with the secondary HRP-conjugated antibody (e.g. Anti-mouse antibody-IgG-HRP 1:3000) diluted in 5% skim milk for one hour, followed by washing three times with 1X PBS-T buffer and then in PBS pH7.4. The anti-FLAG antibody used in this study is a mouse monoclonal antibody for FLAG epitope

conjugated with HRP (Anti-FLAG ®M2, Sigma) therefore only one immuno blotting was applied.

The ECL (enhanced chemiluminescence) Plus Western blotting system (Amersham™ ECL™, GE Healthcare, Buckinghamshire, UK) was used to reveal the proteins. With this method, enzymatic conversion of a luminol-like molecule was performed by the horseradish peroxidase (HRP) conjugated antibody (1mg/ml) (Abcam, Cambridge, Massachusetts, USA), which then generates light in the presence of hydrogen peroxide and the emission is amplified by an enhancer. The emitted light peaks in about 5–20 minutes and decays slowly thereafter. By exposing the membranes to a film, dark bands will indicate the proteins of interest. For reprobing the western blot membranes with different antibodies, the primary and secondary antibodies attached to the western blot membranes have to be removed by using a mild stripping buffer solution (1.5 %Glycine, 0.1% SDS, 1% Tween 20 adjusted pH to 2.2).

3.10 Imaging Methods

3.10.1 Epi Fluorescent Imaging

In order to visualize the fluorescent fusion and reporter proteins expressed in transfected cells or in target cells after infection, different microscopy methods were used.

3.10.1.1 Fluorescence Microscopy on Live Cells

Transfected cells that express fluorescent fusion proteins were analyzed directly in the plastic culture plates after 24 or 48 hours post transfection by a Zeiss Axio Observer Inverted, D1 Epi-fluorescence microscope which has 10x, 40x, and 40x (oil) Neofluor objectives with 0.3,

0.6 and 1.3 apertures (NA) respectively. Images were acquired and analyzed using the AxioVision LE program (Carl Zeiss, released version 4.8.2.0).

3.10.1.2 Fluorescence Microscopy on Fixed Cells

Cover slips placed at the bottom of 6-well or 12-well plates, or glass-bottom cell culture chamber slides (Becton, Dickinson Company) were coated with poly-L-Lysine diluted in ddH2O (1:10) from the stock solution, 0.1% (w/v in water) (Electron Microscopy Sciences, PA, USA). Poly-L-Lysine creates a positive charge on the glass, allowing for increased adhesion between the cells and the surface. This also prevents cells from detachment during washing. A volume of 0.5 ml of a 0.1mg/ml solution poly-L-lysine to coat a $25cm^2$ dish surface was found to be ideal. NIH 3T3 and N2a cells are strongly adhesive, compared to 293T cells. Pre-treatment with poly-L-Lysine is recommended for 293T cells. Cells attached to glass-bottom wells or to coverslips were washed 3 times with 500µl PBS and were then fixed using 4% formaldehyde (in PBS, pH 7.4) for 10 minutes at room temperature (RT) or 30 minutes at 4°C. For fluorescence microscopy, 50% glycerol in PBS was used to cover the cells and then the coverslip was inverted over a glass slide. The coverslips then sealed to the slides with nail polish to protect the cells from drying out. For nuclei staining, commercial mounting solution, Fluoromount-G ™ with Dapi (Electron Microscopy Science, USA), was used.

3.10.2 *In situ* Immunostaining

In situ immunostaining was performed on 293T or N2a cells that were transfected in glass chamber slides. One day before transfection, roughly 50,000 cells were seeded in each chamber that had been previously treated with Poly-L-lysine. The cells were fixed with formaldehyde 24 hours after transfection. Cell membranes were then permeabilized using a mild detergent

solution, 0.5% Triton X-100 in PBS (pH 7.4). Cells were covered for less than 5 minutes at room temperature with buffer, which causes small holes to form in the cell membranes, thereby allowing antibodies to enter the cells. To avoid non-specific antigen-antibody attachment, all buffers including PBS, Triton X-100, primary and secondary antibodies mixes, all contained 1% FBS (fetal bovine serum) or BSA (bovine serum albumin). After permeabilization, the cells were covered with a 5% FBS or BSA blocking solution for 15 minutes at room temperature (RT). To remove the detergent, cells were then washed three times with PBS containing 1% FBS. The anti-prion monoclonal antibody (3F4) (COVANCE, USA) was then applied at a concentration of 1:500 in PBS with 1% FBS for 60 minutes at room temperature (RT). After three washes, the secondary antibody, Alexa Fluor ® 488 was used at a dilution of 1:1000 for 60 minutes at RT in a dark box. The fluorochrome Alexa 488 has an excitation at 495nm and emission at 519nm.

3.10.3 Confocal Microscopy Imaging

Confocal laser scanning microscopy (LSM) provides high-resolution images for fluorescence microscopy. A LSM is conceptually analogous to a modified light microscope supplemented by a laser module that serves as a light source, and a scanning head (attached to the microscope stand) that is used to detect the signal (Introduction for Confocal Laser Scanning Microscopy-Carl Zeiss). The confocal microscope LSM 510 (Carl Zeiss, Germany) was used to obtain high-resolution images to visualize intracellular details. As well, confocal microscopy can provide multifluorescence images by having multi-channel detectors. Transfected and infected live cells inside glass bottom cell culture dishes or fixed cells on glass slides were analyzed by LSM confocal microscopy. The oil objectives at 40X with 1.3 NA and 63X with 1.4 NA were used.

3.10.3.1 Confocal Microscopy on Live Cells

Glass bottom cell culture dishes (Cellview ^{TM}, Greiner bio-one, USA) with one or four compartments were used for seeding 293T, NIH 3T3 and N2a cells for live cell confocal microscopy. For increased attachment of 293T cells, the glass bottom of the dish was pre-coated with 0.5 ml poly L-lysine (0.01%) for 5 minutes at RT. The solution was then removed and left to dry for another 5 minutes. Cells were seeded at 2.0x 10^5 in the "Cellview" dish with one compartment. For 4 compartment dishes, 50 000 cells were seeded in each well. Clear media without phenol red (HyClone Thermo Scientific) was added to the cells to prevent auto-fluorescence. The next day, when the cell confluence had reached 30-40%, the cells were transfected. After 24 hours post-transfection, reporter protein expression was visualized in live cells by confocal microscopy. Cells were visualized while in a closed chamber with a CO2 controller (5%) and heat (37^{o}C), which provided stable and favorable conditions for the cells during the imaging process. The LSM 5 Pascal software (Carl Zeiss, Germany) was used for producing and capturing the images by choosing specific channels related the particular fluorochrome that was expressed.

3.10.3.2 Confocal Microscopy on Fixed Cells

Slides were prepared as detailed above in the *in situ* fluorescence microscopy section. Four-chamber slides were used for seeding 293T, N2a and NIH 3T3 cells (50 000 cells/chamber) a day before transfection. After 24 to 48 hours, depending on protein expression, the cells were fixed with 4% formaldehyde and the chamber was removed. The fixed cells were mounted for direct imaging with the LSM 510 confocal microscope.

3.11 Cell Viability Assays

Dynamin-inhibiting drugs such as Dynasore can inhibit the endocytosis of retroviruses by susceptible target cells (Miyauchi et al., 2009, Permanyer et al., 2010). Studies have indicated that Dynasore hydrate (Sigma-Aldrich, Canada) (80 μM) caused a 90% inhibition of retrovirus uptake in target cells by blocking the function of dynamin and thereby preventing fusion between the virus envelope and the membrane of the endocytic compartments when cells were pre-treated for one hour at 37°C before infection. To determine the toxicity of Dynasore hydrate and the appropriate concentration to use on 293T cells, a 1000 μM stock solution of dynasore in DMSO (Dimethyl sulphate, Hybri-Max ® sterile, Sigma) was made and diluted with cell medium, DMEM 2% FBS, to various concentrations (e.g. 0 μM, 12.5 μM, 25 μM, 50 μM, 80 μM and 100 μM). For all concentrations of Dynasore, DMSO in cell medium did not exceed 0.1% in order to prevent DMSO toxicity. As a negative control, cells were treated by different concentrations of DMSO (e.g. 0%, 0.125%, 0.25%, 0.5%, 0.8% and 0.1%) to determine the effectiveness of dynasore on the cells. After treatment for 48 hours, 293T cells were washed once with PBS and resuspended in 0.5 ml PBS. A cell viability test was performed by using 0.4% trypan blue (Gibco-BRL). To distinguish dead from live cells, an equal volume of cell suspension (e.g. 20 μl) was mixed to trypan blue (e.g. 20 μl) and was incubated for about 1 minute at room temperature. Live cells do not take-up the dye, while dead cells do. To count stained cells (non-viable) from unstained cells (viable), a drop of the trypan blue/cell mixture was applied to a hemocytometer and a light microscope was used for the count. Cell viability was calculated as follows:

$$\text{Viable cells (\%)} = \frac{\text{Total number of viable cells per ml of aliquot}}{\text{Total number of cells per ml of aliquot}} \times 100$$

3.12 Viral Integration Inhibition Assay

A retroviral integrase inhibitor will prevent the integration of retroviral dsDNA into cellular genomic DNA. In order to determine the proper concentration of Raltegravir, an integrase inhibitor (Obtained from the AIDS Research and Reference Reagent Program, Merck & Company, Inc), different concentrations of this drug were made by diluting the stock solution (100 μM) in DMEM, 2% FBS. NIH 3T3 cells were used as target cells for these infectivity assays using MLV (eGFP). When the cells were 50-60% confluent in the 6-well plate, the drug was added at different concentrations (0 nM, 25 nM, 50nM, 100 nM, 300 nM and 500 nM) for three hours prior to infection using MLV (eGFP). The infectious particles were produced in 293T cells by co-transfection and purified as described earlier. Pre-treated NIH 3T3 cells were then infected with 0.3ml of the virus-containing supernatant and cultured for 24 hours. The virus-encoded eGFP transgene will only be expressed in cells where the virus has successfully integrated. Infected cells were analyzed by flow cytometry in order to determine the appropriate concentration of Raltegravir that inhibits integration as measured by eGFP expression.

4.0 RESULTS

4.1 Prion Protein Expression Vectors

In order to examine the relationship between prions and retroviruses, several vectors expressing the human and mouse prion protein were made. Some of these contain the eGFP reporter gene to allow detection and to track the proteins of interest in the *in vitro* infection systems. The human cellular prion gene was cloned from peripheral blood mononuclear cells (PBMC). The cloning primers included additional DNA sequences such as restriction endonuclease (RE) sites and the FLAG-tag epitope to generate internally labeled fusion prion proteins (See Materials and Methods). This was necessary since the N-terminal and the C-terminal of the prion protein are cleaved during post-translational processing. To clone an internal eGFP reporter gene, I used the newly generated EcoRI restriction site created during cloning. I created the EcoRI site in a region of the protein that is predicted to have little impact on protein folding, trafficking and cell localization according to structural and functional studies (Riley et al., 2002, Gauczynski et al., 2002). For the constructs expressing the human cellular prion ($hPrP^{C}$), the EcoRI site was inserted between amino acids 227 and 228 at the end of α helix 3(H3) and before the GPI signal peptide at the C-terminal end of the protein. The plasmid structures of hPrP-EcoRI, hPrP-FLAG and hPrP-eGFP are shown in Figures 4.1, 4.2 and 4.3, respectively.

Figure 4.1 Schematic representation of the hPrP expression vector with an internal EcoRI site (between bases)

The peGFP-N1 plasmid was used as the cloning vector. It contains the CMV promoter and Neomycin/Kanamycin resistance gene. The recombinant human prion protein expressed from this vector has an EcoRI (GAATTC) site between bases 1284-1289 of the plasmid.

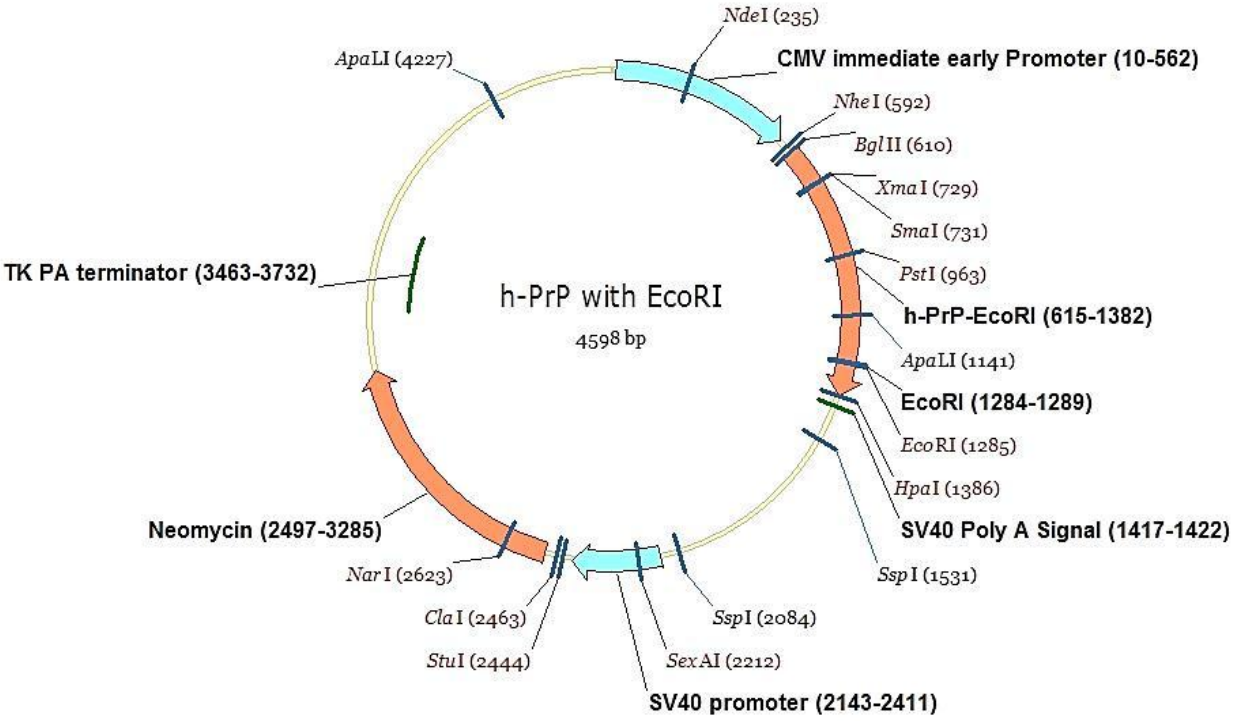

h-PrP with EcoRI
4598 bp
NdeI (235)
CMV immediate early Promoter (10-562)
NheI (592)
BglII (610)
XmaI (729)
SmaI (731)
PstI (963)
h-PrP-EcoRI (615-1382)
ApaLI (1141)
EcoRI (1284-1289)
EcoRI (1285)
HpaI (1386)
SV40 Poly A Signal (1417-1422)
SspI (1531)
SspI (2084)
SexAI (2212)
SV40 promoter (2143-2411)
StuI (2444)
ClaI (2463)
NarI (2623)
Neomycin (2497-3285)
TK PA terminator (3463-3732)
ApaLI (4227)

Figure 4.2 Schematic representation of the hPrP expression vector with an internal FLAG epitope tag (between bases 1284-1316)

The peGFP-N1 plasmid was used as the cloning vector. It contains the CMV promoter and Neomycin/Kanamycin resistance gene. The recombinant human prion protein expressed from this vector has a FLAG sequence between amino acids 227-228 of the molecule.

