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Antibodies directed against the 37 kDa/67 kDa laminin receptor as therapeutic tools for the treatment of prion diseases and cancer

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ABBREVIATIONS

CURRICULUM VITAE

SUMMARY

Transmissible spongiform encephalopathies (TSE), also known as prion diseases, comprise a class of neurological lethal disorders, affecting both humans and animals. TSEs are commonly associated with the formation of abnormal protein aggregates and plaques observed in the central nervous system (CNS) of the affected individual leading to neuronal cell death. The observed aggregates originate from accumulation of the abnormal isoform (PrP^{Sc}) of the host encoded cellular prion protein (PrP^{c}) mainly responsible for the pathology of the disease. Several functions have been described for PrP^{c}, however, little is known about the exact role of PrP^{c} in the disease and the underlying mechanisms of accumulation and neurodegeneration, respectively.

The 37 kDa/67 kDa laminin receptor LRP/LR has been identified as a cellular surface receptor for both PrP^{c} and PrP^{Sc}. In addition, LRP/LR is thought to participate in the prion protein uptake in intestinal cells such as enterocytes suggesting an implication in the oral prion protein uptake. The crucial role of LRP/LR in the prion life cycle indicates new possibilities for the development of an alternative therapy for prion diseases. Tools directed against or downregulating the laminin receptor have been developed. Among them, anti-LRP/LR antibodies have been proven to block the PrP-LRP/LR interaction and their potential in prion disease therapy using experimental prion infection of mice was demonstrated: Passive immunotransfer (intraperitoneal (i.p.) injection) of the polyclonal antibody W3 into scrapie infected mice reduced the peripheral prion propagation and prolonged their survival. Furthermore, i.p. treatment of prion infected mice with single chain antibodies (scFvs) directed against LRP, reduced also the peripheral prion propagation in scrapie infected mice. Delivery of the scFvs via recombinant Adeno-Associated Viral vectors (rAAVs) resulted in an efficient expression of these antibodies and reduced also the peripheral prion propagation of scrapie infected mice, due to trafficking of the i.c microinjected viruses to the spleen.

LRP/LR plays a crucial role in the metastatic process of tumor cells, proliferation and angiogenesis, mainly due to laminin1-LRP/LR interaction. Tools directed against LRP/LR, such as single chain antibodies (scFvs) and full-length IgG antibodies have been proven to interfere with the laminin-1 binding to LRP/LR and hampered the invasion process of tumorigenic cells, therefore providing efficient tools targeting the metastasis of tumor cells.

Anti-LRP/LR antibodies might represent alternative promising therapeutics for the treatment of cancer, especially for preventing metastatic spread, and prion diseases.

CHAPTER I

Introduction

1. Transmissible spongiform encephalopathies

Prion diseases or transmissible spongiform encephalopathies (TSEs) comprise a rare group of neurodegenerative disorders affecting both humans and animals. Histopathological changes in the brain of the affected individuals exhibit vacuolization, also termed spongiosis, reactive changes of astrocytes (gliosis) and variable loss of neurons. The central feature is the conversion of the host encoded cellular prion protein (PrP^{c}) into an abnormal aggregated isoform, designated PrP^{Sc} (Bolton and Bendheim, 1988; Prusiner, 1998; Telling et al., 1996). The mechanism of neurodegeneration that accompanies the accumulation of the PrP^{Sc} is still not clarified. Characteristic neurodegenerative symptoms like dementia, convulations, ataxia, balance and coordination dysfunction and behavioral personality changes occur in most cases after a long incubation phase without clinical signs. In humans, incubation periods can vary from 1.5 to 40 years. To date, there is no therapy available to cure the individuals.

1.1 Human TSEs

In the early 1920s the first human prion-like disease was described by Creutzfeldt and Jakob, (Creutzfeldt, 1920; Jakob, 1921), followed by the discovery of an unusual inherited progressive cerebellar ataxia associated with amyloid plaque formation in the brain by Gerstmann, Sträussler and Scheinker, (Gerstmann, 1936), later termed Gerstmann-Sträussler-Scheinker syndrome (GSS). Kuru and fatal familial insomnia (FFI) have been discovered later and also classified as human prion diseases (Table 1). All of them are related to the abnormal form of the cellular prion protein PrP^{c} and share spongiform degeneration of neurons in the brain concomitant with amyloid plaque formation, which can vary among the prion diseases.

Experimental transmission of prion diseases has been successfully reported (Cuillé, 1936; Gajdusek et al., 1966; Gajdusek et al., 1968; Gibbs et al., 1968), thus justifying the term transmissible spongiform encephalopathy (TSE). Creutzfeldt-Jakob disease (CJD), which is the most frequent human prion disease, can be classified into four categories comprising a sporadic, a genetic, an iatrogenic and a variant type (Prusiner, 1993), respectively.

- **The sporadic form (sCJD)** (Johnson and Gibbs, 1998) represents approx. 85% of all CJD cases and affects women and men equally (Kretzschmar, 1993). Disease outcome occurs at the median age of 60 years with a duration time from onset to death of approx. 5 months

(Collins et al., 2006). sCJD is characterized by rapidly progressive and multifocal dementia and cerebellar degeneration usually with myoclonus (Collinge, 2001). The classical form is supposed to occur through spontaneous misfolding of the cellular prion protein PrP^{c} resulting in a conformational altered form designated PrP^{Sc} (PrP scrapie). Additionally, at codon position 129 in the prion protein gene *PRNP*, homozygosity for amino acid methionine or valine have been identified as a predisposing factor in the majority of sporadic CJD cases (Palmer et al., 1991). sCJD is currently divided into further clinical subtypes depending on the biochemical phenotype (Yuan et al., 2006).

- **The iatrogenic form of CJD (iCJD)** represents a rare disease resulting from neurosurgery, corneal grafting or human dura mater implants which have been contaminated with infectious prions (Boutoleau et al., 2003; Yamada et al., 1994). A correlation between valine homozygosity at position 129 on the *PRNP* gene (Collinge et al., 1991) and susceptibility for iCJD has also been found. Contaminated human growth hormone (hGH) or pituitary derived gonadotropin (hGNH) actually represents the main source of iCJD reported in UK (Swerdlow et al., 2003), Netherlands (Croes et al., 2001) and France (Huillard d'Aignaux et al., 1999). The disease was first discovered in 1974 in a US patient having received a corneal transplant from a donor who had suffered from CJD (Duffy et al., 1974). The incubation period, or latent period (time from exposure to the agent until clinical onset) ranges from two years to more than ten years.

- **Genetic or familial CJD (fCJD)** is associated with mutations within the prion protein gene *PRNP*, sometimes supposed to be inherited (Hsiao et al., 1989). The former used term “inherited” or “familial” CJD is not always appropiate, since not all patients with *PRNP* mutations appear to have affected familiy members. Therefore TSE cases associated with a *PRNP* mutation are termed “genetic TSE” (**gTSE**) (EuroCJD, 2001; Goldman et al., 2004) and include also cases without an apparent family history. The clinical and pathological symptoms as well as the onset age and duration depend on the mutational background into the *PRNP* gene. The majority of familial CJD is associated with a mutation in the *PRNP* gene at codon 200 (E200K) (Bertoni et al., 1992; Chapman et al., 1994; Goldfarb et al., 1991b; Rene et al., 2007) or with a mutation at position 178 (D178N) (Haltia et al., 1991).

- A **new variant of CJD (vCJD)** was first described in the UK in 1996 (Will et al., 1996) and is thought to originate from the consumption of cattle products contaminated with bovine spongiform encephalopathy (BSE) prions (Bruce et al., 1997). Experimental transmission of BSE to mice confirmed that vCJD is caused by the same prion strain that has led to BSE (Bruce et al., 1997). In addition, incubation period and neuropathological changes observed in mice transgenic for bovine PrP displayed similarities between BSE and CJD (Scott et al.,

1999) confirming that the BSE agent has crossed the species barrier and caused a zoonotic disease in humans (Bosque et al., 2002; Butler, 2002). Long incubation periods in prion diseases and the resulting late detection of the disease in an affected individual, respectively, raised concerns of an epidemic (Cousens et al., 1997; Ghani et al., 1998). vCJD can be distinguished from sCJD by a longer disease duration (up to 22 month versus 6 month), and distinct neuropathological features (e.g. earlier onset of the disease (age 19-39)). Despite the UK, where most vCJD cases have been reported, vCJD was also observed in many other countries worldwide, like France (Streichenberger et al., 2000), Spain (EuroSurveillance (Watt et al., 2005)), USA, Japan and Saudi-Arabia (EUROCJD/NEUROCJD). To date, no vCJD case has been reported for Germany (CJD Surveillance Unit Munich/Göttingen). Characteristic features comprise psychiatric symptoms and after a median period of 6 months ataxia, involuntary movements and cognitive impairment become apparent (Will et al., 2000). In contrast to other TSE forms, the abnormal prion protein isoform was additionally detected outside the CNS in tonsils (Hill et al., 1997) and lymphatic tissue (Ironside, 1998). For the development of secondary vCJD, which is distinct from the BSE originated vCJD, another source has been postulated based on sheep transmission experiments. BSE was efficiently transmitted via blood transfusion causing vCJD (Houston et al., 2000; Hunter et al., 2002). Despite blood products, tissue or organ transplantation might also transmit prion disease to humans causing vCJD (Peden et al., 2004; Wroe et al., 2006). Recently, efficient transmission of prion infection was observed in mice transgenic for human PrP inoculated with vCJD rectal tissue (Wadsworth et al., 2007), suggesting a possible risk for disease transmission via transplantation of gastrointestinal tissue. All reported vCJD cases displayed homozygosity for methionine at codon 129 in the *PRNP* gene (Collinge et al., 1996a; Collinge et al., 1996b) (Robert Will, CJD surveillance Unit Edinburgh, may 2008, personal communication). However, it is assumed that in principle all individuals, irrespective of the *PRNP* codon 129, might be susceptible to secondary transmission of vCJD through routes such as blood transfusion (Bishop et al., 2006).

Gerstmann-Sträussler-Scheinker syndrome (GSS) is mostly described in a familial context. To date only few sporadic cases have been reported (Masters et al., 1981). The syndrome was first described in 1928 by the Austrian neurologist Josef Gerstmann followed by a detailed report in collaboration with the colleagues Isaak Scheinker and Erwin Sträussler in 1936 (Gerstmann, 1936). This class of prion disease represents a chronic cerebellar ataxia concomitant with multicentric amyloid plaques. The majority of the affected families display *PRNP* point mutations at codon 102 (Hsiao et al., 1989). However, mutations e.g. on positions 198 (F198S), 117 (A117V) (Piccardo et al., 2001), 131 (G131V) (Panegyres et al., 2001) or 105 (P105L) (Kubo et al., 1995) have been

found in GSS patients. Mice carrying a substitution of leucine for proline at codon 102 (P102L) within the *PRNP* gene, spontaneously developed GSS-like neurologic symptoms of ataxia, lethargy, and rigidity accompanied by spongiform degeneration throughout the brain and served as a model for studying and reproducing GSS (Hsiao and Prusiner, 1991).

Fatal familial Insomnia (FFI) has been found to be worldwide distributed e.g. in Austria, (Almer et al., 1999), Italy (Padovani et al., 1998), Australia (McLean et al., 1997), Japan (Nagayama et al., 1996) and China (Spacey et al., 2004). Its occurrence is associated with a *PRNP* mutation at position 178 (D178N) (Medori et al., 1992a), which has also been observed in a subtype of familiar CJD (fCJD) (Medori et al., 1992b). FFI is characterized by thalamic degeneration, dysautonomia, dementia and insomnia. Although FFI and fCJD are linked to the same *PRNP* mutation at position 178, they can be distinguished by the methionine/valine polymorphism at 129 (Goldfarb et al., 1992). A sporadic form (sFI) has been reported in two patients displaying homozygocity for methionine at position 129 without any mutations in the *PRNP* gene locus (Scaravilli et al., 2000). An atypical case of FFI has been reported recently harbouring neither mutation nor homozygocity for methionine at codon 129 (Piao et al., 2005).

Kuru (means "trembling with fear" in the native language) resulted from transmission of prions by ritualistic cannibalism (Gajdusek, 1977). Restricted to the 'Fore' people in the eastern highlands of New Guinea, the disease occurred mostly in children and women due to consumption of brain from deceased familiy members (Alpers and Gajdusek, 1965). In 1959, the government banned the cannibalistic practice. Lesions observed in the brain of patients were similar to those found in scrapie infected animals (Gajdusek, 1967; Gajdusek et al., 1966).

Table 1. Human prion diseases

Human TSE	Origin	Initial description
sporadic CJD	Somatic mutation in the *PRNP* gene or spontaneous conversion of PrP^{c} to PrP^{Sc}	1921
iatrogenic CJD	Infectious origin (transmission through neurosurgery or contaminated hormons)	1974
genetic/familial CJD	4-9 additional octarepeat insertions in the *PRNP* gene, point mutations at position 178	1924
variant CJD	Infectious origin (transmission through contaminated food)	1996
FFI	Point mutation in the *PRNP* gene at codon 178	1986
GSS	Mutation in the *PRNP* gene at codon 102, 117, 198 or 217	1928
Kuru	Infectious origin (transmission through cannibalistic practice)	1955

1.1.1 Genetic background of human prion diseases

In genetic prion disorders, the disease phenotype seems to be determined by the combined effects of the *PRNP* polymorphism at the coding region 129 (two variant alleles: methionine or valine), other pathogenic mutations and the type of the abnormal prion protein (PrP^{Sc}). Three types of pathogenic *PRNP* mutations can be distinguished (i) point mutations leading to an amino acid substitution (ii) a premature stop codon or (iii) the insertion of additional octarepeats (Collinge, 2001; Collinge et al., 1992). Several subtypes of familial CJD have been associated with variable numbers of octapeptide coding repeats in the region of codon 51 to 91 (Goldfarb et al., 1991a). Point mutations are mainly located in the central and C-terminal region of PrP, and deletion and insertion mutations can be detected in the N-terminal region (Gambetti et al., 2003). The *PRNP* polymorphism at position 129 is suggested to correlate with the different disease types and might influence the clinical course and pathological findings in CJD patients (Gambetti et al., 1995; Miyazono et al., 1992). For instance, patients heterozygous for *PRNP* 129 (M/V) exhibit a later disease onset. CJD patients predominantly displayed homozygosity for either methionine (M/M) or valine (V/V). The incidence of methionine homozygosity was found to correlate with increased susceptibility to the development of sporadic CJD, with the relative risks for the three genotypes M/M: V/V: M/V being 11:4:1 (Windl et al., 1996). Recent studies suggest that a single nucleotide polymorphism at position 101 (C/G) in the regulatory region of the *PRNP* gene might be a risk factor for sCJD among M/V heterozygous patients (Bratosiewicz-Wasik et al., 2007). Additional data originating from transgenic mice suggest that human *PRNP* 129 heterozygotes might be more susceptible to a secondary vCJD infection than to cattle BSE prions and may represent a neuropathological phenotype distinct from vCJD (Asante et al., 2006). The variant CJD phenotype was also observed in mice transgenic for human *PRNP* 129 methionine homozygosity (Wadsworth et al., 2004) suggesting that the presence of valine at 129 may prevent vCJD. Recently, data on worldwide genetic TSEs between 1993 and 2002 have been collected and analysed (EUROCJD group) regarding *PRNP* mutations, pathological findings and phenotype (Kovacs et al., 2005). This study reported for example that (i) the majority of gTSE patients display methionine at codon 129 (M/M 67,9%; M/V 25.8%; V/V 6.3%) in combination with E200K, and (ii) that the distribution of gCJD is geographically heterogenous. Base pair insertions for example, associated with the gCJD phenotype, GSS and FFI cases, revealed longer disease duration compared to cases with point mutations. In addition, the *PRNP* 129 polymorphism seems to influence the phenotype of gCJD (Kovacs et al., 2005).

The polymorphic variation at position 129 on *PRNP* has been supposed to remarkably influence the ability of the protein to form amyloid fibrils spontaneously (Lewis et al., 2006b). Kinetic studies suggest that valine in contrast to methionine accelerates amyloid formation (Baskakov et al., 2005).

Further *in vitro* studies evidenced that polymorphism at 129 strongly influences the conformation of the amyloid fibrils (Jones et al., 2006).

1.2 Animal TSEs

Prion diseases have also been observed in a variety of animal species (Table 2), posing a risk for transmission to humans via prion contaminated food.

Scrapie has been discovered over 300 years ago (Brown and Bradley, 1998). It has been described as a slowly progressive disease causing neurodegeneration in the central nervous system (CNS), mainly in sheep and goats. The term "scrapie" originates from the tendency of affected sheep to scrape off their wool. The first atypical scrapie case was discovered in Norway 1998 (Benestad et al., 2003) newly classified as Nor98. The discovery was followed by reports of further unusual cases displaying distinct neuropathological findings (Klingeborn et al., 2006) for example in Portugal (Orge et al., 2004), in Germany and France (Buschmann et al., 2004), in Sweden (Gavier-Widen et al., 2004) and Belgium (De Bosschere et al., 2004), respectively.

Table 2. Animal prion diseases

Animal TSE	Origin	Initial description
Scrapie in sheep and goats	sporadic	1732
BSE in cattle	Infectious origin (transmission through scrapie contaminated food)	1987
CWD in captive mule, deer and elk	unknown	1967/1978
FSE in captive and domestic cats	Infectious origin (transmission through BSE contaminated food)	1986/1990
TME in minks	infectious origin (transmission through BSE contaminated food)	1947
PSE in non human primates e.g. macaques, lemur	unknown	1996
EUE in zoo antelopes	Infectious origin (transmission through contaminated food, probably BSE)	1986

Although sheep scrapie is the most common animal prion disease, **bovine spongiform encephalopathy (BSE)** has gained prominent attention, since oral uptake of infected meat evoked a

new zoonotic disease in humans. The first BSE case in cattle has been reported 1986 in the UK (Wells et al., 1987). After a long incubation period (four to five years), spongiform degeneration in the brain leads to a neurological disorder. BSE is supposed to originate from scrapie contaminated food prepared from rendered carcasses (Wilesmith et al., 1988). Different "prion" types have been identified, revealing distinct biochemical and histopathological properties concerning the abnormal prion protein. BASE (bovine amyloidotic spongiform encephalopathy) (Casalone et al., 2004), a recently discovered subtype of BSE, displays similarities to the human sCJD suggesting to originate from a distinct prion strain. Experimental serial transmission studies using inbred mouse lines indicated that BASE might represent the origin of BSE (Capobianco et al., 2007).

Chronic wasting disease (CWD) affecting captive and wild cervids including white tailed deer, mule deer, elk and moose has become economically important for North America. The disorder was first recognized in Colorado in 1967 and characterized in 1978 as a TSE (Williams and Young, 1980). In contrast to BSE no evidence from a possible transmission of CWD prions to humans causing a zoonotic disease could be found yet (Belay et al., 2004), but cannot be excluded.

Transmissible mink encephalopathy (TME) appeared in farmed mink in 1947 in Wisconsin and Minnesota (USA), probably through ingestion of scrapie contaminated meat (Marsh and Bessen, 1993).

Ingestion of BSE infected material has obviously caused **feline spongiform encephalopathy (FSE)** in captive cheetah, puma, ocelot, tiger and lion (Kirkwood and Cunningham, 1994). Also domestic cats have been diagnosed with FSE (Ryder et al., 2001). It is widely proposed that the observed FSE cases were caused by BSE contaminated commercial cat food. A ban on bovine spleen and CNS tissue in pet food was initiated in 1990 and all except one of the FSE cases to date occurred in cats born prior to the ban.

In 1996, a first case of spontaneously developed spongiform encephalopathy has been observed in a rhesus monkey (macaca mulatta) (primate spongiform encephalopathy, PSE) in France (Bons et al., 1996). Primates like lemurs infected with BSE displayed similar neuropathology to spontaneous infected zoo lemurs (Bons et al., 1999).

Exotic ungulate encephalopathy (EUE) cases were diagnosed in hoofed zoo species like greater kudu, eland, nyala, gemsbok, Arabian oryx, a scimitar horned oryx and a bison (Kirkwood et al., 1994) in British zoos.

Small animals like hamsters, mice or bank voles (Piening et al., 2006) can be infected experimentally and serve currently as models for the analysis of molecular and biochemical mechanisms in prion pathology.

In parallel to human polymorphisms and genetic susceptibility for the development of a prion disease, association studies of *PRNP* and TSE aquisition have also been reported for animals (Goldmann, 2008). For example, the ovine *PRNP* polymorphism have been found to influence the susceptibility to scrapie and BSE and modulates disease progression in sheep (Goldmann et al., 1990). Single amino acid changes as well as insertions/deletions within the octarepeat region of the N-terminus of the prion protein have been associated with the outcome of prion disease. In sheep, there are three *PRNP* codons (136, 154 and 171) that are of particular interest for both scrapie and BSE susceptibility. Ovine PrP allels comprise $A_{136}R_{154}Q_{171}$ (ARQ) and $V_{136}R_{154}Q_{171}$ (VRQ) (Goldmann, 2008). In elk, a polymorphism at codon 132 (methionine/leucine) of the *PRNP* gene, corresponding to the human 129 polymorphism, has been detected. Elk expressing 132 M/L and 132 L/L seemed to be overrepresented among CWD infected individuals concluding that this polymorphism might also influence susceptibility to prion disease (O'Rourke et al., 1999).

2. The cellular prion protein

The cellular prion protein (PrP^c) represents the central key player in prion diseases, which is during the course of disease proposed to be converted into an abnormal isoform, termed PrP^{Sc} (scrapie), PrP^d (disease associated) or PrP^{TSE}, respectively. Accumulation of the misfolded prion protein occurs mainly in the CNS, associated with neuronal cell death and plaque formation.

PrP^c is a monomeric N-glycosylated protein (30-35kDa) linked to cellular membranes via a glycosyl phosphatidylinositol (GPI) anchor (Stahl et al., 1987). There is evidence that PrP^c occurs also in the chimeric form (Hundt et al., 2003; Kaimann et al., 2008). Like other GPI-linked proteins, it is enriched in detergent-resistant membranes (DRM) or sphingolipid-cholesterol-rich membrane microdomains (SCRMs) (Vey et al., 1996). While expressed in nearly all cell types, PrP^c is abundantly expressed in neurons (Kretzschmar et al., 1986). In addition PrP^c expression has been reported in astrocytes (Moser et al., 1995), in the periphery on lymphoid tissues and leukocytes (Li et al., 2001; Paltrinieri et al., 2004) as well as in follicular dendritic cells (FDCs) (McBride et al., 1992; Thielen et al., 2001). Substantial amounts were also found in heart (Brown et al., 1990) and skeletal muscle (Bosque et al., 2002), intestinal tissues (Morel et al., 2004). In situ hybridization detected PrP^c mRNA also in uterus and testis (Tanji et al., 1995). PrP^c represents a highly conserved protein among mammals (Schatzl et al., 1995) (>85% homology between mouse and human sequence) and is universally expressed in vertebrate species (Wopfner et al., 1999). The corresponding *PRNP* gene was identified in 1986 (Basler et al., 1986; Chesebro et al., 1985) and has been mapped to 20p12-pter on the short arm of human chromosome 20 (Liao et al., 1986) and on mouse chromosome 2 (Sparkes et al., 1986).

2.1 Functional domains of the cellular prion protein

PrP^c (Figure 1) is composed of an N-terminal domain which contains a segment of four repeats of an octameric amino acid sequence (octapeptide repeat region) and one nonarepeat implicated in copper ion binding (Hornshaw et al., 1995). At the very N-terminus a 22 aa secretory signal peptide resides. A highly hydrophobic and conserved region, originally proposed as a transmembrane region (Yost et al., 1990) follows the octapeptide region. The amino acid length as well as position of prion proteins can vary among species to a small extend (Wopfner et al., 1999). In Syrian hamster and mice for example, PrP^c is synthesized as a precursor of 254 amino acids (aa), whereas the human equivalent consists of 253 aa. PrP^c can be present in distinct isoforms depending on proteolytic processing (full length and truncated), topology (GPI-anchored, transmembrane or soluble (see 2.2)) and glycosylation (non-, mono- and di-glycosylated). Glycosylation sites are variably occupied leading to non-, mono- or digylcosylated forms (Stahl et al., 1993).

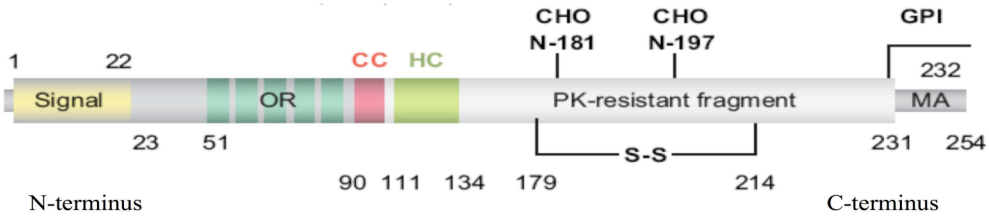


Figure 1. Primary structure of the Syrian hamster prion protein and posttranslational modifications. The hamster prion protein (254 aa) contains at the N-terminus a repeat region of four octarepeats and one nonarepeat (OR) implicated in copper binding. The highly structured C-terminus harbours two glycosylation sites (CHO) at Asparagin 181 (N-181) and 197 (N-197). During processing, a 22 amino acid long signal peptide at the N-terminus is cleaved and at the C- terminus 23 residues is removed upon addition of a GPI anchor to serin residue (Ser-231). Upon proteinase K digestion a resistant core remains designated as PrP^{res}. A loop is generated by the disulfide bond between two cystein residues (C179 - C214). MA, membrane anchor region; HC, hydrophobic core; CC, charged cluster. adopted from (Aguzzi et al., 2007a).

2.2 Trafficking of the prion protein

A newly synthesized PrP^c undergoes cleavage of the hydrophobic N-terminal signal peptide (22 amino acids) and is targeted to the rER, where it is co- and posttranscriptionally modified including

N-glycosylation at two potential sites and the replacement of the hydrophobic carboxy terminal signal peptide by a GPI lipidic moiety.

Due to the lack of a cytoplasmic domain, PrP^c is believed to be endocytosed through clathrin-coated pits (Figure 2) by binding extracellularly to a transmembrane receptor harbouring endocytic motifs on its cytoplasmic domain. In this context it has been recently reported that the low density related protein LRP-1 mediates PrP endocytosis and is important for trafficking of PrP^c in neurons (Parkyn et al., 2008). In addition, the 37 kDa/67 kDa laminin receptor LRP/LR, which is also a receptor for the prion protein (Gauczynski et al., 2001) is thought to be implicated in clathrin mediated prion endocytosis (Rieger et al., 1999).

It has been reported that the chicken homologue of the mammalian prion protein (chPrP) cycles between the cell surface and an endocytic compartment with a transit time of approx. 60 min. (Shyng et al., 1993). More than 95% of the internalized protein is returned to the cell surface intact, and the remainder is proteolytically cleaved within the highly conserved region in the NH_2-terminal half of the molecule (Sunyach et al., 2003). The N-terminal half of the chPrP polypeptide chain is essential for its endocytosis via clathrin-coated pits (Shyng et al., 1995). Studies on primary cultured neurons and N2a neuroblastoma cell line demonstrated also that PrP^c is rapidly and constitutively endocytosed. While it is on the cell surface, prion protein leaves the lipid raft domains to enter non raft-membrane domains from which it enters coated pits (Sunyach et al., 2003). The GPI anchor is thought to be responsible for the lipid raft association of PrP^c (Taraboulos et al., 1995). Cryoimmunogold electron microscopy studies revealed that PrP is also highly enriched in caveolae and caveolae-containing endocytic structures in CHO (Chinese hamster ovary) cells stably transfected with Syrian hamster PrP raising the possibility of another non-clathrin mediated uptake mechanism for PrP^c (Peters et al., 2003) (Figure 2). In addition to the cellular PrP (Harmey et al., 1995), the proteinase K resistant PrP was found in caveolae like domains (CLD) (Vey et al., 1996).

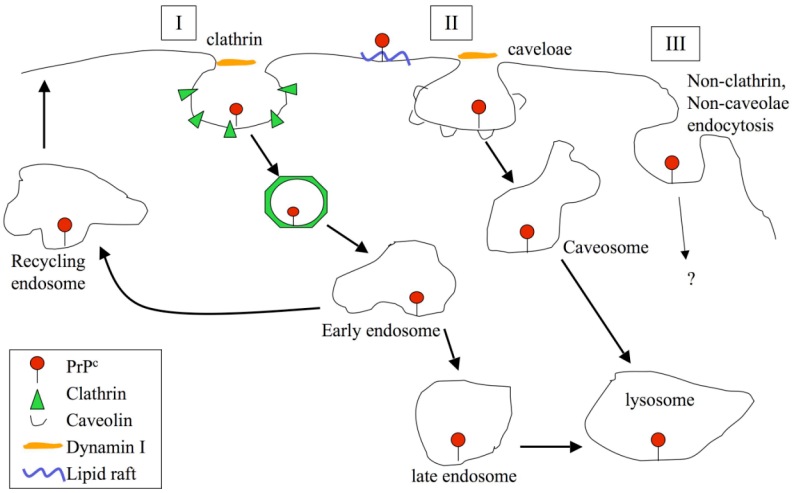


Figure 2. Trafficking of PrP^c demonstrating three possible endocytotic mechanism of PrP^c internalization. Both clathrin-mediated as well as caveolae mediated endocytosis are suggested to be implicated in prion protein life cycle since PrP^c was found to be associated with caveolin and clathrin. PrP^c transits through the secretory pathway to reach the cell surface. Finally, an additional non-clathrin and non-caveolin, but raft dependent endocytotic pathway has been proposed.

In contrast to the majority of glycoproteins which are synthesized in a single orientation with respect to the endoplasmatic reticulum (ER) membrane, PrP^c can be synthesized in different topological forms (Lopez et al., 1990; Yost et al., 1990): the fully translocated form ^{sec}PrP (Hay et al., 1987) and two transmembrane configurations ^{Ntm}PrP and ^{Ctm}PrP, respectively (Hegde et al., 1998; Lehmann and Harris, 1995). ^{Ntm}PrP and ^{Ctm}PrP, span the lipid bilayer once, via a highly conserved hydrophobic region (amino acid residue 11-134), with either the N- or the C- terminus, respectively, on the extracytoplasmatic site of the membrane. In addition, ^{Ctm}PrP has been proposed as a key neurotoxic intermediate for example in Gerstmann-Sträussler-Scheinker syndrome, suggested to cause neurodegeneration (Hegde et al., 1999; Stewart et al., 2001).

2.3 Tertiary structure of PrP^c

NMR measurements on prion proteins from various species including mouse (Riek et al., 1996), Syrian hamster (Donne et al., 1997) human (Zahn et al., 2000), cattle (Lopez Garcia et al., 2000), sheep, cat, dog and pig (Lysek et al., 2005), chicken turtle frog (Calzolai et al., 2005), elk (Gossert

et al., 2005) and african lion (Maj et al., 2008) share a common architecture. The structure of PrP^c is composed of a flexible unstructured N-terminal region (aa 23-124) and a well-ordered C-terminal globular domain (aa 125-228) including three α-helices and a two stranded antiparallel β-sheets that flank the first α-helix (Figure 3).

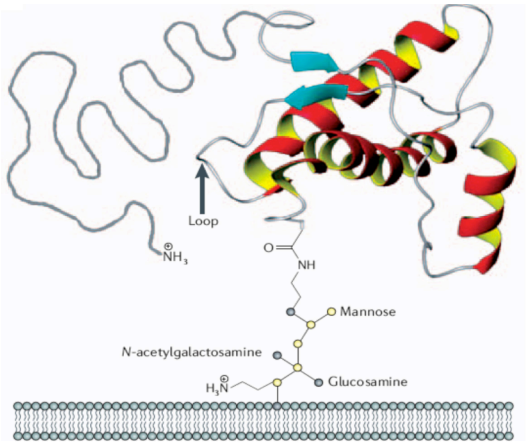


Figure 3. Model of the tertiary structure of the cellular prion protein deduced from NMR spectroscopy inserted into a lipid bilayer, including the unstructured N-terminal tail and the GPI anchor. Two antiparallel β-strands are indicated in blue, three α-helices are shown in red. Sugar residues are depicted as yellow circles. The indicated loop connects the second β-strand and the third α-helix. (adopted from (Aguzzi et al., 2007b)

2.4 Interaction partners for the cellular prion protein

Several PrP^c-mediated functions are related to the interaction with receptors, other proteins or chemical compounds (Vana et al., 2007).

PrP^c is a cell surface receptor for glycosaminoglycans (GAGs) (Gabizon et al., 1993; Pan et al., 2002). Binding of the prion protein to GAGs, laminin and the 37 kDa/67 kDa laminin receptor LRP/LR, along with the neuronal cell adhesion molecule (NCAM) mediates the contact between neurons and other cells and the extracellular matrix (Caughey and Baron, 2006). In addition interaction with LRP/LR together with heparan sulfate proteoglycanes (HSPGs) is an important event in binding and internalization of the cellular prion protein (Hundt et al., 2001).

The **neuroprotective activity** exerted by PrP^c (Chiarini et al., 2002) is associated with the binding to laminin (Graner et al., 2000) and/or to the precursor of the laminin receptor LRP/LR (Rieger et al., 1997). In addition, molecular chaperone and stress protein binding (Edenhofer et al., 1996; Jin et al., 2000; Zanata et al., 2002), or interaction with members of the antiapoptotic Bcl-2 family

(Kurschner and Morgan, 1995) has been reported. Interaction with the stress inducible protein 1 (ST-1) elicits neuroprotective signals that rescue cells from apoptosis (Zanata et al., 2002). Furthermore, the PrP^c-ST-1 interaction modulates short-term memory as well as long-term memory consolidation in rats (Coitinho et al., 2007). Direct interaction at the neuronal cell surface and association of PrP^c with the neural cell adhesion molecule (NCAM) in lipid rafts (Schmitt-Ulms et al., 2001), has been shown to be important for neurite outgrowth (Santuccione et al., 2005). Additionally, interaction with laminin in the hippocampus, suggests a critical role for PrP in memory processing by activation of both PKA and ERK1/2 signaling pathways (Coitinho et al., 2006).

2.5 The physiological function of the cellular prion protein

Although *PRNP* knock out mice ($PrP^{0/0}$) developed normal (Bueler et al., 1992) and display only minor phenotypic anomalies (Tobler et al., 1996), important physiological functions have been attributed to PrP^c including the regulation of immune responses, signal transduction and pro- as well as antiapoptotic functions (Hu et al., 2007) (Table 3 and Table III in (Vana et al., 2007)). Many functions have been studied using transgenic mouse models. Prion protein knock out mice ($PrP^{0/0}$ mice) exhibited no obvious alterations in synaptic transmission and neuronal excitability (Lledo et al., 1996). However, PrP^c knock out mice displayed alterations in nerve fiber organization (Colling et al., 1997), circadian rhythm (Tobler et al., 1996) and spatial learning (Criado et al., 2005), memory formation (Coitinho et al., 2006) or cognitive function. The functions seem to vary and depend on the cell type and location.

A variety of functions have been found concomitant with PrP^c expression. For example it has been suggested that PrP^c acts as a positive regulator of neural precursor proliferation during developmental and adult mammalian neurogenesis (Steele et al., 2006). Present on hematopoetic stem cells (Zhang et al., 2006), PrP^c is supposed to support their self renewal.

A regulatory role in apoptotic pathways within the photoreceptor has been also attributed to the prion protein. Studies demonstrated that the *in vivo* expression level of PrP^c in the retina of mice inversely correlated with the susceptibility of **photoreceptors to light damage.** Therefore PrP^c expression may execute neuroprotective activity for the receptors in the retina (Frigg et al., 2006).

Table 3. Physiological functions of the cellular prion protein PrP^c

Function	interaction partner/mode of action	Reference
Neuroprotection	PrP^c transduces neuroprotective signals	(Chiarini et al., 2002) (Roucou et al., 2004)
Survival and cell to cell interaction	PrP^c on astrocytes interacts with laminin and ST-1	(Lima et al., 2007) (Zanata et al., 2002)
Antiapoptotic	PrP^c inhibits Bax-induced cell death in human primary neurons PrP^c binds to bcl-2	(Bounhar et al., 2001) (Kuwahara et al., 1999)
Proapoptotic	PrP^c increases levels of p53	(Roucou and LeBlanc, 2005)
Modulation of T-cell response	Association with cytokine production	(Bainbridge and Walker, 2005)
T-cell development and activation	PrP^c inducec signal pathway: ZAP 70/Fyn; MAPK association	(Cashman et al., 1990; Mabbott et al., 1997; Mattei et al., 2004)
Synaptic function	Binding of PrP^c to copper	(Brown, 1999; Collinge et al., 1994)
Memory consolidation	PrP^c interacts with ST-1	(Coitinho et al., 2007)
Cell adhesion	PrP^c interacts with NCAM and LRP/LR	(Mange et al., 2002; Schmitt-Ulms et al., 2001)
Oxidative stress	PrP^c protects against ROS species mediated DNA damage	(Watt et al., 2007)
SOD activity	PrP^c binds to copper	(Vassallo and Herms, 2003)
Cell redox homeostasis	PrP^c activates signaling pathway: Fyn/ERK1/2 activation	(Schneider et al., 2003)
Copper uptake	Expression of PrP^c at the synapse	(Brown et al., 1997a; Kretzschmar et al., 2000)
Nervous system development	PrP^c interacts with NCAM	(Santuccione et al., 2005)
Phagocytosis/ inflammatory response	PrP^c expression on leucocytes activation of signaling pathways	(de Almeida et al., 2005)

PrP^c has been demonstrated to play a **role in other neurodegenerative diseases** such as Alzheimers disease or Chorea Huntington. Overexpression of cellular prion protein protects cells from huntingtin aggregation by increasing the proteasomal activity and reducing the reactive oxygen species (ROS) (Lee et al., 2007). Since prion protein deposits have been found in senile plaques in the brain of Alzheimer patients (Ferrer et al., 2001), a role for PrP in Amyloid beta (Aβ) plaque formation has been proposed (Schwarze-Eicker et al., 2005). In addition, it has been shown that the prion protein regulates the ß-secretase activity (Parkin et al., 2007), an enzyme which is responsible for the proteolytic processing of the amyloid precursor protein (APP) leading to beta amyloid plaque formation in Alzheimer patients (Vassar et al., 1999).

It has been demonstrated, that **the octapeptide repeat region** of the prion protein is able to bind metal ions such as Zinc (II) (Gaggelli et al., 2005) or manganese (II) (Jackson et al., 2001) and up to four copper ions (Viles et al., 1999). Furthermore, the binding ability of PrP^c to copper *in vivo* (Brown et al., 1997a) is related to an anti-oxidant activity (Brown et al., 2001) and stimulates endocytosis of PrP^c (Pauly and Harris, 1998) via clathrin coated pits. It has been recently reported that the transmembrane LRP1 (LDL receptor-related protein 1) is required for the Cu^{2+}-mediated endocytosis of PrP^c in neuronal cells (Taylor and Hooper, 2007). A role as a copper sensor in synaptic transmission has also been proposed for PrP^c (Vassallo and Herms, 2003). An additional site within aa residues 91-111 is controversially discussed as the so-called fifth site implicated in copper binding (Burns et al., 2003). Deletion of the copper binding octapeptide region has been reported to impair **SOD like activity of PrP^c** (Brown et al., 1999a), confirming the **neuroprotective effect** of PrP^c (Kuwahara et al., 1999). It has been reported that PrP^c protects cells against reactive-oxygen-species-mediated DNA damage requiring the octapeptide repeat region (Watt et al., 2007). In a process termed beta-cleavage, PrP^c is cleaved at the end of the copper-binding octapeptide repeats through the action of reactive oxygen species (ROS). Lack of ROS-mediated beta-cleavage of PrP has been demonstrated to correlate with the sensitivity of the cells to oxidative stress (Watt et al., 2005). However, the SOD activity is under discussion since *in vivo* experiments did not confirm such an activity (Hutter et al., 2003), as well as recombinant PrP has not been proven to exhibit SOD activity, suggesting that the role in oxidative stress might be an indirect one (Jones et al., 2005).

Recently, it has been demonstrated that PrP^c might serve as a receptor for neurotrophins, mediating additional **neuroprotective effects** (Chiarini et al., 2002) probably through the inhibition of the mitochondrial proapoptotic pathway (Marciano et al., 2004). PrP^c activation on the cell surface through antibodies led to contradictory results. On the one hand, *in vitro*, antibody-mediated PrP^c dimerization led to a rapid phosphorylation of extracellular regulated kinases (ERK) 1 and 2, and promoted neuronal survival (Schneider et al., 2003). On the other hand, cross-linking of PrP^c by monoclonal antibodies *in vivo* was found to induce rapid and extensive apoptotic cell death of

hippocampal and cerebellar neurons suggesting a role of PrP in the control for neuronal survival (Solforosi et al., 2004). Since PrP^c is present in synaptic plasma membrane and in synaptic vesicles (Herms et al., 1999), it appears to be partly responsible for early **synaptic transmission** in neurons and reorganization of neuronal circuitry in the hippocampus (Colling et al., 1997).

Pro as well as **antiapoptotic functions have been attributed to** the prion protein (for review (Roucou and LeBlanc, 2005)). The absence of PrP^c in hippocampal neurons led to apoptotic cell death *in vitro* (Kuwahara et al., 1999). A structural similarity between PrP^c and the BH2 domain of B-cell lymphoma (Bcl)-2 family members have been observed and it could be demonstrated that PrP^c protects human neurons against Bax (Bcl-2-associated X protein)-mediated cell death in an *in vitro* culture system (Bounhar et al., 2001). The pro-apoptotic protein Bax is expressed in neurons and initiates the release of apoptotic factors by mitochondria, accelerating cell death (Martinou and Green, 2001). Expression of Bcl-2 prevents Bax-induced apoptosis in human neurons. Coexpression of PrP^c also completely prevented Bax-induced cell death. Therefore, PrP^c in the CNS might protect neurons against Bax-mediated cell death (Bounhar et al., 2001; Roucou et al., 2004).

PrP^c expression was also found in **correlation to cancer**. For example an antiapoptotic activity of PrP^c has been demonstrated in a human breast carcinoma cell line (Diarra-Mehrpour et al., 2004). Liang et al discovered a Bcl-2 dependent antiapoptotic effect of PrP^c (Liang et al., 2006). Expression of PrP^c in a gastric cancer cell line led to an upregulation of Bcl-2 whereas p53 and Bax were downregulated. In another study the same group demonstrated that PrP^c is overexpressed in metastatic cell lines compared to nonmetastatic ones (Liang et al., 2007). The N-terminal fragment (amino acid 24-90) was suggested to be an indispensable region for signal transduction and invasion-promoting function of PrP^c. It has been suggested that the existence of the N-terminal region of PrP^c might promote the invasive and metastatic abilities of gastric cancer cells. In addition, this group proposed that PrP^c expression might be required for transcriptional activation of Cyclin D1 to regulate the G1/S phase transition, mediated by the octapeptide region.

2.6 The prion protein and its relation to prion diseases

Although the physiological functions of the prion protein remain unclear, it is widely accepted that the pathogenesis of prion diseases requires the expression of PrP^c. The finding that knockout mice carrying a homozygous deletion of the *PRNP* gene fail to develop disease upon inoculation with infectious brain homogenates (Bueler et al., 1993) demonstrated that the presence of the prion protein is important for the development of the disease. In addition, brain tissues of PrP^c deficient

cattle (Richt et al., 2007) were resistant to prion propagation confirming that the presence of PrP^c is crucial for the development of BSE in cattle. Furthermore, all cases of familial TSE cases are characterized by genetic mutations within the *PRNP* gene (Hsiao and Prusiner, 1990), which argues for a major role of the PrP^c in this class of neurodegenerative disorders.

2.6.1 The nature of the prion agent

The theory of a self propagating proteinaceous agent (Prusiner, 1982) was proposed after the isolation of a proteinase K resistant glycoprotein from scrapie infected hamster brain. It was associated with infectivity and identified as the scrapie agent designated the prion protein PrP^{Sc} (Bolton et al., 1982). Due to the observed resistance to UV, high pressure/temperature or nucleases, the agent had been proposed to be devoid of nucleic acids (Alper et al., 1967; Griffith, 1967; Weissmann, 1991). Prusiner, who gained the novel price for medicine in 1997 (Bonn and Ault, 1997), defined the term prion as a "small proteinaceous infectious particle that resists inactivation by procedures which modify nucleic acids" (Prusiner, 1982). It was the first time that a hypothesis suggested that a disease causing agent is composed of a single protein only. The second hypothesis termed "virino hypothesis" (Dickinson and Outram, 1988) postulates that the infectious agent is composed of a scrapie specific nucleic acid associated or coated with the host encoded protein PrP^c. However no striking evidence was found which argues for a TSE specific nucleic acids (Safar et al., 2005). For example virus like particles have been found in neuroblastoma cells infected with Creutzfeldt Jakob disease agent (Manuelidis et al., 2007) which might exclude a solely prionic nature.

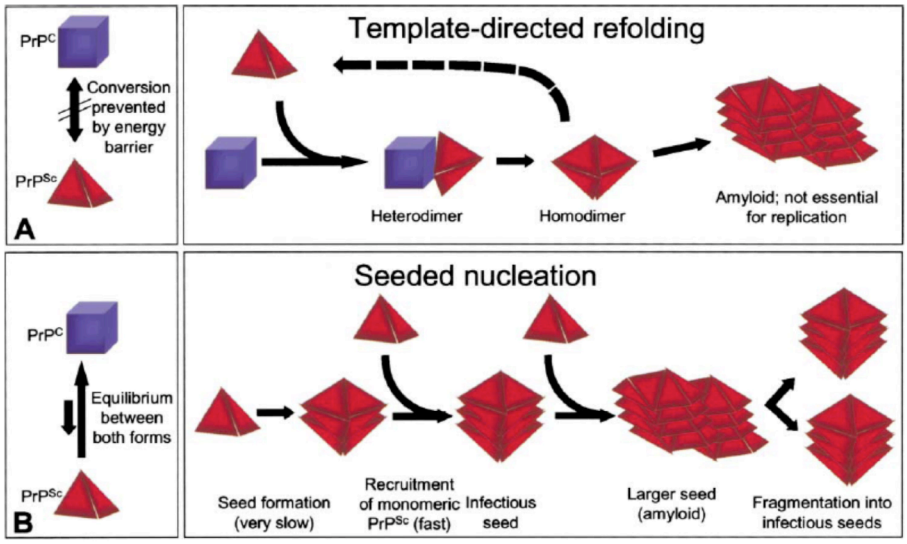
2.6.2 Generation of PrP^{Sc} – mechanisms of conversion

The largely accepted hypothesis for the prion propagation describes that an abnormal prion protein conformation, which propagates itself in an autocatalytic manner, binds to the cellular form (corresponding to the substrate) and converts it to the infectious isoform. Two models have been postulated to explain the mechanism covering the generation of the aggregated prion protein termed PrP^{Sc} or PrP^d.

The template directed refolding model (Figure 4) postulates an interaction between endogenous PrP^c and the exogenously produced PrP^{Sc} resulting in a heterodimer, promoting the conformational

conversion of the cellular prion protein into the PrP^{Sc} conformation (Prusiner, 1991). Although the conversion process is extremely slow in the absence of PrP^{Sc}, it is irreversible. This is due to the extremely stable PrP^{Sc}, which is kinetically inaccessible. PrP^{Sc} initiates a catalytic cascade using PrP^{c} or a partially unfolded intermediate (PrP*) converting it by conformational change into a more stable beta rich structure. The newly formed PrP^{Sc} turns again another PrP^{c} molecule to a PK resistant PrP molecule.

The seeding or nucleation model (Figure 4) proposes that the formation of PrP^{Sc} is initiated by a seed of aggregated PrP^{Sc} triggering a nucleation-dependent polymerization process (Come et al., 1993; Jarrett and Lansbury, 1993). Conversion between the two forms is extremely fast. However, the PrP^{c} conformation is thermodynamically favored. The presence of an aggregated seed, the "nucleus" stabilizes the unfavoured conformation. Recent studies indicate that the seeding model is actually the favoured model for prion conversion (Lee and Caughey, 2007).



(Lansbury and Caughey, 1995)

Figure 4. Models explaining the PrP^{Sc} generation and propagation. The catalytic model (A) proposes that PrP^{Sc} promotes the conversion of PrP^{c} into PrP^{Sc}. The generated stable molecule is now able to bind and catalyse the conversion of further PrP^{c} into the disease associated PrP^{Sc} form. The nucleation-polymerisation (seeding) model (B) postulates that polymerization of PrP^{Sc} molecules result in an infectious seed, which is able to recruit further PrP molecules to build an amyloid structure. Fragmentation of large seeds results in several aggregates, acting themselves as a new seed. (adopted from (Aguzzi and Polymenidou, 2004))

2.6.3 Characteristics of proteinase K resistant PrP fragments

The conversion of the cellular form to the infectivity associated PrP^{Sc} involves changes in the secondary structure characterized by protease resistant, aggregated PrP (multimeric) and higher beta sheet content. Infrared spectroscopy and circular dichroism demonstrated that the secondary structure of PrP^{c} is mainly composed of α-helices (42%), whereas PrP^{Sc} consists mainly of β-sheets (45%) (Cohen et al., 1994) (Table 4). The acquisition of the high beta sheet content enhances the resistance to PK (proteinase K) digestion (Caughey et al., 1991b; Pan et al., 1993) and renders it insoluble, which is the main reason that the three dimensional structure has not yet been resolved. In addition this protein has the tendency to form large, heterogenous aggregates. Based on the higher β-sheet content, several models have been hypothesized, whereas the model of Cohen and colleagues is one of the most favoured (Figure 5). The C-terminal portion of the prion protein, corresponding to the reported protease-resistant core fragment of the abnormal isoform PrP^{Sc}, was found to be essential for prion propagation. This is supported by the finding that antibodies to the C-terminal portion of PrP inhibit PrP^{Sc} accumulation in cells persistently infected with prions (Enari et al., 2001; Peretz et al., 2001).

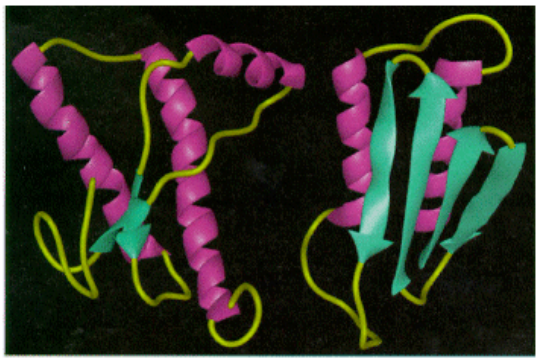


Figure 5. Postulated model of the three dimensional PrP^{Sc} structure (right) compared with already solved PrP^{c} structure) (left) The amino acid region 90-160 has been modeled onto α-helical architecture, while the C-terminal helices are preserved from PrP^{c}. (adopted from http://scienceblogs.com/retrospectacle/2007/2/basic_concepts_prions.php).

It has been reported that Proteinase K digestion of scrapie infected hamster brain extract and, to a lesser extent normal brain homogenate, yielded a fragment designated PrP^{27-30} in infected brain (Figure 6a) (Oesch et al., 1985). Under conditions where PrP^{c} and most other proteins are degraded, PrP^{Sc} is cleaved between aa residues 87 and 91 (depending on the prion protein species). This results

in a fragment (aa90-233) with the molecular weight of 27-30 kDa. PrP^{27-30} displays a characteristic electrophoretic mobility shift of the three bands corresponding to nonn-, mono- and diglycosylated forms, which is resistant to proteinase K digestion (therefore also termed PrP^{res}) (Figure 6a). Depending on the disease, distinct biochemical glycosylation pattern can be observed designating different prion strains (different PrP species) (Figure 6b). The relative ratios of the three glycosylation forms among the various disease phenotypes are used for prion strain phenotyping (Aguzzi et al., 2007a). PrP^{27-30} has been reported to accumulate in rod-shaped polymers (McKinley et al., 1991) that are insoluble in aqueous, organic solvents as well as in nonionic detergents (Caughey et al., 1991b) and is able to build amyloid fibrils. In contrast PrP^{Sc} has a tendency to form aggregates (McKinley and Prusiner, 1991) (Table 4).

Table. 4. Biochemical properties of cellular prion protein and Proteinase K cleaved derivatives

isoform	*α-helix (%)*	*β-sheet (%)*	*solubility*	*Proteinase K digestion*	*aggregation status*
PrP^{c}	43	~3%	soluble	sensitive	monomeric/chimeric
PrP^{Sc}	20	34	insoluble	partially resitant	aggregates
PrP^{27-30}	29	31	insoluble	resistant	amyloid fibrils

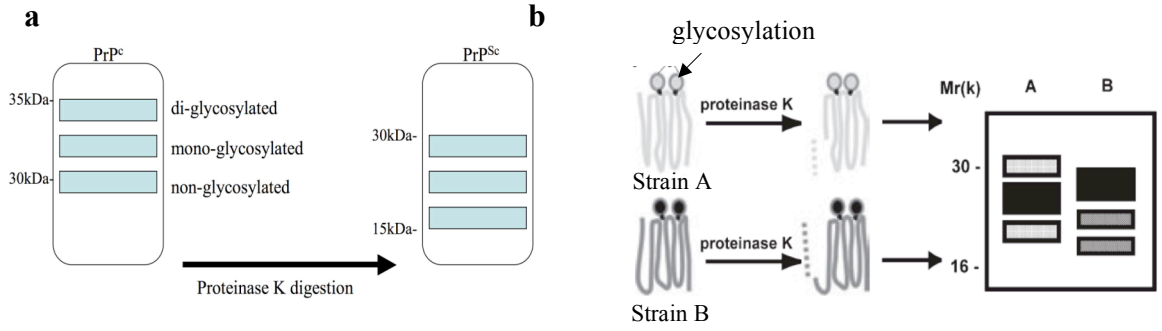


Figure 6. The glycosylation pattern enables discrimination between PrP^{Sc} and PrP^{c}, as well as between different prion strains. PrP^{c} can be distinguished from PrP^{Sc} by limited proteolysis under conditions where PrP^{c} is hydrolyzed and PrP^{Sc} is resistant. (a) To determine the PrP^{Sc} presence, tissues are digested with Proteinase K, which cleaves approx. 70 amino-terminal residues, resulting in a shift in the molecular mass (b) Different "prion" strains reveal distinct fragment pattern prion strains vary in their proteinase K cleavage sites, leading in differences in the molecular mass of the three glycoforms observed by western blotting. (modified from (Lawson et al., 2005))

2.6.4 Methods for the prion conversion mechanism

To elucidate the *in vivo* conversion mechanism as well as the required components, techniques such as the cell free conversion assay or the protein misfolding cyclic amplification (PMCA) have been developed. In 1995, Caughey and colleagues demonstrated that PrP^{c} can be converted into the PK resistant form PrP^{res}, in the presence of PrP^{Sc} in a cell free system (Caughey et al., 1999; Horiuchi and Caughey, 1999). The *in vitro* generated PrP^{res} has been confirmed to be infectious. Therefore, it has been concluded that PrP^{Sc} is formated through a nucleation dependent polymerization mechanism, which is inconsistent with a heterodimer mechanism (Caughey et al., 1995). The invention of the PMCA method in 2001 (Saborio et al., 2001) provided strong evidence for the protein-only hypothesis. PrP^{Sc} is amplified by incubation with brain homogenates followed by cycles of sonication (Figure 7a). Sonication is required to fractionate PrP^{Sc} aggregates into smaller units, which serve themselves as a seed. Interestingly, these small seeds were reported to be more infectious than larger amyloid fibrils (Silveira et al., 2005). During PMCA, disruption of growing PrP^{Sc} molecules by repeated sonication in the presence of detergent occurs. In addition, PMCA reactions have been compared with reactions lacking the sonication step concluding that aggregate breakage is essential for prion propagation (Piening et al., 2005). Soto and coworkers employed the PMCA method for bioassays (Figure 7b) and found that *in vitro* generated PrP^{res} by PMCA led to a scrapie disease in hamster identical to the illness produced by brain infectious material (Castilla et al., 2005a). This cell free amplification method is currently used for the detection of extremely low PrP^{Sc} amounts in different tissues (e.g. in blood (Castilla et al., 2005b)) and for diagnostic purposes (Atarashi et al., 2007; Jones et al., 2007). Since brain homogenate as a template is required for PMCA, other factors involved in the conversion process are not excluded.

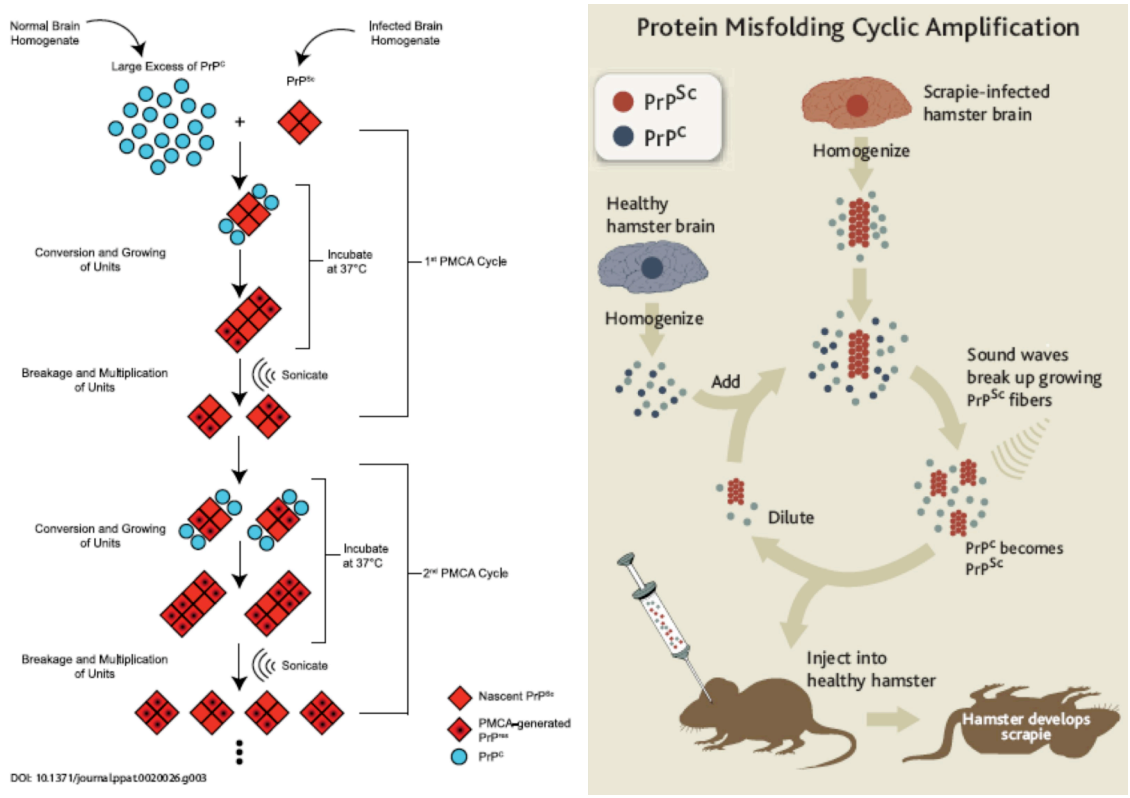


Figure 7. Schematic presentation of the procedure of the protein misfolding cyclic amplification (PMCA) method. A) (adopted from (Watts et al., 2006)) This method is used to amplify prion proteins which are below detection level. PrP^{Sc} can be subsequently determined by western blot analysis or infection with animals. Brain homogenate from a healthy donor is first incubated with a PrP^{Sc} seed which might derive from a TSE infected brain. After alternating cycles of incubation followed by sonication (should mimic fragmentation) the infectivity can be determined by injection of the PMCA mix into animals (bioassay) (b) adopted from (Enserink, 2005). For subsequent serial amplification the PMCA product is diluted into a new brain homogenate.

2.6.5 Prion conversion – involvement of cofactors and localisation

Posttranslational modifications of PrP^{c} such as the GPI anchor (Campana et al., 2007) or glycosylated site chains have been proposed to influence the conversion ability of PrP^{c} into PrP^{Sc} (Ermonval et al., 2003). The conversion process itself is thought to take place at the cell surface, in endosomes or lysosomal compartments (Arnold et al., 1995; Magalhaes et al., 2002). In 1992, abnormal high numbers of lysosomes and lysosome related structures (multivesicular and tubulovesicular dense bodies) have been detected in neuronal cells of scrapie infected brains, containing PrP (Laszlo et al., 1992). Cell culture studies using scrapie infected neuroblastoma cells indicate that conversion occurs in the plasma membrane or along with the endocytic pathway (Caughey et al., 1991a). Both PrP^{c} and PrP^{Sc} have been found in caveolae like domains, suggesting that the conversion may occur within these compartments (Vey et al., 1996). However, since

immunogold labeling of disease specific PrP in scrapie infected mice revealed the presence of the prion protein in the extracellular space (Jeffrey et al., 1994), it has been suggested that conversion does not exclusively occur inside the cell. Lipid rafts have been reported to be involved in conformational conversion of PrP^{c} to PrP^{Sc}. In the brain of different mammalian species a PrP^{c} C-terminal fragment was present in detergent-resistant raft domains (DRM) (Laffont-Proust et al., 2006). Raft domains are recently proposed to play a critical role in the conversion process (Baron et al., 2002). For example it has been demonstrated that sphingolipid-rich rafts play an essential role in the posttranslational (Borchelt et al., 1990) formation of the scrapie prion protein PrP^{Sc} (Taraboulos et al., 1995). It has been proposed that for conversion both the PrP^{c} and the infectious PrP^{Sc} has to be inserted into the raft membrane (Baron et al., 2002). Recent biophysical studies indicate that for conversion not the raft itself is required but rather other factors, e.g. increased local PrP concentrations or high concentrations of membrane-associated conversion factors (Eberl et al., 2004).

To date no report confirmed that the TSE agent is exclusively composed of the protein alone, pointing out that other molecules might be crucial for prion conversion and infectivity propagation. Several groups reported the involvement of additional factors in the conversion process. A potential factor termed protein X, which might act as a chaperone participating in prion formation, has been postulated for an involvement in the conversion process (Telling et al., 1995).

Several reports argue for participation of metal ions in the conversion process. For example manganese has been demonstrated to reveal a pro-aggregatory effect on PrP^{c}, whereas copper seem to counteract aggregation of PrP (Giese et al., 2004). Host encoded factors proposed to be involved in the conversion process should stabilize PrP^{Sc} structure on the one hand or might help triggering the conversion on the other hand. For example polysaccharides are found as prion rod components in scrapie infected brain (Appel et al., 1999). In addition, lipids have been observed as components surrounding the "prion environment" (Klein et al., 1998). Scrapie associated PrP was captured by an anti-DNA antibody suggesting that nucleic acids represent possible components in the infectious prion agent (Zou et al., 2004). Indeed, DNA has been reported to convert PrP into a beta sheet conformation (Cordeiro et al., 2001) proposing a catalytic action of host derived nucleic acids (Cordeiro and Silva, 2005). Recently, find nucleic acids, which might trigger infection, were not confirmed to be prion specific but host derived (Safar et al., 2005).

Several studies demonstrated that polyanionic compounds such as host encoded RNA (Deleault et al., 2003) or proteoglycan molecules (e.g. heparan sulfate) (Wong et al., 2001) were able to support prion seeded conversion of PrP^{c} to PrP^{Sc} *in vitro*. Recently, PMCA triggered de novo formation of PrP^{Sc} from purified brain derived hamster PrP was reported to require only synthetic poly(A)+ RNA and lipid molecules (Deleault et al., 2007). In addition, these *in vitro* generated purified PrP^{Sc}

molecules were infectious *in vivo*. Infectivity could be generated spontaneously, without the addition of brain derived PrP^{Sc} seeds, which might explain the spontaneous development of sporadic Creutzfeldt Jakob disease. Since the conversion process is suggested to occur at the plasma membrane and/or in endocytic vesicles of infected cells (Borchelt et al., 1992; Caughey and Raymond, 1991), Glycosaminoglycans (GAGs), for example, present at the plasma membrane, are likely to be the *in vivo* counterparts of polyanionic compounds. In contrast single stranded nucleic acids are usually not present at the cell surface or in endosomes. In addition, GAGs are found in PrP^{Sc} deposits *in vivo* (Snow et al., 1990), and heparan sulfate proteoglycanes (HSPGs) act as receptors for PrP^{Sc} (Ben-Zaken et al., 2003; Hijazi et al., 2005; Horonchik et al., 2005). Since the 37 kDa/67 kDa laminin receptor LRP/LR represents a cell surface receptor for PrP^{c} (Gauczynski et al., 2001) and PrP^{Sc} (Gauczynski et al., 2006), the hypothesis that conversion takes place at the cell surface is supported. However, direct participation of LRP/LR has not yet been proven.

2.6.6 Infectivity does not always correlate with proteinase K resistant PrP

Infectivity is not in all cases associated with PrP^{Sc} depositions. In 1997, Lasmezas and colleagues demonstrated that upon intracerebral BSE infection, more than half of the infected mice exhibited no detectable PrP^{res} although displaying neurological disease (Lasmezas et al., 1997). In addition, Barron and coworkers confirmed that tissues containing little or no proteinase K-resistant PrP are also infectious and harbour high titers of TSE infectivity (Barron et al., 2007), suggesting that, supplementory transmissible infectious agents in addition to PrP^{res} might exist. Beside PrP^{Sc}, aberrant regulation of protein biogenesis and topology at the endoplasmic reticulum can also result in neurodegeneration as demonstrated by Hegde and colleagues, who found that ^{ctm}PrP is responsible for the pathology in the absence of proteinas K resistant PrP^{Sc} (Hegde et al., 1998).

A reliabable test for infectivity remains to be the bioassay in animals. For example, in 2005, Nazor *et al* could demonstrate that a protease sensitive PrP^{Sc} is associated with the GSS disease in transgenic mice (Nazor et al., 2005).

2.6.7 Mechanisms of neurodegeneration

Several mechanisms or a combination of different processes are proposed to be responsible for neurodegeneration in prion diseases, (i) the loss of PrP^c function, (ii) toxic intermediates and/or side effects involved in/resulting from conversion of PrP^c, and (iii) the toxic gain of function (Figure 8).

It is suggested that the normal **neuroprotective function of PrP^c is lost** (Figure 8) during conversion or interaction with PrP^{Sc} and is involved in the mechanism of neurodegeneration.

Overexpression of PrP was demonstrated to rescue cells from death (Kuwahara et al., 1999) and to protect cells from oxidative stress (Brown et al., 2002). Due to the suggested action as an effective anti-apoptotic protein, the **loss of function** might result in a reduction of the anti-apoptotic capacity of PrP^c contributing to the pathogenic mechanism (Kim et al., 2004). In addition, the prion protein is necessary for normal synaptic transmission, a loss of function may contribute also to the early synaptic loss and neuronal degeneration (Collinge et al., 1994).

The Cu^{2+} binding ability (Brown et al., 1997a) plays a critical role regarding the function of PrP^c. Copper binding to the octarepeat region of PrP has been suggested to inhibit prion pathogenesis (Harris and True, 2006; Hijazi et al., 2003). In addition the *in vitro* conversion of the prion protein into amyloid fibrils was inhibited through Cu^{2+} binding indicating that the copper mediated physiological PrP function contributes to the neuropathogenesis of prion disease (Bocharova et al., 2005). The role of copper in prion disease pathology is not entirely clear, since it was also shown that copper ions stabilize the disease-specific isoform against proteolytic clearance and enhanced the resistance of amyloid (Kuczius et al., 2004). In addition, PrP^c has been implicated in higher resistance of cells to oxidative stress or copper toxicity (Brown et al., 1997b), suggesting that the loss of PrP^c correlates with the lack of prevention of copper toxicity due to an impaired Cu^{2+} metabolism. The octarepeat region has been reported to play a role in generation of the proteinase K resistant fragment PrP^{Sc} since cells expressing PrP^c devoid of this region did not produce PrP^{Sc} (Sakudo et al., 2008). Transgenic mice expressing an N-terminally truncated form of PrP spontaneously develop a neurodegenerative phenotype (Li et al., 2007b). Another study reported that transgenic mice expressing the mouse homolog of a mutant human PrP which contained a nine octapeptide insertion, display a prion disease accompanied by neurological disorders (Chiesa et al., 1998).

An additional mechanism contributing to the neurodegenerative phenotype represents the **toxic gain of function**. This implicates that after conversion of the prion protein to the abnormal form the prion agent receives toxic properties for example a neurotoxic activity (Figure 8). Additional deposition of amyloid plaques and blockage of axonal transport might result from PrP^{Sc} aggregates, likely leading

to perturbation of the normal physiological activity. The central region of the prion protein stretching from 105-125 (in humans 106-126) is suggested to be implicated in neurotoxicity. Mice carrying deletions in this region spontaneously developed a neuropathological phenotype (Shmerling et al., 1998) and neonatal lethality (Li et al., 2007a). The corresponding synthetic peptide PrP 106-126 shares many characteristics with PrP^{Sc} and is largely used to elucidate the toxic mechanisms underlying prion diseases. PrP 106-126 reveals a high tendency to aggregate into fibrils and lead to neuronal death of primary rat hippocampal cultures (Forloni et al., 1993). Furthermore, the peptide has been demonstrated to induce oxidative stress by free radicals (Brown, 2005) and trigger endoplasmic reticulum stress-induced apoptotic cell death (Ferreiro et al., 2008). However, small amounts of detergent-insoluble proteinase K resistant PrP aggregates were also found in healthy human brains suggesting that this PrP form is not necessarily neurotoxic (Yuan et al., 2006). Transgenic mice expressing PrP lacking the C-terminal GPI anchor displayed abnormal protease-resistant PrP^{res} deposition in amyloid plaques but only minimal scrapie neuropathology proposing that membrane attachment of PrP^{c} is essential for transducing PrP^{Sc} derived neurotoxic signal (Chesebro et al., 2005) (Figure 8). A contradictory study demonstrated that the cleavage of the GPI anchor did not alter infectivity in a mouse bioassay (Lewis et al., 2006a).

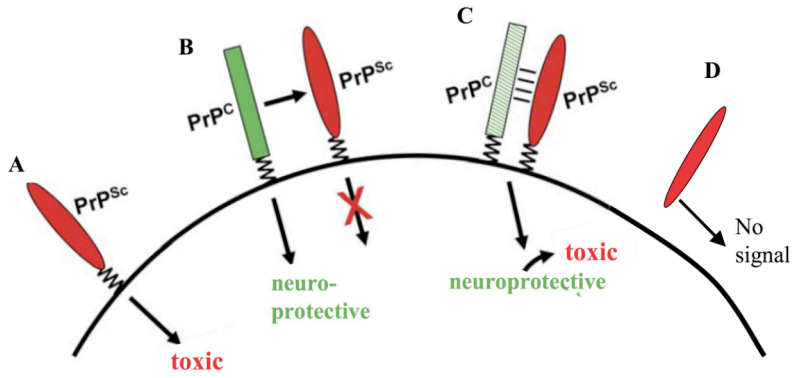


Figure 8. Possible relationship between PrP^{c} and PrP^{Sc} interactions on the cell surface. Neuronal loss is suggested to result from toxic signal delivered by PrP^{Sc} (toxic gain of function) (A), PrP^{c} looses neuroprotective activity upon conversion top PrP^{Sc} (loss of function) (B), or subversion of PrP^{c} by PrP^{Sc} generating a toxic signal of the original protective one (C). Data from GPI anchorless mice suggest that PrP^{c} lacking the GPI anchor delivers no signal and is therefore not toxic (D). (modified from (Harris and True, 2006))

Another proposal for the pathogenic mechanism involves **subversion of the normal neuroprotective functions** of PrP^{c} (Figure 8). PrP^{c} is converted by the action of PrP^{Sc} into a transducer of neurotoxic signals (Harris and True, 2006). This might probably result from PrP^{Sc} induced aggregation of PrP on the cell surface. Consistent with this, it has been demonstrated that

cross-linking of PrP^c on the cell surface by antibodies results in apoptotic death in CNS neurons (Solforosi et al., 2004). Another possibility would be that PrP^{Sc} bind and block specific regions on PrP^c, which are required for the delivery of a neuroprotective signal or just alter the normal signaling properties (Westergard et al., 2007).

Astrocytosis as well as microglial activation are observed features in prion diseases, since these cells are for example increased in the brain of CJD patients (Van Everbroeck et al., 2002). Microglia are associated with amyloid plaques and supposed to contribute to the development of spongiform vacuoles (Rezaie and Lantos, 2001). In addition, in scrapie infected mice microglial activation has been demonstrated to be involved in the neurotoxicity of PrP^{Sc} (Giese et al., 1998). Neuronal cell death in the brain is associated with chronic inflammatory response dominated by microglia, which is suggested to contribute to the spread of infection for the whole symptomatic period of the disease (Szpak et al., 2006). Through microglial activation, which has been found to be upregulated in CJD or GSS, inflammatory proteins such as prostaglandin E are released and elevated in the cerebrospinal fluid (CSF) of CJD patients (Minghetti and Pocchiari, 2007). Astrocytes have also been reported to participate in the formation of amyloid plaques (Liberski and Brown, 2004).

Neuronal loss is suggested to occur through an apoptotic process. Examination of brain areals originating from different CJD patients revealed apoptotic neurons in all disease types, found mostly in damaged regions (Gray et al., 1999). Their abundant presence seemed to correlate closely with neuronal loss. Neuronal autophagy, like apoptosis, is one of the mechanisms of the programmed cell death (PCD). It is also implicated in TSE pathogenesis and might participate in the formation of spongiform changes (Liberski et al., 2004). Autophagy is an important step in the cellular turnover of proteins and organelles. It is known to occur in neurons under physiological as under pathological conditions. Large autophagic vacuoles have been observed in hamsters experimentally infected with scrapie (Boellaard et al., 1991) and in CJD infected mice (Boellaard et al., 1989).

Several groups demonstrated the involvement of endoplasmatic reticulum (ER) stress in prion replication (Hetz et al., 2007). Kristiansen et al provided evidence that soluble aggregates of a toxic might cause prion disease by the inhibition of the proteasomal activity, concluding a strong involvement of the degradation machinery. In addition the authors demonstrated that scrapie infected neurons display a reduced proteasomal activity confirming that this is one toxic mechanism contributing to neuronal loss (Kristiansen et al., 2007).

2.6.8 Route of prion infection

The spread of prions depends on the site of entry, strain and species as well as the PrP genotype of the host. Following exposure to the infectious agent PrP^{Sc}, infection is thought to propagate in lymphoid tissues (Fraser, 1996) prior to neuroinvasion and spread within the central nervous system.

BSE, CWD Kuru or vCJD are assumed to originate from oral infection. After oral ingestion, the infectious agent has to cross the intestinal epithelium to accumulate in lymphoid tissues (Kimberlin and Walker, 1989a). Subsequently it spreads to the peripheral nervous system (Kimberlin and Walker, 1980), termed neuroinvasion (Figure 9). Kimberlin and Walker reported very early the prion spread in mice after intragastric challenge and observed propagation in the cervical lymph nodes and spleen (Kimberlin and Walker, 1989a). However, splenectomy of these mice did not alter the incubation time of the infected mice, suggesting that the spleen seems to play rather a minor role in prion spread after oral infection. A more prominent involvement of the spleen has been observed in other infection routes, such as intraperitoneal (i.p.) or intravenous (i.v.) infection (Fraser and Dickinson, 1978; Kimberlin and Walker, 1989b). Tissue examination of vCJD patients revealed PrP^{Sc} presence in the spleen (Hill et al., 1999), concluding that the spleen is important for prion propagation but not always required for an efficient spread of prions.