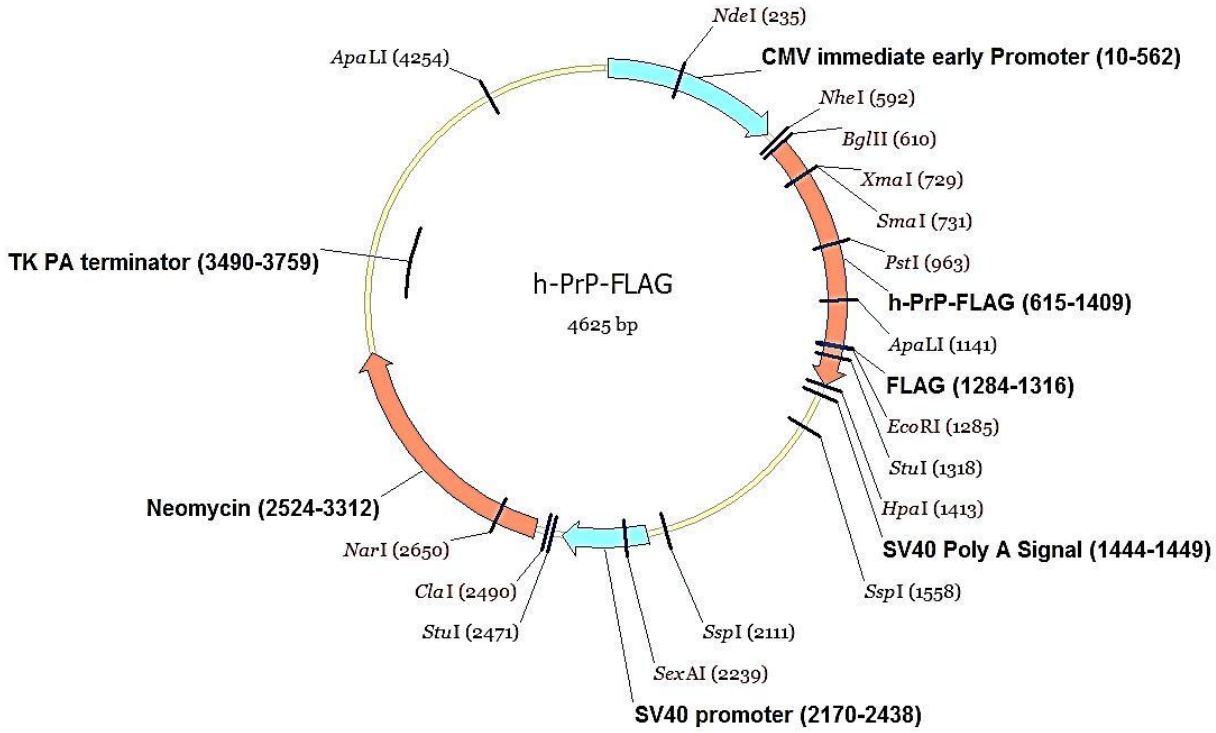
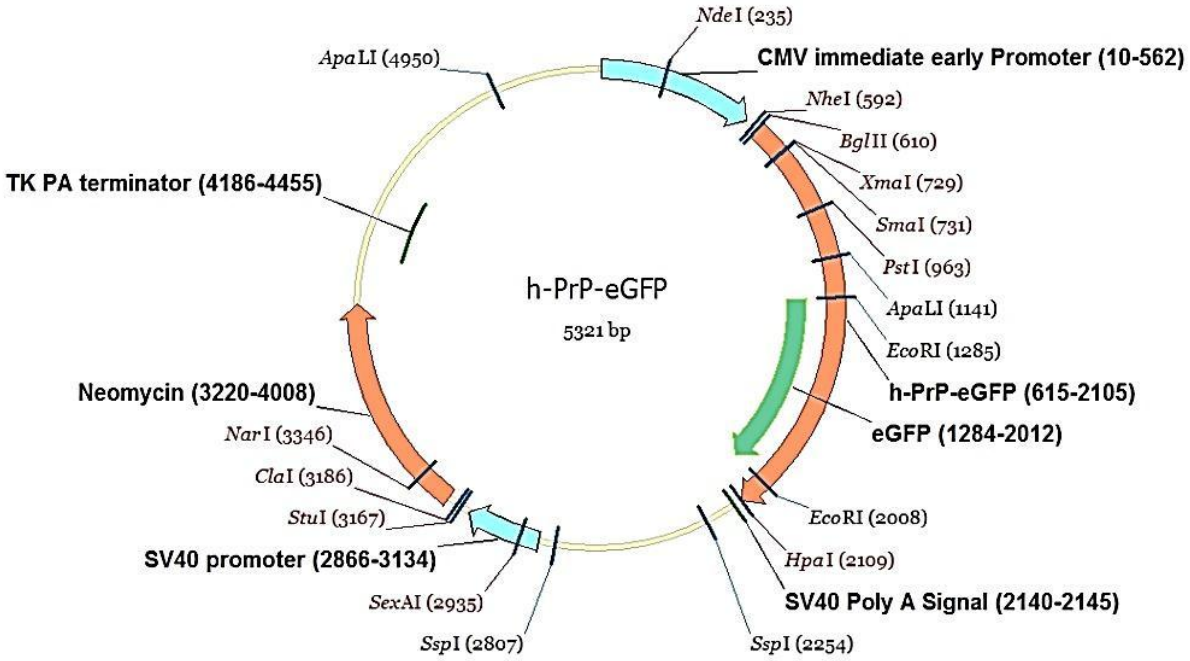

NdeI (235)
CMV immediate early Promoter (10-562)
NheI (592)
BglII (610)
XmaI (729)
SmaI (731)
PstI (963)
h-PrP-FLAG (615-1409)
ApaLI (1141)
FLAG (1284-1316)
EcoRI (1285)
StuI (1318)
HpaI (1413)
SV40 Poly A Signal (1444-1449)
SspI (1558)
SspI (2111)
SexAI (2239)
SV40 promoter (2170-2438)
StuI (2471)
ClaI (2490)
NarI (2650)
Neomycin (2524-3312)
TK PA terminator (3490-3759)
ApaLI (4254)
h-PrP-FLAG
4625 bp

Figure 4.3 Schematic representation of the hPrP-eGFP expression vector with an internal eGFP reporter sequences (between bases 1284-2012)

The peGFP-N1 plasmid was used as the cloning vector. It contains the CMV promoter and Neomycin/Kanamycin resistance gene. The recombinant human prion protein expressed from this vector has internal EcoRI-eGFP reporter protein between amino acids 227-228 of the molecule.


NdeI (235)
CMV immediate early Promoter (10-562)
NheI (592)
BglII (610)
XmaI (729)
SmaI (731)
PstI (963)
ApaLI (1141)
EcoRI (1285)
h-PrP-eGFP (615-2105)
eGFP (1284-2012)
EcoRI (2008)
HpaI (2109)
SV40 Poly A Signal (2140-2145)
SspI (2254)
SspI (2807)
SexAI (2935)
SV40 promoter (2866-3134)
StuI (3167)
ClaI (3186)
NarI (3346)
Neomycin (3220-4008)
TK PA terminator (4186-4455)
ApaLI (4950)
h-PrP-eGFP
5321 bp

4.2 Analysis of Prion Protein Expression

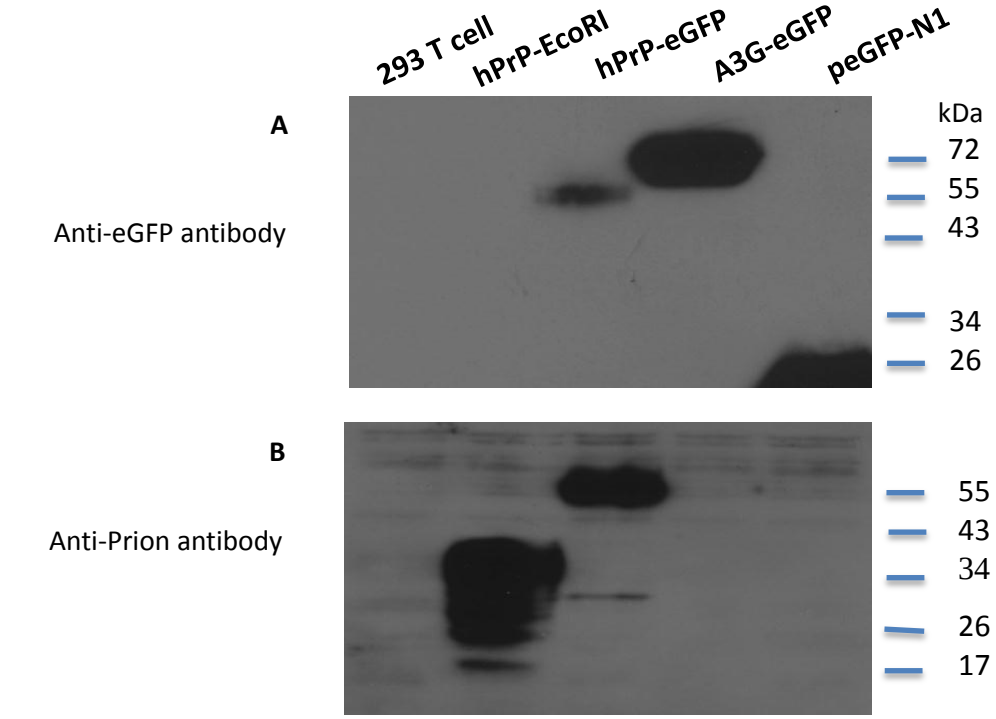
4.2.1 Expression of Recombinant hPrP-EcoRI and hPrP-eGFP

Forty eight hours after transfection of the constructs, 293T cells were harvested and protein lysates were prepared and analysed by Western blot (WB). The 3F4 monoclonal antibody was used to detect the human prion protein and monoclonal antibodies were used to detect the internal eGFP reporter. The experiments show the detection of the hPrP-eGFP fusion protein and the A3G-eGFP control using anti-eGFP antibody (Fig. 4.4A). The 3F4 antibody detected the expression of both the hPrP-eGFP fusion protein and the untagged hPrP-EcoR1 protein (Fig. 4.4B). The hPrP-eGFP fusion protein was also detected by fluorescence microscopy using a 10X objective (Fig. 4.4 D).

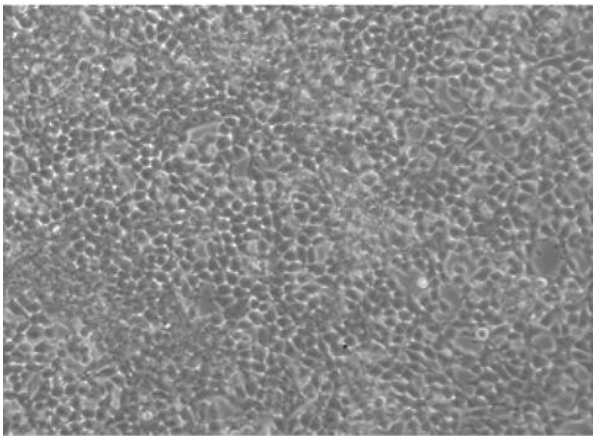
The expected molecular weight for hPrP-EcoRI is ~21 kDa for the non-glycosylated form and 27 kDa for the mono- and 34 kDa for di-glycosylated forms. Glycosylation of human prion proteins is achieved through two stages of glycosylation that happens in the endoplasmic reticulum and in the Golgi complex (Capellari et al., 1999, De Keukeleire et al., 2007). The expected molecular weight for hPrP-eGFP is approximately 27 kDa more because of the insertion of the eGFP transgene; therefore the bands appear at ~47, 54 and 61 kDa. The control proteins, A3G-eGFP (~73kDa) and peGFP-N1 (~27kDa), were also detected as shown in Fig 4.4 (Panel A and B).

Figure 4.4 Detection of overexpressed recombinant prion proteins

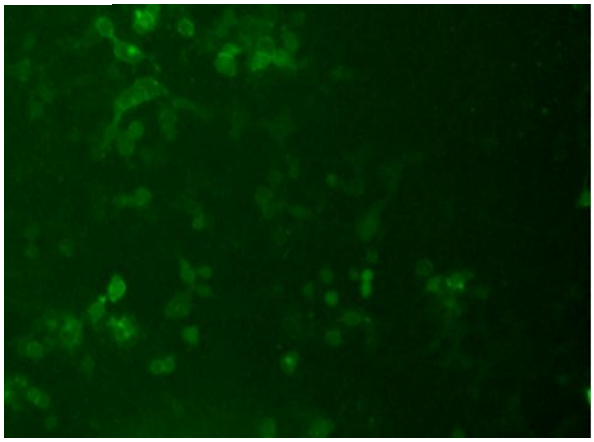
Cell lysates were prepared from transfected 293T cells and loaded on gel in this sequence; untransfected 293T cells, hPrP-EcoRI, hPrP-eGFP, A3G-eGFP, and eGFP-N1. Panel A and B is the same blot that was probed, stripped and re-probed with a different antibody. **A)** Immunoblotting was carried out with a mouse monoclonal anti-eGFP antibody (1:3000) and a secondary anti-mouse–HRP antibody for detection. **B)** Immunoblotting was performed using a mouse monoclonal anti-prion antibody (3F4) (1:2000) as primary and an anti-mouse–HRP as secondary antibody (1:3000). Molecular weights are in kDa. Panel C and D represent 293T cells transfected with the Prion-eGFP expression vector. Live cells were analysed by fluorescence microscopy using a 10X objective. **C)** Phase contrast of hPrP-eGFP transfected cells. **D)** Fluorescent imaging of over-expressed hPrP-eGFP in 293T cells.


293 T cell
hPrP-EcoRI
hPrP-eGFP
A3G-eGFP
peGFP-N1
A
Anti-eGFP antibody
kDa
72
55
43
34
26
B
Anti-Prion antibody
55
43
34
26
17