Feeding of the scrapie agent lead to accumulation of prions in peyers patches and mesenteric lymph nodes followed by the lymphoreticular system (LRS) as a secondary site of replication (Maignien et al., 1999). The gut associated lymphoid tissue (GALT) was found to play a role in the early stages of a prion infection (Beekes and McBride, 2000). Different types of cells, e.g. dendritic cells, macrophages, microfold cells (M-cells) and follicular dendritic cells (FDC) of the GALT and are implicated in the transfer of the prion agent. In addition, enterocytes representing the major population in the intestine, expressing PrP^{c} (Morel et al., 2004), are also implicated in prion uptake (Morel et al., 2005); Kolodziejczak et al., submitted). M-cells have been demonstrated to transcytose the prion agent (Heppner et al., 2001), whereas intestinal dendritic cells are able to transport the PrP^{Sc} to the mesenteric lymph nodes (Huang et al., 2002). Especially FDCs seem to be important for peripheral prion propagation. In transgenic mice, replication of the scrapie agent in lymphoid tissues such as the spleen, was demonstrated to depend on PrP^{c}-expressing FDCs (Brown et al., 1999b). Depletion of FDCs from the spleen of mice hampered neuroinvasion (Mackay and Browning, 1998), suggesting that FDCs transport the prion agent to sympathetic nerves.

The peripheral nervous system (PNS) is suggested to be involved in further prion neuroinvasion (Blattler et al., 1997). It has been confirmed that transmission of the prion agent within the PNS requires prion protein expression (Glatzel and Aguzzi, 2000). Recently, in cattle orally infected with BSE, PrP^{Sc} was found to accumulate in parts of the peripheric nervous system (Masujin et al., 2007).

It has been suggested that prion contact between immune cells and nerve tissue occurs in the innervated lymphoid tissue (Defaweux et al., 2005). Detailed analysis of the disease specific PrP distribution within the nervous system of orally infected rodents indicated that the scrapie agent spreads from the GALT to the CNS through the enteric nervous system (Beekes and McBride, 2000).

Several possible routes from the PNS to the brain have been implicated (Figure 9). Immunocytochemistry data from scrapie infected hamsters indicate that both parasympathetic (e.g. nervus vagus) as well as sympathetic nerves are suggested to provide a pathway to the brain (McBride et al., 2001). In the hamster scrapie model, a spinal cord independent infection route has been discovered, most likely terminating in the medulla oblongata (Baldauf et al., 1997) occuring via the vagus nerve (Beekes et al., 1998). A permanent depletion of sympathetic nerves has been demonstrated to hamper scrapie agent neuroinvasion arguing for a major role of sympathetic nervous system in prion neuroinvasion (Glatzel et al., 2001).

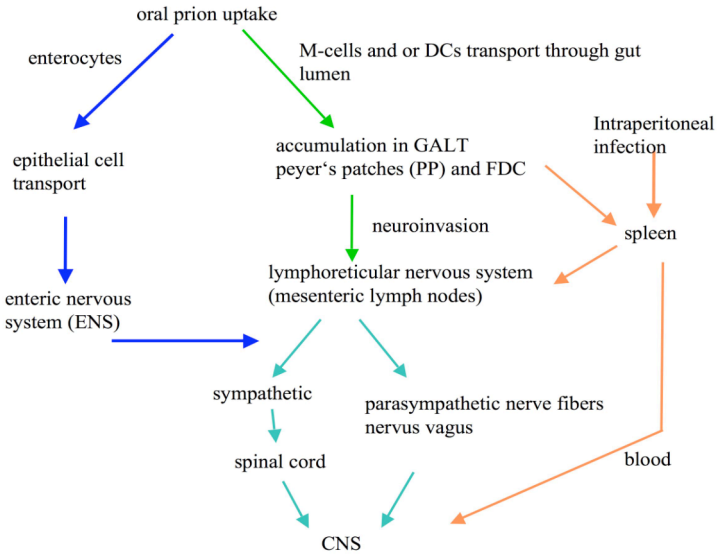


Figure 9. Model of the prion transport through the body. Orally ingested prions are intestinally absorbed via enterocytes or M-cells/Dendritic cells (DC) and transported through different pathways lymphoid tissues or blood. Peripheral replication/propagation takes place in the spleen, appendix, tonsils or other lymphoid tissues. Subsequently, prions are transported through the peripheral nervous system via two pathways (sympathetic or parasympathetic) to the CNS, independently or dependently of the spinal cord. Since the spleen has no afferent lymphatic connection, hematogenous spread seem to be most likely, either directly or via superficial cevical lymph nodes. Intraperitoneal infection with prions results in PrP^{Sc} accumuation in the spleen. It is not elucidated how prions get access from the spleen to the CNS, probably either via the blood stream, through B-or T-cells, or via other lymphatic routes e.g. FDCs (folicular dendritic cells). GALT = gut associated lymphoid tissue; M-cells (microfold cells).

An alternative prion spread may involve blood constituents, since PrP^c was detected in the blood of different individuals, e.g. scrapie infected hamsters during presymptomatic phase of the disease (Saa et al., 2006), or in vCJD patients (Ramasamy et al., 2003). The TSE agent might pass the blood brain barrier (BBB) through lymphocytes as possible TSE carriers. During preclinical TSE stages it has been observed that T-cells (Lewicki et al., 2003) as well as DCs (Rosicarelli et al., 2005) cross the BBB. However there is no striking evidence for the importance of these cells as so called prion transporters responsible for the spread (Armstrong et al., 2003). Blood is rather suggested to act as a TSE source through transmission (Llewelyn et al., 2004).

Muscle tissue has been implicated in the centrifugal spread of prions after CNS infection and provides a possible infectious source. Since PrP^{TSE} has been detected in muscle spindles, which act as mechanoreceptors, sensory nerve fibers may also provide an additional pathway for infection (Beekes and McBride, 2007). Thomzig and coworkers demonstrated that hamsters orally infected with scrapie displayed substantial amounts of PrP^{TSE} in a variety of muscles providing evidence for the spread of infection to muscle tissue after oral infection (Thomzig et al., 2003). Prion invasion of muscle tissue was also observed in TSEs with an origin other than oral infection e.g. in sCJD patients and iatrogenic CJD patients (Peden et al., 2006) suggesting a potential risk of iatrogenic spread via contaminated surgical instruments.

2.7 Transmission, Prion strains and transmission barriers

Prion disease transmission can occur horizontal, between members of the same species, (intraspecies transmission) as well as between different species (interspecies transmission). Efficient horizontal transmission has been suggested to occur through contaminated saliva (Vascellari et al., 2007), blood, urine (Murayama et al., 2007) or feces. In case of the latter, it has been recently demonstrated that cohabitation of uninfected hamsters to the bedding of hamsters orally infected with Sc237 lead to horizontal infection (Safar et al., 2008). This was most likely due to consumption of prion contaminated feces (coprophagy), since substantial amounts of PrP^{Sc} have been detected. It has been reported that CWD is efficiently transmitted horizontally and maternally among captive mule deer (Miller and Williams, 2003).

It has been discovered that clinical TSE symptoms and biochemical PrP properties differ among infected individuals (Ironside et al., 2005; Lowenstein et al., 1990) suggesting that different prion strains exist. They can be distinguished by neuropathological profiles, sensitivity to proteinase K digestion (Kuczius and Groschup, 1999), plaque morphology, incubation period and differences in the PrP^{Sc} tissue distribution and time point of occurrence (Farquhar et al., 1996). Different PrP^{Sc}

types displaying distinct glycosylation pattern, have been detected in humans associated with different phenotypes of CJD (Collinge et al., 1996b; Parchi et al., 1996). Molecular strain typing, including glycosylation pattern and PrP^{Sc} types, is used for diagnosis of the different types in CJD and subtypes in sporadic CJD (Cali et al., 2006; Heinemann et al., 2007; Notari et al., 2004). New prion strains have been generated over the past years by passaging prion strains from one animal species to another (experimental interspecies transmission). For example, mouse scrapie has been transmitted to hamster and passaged resulting in hamster adapted scrapie prions (Kimberlin et al., 1987). Several TSE strains have been generated upon transmission to mice or hamsters. RML, Chandler (Chandler, 1961), 22L or ME7 (Kuczius et al., 1998) are for example commonly used scrapie strains adopted in mice. Other TSE species like BSE, CJD or TME have been adapted in both mice and hamster. It has been early proposed that the infected tissue used for transmission of the disease, contains a prion strain mixture and adaptation from one species to another may involve the selection of a single strain which is specifically pathogenic for the infected species (Kimberlin and Walker, 1978). Upon transmission of prion diseases between different mammalian species, altered incubation periods or even a resistance to the disease has been observed. This phenomenon has been described as the so-called **species barrier** (Pattison, 1965). Mice that are normally resistant to a hamster adapted scrapie strain 236K have been demonstrated to become susceptible by the introduction of a hamster PrP gene (Scott et al., 1989). Interspecies transmission is widely studied using transgenic mouse models. For example mice expressing either human PrP (Telling et al., 1994), bovine PrP (Buschmann et al., 2000) or ovine PrP (Vilotte et al., 2001) displayed reduced incubation times and survival following inoculation with human, bovine or sheep prions, respectively. Furthermore, a reduction of the incubation period upon scrapie transmission has been observed in transgenic mice (tga20) expressing a 10-fold higher level of PrP^{c} than wild type mice (Thackray et al., 2002). Beside the prion protein sequence (Kupfer et al., 2007) and the infectivity dose, also the route of infection (Beekes and McBride, 2007) is supposed to determine interspecies transmission ability (Beringue et al., 2008).

Current studies focus on the oral transmission (Davies et al., 2006; Sales, 2006), since the natural route of prion infection occurs mostly orally. Recently, it has been suggested that human intestinal cells (Caco-2/TC7) play a crucial role in the species barrier via the oral prion uptake and therefore might influence the susceptibility for prion infection (Mishra et al., 2004). Prion protein binding studies on different enterocyte species provided evidence for a LRP/LR dependent prion uptake (Kolodziejczak et al., submitted;) Moreover, data obtained from immunofluorescence colocalistion studies of CWD prions on human enterocytes, suggest a possible transmission of CWD via the oral route to humans, leading to a possible new zoonotic disease.

2.8 Prion like proteins

Two paralogs of the prion protein have been discovered, however the complete function is not clarified yet. Doppel (Dpl) is a testis expressed protein which appears to play an important role in the proper function in the male reproduction system (Behrens et al., 2002). The tertiary structure is very similar to the C-terminus of PrP^c sharing 25% sequence identity. Doppel resembles the C-terminal domain of PrP^c but lacks the hydrophobic domain and the N-terminal octapeptide regions (Figure 10) (Watts et al., 2007). Like PrP^c, Doppel is localized to lipid rafts harbouring a GPI anchor. It has been reported that Bax signaling associated apoptosis of Purkinje cells can be induced by Doppel (Heitz et al., 2007). In contrast to PrP^c, Dpl does not seem to support prion replication. Furthermore, expression in the brain caused a neurodegenerative phenotype distinct from that of PrP^c (Mo et al., 2001). There is no evidence for a role of doppel in prion pathogenesis (Watts and Westaway, 2007). The second prion protein paralog, Shadoo (Sho) is a hypothetical GPI-anchored protein encoded by the *SPRN* gene (shadow of prion protein) (Premzl et al., 2003) exhibiting homology and domain organization similar to the N-terminus of PrP (Figure 10). Shadoo represents a CNS-expressed protein harbouring similar properties as PrP^c and is thought to retain overlapping activity (Premzl et al., 2003). It has been found to be significantly reduced in prion infected mice (Watts et al., 2007). However, the exact physiological role in the CNS as well as a potential role in prion pathogenesis remains still unclear.

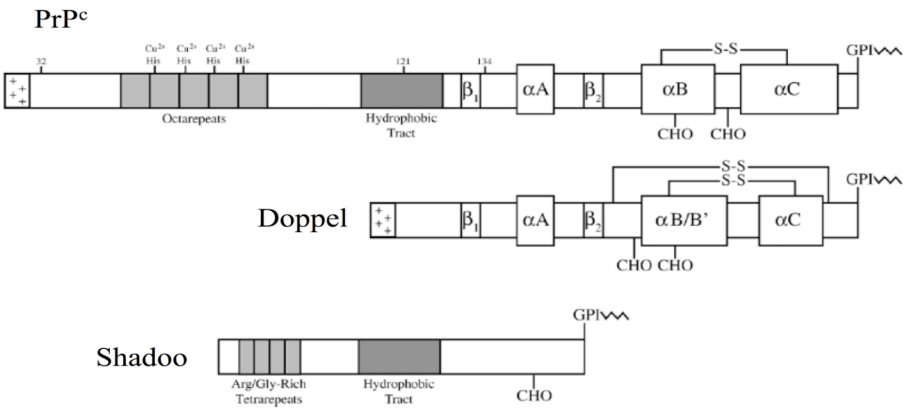


Figure 10. Domain architecture of the prion protein family members. Both PrP paralogs Doppel (Dpl) and Shadoo (Sho) contain a GPI anchor. Dpl reveal similar C-terminal domains to PrP^c consisting of three α-helices and two short β-strands. Disulfide bridges and N-glycosylation sites are depicted. Sho exhibits repetitive elements, similar to the octapeptide region of PrP. However, in contrast to PrP^c the repetitive motif consists of arginin and glycine rich tetrarepeats. The hydrophobic region is conserved in both proteins. (adopted from (Watts and Westaway, 2007)

3. The 37 kDa/67 kDa Laminin receptor (LRP/LR)

In 1983, several groups isolated the laminin receptor as a laminin binding protein from different cell lines, including human breast carcinoma (Terranova et al., 1983), murine melanoma cells (Rao et al., 1983) and normal muscle cells (Lesot et al., 1983). Purification and isolation identified a protein with an apparent molecular mass of approx. 67 kDa (according to SDS-PAGE) and was therefore termed high affinity 67 kDa Laminin receptor (LR), due to its strong binding to laminin . The full length protein sequence could not yet been identified, however, cleavage of the purified protein with cyanogen bromide followed by microsequence analysis revealed an octapeptide fragment (MLAREVLR) (Wewer et al., 1986), which enabled the identification of the cDNA encoding a 295aa long peptide with an approx. molecular mass of 32.8kDa. It appeared at approx. 37 kDa in western blot analysis of cell lysates. The remarkable discrepancy between the observed mass isolated from tumors and the protein resulting from its corresponding gene is still not clarified. A pulse chase experiment using 37 kDa LRP from ^{35}S methionine labeled human melanoma cells indicated that the 37 kDa form represents the precursor of the laminin receptor (LRP), which might undergo modifications prior to maturation to the surface protein 67 kDa LR (Rao et al., 1989). Fatty acylation might occur during the proposed conversion of LRP to LR (Buto et al., 1998; Landowski et al., 1995). A suggested homodimerization was not confirmed by yeast two hybrid studies and size exclusion chromatography of recombinant LRP (Hundt et al., 2001). Since the 67 kDa form harbours lectin epitopes, heterodimerization with another laminin binding protein carrying epitopes from galectin-3 (Hinek et al., 1993) has been proposed. However, the finding that both receptor forms are present on the surface of N2a (mouse neuroblastoma) cells which do not express galectin-3, (Gauczynski et al., 2001), suggested that heterodimerization between gal-3 and LRP is unlikely.

Fluorescent in situ hybridization localized the 37LRP/p40 active gene on chromosome 3 in the locus 3p21.3 which represents a hot spot for genetic alterations in several cancers and particularly in small cell lung carcinoma (Jackers et al., 1996b). Multiple copies of the LRP gene exist among mammals, whereas the human genome harbours 26 copies and the mouse contains 6 copies, respectively (Jackers et al., 1996a).

3.1 The 37 kDa/67 kDa LRP/LR and its correlation to prion disease

A yeast two hybrid screen employing a complex HeLa cDNA library, identified the 37 kDa laminin receptor precursor (LRP) as an interaction partner for the prion protein (Rieger et al., 1997).

Additional *in vitro* studies in neuronal and non-neuronal cells revealed that LRP/LR acts as the receptor for PrP^c (Gauczynski et al., 2001). Mapping analysis identified two PrP binding domains for LRP (Hundt et al., 2001): a direct binding domain (PrPLRPbd1, aa144-179) and an indirect one (PrPLRPbd2, aa53-93), which is dependent on the presence of heparan sulfate proteoglycanes (HSPGs) (Hundt et al., 2001; Warner et al., 2002)). Further binding sides on PrP^c for HSPGS have been identified (Warner et al., 2002). Hence, HSPGs, known as interaction partners for PrP^c (Gabizon et al., 1993), have been proposed to act as co-receptors for PrP^c (Hundt et al., 2001) and the glycosaminoglycane moiety heparan sulfates act as receptors for the scrapie prion protein PrP^{Sc}, since heparinase III treatment prevents binding of prion rods to neuronal cells(Horonchik et al., 2005). In addition to HSPGs LRP/LR has been demonstrated as coreceptor for PrP^{Sc} (Gauczynski et al., 2006). The corresponding binding sites for PrP on LRP have been mapped between aa residues 161 and 179, whereas the second indirect binding domain is located between aa180-285 (Figure 11) (Hundt et al., 2001). PrP has been confirmed to specifically bind to LRP/LR and becomes internalized mediated by LRP/LR (Gauczynski et al., 2001). Several isoforms corresponding to different maturation stages of the receptor have been detected in mouse brain, including a 42 kDa, a 60 kDa, a 67 kDa and a 220 kDa form (Simoneau et al., 2003). In addition, all isoforms were able to bind to PrP. Immunocytochemistry studies demonstrated that the 67 kDa LR is expressed in adult rat brain and spinal cord within the cytoplasm and at the plasma membrane of most neurons and in a subset of glial cells (Baloui et al., 2004). The overall distribution of LR was found to correlate with brain regions classically associated with prion-related neurodegeneration (Baloui et al., 2004). In contrast, the 37 kDa form was detected in cortical interneurons, which are known to degenerate during the early stages of Creutzfeldt-Jakob disease (Belichenko et al., 1999). Interestingly, LRP levels have been found to be elevated in scrapie infected mouse and hamster brain and splenic tissues (Rieger et al., 1997). LRP/LR acts also as a receptor for PrP^{Sc} supporting prion replication, due the binding of PrP^{res} (Gauczynski et al., 2006) and the observed hampererd PrP^{Sc} propagation by the usage of tools directed against or downregulating LRP/LR, including polysulfated glycanes, such as pentosan polysulfate or heparan mimetics (for reviews (Vana et al., 2007); (Ludewigs et al., 2007; Vana, in press; Zuber et al., 2007a). Especially, antibodies directed against the 37 kDa/67 kDa LRP/LR have been demonstrated in mice experiments to be promising for prion disease therapy (Zuber et al., 2008a; Zuber et al., 2007a; Zuber et al., 2007b; Zuber et al., 2008c). In addition, LRP/LR was found to bind and internalize PrP^{BSE} in human enterocytes (Kolodziejcak et al., submitted).

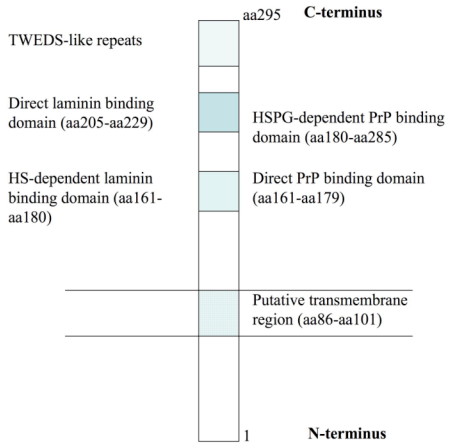


Figure 11. Schematic model of the 37 kDa LRP. The human LRP represents a 295 aa long peptide, displaying the C-terminal part on the extracellular site. A hydrophobic region stretching from aa 86-101 is proposed to serve as the transmembrane region. LRP has been demonstrated to bind laminin via three different regions, including (i) a heparan-dependent binding region, termed Peptide G (aa161-180), which contains the palindromic sequence LMWWML, (ii) TWEDS like repeats at the C-terminal part and (iii) a direct laminin-binding domain (205-229). Two prion protein (PrP^c) binding sites exist on LRP, whereas one represents a heparan sulfate proteoglycan-dependent region (aa180-285) and the other stretches from aa161-179.

3.2 Localization of LRP/LR

In 1988, a polypeptide identified from mouse tumor cells, termed p40 (an acidic ribosomal protein with a molecular mass of approx. 40 kDa) (Makrides et al., 1988) was found to share identical sequence to human 37 kDa LRP (Auth and Brawerman, 1992). Sucrose gradient sedimentation of cytoplasmic fractions from murine sarcoma and erythroleukaemia cells detected the 37 kDa form associated with ribosomal 40S subunits. Gene expression analysis of different human tissues detected the 37 kDa LRP gene product (RPSA) in ribosomes of all investigated organs, such as brain, liver, skeletal muscle, ovary, retina and uterus (Bortoluzzi et al., 2001). LRP was also found in heart tissue associated with the mitochondrial proteome (Taylor et al., 2003). Comparative modeling of the N-terminal domain using crystal structures from prokaryotic p40 homologues suggests that the N-terminal domain is implicated in ribosomal function (Kazmin et al., 2003). In addition to mammals, LRP was found in mitochondrial ribosomes in other species such as budding yeast (*Saccharomyces cerevisiae*) (Davis et al., 1992). Interestingly, the disruption of the corresponding yeast genes encoding for proteins similar to 37 kDa LRP, resulted in reduced cell proliferation and deletion of both was lethal, suggesting that p40/LRP homologues support ribosomal stability or assembly of the 40S subunit (Demianova et al., 1996).

LRP has been also detected in cytoplasmic (Grosso et al., 1991), perinuclear and perichromosomal regions of the cell (Sato et al., 1996). In addition, *in vitro* analysis using cellulose affinity column revealed that p40/LRP is tightly associated with histone H1 and in particular the core histones H2A, H2B, and H4 (Kinoshita et al., 1998). However, the exact role of LRP in the nucleus as well as on ribosomal structures is not clear. Introduction of antisense cDNA against p40 has been demonstrated to induce apoptosis (Kaneda et al., 1998), demonstrating a protective role of LRP. It has been suggested that 37 kDa/p40 might be required for translation or stabilization of chromatin. A role for LRP in cell viability has been proposed via interaction with midkine, a cell survival and migration mediating protein (Salama et al., 2001). Midkine binding to the 37 kDa LRP leads to nuclear translocation. Furthermore, downregulation of LRP via siRNAs demonstrated an anti-apoptotic function of LRP (Susantad and Smith, 2008), confirming that LRP/LR contributes to cell viability.

3.3 Function of LRP/LR

Besides the described function in prion protein binding and cell viability, LRP/LR binds to laminin, elastins and carbohydrates (Mecham, 1991) and acts as a receptor for a variety of viruses, such as the Sindbis Virus (Wang et al., 1992), Dengue Virus (Thepparit and Smith, 2004), Venezuelan Enquine Encephalitis Virus (Ludwig et al., 1996) and Adeno Associated Virus serotype 2, 3, 8 and 9 (Akache et al., 2006). The receptor might also be important for bacterial infection, since 37 kDa LRP binds to the bacterial toxin cytotoxic necrotizing factor 1 (CNF1) (Chung et al., 2003; McNichol et al., 2007). The 67 kDa form has also been proven to promote internalization of a CNF1-expressing E.*coli* strain into human brain microvascular endothelial cells (HBMEC) (Kim et al., 2005).

Vascular smooth muscle cells have been demonstrated to produce and secrete LRP into the extracellular matrix for cell attachment (Hu et al., 2001). LRP is supposed to mediate the interaction between smooth muscle and endothelial cells and might therefore contribute to angiogenetic processes like blood vessel formation.

Furthermore, LRP/LR is strongly implicated in the metastatic process of tumor cells discussed in section 3.5.

In addition a differential expression of the 67 kDa LR between dividing and contact-inhibited cells has been detected indicating a role for this receptor during proliferative processes (Donaldson et al., 2000; Stitt et al., 1998). An involvement in mobilization of hematopoetic stem cells (HSCs) by the

granulocyte–colony-stimulating factor (G-CSF) has been suggested, since LR expression correlated with mobilization efficiency of HSCs (Selleri et al., 2006).

3.4 Functional laminin binding domains and structure of LRP/LR

LRP/LR is a type II receptor, whereas the C-terminus is suggested to represent the extracellular laminin binding domain and the N-terminal part locates inside the cell (Figure 11). A hydrophobic domain stretching from aa 86-101 has been proposed as a putative transmembrane region (Castronovo et al., 1991; Menard et al., 1997). Phylogenetic protein sequence analysis revealed that the C-terminal region, containing the laminin binding palindromic sequence LMWWML, appeared during evolution concomitant with laminin and the basement membrane (Ardini et al., 1998). Thus, the original 37 kDa ribosomal protein acquired during evolution a laminin receptor function, which is linked to multicellular tissue formation of higher organisms. Additional laminin binding sites in the C-terminal domain of LRP/LR have been identified (Figure 11). A sequence YIGSR, corresponding to the region on LRP between aa 205-229 has been proven to bind laminin directly (Graf et al., 1987; Starkey et al., 1999). A heparan sulfate dependent laminin binding region stretching from aa 161-180 has been identified (Castronovo et al., 1991). The palindromic motif LMWWML is also termed Peptide G and is strongly implicated in laminin binding on cancer cells (Magnifico et al., 1996). In addition, a third laminin binding site has been proposed at the very ultimate C-terminus involving TWEDS like repeats (Kazmin et al., 2000).

Recently, the crystal structure of the aa region 1-220 of the human 37 kDa LRP has been solved (Figure 12) (Jamieson et al., 2008). According to this, the former proposed transmembrane region (86-101) is unlikely to serve as a transmembrane region. In addition, the peptide G epitope has been discussed to be only in part accessible for the binding to laminin-1.

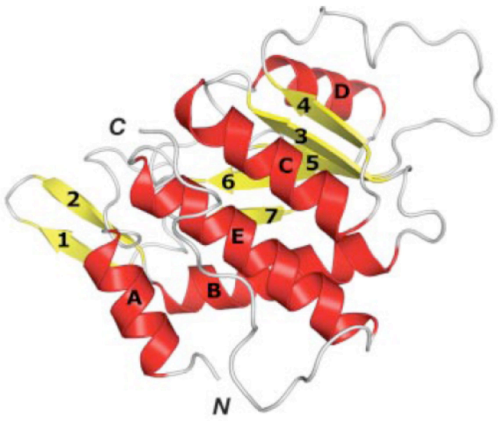


Figure 12. Structure model of human 37 kDa LRP peptide 1-220. The structure was determined based on the *Archeoglobus fulgidus* homologue, the 30S ribosomal protein S2p. The overall structure is composed of 5 parallel β and 2 antiparallel β sheets (colored yellow). The N-terminal region (residue 1-8) and the C-terminus (residues 206-220) are disordered. Five α-Helices are indicated in red (A-E).

3.5 The role of the LRP/LR in cancer

3.5.1 Cancer

Cancer describes a class of diseases in which host cells start to display uncontrolled growth and invasive potential. Most of the affected individuals die from metastasis designating cancer spread to other locations via lymph or blood and its formation of metastases at the secondary sites. Cancer is usually classified according to the tissue from which the cancerous cells originate, the primary tumor, as well as the normal cell type they most resemble (Table 4). Tumorigenic cells describe malignant neoplasms, designating abnormal proliferation of genetically altered cells. They are distinguished from benign neoplasms, which are able to stop growing by themselves and do not invade other tissues and do not form metastases, respectively.

Table 4. Cancer types and their original tissue

Type	Derived tumor	Location/tissue
Epithelium	adenocarcinoma, squamous cell carcinoma	breast, liver, skin, intestinal mucosae, bladder, lung, cervix
Connective tissue	osteosarcoma, chondrosarcoma, rhabdomyosarcoma, angiosarcoma	chondrae, bone, muscle, blood vessels
Haematopoetic system	lymphoma, myeloma, erythroleukaemia, lymphoid or myeloid lymphoma	bone marrow, spleen
Nervous tissue	neuroblastoma, glioma, astrocytoma, schwannoma, neuroma, medulloblastoma	PNS; CNS

Tumorigenesis is a multistep process involving genetic alterations leading to transformation of normal human cells into highly malignant derivates. Tumor development and progression is accompanied by the change in the genomic and proteomic expression profile resulting in physiological changes: (i) self-sufficiency in growth signals, (ii) insensitivity, to growth-inhibitory (antigrowth) signals, (iii) evasion of apoptosis, (iv) limitless replicative potential, (v) sustained angiogenesis, and (vi) tissue invasion and metastasis (Hanahan and Weinberg, 2000). Tumorigenic cells aquire defense mechanisms and display reduced dependence on exogenous growth stimulation

disrupting the critically important homeostatic mechanism that normally operates to ensure proper behavior of a cell type within a tissue.

3.5.1.2 Metastatic process of tumorigenic cells – involvement of the laminin receptor and binding to laminin

The metastatic process is a highly complex process including attachment of cells to the extracellular matrix components through several receptors, degradation of extracellular matrix (ECM) components by local secretion of proteolytic enzymes and migration into adjacent tissues (Makale, 2007; Pauli et al., 1983). Components of the ECM comprise laminin, fibronectin, type IV collagens and proteoglycanes (Vracko, 1974). An important feature of a tumorigenic cell is the initiation of local invasion and neovascularization, which marks the beginning of the metastatic process (Meyer and Hart, 1998). Formation of new blood vessels is important for nutritional support of the tumor cell and for entering the blood circulation, respectively (Bikfalvi, 1995). A variety of molecules are implicated in the angiogenetic process including fibroblast growth factors (bFGF), angiogenin, vascular permeability factor (VPF) and transforming growth factor α and β (TGF α and β) (Folkman and Klagsbrun, 1987). Several reports argue for a role of LRP/LR in the angiogenetic process (Tanaka et al., 2000), observed for example in melanocyte tumor progression (Vacca et al., 1993) and in retinal angiogenesis (McKenna et al., 2001; Stitt et al., 1998). Interaction of 67 kDa LR with laminin has been suggested to be crucial for adhesion during angiogenesis (Berno et al., 2005). Expression analysis of mice revealed a bi-phasic expression pattern of LR in retinal vasculature, which correlated with key stages of retinal vascular development in the murine retina (McKenna et al., 2001). In addition, shedding of 67 kDa LR from malignant cells has been observed, concomitant with laminin-1 binding suggesting that the laminin receptor modulates angiogenesis (Moss et al., 2006). This process enables the invasion of adjacent tissues along with the formation of new colonies distant from the origin tumor.

LRP/LR plays a major role in cancer, since increased expression of the 67 kDa laminin receptor correlates with cell proliferation (Stitt et al., 1998), migration (Chen et al., 2002; Vande Broek et al., 2001) and invasion capacity (van den Brule et al., 1994b). Overexpression of 67 kDa LR has been observed in several human cancer cell type associated with metastasis (Table 5) and implicated in tumor progression (Givant-Horwitz et al., 2005).

Due to the strong binding capacity to the glycoprotein laminin, the 67 kDa form is termed the high affinity laminin receptor. The binding of laminin is concomitant with many functions attributed to the receptor, e.g. cell attachment to the basement membrane, cell adhesion, migration (Wewer et al., 1987) and invasion of tumorigenic cells (Menard et al., 1997). Laminin is the major noncollagenous component of the extracellular matrix mediating cell adhesion, movement, extravasation, growth, differentiation, maintainence of tissue phenotype and survival (Aumailley and Krieg, 1996; Kleinman et al., 1985). More than 14 laminin isoforms exist (Patarroyo et al., 2002), whereas laminin-1 has been prominently implicated in LRP/LR binding (Wewer et al., 1987). Laminin is a cross-shaped heterotrimer of three subunits (α, β and γ chains) held together by a disulphide bond (Nomizu et al., 1994). Different laminin isoforms exhibit different tissue distribution and distinct developmental functions (Engvall and Wewer, 1996). Laminin-1–LRP/LR interaction is a crucial step in tumor cell migration (Wewer et al., 1987). In addition, it has been demonstrated that the 67 kDa LR functions in the proteolytic cleavage of laminin-1, resulting in basement membrane degradation and tumor dissemination (Ardini et al., 2002; Berno et al., 2005). It has been reported that LR mediates laminin-1 induced proteolytic activity of extracellular matrix degrading enzymes (Givant-Horwitz et al., 2004), such as matrix metalloproteinases (MMPs) (Chambers and Matrisian, 1997) which are implicated in the destruction of adjacent tissue. Analysis of laminin induced receptor signaling demonstrated an involvement of the MAP kinase pathway as well as the initiation of MMP-2 (Givant-Horwitz et al., 2004).

Table 5. Human tumors reported to overexpress 67 kDa LR

Human tumors with LR expression	**Reference**
Colorectal carcinoma	(Sanjuan et al., 1996)
Colon cancer	(Campo et al., 1992)
Cervical preneoplastic and neoplastic squamous epithelial lesions	(al-Saleh et al., 1997)
Gastric adenocarcinoma	(Lee et al., 1996)
Breast carcinoma	(Martignone et al., 1993; Nadji et al., 1999)
Acute myeloid leukemia (AML)	(Montuori et al., 1999)
Human laryngeal sqamous cell carcinoma	(Zhou et al., 2006)
Human small cell lung cancer	(Satoh et al., 1992)
Prostate cancer	(Waltregny et al., 1997)
Ovarian carcinoma	(van den Brule et al., 1994a)
Uterine adenocarcinoma	(van den Brule et al., 1996)

The 37 kDa LRP was found to interact with Midkine, a protein which is well correlated with a poor cancer prognosis, when overexpressed (O'Brien et al., 1996). Through binding to LRP, Midkine is transported into the nucleus (Salama et al., 2001). The complex of both proteins might therefore also contribute to tumor progression.

3.5.1.3 LRP/LR as a prognostic marker for tumor progression

The 67 kDa LR has been proposed to be one important determinant in the invasive progression of cancer. Some tumorigenic cell lines isolated from cancer patients are currently studied for the presence of LRP/LR and its correlation to metastasis and/or progression (Menard et al., 1998). A prognostic significance has been discussed in several human cancers (Menard et al., 1998). In human breast cancer for example, a correlation between tumor size as well as onset age and the 67 kDa LR was found (Martignone et al., 1993). In addition, the presence of LR correlated with poor prognosis. Immunohistochemistry analyses following the morphological phases in breast carcinoma progression suggested that the expression is related to tumor progression (Viacava et al., 1997). A correlation has been also demonstrated for colorectal carcinoma (Sanjuan et al., 1996). Furthermore, 67 kDa LR expression in primary tumors has been found to correlate with bone marrow metastases (Menard et al., 1994). Proteomic analyses of differentially expressed proteins on tumor tissues from different esophageal squamous cell carcinoma patients identified the 67 kDa LR as a stage specific marker (Fu et al., 2007). Although expression did not correlate with lymph node metastases or patients age, a clinical significance of the association with the tumor stage was found. A significant correlation between 67 kDa LR overexpression and tumor progression has also been found with lymph node metastasis in gastric carcinoma (Lee et al., 1996).

However, a recent study on cervical carcinoma did not support the prognostic relevance of the receptor in cervical carcinoma (Branca et al., 2006). Upon immunohistochemical staining of lesions, Branca and coworkers failed to prove that upregulation of the LR is a predisposing factor for an invasive phenotype. However, elevated expression was found to correlate with an increasing grade of lesions (Branca et al., 2006). During transition to an invasive phenotype, LR was upregulated implicating a role in cell proliferation. In addition, 67 kDa LR expression did not predict the outcome of cervical cancer (Branca et al., 2006). Contradictory studies with prostate cancer have been conducted, whereas a first study found that 67 kDa LR expression independently predicted poor prognosis for relapse (Waltregny et al., 1997). In contrast, in a second study, no correlation between the 67 kDa LR expression and the prognosis of relapse has been detected (Waltregny et al., 2001).

3.5.1.4 Therapy of cancer through targeting LRP/LR

Therapeutic strategies in cancer describe surgery, chemotherapy, radiation therapy and actually monoclonal antibody therapy (Chapter 4, Table 7) depending on the tumor. A prominent example for currently used antibodies represents the anti-HER2/neu antibody trastuzumab (Herceptin) (Chapter 4) used in breast cancer, targeting the epidermal growth factor receptor (EGFR) to inhibit uncontrolled tumor cell growth. Since a variety of antibodies are available directed against surface receptors, anti-LRP/LR antibodies might be promising therapeutic tools for cancer therapy. Since LRP/LR plays a prominent role in tumor cell migration, proliferation, angiogenesis and metastasis, it exhibits a valuable target for the development of therapeutics in cancer. In addition, LRP/LR seems to play a role in apoptotic processes, since downregulation of LRP via siRNAs was concomitant with apoptosis (Susantad and Smith, 2008). Together with the finding that LRP/LR is overexpressed in metastatic tumors, it can be concluded, that tumor cells prevent apoptosis by increasing the LRP/LR level. Specific targeting of LRP/LR on tumor cells to induce apoptosis, might therefore be an additional therapeutic strategy besides other approaches in cancer targeting apoptosis (Fesik, 2005; Fulda and Debatin, 2006).

Inhibition of laminin-1 - LRP/LR interaction has been achieved by incubation of cells with anti-LRP antibodies (Zuber et al., 2008b). Single chain antibodies (scFv) as well as full length monoclonal IgG1 antibodies directed against LRP/LR are directed against the TWEDS region at the C-terminus on LRP, which have been reported to be implicated in tumor cell adhesion (Kazmin et al., 2000). Therefore, these antibodies were able to reduce invasion of tumorigenic cells (Zuber et al., 2008b). The inhibition of LRP/LR-laminin binding represents therefore a promising strategy for the treatment of invasive cancers. Blocking of the LRP/LR via antibodies has been reported to be efficient in mice. For example, experimental lung metastasis was inhibited by preincubation of tumorinducing cells with the anti-LRP antibodiy P4G prior to injection (Narumi et al., 1999). Furthermore, downregulation of 37 kDa LRP has been confirmed to reduce the capability of lung tumor formation in balbc *nu/nu* mice (Satoh et al., 1999).

In summary, targeting LRP/LR for cancer intervention may act through several mechanisms due to the multifunctional properties of LRP/LR.

First, tumorigenic cells exhibit remarkably elevated 67kDa LR levels on the cell surface, which enables discrimination from normal cells. LR can be therefore used as a marker for specific targeting of cancer cells, for example in gene therapeutic delivery approaches.

The laminin1-LRP/LR interaction exhibits a second application point interfering with the metastatic process of tumorigenic cells. Antibodies recognising specifically the laminin binding TWEDS

region on the C-terminus were able to reduce the invasion of tumorigenic fibrosarcoma cells (Zuber et al., 2008a; Zuber et al., 2008b).

Third, the observed overexpression of 67kDa LR in tumorigenic cells together with the proposed protective role in apoptosis (Susantad and Smith, 2008), suggests that the increased LR level on tumor cells serves as a protective mechanism of the cancer cell to prevent apoptosis. Targeting LRP/LR, for example by downregulation of LRP/LR, might re-induce apoptosis of the tumorigenic cell

4. Antibodies

Antibodies are synthesized and secreted during humoral immune response by plasma cells, which are derived from B-lymphocytes (B-cells). They recognize and bind selectively foreign molecules and serve as markers for signaling and defense of pathogenic invasion. Antibodies exhibit specific affinity for a particular part of the target molecule, which is called epitope or antigenic determinant.

4.1 Structure and function of antibodies

The first immunoglobulin (Ig) structure was published in 1979 (Amzel and Poljak, 1979). A typical antibody, or immunoglobulin (Figure 13) represents a compactly folded globular unit of approx. 110 amino acids. The monomer is composed of two identical polypeptide light and heavy chains folded into a immunoglobulin like structure (Figure 13). The whole molecule consists of two antigen binding fragments termed Fabs (**F**ragment **a**ntigen **b**inding), which are linked via a flexible hinge region to the Fc part (**c**rystallizable). The Fc portion determines the Ig isotype (IgG and subclasses, IgA, IgD, IgE and IgM) and is able to bind to various effector molecules of the immune system e.g. Fc receptors, complement receptors or other compounds that influence the biodistribution and clearance of the antibody.

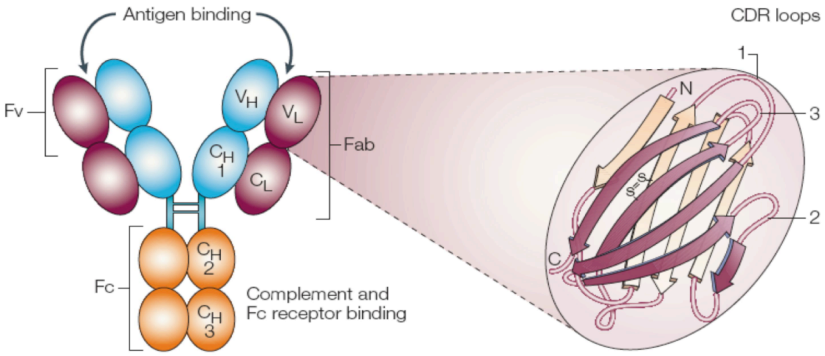


Figure 13 Monomer structure and domain organization of an immunoglobulin. Light chains consist of one constant (C_L) domain and one variable (V_L), whereas the heavy chain is composed of three constant region (C_H1, C_H2, C_H3) and one variable chain (V_H). Two variable domains are responsible for the antigen binding and harbour three loops (hypervariable loops) designating complementary determining regions (CDR) 1, 2 and 3. These domains confine the specifity of the antibody for epitope recognition and the highest diversity is found within CDR 3.The immunoglobulin fold consist of a pair of antiparallel beta sheets Fab = fragment antigen binding; Fc = crystallizable. (adopted from (Brekke and Sandlie, 2003)).

The ability of antibodies to activate complement cascade or the antibody dependent cellular cytotoxicty (ADCC) depends on the isotype. In humans, the IgG1 is the most prominent one. Through binding to a receptor the antibody is able to induce signaling cascades e.g. induction of apoptotic pathways.

4.2 Passive Antibody therapy

Passive antibody therapy was used since 1890s, after the discovery, that immune sera are able to prevent certain infections in experimental animals (Casadevall and Scharff, 1994). In 1920 and 1930, several sera, originating from rabbit or horse, were used for treatment of pneumococcal pneumonia, meningococcal meningitis, diphtheria, scarlet fever or measles (Casadevall and Scharff, 1994). However, administration was associated with toxic side effects, due to contaminations and animal origin, which elicited immune respone. In addition, a polyclonal serum contains a variety of antibodies with different specifities and affinities. The invention of the hybridoma technology (Kohler and Milstein, 1975) allowed the production of monoclonal antibodies, comprising a specific isotype with one specifity and marked an important step in the development of antibody therapies. A splenic derived B-cell is fused to a plasmacytoma to combine the antibody synthese function with immortality. The potential of a produced mouse monoclonal antibody (mAb) (Figure 14) was limited due to a short half life, the inability to trigger human effector functions and the HAMA

response (Schroff et al., 1985), which results in the production of reactive human anti-mouse antibodies. In the following, genetically engineered antibodies were designed (Morrison et al., 1984; Wright et al., 1992) comprising a human constant part and a mouse variable region. However, a so-called HACA response, a human anti-chimeric antibody response, was observed after application (Kuus-Reichel et al., 1994; Sandborn and Hanauer, 1999). To minimize the mouse component, further CDR (complementary determining region) grafting has been used (Jones et al., 1986). Hence, mouse CDR regions are inserted into human constant and variable regions of the antibody construct, resulting in humanized antibodies (Figure 14). The selection of human antibody fragments from *in vitro* libraries or transgenic mice finally enabled the construction of complete human antibodies (Figure 14). Transgenic mice able to express human immunoglobulins, can be immunized against the desired antigen and the B-cells are subsequently isolated and fused to a myeloma cell. The resulting hybridomas are screened for specific human antibodies (hu mAb).

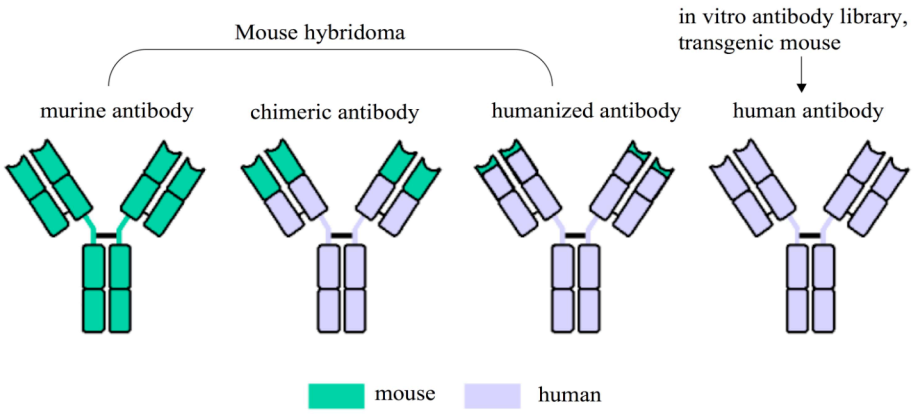


Figure 14. Schematic representation of modified monoclonal antibodies. Antibodies can be generated through mouse hybridoma technology resulting in murine antibodies (ending: -mumab), chimeric (-ximab) and humanized (-zumab) antibodies (Table 6). Cloning of mouse variable regions into human constant region genes generates chimeric antibodies. Humanized antibodies can be generated by insertion of mouse CDRs into human constant and variable domain frameworks. Entire or full length human antibodies are generated by selection of human antibody fragments from *in vitro* libraries or transgenic mice. (modified from http://www.biologie.de/biowiki/ Monoklonaler_Antik%C3%B6rper)

4.2.1 Antibodies and derived fragments – therapeutic implications

Besides monoclonal antibodies, different antibody fragments have been developed for therapeutic purposes. Fab fragments and single chain antibodies (scFvs) (Figure 15), represent also promising

therapeutic antibody formats, lacking the Fc region and exhibit in contrast to full length IgGs a better tissue penetration as well as possible faster distribution in the organism due to the smaller size (approx. 35kDa). ScFvs or Fabs can be polymerized into dia-, triabodies or modified through PEGylation (Yang et al., 2003). The chemical coupling of a polyethylene glycol group (PEG) reduces the immunogenicity and prolongs the serum half life. Bispecific antibodies can consist of two different Fab fragments, one binding to the host immune complement component, which is often a receptor. The other fragment recognizes the pathogen (microbial epitope). A bispecific mAb crosslinking the erythrocyte complement receptor with an anti-bacterial antigen specific for *Pseudomonas aeroginosa* has been reported to clear primates effectively from bacterial infection (Lindorfer et al., 2001). In contrast to scFv, which cannot be rescued from degradation due to the lack of the Fc part, full-length antibodies are more stable and exhibit a longer serum half life. The long survival of IgG relative to the other plasma proteins has been associated with the binding to the FcRn or Brambell receptor (Brambell et al., 1964; Junghans and Anderson, 1996). The Fc portion of a full length IgG binds to this neonatal Fc receptor (FcRn), expressed on human placenta and blood vessels, which is able to transport IgGs. It has been demonstrated that upon binding to FcRn in endosomes, IgGs can be prevented from lysosomal degradation and recycled to the blood circulation (Hinton et al., 2006). Approaches to modify the Fc region of therapeutic antibodies for a higher binding affinity to FcRn have been reported, aiming at an increase in the serum half life (Petkova et al., 2006).

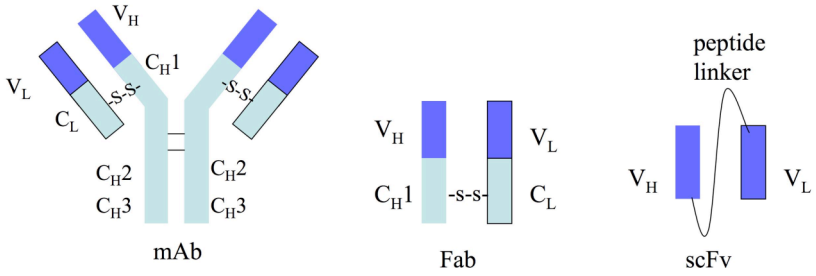


Figure 15. Schematic representation of antigen-binding fragments derived from Ig. The Fab fragment represents the antigen-binding portion without the Fc part. A single chain antibody (scFv) consists of the variable regions of the heavy and light chains which are linked by a small peptide linker molecule e.g. Gly4Ser3 or yol consisting of 8-15 amino acids.

4.2.2 Manufacture of antibodies and antibody fragments

For antibody selection, the prominent *in vitro* technology describes the phage display technique (McCafferty et al., 1990) (Figure 16), which enabled the design of antibodies composed only of the

variable regions, termed single chain antibodies scFv (single chain Fragment variable). Both human and synthetic gene repertoires can be cloned into specific expression vectors, which are subsequently transfected into phages producing *E.coli* (*in vitro* library) (Gram et al., 1992). The variable genes encoding antibody variable domains are expressed on the surface of filamentous bacteriophages as fusion products to the coat proteins of the phage (Figure 16). The fragments are selected for the antigenbinding, the corresponding gene is cloned into an expression vector and produced in *E.coli* (scFv) or in mammalian cells (Fab-IgG), respectively. The first monoclonal antibody, which was generated by phage display was adalimumab (Humira®, anti-TNFα) (Table 6) approved at the end of 2002 for the treatment of rheumatoid arthritis. Chain-shuffling can be additionally used to improve the binding affinity of an antibody, through the introduction of point mutations in the CDR. The light chain repertoire is substituted by a new light chain repertoire, retaining the variable heavy chain. Panning of such chain-shuffled libraries by stringent conditions generates antibodies with higher affinities than the original one. Further approaches generate an antibody library, with mutations within the variable regions through error prone polymerase chain reaction (Pannekoek et al., 1993) or site specific mutagenesis (Miyazaki et al., 1999).

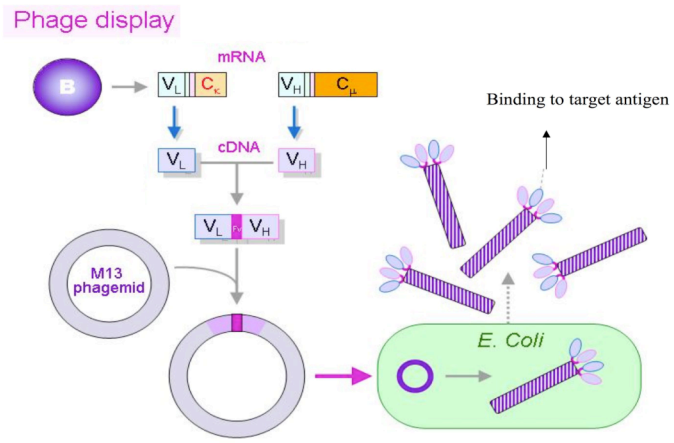


Figure 16. Schematic overview of the phage display technique. From a large pool of lymphocyte DNA a library of variable region genes is isolated and inserted by random combination into the phage DNA. Following infection and amplification of the phage in *E. coli*, a huge number of different specificities are expressed on the surface. After binding to the purified antigen, the phage can be amplified by replication in the bacterial host. Following several cycles of selection and amplification, a population of antigen-specific phages is obtained, each containing the specific DNA, which encodes for the expressed Fv fragment. The DNA is subsequently cloned into vectors for amplification and expression in bacteria (in case of scFv) or mammalian cells (full-length IgG).
(adopted from http://juang.bst.ntu.edu.tw/ECX/images/phage%20display.jpg)

4.2.3 Features of antibodies for therapy

A striking feature of antibodies is the high specificity, targeting only the microorganism that causes the disease or the tumorigenic cell, which displays distinct properties in contrast to normal cells. The design of an antibody (isotype, coupling of chemical compounds, scFv of IgG) depends on the target, disease and mode of action. Antibodies can function through several mechanisms: selective killing of the target cell by either delivery of toxic payload (radioisotope, catalytic toxins, drugs, cytokines and enzymes) (i), or inducing apoptotic signaling (ii), antibody dependent cellular cytotoxicity (ADCC) (iii), complement dependent cellular cytotoxicity (CDC) (iv), or simply blocking the binding of molecules to a target e.g. a receptor (v).

The isotype offers a variety of possibilities for the design of antibodies to achieve a specific effect. Most therapeutic monoclonal antibodies (mAb) contain the IgG1 isotype, which is able to mediate Fc domain-based functions, such as ADCC and complement fixation. Other isotypes e.g. IgG2 act only through antigen-binding e.g. mAb directed against growth factor receptors on tumor cells.

Since antibodies are produced in cell lines or other expression systems, a possible contamination with viruses or other infectious agents cannot be excluded. A further challenge is the appropriate therapeutical antibody concentration, which is crucial for a successful therapy, since the so-called prozone effect occuring after excessive antibody administration, can be detrimental for the host (Taborda et al., 2003). The prozone effect describes the lack of agglutination at high concentrations of antibodies. Since protection against bacterial infection is hampered as a result of high antibody concentration, the appropriate antibody concentration has to be considered for the treatment for infectious diseases.

4.2.3.1 Antibodies for cancer therapy

Several monoclonal antibodies are currently used for therapy of several cancer types (Table 6). The first humanized antibody was alemtuzumab (Campath® anti-CD52) (Albitar et al., 2004; Osterborg et al., 1997), which is now used for the treatment of chronic lymphocytic leukemia (Alinari et al., 2007). In contrast to most anti-cancer antibodies, which are coupled to toxic substances, so-called naked antibodies are in therapeutic use, acting through Fc receptor or complement activation, which induces crosslinking of surface receptors leading to apoptotic signaling. Many solid tumors overexpress growth factor receptors such as EGFR (epidermal growth factor receptor) stimulating cell growth in an autocrine manner. A series of mAbs were shown to inhibit ligand binding and

therefore, receptor activation (Teramoto et al., 1996). Healing from established tumors was achieved when anti-EGFR mAbs were combined with chemotherapeutic agents such as cisplatin (Fan et al., 1993). A novel strategy in mAb tumor therapy describes the antibody-directed enzyme prodrug therapy (ADEPT). Here, an enzyme is conjugated to the antibody. After antibody binding to the tumor cell, the enzyme cleaves a subsequently delivered prodrug, which becomes then active in the tumor cell. This leads to highly specific deposition of an active drug solely in the tumor cell. Clinical study, employing this strategy, have been investigated for colorectal cancer (Francis et al., 2002) (Mayer et al., 2004).

Lymphomas are targeted by mAbs directed against cell surface receptors like e.g. CD20 acting through mechanisms like activation of ADCC, CDC and apoptosis. The affinity of an antibody can also be modified according to the desired effect. For example, in tumor therapy a higher affinity of the antibody to the antigen is not recommended since this correlates with an impeded penetration ability into the tumor mass (van Osdol et al., 1991; Weinstein et al., 1987). This finding was proposed as the binding site barrier, which describes the fact that high affinity interactions between the antibody and its target block the diffusion of the antibody through the tumor mass. The use of a high affinity scFv directed aginst EGF (epidermal growth factor) which exhibited diminished penetration into solid tumor compared to their lower affinity counterparts (Adams et al., 2001) supported this effect. In contrast, if an ADCC response should be achieved, a higher affinity is required. In combination with multivalency, which is also an important feature, the ADCC response can be more efficient. For example bispecific antibodies targeting both EGFR and a distinct Fc receptor reveal more effective mediators of ADCC, in case the antibody affinity is higher (Weiner and Carter, 2005).

4.2.3.2 Antibody based therapy for infectious diseases and other implications

Passive antibody therapy is currently used to treat and prevent diseases caused by severeal viruses and bacteria. Besides the general properties such as ADCC or CDC, antibodies exhibit also direct toxin neutralization and microbial properties (e.g. generation of oxidants). Human mAb have been generated against e.g. fungal antigens (Matthews et al., 2003). Antiviral active antibodies have been designed against respiratory syncitical virus (RSV) infection, available for prophylaxis (Wang and Tang, 2000) and for treatment (Domachowske and Rosenberg, 1999). Prophylactic antibodies against Hepatitis B virus are currently used (Zuckerman, 2007). A protective effect against the tetanus toxin has been discovered 1993 after the isolation of toxin-neutralizing human monoclonal

antibodies (Lang et al., 1993). Recently, immunization of transgenic mice resulted in the generation of shiga-toxin 1 neutralizing human mAbs (Mukherjee et al., 2002). Additionally, in mice, high affinity scFvs have been demonstrated to be protective against anthrax toxin in mice (Maynard et al., 2002). Furthermore, neutralizing Fab fragments against measles virus have been isolated by phage display (de Carvalho Nicacio et al., 2002). Moreover, some antibodies seem to be able to kill bacteria directly by altering their surface structures (Connolly and Benach, 2001). Radioimmunotherapy, succesfully used in cancer treatment, has also been described for treatment of fungal infection (Dadachova et al., 2003).

Antibodies encompassing high affinity can be developed to bind cytokines and their receptors to reduce the inflammatory response. Patients with rheumatoid arthritis for example, can be treated with chimeric anti-TNF α antibodies (e.g. Infliximab; Table 6), which are also available for the treatment of Crohn's disease (Mikula, 1999).

Fc-IgG interaction has been reported to reduce the inflammatory response and diseasae related damages (Samuelsson et al., 2001).

Antibodies can be applied not only for disease treatment or vaccination, but also for other implications (Table 6). For instance, in organ transplantation, immunsuppression is required and achieved by targeting CD3 (Wilde and Goa, 1996), a T-cell surface receptor required for T-cell activation. Furthermore, antibodies directed against the 37 kDa/67 kDa LRP/LR block both the binding of the prion protein (Leucht et al., 2003; Zuber et al., 2008a; Zuber et al., 2007b) and the binding of laminin-1 to the laminin receptor (Zuber et al., 2008b). Thus, these antibodies can be used for therapeutic application in prion disease (Chapter II-IX) and intervention of metastatic potential of tumor cells (CHAPTER XI).

4.2.3.3 Rediscovery of polyclonal antibodies for therapy

Although designed monoclonal antibodies, Fabs and scFvs are widely used and generated for therapeutic purposes, also the polyclonal format becomes again interesting for therapeutic applications. Polyclonal antibodies (pAb) are recommended for the treatment of diseases associated with complex antigens such as sepsis (Kreymann et al., 2007). A polyclonal ovine anti-TNF fragment antigen binding (Fab) fragment was effective for sepsis (Rice et al., 2006). Additionally, an oligoclonal antibody cocktail has been proven to efficiently neutralize and clear patients from botulinum neurotoxin (Nowakowski et al., 2002). The polyclonal anti-LRP/LR antibody displayed also a more prominent therapeutic effect compared to the single chain format (Zuber et al., 2007b).

Intraperitoneal (ip.) administration in scrapie infected mice prolonged the survival of these mice compared to another study where scFvs were delivered i.p. into prion infected mice. For cancer therapy the polyclonal format is believed to be more efficient in mediating effector functions and escape variants are thought to be minimized by multi-targeting through the polyclonal format (Sharon et al., 2005). Polyclonal antibodies can be produced in transgenic animals or via the combination with mAb production technology. For this, polyclonal antibody libraries (pALs) have been generated (McNichol et al., 2007; Sarantopoulos et al., 1994). Heavy and light chain variable region genes are obtained from immune B cell-containing tissues, by RT-PCR, and cloned in pairs in a Fab phage display vector. The resulting Fab phage display library is then selected for desired specificities and the selected V_L–V_H region gene pairs are transferred from the phage display vector to a mammalian expression vector. Full-length antibodies can be produced by transfection of the mammalian expression vector library into mammalian cells (Sharon et al., 2005; Sharon et al., 2002).

Table 6. Available therapeutic antibodies

Generic name / Trade name	**type**	**target**	**indication**
Cancer			
Alemtuzumab / MabCampath®	humanized	CD52-antigen on lymphocytes	Chronic lymphatic leukemia, T-cell-lymphoma
Bevacizumab / Avastin®	humanized	VEGF (Vascular Endothelial Growth Factor)	colorectal cancer, lung cancer
Cetuximab / Erbitux®	chimeric	EGF-receptor (Epidermal Growth Factor Receptor)	colorectal cancer
[2]Epratuzumab / LymphoCide®	humanized	CD22-antigen	Non-Hodgkin-Lymphoma, Autoimmune diseases
[1]Gemtuzumab / Mylotarg®	humanized, conjugared to calicheamicin	CD33-antigen	acute myelogenous leukemia
Ibritumomab / Zevalin®	murine, ^{90}Y-radiolabeled	CD20 on B-lymphocytes	non-Hodgkin-lymphoma
Panitumumab / ABX-EGF	humanized	EGF (Epidermal Growth Factor Receptor)	non-small cell lung cancer (not approved yet) (Phase I/II-Studien)
Rituximab / MabThera®	chimeric	CD20 on B-lymphocytes	non-Hodgkin-lymphoma
[1]Tositumomab /	murine, ^{131}I-	CD20 on B-lymphocytes	non-Hodgkin-lymphoma

Bexxar®	labeled		
Trastuzumab / Herceptin®	humanized	HER2/neu-Rezeptor	breast cancer
Autoimmune diseases			
Adalimumab / Humira®	humanized	Tumor Nekrose factor α (TNF-α)	rheumatoid arthritis
Basiliximab / Simulect®	chimeric	CD25-antigen (Interleukin-2-receptor)	Prophylaxis in kidney transplant rejection
Daclizumab / Zenapax®	humanized	CD25-antigen (Interleukin-2-receptor)	Prophylaxis in kidney transplant rejection
Infliximab / Remicade®	chimeric	tumor Nekrose factor α (TNF-α)	Crohn's disease, rheumatoid arthritis
Muromonab / Orthoclon OKT3®	murine	CD3-antigen (T-lymphocytes)	Prophylaxis in kidney/heart/liver transplant rejection
[1]Natalizumab / Tysabri® (FDA appproved)	humanized	CD49d (VLA-4, integrin)	multiple sclerosis
Cardiovascular disease			
Abciximab / ReoPro®	chimeric, Fab2-fragment, anti-platelet	GPIIb/IIIa on thrombocytes	Coronary intervention and angioplasty
Infectious disease			
Palivizumab / Synagis®	humanized	anti-F protein, component of respiratory syncytial virus (RSV)	Prophylaxis of RSV-pneumonia
others			
Efalizumab / Raptiva®	humanized	CD11a-antigen	psoriasis
[1]Omalizumab / Xolair ®	humanized	IgE (Fc-Teil)	Allergy, severe asthma bronchiale

([1]not approved for Germany), indicated types are all full length Igs except Abciximab

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Chapter II

Therapeutische Ansätze zur Behandlung von Prionenerkrankungen – Therapeutic approaches for the treatment of Prion diseases

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Therapeutische Ansätze zur Behandlung von Prionenerkrankungen[1]

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Mit 2 Abbildungen und 2 Tabellen

Zusammenfassung

Prionenerkrankungen bilden eine Gruppe neurodegenerativer Krankheiten, welche mit einer Umfaltung eines Wirtsproteins, des Prionproteins PrP^C, einhergehen. Trotz zunehmendem Wissen über diese Gruppe von Krankheiten konnte bis jetzt kein therapeutischer Ansatz zur Heilung oder Stabilisierung der Krankheit beim Menschen gefunden werden. Eine Reihe potentieller therapeutischer Agenzien wurde in experimentellen Modellen überprüft, wobei sich gezeigt hat, daß einige davon bei Nagetieren eine Prioneninfektion verhindern konnten. Jedoch vermochte keine dieser Substanzen die Krankheit in einem fortgeschrittenen Stadium zu heilen. Dennoch haben die Wissenschaftler seit der BSE-Krise, durch die möglicherweise eine große Anzahl von Menschen auf dem Nahrungsweg infiziert wurde, ihre Anstrengungen sowohl in Erarbeitung experimenteller Therapien als auch in der Grundlagenforschung verstärkt. Wir stellen in diesem Artikel verschiedene Therapiemöglichkeiten zur Behandlung von TSEs vor, wobei alternative Ansätze unter Verwendung von Antikörpern und siRNAs, gerichtet gegen das Prionprotein und seine Rezeptoren, diskutiert werden.

Abstract

Prion diseases are a group of lethal neurodegenerative disorders associated with misfolding of a host protein, the prion protein PrP^C. But despite increasing knowledges concerning this class of disease, up to now no therapeutic approach has been found to cure the disease or stabilize the pathogenesis in humans. A variety of potential therapeutic compounds have been tested in experimental models and some of them have been proven to prevent prion infection in rodents. However, none of them was successfull to cure the disease at a late stage. Nevertheless since the BSE crisis that might have infected a number of humans by dietary exposure, increased efforts have been done in the scientific community both in experimental therapy and basic research. We present in this report different possibilities for a TSE-therapy and discuss alternative approaches employing antibodies and siRNAs directed against the prion protein and its receptor molecules.

1. Einführung

Übertragbare spongiforme Enzephalopathien (TSEs) oder Prionenerkrankungen sind tödlich verlaufende neurodegenerative Krankheiten. Dazu gehören beim Menschen die Creutzfeldt-Jakob-Krankheit (CJD) und im Tierreich die Bovine Spongiforme Enzephalopathie (BSE). Prionen stellen als infektiöse Proteine eine neue Klasse von Krankheits-

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erregern dar und unterscheiden sich grundsätzlich von klassischen Krankheitserregern wie Viren, Bakterien oder Pilzen, welche alle eine Nukleinsäure aufweisen. Das infektiöse Protein (PrP^{Sc}) entsteht durch eine Konformationsänderung des wirtseigenen zellulären Prionproteins PrP^{C}. Dieser Konversionsprozeß ist zentral für die Entstehung einer TSE. Wegen seiner Proteaseresistenz wird PrP^{Sc} nicht vom Proteolysesystem des Wirts (d. h. über proteasomale oder lysosomale Degradation) abgebaut und reichert sich im Zentralnervensystem (ZNS) an. Beim Menschen treten diese Krankeiten in infektiösen, sporadischen und genetischen Formen auf. Bestrebungen, eine effektive Behandlungsmöglichkeit zu entwikkeln, begannen nach der britischen BSE-Epidemie, die sich vermutlich durch die Verfütterung von Fleisch und Knochenmehl scrapieinfizierter Schafe an Rinder verbreitet hat. BSE ist nach dem heutigen Kenntnisstand auf den Menschen übertragbar, wobei sie die neue Variante der Creutzfeldt-Jakob-Krankheit (vCJD) verursachte. Da zahlreiche Aspekte der Prionenbiologie bis heute noch ungeklärt sind, stellen Prionenerkrankungen ein faszinierendes biologisches Rätsel dar. So weiß man über die physiologische Funktion des Prionproteins und die zur neuronalen Zerstörung führenden Mechanismen noch sehr wenig.

2. Therapie von Prionenerkrankungen: Eine Herausforderung?

Bis jetzt wurden nur wenige Substanzen an Patienten, welche an CJD erkrankten, erprobt, und bis heute gibt es keine effiziente Therapie, so daß die TSEs ausnahmslos letal enden. Die Krankheit verläuft rasch fortschreitend, und bereits nach dem Auftreten der ersten Symptome sind schon einige Hirnregionen irreversibel geschädigt. So ist das Fehlen einer frühen Diagnosemöglichkeit der erste limitierende Faktor für eine mögliche Therapie. Überdies gestalten sich klinische Studien schwierig, da diese Krankheiten extrem selten sind. Wegen des tödlichen Ausgangs ist die Verabreichung von Placebos ethisch kaum vertretbar. Auf Grund der außergewöhnlichen Eigenschaften der TSEs können Standardmethoden (Verabreichung von antiviralen oder antibakteriellen Substanzen) nicht angewendet werden. Folglich ist es nötig, neue Behandlungsstrategien für Prionenerkrankungen zu entwickeln.

Bei peripherer Infektion infiltriert der Scrapie-Erreger zuerst die Lymphorgane, bevor er in das ZNS gelangt (JEFFREY et al. 2000). In diesem Fall könnte eine post-expositionelle Prophylaxe eingesetzt werden, um die neurale Invasion zu verhindern. Nach der neuronalen Invasion müssen die therapeutischen Substanzen entweder die Blut-Hirn-Schranke überwinden oder direkt ins Gehirn appliziert werden.

Die Herausforderung, TSEs zu heilen oder zumindest die Überlebenszeit zu verlängern, besteht in zweifacher Hinsicht, sowohl in der Verbesserung der Diagnostik als auch in der Entwicklung von effizienten, nicht-toxischen Substanzen, welche die Blut-Hirn-Schranke überwinden können. Bis jetzt kennt man über 20 Agenzien, welche die PrP^{Sc}-Propagation in Zellkultur verhindern können (engl. *clearance*). Einige davon konnten die Inkubationszeit bei Mäusen und Hamstern verlängern, jedoch nur, wenn sie zum Zeitpunkt der Prioneninokulation verabreicht wurden. In Anbetracht verstärkter Forschungsanstrengungen und eines wachsenden Wissens über TSEs könnte eine Prionentherapie in Zukunft möglich sein, wobei eine Kombinationstherapie, wie sie z. B. bei der Behandlung von HIV eingesetzt wird, auch für die Therapie von Prionenerkrankungen der Schlüssel zum Erfolg sein könnte.

3. Modelle für die Therapieforschung

3.1 In-vitro-Modelle

Die Entwicklung zellfreier Testsysteme (*Assays*) für die Konversion, Bindung und Polymerisation des Prionproteins gestattet die Evaluierung eines möglichen inhibitorischen Effekts von Substanzen, die entweder mit PrP^{C} oder mit PrP^{Sc} interagieren. Da *Protein-misfolding-cyclic-amplification*(PMCA)-Reaktionen in Hirn-Homogenaten eine höhere Ausbeute an falsch gefalteten PrP erzielen als die *In-vitro*-Konversion (SUPATTAPONE 2004), könnten sie für ein *High-throughput*-Screening adaptiert werden.

3.2-Zellkulturmodelle

Zellkultursysteme stellen bedeutende experimentelle Modelle für TSEs dar. Der Scrapie-Erreger ist in der Lage, neuronale Zellinien chronisch zu infizieren. Dies wurde an murinen Neuroblastom-Zellinien (z. B. N2a) und an neuronalen Zellinien aus dem Hypothalamus (z. B. GT1) gezeigt (SOLASSOL et al. 2003). Man hat diese Zellen zur Überprüfung und Erforschung potentieller therapeutischer Präparate verwendet. Dabei wurden sie mit einem Inokulum aus Hirngewebe von an Scrapie erkrankten Individuen derselben Spezies infiziert. Trotzdem zeigen auch diese Modelle Grenzen:

– die Infektiosität ist nicht immer stabil und kann nach einigen Passagen aus ungeklärter Ursache verloren gehen;
– sie stellen ein stark vereinfachtes Modellsystem im Vergleich zu Tierexperimenten dar (Rolle des Immunsystems, Nervensystems) mit der Folge, daß sich *in vitro* wirksame Substanzen *in vivo* häufig als wirkungslos erweisen.

3.3 Tiermodelle

Die Übertragung von TSE-Erregern auf Labortiere wie Maus und Hamster erfolgt durch die Verwendung Nager-adaptierter Stämme. Diese Tiermodelle sind für die Evaluation möglicher Wirkstoffe trotz relativ langer Inkubationszeiten und einer unterschiedlichen Physiologie im Vergleich zum Menschen von großer Bedeutung. Die Inkubationszeiten sind von der Art der Applikation des Inokulats abhängig und betragen bei intrazerebraler Inokulation bei Mäusen ca. 150 Tage, bei intraperitonealer Verabreichung ca. 200 Tage. Erste Anzeichen einer Scrapie-Infektion können in der Milz je nach Art der Applikation zwischen 70 und 90 Tagen verifiziert werden. Primaten gelten als die dem Menschen am ähnlichsten Organismen, können sogar mit CJD infiziert werden, verursachen aber hohe Tierhaltungskosten und zeigen sehr lange Inkubationszeiten von mehreren Jahren.

4. Angriffspunkte und Strategien für die Behandlung der TSEs

Für medizinische Interventionen bei TSEs gibt es mehrere Ansatzpunkte. Viele Forscher zielen direkt auf die Prionproteine PrP^{C} oder PrP^{Sc}, aber es gibt auch Ansätze, welche auf das Immunsystem abzielen und hier die Infiltration der Lymphorgane verhindern. Alternative Therapiestrategien zielen auf die Ausschaltung von PrP-Rezeptoren oder -Korezeptoren (Tab. 1).

Tab. 1 Therapeutische Strategien für die Behandlung von Prionenkrankheiten, die am experimentellen Modell erforscht wurden

Targets/Agenzien	Effekt in Scrapie-infizierten Zellen	Effekt in Nager-Modellen		Anmerkungen
		prophylaktisch	therapeutisch	
PrP^{C}				
– Anti-PrP-Antikörper	ja	ja	nein	passive Immunisierung
– Anti-PrP-scFv-Antikörper	ja	ja	?	transgene Expression
– Immunisierung gegen PrP		ja	?	
PrP^{Sc}				
– bindende Stoffe				
↙ IDX		ja		Medikament wird mit dem
↙ Tetrazyklin		ja		Scrapie-Inokulum prä-
↙ Kongo-Rot	ja	ja	?	inkubiert
– β *sheet breaker*		ja	?	Peptide, die dem Scrapie-
– Erhöhung der Clearance (verzweigte Polyamine)	ja	?	?	Inokulum zugesetzt werden
Raft-Domänen				
– Polyen-Antibiotika				
↙ Amphotericin B	ja	ja	ja	Effekt auf bestimmte Scrapie-Stämme beschränkt
↙ MS-8209	?	ja	ja	weniger toxisch als Amphotericin B
Immunsystem				
– FDC-Reifungsinhibition (löslicher Lymphotoxin-Rezeptor)		ja	?	
– Komplementinhibition		ja	?	
HSPG				
– Heparanmimetika (DS500, Pentosanpolysulfat)	ja	ja	?	DS500 ist toxisch
LRP				
– Anti-LRP-Antikörper	ja	?	?	
Quinacrin	ja	nein	nein	gegensätzliche Ergebnisse
Chlorpromazin	ja	?	?	in klinischen Studien

4.1 PrP als Angriffspunkt

4.1.1 Moleküle, welche PrP^C inaktivieren

Wie an PrP^C-Knockout-Mäusen gezeigt werden konnte, verhindert das Ausschalten von PrP^C eine Scrapie-Infektion (Bueler et al. 1993). Folglich stellt PrP^C einen potentiellen Angriffspunkt für Interventionen gegen TSEs dar. Der Beweis dafür konnte mit transgenen Mäusen, die Anti-PrP-Antikörper exprimieren, erbracht werden. Diese sind nämlich gegen eine periphere Prioneninfektion immun (Heppner et al. 2001). Zusätzlich konnte gezeigt werden, daß Anti-PrP-Antikörper in Zellkultur die PrP^{Sc}-Propagation verhindern konnten (Enari et al. 2001, Peretz et al. 2001, Perrier et al. 2004). Eine andere Forschergruppe berichtete, daß nach passiver Immunisierung peripher infizierter Mäuse mit monoklonalen Anti-PrP-Antikörpern, die Infektiosität der Erreger in der Milz herabgesetzt werden konnte (White et al. 2003). Allerdings zeigte sich, daß die Injektion einer großen Menge monoklonaler Anti-PrP-Antikörper eine massive neuronale Apoptose hervorrief. Diesen Effekt erklärt man durch eine antikörpervermittelte Vernetzung (*cross-linking*) des PrP (Solforosi et al. 2004). Eine aktuelle Studie schlägt als Alternative die Verwendung von Einzelketten-Antikörpern (*single chain antibodies; miniantibodies*) vor (Donofrio et al. 2005). Obwohl die Infektion mit TSEs bei Tieren keine Immunantwort gegen PrP^{Sc} hervorruft, wurden Strategien zur aktiven Immunisierung und Vakzinierung erforscht. Dieser Ansatz impliziert die Aufhebung der Immuntoleranz gegen PrP, welche durch verschiedene Strategien erreicht werden kann (PrP-Dimerisierung, PrP-*cross-linking* mit einem Bakterienprotein) (Gilch et al. 2003, Koller et al. 2002, Rosset et al. 2004). Bei infizierten Mäusen konnte aber nur ein geringer protektiver Effekt nachgewiesen werden (Polymenidou et al. 2004, Schwarz et al. 2003, Sigurdsson et al. 2003). Trotzdem gibt es auch neue Strategien, wie z. B. die Expression von PrP auf der Oberfläche viraler Partikel, um eine Erhöhung des Titers von Autoantikörpern zu bewirken, der möglicherweise den limitierenden Faktor darstellt.