C



D

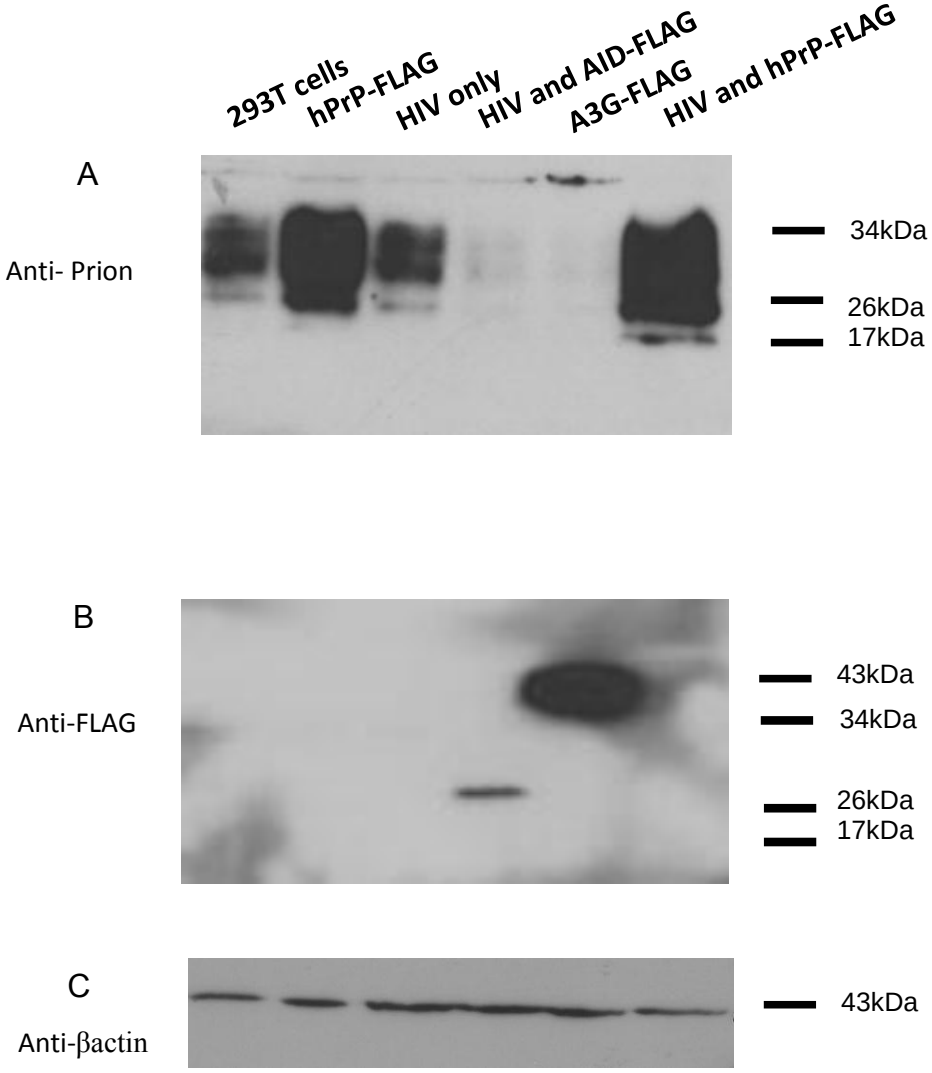


4.2.2 Co-Expression of Recombinant hPrP-FLAG with HIV

To verify that there aren't unexpected consequences of overexpressing human prion proteins and retroviral proteins in the same cells, I performed the co-transfection of hPrP-FLAG and HIV expression plasmids in 293T. The expression of hPrP-FLAG in virus producer cells was detected by two different immunoblotting strategies: Cellular prion protein and hPrP-FLAG with multiple bands (~ 17-34 kDa) were detected using an anti-prion antibody (3F4) followed by an anti-mouse-HRP and (Panel A in Fig 4.5). After stripping the membrane; an anti–FLAG antibody-HRP (M2 antibody, 1:3000) was used to detect the FLAG epitope tag within the fusion protein. The monoclonal anti–FLAG antibody (M2) from Sigma is not calcium-dependent and has been designed to work on FLAG epitopes even in solutions containing the EDTA chelating reagent, as is the case in the SDS lysis buffer used to prepare the cell extracts. Moreover, according to the FLAG-M2 antibody technical information, this antibody should be able to detect internal FLAG epitopes as well as N- or C- terminal FLAG fusions. But for an unknown reason, I was not able to detect the internal FLAG epitope in my hPrP-FLAG fusion proteins, while the anti-prion antibody (3F4) clearly shows the presence of the prion protein (Panel B in Fig 4.5). The anti-FLAG antibody did however detect the A3G-FLAG (~50 kDa) and AID-FLAG (~28kDa) controls and anti-β actin antibody was able to detect β actin protein (~43 kDa) in the cell lysates (Panel C, Fig. 4.5). Another interesting observation with this immunoblot is that there appears to be an up regulation of endogenous PrP^C levels when the cells are co-transfected with HIV-producing vectors. What is clear, however, is that HIV protein and the hPrP-Flag protein can be co-expressed by transfection in 293T cells.

Figure 4.5 Detection of overexpressed recombinant human prion proteins with or without HIV in co-transfected 293T cells

Cell lysates were loaded in this sequence: un-transfected 293T cells, hPrP-FLAG, HIV-1, AID-FLAG, A3G-FLAG and HIV with hPrP-FLAG. Panels A and B are the same blot that was reused with a different antibody after stripping. **A)** Immunoblot using an anti-prion antibody (3F4) as primary antibody (1:2000) and an anti-mouse–HRP antibody as secondary antibody (1:3000). **B)** Immunoblot using anti-FLAG-HRP (1:3000). **C)** Immunoblot using anti-β actin (1:3000)

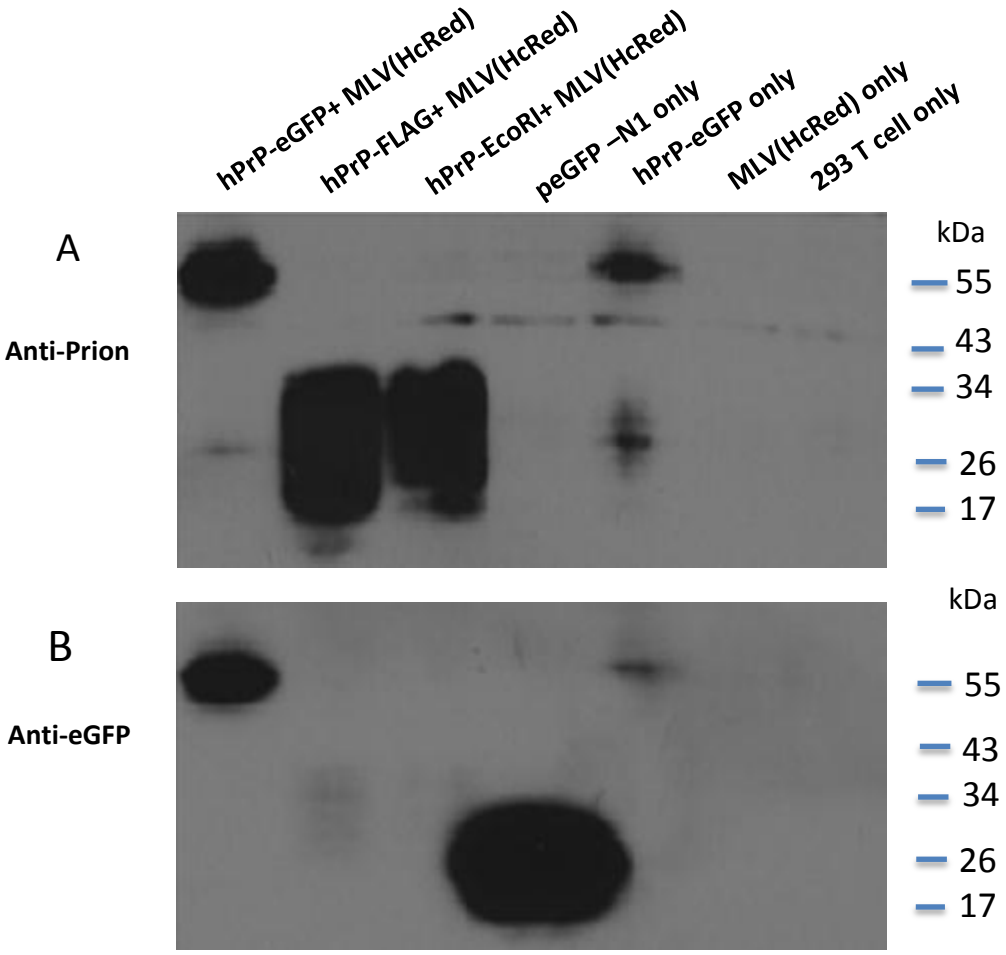

293T cells
hPrP-FLAG
HIV only
HIV and AID-FLAG
A3G-FLAG
HIV and hPrP-FLAG
A
Anti- Prion
34kDa
26kDa
17kDa
B
Anti-FLAG
43kDa
34kDa
26kDa
17kDa
C
Anti-βactin
43kDa

4.2.3 Co-Expression of Recombinant hPrP with MLV

I next wanted to assess the co-expression levels of the various recombinant prion proteins, hPrP-EcoRI, hPrP-FLAG and hPrP-eGFP, with MLV. Here I used the MLV molecular clone that expresses HcRed in infected cells, henceforth named MLV (HcRed) for simplicity (Fig 4.6). Cell lysates were run on SDS-PAGE and a WB was performed first with an anti-prion antibody (3F4, 1:2000) (Fig 4.6 panel A). The membrane was then stripped and re-blotted with an anti-eGFP antibody. The results are shown in panel B of figure 4.6, which demonstrates a weak band for the hPrP-eGFP fusion protein alone but all 3 PrP constructs appear to be well expressed when in presence of MLV (HcRed) (Fig 4.6). For unknown reasons, I was unable to detect endogenous prion proteins in WB of MLV (HcRed) only (panel A of figure 4.6) contrary to HIV only (panel A of figure 4.5), where I could clearly detect endogenous prions expression.

Figure 4.6 Detection of overexpressed recombinant human prion proteins with or without MLV (HcRed) in co-transfected 293T cells

Panels A and B are the same blot that was reused with a different antibody after stripping. Cell lysates were loaded in this sequence: hPrP-eGFP and MLV (HcRed), hPrP-FLAG and MLV (HcRed), hPrP-EcoRI and MLV (HcRed), peGFP-N1 only, hPrP-eGFP only; MLV (HcRed) only and untransfected 293T cell lysate. **A)** Immunoblot using an anti-prion antibody (3F4) as primary antibody (1:2000) and anti-mouse–HRP antibody as secondary antibody (1:3000) for detecting human prion proteins. **B)** Immunoblot using anti- eGFP antibody as the primary antibody (1:3000) and anti-mouse–HRP antibody as the secondary antibody (1:3000).


hPrP-eGFP+ MLV(HcRed)
hPrP-FLAG+ MLV(HcRed)
hPrP-EcoRI+ MLV(HcRed)
peGFP –N1 only
hPrP-eGFP only
MLV(HcRed) only
293 T cell only
A
Anti-Prion
kDa
55
43
34
26
17
B
Anti-eGFP
kDa
55
43
34
26
17

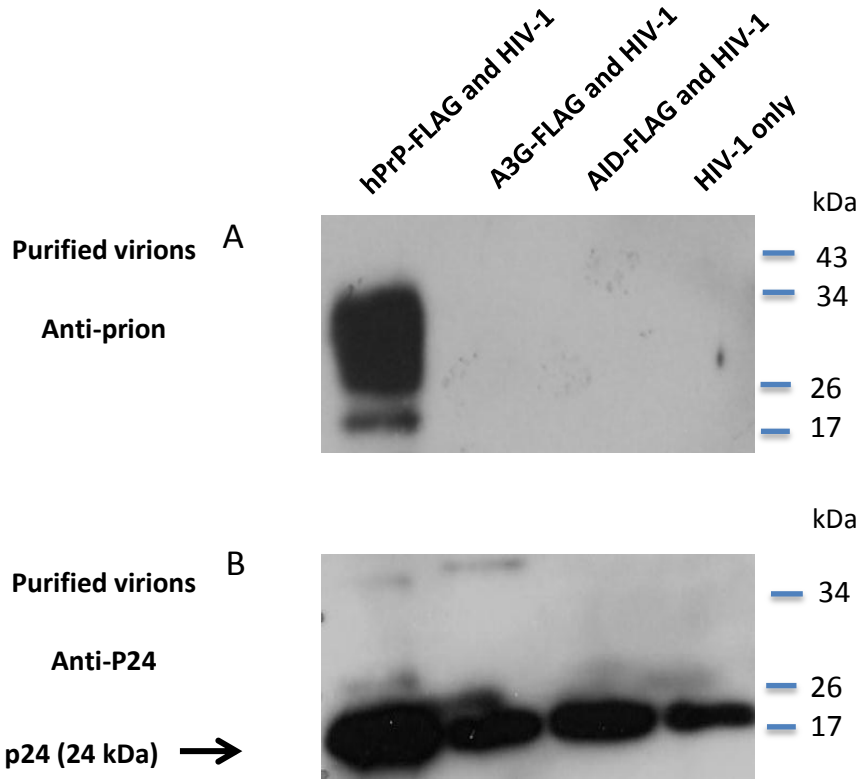
4.2.4 Potential Association of Recombinant hPrP with HIV virions

Here I investigated whether cell-free HIV virions associate with recombinant hPrP. 293T cells were co-transfected with HIV and hPrP-FLAG expression plasmids (Figure 4.7). Cell culture supernatants were harvested 48 hours after transfection, centrifuged at 2000 rpm for 5min and filtered through a micro filter (0.22μm) in order to remove cell debris. Viral particles were then pelleted by ultracentrifugation at 175000x g and the pellets were lysed in RIPA buffer (see Material and methods). The viral lysates were then analysed by WB using an anti-prion antibody (3F4) A strong band, spanning approximately between 17-34kDa, was detected for hPrP-FLAG associated with HIV particles.

Presence of viral particles was confirmed by stripping the membrane and re-staining with an anti-p24 antibody that recognized the p24 HIV capsid protein. Endogenous prion proteins expressed in 293T cells were not detectable in the viral particles (last lane). Although it may appear that hPrP-FLAG associates with HIV (first lane of Fig 4.7), but unfortunately this experiment lacked proper controls in the same WB assay to allow for clear conclusion to be drawn. First, I did not have a WB of the producer cells to show that the proteins were in fact being expressed. Second, I was missing an essential control, which was the expression of hPrP-FLAG alone without virus because it could very well be that hPrP-FLAG is excreted in the supernatant and is detectable in absence of viruses. Third, I did not perform Anti-FLAG antibody to ensure that the positive controls for FLAG TAG domain, A3G-FLAG and AID-FLAG constructs (see Material and method) that are known to associate with HIV are actually being detected in my experiment.

Figure 4.7 Potential associations of hPrP-FLAG with HIV virions

Western blot performed on lysates of HIV virions pelleted from the supernatant of co-transfected 293T cells. Purified virions were loaded from left to right in this sequence: HIV and hPrP-FLAG, HIV and A3G-FLAG, HIV and AID-FLAG, and HIV only. **A)** Immunoblotting was performed using an anti-prion antibody (3F4) as primary antibody (1:2000) and an anti-mouse–HRP antibody as secondary antibody (1:3000). **B)** After stripping the WB membrane, an immunoblot was performed using an anti-HIV Gag protein (p24) primary antibody (1:200) and anti-mouse-HRP antibody as secondary antibody (1:3000).


hPrP-FLAG and HIV-1
A3G-FLAG and HIV-1
AID-FLAG and HIV-1
HIV-1 only
kDa
A
Purified virions
Anti-prion
43
34
26
17
kDa
B
Purified virions
Anti-P24
p24 (24 kDa)
34
26
17

4.2.5 Potential Association of Recombinant hPrP with MLV virions

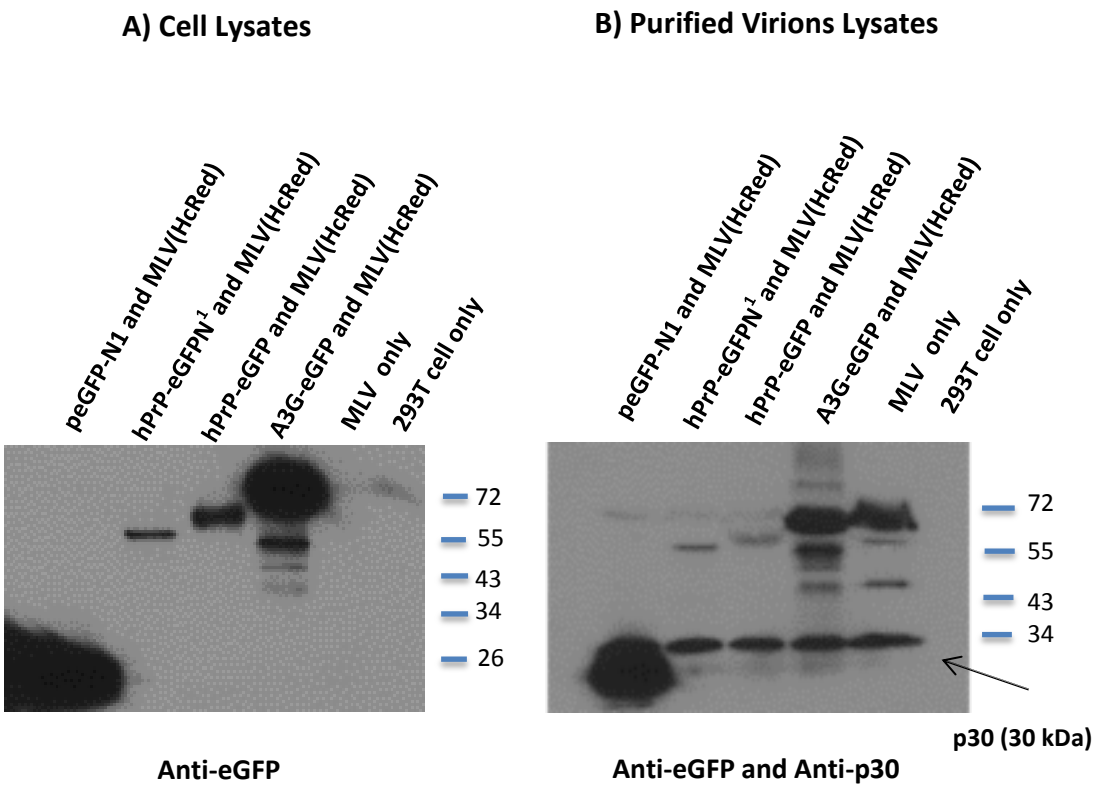
Here I attempted to analyze whether human prions associate with another class of retroviruses, murine leukemia viruses (MLVs). 293T cells were co-transfected with MLV (HcRed) and hPrP-FLAG expression plasmids (Figure 4.8). Cells and cell culture supernatants were harvested 48 hours after transfection and prepared as described in the materials and methods section. In this experiment, I also used hPrP-eGFPN[1], which is another hPrP construct that contains an N-terminal eGFP fusion. This recombinant protein did not display an appropriate cell distribution nor did it have the expected molecular weight on gel. This was due to post-translational proteolytic cleavage of the N-terminal signal peptide of the prion protein. Other than appearing in this figure, it was excluded from the study. Panel A of Figure 4.8 shows a WB using an anti-eGFP antibody performed on the lysates of co-transfected cells. Panel B shows a WB performed on purified MLV virions using simultaneously an anti-eGFP antibody and an anti-p30 antibody to detect the p30 viral capsid protein of MLV.