4.1.2 Moleküle, welche mit PrP^{Sc} interagieren

Ein Hauptcharakteristikum von Prionenerkrankungen ist die Konversion von PrP^C zu PrP^{Sc}. Substanzen, mit der Fähigkeit an PrP^{Sc} zu binden, könnten verhindern, daß PrP^{Sc} als *Template* für seine Replikation dienen kann. Verschiedene Moleküle, welche spezifisch mit PrP^{Sc} oder Amyloid reagieren, wurden bereits getestet: Iodoxorubicin (Tagliavini et al. 1997), Tetrazyklin (Forloni et al. 2002, Tagliavini et al. 2000) und Kongo-Rot (Caughey und Race 1992, Ingrosso et al. 1995). Der Wirkmechanismus dieser Substanzen könnte darin bestehen, PrP^{Sc} als *Template* für die Konversion unbrauchbar zu machen.

Eine weitere Gruppe von Anti-TSE-Substanzen besteht aus speziellen synthetischen PrP-Peptiden, sogenannten »β-sheet breakers«. Diese Peptide mit einer dem PrP^C homologen Aminosäuresequenz plus erhöhtem Prolinanteil reagieren mit PrP^{Sc} und verändern so dessen Sekundärstruktur (Chabry et al. 1998). Dies führt zu einer Herabsetzung der Proteinaseresistenz, was wiederum eine bessere PrP^{Sc}-Clearance ermöglicht. Werden Mäuse mit einem Inokulum, dem solche »β-sheet breakers« zugesetzt sind, infiziert, so führt dies zu einer Verlängerung der Überlebenszeit (Soto et al. 2000).

4.1.3 Steigerung der PrP^{Sc}-Clearance

PrP^{Sc} ist teilweise gegen den proteolytischen Abbau resistent, wie durch *In-vitro*-Versuche mit Proteinase K gezeigt werden konnte. Es gibt aber offensichtlich noch andere natürliche Mechanismen, die seine Zerstörung bewirken können. So hat sich gezeigt, daß in PrP-Knockout-Mäusen, die mit einer hohen Prionendosis inokuliert wurden, das PrP^{Sc} innerhalb von zwei Wochen verschwindet. Die Halbwertszeit des PrP^{Sc} in Scrapie-infizierten Zellen liegt bei ca. 24 Stunden (Ertmer et al. 2004).

Die Steigerung des PrP^{Sc}-Abbaus scheint eine vernünftige Abwehrstrategie der Lebewesen zu sein. Verzweigte Polyamine, die eine Stimulierung des endolysosomalen PrP^{Sc}-Abbaus bewirken sollen, können chronisch infizierte Zellen heilen (Supattapone et al. 1999, 2001). Auch ein Inhibitor der Tyrosin-Kinase c-abl aktiviert den lysosomalen Abbau von PrP^{Sc} (Ertmer et al. 2004).

4.1.4 Indirekter Effekt: Störung der *Raft*-Biologie

Einige Polyene-Antibiotika, wie Amphotericin B und MS 8209, die normalerweise als Fungizide verwendet werden, können mit Cholesterin reagieren (»interactors«) und so die *Raft*-Integrität stören. Man glaubt, daß sie die Endozytose von PrP^{C} oder PrP^{C}/PrP^{Sc} beeinträchtigen und auf diese Weise die Akkumulation des PrP^{Sc} verlangsamen, wie an chronisch infizierten Zellen gezeigt werden konnte (Mange et al. 2000). MS 8209 erwies sich dabei als besonders effizient bei Hamstern (Adjou et al. 1999, 2000, Demaimay et al. 1997).

4.2 Wirkstoffe, welche die Blut-Hirn-Schranke überwinden

Medikamente, welche für andere Indikationen am Menschen zugelassen sind, wurden auf ihre potentielle Wirkung als Anti-TSE-Therapeutika überprüft. Dabei zeigte sich, daß Chlorpromazin und Quinacrin in infizierten Zellen effizient wirken (Korth et al. 2001), jedoch in Nager-Tiermodellen die Überlebenszeit nicht verlängern konnten (Barret et al. 2003, Collins et al. 2002). Quinacrin wurde auch in kleinen klinischen Studien an CJD-Patienten erprobt, allerdings ohne signifikanten Effekt. Lediglich in einigen Studien wurde eine vorübergehende Verbesserung der visuellen Stimulation beobachtet (Benito-Leon, 2004, Furukawa et al. 2002, Kobayashi et al. 2003, Nakajima et al. 2004). Außerdem kann die Behandlung mit Quinacrin Fehlfunktionen der Leber nach sich ziehen.

4.3 Immunsystem

Follikuläre Dendritische Zellen (FDC) spielen in der peripheren Infektion eine wichtige Rolle, da sie für die PrP^{Sc}-Akkumulation in den Keimzentren der sekundären Lymphorgane verantwortlich sind. In nächster Umgebung von sympathischen Nervenendigungen gelegen, könnten sie direkt als Schnittstelle zwischen Lympho- und Neuroinvasion fungieren. Differenzierung und Reifung der FDCs, welche durch einen molekularen Dialog mit

B-Zellen vermittelt werden, stellen zwei der für die PrP^{Sc}-Akkumulation in der Milz erforderlichen Schritte dar. Sowohl Lymphotoxin β als auch TNF sind in diese Prozesse verwickelt, so daß es nahe liegt, daß sie potentielle »Targets« darstellen (Aguzzi und Heikenwälder 2005, Brown et al. 2000). Der Reifungsprozeß der FDCs kann beeinträchtigt werden, wenn der Lymphotoxin-β-Rezeptor durch Verwendung seiner löslichen Form unwirksam gemacht wird (Mabbott et al. 2003, Mohan et al. 2005). Bei Anwendung an intraperitoneal infizierten Mäusen kann eine einzige Injektion eines Lymphotoxininhibitors effizient die frühe PrP^{Sc}-Akkumulation in der Milz verhindern, was allerdings nicht durch »orales challenging« gelingt.

4.4 Das Aufhalten der neuronalen Zerstörung

4.4.1 Transplantation von Neuronen

Therapeutische Ansätze, die den Ersatz oder die Heilung zerstörter Neuronen zum Ziel haben, könnten zu einer Verzögerung des Auftretens klinischer Symptome beitragen. Brown und Mitarbeiter (2001) berichteten, daß die Transplantation PrP-freier embryonaler Zellen in den Hippocampus vor neuronalem Verlust bei Scrapie schützt. Obwohl die Inkubationszeit nicht verlängert werden konnte, wurde bei Mäusen, welche erst in einem relativ späten Krankheitsstadium transplantiert wurden, beobachtet, daß das behandelte Gebiet 50 % mehr Neuronen behielt als bei den Kontrollen. Die gleichzeitige Transplantation an verschiedenen Stellen des Gehirns könnte dabei zu einer Verlängerung der Inkubationszeit führen.

4.4.2 Neuroprotektive Moleküle

Da ein Hauptcharakteristikum der TSEs das Absterben von Neuronen darstellt, könnte jede Substanz, welche diesen Ablauf hemmt, von Interesse sein.

4.5 PrP-Rezeptoren

4.5.1 Heparansulfat-Proteoglykane (HSPGs)/Glykosaminoglykane (GAGs)

HSPGs bestehen aus einem *core*-Protein, an das GAGs kovalent gebunden sind. Sie werden entweder sezerniert oder in die Plasmamembran eingebaut und stellen eine wichtige Komponente der extrazellulären Matrix dar, wobei eine Reihe von ihnen als Korezeptoren fungieren.

Es konnte gezeigt werden, daß HSPGs, die eine Bindungsaffinität für PrP besitzen, eine aktive Rolle bei der PrP^{C}- (Hundt et al. 2001) und PrP^{Sc}-Aufnahme spielen (Hijazi et al. 2005, Horonchik et al. 2005). Aus diesem Grund könnten »Mimetics« (Nachahmer) von Heparansulfaten gute Kandidaten für eine therapeutische Intervention darstellen (Caughey und Raymond 1993). Leider erwiesen sich diese Stoffe *in vivo* als toxisch wie Dextransulfat 500 (Farquhar und Dickinson 1986) und Pentosanpolysulfat (Diringer und Ehlers 1991). Neue chemische Verbindungen der »Heparan Mimetics«, welche durch Gruppensubstitution optimiert wurden, konnten sowohl in Zellkultur als auch im Tier die PrP^{Sc}-Bildung hemmen (Adjou et al. 2003), vermutlich durch die Blockierung der PrP^{Sc}-Bindung an den 37kDa/67kDa-Lamininrezeptor (LRP/LR) (Gauczynski et al. 2006, in press).

4.5.2 Der 37kDa/67kDa-Lamininrezeptor als Angriffsziel

Der 37kDa/67kDa-Lamininrezeptor (LRP/LR) wurde von uns als Zelloberflächen-Rezeptor für PrP^C identifiziert (Gauczynski et al. 2001, Rieger et al. 1997). Gemäß unserem Arbeitsmodell (Abb. 1) postulieren wir, daß ein Eingriff in die LRP-PrP^C/PrP^{Sc}-Interaktion zuerst den Eintritt von Prionen in den Verdauungstrakt vermindern und gleichzeitig die Internalisierung von PrP^C hemmen würde, was zu einer Reduktion der PrP^{Sc}-Bildung im endozytotischen Weg führt. Wir konnten mit französischen Kollegen zeigen, daß der gegen LRP gerichtete polyklonale Antikörper W3 die Internalisierung des bovinen PrP^C in humane Erythrozyten blockiert (Morel et al. 2005). In einer aktuellen Arbeit bestätigten wir, daß infektiöse Prionen an den 37kDa/67kDa LRP/LR binden (Gauczynski et al. 2006). Beide Studien zeigen, daß LRP/LR als Rezeptor für infektiöse Prionen fungiert. Wir vermuten weiterhin, daß LRP/LR auch die PrP^{Sc}-Übertragung von Zelle zu Zelle be-

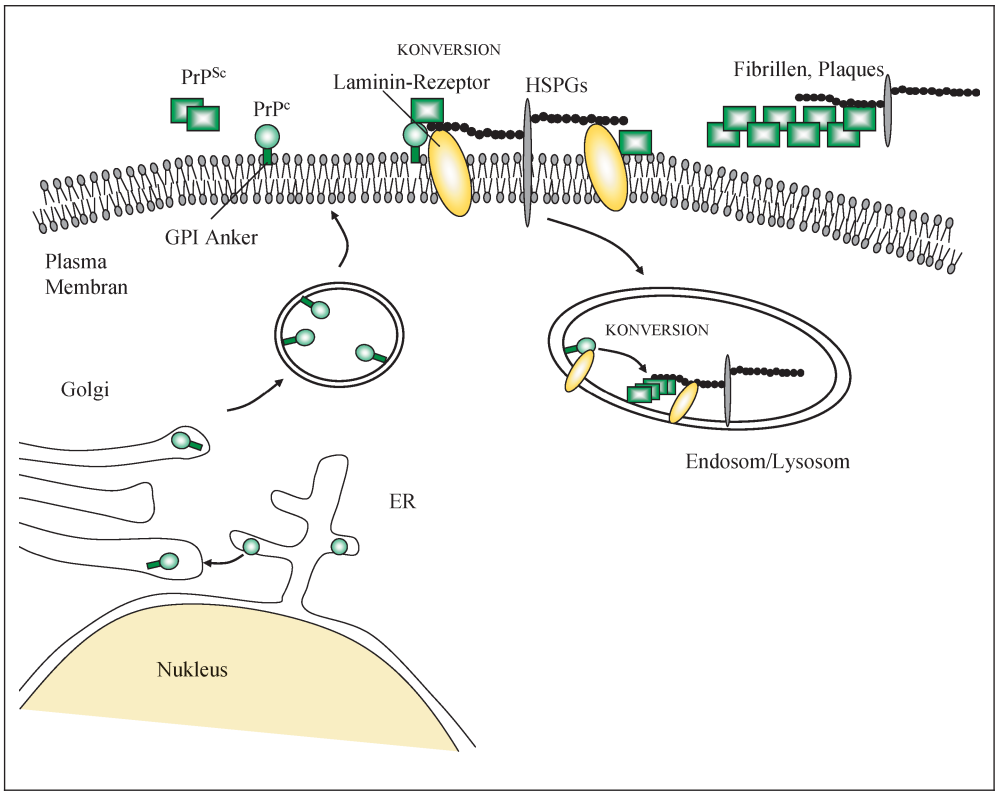

Abb. 1 Die Rolle des Lamininrezeptors in der Biogenese von Prionen. Die zelluläre Isoform des Prionproteins PrP^C wird im ER synthetisiert, glykosiliert und mit einem GPI-Anker versehen. PrP^C wird zur Plasmamembran transportiert, wo es mit dem Lamininrezeptor interagiert. Während der Prioneninfektion bindet die abnorme Isoform PrP^{Sc} an PrP^C, welches durch eine Konformationsänderung zu PrP^{Sc} konvertiert wird. Dieser Prozeß läuft an der Zelloberfläche oder in endozytotischen Vesikeln ab. Die Ausbreitung der Prionen wird von verschiedenen Faktoren beeinflußt, wie z. B. dem Lamininrezeptor und den Proteoglykanen, welche zur Bindung des Prionproteins und auch zu seiner Internalisierung beitragen. PrP^{Sc} akkumuliert in der Plasmamembran, in Lysosomen und im extrazellulären Raum. Die Abbildung wurde modifiziert nach Gauczynski et al. 2006.

günstigt. LRP/LR ist für die Propagation von PrP^{Sc} in Scrapie-infizierten Zellen nötig (LEUCHT et al. 2003). Daher verhindert die Blockierung von LRP/LR mit Hilfe von *Antisense*-RNAs oder der siRNA-Technologie (beide zielen auf die Blockierung der LRP mRNA) die PrP^{Sc}-Propagation in chronisch infizierten Zellen. In einer aktuellen Studie konnten wir zeigen, daß auch die Sekretion einer Transmembrandeletionsmutante des LRP die Prionpropagation in Zellkultur unterbinden kann (VANA und WEISS 2006). Der gleiche Effekt läßt sich auch durch polyklonale Antikörper gegen LRP erzielen, was Möglichkeiten zur Entwicklung neuer auf Antikörper gestützter, experimenteller Therapien eröffnet (LEUCHT et al. 2003). Da polyklonale Antikörper für therapeutische Zwecke kaum in Frage kommen, kann man sich einer neuen Technik bedienen, nämlich der Herstellung und Anwendung von *single chain antibody fragments* (scFvs), sogenannten Einzelkettenantikörpern oder »Miniantikörpern« (BIRD et al. 1988). Die Entwicklung einer TSE-Therapie, die auf gegen LRP/LR gerichtete scFvs basiert, stellt eine vielversprechende Alternative bzw. Ergänzung zum Einsatz von Anti-PrP-Antikörpern dar.

5. *Single Chain Antibodies* als therapeutische Werkzeuge

Auf Antikörper basierende Therapeutika scheinen sich tatsächlich als die »Magic bullets« zu erweisen, für die sie anfangs gehalten wurden.

Monoklonale Antikörper werden in Diagnose und Therapie verschiedenster Erkrankungen, einschließlich Krebs, eingesetzt. Etwa 20 davon sind für die Anwendung am Menschen zugelassen, wie z. B. Rituximab® (LEGET und CZUCZMAN 1998) bei Lymphomen und Herceptin® bei Brustkrebs (DE LORENZO et al. 2004). Dennoch hat man, um diese Technologie zu verbessern, kleinere Antikörper konstruiert, die besser ins Gewebe eindringen können und eine spezifischere Bindungsaffinität besitzen, da sie nur von einem Gen exprimiert werden, welches für ein einziges Polypeptid kodiert.

Unter diesen neuartigen Antikörpern sind die Einzelketten-Antikörper (s*ingle chain antibodies*) die kleinsten: Sie weisen nur Molekulargewichte von ca. 30kDa auf gegenüber 150kDa für vollständige Immunglobuline. Ein *single chain antibody fragment* (scFv) besteht aus den V-Domänen der schweren und leichten Kette (V_H und V_L) eines monoklonalen Antikörpers, welche durch eine *Linker*-Sequenz miteinander verbunden sind (Abb. 2). Die Vorteile der scFvs gegenüber den IgGs machen sie zu einem interessanten Werkzeug für die Therapie neurodegenerativer Krankheiten:

- Sie sind einfach durch Verfahren, wie den »Phage Display«, zu selektieren und in Bakterien zu exprimieren;
- sie können besser in das Hirngewebe eindringen;
- sie können in Form einer Gentherapie appliziert werden; und
- durch das Fehlen des Fc-Anteils lösen sie keine Immunantwort aus.

scFvs haben ihr hohes Potential schon in zahlreichen Publikationen und klinischen Studien, hauptsächlich auf dem Gebiet der Krebserkrankungen, gezeigt. Es werden aber auch neue scFvs für neurodegenerative Krankheiten entwickelt. Als Alternative zu monoklonalen Antikörpern, welche bei Patienten schwere Nebenwirkungen hervorrufen, wurden bereits scFvs gegen β-Amyloid generiert (LIU et al. 2004). Diese scFvs können toxische Effekte von aggregierten A-β-Peptiden in Zellen eliminieren. Desgleichen wurden scFvs gegen Huntingtin hergestellt, welche sich in der Behandlung von Chorea Hun-

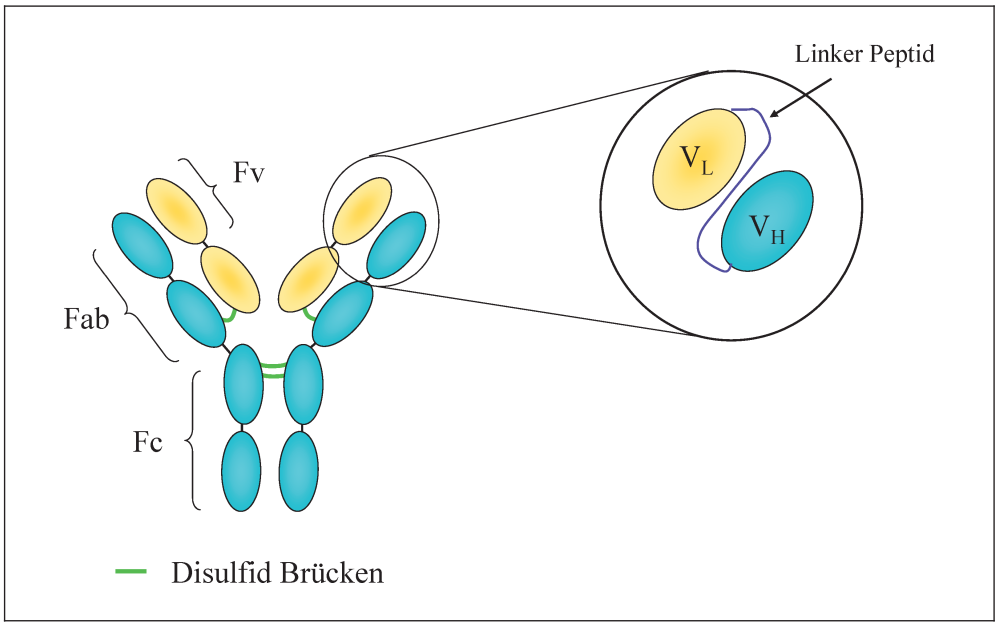

Abb. 2 Schematische Darstellung eines vollständigen IgG-Moleküls und eines Einzelketten-Antikörpers (*single chain antibody*). IgGs bestehen aus 2 ähnlichen leichten Ketten (L) und 2 ähnlichen schweren Ketten (H). Die leichten Ketten enthalten eine konstante Domäne und eine variable Domäne (V_L), während die schwere Kette aus 3 konstanten und einer variablen Domäne (V_H) besteht. Das Heterodimer aus V_H und V_L, verbunden durch ein Peptid, heißt *single chain Fv* (für *fragment variable*). Es hat noch die Fähigkeit der Antigen-Bindung. Gegen PrP oder LRP/LR gerichtete *single chain antibodies* könnten besondere Werkzeuge in der Therapie bei Prionenerkrankungen darstellen.

tington als hilfreich erweisen könnten (Khoshnan et al. 2002). Transgene Tiere, welche scFv gegen PrP^C überproduzieren, sind gegen TSEs geschützt. Erst kürzlich wurde gezeigt, daß Anti-PrP-scFvs, welche aus genetisch modifizierten Zellen stammen, dazu beitragen können, die Ausbreitung der Prionen, von in Kokultur gehaltenen chronisch infizierten Zellen, zu verhindern (»parakriner Effekt«) (Donofrio et al. 2005).

6. Verabreichung (»Delivery«) von Antikörpern gegen TSEs

6.1 Passiver Immunotransfer

Der einfachste Weg zur Verabreichung von Antikörpern stellt die intraperitoneale oder intravenöse Injektion dar. Dafür ist jedoch eine große Menge rekombinanter Antikörper erforderlich, von denen nach der Passage der Blut-Hirn-Schranke nur ein geringer Anteil im Gehirn wirksam werden kann, wo das meiste PrP^{Sc} akkumuliert. Frühere Schätzungen kamen zu dem Schluß, daß nur 0,1 % der injizierten IgGs ins Gehirn gelangen konnten (Bard et al. 2000), für scFvs gibt es dazu noch keine Daten. Daher wird dieser Ansatz für die prophylaktische Behandlung oder eine Behandlung im Anfangsstadium der Krankheit bevorzugt, um die periphere Invasion zu verzögern. Dennoch könnte die intrazerebrale

Injektion ein alternativer Weg der Applikation sein. Eine einzige Injektion in den 3. Ventrikel des Mausgehirns führt innerhalb von 24 Stunden zu einer Diffusion in das gesamte Gehirn (CHAUHAN et al. 2001). Diese Strategie wurde mit dem Anti-Prionenmedikament Pentosan-Polysulfat erprobt, welches über eine dauerhaft gelegte intra-ventrikulare Pumpe infundiert wurde (DOH-URA et al. 2004).

6.2 Gentransfer mittels viraler Vektoren

Die Verwendung von scFvs für TSEs ist wegen der notwendigen großtechnischen Herstellung und der biologischen Verfügbarkeit nur begrenzt möglich. Um diese Einschränkungen zu umgehen, kann eine antikörperbasierte Gentherapie z. B. als *In-vivo*-Gentransfer über virale Vektoren angewandt werden. Mit einem therapeutischen Gen beladene Vektoren können in eine Virushülle verpackt werden und erlauben einen effizienten Gentransfer in der Zielzelle, in Abwesenheit von viraler Genexpression. Mehrere virale Vektorsysteme sind für die Infektion von ZNS-Zellen geeignet. Darunter sind Adenoassoziierte Viren, Lentiviren, Herpesviren und Adenoviren (Tab. 2). Eine einzige Mikroinjektion mit einem rekombinanten Virus in das Gehirn kann dabei die kontinuierliche transgene Expression und Sekretion des Antikörpers gewährleisten.

Tab. 2 Übersicht über Vor- und Nachteile der viralen Vektoren, die für das *Targeting* im Gehirn geeignet sind. Die experimentelle Verabreichung der therapeutischen Moleküle über virale Vektoren ist für drei neurodegenerative Krankheiten in dieser Tabelle aufgeführt. Virale Vektoren werden oft verwendet, um neurotrophe Faktoren im ZNS zu exprimieren, wie z. B. BDNF (»brain derived neurotrophic factor«), GDNF (»glial cell line-derived neurotrophic factor«), CNTF (»ciliary neurotrophic factor«), ApoE (Apolipoprotein E)

Vektor	entzündungs-auslösendes Potential	Nachteile	Vorteile	Verwendung im experimentellen Modell für neurodegenerative Krankheiten		
				Parkinson	Alzheimer	C. Huntington
Lenti-virus	niedrig	Integration kann Onkogenese auslösen	persistenter Gentransfer	GDNF[1]	ApoE[2]	GDNF[3] CNTF
Herpes-virus	hoch	entzündliche Reaktion	selektiver Tropismus für Neuronen	GDNF[4]	–	–
Adeno-assoziiertes Virus	niedrig	entzündliche Reaktion	persistenter Gentransfer, keine Entzündungsinduktion, nicht pathogen	GDNF[5]	ApoE[6]	GDNF[7]
Adeno-virus	hoch	geringe Ladekapazität 5 kb		GDNF[8]	–	BDNF[9]

[1] AZZOUZ et al. 2004; [2] DODART et al. 2005; [3] REGULIER et al. 2003; [4] FINK et al. 2003; [5] WANG et al. 2002; [6] FENG et al. 2004; [7] KELLS et al. 2004; [8] CHEN et al. 2003; [9] BEMELMANS et al. 1999

6.3 Antikörper produzierende Zellen

Genetisch veränderte Zellen könnten die Quelle dauerhaft hoher Konzentrationen von löslichem Antikörperfragment sein, fähig, eine Langzeitexpression zu erreichen. Die Zellen können in immunprotektive Strukturen eingekapselt werden, um Abstoßungsreaktionen zu vermeiden. Die Verwendung von Hybridoma-Zellen, welche monoklonale Antikörper produzieren, ist eine naheliegende Option. Jedoch wird dieser Ansatz durch die kurze Lebenszeit der Hybridoma-Zellen begrenzt. Hingegen sind Muskelzellen aussichtsreichere Kandidaten, da sie langlebig und *in vivo* fähig sind, die Sekretion monoklonaler Antikörper mehrere Monate lang aufrechtzuerhalten (Noel et al. 1997).

7. Ausblick

Obwohl wir noch weit davon entfernt sind, TSEs bei Menschen heilen zu können, stimmen die Interventionen gegen das Fortschreiten der TSEs im Tiermodell – insbesondere durch die Entwicklung der Immuntherapie mit dem Prionprotein und seiner Rezeptoren im Fokus – optimistisch. Es ist anzunehmen, daß die weiteren experimentellen Befunde auf dem Gebiet der Prionenbiologie dazu beitragen werden, in Zukunft weitere Therapieansätze zu entwickeln.

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Chapter III

Novel aspects of prions, their receptor molecules, and innovative approaches for TSE therapy

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Novel Aspects of Prions, Their Receptor Molecules, and Innovative Approaches for TSE Therapy

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SUMMARY

1. Prion diseases are a group of rare, fatal neurodegenerative diseases, also known as transmissible spongiform encephalopathies (TSEs), that affect both animals and humans and include bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, chronic wasting disease (CWD) in deer and elk, and Creutzfeldt–Jakob disease (CJD) in humans. TSEs are usually rapidly progressive and clinical symptoms comprise dementia and loss of movement coordination due to the accumulation of an abnormal isoform (PrP^{Sc}) of the host-encoded prion protein (PrP^{c}).

2. This article reviews the current knowledge on PrP^{c} and PrP^{Sc}, prion replication mechanisms, interaction partners of prions, and their cell surface receptors. Several strategies, summarized in this article, have been investigated for an effective antiprion treatment including development of a vaccination therapy and screening for potent chemical compounds. Currently, no effective treatment for prion diseases is available.

3. The identification of the 37 kDa/67 kDa laminin receptor (LRP/LR) and heparan sulfate as cell surface receptors for prions, however, opens new avenues for the development of alternative TSE therapies.

KEY WORDS: bovine spongiform encephalopathy; Creutzfeldt–Jakob disease; heparan sulfate; 37 kDa/67 kDa laminin receptor; LRP/LR; prion; PrP therapy; transmissible spongiform encephalopathy.

INTRODUCTION

Prion diseases or transmissible spongiform encephalopathies are incurable neurodegenerative disorders, which occur both in humans and animals. Human TSEs include Kuru (Gajdusek and Zigas, 1957), Gerstmann–Sträussler–Scheinker syndrome (GSS) (Gerstmann *et al.*, 1936), fatal familial insomnia (FFI) (Lugaresi *et al.*, 1986), and Creutzfeldt–Jakob disease (CJD) (Creutzfeldt, 1920), which is the most prominent prion disease in humans. However, all of them (as summarized in Table I) are a group of rapidly progressive disorders characterized by a defined spectrum of clinical abnormalities. They share a spongiform degeneration of the brain

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Table I. Summary of the Initial Description of Human TSEs

TSE	Initially described	Reference
Creutzfeldt–Jakob disease (CJD)	1920	(Creutzfeldt, 1920)
Sporadic Creutzfeldt–Jakob disease (sCJD)	1921	(Jakob, 1921)
Familial Ceutzfeldt–Jakob disease (fCJD)	1924	(Kirschbaum, 1924)
Iatrogenic Creutzfeldt–Jakob disease (iCJD)	1974	(Duffy *et al.*, 1974)
New variant Creutzfeldt–Jakob disease (vCJD)	1996	(Will *et al.*, 1996)
Gerstmann–Sträussler–Scheinker syndrome (GSS)	1928	(Gerstmann *et al.*, 1936)
Kuru	1957	(Gajdusek and Zigas, 1957)
Fatal familial insomnia (FFI)	1986	(Lugaresi *et al.*, 1986)
Sporadic fatal insomnia (sFI)	1999	(Mastrianni *et al.*, 1999; Parchi *et al.*, 1999)

and a variable amyloid plaque formation and can appear as sporadic, inherited, or iatrogenic disorders.

Transmissible spongiform encephalopathies (TSEs) also frequently occur in different animal species. Scrapie in sheep and goats (McGowan, 1922), feline spongiform encephalopathy (FSE) in cats (Wyatt, 1990), transmissible mink encephalopathy (TME) (Burger and Hartsough, 1965), chronic wasting disease in wild ruminants (CWD) (Williams and Young, 1980), bovine spongiform encephalopathy (BSE) in cattle (Wells *et al.*, 1987), and encephalopathies of a number of zoo animals (exotic ungulate encephalopathy, EUE) (Jeffrey and Wells, 1988; Kirkwood *et al.*, 1990) have been described. A hallmark of all prion diseases is the accumulation of an abnormal, partially proteinase-resistant isoform of the cellular prion protein (PrP^{c}), which represents a cell-surface glycosylphosphatidyl inositol (GPI) anchored protein (Stahl and Prusiner, 1991). PrP^{c} is highly conserved among mammals (Schätzl *et al.*, 1995) and is expressed in many tissues with notably high levels in the brain of animals and humans (Kretzschmar *et al.*, 1986; Moudjou *et al.*, 2001). The conversion of the host-encoded PrP^{c} into the abnormal disease-inducing isoform (PrP^{Sc}) involves a conformational change and is important in the pathogenesis of these diseases (Prusiner, 1994). Several hypotheses about the nature of the infectious agent have been proposed. Initially, the agent was thought to be a slow virus (Sigurdsson, 1954; Thormar, 1971). In 1967, J. S. Griffith postulated the hypothesis that the causative agent might be a protein (Griffith, 1967). The theory of a self-propagating proteinaceous agent (Bolton *et al.*, 1982) was proposed after the isolation of a protease-resistant sialoglycoprotein specifically associated with infectivity, designated the prion protein (PrP) (Bolton *et al.*, 1982). The term prion, which was devised by Stanley Prusiner, is the abbreviation for "proteinaceous infectious particle" and was defined as "small proteinaceous infectious particle that resists inactivation by procedures which modify nucleic acids" (Bolton *et al.*, 1982; Prusiner, 1982).

THE CELLULAR PRION PROTEIN PrP^{c}

The prion protein (PrP^{c}) is a normal cellular glycosylphosphatidyl inositol (GPI) anchored protein and is highly conserved among mammalian species (Schätzl

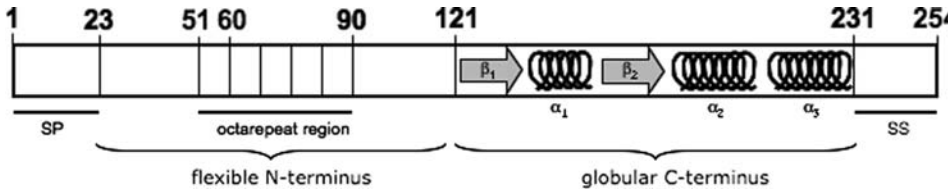

Fig. 1. Schematic view of structural elements of the murine cellular prion protein. The depicted murine cellular PrP (PrP^c) is a GPI-anchored protein of 254 amino acid residues. During PrP^c processing, a 22 amino acid N-terminal signal peptide (SP) is removed and 23 carboxy-terminal amino acid residues (signal sequence (SS)) are cleaved upon the addition of the glycosylphosphatidyl inositol anchor to Ser-231. The N-terminal region contains a series of four octapeptide repeats that have been implicated in the binding of metal ions (Whittal *et al.*, 2000). The first repeat represents a nonarepeat due to an additional glycin residue. The repeat has a histidine residue substituted by a glutamine and, therefore, fails to bind copper (Leliveld *et al.*, 2006). The globular C-terminus of the molecule folds into three α-helices and an antiparallel ß-sheet, whereas the N-terminal part of the protein is flexible as determined in solution. The structure of the murine PrP 121-231 was initially solved by Riek and colleagues (Riek *et al.*, 1996), and that of hamster PrP 29-231 by Donne and colleagues (Donne *et al.*, 1997).

et al., 1995; Wopfner *et al.*, 1999). It has been identified in various animals including birds (Harris *et al.*, 1993), pisces (Gibbs and Bolis, 1997), and marsupials (Windl *et al.*, 1995), and may be present in all vertebrates. PrP mRNA is constitutively expressed in the brains of adult animals with a high expression in neurons (Kretzschmar *et al.*, 1986). Substantial amounts have also been found in heart (Brown *et al.*, 1990), skeletal muscle (Brown *et al.*, 1998; Bosque *et al.*, 2002), lymphoid tissue and leukocytes (Liu *et al.*, 2001; Paltrinieri *et al.*, 2004), intestinal tissues (Morel *et al.*, 2004), and uterus and testis (Tanji *et al.*, 1995). In 1986, the human PrP gene (*Prn-p*) has been mapped to 20p12-pter (Liao *et al.*, 1986; Robakis *et al.*, 1986; Sparkes *et al.*, 1986). The conformation of the cellular isoform of murine PrP was first determined by nuclear magnetic resonance (NMR) studies (Riek *et al.*, 1996). Since then, NMR measurements on the prion protein from various species were performed and revealed that they all have global architecture similarities. The prion protein has a flexible, unstructured N-terminal region and a well-ordered C-terminal globular domain, which includes three α-helices and two antiparallel β-sheet structures (Riek *et al.*, 1996). The N-terminal region contains a segment of several octapeptide-repeat regions that preferentially bind copper (Hornshaw *et al.*, 1995) (Fig. 1). Infrared spectroscopy and circular dichroism demonstrated that the secondary structure of PrP^c is mainly composed of α-helices (42%), whereas PrP^{Sc} consists mainly of β-sheets (Table II) (Cohen *et al.*, 1994). In Syrian hamster and

Table II. Comparison of Biochemical Features of PrP^c, PrP^{Sc}, and PrP27-30

PrP isoform	PrP^c	PrP^{Sc}	PrP27-30
Infectivity	Noninfectious	Infectious	Infectious
Protease status	Sensitive	Partially resistant	Resistant
Solubility	Soluble	Insoluble	Insoluble
Aggregation status	Monomer/dimer/oligomer	Aggregates	Amyloid fibrils
Secondary structure	α-helices (42%), β-sheets (3%)	α-helices (30%), β-sheets (43%)	α-helices (21%), β-sheets (54%)

mice, PrP^c is synthesized as a precursor of 254 amino acids while the human *Prn*-p encodes a prion protein of 253 amino acids in length. During trafficking through the secretory pathway of the cell, the N-terminal signal peptide is cleaved off in the endoplasmatic reticulum (Hope *et al.*, 1986) and 23 C-terminal residues demerge upon addition of the GPI anchor at serine (Ser) 231 (Stahl *et al.*, 1987). Cell culture studies revealed that PrP^c constitutively cycles between the cell surface and an endocytic compartment with a transit time of approx. 60 min and more than 95% of the internalized protein is recycled back to the cell surface (Shyng *et al.*, 1993).

THE PATHOGENIC ISOFORM PrP^{Sc} AND REPLICATION MECHANISMS

In TSEs, the cellular prion protein PrP^c can be converted into a pathogenic isoform referred to as PrP^{Sc} that shows great resistance to radiation and nucleases (Alper *et al.*, 1967). The high proportion of β-sheets in PrP^{Sc} renders it insoluble and markedly resistant to proteases (Table II) (Cohen and Prusiner, 1998). Digestion with proteinuse K results in a 27–30 kDa fragment, termed PrPres (Bolton *et al.*, 1982). PrP27-30 is unusually stable at high temperatures and can only be inactivated by protein denaturants that modify the structure of PrP27-30 (Prusiner *et al.*, 1993). After detergent and protease treatment, PrP27-30 was found to accumulate into rod-shaped polymers that are insoluble in aqueous and organic solvents as well as nonionic detergents. In contrast, PrP^{Sc} (the full-length infectious conformer of PrP^c) has a tendency to form aggregates but not amyloid fibrils (McKinley *et al.*, 1991) (Table II).