EGFP expressed from eGFP-N1, hPrP-eGFP variants (~47, 54 and 61 kDa) and the A3G-eGFP control (~73 kDa) could all be detected in the cell lysates (Figure 4.8, panel A). These same proteins could also be detected at varying intensities in virion lysates (Figure 4.8, panel B). Unfortunately, this experiment is again inconclusive for several reasons. First, as in the previous experiment with HIV (Figure 4.7), I am missing the crucial control of 293T cells transfected with the hPrP-eGFP expression plasmid in absence of MLV viral particles. Second, the cell lysates do not have a loading control such as β-actin or β-tubulin.

Figure 4.8 Potential association of hPrP-eGFP with MLV virions

Western blot assays performed on lysates of co-transfected 293T cells and on lysates of MLV particles pelleted from cell supernatants. Cell lysates are shown in panel A and MLV virions lysates in panel B. Lysate loading sequence is as follows: peGFP-N1 and MLV(HcRed), hPrP-eGFPN[1] and MLV(HcRed), hPrP-eGFP and MLV(HcRed), A3G-eGFP and MLV(HcRed), MLV(HcRed) only and non-infected 293 T. Immuno-blots in panels A and B were performed using an anti-eGFP antibody (1:2000) as primary antibody and an anti-mouse–HRP antibody as secondary antibody (1:3000). The immunoblot in panel B was additionally stained with an anti-MLV Gag protein (p30) as primary antibody (1:150) and anti-Rat-HRP antibody as secondary antibody (1:10000).Molecular weights are in kDa.

[1] An early protein construct of human prion protein with N-terminal eGFP which didn't have the proper size and cell localization due to cleavage at the N-terminal signal peptide


A) Cell Lysates
B) Purified Virions Lysates
peGFP-N1 and MLV(HcRed)
hPrP-eGFPN1 and MLV(HcRed)
hPrP-eGFP and MLV(HcRed)
A3G-eGFP and MLV(HcRed)
MLV only
293T cell only
72
55
43
34
26
p30 (30 kDa)
Anti-eGFP
Anti-eGFP and Anti-p30

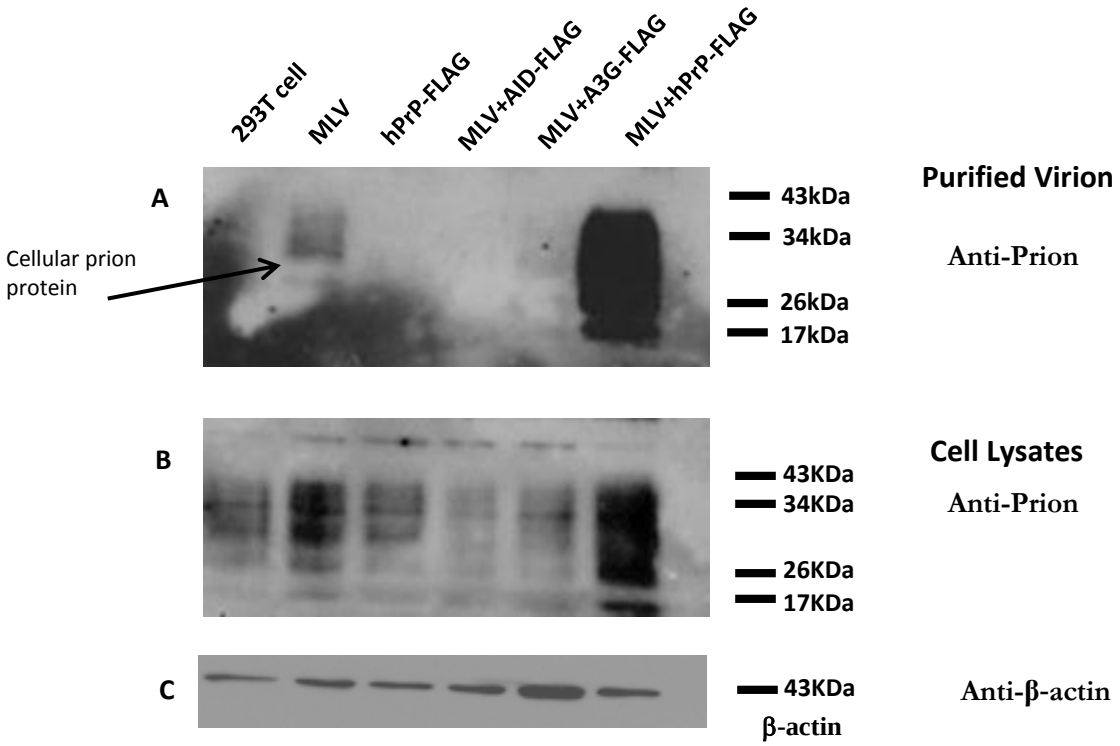
4.2.6 Confirmation of the Potential Association of Recombinant hPrP with MLV and HIV virions

Here I have attempted to confirm whether recombinant PrP can associate with MLV and HIV virions in repeat experiments that include the required controls. Figure 4.9 consists of the co-transfection of MLV (eGFP) and three different FLAG protein constructs (either hPrP-FLAG, AID-FLAG or A3G-FLAG) in 293T cells. As done previously, the virus-containing supernatants were collected and the virions were pelleted by ultracentrifugation. Virions and cell lysates were analysed by WB (Figure 4.9 panels A and B respectively). Immunoblots were performed with an anti-prion antibody (3F4) for both cell and purified virion lysates. Additionally, the blot for the cell lysates was striped and re-probed with a β-actin antibody (Figure 4.9, panel C).

As mentioned before, the anti-FLAG antibody was not able to detect the internal FLAG epitope tag in hPrP-FLAG construct; therefore I didn't use the anti-FLAG (M2) antibody for this experiment. Here I could detect endogenous PrP^C in all 293T cell lysates, but the expression of the recombinant hPrP-FLAG protein did not appear to be significantly more than that of controls (Figure 4.9, panel B). However can see that transfection of MLV appears to increase the expression levels of endogenous PrP^C and also that of hPrP-FLAG (Figure 4.9; panel B). Analysis of the purified virion lysates clearly shows the presence of hPrP-FLAG and also that of endogenous PrP^C with MLV particles (Figure 4.9, panel A) and hPrP-FLAG alone was not detected in the supernatant in absence of MLV, which argues in favour that prions do fact interact with MLV. This experiment still lacked important controls such as MLV p30 capsid detection and also the re-probing of both membranes for panels A and B with an anti-FLAG antibody to confirm that the A3G-FLAG and AID-FLAG control proteins were expressed and packaged into the MLV virions.

Figure 4.9 Association of hPrP-FLAG with MLV virions

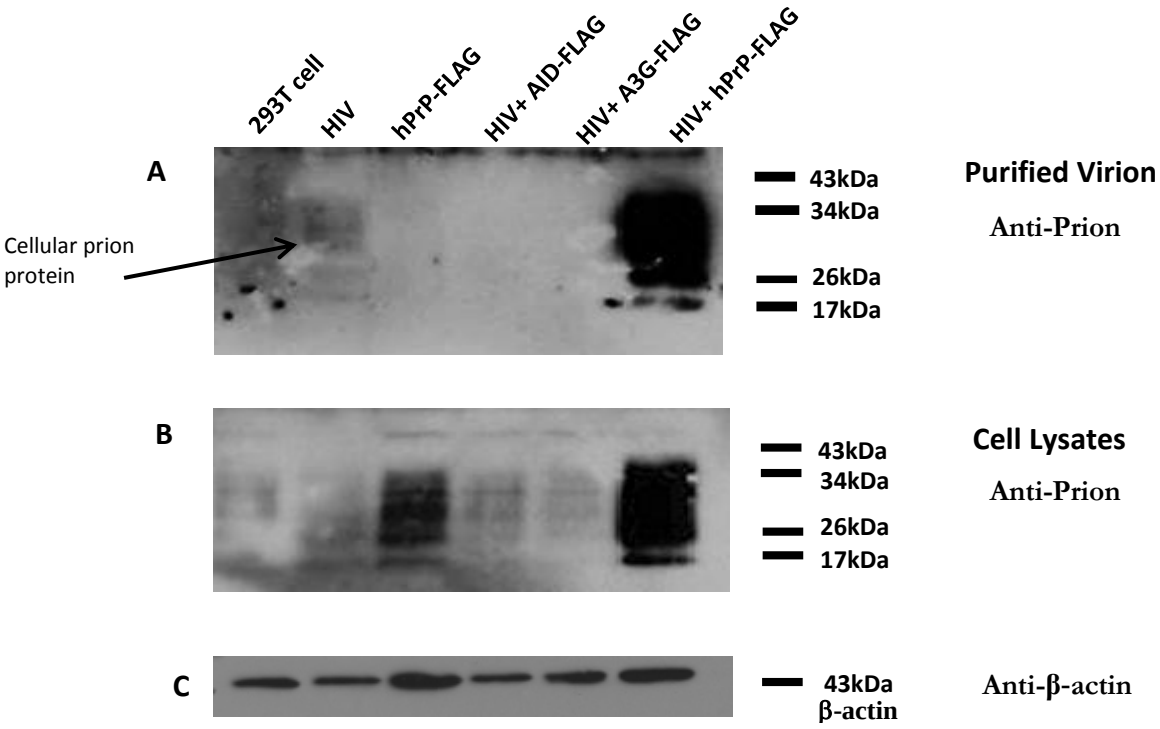
293T cells were co-transfected with MLV (eGFP) and hPrP-FLAG expression vectors and controls. Western blot analysis was performed on lysates of co-transfected 293T cells and on lysates of MLV particles pelleted from cell supernatants. MLV virions lysates are shown in panel A and cell lysates are shown in panel B. Immunoblots were carried out using an anti-prion antibody (3F4) (1:2000) followed by an anti-mouse-HRP antibody (1:3000) for detection. Panel C is an immunoblot using anti-β-actin-HRP (1:3000) performed on the cell lysate membrane of panel B.


293T cell
MLV
hPrP-FLAG
MLV+AID-FLAG
MLV+A3G-FLAG
MLV+hPrP-FLAG
A
Cellular prion protein
43kDa
34kDa
26kDa
17kDa
Purified Virion
Anti-Prion
B
43KDa
34KDa
26KDa
17KDa
Cell Lysates
Anti-Prion
C
43KDa
β-actin
Anti-β-actin

Next, I attempted to confirm whether recombinant PrP can associate with HIV virions in a similar fashion to MLV virions. Figure 4.10 was generated according to the same experiment scheme as that of Figure 4.9, with the exception that HIV (eGFP) expression plasmids were used instead of MLV (eGFP). This time, however, we can clearly see expression of the recombinant hPrP-FLAG protein in the cell lysates (Figure 4.10, Panel B). Also, a faint signal for the endogenous PrP^{C} protein can be detected in all lanes of the cell lysates. Purified virions lysates show a very strong signal for hPrP-FLAG produced alongside HIV, and also a weak signal in HIV viral particle only, which could be attributable to the detection of endogenous PrP^{C} associated with HIV particles (Panel A). Although the results of this figure are in full agreement with the results presented in Figure 4.9, this experiment suffers from the same deficiencies as the latter. The immuno-blot was not probed with anti-FLAG antibody to confirm the packaging of the protein controls, or with anti-p24 to detect for the presence of the HIV capsid protein. WB assay results of figures 4.9 and 4.10 showed that prion proteins are detected by anti-prion antibody (3F4) in purified virion lysates in association with retroviruses however human prion protein construct without retroviruses are not identified the same assay. Altogether these results, suggest despite the missing controls, that both recombinant and endogenous prions can co-sediment with retroviral particles.

Figure 4.10 Association of hPrP-FLAG with HIV virions

293T cells were co-transfected with HIV (eGFP) and hPrP-FLAG expression vectors and controls. Western blot analysis was performed on lysates of co-transfected 293T cells and on lysates of HIV particles pelleted from cell supernatants. HIV virions lysates are shown in panel A and cell lysates are shown in panel B. Immunoblots were carried out using an anti-prion antibody (3F4) (1:2000) followed by an anti-mouse-HRP antibody (1:3000) for detection. Panel C is an immunoblot using anti-β-actin-HRP (1:3000) performed on the cell lysate membrane of panel B.


293T cell
HIV
hPrP-FLAG
HIV+ AID-FLAG
HIV+ A3G-FLAG
HIV+ hPrP-FLAG
A
Cellular prion protein
43kDa
34kDa
26kDa
17kDa
Purified Virion
Anti-Prion
B
43kDa
34kDa
26kDa
17kDa
Cell Lysates
Anti-Prion
C
43kDa
β-actin
Anti-β-actin

4.3 Fluorescence Microscopy Imaging

Preliminary results presented in the previous sections indicate that the recombinant hPrP-eGFP protein is well expressed and appears to associate with MLV and HIV pseudoviruses. An important component of this project is not only to show that prions and retroviral virions associate, but most importantly, to show that prions are delivered to target cells. This is an essential requirement for positing that retroviruses could act as vectors for prion dissemination. In this section, I have attempted to design fluorescence microscopy approaches to visualize the uptake of fluorescent prion proteins (hPrP-eGFP) by retroviruses and track their delivery into target cells.

4.3.1 Fluorescence Microscopy Imaging on Live Cells

In this section, I set out to confirm that the eGFP reporter inserted within the hPrP coding sequence was being expressed. Some of the retroviruses used in the imaging experiments express the far-red fluorescent protein HcRed. HcRed is expressed from the 5'LTR viral promoter of the MLV (HcRed) molecular clone. It can therefore be expressed from the plasmid itself in transfected cells or following proviral integration in target cells. HcRed is not expressed however at measurable levels in these imaging assays from unintegrated proviral DNA following viral infection.