Given the same primary sequence of PrP^c and PrP^{Sc} (Basler *et al.*, 1986), the different properties of PrP^c and PrP^{Sc} seemed likely to involve posttranslational modifications. Extensive biochemical investigations have failed to reveal any covalent differences between PrP^c and PrP^{Sc} (Stahl *et al.*, 1993). By contrast, spectroscopic studies demonstrated a conformational difference between PrP^c and PrP^{Sc}. PrP^c has a high α-helical content of approx. 42%, with little or no β-sheets (approx. 3%), whereas PrP^{Sc} contains approx. 30% α-helices and approx. 45% β-sheets (Pan *et al.*, 1993) (Table II). PrP^{Sc} formation is supposed to occur via the interaction between PrP^c and PrP^{Sc}, which is able to convert the host protein into a likeness of itself (Griffith 1967; Bolton *et al.*, 1982). The mechanism by which PrP^{Sc} triggers further PrP^{Sc} production is unknown, although two major models have been proposed. The catalytic model (Prusiner, 1991) proposes that the presence of PrP^{Sc} catalyzes the conversion of PrP^c to PrP^{Sc}. Alternatively, it has been proposed that the formation of PrP^{Sc} is a nucleation-dependent process (Lansbury and Caughey, 1995). The cellfree *in vitro* conversion process was shown to be consistent with the nucleation-dependent polymerization mechanism of PrP^{Sc} formation and inconsistent with the heterodimer mechanism (Caughey *et al.*, 1995). Regardless of the underlying mechanisms, there is more and more evidence supporting the initial idea of self-replicating prions consisting of protein-only, which has in fact been long debated. Although compelling evidence supports this hypothesis, generation of infectious prion particles *in vitro* has not been convincingly demonstrated for a long period of time. Thus, it was shown in 2004, by Legname and colleagues, that

recombinant murine prion protein (including amino acid residues 89–230) produced in *E. coli* can be converted into an infectious PrP form being able to cause a prion disease-like phenotype in transgenic (PrP89-230 expressing) and, in the second round, in wild-type mice (Legname *et al.*, 2004). In addition, Castilla and colleagues showed that PrP conversion can be mimicked *in vitro* by protein misfolding cyclic amplification (PMCA), resulting in indefinite amplification of infectious PrPres as shown by bioassays in hamsters (Castilla *et al.*, 2005).

The precise subcellular localization of PrP^{Sc} propagation remains controversial. There is evidence, however, that either late-endosome-like organelles or lysosomes are involved (Mayer *et al.*, 1992; Arnold *et al.*, 1995). A role for lipid rafts in the formation of PrP^{Sc} is deduced from the finding that both PrP^{c} and PrP^{Sc} are present in rafts isolated from infected cells (Baron and Caughey, 2003; Botto *et al.*, 2004). It was also shown that PrP^{c} lacking the GPI anchor is converted into PrP^{Sc} (Chesebro *et al.*, 2005)

THE FUNCTION OF PrP

The exact physiological role of the cellular prion protein PrP^{c} still remains obscure, although some possible biological functions have been described. The proposed functions include a neuroprotective function due to antiapoptotic activity (Bounhar *et al.*, 2001; Diarra-Mehrpour *et al.*, 2004), a functional role in copper metabolism due to its copper-binding capacity (Brown *et al.*, 1997), involvement in signal transduction (Koch *et al.*, 1991; Mouillet-Richard *et al.*, 2000), memory formation (Collinge *et al.*, 1994), and neuritogenesis (Graner *et al.*, 2000). Mice lacking PrP ($Prnp^{0/0}$) showed no obvious phenotype (Bueler *et al.*, 1992), although they have abnormalities in synaptic physiology (Collinge and Palmer, 1994) and in circadian rhythm and sleep (Tobler *et al.*, 1996). $Prnp^{0/0}$ mice were shown to be completely resistant to prion disease (Bueler *et al.*, 1993). Several lines of PrP knockout mice have been generated to unveil the function of PrP^{c} (Weissmann and Flechsig, 2003).

The finding that lymphocytes express PrP^{c} on the cell surface implicates a role in lymphocyte activation (Cashman *et al.*, 1990). Anti-PrP^{c} antibodies cause partial inhibition of mitogen driven T-cell proliferation giving evidence for a role in modulating T-cell responses (Bainbridge and Walker, 2005). The fact that PrP^{c} is abundantly expressed in the lymphoid tissue and acts as a signaling molecule on T-cells implicates a role in the development and normal function of the immune system (Mazzoni *et al.*, 2005; Ballerini *et al.*, 2006).

The molecular mechanism of PrP protection against oxidative stress is still unclear, but PrP may reduce copper-mediated oxidative stress due to its copper-binding activity (Vassallo and Herms, 2003). Since PrP knockout mice exhibit approx. 50% lower copper concentration in synaptosomal fractions than wild-type mice, it was suggested that PrP^{c} might regulate the copper concentration in the synaptic region and may play a role in the reuptake of copper into the presynapse (Kretzschmar *et al.*, 2000).

Furthermore, it has been shown that PrP^{c} harbors a copper/zinc-dependent superoxide-dismutase (SOD) that provides PrP^{c} with antioxidant activity. By deletion of the octapeptide repeat region involved in copper binding, the SOD activity

was abolished (Brown *et al.*, 1999). *In vivo* experiments revealed that protein and lipid oxidation is increased in skeletal muscle, heart, and liver in $Prnp^{0/0}$ mice suggesting a PrP^c function related to cellular antioxidant defenses (Klamt *et al.*, 2001).

PrP^c is known to be attached to the plasma membrane through a glycosylphosphatidyl inositol (GPI) anchor and may act as a cell-surface receptor mediating cell-surface signaling or cell adhesion. Recently, a coupling of PrP^c to the nonreceptor tyrosine kinase Fyn was observed (Mouillet-Richard *et al.*, 2000). Furthermore, PrP^c has been described to regulate serotonergic receptor signaling and, thus, acting as a protagonist for the homeostasis of serotonergic neurons (Mouillet-Richard *et al.*, 2005).

In addition, several experimental findings suggest a major role for PrP^c in cell survival or cell death. In a yeast two-hybrid system, PrP^c was demonstrated to interact selectively with the Bcl-2 protein (Kurschner and Morgan, 1995), that is a suppressor of the programmed cell death. Recently, the antiapoptotic activity of PrP has been shown in a human breast carcinoma cell line (Diarra-Mehrpour *et al.*, 2004). Cross linking of PrP^c using monoclonal antibodies resulted in rapid and extensive apoptosis in hippocampal and cerebellar neurons suggesting that PrP^c acts in the control of neuronal survival (Solforosi *et al.*, 2004). Expression of PrP^c in gastric cancer cell line led to an upregulation of Bcl-2 whereas p53 and Bax were downregulated (Liang *et al.*, 2006). However, coaggregation of cytosolic PrP with Bcl-2 leads to the induction of apoptosis (Rambold *et al.*, 2006).

Despite all knowledge, there is some controversy on the protective function of PrP^c. It has been demonstrated that in some cell lines, the overexpression of PrP^c increases the susceptibility of these cells to staurosporine-induced apoptosis (Paitel *et al.*, 2002, 2003). In addition, it was proposed that endogenous cellular prion protein sensitizes neurons to apoptotic stimuli through a p53-dependent caspase 3 mediated activation (Paitel *et al.*, 2003, 2004).

INTERACTION PARTNERS OF THE CELLULAR PRION PROTEIN

More than 10 years ago, the existence of a cellular receptor for prions was proposed. It was reasoned that the cellular prion protein PrP^c would require a transmembrane protein to trigger intracellular events (Shyng *et al.*, 1994). Different proteins (Table III) have been shown to interact with the cellular prion protein including laminin (Graner *et al.*, 2000), which is an extracellular matrix protein, N-CAM (Schmitt-Ulms *et al.*, 2001), a cell surface component with an important role in neuronal aggregation, and tyrosin kinase Fyn implicating a role of PrP in cell signaling (Mouillet-Richard *et al.*, 2000).

Employing complementary hydropathy, a 66 kDa membrane protein that binds PrP^c both *in vitro* and *in vivo* was found (Martins *et al.*, 1997) and it was reasoned that this protein might act as a cellular prion protein receptor. However, the same group identified this 66 kDa protein as stress-inducible-protein1 (STI1), playing a role in neurite outgrowth and neuroprotection (Zanata *et al.*, 2002). Parallel to this study, we identified in a yeast two-hybrid screen, the 37 kDa laminin receptor precursor (LRP) as an interaction partner for the prion protein (Rieger *et al.*, 1997).

Table III. Summary of Major Binding Partners for the Cellular Prion Protein, Their Proposed Function, and Subcellular Localization

PrP^c binding molecules	Proposed function	Subcellular localization	Reference
Synapsin 1b	Signal transduction	Intracellular vesicles	(Spielhaupter and Schätzl, 2001)
Grb2	Signal transduction	Intracellular vesicles	(Spielhaupter and Schätzl, 2001)
Pint 1	Unknown	Unknown	(Spielhaupter and Schätzl, 2001)
Tyrosine kinase Fyn	Binding/internalization	Plasma membrane	(Mouillet-Richard *et al.*, 2000)
Caveolin-1	Binding/internalization	Caveolae/rafts	(Mouillet-Richard *et al.*, 2000)
Clathrin	Binding/internalization	Clathrin-coated pits	(Mouillet-Richard *et al.*, 2000)
CK2	Binding/internalization	Caveolae/rafts	(Meggio *et al.*, 2000)
STI 1	Neuroprotection, neuronal outgrowth	Cell surface	(Zanata *et al.*, 2002)
Bcl-2	Antiapoptotic/proapoptotic function	Cytoplasm	(Kurschner and Morgan, 1995; Sakudo *et al.*, 2003; Rambold *et al.*, 2006)
p75	Binding/internalization	Caveolae/rafts	(Della-Bianca *et al.*, 2001)
Laminin	Cell differentiation/cell growth/movement ECM formation	Cell surface	(Graner *et al.*, 2000)
GAGs	Proposed role in prion pathogenesis/receptor for prions	Cell surface	(Pan *et al.*, 2002; Hijazi *et al.*, 2005)
HSPGs/HS	Cofactor for PrP^{Sc} synthesis/receptor for prions	Cell surface	(Gabizon *et al.*, 1993) (Horonchik *et al.*, 2005)
	Co-receptor for PrP^c		(Hundt *et al.*, 2001)
N-CAM	Caveolae-like domain	Caveolae-like domain	(Schmitt-Ulms *et al.*, 2001)
Hsp60	Might influence PrP conversion	Mitochondria (main) (ER, Golgi, secretory granules, membrane fractions)	(Edenhofer *et al.*, 1996)
Nrf2	Unknown	Unknown	(Yehiely *et al.*, 1997)
Aplp1	Unknown	Cell surface	(Yehiely *et al.*, 1997)
37 kDa/67 kDa Laminin Receptor (LRP/LR)	PrP^c binding and internalization (prion protein receptor)/PrP^{Sc} binding and internalization (PrP^{Sc} receptor)	Cell surface	(Rieger *et al.*, 1997; Gauczynski *et al.*, 2001) (Morel *et al.*, 2005; Gauczynski *et al.*, 2006)
NRAGE	Neuronal viability	Cytosol	(Bragason and Palsdottir, 2005)
Tubulin	Intracellular trafficking	Microtubular network cytoskeleton	(Nieznanski *et al.*, 2005)
ZAP-70	T-cell activation	Glycosphingolipid-enriched microdomains	(Mattei *et al.*, 2004)

37 kDa LRP/67 kDa LR AND HEPARAN SULFATE PROTEOGLYCANES AS RECEPTORS/CORECEPTORS FOR PrP^{c} AND PrP^{Sc}

Further *in vitro* studies on neuronal and nonneuronal cells validated that both forms of the laminin receptor, the 37 kDa LRP and the 67 kDa high affinity laminin receptor act as the receptor for the cellular prion protein. Corresponding binding domains on LRP/LR as well as on PrP were identified by yeast-two hybrid technology (Hundt *et al.*, 2001), revealing both a direct and an indirect, HSPG-dependent binding site.

The 37 kDa LRP is thought to be the precursor of the 67 kDa LR, which was first isolated from melanoma cells due to its high binding capacity to laminin (Rao *et al.*, 1983). Although LRP consists of a transmembrane domain (amino acid residue 86–101, (Castronovo *et al.*, 1991)), it is abundantly localized in the cytoplasm (Romanov *et al.*, 1994). In mammalian cells, it has been demonstrated that both the 37 kDa LRP and the 67 kDa LR are present in plasma membrane fractions (Gauczynski *et al.*, 2001). The exact mechanism by which the 37 kDa precursor forms the mature 67 kDa isoform is up to now speculative. Data from the yeast two-hybrid analysis showed that LRP failed to interact with itself (Hundt *et al.*, 2001), which would argue against homodimerization. Analysis of the membrane-bound 67 kDa LR indicated that acylation of LRP is involved in the processing of the receptor (Landowski *et al.*, 1995) and the authors suggest that the 67 kDa form consists of a homodimer of the LRP polypeptide modified by fatty acid chains. On the contrary, a later study postulated that the 67 kDa LR is a heterodimer stabilized by fatty acid-mediated interactions (Buto *et al.*, 1998). Interestingly, mammalian genomes contain multiple copies of the LRP gene, particularly 6 copies in murine and 26 copies in the human genome (Jackers *et al.*, 1996). Sequencing revealed that over 50% of the 37 kDa LRP gene copies were pseudogenes most probably generated by retropositional events suggesting that the accumulation of several copies of this gene might have given a survival advantage to the cell in the course of evolution (Jackers *et al.*, 1996).

Regarding the function of LRP/LR, the 37 kDa LRP appears to be a multifunctional protein (Fig. 2) involved in the translational machinery (Auth and Brawerman, 1992) and has also been identified as p40 ribosome-associated protein (Makrides *et al.*, 1988). LRP has also been found in the nucleus, where it is tightly associated with nuclear structures (Sato *et al.*, 1996) and binds to DNA through associations with histones H2A, H2B, and H4 (Kinoshita *et al.*, 1998). The 37 kDa/67 kDa LRP/LR has been described to act as a receptor for laminin, elastin, and carbohydrates (Ardini *et al.*, 1998), as well as a receptor for Venezuelean equine encephalitis virus (VEE) (Ludwig *et al.*, 1996), Sindbis virus (Wang *et al.*, 1992), and Dengue virus (Tio *et al.*, 2005) (Table III and Fig. 2). Very recently, LRP/LR has been identified as a receptor for Adeno-associated Virus (AAV) serotypes 8, 2, 3, and 9 (Akache *et al.*, 2006). Due to the colocalization of LRP/LR and PrP on the surface of mammalian cells, a possible role of LRP/LR for PrP binding and internalization was assumed. Cell-binding assays revealed, that the PrP^{c} internalization process represents an active LRP/LR-mediated event (Gauczynski *et al.*, 2001). Due to the identification of various LRP/LR isoforms, additional studies have been performed to detect the isoforms that are present in the central nervous system and bind PrP.

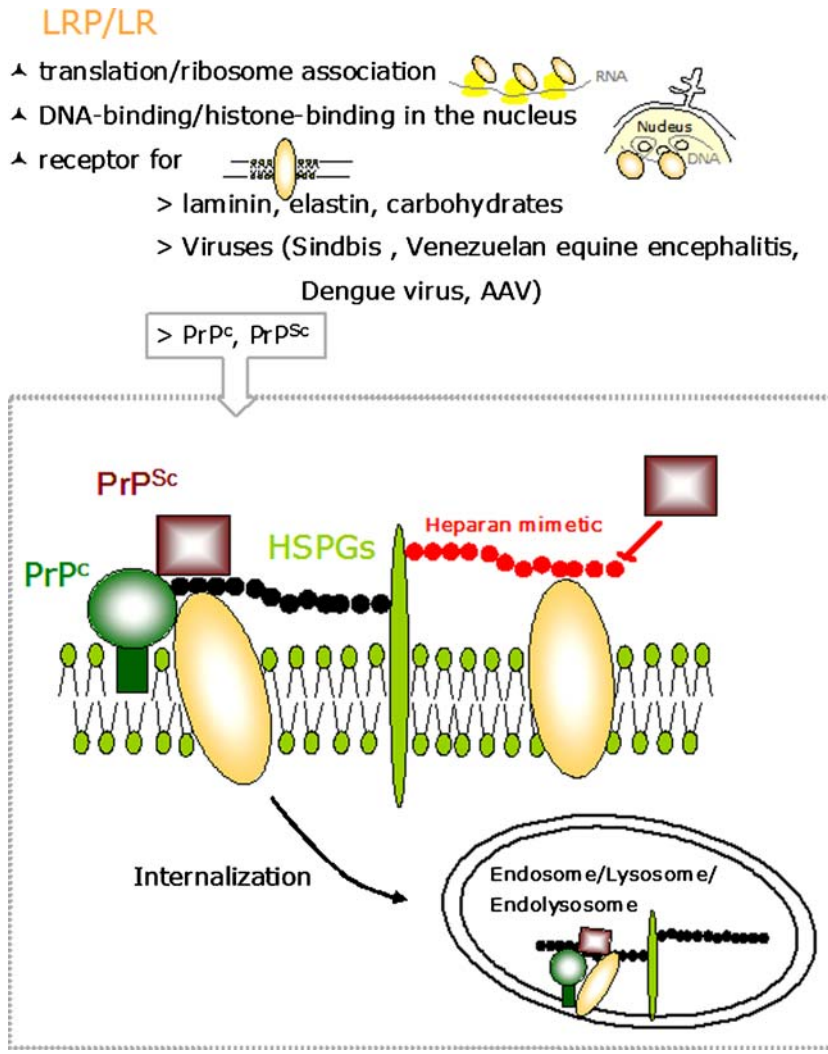

Fig. 2. Cellular functions of LRP/LR and model of the LRP/LR-/HSPG-dependent PrP^{c} and PrP^{Sc} binding and internalization. *Upper panel*: LRP/LR is associated to ribosomes and is involved in the translation machinery (i), binds to nuclear DNA through its association with histones contributing to the maintenance of nuclear structures (ii), and acts as a receptor for laminin, elastin, and carbohydrates, as well as viruses and PrP^{c} and PrP^{Sc}. *Lower panel:* PrP^{c} (*green circle*) anchored by glycosylphosphatidyl inositol (GPI) (*green bar*) becomes internalized by LRP/LR (*yellow ovals*) (Gauczynski *et al.*, 2001) utilizing HSPGs (*light green bars* and *black chain*) as cofactors/coreceptors (Hundt *et al.*, 2001). PrP^{Sc} (*purple squares*) binds to the cell surface in a LRP/LR-(Gauczynski *et al.*, 2006), and heparan sulfate (*black chain*)/ HSPG- (Horonchik *et al.*, 2005) dependent manner. The PrP/LRP-LR/HSPG complex becomes internalized into endo-/lysosomes. LRP/LR (Morel *et al.*, 2005; Gauczynski *et al.*, 2006) and heparan sulfates (Horonchik *et al.*, 2005) mediate presumably in synergy PrP^{Sc} internalization. Polysulfated glycans such as the heparan mimetics HM 2602 and HM 5004 (*red chain*) block PrP^{Sc} binding to the cells by competing with the binding to heparan sulfate and LRP/LR (Gauczynski *et al.*, 2006). Pentosan polysulfate (SP-54) and phycarin sulfate may have similar effects as the HMs (adopted from (Gauczynski *et al.*, 2006)). AAV denotes Adeno-Associated Virus.

Several LRP/LR isoforms corresponding to different maturation states of the receptor were identified, including a 44 kDa, 60 kDa, 67 kDa, and a 220 kDa form. All of these isoforms were able to bind PrP, supporting a physiological role for the laminin receptor/PrP interaction in the brain (Simoneau *et al.*, 2003). A closer insight into the fine cellular distribution of LRP/LR in the central nervous system was obtained by using immunohistochemistry in adult rat brain (Baloui *et al.*, 2004). It has been shown that the 67 kDa LR is the major receptor form, which is expressed within the cytoplasm and at the plasma membrane in most neurons and in a subset of glia cells. In contrast, the 37 kDa LRP is much less abundant in adult than in postnatal central nervous system and its expression is restricted to a subclass of cortical interneurons known to be particularly sensitive to abnormal prion accumulation and rapidly degenerate during early stages of CJD (Belichenko *et al.*, 1999). In addition, recent studies showed that LRP/LR is not only involved in the PrP^{c} metabolism, but has also a crucial role in prion propagation. Using antisense LRP RNA and small interfering (si) RNAs specific for LRP mRNA, PrP^{Sc} levels in scrapie-infected neuronal cells were reduced demonstrating the necessity for the laminin receptor LRP/LR for PrP^{Sc} propagation in cultured cells (Leucht *et al.*, 2003). Moreover, in a recent study, it has been shown that bovine PrP^{Sc} is internalized by human enterocytes via LRP/LR-mediated endocytosis (Morel *et al.*, 2005) using the Caco-2/TC7 cell model system. Analysis of the presence of PrP^{Sc} after supply of prion-contaminated brain homogenate from different sources in Caco-2/TC7 enterocytes revealed that BSE prions were specifically internalized and accumulate in human enterocytes, whereas murine-adapted scrapie-prions were not endocytosed. PrP^{BSE}-containing vesicles visualized in these cells colocalized with LRP/LR in the subapical compartment. PrP^{BSE} internalization was blocked by the anti-LRP antibody W3 approving that prion endocytosis in human enterocytes is mediated by the 37 kDa/67 kDa laminin receptor LRP/LR. Even more recently, the specificity of prion binding in dependency of the 37 kDa/67 kDa LRP/LR has been shown on BHK cells overexpressing LRP (Gauczynski *et al.*, 2006). This effect can be inhibited efficiently by the LRP-specific polyclonal antibody W3 as well as by polysulfated glycans such as pentosan polysulfate and heparan mimetics (Gauczynski *et al.*, 2006). Moreover, it has been shown that GAGs (Hijazi *et al.*, 2005), especially heparan sulfate (Horonchik *et al.*, 2005) act also as receptors for PrP^{Sc}. Taken together, prions like other infectious agents such as viruses may use LRP/LR and heparan sulfate as receptors, presumably in synergy as suggested also in case of PrP^{c} (Gauczynski *et al.*, 2001; Hundt *et al.*, 2001), which also employs LRP/LR and HSPGs as receptors and coreceptors, respectively (Fig. 2).

THERAPEUTIC STRATEGIES FOR THE TREATMENT OF PRION DISEASES

Since variant Creutzfeldt–Jakob disease appeared, numerous strategies and targets have been proposed for a therapy of prion diseases, including:

1. stabilization of the structure of PrP^{c} to prevent the transconformation from PrP^{c} to PrP^{Sc}

2. interference of the binding of PrP^{Sc} to PrP^{c}
3. inhibition of the formation of the abnormal form of PrP
4. prevention of PrP synthesis
5. destruction of PrP^{Sc} aggregates
6. inhibition of the prion protein receptor(s)

The inhibition of the PrP^{Sc} accumulation, however, is the most studied target. There are a number of compounds that have been shown to efficiently interfere with the PrP^{Sc} accumulation, such as Congo red (Ingrosso *et al.*, 1995) and analogs (Demaimay *et al.*, 1997), certain cyclic tetrapyrrols such as porphyrins and phtalocyanines (Priola *et al.*, 2000), sulfated polyanions such as dextran sulfate 500 (Farquhar and Dickinson, 1986), pentosan polysulfate (Caughey and Raymond, 1993) and suramin (Gilch *et al.*, 2001), as well as polyene antibiotics such as AmB and its derivative MS 8209 (Adjou *et al.*, 1995) (Table IV). Many other compounds have been identified to have an effect on the formation of pathological PrP^{Sc} *in vitro* and *in vivo*, but only flupirtine, an analgetic, is possibly beneficial in humans (Otto *et al.*, 2004). To identify novel substances regarding a therapeutic potency, assays for the screening of large compound libraries, e.g., a high-throughput assay for the identification of drugs, which interfere with the PrP^{c}/PrP^{Sc} interaction, were developed (Bertsch *et al.*, 2005). Although several substances have been identified to date, which inhibit PrP^{Sc} formation, unfortunately most of them show only

Table IV. Summary of Major Components Exhibiting Therapeutic Antiprion Effects

Class of compounds	Example	Reference
Polysulfonated, polyanionic substances	Dextran sulfate,	(Farquhar and Dickinson, 1986; Diringer and Ehlers, 1991)
	suramin,	(Gilch *et al.*, 2001)
	pentosan polysulfate,	(Caughey and Raymond, 1993; Farquhar *et al.*, 1999; Gauczynski *et al.*, 2006)
	heparan sulfate mimetics	(Adjou *et al.*, 2003; Gauczynski *et al.*, 2006)
Amyloidotropic intercalators	Congo red	(Caughey and Race, 1992; Poli *et al.*, 2004)
Polyene antibiotics	Amphotericin B (AmB),	(Pocchiari *et al.*, 1987)
	MS 8209	(Adjou *et al.*, 1995)
	Filipin	(Marella *et al.*, 2002)
Cyclic tetrapyrrols	Porphyrines, phtalocyanines	(Caughey *et al.*, 1998; Priola *et al.*, 2000)
Polyamines	DOSPA,	(Winklhofer and Tatzelt, 2000)
	SuperFect, polyethyleneimine	(Supattapone *et al.*, 2001)
Anthracyclines	IDX	(Tagliavini *et al.*, 1997)
Phenothiazines	Chlorpromazine	(Achour 2002; Benito-Leon 2004)
Acridines/bis-acridines	Quinacrine	(Korth *et al.*, 2001)
Designer peptides	β-sheet breaker	(Reilly 2000; Oishi *et al.*, 2003)
RNA aptamers	RNA aptamer Ap1/2/3 (antiprion effect not proven)	(Weiss *et al.*, 1997)
	RNA aptamer DP7	(Proske *et al.*, 2002)
	RNA aptamer 60-3 (antiprion effect not proven)	(Sekiya *et al.*, 2006)

significant effects when administered long before the clinical onset. At present, there is no effective therapy for clinically affected TSE patients available, so that TSEs usually culminate in death.

Another strategy was based on the finding that PrP-specific antibodies antagonize prion propagation both *in vitro* and *in vivo* (for a review, see (Buchholz *et al.*, 2006)). It was proven, that chronically scrapie-infected neuroblastoma cells have been cured by a monoclonal antiprion protein (PrP) antibody (Enari *et al.*, 2001). In a murine model, treatment using this monoclonal antibody has delayed the development of prion disease (White *et al.*, 2003). Application of monoclonal antibodies raised against recombinant PrP resulted also in a reduction of PrP^{Sc} level in mouse neuroblastoma cells (Pankiewicz *et al.*, 2006).

Since active immunization suffers from high costs, researchers have developed passive immunization strategies although this issue deals with the problem of autotolerance. Recently, it became obvious that the induction of a native PrP^{c}-specific antibody response (in contrast to a response against recombinant PrP^{c} produced in bacteria) may help to overcome tolerance. Novel strategies have been developed to obtain such responses by passive immunization (Goni *et al.*, 2005; Nikles *et al.*, 2005). Alternatively, single chain antibodies, which usually can be produced easier and faster than full-length antibodies, revealed an antiprion effect in neuro-blastoma cells (Donofrio *et al.*, 2005).

For the treatment of neurodegenerative diseases such as Alzheimer's disease, AAV-mediated gene delivery has already been established (Zhang *et al.*, 2003; Feng *et al.*, 2004; Hara *et al.*, 2004; Sanftner *et al.*, 2005).

THERAPEUTIC APPROACHES TARGETING LRP/LR

Polysulfated Glycanes

Polysulfated glycanes such as heparan mimetics (HMs) or pentosan polysulfate block PrP^{Sc} binding to target cells by interfering with the PrP^{Sc}-LRP/LR-HSPG binding and internalization complex (Gauczynski *et al.*, 2006) and prolong the survival times of scrapie-infected mice (Farquhar *et al.*, 1999; Adjou *et al.*, 2003). Therefore, these substances are promising tools for the treatment of TSEs.

Transdominant Negative LRP Mutants

Recently, it has been shown that a LRP mutant encompassing only the extracellular domain of LRP/LR (LRP102-295::FLAG) might act in a transdominant negative manner as a decoy by trapping PrP molecules (Vana and Weiss, 2006). *In vitro* studies revealed that the LRP mutant is able to reduce the PrP^{Sc} accumulation in scrapie-infected neuronal cells (Vana and Weiss, 2006) and, thus, might have potential for the development of a TSE therapy.

Antibodies

The PrP binding capacity of LRP offers strategies in therapeutic approaches against prion diseases. Antibodies directed against LRP/LR such as W3 are able to

block PrP^{Sc} propagation in cultured cells (Leucht *et al.*, 2003), prohibit PrP^{BSE} internalization by human enterocytes (Morel *et al.*, 2005), and interfere with PrP27-30 binding to mammalian cells (Gauczynski *et al.*, 2006). Therefore, antibodies directed against the receptor might be powerful tools in the treatment of prion diseases.

However, this antibody format might not be suitable for a therapy in animals or humans. Single chain antibodies are a promising alternative, which are already in use for cancer treatments such as Herceptin® (De Lorenzo *et al.*, 2004) for the treatment of breast cancer (Zhou and Zhong, 2004). Although they consist of a lower molecular weight (30 kDa) compared to the complete immunoglobulins, they reveal a better tissue penetration and higher binding affinity. For therapeutic application, scFv directed against 37 kDa/67 kDa LRP/LR might be passively delivered by intracerebral injection directly into brain regions where massive PrP^{Sc} propagation takes place. In addition, permanent expression and secretion of scFvs might be achieved by gene therapeutic strategies employing lentiviral or AAV-based vector systems.

RNA Interference and Antisense RNA

A further strategy to interfere with PrP^{Sc} propagation is the knockdown of LRP/LR by siRNA and antisense RNA technology. Successfull knockdown has already been shown for PrP using *Prn-p*-specific sequences. Thus, the transfection of siRNA duplices corresponding to the murine *Prn-p* triggered specific *Prn-p* gene silencing in scrapie-infected neuroblastoma cells and caused a rapid loss of their PrPres content (Daude *et al.*, 2003). Accordingly, it was shown in scrapie-infected neuronal cells that transfection of either LRP antisense RNA or LRP-specific siRNAs ablated LRP/LR expression and prevented PrP^{Sc} propagation in scrapie-infected neuronal cells (Leucht *et al.*, 2003), confirming a requirement of LRP/LR for PrP^{Sc} propagation in cultured cells.

However, a permanent effect using RNAi may be achieved by lentivirus-mediated gene transfer to specifically knockdown disease-relevant genes (Ralph *et al.*, 2005; Raoul *et al.*, 2005). Therefore, a lentivirus-based RNAi gene therapy strategy using HIV-derived vectors expressing LRP-specific siRNAs represents an innovative approach in TSE treatment.

CONCLUSIONS

Prion diseases are rare diseases and the protein-only hypothesis (Bolton *et al.*, 1982; Prusiner, 1982) is an approved theory to explain the characteristics of TSEs. However, the natural function of the cellular prion protein (PrP^{c}) remains enigmatic. A series of strategies for TSE treatment are currently under consideration. The majority of these therapeutics target the prion protein itself, destabilize the PrP^{Sc} structure, or interfere with the binding of PrP^{c} to PrP^{Sc}. Nevertheless, no treatment has been shown to prevent the appearance of clinical symptoms and death in animal models or in CJD patients. In June 2004, the PRION-1 clinical trial (3 years) was started to assess the activity and safety of quinacrine in human prion disease

since there are no other drugs available that are considered suitable for human evaluation. Thus, further work is essential to establish treatments that efficiently medicate prion diseases.

The discovery of the 37 kDa/67 kDa laminin receptor (LRP/LR) as the cell surface receptor for the cellular (PrP^c) (Gauczynski *et al.*, 2001) and a receptor for infectious prions (Morel *et al.*, 2005; Gauczynski *et al.*, 2006) as well as GAGs (Hijazi *et al.*, 2005)/heparan sulfate (Horonchik *et al.*, 2005) as receptors for PrP^{Sc} and HSPGs as cofactors/coreceptors for PrP^c (Hundt *et al.*, 2001) opens new avenues for the development of alternative antiprion therapies. Antibodies directed against LRP/LR (Leucht *et al.*, 2003), small interfering RNAs directed against LRP mRNA (Leucht *et al.*, 2003), LRP mutants (Vana and Weiss, 2006), and polysulfated glycanes interfering with the PrP^{Sc}/LRP/LR interaction process (Gauczynski *et al.*, 2006) represent alternative promising tools for the treatment of prion diseases.

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Chapter IV

Therapeutic approaches targeting the prion receptor LRP/LR

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Therapeutic approaches targeting the prion receptor LRP/LR

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Abstract

Transmissible spongiform encephalopathies known as prion diseases are a group of fatal neurodegenerative disorders that affect both humans and animals. The generally accepted principle of the disease is that the conversion of the cellular prion protein (PrP^c) into the disease associated isoform PrP^{Sc} leads to spongiform degeneration of the brain and amyloid plaque formation. Until now no therapy leading to potential alleviation or even cure of the disease exists. It is therefore important to develop therapeutic approaches for the treatment of TSEs since these infections are inevitably fatal and, especially in the case of vCJD, they affect youngsters. Besides current conventional therapeutic strategies, this review summarizes new therapeutic tools targeting the prion receptor LRP/LR.

Keywords: Bovine spongiform encephalopathy; 37 kDa/67 kDa laminin receptor; LRP/LR; Prion; PrP; Transmissible spongiform encephalopathy; Therapy

1. Introduction

Prion diseases or TSEs are neurological disorders associated with the aggregation of a pathologic isoform of a host-encoded prion protein (PrP). Conversion of the cellular prion protein (PrP^c) into the disease-associated form PrP^{Sc} leads to conformational changes resulting in aggregation and accumulation. Deposition of this abnormal protein takes place mainly in the brain and the lymphoreticular system, accompanied with neuronal vacuolation (spongiosis) and neuronal death. After extremely long incubation times, affected individuals show progressive neurological symptoms terminating in death. Conventional therapeutic approaches use anti-prion compounds which can prolong incubation times but do not lead to a cure. It has been demonstrated that prion propagation *in vitro* requires the laminin receptor (Leucht et al., 2003) implicating that approaches downregulating LRP/LR are a promising alternative strategy for the treatment of prion diseases.

2. Prion diseases in humans and animals

Prion diseases involve rapid neurological decline, accompanied by neuronal loss and spongiform changes caused by accumulation of the aggregated and misfolded prion protein. The most common type

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of human prion diseases, termed Creutzfeldt-Jakob disease (CJD), can be classified into four categories: sporadic (sCJD), inherited/familial (fCJD), iatrogenic (iCJD) and variant (vCJD). Whereas it has been suggested that the latter results from ingestion/consumption of BSE-contaminated food (Bruce et al., 1997), familial disorders (fCJD) are the inheritance of autosomal-dominant mutations within the *Prn*-p locus. Transplantation of tissues or injection of hormones originating from individuals suffering from CJD or the use of contaminated surgical instruments resulted in the iatrogenic form of CJD. Gerstmann–Sträussler–Scheinker syndrome (GSS), fatal familial insomnia (FFI), its sporadic form (sFI) and kuru are other human prion diseases.

Animal TSEs have been observed in different species: Bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goat, chronic wasting disease (CWD) in cervids such as deer, elk or captive mule and feline spongiform encephalopathy (FSE) in cats or transmissible mink encephalopathy (TME). In addition, some exotic diseases were observed including exotic ungulate encephalopathy (EUE) and primate spongiform encephalopathy (PSE). Transmission of BSE to pigs has been experimentally proven (Wells et al., 2003). Recently, new forms of TSEs with unusual characteristics e.g. an atypical scrapie case (Nor98) (Benestad et al., 2003) have been discovered. Apart from the existing species barrier, the different modes of transmission are not yet understood.

3. Prions and different forms of prion proteins

The term prion was defined by Stanley Prusiner as a ‘‘small proteinaceous particle that resists inactivation by procedures which modify nucleic acids’’, suggesting that a new agent exists beside the commonly known pathogenic organisms such as bacteria, virus or fungi (Prusiner, 1982). The infectious ‘‘agent’’, the prion, and the exact infectious mechanism for prion disorders is just as little understood as the mechanism by which they kill neurons.

PrP^{c} is an ubiquitous membrane-bound glycoprotein attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor, expressed in many tissues and cell types. Its conversion leads to the disease-associated form PrP^{Sc}, which exhibits a higher β-sheet content correlating with a high tendency to form aggregates. PrP^{Sc} is characterized as insoluble and partially resistant to proteases (Cohen and Prusiner, 1998). Digestion of PrP^{c} with protease K results in the truncated form PrPres (a 27–30 kDa fragment) demonstrating insolubility in aqueous and organic solvents as well as in non-ionic detergents. Additionally, it is completely resistant to proteases. PrP^{Sc} and PrP27-30 both have the tendency to form amyloid fibrils.

4. The 37 kDa/67 kDa LRP/LR as the receptor for PrP^{c}

In a yeast two-hybrid screen we identified the 37 kDa laminin receptor precursor (LRP) as an interaction partner for the prion protein (Rieger et al., 1997). Further *in vitro* studies on neuronal and non-neuronal cells proved that both the 37 kDa LRP and the 67 kDa high affinity laminin receptor function as the receptor for the cellular prion protein (Gauczynski et al., 2001). Direct and indirect heparan sulphate proteoglycane (HSPG)-dependent binding domains on LRP/LR and on PrP have been identified suggesting that HSPGs act as co-factors or co-receptors for PrP^{c} (Hundt et al., 2001). It has been suggested that the 37 kDa LRP is the precursor of the 67 kDa form which was first isolated 1983 from melanoma cells due to its high binding capacity to laminin (Rao et al., 1983). The relationship between the 37 kDa precursor form and the mature 67 kDa isoform is still unknown. Regarding the function of LRP/LR, the 37 kDa LRP appears to be a multifunctional protein involved in the translational machinery (Auth and Brawerman, 1992) and has also been identified as a ribosome-associated protein termed p40 (Makrides et al., 1988). LRP has also been localized in the nucleus, where it is closely associated with nuclear structures (Sato et al., 1996) and binds to DNA through connections with histones H2A, H2B and H4 (Kinoshita et al., 1998). The 37 kDa/67 kDa LRP/LR has been described as a receptor for laminin, elastin and carbohydrates (Ardini et al., 1998), as well as a receptor for Venezuelan equine encephalitis virus (VEE) (Ludwig et al., 1996), Sindbis virus (Wang et al., 1992), Dengue virus (Tio et al., 2005) and Adeno-Associated Viruses (Akache et al., 2006). In addition, studies have been carried out

in order to detect the isoforms that are present in the central nervous system and that bind PrP. Several maturation states of the receptor were identified, including a 44 kDa, 60 kDa, 67 kDa and a 220 kDa form. All of these isoforms were able to bind PrP, suggesting a physiological role for the laminin receptor/PrP interaction in the brain (Simoneau et al., 2003). Although LRP consists of a transmembrane domain (amino acid residue 86–101 (Castronovo et al., 1991)) it is abundant in the cytoplasm (Romanov et al., 1994). In mammalian cells both the 37 kDa LRP and the 67 kDa LR are present in plasma membrane fractions (Gauczynski et al., 2001).