Here I have conducted two experiments. First, I have transfected hPrP-eGFP, A3G-eGFP and MLV (HcRed) expression plasmids separately into 293T cells (Figure 4.11) and in the second I co-transfected 293T cells with hPrP-eGFP and MLV (HcRed) (Figure 4.12). Images of live cells

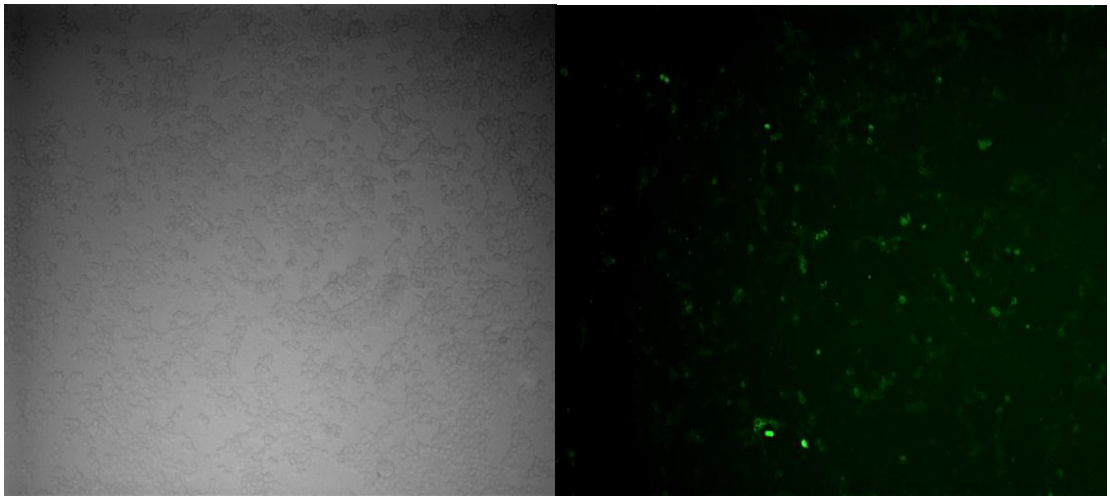
were taken directly through the bottom of a plastic 6-well plate using an inverted fluorescence microscope 48 hours after transfection or co-transfection. A first image was taken in phase contrast to visualize cell density; images were then taken to observe eGFP and HcRed fluorescence in the samples (see materials and methods).

Figure 4.11 Fluorescence microscopy images of transfected 293T cells using a 10X objective

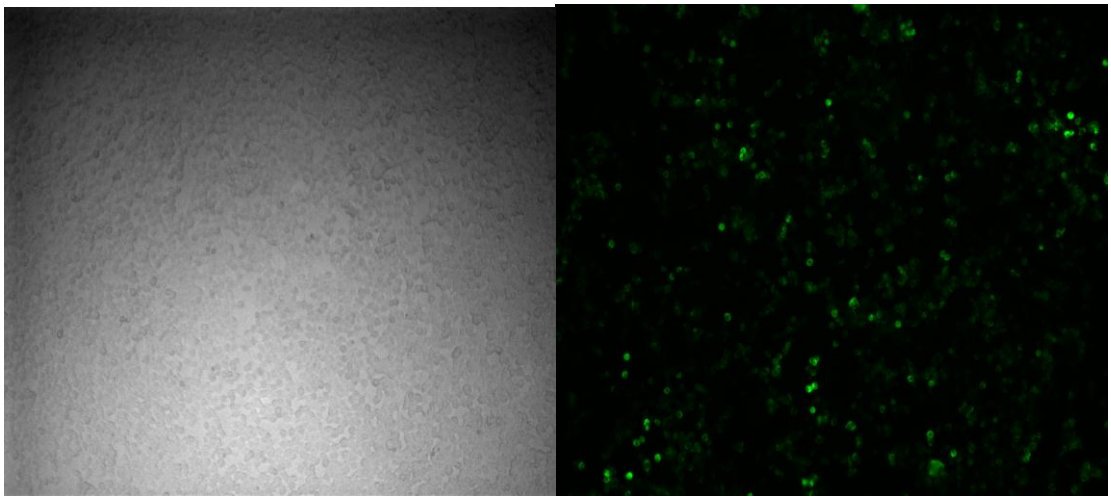
Cells were analysed directly through the bottom of 6-well plates 48 hours post transfection of the different expression plasmids: hPrP-eGFP (panel A), human A3G-eGFP (panel B) and MLV (HcRed) (panel C). An image was captured in phase contrast and using a fluorescent light source for eGFP and for HcRed.

Phase Contrast Fluorescent Images

A) hPrP-eGFP



B) A3G-eGFP



C) MLV (HcRed)

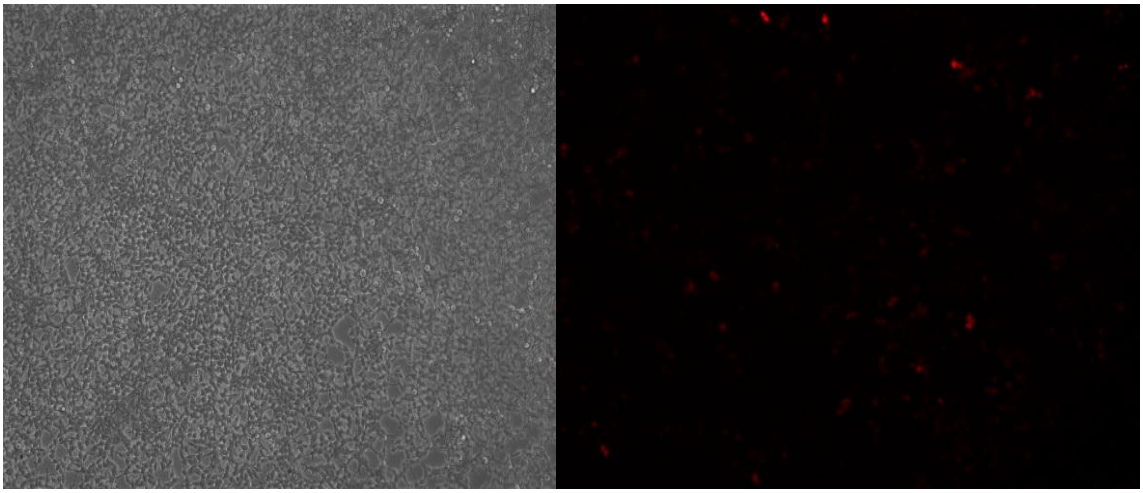
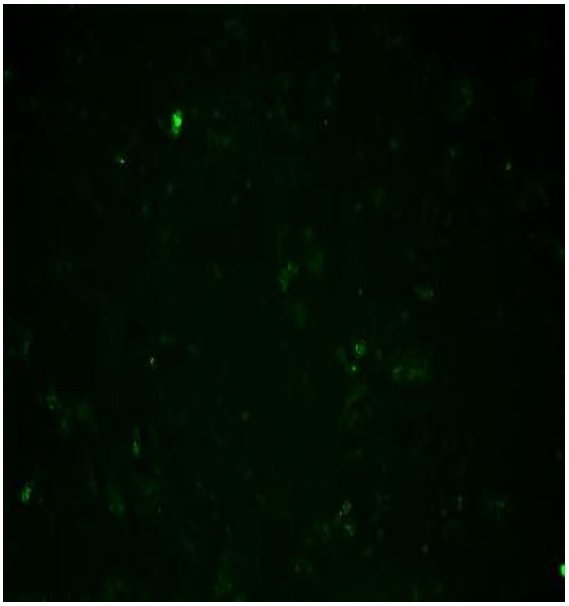


Figure 4.12 Fluorescence microscopy images of co-transfected 293T cells using a 10X objective

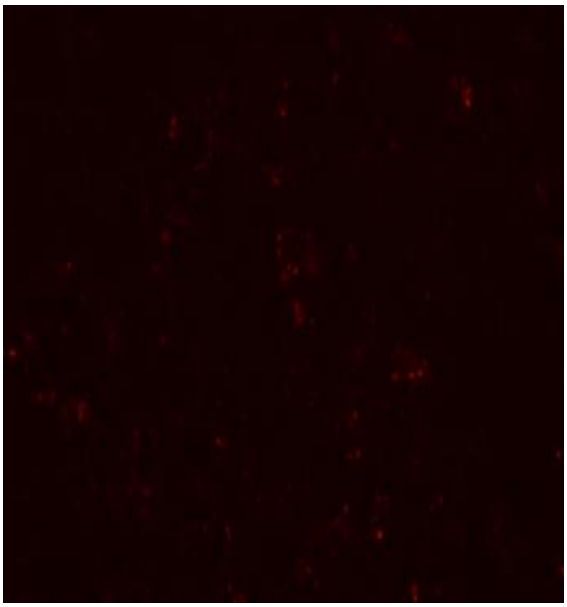
Cells were analysed directly through the bottom of 6-well plates 48 hours post co-transfection of hPrP-eGFP and MLV (HcRed). **A)** Phase contrast of 293T cells co-transfected with MLV (HcRed) and hPrP-eGFP. **B)** Epi fluorescent image of hPrP-eGFP expressing fluorescent eGFP-green light. **C)** Epi fluorescent image of MLV (HcRed) expressing red fluorescent light 48 hours after co-transfected with human prion recombinant in the same cells.

A) Phase contrast

B) eGFP (green) filter for hPrP-eGFP



C) Hc-Red (red) filter for MLV



4.3.2 Confocal Microscopy Imaging on Live Cells

In order to obtain better magnification and resolution for visualizing the various fluorescent proteins, imaging using a confocal microscope with a 40X oil-immersion objective was chosen for this part of the study. I also used glass-bottom culture dishes to increase the clarity of the images.

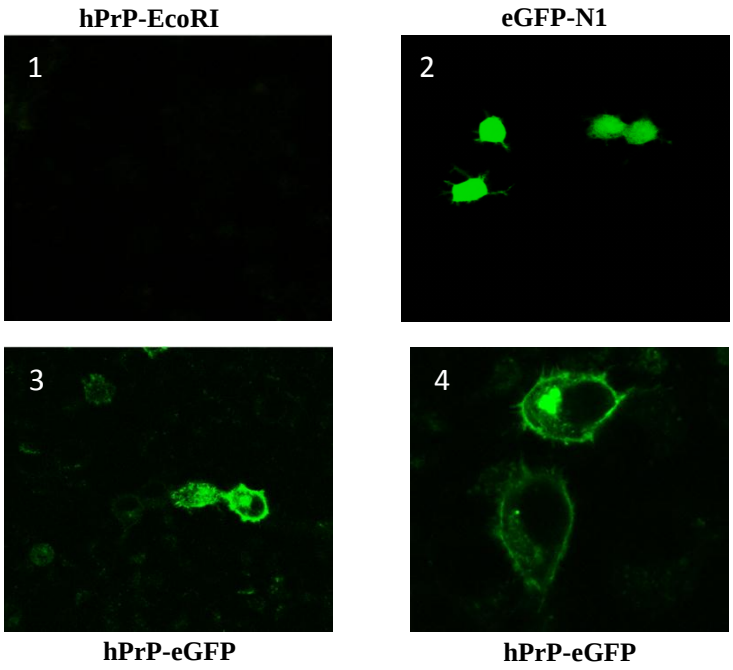
293T and N2a cells were transfected with hPrP-EcoRI, hPrP-eGFP and the control plasmid peGFP-N1. Figure 4.13 shows images of co-transfected N2a (Set A) and 293T cell lines (set B). Each set comprises 4 images: human prion-EcoRI (panel 1) and peGFP-N1 (panel 2), hPrP-eGFP (panels 3 and 4). Panel 4 represents a 10X digital magnification of panel 3 achieved using the analysis software.

These results show that the cellular localization of hPrP-eGFP in 293T and N2a cells is very similar (Figure 4.13, Set A and B, Panel 4). Human prion protein appears to localize to the cell membrane and it also accumulates in a dense cytoplasmic structure of unknown identity. Cell distribution of hPrP-eGFP is very different to the free eGFP protein that appears diffuse throughout the cell's cytoplasm.

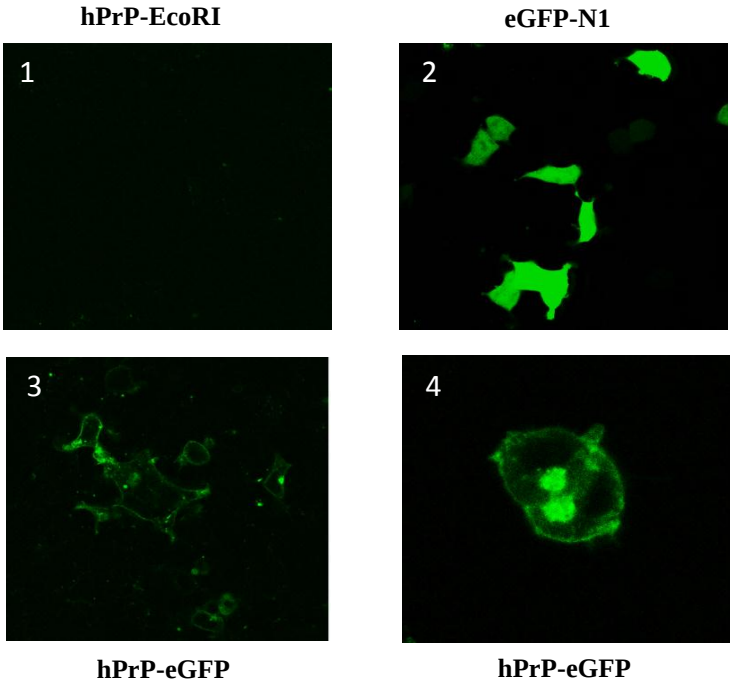
Figure 4.13 Live cell confocal microscopy imaging of hPrP-eGFP transfected cells

Images were taken 24 hours post-transfection in N2a (Set A: 1-4) and 293T (Set B: 1-4) cells. Panel 1: hPrP-EcoRI (without eGFP); panel 2: peGFP-N1 (as a positive control); panel 3: hPrP-eGFP; and panel 4: a 10X magnification of hPrP-eGFP. Images were taken using a 40X oil-immersion objective.

A) N2a cells



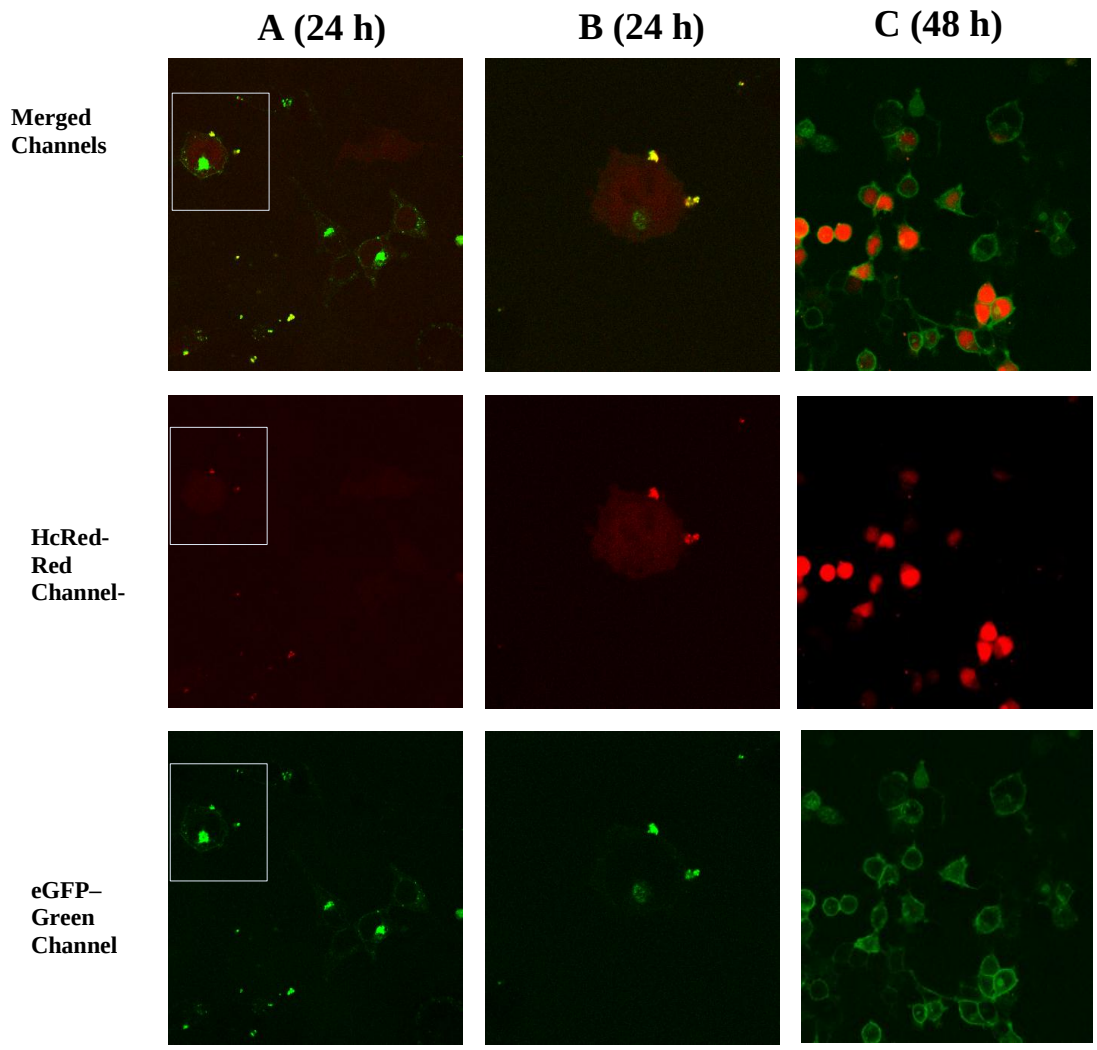
B) 293T cells



Next, I wanted to simultaneously visualize the intracellular expression of hPrP-eGFP and HcRed using confocal microscopy imaging. 293T cells were co-transfected with expression plasmids coding for hPrP-eGFP and MLV (HcRed). Images of the live cells were acquired 24 and 48 hours after transfection (Figure 4.14). Column A of Figure 4.14 shows transfected cells after 24 hours; column B shows a 10X magnification of a selected field of the corresponding panel in column A; column C is an image of the cells 48 hours post-transfection. The first row of Figure 4.14 is a merged image of the channel for HcRed (second row, red) and for eGFP (third row, green). These images show that hPrP-eGFP and HcRed can be detected in my assays and therefore allow for simultaneous detection and tracking of both fluorescent proteins.

Figure 4.14 Live cell confocal microscopy imaging of hPrP-eGFP and MLV (HcRed) transfected cells

Images were taken 24 and 48 hours post-transfection. A merge of red (HcRed) and green (eGFP) channels are depicted in the top row, HcRed expressed by MLV(HcRed) is in the middle row (red), hPrP-eGFP transfected cells are shown on the bottom row (green). Images were taken using a 40X oil-immersion objective.


A (24 h)
B (24 h)
C (48 h)
Merged Channels
HcRed-Red Channel-
eGFP–Green Channel

4.3.3 Fluorescence and Confocal Microscopy on Fixed Cells

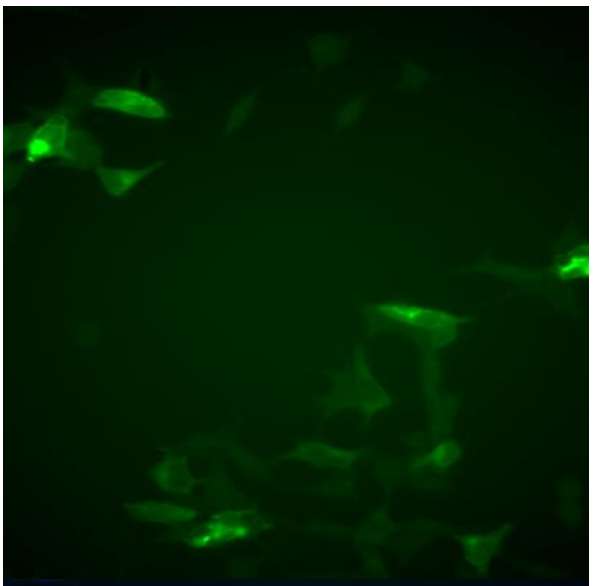
In order to acquire high-resolution pictures of the cell distribution of hPrP-eGFP, 293T cells were grown on coverslips, transfected with hPrP-eGFP, fixed with formaldehyde and examined under a fluorescent microscope using an oil-immersion 40X objective. Figure 4.15 shows a preparation of formaldehyde-fixed cells that was mounted with Dapi, which stains the nuclei of cells.

Figure 4.16 shows live images of 293T cells either transfected with hPrP-eGFP (Panel A) or peGFP-N1 (Panel B), and co-transfected with hPrP-eGFP and MLV (HcRed) (Panel C). Glass bottom dishes were used for live fluorescence imaging (see materials and methods). Images were acquired on a confocal microscope using a 63X oil-immersion objective. The high resolution of the confocal microscope allows to clearly distinguish the accumulation of hPrP-eGFP at the cell membrane and in large cytoplasmic structures (Panel A), as opposed to peGFP-N1 where it is in the whole cell including; nucleus, cytoplasm and cellular membranes (Panel B).

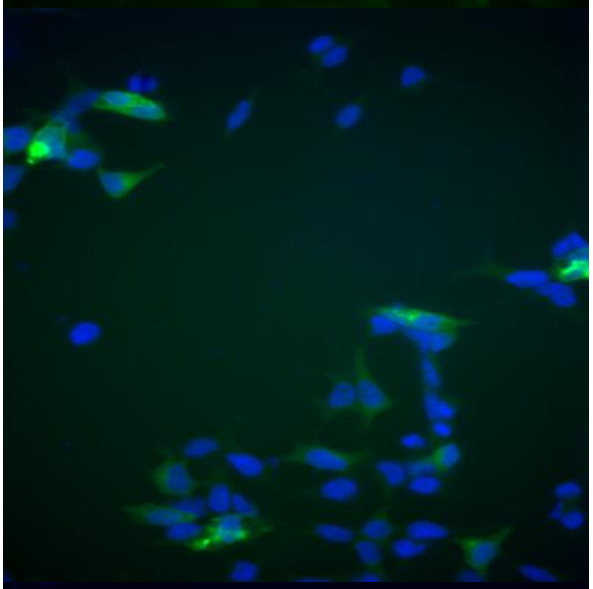
Figure 4.15 Fluorescence microscopy imaging performed on 293T cells 48 hours post-transfection

A) hPrP-eGFP (green). B) Merged images of Dapi (blue) and hPrP-eGFP (green). C) Image of cell nuclei stained with Dapi (blue). Images were taken using a 40X oil-immersion objective.

A



B



C

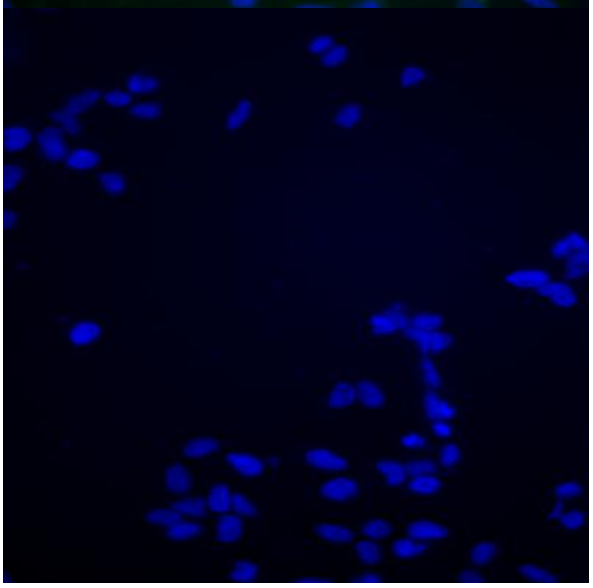
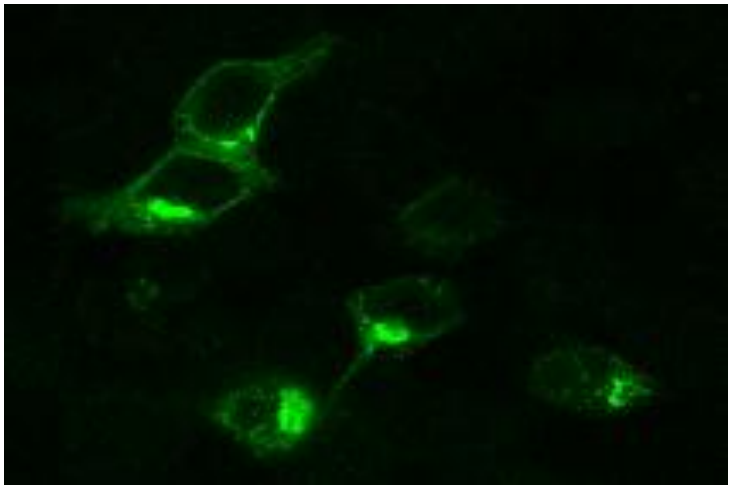


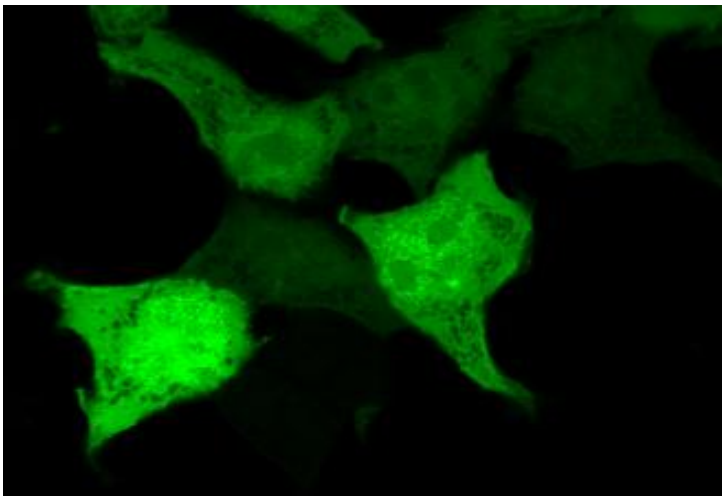
Figure 4.16 Confocal microscopy live imaging performed on 293T cells 48 hours post-transfection

A) hPrP-eGFP; B) peGFP-N1; C) Co-transfection of hPrP-eGFP and MLV (HcRed) Images were taken using a 63X oil-immersion objective.

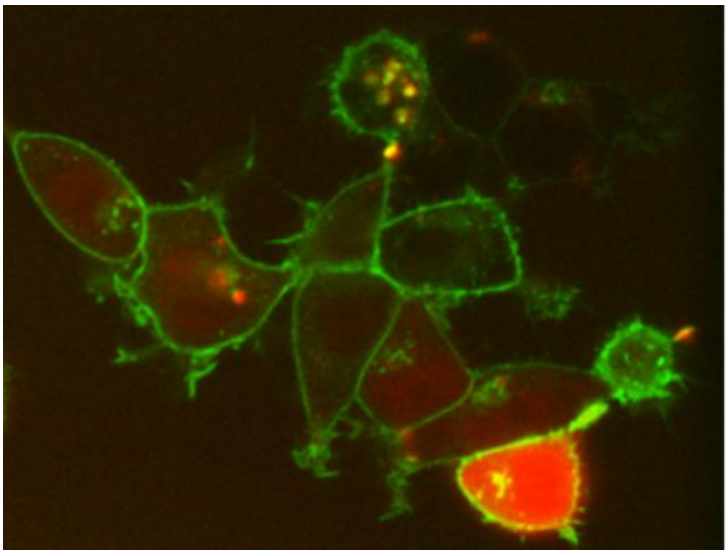
A



B



C



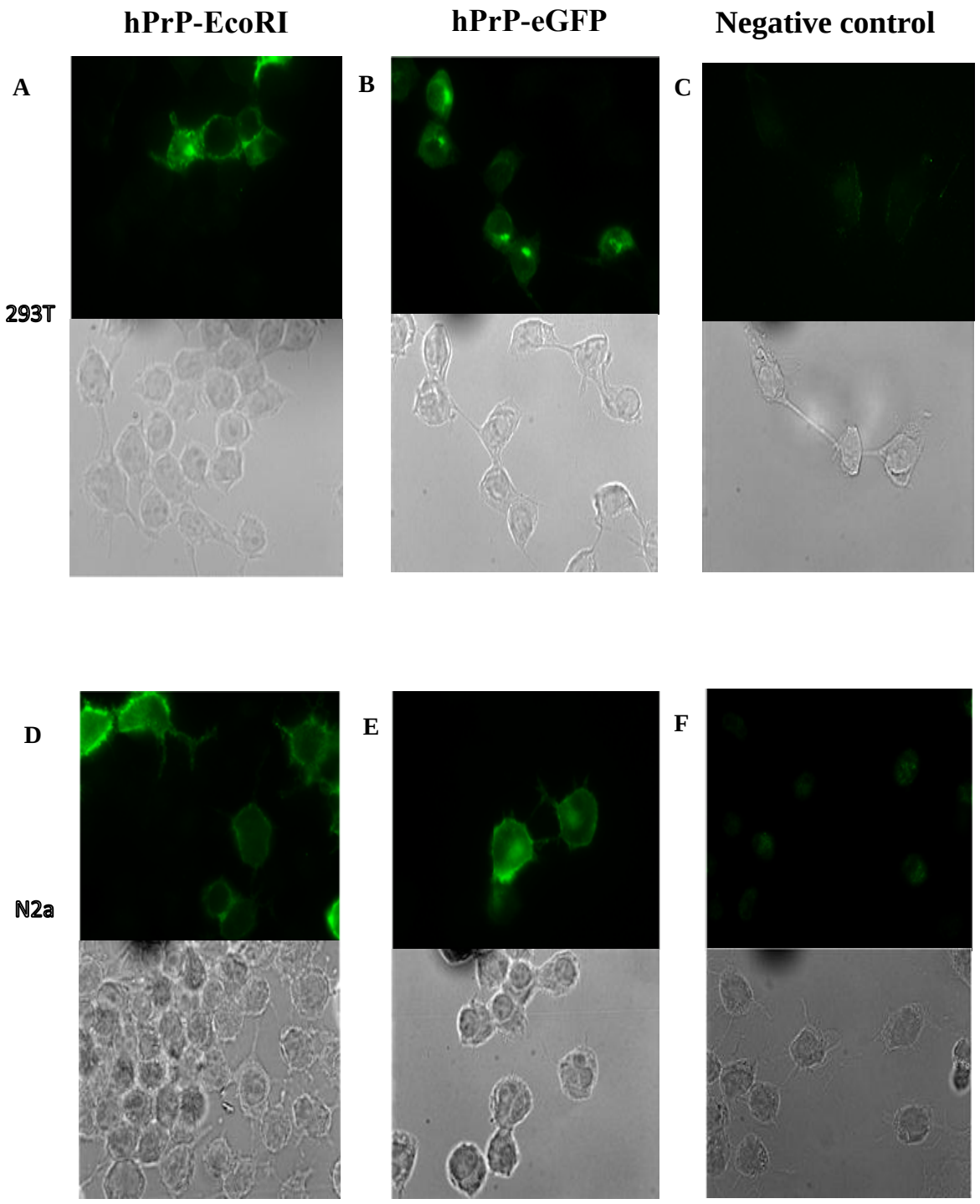
4.3.4 *In situ* Immunostaining

Expression of the eGFP reporter within the PrP protein could potentially alter cell localization. Here we attempted to assess if or how eGFP affects hPrP-eGFP cell distribution and whether our recombinant hPrP protein displays a similar pattern to that of the endogenous PrP^C protein. I*n situ* immunostaining was performed on formaldehyde-fixed HEK 293T or N2a cells transfected with either the hPrP-EcoRI or hPrP-eGFP constructs (Figure 4.17). A human-specific anti-prion monoclonal antibody (3F4) was used for probing and a secondary anti–mouse monoclonal antibody conjugated to the fluorochrome Alexa 488 (green) was used for the detection. The images were taken with a confocal microscope using a 40X oil-immersion objective (see materials and methods for complete details).

The two human prion proteins, hPrP-EcoRI and hPrP-eGFP displayed very similar cell distribution patterns in both transfected HEK 293T and N2a cells (Fig 4.17). The anti-prion 3F4 antibody only recognizes the human form of the protein. Although 293T cells are of human origin and should express detectable levels of PrP^C, only a faint signal could be seen in these cells (Figure 4.17, panel C). It is unlikely that this signal constitutes a specific interaction with the endogenous prion protein because a weak signal could also be seen in mouse N2a cells (Figure 4.17, panel F). In summary, expression of eGFP as an internal protein fusion reporter did not appear to significantly affect cell localization. However, I was unable to stain the endogenous cellular prion protein in 293T cells and thereby unable to compare the distribution of the recombinant hPrP protein with that of the endogenous PrP^C protein.