5. The role of LRP/LR in PrP^{Sc} propagation

LRP/LR not only acts as a receptor for the cellular prion protein but also for the infectious PrP27-30, an N-terminal truncated version of PrP^{Sc} (Gauczynski et al., 2006). The importance of LRP/LR in PrP^{Sc} propagation was verified using a polyclonal anti-LRP/LR antibody termed W3 which was able to block and prevent the binding of PrP^{Sc} and to cure scrapie-infected neuroblastoma cells (ScN2a) from PrP^{Sc} (Leucht et al., 2003).

LRP/LR-dependent binding of PrP^{c} and PrP^{Sc} to the cell surface (either alone or together with other co-factors) is accompanied by internalisation which is thought to occur in clathrin-coated pits. After this receptor-mediated endocytosis the conversion of PrP^{c} molecules into the disease-associated form probably takes place in endosomes, lysosomes or endolysosomes. Heparan sulphates also play an essential role in prion uptake and cell infection (Horonchik et al., 2005) suggesting that both the LRP/LR and heparan sulphates act presumably in synergy for PrP^{Sc} binding and internalisation.

The fact that LRP/LR is present in higher amounts in several organs and tissues of scrapie-infected mice and hamsters suggests a correlation between LRP/LR levels and PrP^{Sc} propagation (Rieger et al., 1997). Furthermore, expression studies revealed distribution of the laminin receptor in the intestinal epithelial/brush border confirming that the prion protein uptake and therefore the infection is mediated and supported by this receptor (Shmakov et al., 2000). After oral exposure, TSE agents accumulate in lymphoid tissue, spleen, lymph nodes, tonsils, appendices and Peyer's patches. For this reason prion particles have to cross the intestinal epithelial barrier. Besides the proposition that M-cells are responsible for the uptake of prions (Heppner et al., 2001) has also been suggested that enterocytes are involved in this process, due to the fact that bovine prions are rapidly endocytosed in the presence of LRP/LR (Morel et al., 2005). By preincubating the human enterocytes with the polyclonal anti-LRP/LR antibody, endocytosis of PrP^{BSE} was reduced.

Distribution studies in adult rats revealed that the 67 kDa LR form is highly present in brain regions, classically associated with prion-related neurodegeneration, whereas the 37 kDa form was detected in a subclass of interneurons known to be particularly sensitive to abnormal prion accumulation and cell death during the early stages of CJD (Baloui et al., 2004).

6. Conventional therapeutic strategies for the treatment of TSEs

In recent years, various studies gave evidence that substantial neuropathological changes (e.g. nerve cell degeneration) already occur prior to the onset of symptoms and might be related to PrP^{Sc} accumulation. Accordingly, any effective intervention must aim to start directly after inoculation. Unfortunately, no diagnostic tests are available to detect the disease prior to the onset of symptoms, except for individuals carrying pathogenic mutations within the *Prn*-p gene. Inhibition of PrP^{Sc} formation is the most studied target and can be achieved through (i) inhibition of PrP^{c} synthesis or prevention of its transport to the cell surface, (ii) stabilization of the PrP^{c} structure to make its conformational change unfavourable, (iii) destruction of PrP^{Sc} aggregates, (iv) reversion of PrP^{Sc} to a protease-sensitive form and (v) inhibition of the prion protein receptor(s).

A series of compounds efficiently interfere with PrP^{Sc} accumulation, such as Congo red (Ingrosso et al., 1995) and analogs (Demaimay et al., 1997), certain cyclic tetrapyrrols (Priola et al., 2000) and sulphated polyanions. Although many other compounds have been identified, only flupirtine, an analgetic, had beneficial effects on cognitive function

for human CJD patients (Otto et al., 2004). The anthracycline 4′-iodo-4′-deoxy-doxorubicin (IDX) was able to delay clinical signs of the disease and prolong the survival time in scrapie-infected hamsters (Tagliavini et al., 1997). It was also shown that quinacrine reduces the protease resistance of PrP peptide aggregates and is able to inhibit the *in vitro* conversion of the normal prion protein (PrP^c) to the abnormal form (PrPres) (Barret et al., 2003). Chlorpromazine was reported to increase incubation time in mice after intracerebral, but not intraperitoneal, injection (Roikhel et al., 1984), but was less effective in cell culture than quinacrine (Korth et al., 2001). Unfortunately, most substances that inhibit PrP^{Sc} formation show only significant effects when administered long before the clinical onset of the disease. Since no effective therapy for clinically affected TSE patients is available these diseases are inevitably fatal.

PrP-specific antibodies, a promising alternative tool in TSE treatment, counteract prion propagation both *in vitro* (Enari et al., 2001; Peretz et al., 2001; Perrier et al., 2004) and *in vivo* (Buchholz et al., 2006). In a murine model, treatment with a monoclonal anti-PrP antibody delayed the development of prion disease (White et al., 2003). Application of monoclonal antibodies raised against recombinant PrP also resulted in a reduction of PrP^{Sc} levels in infected mouse neuroblastoma cells (Pankiewicz et al., 2006). Paracrine secretion of single chain antibodies (scFv) directed against PrP^c revealed an anti-prion effect in neuroblastoma cells (Donofrio et al., 2005).

7. Therapeutic approaches targeting LRP/LR

7.1. Trans-dominant negative LRP mutants

Recently, it has been shown that an LRP mutant encompassing the extracellular domain of LRP/LR (LRP102-295::FLAG) might act in a trans-dominant negative way as a decoy by trapping PrP molecules (Fig. 1, I) (Vana and Weiss, 2006). *In vitro* studies revealed that the LRP mutant is able to reduce the

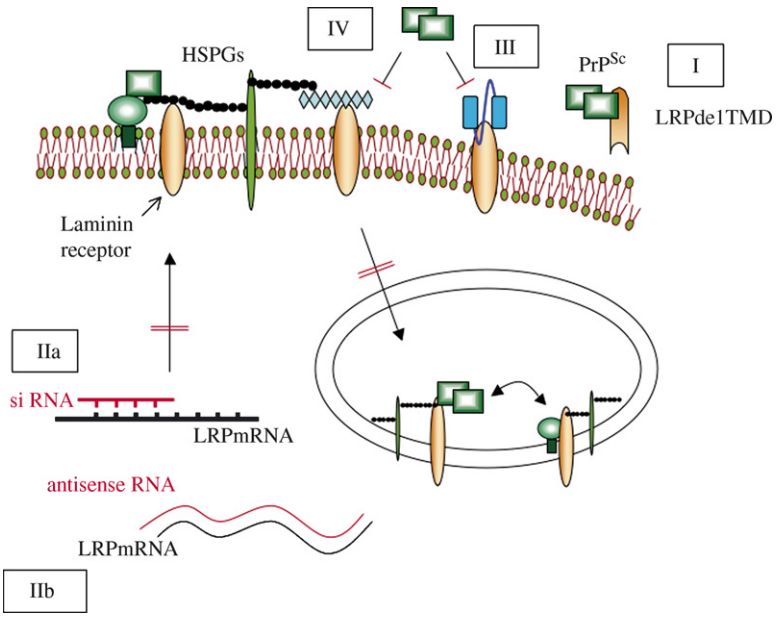

Fig. 1. Molecules inhibiting the PrP-LRP interaction and reducing the LRP/LR levels. LRP/LR (orange) is able to bind both the cellular prion protein (green circle) and the infectious PrP^{Sc} (green rectangle). After binding to the LRP/LR–HSPG complex the prion protein becomes internalized into endo-/lysosomal compartments where the conversion of PrP^c to PrP^{Sc} might take place. Prevention of the binding to LRP/LR is achieved by (I) trapping PrP^{Sc} by a LRP mutant (delTMD) encompassing the extracellular domain (LRP102-295), downregulation of LRP/LR by (IIa) siRNAs and (IIb) antisense RNA directed against LRP mRNA, (III) anti-LRP/LR antibodies (blue rectangles) competing with PrP for LRP binding sites and (IV) heparan mimetics interfering with the binding of PrP^{Sc} to the LRP/LR/HSPG complex.

PrP^{Sc} formation in scrapie-infected neuronal cells (Vana and Weiss, 2006) and might therefore represent a promising novel tool in TSE therapy.

7.2. RNA interference and antisense RNA

A further strategy used to influence the PrP^{Sc} propagation level is the knockdown of LRP/LR by siRNA and antisense RNA technology. This has already successfully been shown for PrP using *Prn*-p-specific sequences. Thus, the transfection of siRNAs corresponding to the murine *Prn*-p triggered specific *Prn*-p-gene silencing in scrapie-infected neuroblastoma cells. This caused a rapid loss of their PrPres content (Daude et al., 2003). Accordingly, it was shown that transfection of either LRP antisense RNA or LRP-specific siRNAs in scrapie-infected neuronal cells results in downregulation of LRP/LR expression and prevention of PrP^{Sc} propagation (Fig. 1, II) (Leucht et al., 2003).

Furthermore, a permanent effect of knockdown of disease-relevant genes using RNAi has been achieved using a lentivirus-mediated gene transfer (Ralph et al., 2005; Raoul et al., 2005). Recently it was shown that lentivector-mediated RNAi efficiently suppressed the prion protein and prolonged survival of scrapie infected mice (Pfeifer et al., 2006). This suggests that an alternative lentivirus-based RNAi gene therapy approach using HIV-derived vectors expressing LRP-specific siRNAs might represent another promising approach in TSE treatment.

7.3. Antibodies directed against the LRP/LR

The PrP binding capacity of LRP offers strategies in therapeutic approaches against prion diseases. The curative effect of the polyclonal anti-LRP/LR antibody (W3) on scrapie infected N2a cells recommends anti-LRP antibodies as therapeutic tools for the treatment of prion diseases (Leucht et al., 2003). On the molecular level this antibody (i) prevents the binding of infectious prions to mammalian cells (Fig. 1, III) (Gauczynski et al., 2006) and (ii) blocks endocytosis of PrP^{BSE} by enterocytes mediated by the LRP/LR is inhibited after treatment with W3 (Morel et al., 2005). Moreover W3 was able to prolong the incubation/survival time in scrapie mice (Zuber et al., submitted).

Since a polyclonal antibody format is not suitable for a therapy in animals or humans the development of single-chain antibodies directed against LRP/LR provides a promising alternative therapeutic strategy. Smaller (30 kDa) and with better tissue penetration, they can be delivered via passive immunotransfer for example intracerebrally into the brain region where massive prion propagation takes place. So far, no immune response or side reactions have been observed after application of scFvs. In a murine scrapie model passively delivered anti-LRP/LR single chain antibodies reduced peripheral prion propagation (Zuber et al., in press). To circumvent the problem of the short half-life in organisms a permanent delivery of single chain antibodies directed against LRP/LR may be achieved by gene therapeutic strategies employing AAV-based or lentiviral vector systems.

7.4. Polysulphated glycans

Polysulphated glycans such as heparan mimetics (HM) or pentosan polysulphate interfere with the binding of the infectious PrP27-30 to the LRP/LR–HSPG complex and are therefore alternative promising therapeutic tools (Fig. 1, IV) (Gauczynski et al., 2006). Treatment of scrapie-infected mice with pentosan polysulphate resulted in a prolonged incubation time and even in the cure of two mouse strains infected with two different scrapie strains (Farquhar and Dickinson, 1986). Moreover, it has been shown that GAGs (Hijazi et al., 2005), especially heparan sulphate, (Horonchik et al., 2005) also act as receptors for the infectious PrP^{Sc}. Polysulphated glycans such as SP54 and PS3 (phycarin sulphate) also show an inhibitory effect on the binding of PrP27-30 to LRP hyperexpressing BHK cells (Gauczynski et al., 2006). Both pentosan polysulphates and heparan sulphate mimetics are able to prolong the incubation time in rodent models and interfere with PrP^{Sc} propagation in neuronal cells due to the inhibition of the LRP/LR dependent binding of prions to target cells.

8. Conclusions

So far, there is no TSE treatment available that is able to cure affected individuals. Alternative therapeutic strategies targeting LRP/LR might be promising since it

acts as the receptor for PrP^{c} and PrP^{Sc}. Molecules targeting the LRP–PrP interaction such as LRP mutants, LRP/LR-specific antibodies and polysulphated glycans or tools downregulating the LRP/LR levels such as siRNAs and antisense RNAs might be effective in the treatment of prion disorders.

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Chapter V

Therapeutic approaches for prion disorders

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Therapeutic approaches for prion disorders

Heike Ludewigs, Chantal Zuber, Karen Vana, Daphne Nikles, Inga Zerr and Stefan Weiss[†]

Prion diseases are lethal for both humans and animals, and affected individuals die after several months following a rapid disease progression. Although researchers have attempted for decades to develop effective therapeutics for the therapy of human prion disorders, until now no efficient drug has been available on the market for transmissible spongiform encephalopathy (TSE) treatment or cure. Approximately 200 patients worldwide have died or suffer from variant Creutzfeldt–Jakob disease (CJD). Incidences for sporadic and familial CJD are approximately 1.5–2 per million per year and one per 10 million per year, respectively, in Europe. This review summarizes classical and modern trials for the development of effective anti-TSE drugs, introduces potential effective delivery systems, such as lentiviral and adeno-associated virus systems for antiprion components, including antibodies and siRNAs, and presents vaccination trials. Most of the antiprion drugs target prion protein PrP^{c} and/or PrP^{Sc}. Alternative targets are receptors and coreceptors for PrP, that is, the 37/67-kDa laminin receptor and heparan sulfate proteoglycanes. We review clinical trials for the treatment of TSEs and describe hindrances and chances for a breakthrough in therapy of prion disorders.

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Prion diseases & therapy

Prion diseases are a group of neurodegenerative diseases affecting animals and humans [1]. Animal transmissible spongiform encephalopathies (TSEs) occur as scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, transmissible mink encephalopathy in mink (TME), exotic ungulate encephalopathy (EUE) in zoo animals and chronic wasting disease (CWD) in elk and deer [1]. Human prion diseases include kuru, Gerstmann–Sträussler–Scheinker syndrome (GSS), fatal and sporadic familial insomnia (FFI/sFI) and Creutzfeldt–Jakob disease (CJD) [1]. CJD can occur in sporadic (s), familial (f), iatrogenic (i) and variant (v) forms (TABLE 1). vCJD is thought to be caused by BSE and represents a zoonotic disease [1]. The incidence for sCJD is 1.5–2 per million per year [2]. Until April 2007, approximately 200 suspected and confirmed cases of vCJD occurred worldwide, most of them in the UK (164) and France (22). Countries showing between one and four vCJD cases include the Republic of Ireland (4), the USA (3), Canada (1), Japan (1), Portugal (1), Spain (1) and The Netherlands (2) [201]. A clear correlation between the numbers of BSE cases and the numbers of vCJD cases in individual countries is not visible, since Germany with no reported case of vCJD has 405 BSE cases (June 2007) [202]. Recent findings on iatrogenic vCJD transmission via blood and blood products caused major concern in various countries and effective therapeutic or postexposure measurements are urgently needed.

Human TSEs, such as CJD, are lethal, with long incubation times (i.e., the time from the day of infection to the first day TSE-relevant symptoms occur). Survival (i.e., the time from the day the first symptoms occur to the day of death) varies between approximately 6 months for sCJD and 15 months for vCJD. The average age for vCJD patients is approximately 27 years [201]. The youngest patient to date died at the age of 14 years, the eldest at the age of 74 years [3,4].

Until now, no therapeutic is on the market for the treatment or cure of prion diseases. In addition, no vaccination against prion diseases

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is available. This article reviews therapeutic and vaccination approaches for the treatment and prevention of prion diseases. The effectiveness of a potential drug is, in principal, tested by three systems: *in vitro* systems representing mainly scrapie propagating cells, *in vivo* systems representing scrapie-infected rodents or macaques, and finally clinical studies. Although a series of drugs demonstrated convincing effects *in vitro* and *in vivo*, those tested in clinical trials failed to show significant effects on the prolongation of the incubation time or survival in patients suffering from a TSE. The development of an effective antiprion compound is challenging because the drug has to penetrate the blood–brain barrier. Although many of the compounds are effective in a cell culture system, they lack any effect *in vivo* because of their low penetration into the CNS. Moreover, a prerequisite for an effective treatment, however, represents the development of a reliable preclinical screening test to diagnose the disease at the preclinical stage and initiate the treatment at an early stage of the disease. Some progress has been made toward this, as shown in the work by Malucci *et al.*, suggesting that disease pathology and symptoms might be reversible [5,6].

Polysulfonated, polyanionic substances

Dextran sulfate

The sulfated polyanion dextran sulfate 500 (DS500) is proven to be an effective substance against TSEs *in vivo* (TABLE 2). A single intraperitoneal injection leads to a dose-dependent reduction of susceptibility to scrapie in mice and to a lengthening of incubation time. The effective scrapie titer has even been reduced by 90% [7]. However, DS500 is toxic in mice, which leads to the question of whether this substance should be tested in human prion diseases.

Suramin

Suramin is a polysulfonated aromatic urea derivative (TABLE 2). It inhibits the scrapie prion protein (PrP^{Sc}) formation *in vitro* and *in vivo* by inducing misfolding of cellular PrP (PrP^{c}) in a post-endoplasmic reticulum (ER)/Golgi compartment (FIGURE 1). The protein is retargeted to acidic compartments, thereby preventing PrP^{c} from reaching the cell surface and the cellular compartment(s) of conversion [8]. The derivatives of suramin are also able to inhibit the PrP^{Sc} *de novo* synthesis and to induce aggregation and reduction of the half-life of PrP^{c} without downregulating the PrP^{c} cell surface expression. Therefore, a symmetrical bipolar structure might be necessary [9].

Table 1. Human prion disorders.

Disorder	Cause
Sporadic CJD	Spontaneous?
Familial CJD	*PRNP* mutation
Gerstmann-Sträussler-Scheinker syndrome	*PRNP* mutation
Fatal familial insomnia	*PRNP* mutation
Variant CJD	Acquired
Iatrogenic CJD	Acquired
Kuru	Acquired

CJD: Creutzfeldt–Jakob disease; *PRNP*: Prion protein gene.

Heparan mimetics

Heparan sulfate mimetics (HMs) belong to the group of polysulfated glycans (TABLE 2). The HMs 2602 and 5004 block the PrP^{Sc}-37/67-kDa laminin receptor (LRP/LR)-HSPG binding and internalization into target cells (FIGURE 1) [10]. HM2602 was able to prolong the survival time in scrapie-infected hamsters, whereas HM5004 does not have any effects *in vivo* [11]. CR36 was efficient in reducing proteinase K (PK)-resistant PrP (PrPres) in scrapie-infected cells but had only a marginal effect on the survival time of mice infected with BSE [12].

Pentosan polysulfate (SP54)

Pentosan polysulfate is a large polyglycoside molecule with weak heparin-like activity (TABLE 2). It is able to prevent PrP^{Sc} propagation in cell culture models (FIGURE 1) [13]. It may inhibit the binding of PrP^{Sc} to LRP/LR by competing with the coreceptor heparan sulfate [10]. *In vivo*, SP54 is able to prolong the survival of scrapie-infected mice and even cures two mouse strains (VM and CBA) infected with two defined prion strains (22A and ME7) [14]. Orally or intraperitoneally administered SP54 may not be very effective since the drug may not cross the blood–brain barrier. Case studies for treatment of vCJD in humans were published in which SP54 was delivered by chronic intraventricular infusion. However, no definite clinical benefit was observed [15,16]. Further clinical studies are required to assess the efficacy of SP54 administration in vCJD and other prion diseases (see 'Antiprion drugs in clinical trials' section).

Phosphorothioate oligonucleotides

Nucleic acids are known to interact with PK-sensitive PrP (PrPsen) molecules. In cell-free assays, DNA binds to recombinant PrP and, depending on their concentrations, PrPsen aggregation is promoted or inhibited [17–19]. Natural nucleic acids do not affect PrPres formation in scrapie-infected neuroblastoma cells (ScN2a), but their degenerate single-stranded phosphorothioated analogs (TABLE 2) (FIGURE 1) are found to bind PrP^{c} and inhibit PrPres accumulation [20]. Phosphorothioate oligonucleotides (PS-ONs) administered prophylactically significantly prolong survival of scrapie-infected rodents [20]. Hydrophobicity and molecular size, but not the base composition of PS-ONs, are important to efficiently interact with PrP^{c}, implying an interaction with an amphipathic site that influences conversion. Thus, PS-ONs are not only an attractive new group of agents in TSE therapy but may also help to identify the mechanism for PrPres formation.

Table 2. Summary of anti-infective drugs against prion disorders, their effects *in vitro* and *in vivo* and their suggested mode of action.

Class of compounds	Example	Effect *in vitro*/*in vivo*	Suggested mode of action	Ref.
Polysulfonated, polyanionic and polycationic substances	Dextran sulfate	+/+	Not known	[7]
	Suramin	+/+	Induces aggregation and retargeting of PrP^{c} to endocytic compartment	[8,9]
	Pentosan polysulfate	+/+	May inhibit internalization of PrP^{Sc} by competing with heparan sulfate and blocking the binding to LRP/LR	[10,13–16]
	Heparan sulfate mimetics e.g., HM2602 HM5004 CR36	+/+ +/- +/-	Inhibits PrP^{Sc} internalization by competing with heparan sulfate and blocking the binding to LRP/LR	[10–12]
	Phosphorothioate oligonucleotides	+/+	Binds to PrP and inhibit PrPres formation	[20]
Amyloidotrophic intercalators	Congo red	+/+	May inhibit PrP^{Sc} propagation by overstabilization of the PrP^{Sc} molecule	[23–25, 27,28]
Polyene antibiotics	Amphotericin B	+/+	May inhibit endocytosis of PrP^{Sc}	[29–35]
	MS 8209	+/+	May inhibit endocytosis of PrP^{Sc}	[29–35]
	Filipin	+/n.d.	Reduces endocytosis of PrPsen	[36]
Tetracyclines	Tetracycline, Doxycycline*	+/+	Direct interaction with PrP^{Sc} leads to reduction of infectivity	[37,38]
Cyclic tetrapyrrols	Porphyrines, Phtalocyanines	+/+	May inhibit PrPres formation	[12,39–42]
Polyamines	DOSPA	+/n.d.	Interferes with the accumulation of PrP^{Sc}	[44]
	SuperFect, Polyethyleneimine	+/n.d.	Interferes with PrP^{Sc} propagation	[43,142]
	Spermine, Spermidine	+/n.d.	Prevents polymerization of recombinant human PrP to PrP^{Sc}	[45]
Anthracyclines	IDX	+/+	May prevent polymerization by binding to amyloid PrP	[48,49]
Phenothiazines	Chlorpromazine	+/n.d.	Prevents PrP^{Sc} formation	[50]
Acridines/bisacridines	Quinacrine†	+/-	May prevent PrP^{Sc} formation	[50–52]
Designer peptides	β-sheet breaker	+/+	Reverses conformational changes of pathogen PrP	[54]
RNA aptamers	RNA aptamer Ap1/2 (antiprion effect not proven)	+/-	Specifically binds PrP^{c}, but not PrP^{Sc}	[55]
	RNA aptamer DP7	+/n.d.	Interferes with the *de novo* synthesis of PrP^{Sc}	[56]
Tyrosine kinase inhibitors	STI571	+/n.d.	Induces of the lysosomal degradation of PrP^{Sc}	[57]

*Currently recruiting for a placebo-controlled trial in Italy (Milan) and Germany (Göttingen) (A Randomized, Double-Blinded, Placebo-Controlled Study of the Efficacy of Doxycyclin in the Treatment of Sporadic Creutzfeldt-Jakob Disease).
†Currently recruiting for a Phase II clinical trial (Novel Therapeutics For Prion Diseases: A Randomized, Double-Blinded, Placebo-Controlled Study of the Efficacy of Quinacrine in the Treatment of Sporadic Creutzfeldt-Jakob Disease).
IDX: Iododeoxydoxorubicin; LRP/LR: Laminin receptor; n.d.: Not determined; PrP: Prion protein.

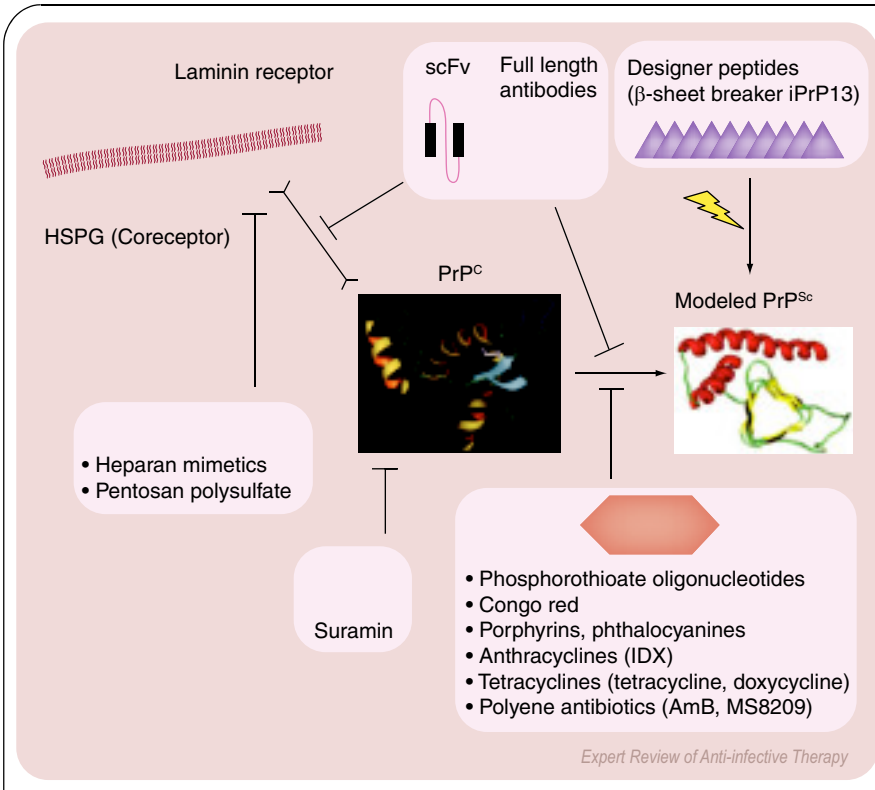

Figure 1. Schematic view of the proposed mode of actions of antiprion drugs, which are effective *in vivo* and *in vitro*. Heparan mimetics and pentosan polysulfate interfere with the PrP^{c} and PrP^{Sc} binding to the 37/67-kDa laminin receptor (LRP/LR). Antibodies directed against PrP^{c} or LRP/LR interfere with the PrP^{c} binding to LRP/LR or PrP^{Sc}. Many anti-transmissible spongiform encephalopathy (TSE) drugs target the PrP^{c}–PrP^{Sc} conversion, such as phosphorothioate oligonucleotides, Congo red, porphyrins, phtalocyanines (cyclic tetrapyrrols), anthracyclines (IDX), tetracyclines and polyene antibiotics. Some of them (e.g., polyene antibiotics) are thought to interfere with the PrP internalization process. The PrP^{c}–PrP^{Sc} conversion process might take place either on the cell surface or within compartments of the endocytic pathway, such as endosomes, lysosomes and endolysosomes. Designer peptides might reverse conformational changes of PrP^{Sc} by breaking β-sheets. Suramin aggregates PrP^{c} to a β-sheet PrP^{C} structure, which is no longer available as a template for conversion to PrP^{Sc}.
HSPG: Heparan sulfate proteoglycane; PrP: Prion protein; scFv: Single chain antibody.
The image of PrP^{c} (NMR structure) was modified from [141]. The model of PrP^{Sc} appears courtesy of Fred E Cohen, Department of Cellular & Molecular Pharmacology, University of California San Francisco (UCSF), USA [203].

Congo red as an amyloidotrophic intercalator

Congo red (CR) (TABLE 2), a sulfonated azo dye that was originally used as a histologic stain for amyloids [21,22], was identified to potently inhibit the PrPres accumulation in scrapie-infected cells (FIGURE 1) without affecting the metabolism of PrP^{c} [23]. Thus, the exact molecular mechanism of CR is not clearly established. However, since CR binds to protein aggregates, it has been proposed that CR inhibits prion propagation by overstabilization of the PrP^{Sc} molecule [24]. Ingrosso and colleagues first reported that *in vivo* administration of CR prolongs the incubation period of hamsters experimentally infected with scrapie. In these studies, the maximal effect was observed when the scrapie agent and CR were coinjected, suggesting that the timing of drug administration is a key determinant [25]. Nevertheless, CR has some properties that make it inappropriate as a therapeutic candidate; for example, CR is not able to cross the blood–brain barrier and it may be toxic after oral administration [26]. Therefore, a number of CR derivatives were synthesized to overcome these unfavorable properties and were tested for their antiprion activity *in vitro* [27] and *in vivo* [28]. In scrapie-infected hamsters, subcutaneous injection of the CR analog CR-A was shown to prolong the survival time comparable to CR [28]. Nevertheless, the adverse effects of these substances make them currently unprofitable for the treatment of human TSEs.

Polyene antibiotics

Polyene antibiotics, such as amphotericin B (AmB) and its synthetic, less-toxic derivative MS-8209 (TABLE 2), are antifungal agents and have been evaluated as anti-TSE agents in scrapie-infected hamsters, delaying disease and PrP^{Sc} accumulation [29,30]. In a murine model of BSE and scrapie, both AmB and MS-8209 were shown to prolong survival times and delay the PrPres and glial fibrillary acidic protein accumulation [31,32]. These substances are thought to interact with cholesterol present in mammalian cell membranes disturbing the PrP^{Sc} endocytosis [33], prevent the propagation of the scrapie agent to the peripheral nervous system [34] or directly affect the conversion of PrP^{c} to PrP^{Sc} (FIGURE 1) [35]. Another polyene antibiotic, filipin (TABLE 2), has also been shown to inhibit PrPres formation *in vitro*. Filipin reduced the endocytosis of PrPsen and led to a massive secretion of fully matured PrPsen and, thus, decreased the amount of PrP^{c} for the conversion process [36].

Tetracyclines

This antibiotic, which is produced in streptomyces, and its derivative doxycycline (TABLE 2) have been shown to revert abnormal physicochemical properties and to abolish neurotoxicity of PrP peptides *in vitro* [37]. Based on these findings, it was tested whether tetracyclines interact with PrP^{Sc} derived from vCJD patients and from cattle infected with BSE. Incubation of the infectious material with tetracycline and doxycycline leads to a dose-dependent decrease in PK-resistant PrP^{Sc} (FIGURE 1) [38]. Syrian hamsters injected with scrapie-infected brain homogenate coincubated with tetracycline or doxycycline showed a delayed onset of disease and a prolonged survival [38]. These data suggest a reduction of prion infectivity through a direct interaction of the antibiotics with PrP^{Sc}. Thus, they are potentially useful for the development of inactivation strategies for BSE- or vCJD-contaminated material.

Cyclic tetrapyrroles

Tetrapyrrole compounds, which include porphyrins and phtalocyanines, are known to bind selectively to proteins and induce conformational changes (TABLE 2). Certain molecules have been identified to inhibit the formation of PrPres *in vitro* [39] and increased the survival of TSE-infected animals [40]. Among different tested compounds, phtalocyanine tetrasulfonate [40,41], *meso*-tetra(4-*N*-methylpyridyl)porphine iron (III) [40], deuteroporphyrin IX 2,4-bis-(ethylene glycol) iron (III) [40] and Fe(III)*meso*-tetra(4-sulfonatophenyl)porphine [42] were the most potent inhibitors. Tetrapyrroles (FIGURE 1) have also been shown to inhibit PrPres formation in a cell-free conversion reaction, suggesting a direct interaction of the tetrapyrrole with PrP molecules [39].

Polyamines

The finding that transfection of scrapie-infected mouse neuroblastoma (ScN2a) cells using the SuperFect reagent, which is a mixture of branched polyamines, reduced the level of pre-existing PrP^{Sc} and led to the testing of several other polyamines (TABLE 2) for their ability to interfere with PrP^{Sc} propagation in chronically infected ScN2a cells. The branched polymers investigated include polyethyleneimine, polypropyleneimine and polyamidoamide dentrimers [43]. All these compounds have been demonstrated to eliminate PrP^{Sc} in an *in vitro* assay using prion-infected cells. Furthermore, the cationic lipopolyamine DOSPA was shown to interfere with the accumulation of PrP^{Sc} in scrapie-infected neuroblastoma cells [44]. Recently, it has been reported that physiological concentrations of the aliphatic polyamines spermine and spermidine (TABLE 2), which are involved in the cellular metabolism and the stabilization of nucleic acids, prevent the polymerization of human recombinant PrP to PrP^{Sc} in solution [45]. Since nucleic acids have been found to catalyze the conversion of PrP^{c} to the β-sheet conformation [17,46] and, most recently, to infectious PrP^{Sc} employing protein misfolding cyclic amplification and polyA RNA [47], the diminishment of biological amines might lead to oligomerization and polymerization of the PrP^{c} and thus, application of polyamines *in vivo* might be a promising therapeutical approach in TSEs.

Anthracyclines

Anthracyclins are a class of chemotherapeutic agents normally used to treat a wide range of cancers (TABLE 2). Administration of a derivative of doxorubicin, 4′-iodo-4′-deoxy-doxorubicin (IDX), in a murine model of reactive amyloidosis reduced amyloid deposits [48]. In prion diseases, IDX was shown to bind to PrP amyloid and prolonged the survival of scrapie-infected hamsters, when administered intracerebrally at the same time as the scrapie agent [49]. It has been proposed that IDX binds to the abnormal form of PrP, decreasing the availability of template molecules for the conversion process of the normal PrP (FIGURE 1) [49]. Owing to its cytotoxcity and limited ability to cross the blood–brain barrier, IDX is inappropriate for treatment of TSEs. However, owing to its antiprion activity, this substance may act as a template for further compound development.

Phenothiazines

Phenothiazines represent compounds consisting of a tricyclic structure and an aliphatic side chain at the central ring (TABLE 2). Chlorpromazine was shown to inhibit PrP^{Sc} formation in ScN2a cells at micromolar concentrations. After a 6-day treatment, PrP^{Sc} was not detectable in ScN2a cells [50]. Since phenothiazines have been used as antipsychotic drugs in humans for approximately 50 years, their pharmacological profile is well documented. Their ability to cross the blood–brain barrier designates these drugs as good candidates for clinical trials for the treatment of CJD.

Acridines/bisacridines

Quinacrine is one of the most prominent drugs tested as a therapeutic tool against prion diseases (TABLE 2). It is a tricyclic antimalaria drug that has been used in humans for more than 60 years. Therefore, long-term experience in treatment is available and its pharmacological properties are well documented. It is able to cross the blood–brain barrier and can be administered orally. Quinacrine has an approximately tenfold higher antiprion potency than chlorpromazine in a ScN2a cell assay [50] and the S-quinacrine-enantiomer was found to have a higher antiprion activity in preventing PrP^{Sc} formation than the S-enantiomer [51]. However, *in vivo* studies of intraventricular-administered quinacrine into Tg7 mice did not show any prolongation effects [52] and even a paradoxical increase of PrPres was observed when mice were treated with quinacrine by the intraperitoneal route [53].

Nevertheless, it was under investigation in a Safety and Efficacy Study (PRION-1: Quinacrine for Human Prion Disease), which ended in March 2007 and, currently, patients are recruited for a Phase II clinical trial (Novel Therapeutics For Prion Diseases: a Randomized, Double-Blinded, Placebo-Controlled Study of the Efficacy of Quinacrine in the Treatment of Sporadic Creutzfeldt–Jakob Disease).

Designer peptides

β-sheet breaker peptides are a prominent group of designer peptides that are known to interfere with the dimensional structure of PrP (FIGURE 1 & TABLE 2). iPrP13, a 13-residue β-sheet breaker peptide, is able to reverse the structural state of PrP^{Sc} to a similar structure of PrP^{c} in a chinese hamster ovary cell culture assay [54]. *In vivo*, the infectivity of PrP^{Sc}-containing material was decreased by 90–95% and the appearance of clinical symptoms in scrapie-infected mice was delayed when the infectious agent was incubated with iPrP13 prior to injection [54]. Thus, these peptides might provide a useful tool to study the PrP conformational changes during pathogenesis.

RNA aptamers

RNA aptamers were selected from large combinatorial libraries in order to recognize target molecules with high affinity (TABLE 2). The two aptamers AP1 and AP2 were identified to bind specifically to murine, hamster and cattle PrP^{c} but not to native PrP27–30 from mice, suggesting that they recognize the

PK-sensitive N-terminus of PrP^c [55]. This finding might contribute to the development of diagnostic tools to distinguish between cellular and pathogenic PrP. Another promising aptamer DP7 significantly reduced *de novo*-synthesized PK-resistant PrP^{Sc} in ScN2a cells that express a chimeric mouse–hamster–mouse PrP [56] and could, therefore, provide a new class of therapeutic agents against TSEs.