Figure 4.17 Cell distribution of the recombinant hPrP protein in transfected HEK 293T and N2a cells

In situ immunostaining images were captured on a using a fluorescence microscope 48 hours post transfection in the HEK 293 T (panels A, B and C) and N2a cell lines (panel D, E and F). Top panels in each set are immunofluorescence images of the transfected cells and bottom panels are phase contrast images of the field analysed. The primary an anti-prion 3F4 was used at a dilution of 1:500 and secondary anti-mouse IgG (Alexa 488) antibody was used at 1:1000. Images were taken using a 40X oil-immersion objective.

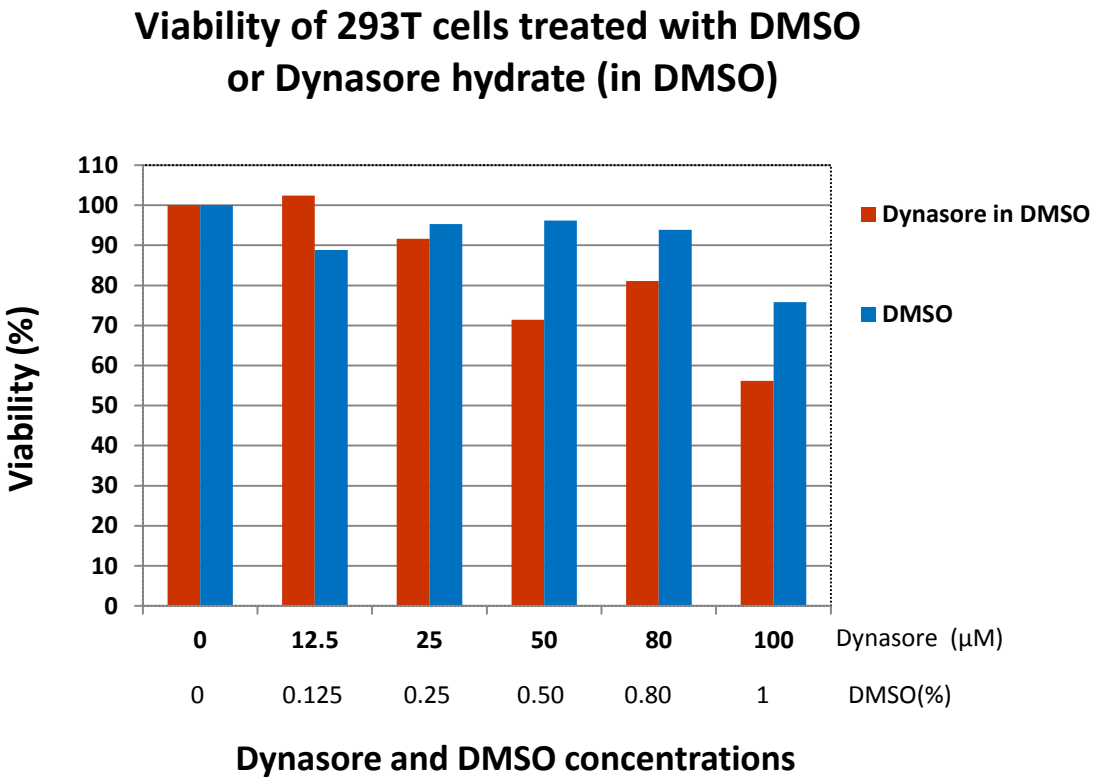

hPrP-EcoRI
hPrP-eGFP
Negative control
A
B
C
293T
D
E
F
N2a

4.4 Cell Viability Assay after treatment with Dynasore

In preparation for infections assays designed to track the delivery of prion cargo to target cells by retroviruses, here I measured the toxicity of an endocytosis inhibitor drug called dynasore hydrate. Although retroviruses, like MLV, can enter susceptible target cells using different mechanisms, the most common mode of entry is through receptor-mediated fusion of the retroviral envelope with the host's endosomal membrane in endosomal vesicles (Miyauchi et al., 2009, Permanyer et al., 2010)(Campbell and Hope, 2008). An important control for future experiments is one where retroviruses carrying prions are unable to enter the target cell by endosomal fusion, and therefore do not have the opportunity to deliver their prion cargo into the cytoplasm of the target cell. Internalisation of endosomes is dynamin-dependent; inhibition of dynamin will therefore paralyse the endosomal machinery and prevent viral entry. In this optimization experiment, I controlled for DMSO and Dynasore toxicity by measuring cell viability using Trypan blue exclusion (Figure 4.18). Using 80μM of dynasore (containing 0.8% DMSO) is recommended for endosomal fusion inhibition assay where 81 % of target 293T cells were viable at this concentration (Fig 4.18).

Figure 4.18 Viability of 293T cells 48 hours post-treatment with increasing concentrations of Dynasore-DMSO or DMSO alone

Values were obtained by compiling viable cells over total cells after staining with trypan blue (0.4%) and normalizing to the viability of untreated cells. The values represent the average +/- standard deviation from a triplicate count.


Viability of 293T cells treated with DMSO or Dynasore hydrate (in DMSO)
Viability (%)
110
100
90
80
70
60
50
40
30
20
10
0
Dynasore in DMSO
DMSO
0
12.5
25
50
80
100
Dynasore (μM)
0
0.125
0.25
0.50
0.80
1
DMSO(%)
Dynasore and DMSO concentrations

4.5 Integration Inhibition of MLV

Another important control for future work is one where retroviral integration is inhibited. This control will allow us to determine whether a productive infection is required for the potential delivery of prions to target cells.

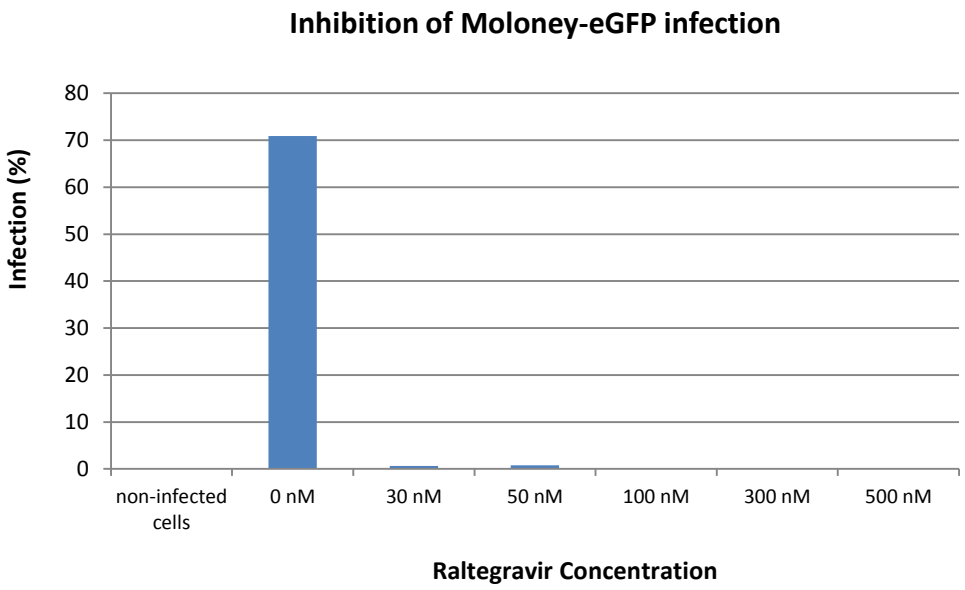
Raltegravir is a viral integrase inhibitor. Here I pre-treated NIH 3T3 target cells with increasing concentrations of Raltegravir for 3 hours and then infected the cells with VSV-G-pseudotyped MLV (eGFP) with the replicative ecotropic Moloney MLV virus that also expresses the eGFP reporter protein (Moloney-eGFP). Moloney-eGFP is produced by transfecting 293T cells with a single expression vector coding for the proviral DNA. Raltegravir at the indicated concentration was maintained in the media throughout the experiment. Infection was assessed by evaluating the percentage of eGFP-positive target cells by flow cytometry analysis 24 hours after infection.

Panel A of Figure 4.19 shows the inhibition of replicative Moloney-eGFP infection by increasing concentrations of Raltegravir. Only 30 nM of the drug was required to completely inhibit infection. On the other hand, Raltegravir had a marginal effect on MLV (eGFP) particles that are produced by co-transfecting 3 plasmids (see matrials and methods) (Figure 4.19, panel B). EGFP in this system is expressed from an internal CMV promoter. Because infection was measured as eGFP expression in the target cells, lack of apparent inhibition of infection could be due to high levels of episomal expression of the eGFP reporter protein from structures such as 1-LTR or 2-LTR circles. A viral integration assay to measure the incorporation (or lack) of proviral DNA into the host genome would confirm this hypothesis.

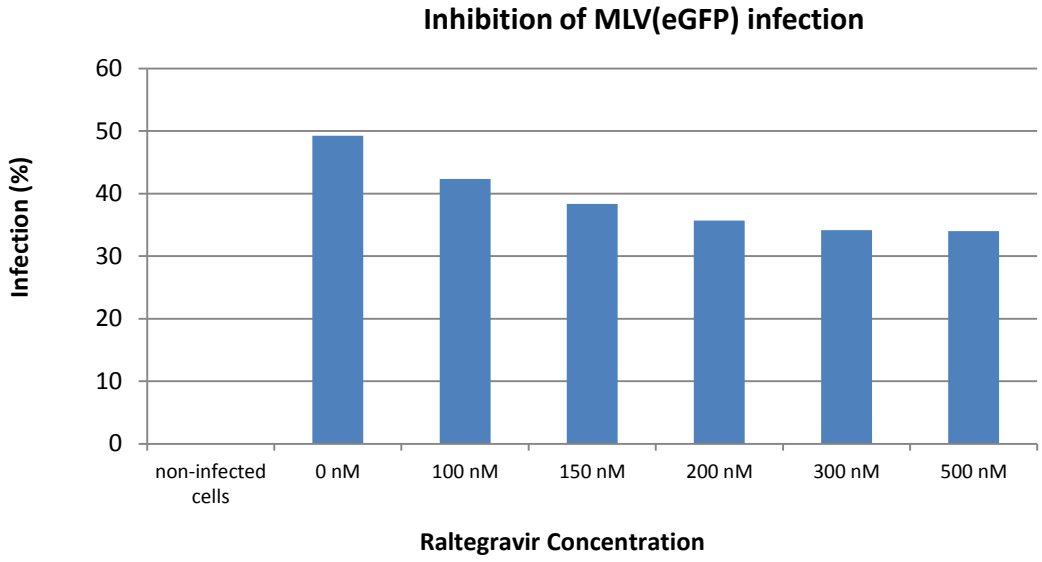
Figure 4.19 Inhibition of viral infection by using the integrase inhibitor Raltegravir

NIH 3T3 target cells were pre-treated for 3 hours with increasing concentrations of Raltegravir and then infected with either replicative Moloney-eGFP (Panel A) or VSVG-pseudotyped MLV (eGFP) (Panel B). Infection was quantified by flow cytometry analysis 24 hours later as a measure of the percentage of cells expressing eGFP.

A)


Inhibition of Moloney-eGFP infection
Infection (%)
80
70
60
50
40
30
20
10
0
non-infected cells
0 nM
30 nM
50 nM
100 nM
300 nM
500 nM
Raltegravir Concentration

B)


Inhibition of MLV(eGFP) infection
Infection (%)
60
50
40
30
20
10
0
non-infected cells
0 nM
100 nM
150 nM
200 nM
300 nM
500 nM
Raltegravir Concentration

5.0 DISCUSSION

Prion diseases are fatal brain disorders, whose infectious agent is an abnormal and converted isoform of the cellular prion protein. Because PrP^{C} is highly expressed in brain cells, if a small amount of pathogenic PrP^{Sc}, ingested via contaminated food for example, were to find its way through the blood brain barrier (BBB) to the brain, prion diseases could occur. How these infectious prions are transported from their site of entry to the central nervous system (CNS), or why this tissue rather than others that also expressed high levels of PrP^{C} is specifically targeted remains a mystery.

According to recent studies, retroviral particles are able to infect and replicate inside mature monocytes ($CD14^{+}CD16^{+}$) and access the CNS via diapedesis of the monocytes through the BBB (Williams et al. 2012). Moreover, several studies have shown that there exists a strong relationship between retroviruses and prion proteins since they can co-localize or co-assemble in the same intracellular endosomal vesicles such as early and late endosomes, and multi-vesicular bodies (MBV) during their maturation in the cytoplasm. Furthermore, prions can be incorporated into nascent retroviral particles during their budding and releasing from infected cells (Leblanc et al., 2006) or they can also transfer from one cell to another by up taking in the exosomes that are releasing from cells by exocytosis (Fevrier et al.,2005). It is currently unknown whether prions accumulate in the envelope, the capsid, or both of nascent retroviral particles (Leblanc et al., 2006, Ashok and Hedge, 2006). Prions could also contaminate retroviral preparations by associating with exosomal vesicles released from producer cells that co-purify with retroviruses (Fevrier et al., 2004, Porto-Carreiro et al., 2005). By consolidating these observations, it is hypothesized that retroviruses (either exogenous or endogenous) could act as vectors for prion disease by capturing pathogenic prion proteins from an infected cell,

shuttling them through the host and across the BBB, and delivering them to neuronal cells. It is now well established that retroviruses can cross the intact BBB, and that free-floating oligomeric or protofibrillar infectious prion proteins in the blood cannot cross (Heikenwalder et al., 2007, Begley, 2009,). In an attempt to validate this hypothesis, I began my study by examining whether retroviruses were able to capture cellular prions from a retrovirus-infected cell and deliver them to susceptible target cells in an *in vitro* infection model.

To carry out my study, I generated several vectors expressing the human and mouse prion protein with or without an internal FLAG epitope tag or the fluorescent reporter protein eGFP. The human cellular prion cDNA was cloned from the mRNA of peripheral blood mononuclear cells (PBMC). An important obstacle for the cloning was that prion proteins undergo both N-terminal and C-terminal post-translational cleavage and maturation. It is therefore undesirable to add a reporter protein or an epitope tag fused to extremities of the protein. An EcoRI site was designed and inserted in a region of the prion protein that is predicted to have little effect on folding, trafficking and cell localization according to structural and functional studies (Riley et al., 2002, Gauczynski et al., 2002). The eGFP reporter gene sequence was inserted in this EcoRI site to produce an internally eGFP-labeled prion protein that can be tracked and detected in fluorescence microscopy assays. The DNA sequence coding for the FLAG epitope tag was also inserted in this restriction site. The structures of the three human prion expression vectors used in this thesis are shown in figures 4.1, 4.2 and 4.3.

HEK 293T, an easily transfectable human cell line, was used for protein expression, which was monitored by WB using either a monoclonal antibody(mAb) that is specific for the human prion protein (3F4), or anti-eGFP and anti-FLAG antibodies. The anti-prion antibody revealed bands of the expected sizes for all three human prion constructs (Fig 4.4, 4.5 and 4.6). In the

same way, the anti-eGFP antibody was able to detect the internal eGFP fusion sequence in the recombinant hPrP-eGFP protein (Fig 4.4). Surprisingly, immunoblots of hPrP-FLAG using the anti-Flag-HRP antibody did not detect the internal FLAG epitope though it was able to detect the N-terminal FLAG tags of our A3G-FLAG and AID-FLAG controls (Fig 4.5). Thus, work with the anti-FLAG antibody was postponed because firstly, the eGFP reporter in hPrP-eGFP can be detected by the anti-eGFP antibody by WB and it can also be clearly visualized in transfected cells. Furthermore, the anti-prion 3F4 antibody can readily detect the prion protein that is expressed from all three of my constructs, including hPrP-FLAG (Fig 4.6).

The interaction between retroviruses and the human prion protein was successfully confirmed when co-transfection of different human prion expression constructs was carried out with the HIV-1(eGFP) and MLV (HcRed) virus expression vectors. The association between the recombinant human prion protein and HIV-1 virions is shown in figure 4.7. In that experiment, viral lysates were generated from the supernatant of co-transfected virus-producing cells. These cells gave positive signals for the anti-prion (3F4) and the anti-p24 (HIV-capsid protein) monoclonal antibodies by WB analysis, which confirmed the co-expression of our prion protein and viral proteins. Similar results were obtained from the supernatants of cells expressing MLV (HcRed) and hPrP-eGFP (Fig 4.8). To confirm the relationship between retroviral particles and prion proteins, and to exclude that the interaction observed by WB was a direct consequence of an interaction with the eGFP reporter protein and not the prion protein itself, additional co-transfection assays were performed with hPrP-FLAG. Figure 4.9 shows the association of hPrP-FLAG with MLV (HcRed) and figure 4.10 displays same association with HIV(eGFP).

Moreover, confocal microscopy on live or fixed cells expressing recombinant prion proteins provided details on the intracellular localization of these proteins in transfected cells. To determine if the type of cell line would affect the cellular localization of the prions, two different cell lines, 293T and N2a, were used for expressing hPrP-eGFP. Live cell imaging showed that there is in fact no significant difference in cell localization of the labeled prion protein between both these cell lines (Fig 4.13). In addition, the cell distribution of hPrP-eGFP is very different to that of free eGFP, which appears diffused throughout the cell's cytoplasm. Figures 4.13, 4.14 and 4.16 showed that hPrP-eGFP expression appears to be mainly restricted to the cell membrane and to dense cytoplasmic structures of unknown identity. These results are in accord with the results of others (Negro et al), whereby they have also observed cell membrane accumulation of the prion protein and an association with the mid–Golgi marker mannosidase II. In our study, the Golgi system was not stained but it is possible that the dense green fluorescent speckles near the nucleus are indicative of the Golgi complex. We also confirmed by performing comparative *in situ* immunostainings of hPrP-eGFP and hPrP-EcoRI that the eGFP reporter protein did not interfere with cellular localization of hPrP in the 293T and N2a cell lines (Fig 4.17).

The next step of the project was to track hPrP-eGFP-loaded retroviruses and determine whether they deliver their fluorescent cargo to susceptible target cells. In order to visualize hPrP-eGFP associated with retroviruses binding to a cell surface, it is necessary to use a very high multiplicity of infection (MOI). This measurement represents the ratio of infectious viruses-to-target cells in the infection assay; the higher the MOI, the greater the number of viruses infecting a single cell. Unfortunately, despite many attempts, I was unable to visualize eGFP fluorescence at the surface of the target cells either by flow cytometry, immunostaining

or by confocal fluorescence microscopy (data not shown). I believe that the reason why these assays failed was that I used an MOI that was far too low to allow for signal detection (MOI of 1.2). Although the WB assays determined that an association between retroviruses and hPrP-eGFP exists, preliminary results for prion delivery to the target cells in an *in vitro* infection model remain inconclusive.

In the instance where delivery of hPrP-eGFP would be successfully detected in the target cells, it would then have to be determined whether endocytosis of retroviral particles and a productive infection are required for this delivery. To prepare for such an eventuality, I determined the optimal amounts of the endocytosis inhibitor Dynasore that can be used on the target cells without being detrimental to cell viability. I also determined the concentration of the integrase inhibitor Raltegravir that is required for inhibiting infection by the retroviruses used in my assays. Hence, I established that 81% of target 293T cells were viable when treated with Dynasore at a concentration of 80 μM (Fig 4.18), and that 30 nM of Raltegravir was sufficient to completely inhibit the infection of replicative Moloney-eGFP in NIH 3T3 cells (Fig 4.19).

To be able to take this project forward, more sensitive detection strategies must be developed to visualize labeled or fluorescent prion protein being delivered by retroviruses. Such strategies may employ prion proteins labeled with radioisotopes or brighter fluorescent dyes or reporter proteins. This thesis constitutes a first step in assessing the potential role of retroviruses in transporting and spreading prions between cells. If a link were to be confirmed between retroviruses and prion dissemination, treatment strategies could be developed in the future that would target the vector rather than the pathogen.

6.0 CONCLUSION

In this work, I have cloned both the human and mouse *PRNP* cDNA and have generated expression plasmids allowing for the production of recombinant prion proteins containing either an internal FLAG epitope tag, the eGFP reporter, or no tag at all. I have shown that these recombinant proteins are expressed in human and mouse cells following transfection and that their cellular localization appears to be unaffected by the modifications I have introduced into their coding sequence. Unfortunately there are no functional assays that could allow me to test the biological activity of these recombinant proteins.

Here, I also present evidence that these recombinant prion proteins can easily be detected in the supernatant of retrovirus-producing cells. This is of particular interest in light that retroviruses can cross the blood-brain-barrier and gain access to neuronal cells, which are the main targets of pathogenic prions.

This work and the tools that I have developed here will now serve as stepping stones for further research that will investigate whether prions are delivered to target cells upon infection, and determine whether PrP^{Sc} can also be hijacked by retroviruses in a similar way to PrP^{c}. The implications of demonstrating that retroviruses can act as vectors for PrP^{Sc} dissemination are tremendous because this would provide the rationale for treating infected or at risk subjects with antiretroviral medications to halt disease progression and prevent transmission to uninfected hosts. There are currently no cures or effective treatments for prion disease.

7.0 REFERENCES

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8.0 CURRICULUM VITAE

SHABNAM RAHIMI KHAMENEH

PERSONAL PROFILE:

- Trained in a variety of professional research and development subjects including virology, biochemistry, molecular biology, genetic, epidemiology, statistical analysis and public health and etc.
- Expertise in molecular biology/virology research projects and clinical studies
- Trained in different professional including; laboratory-safety, hazardous material handling and biosafety, WHMIS, Confocal laser microscopy and level three laboratories training. I have also Secrete security clearance
- Good in searching, finding and entering data and using programs from network and soft-wares
- Dynamic, organized, motivated professional with a proven track record in employer satisfaction
- Created and implicated plans and trouble shooting in difficulties and successfully independent in completing assigned tasked
- Leadership, project management, time management and multiple priorities and coordination skills
- Strong drive and motivation and fast in learning new skills and methods
- Excellent ability to manage the financial aspects of complex transactions and research projects
- Excellent co-operation abilities, supporting, accepting authority, respecting differences, working on a team and stating opinions

ACADEMIC EDUCATION:

04/2010-09/2012 **M.Sc.** in microbiology, Department of Biochemistry, Microbiology and Immunology (BMI), Ottawa University, Ottawa, Canada

09/1995-02/2001 **Ph.D.** in Virology, Department of Pathobiology, Health Faculty, Tehran University of Medical Sciences, Tehran, Iran

02/1991-02/1994 **M.S**.**P.H** in Virology, Department of Pathobiology, Health Faculty, Tehran University of Medical Sciences, Tehran, Iran

09/1986-02/1991 **B.Sc.** in Cell and Molecular Biology, Department of Biology, Science Faculty, Tehran University, Tehran, Iran

SECTORS OF EXPERTISE:

- Up to date experiences in new molecular virology techniques and research methods in biotechnology and molecular biology, for example gene cloning, immunofluorescent microscopy, insitu immunostaining and Flowcytometry
- Expertise of working in level three laboratory for sampling infectious prion protein
- Expertise in prion protein and disease and also their relation with retroviruses
- Experience in infectious disease surveillance, collecting information, data entry and analyses surveillance for influenza and other respiratory infectious diseases
- Experience in writing and preparing reports, graphs and data tables in influenza surveillance and send them to the appropriate partners in different provinces and territories
- Familiar to medical terminology including diseases, micro-organisms, treatments, lab tests, etc.

- Operating with computer and excellent knowledge and skills in word perfect, MS Word, MS Office, Lotus Notes, PowerPoint, Excel, Access, Harvard graphic and SPSS (statistic software) and familiar with EPI -Info and SAS software
- Respiratory infections including Influenza A, Para influenza ,and SARS
- Experience in detecting ,isolating and quantifying infectious disease viruses including Measles, Rubella, HCV/HBV/ TTV/HIV and other blood borne viruses
- Expertise in different molecular biology techniques.
- Literature review and using reference manager for preparing references
- Presentation skills by using Power point and graphics
- Teaching virology and molecular biology to the undergraduate and graduate students, and lab staff at the university

PROFESSIONAL MEMBERSHIP:

American Association of Microbiology	2004-Present time
Member Faculty of Medical Zandjan University-IRAN	1994-2002
Iranian Association of Virology	2001-Present time
Member of Iranian Medical laboratory Council	2001-Present time

PROFESSIONAL EXPERIENCES:

Second Master degree at the University of Ottawa, BMI department (1April 2010 –28 September 2012)

Up to date my molecular biology and virology knowledge and I have a very interesting project about

Prion protein and disease and how retroviruses may transfer and spreading the infectious protein

from gut where we eat it to the brain and produce transmissible spongiform encephalopathy

Master in Science thesis title: **"ASSESSMENT OF RETROVIRUSES AS POTENTIAL VECTORS FOR THE CELL DELIVERY OF PRIONS" 28 SEPTEMBER 2012**

Research officer -Public Health Agency of Canada

Immunization and Respiratory Infections Disease Department, FluWatch program (31Dec 2007-1 April 2010)

Working with Access and excel programs for preparing influenza data sheet and analyzing them for Fluwatch program. I am using these data for preparing weekly reports, graphs and tables about influenza virus and infection in Canada.

Research Analyst ES-4 (short contract) Public Health Agency of Canada

Immunization and Respiratory Infections Disease Department, Pandemic program (21Oct 2007-14Dec 2007)

Working on the scientific documents and references in Pandemic section of IRID, preparing report for antiviral survey in provinces and territories, revising Annex G part of pandemic influenza plan and producing Reference Manager Data Base for Emerging Respiratory Infections Disease

Acting in ES-4 position as an Epidemiologist (casual contract) Public Health Agency of Canada, Immunization and Respiratory division, Vaccine Safety section (Dec2006-May2007)

Working with SAS program and CAEFI database for reporting Adverse Events Following Immunization (AEFI) for Influenza and the other vaccines

Post-doctoral fellowship at the R&D Group, Canadian Blood Services (10/2002-7/2005)

Postdoctoral fellow in virology, under the supervision of Dr Yu-Wen Hu

I studied blood borne viruses such as HCV, TTV, TLMV and HTLV-I/II and learned advanced molecular biology methods such as: **Gene cloning**, **Sequencing, Protein expression** in **prokaryotic** and **eukaryotic systems** and new **protein purification methods**. Two papers based on my work on TTV and TLMVs has been submitted and published.

Participated in a collaborative study about SARS-CoV with Dr. Brown at the Ottawa University

Developing single and **multiplex real-time or quantification PCR and RT-PCR (Q-PCR or QRT-PCR)** assays for HBV, HCV, HAV, B19 viruses under the super vision of Dr. John Saldanha

Assistant Professor of Microbiology, Zandjan University-Medicine Faculty, Microbiology Department (02/2001-08/2002)

Assistant professor in the Microbiology Department and teaching undergraduate students and have set-up some simple virology techniques **(chick embryo techniques**, **Hemagglutinaion test)**

Ph.D. Virology, Tehran University of Medical Sciences -Health Faculty, Virology Department (09/1995-02/2001)

Advanced in molecular virology methods such as: **Cell Culture, virus isolation, IF, RT-PCR and PCR, Nucleic acids extraction,** and **purification** methods.

Analyzing isolated measles sequences and providing phylogenetic studies using the **Megalign** program of **DNASTAR software.**

Ph.D. thesis title **"Detection of Measles RNA from clinical samples and determination of their genetic Differences".**

Virology Instructor, Zandjan University-Medicine Faculty, Microbiology Department (04/1994-02/2001)

Member of faculty at the Microbiology Department of Zandjan Medical University

Received a scholarship from Zandjan University and registered for a PhD in Virology from Tehran University

Grant award from Zanjan University to work on the following **sero-epidemiology** project, **"A study of rubella Immunity in girls of marrying age in Zandjan and its suburbs"**. Which published in 1998 in the Journal of Zandjan medical University (by using **HI** and **HA** tests for rubella and SPSS and Harvard Graphic software)

MSc Virology, Tehran University of Medical Sciences-Health faculty-Virology Department (02/1991-01/1994)

The title of my thesis was **"Primary Rubella Infection Identification by Indirect Immunofluorescence (IIF) and ELISA tests"**. This study is important for public health

For statistical analysis SPSS soft-ware has been used

BSc in Cell and molecular Biology, Tehran University-Science Faculty-Biology Department (09/1986-02/1991)

I studied cell and molecular biology and got good experiences in theory and practical approaches. I was a student of the year and got an award from Tehran University

Summer Research Student, Tehran University (06/1989-09/1989)

Molecular biology and characteristics of a new anti-cancer herbal mixture on cancer cells by using an erythropoietin stem cell line to determine if it can suppress growing cells and validate its potential as anti-cancer drug.

TRAINING:

- **Laboratory-safety, hazardous material handling and biosafety, WHMIS, Confocal laser microscopy and level three laboratories at** **04-2010**

the University of Ottawa BMI department

- **The Blood Borne Pathogen training, Canadian Blood Services 28-01-2004**
- **The Basic WHMIS training, Canadian Blood Services 12-11-2003**
- **The Fire Safety training, Canadian Blood Services 06-11-2002**
- **Research Writing Skills, Zandjan University 21-06-2002**
- **Advance Research Methods workshop, Zandjan University 07-11-2001**
- **Polymerase Chain reaction (PCR) workshop, Tehran University 03-03-1998**
- **Primary Research Methods workshop, Tehran University 19-11-1997**
- **Advance Molecular Biology of viruses, theoretical 08-03-1995**
- **National Centre of Genetic Engineer and Biotechnology Investigation 29-02-1992**
- **Primary Public Health Care workshop, Tehran University, 06-03-1992**

FELLOWSHIPS AND AWARDS:

1. 2010. Admission scholarship from University of Ottawa, BMI department
2. 2011. Ontario Graduate Scholarships in Science and Technology (OGSST) Award and excellent award from University of Ottawa
3. 1999. Student of the year award (PhD), Tehran University of Medical Sciences, Faculty of Health, Tehran, Iran
4. 1995. PhD Scholarship award from Zandjan University of Medical Sciences, Faculty of medicine, Zandjan, Iran
5. 1994. Student of the year award (MSc), Tehran University of Medical Sciences, Faculty of Health, Tehran, Iran
6. 1991. Student of the year award (BSc), Tehran University of Medical Sciences, Faculty of Science, Tehran, Iran

PUBLICATION:

1. Hu YW, Al-Moslih MI, Al Ali MT, Uzicanin S, Perkins H, Yi QL**, Rahimi Khameneh S,** Wu J, Brown EG. Clinical outcome of frequent exposure to Torque Teno virus (TTV) through blood transfusion in thalassemia patients with or without hepatitis C virus (HCV) infection. Journal of Medical Virology, (2008) Feb;80(2):365-71.

2. Hu Y.W., Al- Moslih MI., Al-Ali MT., **S. Rahimi Khameneh**. *et.al*. Molecular Detection method for all known genotypes of TT virus (TTV) and TTV-like viruses in thalassemia patients and healthy individuals, Journal of Clinical Microbiology(2005), Aug; 43 (8):3747-54 .

3. Hu Y.W., **S Rahimi Khameneh**, *et-al,* The Clinical Significance of Antibody Cross-reactivity of E2 and NS5A Quasispecies in Diagnosis of Hepatitis C Virus Infection for Blood Screening

4. **Rahimi Khameneh S.**, C. Camerone, and J. Saldanha; Quantitative multiplex real Time NAT assays for blood borne viruses. (Submitted to Journal Virological Methods, (submitted to Transfusion-2007-0488)

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3-**Rahimi Khameneh S.**, The Genotype of the wild Measles viruses isolated in Iran, Poster presentation in the 12th European Congress of Clinical Microbiology and Infectious Diseases (12th ECCMID), J Clin Microbiol Infect, abstract Number P999. 24-27 April, 2002, Milan-Italy

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