Tyrosine kinase inhibitors

Interference with intracellular signaling has been assumed to play a role in the conversion process of PrP^c to its pathogenic isoform. A screening of inhibitors of specific signaling pathways in prion-infected cells revealed that tyrosine kinase inhibitor STI574 (Gleevec®, imatinib mesylate) (TABLE 2) was effective against PrP^{Sc} propagation [57]. It was shown that STI574 decreases the half-life of PrP^{Sc} in ScN2a cells by inducing its lysosomal degradation and, furthermore, the tyrosine kinase c-Abl was proposed to be probably responsible for the anti-prion effect of STI574 [57]. This novel class of compounds might be a promising tool to investigate the role of cellular signaling pathways in PrP^{Sc} propagation, as well as to develop new therapeutic approaches with substances that are already used for treatment of other human diseases. In this regard, Gleevec is successfully established in the treatment of chronic myeloid leukemia.

Antibodies as immunotherapeutics

Since antibodies are the most rapidly growing class of human therapeutics, the development and design of these tools for antagonizing prion infection have been investigated (TABLE 3). There are only a few antibodies specifically targeting PrP^{Sc}; for example, the 15B3 monoclonal antibody (mAb), which is able to discriminate between the normal PrP^c and the disease-associated form PrP^{BSE} [58]; and 8G8, which also shows a species-specific recognition for bovine PrP^{BSE} [59,60]. Antibodies directed against the repeat motif tyrosine–tyrosine–arginine were able to recognize the pathological isoform of PrP [61]. However, none of the mentioned antibodies were effective in interfering with prion propagation, although, antibodies directed against PrP^c aiming to interfere with PrP^{Sc} propagation are permanently under investigation. Primarily, the use of prion-knockout mice enabled the generation of anti-PrP antibodies that were formerly restricted to immunotolerance. A difficulty for the design of antibodies constitutes the PrP structure. While the C-terminus is well defined (structured), the structure of the N-terminus part remains poorly defined (nonstructured). Furthermore, it is still unclear which PrP epitope exhibits the appropriate target interfering with prion propagation.

Antibodies directed against the C-terminal domain of PrP, such as 6H4 (recognizing PrP epitope amino acids [aa]144–152) [62], or the antigen binding fragment F_{ab} D18/D13 (recognizing aa132–156 and aa95–103, respectively) [63] have been reported to inhibit PrP^{Sc} accumulation in neuroblastoma cells, suggesting that the binding of mAb to the corresponding epitope on PrP^c inhibits the PrP^c–PrP^{Sc} interaction occupying their binding domains. In addition to a complete cure, 6H4 protected N2a cells from a scrapie infection. By contrast, antibodies recognizing the octapeptide repeat region that is suggested to be nonessential for prion propagation [64] are able to antagonize PrP^{Sc} formation [65,66]. It can be concluded that the binding to mature PrP^c on the cell surface is necessary for inhibition of prion propagation. Studies with polyclonal antibodies generated against dimeric recombinant murine PrP in mice confirmed that crosslinking of PrP^c on the cell surface is important for PrP^{Sc} formation [67]. Adjacent to therapeutic approaches, antibodies recognizing different epitopes have been generated to clarify the mechanism of PrP^{Sc} conversion and prion propagation, respectively. One study dealing with hybrid-PrP antibody reagents suggests that the PrP regions aa89–112 and aa136–158 are critical for PrP^c–PrP^{Sc} interaction [68].

Based on cell culture models, several *in vivo* studies have been investigated. Prion pathogenesis has been prevented in transgenic mice expressing anti-PrP 6H4 µ-chain [69]. Treatment of scrapie-infected mice with mAbs termed ICSMs by intraperitoneal injection resulted in a markedly reduced peripheral PrP^{Sc} level and prion activity [70]. ICSM35 was raised against the region aa91–110, whereas ICSM18 was directed against the PrP epitope stretching from aa146 to aa159. Treated mice remained healthy for more than 300 days after continued administration starting from the point of maximal PrP^{Sc} accumulation. However, passive immunotransfer of anti-PrP antibodies revealed no effect in the late incubation period when clinical signs already have developed. In addition, this therapy is efficient only if prions are inoculated into mice by the intraperitoneal route. A postexposure prophylaxis using mAbs 8B4 (residues aa34–52) and 8H4 (aa175–185) resulted in a prolongation of the incubation period in scrapie-infected mice [71].

Several groups reported the use of combinations of different antibodies in cell culture. For instance, SAF34 directed against the octapeptide repeat region and SAF61 recognizing residues aa144–152 resulted in an inhibited PrP^{Sc} formation [66]. The observed acceleration of PrP^c degradation suggests that the disappearance of PrP^{Sc} in cells is directly coupled to PrP^c degradation by reducing its half-life [66]. Since the epitopes for SAF34 and SAF61 correspond to the two different binding sites on the PrP for the 37/67-kDa LRP/LR [72], one possible mechanism might be a disruption of the PrP–LRP/LR interaction resulting in an impaired life cycle and degradation of PrP^c. In addition, the 37/67-kDa LRP/LR was confirmed as a receptor for normal PrP^c [73] and infectious PrP^{Sc} [10], suggesting that antibodies blocking the LRP–PrP interaction might be able to interfere with prion propagation. Indeed, the polyclonal anti-LRP/LR antibody W3 abrogates PrP^{Sc} propagation in scrapie-infected N2a cells [74] and prolongs the survival time in rodents infected intraperitoneally with scrapie [ZUBER C ET AL, UNPUBLISHED DATA]. A single chain anti-LRP/LR antibody (scFv), termed S18, selected by phage display reduced peripheral PrP^{Sc} propagation

Table 3. Antiprion antibodies, passive and active immunotherapeutic strategies.

Antibody	*In vitro*/*in vivo* studies	Suggested mode of action	Ref.
Monoclonal anti-PrP 6H4	+/+ (passive immunotherapy)	Inhibition of the PrP^{c}–PrP^{Sc} interaction by occupying their binding domains	[62,69]
scFv antibody 6H4	+/n.d.	Inhibition of the PrP^{c}–PrP^{Sc} interaction by occupying their binding domains	[81]
Anti-PrP mAb 8B4/8H4	n.d./+ (passive immunotherapy)	Interaction with PrP^{c} and/or PrP^{Sc}	[71]
Antigen binding fragment Fab D18/D13	+/n.d.	Inhibition of the PrP^{c}–PrP^{Sc} interaction	[63]
Antiprion mAb ICSM 18	+/+ (passive immunotherapy)	Almost exclusive binding to PrP^{c}	[70]
Antiprion mAb ICSM 35	+/+ (passive immunotherapy)	Recognize PrP^{Sc} in addition to PrP^{c}	[70]
SAF 34/61	+/n.d.	Inhibition of PrP^{Sc} formation	[66]
Antiprion intrabodies derived from 8H4	+/+ (cell therapy)	Inhibition of the transport of PrP^{c} to the cell surface thus blocking of PrP^{Sc} accumulation	[81]
Anti-LRP/LR polyclonal antibody W3	+/+ (passive immunotherapy)	Interference with PrP-LRP/LR interaction	[74]
scFv S18/N3	+/+ (passive immunotherapy)	Interference with PrP-LRP/LR interaction	[75]

LRP/LR: Laminin receptor; mAb: Monoclonal antibody; n.d.: Not determined; PrP: Prion protein; scFv: Single chain antibody.

in a murine scrapie model [75]. Considering essential functions of PrP, for example, within neuroprotection or within signal transduction pathways that might be impaired by anti-PrP antibodies, inhibition of LRP/LR and not PrP by antibodies might be an alternative strategy for therapeutic intervention in prion diseases.

The exact mechanisms by which anti-PrP antibodies interfere with PrP^{Sc} replication are still unclear. A recent screening of mAbs for their capacity to antagonize prion infection in cell culture suggests an epitope-independent mechanism concerning more global effects on the PrP trafficking and/or transconformation process [59]. Antibodies raised against recombinant PrP^{c} were able to persistently clear neuroblastoma cells (N2a) from PrP^{Sc} and to prevent *de novo* infection, due to the interference in PrP^{Sc} formation both on the cell surface and after internalization in the cytosol [76].

One has to consider that the induction of signal transduction pathways by PrP may be disrupted by the application of anti-PrP antibodies. Therefore, the generation of antibodies recognizing a PrP region that is not implicated in cell signaling might be more favorable. Generally, no toxicity was observed in cell culture studies involving anti-PrP antibodies. However, it was reported recently that crosslinking of PrP^{c} by mAbs directed against the central amino sequence induced neuronal apoptosis, suggesting that PrP^{c} might act as an essential protein in the cell [77]. Furthermore, PrP^{c} itself might play a critical role in prion neuropathology and neuronal cell death.

In addition to polyclonal and monoclonal anti-PrP antibody strategies, alternative approaches are undertaken for novel antibody designs. Phage display-based scFvs directed against PrP were effective in cell culture models [78,79]. Moreover, scFvs derived from 6H4 expressed in a eukaryotic system exert paracrine antiprion activity when cocultured with ScN2a cells [80]. Antiprion intrabodies originated from 8H4 targeted to the ER lumen inhibited the transport of PrP^{c} to the cell surface, which resulted in blockage of PrP^{Sc} accumulation [81]. Mice injected intracerebrally with PC12 cells expressing this intrabody developed no clinical scrapie signs [81].

Active immunization/vaccination trials

Active immunization against prion disease has been at the center of a variety of studies since it became evident that humoral immune responses to the PrP can antagonize prion disease, as described in detail in the previous section. The studies using active immunization approaches, few of which were able to prolong incubation times in animals, have been summarized in detail previously [82]. However, the major aspect of prion immunization has recently turned out to be the induction of a response against the native form of PrP as it is expressed, for example, on the surface of murine T cells. Such responses have been predicted to be the key necessity in successful vaccination against prion disease [83]. By contrast, responses to recombinant PrP often failed to prolong incubation times in animals. The need to break tolerance thus prompted researchers to develop elegant strategies starting from antigen presentation via foreign immunogens, such as retrovirus-derived particles [84] or an attenuated *Salmonella* vaccine strain [85]. Recently, a DNA vaccine that combines antigen expression with lysosomal targeting has been developed [86]. Handisurya and colleagues have recently published a study in which papillomavirus-like particles were generated that display a nine amino acid B-cell epitope of PrP, DWEDRYYRE, resulting in potent conversion inhibitors *in vitro* [87]. Taken together, the new generation of antiprion

vaccines raise the promise to be of complete effectiveness, compared with first-level vaccines developed on the basis of recombinant PrP, by inducing a, so far barely understood, antibody response against native PrP.

An alternative strategy for the development of a vaccine to reduce or prevent spread of CWD in domestic and wild populations of cervids was presented by Miller [88]. Five prion peptides were tested in mice for their suitability to act as possible CWD vaccines. Two of the peptides revealed a significant prolongation of survival in Rocky Mountain Laboratories-infected mice. Since recently infectious prions have been detected in body fluids of deer suffering from CWD [89], a vaccination approach seems to be desirable.

Transdominant negative mutants

Several reports have discussed strategies for inhibiting PrP^{Sc} accumulation. One major problem with drugs shown to exert antiscrapie effects is their intrinsic property to induce a wide variety of side effects [49,90]. Several PrP mutants have been investigated for their therapeutic antiprion potential *in vitro* [91]. Kaneko and collegues first showed that substitution of a basic residue at position 167, 171 or 218 in the PrP prevents PrP^{Sc} formation [91] and claimed that these PrP mutants appear to act as dominant negative mutants by binding to protein X more avidly than the wild-type PrP and thus rendering it unavailable for the prion replication process. Mechanistically, Kaneko *et al.* argued that PrP^{c} initially forms a complex with protein X that might act as a molecular chaperone following PrP^{Sc} binding [91]. Alternatively, deleted PrP molecules inhibiting the accumulation of PrP^{Sc} in a transdominant fashion were designed [92]. Employing scrapie-infected mouse N2a cells as a model system, it has been shown that a deletion of eight amino acids in mouse PrP^{c} (PrP^{c}del114–121) abrogates the conversion of the mutant protein into PrP^{Sc}. In addition, PrP^{c}del114–121 overexpression resulted in inhibition of PrP^{Sc} accumulation [92]. Assuming that any side effects of PrP^{c}del114–121 should be minimal compared with those of the chemical compounds, an alternative therapeutic approach can be envisaged using transdominant PrP mutants.

Further studies using transgenic mice expressing dominant negative PrP mutants (Q167R and Q218K) demonstrated that expression of dominant negative PrP strongly reduced PrP^{Sc} accumulation *in vivo* [93]. Here, Perrier *et al.* generated transgenic mice expressing PrP with either the Q167R or Q218K mutation in wild-type mice or on a $Prnp^{0/0}$ background. Following intracerebral prion inoculation into these mice, $Tg(Q167R)Prnp^{+/+}$ and $Tg(Q218K)Prnp^{+/+}$ mice exhibit only low PrP^{Sc} levels or even no PrP^{Sc} accumulation in the brain [93], supporting the possibility of producing prion-resistant livestock.

Recently, in an animal model for iCJD, it has been shown that after a 7-day-lasting intracerebroventricular administration of the dominant negative PrP mutant rPrP-Q218K, the incubation period was prolonged from 117 to 131 days [94].

Delivery of PrP containing dominant negative mutations has been achieved using lentiviral gene transfer [95]. By taking advantage of 'prion-resistant' polymorphisms that naturally exist in sheep and humans (Q171R and E219K), corresponding residues were mutated in the murine *Prnp* gene (Q167R and Q218K) and subcloned into lentiviral vectors. Transduction of prion-infected N2a cells with lentiviral vectors carrying the dominant negative PrP mutants showed a strong expression of the transgene and a potent inhibition of PrP^{Sc} accumulation [95].

Since PrP^{c} is a major cellular requirement for the propagation of infectivity [96], it represents an attractive therapeutic target. However, identification of the 37/67-kDa LRP/LR as the receptor for prions [10,60,73] suggested an alternative target for the development of TSE therapeutics. Recently, it has been shown that a LRP mutant encompassing only the extracellular domain of LRP/LR (LRP102–295::FLAG) might act in a transdominant negative manner as a decoy by trapping PrP molecules [97]. *In vitro* studies revealed that the LRP mutant is able to reduce the PrP^{Sc} accumulation in scrapie-infected neuronal cells [97] and, thus, might have potential for the development of a TSE therapy.

RNA interference approaches

Reduction of the 37/67-kDa LRP/LR level by RNA interference (RNAi) inhibits PrP^{Sc} accumulation in ScN2a and ScGT1 cells [74], suggesting that LRP/LR represents a promising target for a gene delivery approach using siRNAs directed against LRP mRNA.

Another target for siRNAs is the *Prnp* gene. Transfecting scrapie infected N2a cells with siRNA duplexes directed against the *Prnp* mRNA leads to downregulation of both PrPsen and PrPres levels [98]. Recently, lentiviral gene transfer was used to downregulate PrP^{c} expression by delivering shRNAs into goat fibroblasts, which were used to produce a cloned goat fetus by nuclear transplantation. Analysis of the fetal brain tissue revealed a significant downregulation of PrP^{c} (>90%) [99]. Delivery of the shRNAs was even successful when the recombinant lentiviral vectors were injected into the bovine ova prior to *in vitro* fertilization [99].

The group headed by Kretzschmar reported recently that chronic ScN2a cells revealed an efficient reduction of PrP^{Sc} levels after transduction with lentiviral shRNA512 [100]. Most notably, the group mimicked the clinical situation by generating chimeric mice derived from lentivector-transduced embryonic stem cells. These animals carried the lentiviral shRNAs in a defined percentage of brain cells and expressed reduced PrP^{c} levels. Most importantly, in highly chimeric mice, survival was significantly extended after scrapie infection, strongly suggesting that lentivector-mediated RNAi represents an important approach for the treatment of prion disorders [100].

Disablement of both *Prnp* alleles does not impair normal development and behavior in mice [101]. Recently, PrP^{c}-deficient cattle were generated that appeared to be physiologically,

histopathologically, immunologically and reproductively normal [102]. In addition, transgenic mice [tgN(NSEasLRP)2] were constructed that showed a reduced LRP/LR level in hippocampal and cerebellar brain regions and no abnormal behavior compared with control mice [103]. In addition, mice treated with scFv S18 [75] or polyclonal antibody W3 (ZUBER C *ET AL*, UNPUBLISHED DATA), both directed against LRP/LR by immunotransfer, revealed no side effects, suggesting that ectopic downregulation of LRP/LR in the brain or blocking the prion receptor by antibodies has no phenotype. These findings lead to the conclusion that gene silencing by RNAi targeting PrP and LRP is a promising approach to retard disease progression, especially in familial forms of prion diseases where an early treatment is reasonable owing to a possible diagnosis at an early stage of disease.

The 37/67-kDa LRP/LR as a target in therapy of prion diseases

37/67-kDa LRP/LR – originally identified as a PrP^c interacting protein [104] – acts as a receptor for the PrP^c [73] and the infectious PrP^{Sc} [10]. LRP/LR is required for the internalization of BSE prions by human enterocytes [60]. We are developing a series of therapeutics for the treatment of TSEs targeting LRP/LR (reviewed in [1,105]): the polysulfated glycanes HMs and pentosan polysulfate (SP54) (FIGURE 1) both interfere with the binding of PrP27–30 to LRP/LR at the cell surface [10]. Antisense LRP RNA expressed in scrapie-infected neuronal cells reduced PrP^{Sc} propagation [74]. siRNAs directed against LRP mRNA also hamper PrP^{Sc} propagation in scrapie-infected neuronal cells [74]. The lentiviral gene delivery system will be employed to transfer siRNAs into animals. A transdominant negative LRP decoy mutant termed LRP102–295::FLAG impaired PrP^{Sc} propagation in scrapie-infected neuronal cells [97]. Finally, antibodies directed against LRP/LR seem to be powerful tools for the treatment of TSE. The polyclonal antibody W3 cured PrP^{Sc}-propagating cells from scrapie [74] and prolonged the survival time in scrapie infected mice (ZUBER C ET AL, UNPUBLISHED DATA). scFv antibodies directed against LRP/LR (FIGURE 1) reduced peripheral PrP^{Sc} propagation by passive immunization of mice [75] and through delivery of transgenes encoding for scFvs into mice by recombinant adeno-associated viruses (AAVs) (ZUBER *ET AL*. UNPUBLISHED DATA).

Antiprion drugs in clinical trials

Symptomatic treatment

Given the wide range of symptoms and signs in CJD and current limitations of causal therapy, symptomatic treatment becomes extremely important. Antidementive drugs, such as acetylcholinesterase inhibitors, were not tested systematically in CJD patients, probably because, in most cases, the diagnosis is made in advanced disease stages when dementia is severe. Many CJD patients suffer from psychiatric symptoms, such as depression, anxiety, psychosis and hallucinations. Symptomatic treatment spans a wide range of anxiolytic and antipsychotic drugs, such as benzodiazepines or neuroleptics. Since muscle rigidity and akinesia are frequent in the middle and advanced disease stages, atypical neuroleptics should be used to minimize the adverse effects. A symptomatic therapy exists for CJD-typical myoclonus that responds well to clonazepam or valproate at a standard dose.

Observational trials

So far, only a few case reports are available on therapeutic measures that have an effect on the prognosis and course of the disease [106]. Several compounds have been tested for their potential as an antiprion drug. They belonged to distinct classes, such as analgesic, antidepressant, antipsychotic, antimicrobial and anticoagulant drugs. Most of them were tested in observational trials only on a small number of individuals and mainly case reports for these patients are available. These studies are listed in TABLE 4 [107,109].

In vitro experimental results indicate that pentosan polysulphate (PPS) has an effect on PrP production, replication and associated cell toxicity [110]. In animal experiments, PPS has a prophylactic effect [52]. A major problem is that PPS is believed not to cross the blood–brain barrier; therefore, the compound has to be administered intraventricularly. This has been performed in a number of individuals with vCJD and sCJD [15,16,111]. There are controversial results of PPS in clinical studies. Although prolongation of survival was not always reported, this finding is hard to judge because this compound has not been tested in a case–control study. PPS might still be a candidate for a clinical trial since a significant effect was observed in a murine model of scrapie, BSE and vCJD after oral, intraventricular, intraperitoneal and intracerebral administration of PPS [112].

Case–control trials

Controlled clinical trials using a prospective double-blinded approach were conducted for flupirtine only [113]. Other studies reported to date in the literature compared clinical variables and survival times in treated patients and cohorts with untreated CJD patients. Such trials are available for sporadic and vCJD patients using quinacrine and sCJD patients using doxycycline.

Table 4. Summary of case reports with anti-infective drugs used in prion diseases.

Drug	Ref.
Acyclovir	[144,145]
Amantadine	[109,146–151]
Amphotericin B	[152]
Interferon	[107,153]
Pentosan polysulfate	[15]
Quinacrine	[154–157]
Vidarabin	[158]

The flupirtine trial was conducted in 26 sCJD patients and two iCJD patients [113]. A total of 13 patients were treated with 100 mg (300–400 mg) flupirtine daily; 15 controls received placebo. There was no difference regarding survival, that is, 107 days for the treatment group and 106 for the controls. One of the parameters used to monitor the disease progression was the change in the ADAS-Cog score. Patients treated with flupirtine showed significantly less deterioration in the dementia tests than patients treated with placebo. The mean change in ADAS-Cog (baseline to best) was +8.4 (±15.3) in the flupirtine group and +20.6 (±15.1) in the placebo group (p = 0.02, one-sided t-test) [113].

Disease progression was evaluated in 30 sCJD patients and two vCJD patients using 100 mg quinacrine three-times daily. The data were compared with untreated 125 sCJD patients. There was no significant difference on the mean survival time between the treated and untreated groups [114].

According to cell culture and animal experiments, doxycyclin may bind PrP^{Sc} directly, thus preventing further conformational changes [38,115]. A limited number of patients with sCJD were treated with doxycycline and an increased survival in these patients compared with historical sCJD cases was reported [116]. These preobservations have to be confirmed in a prospective multinational study in Italy and Germany, which is currently underway (TAGLIAVINI F, ZERR I, PERS. COMM.). No systematic studies are reported to date for establishment of this treatment in routine clinical practice.

Problems of clinical trials in CJD

Clinical diagnosis

There is a wide scope of clinical phenomenology in human prion disease, regarding the age of onset, presenting features, rate of progression and appearance of other clinical manifestations [117–119]. Owing to clinical heterogeneity, the diagnosis at early stages might be difficult. However, recent advances in clinical diagnostic techniques, such as cerebrospinal fluid (CSF) tests and MRI, enable the recognition of the disease at earlier stages, but better tests are needed to identify patients as early as possible, at best at the preclinical stage [120,121]. On the other hand, clinical studies in patients with prion diseases are hampered by various clinical presentations and variability in the disease course, which are influenced by several factors, such as age at onset, gender, molecular disease subtype and *Prnp* codon 129 genotype [117,122].

At disease onset, the symptoms and signs are not specific; however, during the disease course, almost all patients develop rapid progressive dementia, ataxia, myoclonus and muscle tone abnormalities. The prodromal phase is mainly characterized by unspecific behavioral changes, fear, adynamia and dizziness, which are frequently regarded as unspecific symptoms and no suspicion of a prion disease is raised at that time. Many patients complain of sleep disturbances or relatives might notice eating abnormalities and weight loss. These signs, together with altered social behavior and depressive mood changes, might point toward an organic depression at this phase. Depression, fear and aggression were reported as early psychiatric symptoms [123–127].

With the progression of the disease, patients develop cognitive deficits and focal neurological signs, such as hemianopsia, cerebellar ataxia, pyramidal and extrapyramidal signs, myoclonus and akinetic mutism. In the advanced stage of the disease, the patients have lost contact with the environment, are usually bedridden, akinetic and mute [128]. Muscle tone abnormalities (rigor and/or spasticity) and myoclonus are mostly present. Patients of the classical sCJD variant usually die within months or weeks (median 6 months), whereas others (atypical cases) may survive in this state for 1–2 years [122,129–131].

Differential diagnosis

Owing to the nature of the clinical symptomatology, the differential diagnosis of sCJD includes a large number of neurological and psychiatric diseases. The most frequent differential diagnosis is Alzheimer's disease [132–136]. The rapid disease course, in particular, can rarely be discriminated from CJD when myoclonus is present. Dementia with Lewy bodies and vascular dementias are further diagnoses that are frequently found as a differential diagnosis of CJD in elder patients [118]. Chronic encephalitis is often the differential diagnosis among patients of younger age in both sCJD and vCJD. CSF tests and MRI help to differentiate CJD from other neurodegenerative and inflammatory conditions.

Phenotypic heterogeneity

Phenotypic variability in the clinical syndrome and neuropathological changes in sCJD were recognized a long time ago and some attempts were made to define disease subtypes (such as Heidenhain variant or Brownell–Oppenheim variant). Based on new molecular diagnostic criteria, distinct clinicopathological phenotypes are defined by the codon 129 genotype of *Prnp* together with the type of PK-resistant core of the PrP (either as type 1 or 2). Some of clinicopathological subtypes, formerly known as Heidenhain variant, could be classified as homozygous for methionine (MM)-1/heterozygous (MV)-1 subtype and Brownell–Oppenheim variant as homozygous for valine (VV)-2 subtype [117].

The most frequent subtypes of sCJD are designated as MM-1/MV-1 (the latter are often summarized as one subtype because of clinicopathological similiarities), VV-2 and MV-2 (TABLE 5). Patients with MM1 (median age at onset: 65 years [range 31–81 years]) subtype usually display a short clinical disease course of several months and present with rapid progressive myoclonic dementia. Patients with VV2 subtype usually develop cerebellar ataxia for several months and dementia is usually mild and becomes apparent as the disease progresses, often at late disease stages. The disease course is prolonged (median 7 months). In MV2 subtype, the clinical presentation might vary between extrapyramidal syndrome, ataxia and dementia. The slowly progressive disease, which might span a period of up to 3 years, often hampers the diagnosis, since many other neurodegenerative conditions might be taken into account before CJD is suspected [117,126].

Table 5. Molecular subtypes of sporadic Creutzfeldt–Jakob disease (clinical and pathological characteristics).

Occurence	Molecular disease subtype	Median age at onset, years (range)	Median duration, months (range)	Most prominent clinical signs/symptoms	Neuropathological features
Frequent	MM1/MV1	65 (31–82)	4 (1–18)	Dementia, cortical anopsia and myoclonus	Prominent involvement of occipital cortex, 'synaptic type' PrP staining
	MV2	64 (53–76)	12 (4–27)	Ataxia, dementia and extrapyramidal	Similiar to VV2, focal involvement of the cortex, amyloid-kuru plaques in the cerebellum, plaque-like focal PrP deposits
	VV2	61 (40–76)	7 (3–18)	Ataxia at onset and late dementia	Prominent involvement of subcortical structures, including brain stem nuclei, spongiosis often limited to deep cortical layers, plaque-like PrP staining, prominent perineuronal staining
Rare	MM2-thalamic MM2-cortical	52 (36–71) 64 (49–77)	16 (8–24) 16 (9–36)	Insomnia, dysautonomia at onset, later ataxia and cognitive impairment. Progressive dementia for several months	Atrophy of the thalamus and inferior olive, spongiosis may be absent or focal. Large confluent vacuoles with perivacuolar PrP staining
	VV1	44 (19–55)	21 (17–42)	Dementia at onset, later ataxia and extrapyramidal	Severe pathology in the cerebral cortex and striatum with sparing of brain stem nuclei and cerebellum

CJD: Creutzfeldt-Jakob disease; MM: Homozygous for methionine; MV: Heterozygous for methionine or valine; PrP: Prion protein; VV: Homozygous for valine.
Data from German CJD Surveillance and according to [117,119,129–131].

The median disease duration reported for all forms of sCJD is approximately 6 months [118,122,127]. However, the disease duration appears to vary with respect to the subtypes. The influence of the genotype at codon 129 on disease duration is shown in FIGURE 2. Patients who are homozygous for methionine have the most progressive disease course, followed by those who are homozygous for valine and heterozygous. The latter individuals have the longest survival. In addition, patients who are younger at disease onset might display a disease duration three-times longer than elder individuals with the same genotype (FIGURE 3) [122,137]. Thus, age and codon 129 genotype have to be taken into account when controlled clinical trials are undertaken in CJD patients.

Case identification

Although single prospective, double-blinded trials in CJD were performed [113,114], clinicians face several difficulties with the design. First, CJD is still a rare condition and the incidence rate is 1.5–2 per million per year [138]. Therefore, a monocentric approach has to be chosen, ideally by centralizing the data in one center experienced with case identification and diagnosis. Another major problem is the rapid disease progression. Given a short survival in most patients, an early case identification is crucial to start treatment. A specific problem has to be addressed when a clinical trial has to be carried out in patients with dementia, which might be severe at the time when the CJD diagnosis is made. It is important that ethical issues concerning the trials, which will be carried out on patients unable to give an informed consent, are addressed.

Disease progression

Dementia syndrome in CJD differs from other more common neurodegenerative dementia, such as Alzheimer's disease, but has been poorly characterized and neuropsychological assessment data are limited [124]. Commonly used dementia scales were designed to monitor disease progression in Alzheimer's disease and scales for vascular dementia and frontotemporal dementia are also available [139]. However, these scales and scores might not be suitable to monitor progression of the cognitive decline in CJD patients; therefore, disease-specific scores have to be developed. In addition, scores for monitoring other neurological abnormalities, such as ataxia, rigidity or myoclonus, also have to be applied in clinical trials. Owing to the variability in clinical syndromes and survival across molecular CJD subtypes, scales might be weighted for single subtypes: ataxia is more prominent in VV2 subtype and an ataxia scale might be more useful to monitor the disease progression in this subtype, whereas a score that involves neuropsychiatric assessment might reflect disease progression for the MM1 subtype.

Conclusions

A series of anti-TSE drugs were effective *in vitro* (scrapie-infected neuronal cells) and *in vivo* (mainly scrapie-infected rodent models). However, when administered in clinical studies, none of them so far have revealed significant effects regarding a prolongation of survival times in CJD patients or an improvement of the state of health. A symptomatic therapy for CJD-typical myoclonus exists

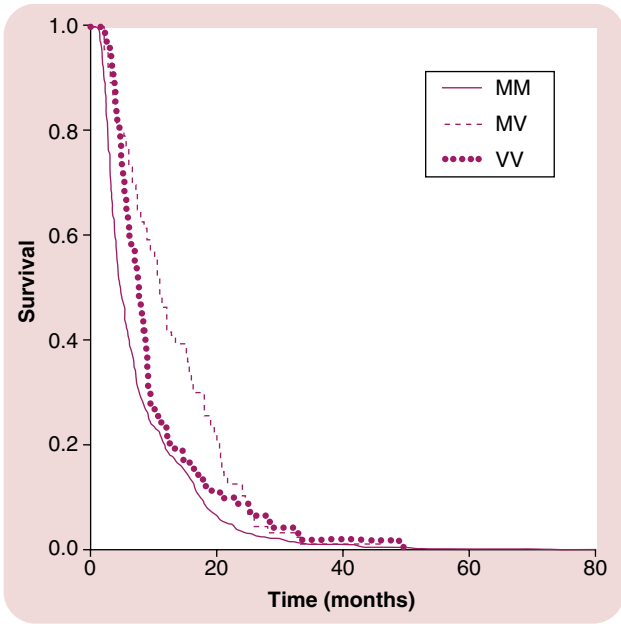

Figure 2. Kaplan–Meyer survival time in Creutzfeldt–Jakob disease patients stratified by codon 129 genotype.
MM: Homozygous for methionine; MV: Heterozygous for methionine and valine; VV: Homozygous for valine.
Reproduced from National TSE reference center, Göttingen, Germany.

that responds well to clonazepam or valproate at a standard dose. A safety and efficacy study with quinacrine ended in March 2007 and patients are currently being recruited for a Phase II clinical trial. Pentosan polysulfate (SP54) was able to cure two mouse strains from two scrapie strains. Clinical trials so far have led to controversial results. HMs might represent alternatives to SP54. Recently, Doh-ura reported prolonged survival times in CJD patients after long-term cerebroventricular administration of the drug [140]. Antibodies directed against PrP and LRP/LR revealed significant effects *in vivo* and might be suitable for passive immunization and vaccination strategies. Promising vaccination trials were reported, including papilloma-like particles that display a nine amino acid B-cell epitope of PrP resulting in potent conversion inhibitors *in vitro*. Prion peptides may act as possible CWD vaccines, which might protect exposed individuals from a CWD infection in the USA. Each attempt to develop a therapeutic strategy considers the development of an early (preclinical screening) test. Until such a test is available, clinical studies in humans will be hampered by the late diagnosis, when extensive neuronal damage has already taken place. On the other hand, many factors have to be taken into account for the analysis of the treatment outcome, such as genetic background and heterogeneous disease course with variable survival times. Disease-specific scores have to be developed to monitor disease progression in single molecular disease subtypes.

Expert commentary

vCJD patients are young and die at a mean age of approximately 27 years. All human TSEs, including the sCJD forms with an incident rate of 1.5–2 cases per million per year, iCJD and the familial forms, fCJD, fatal familial insomnia and GSS syndrome, are lethal and survival times range between approximately 6 months in sporadic and a few years in genetic cases. No therapeutic for the causal treatment of human prion disorders is available on the market worldwide, and TSE patients are treated only symptomatically. Even if the number of vCJD cases declines further, the numbers of sporadic and familial human TSE cases will stay constant or will even increase. Therefore, the development of effective antiprion drugs is essential.

It is hard to predict which of the drugs will show a significant effect on the prolongation of survival in patients. Most of the drugs target PrP. Since PrP does not seem to have essential functions for the organism (PrP-knockout cows reveal no phenotype [102]), depletion of PrP might also be tolerable for the human body. An alternative target is LRP/LR, identified as a receptor for PrP^{c} and PrP^{Sc}. This receptor appears to be necessary for organisms owing to its multifunctional role, such as cell adhesion, growth and development (reviewed in [1,105]). However, ectopic downregulation of LRP in the brain by using an antisense RNA strategy revealed no phenotype [103], suggesting that depletion of LRP in the brain is tolerable for the organism. In addition, intraperitoneal treatment of mice with antibodies directed against LRP/LR, such as W3 and scFv S18 [75], did not reveal any side effects. Therefore, tools blocking LRP/LR might be promising for the treatment of TSEs, especially when delivered by passive immunotransfer, *ex vivo* approaches (grafting of antibody secreting myotubes) and gene delivery systems, including recombinant lentiviral vectors. Here, antibodies directed against LRP/LR, siRNAs directed against LRP mRNA, transdominant negative LRP mutants and polysulfated glycanes, such as SP54 or heparan mimetics, might be interesting tools. In addition, siRNAs directed against PrP mRNA

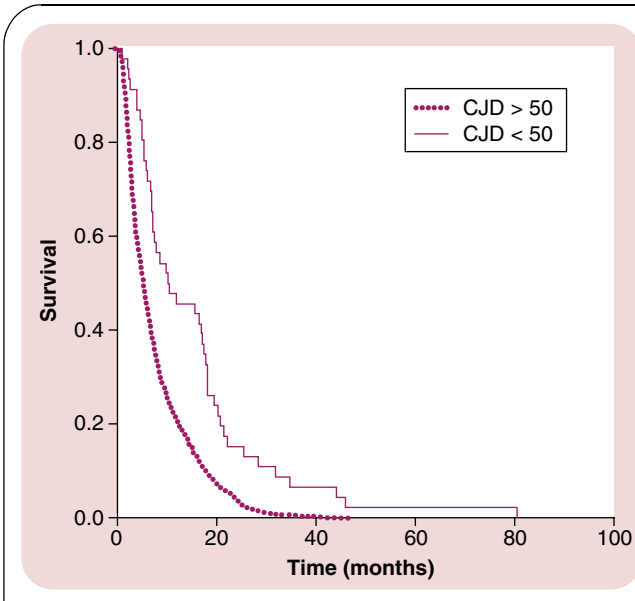

Figure 3. Kaplan–Meyer survival time in CJD patients stratified by age at onset.
CJD: Creutzfeldt-Jakob disease.
Reproduced from National TSE reference center, Göttingen, Germany.

delivered by lentiviral vectors [100] might be promising. The development of preclinical screening tests is a prerequisite for successful treatment of TSE patients. New approaches for the identification of novel drugs for the treatment of TSEs might be required, which target either novel components of the life cycle of prions or well-known interaction partners of the PrP [1]. As long as it is unclear whether CWD and sheep scrapie might cause a zoonotic disease in humans, vaccination trials might be of interest for those people coming in contact with cervids suffering from CWD and/or sheep suffering from scrapie.

Five-year view

Within the next 5 years, we will know whether classical antiprion drugs, such as SP54, HMs, quinacrine, doxycycline, or others, will have the potential to prolong the survival time in TSE patients. We consider the development of novel antiscrapie drugs focusing on further essential components of the life cycle of prions as important. One of the targets is the 37/67-kDa LRP/LR identified as a receptor for PrP^{c} and PrP^{Sc}. Here, antibodies, siRNAs and transdominant negative mutants are currently tested for their antiprion activity in animal models and, in case they are active, also in clinical studies.

A bottleneck in TSE therapy is the delivery system. Antibodies, for instance, can be delivered by passive immunization, *ex vivo* approaches (e.g., grafting of antibody-secreting muscle cells) and gene delivery systems, including AAV and lentiviral vectors. Vaccination approaches include active immunization with PrP and prion peptides, as well as DNA vaccination. Vaccination trials are important, especially for those individuals in contact with animals suffering from a TSE to avoid the development of a zoonotic disease.

siRNAs might represent further powerful therapeutic tools, which can be directed against *Prnp* mRNA, LRP mRNA and mRNAs encoding for further important proteins of the life cycle of prions. Cutting-edge gene delivery systems might include lentiviral vectors, the AAV system or papilloma-like virus particles. A better understanding of the life cycle of prions via identification of all essential components for prion replication will reveal potential alternative targets for development of a powerful antiprion drug. The development of preclinical screening systems seems to be realistic within the next 5 years to enable an early preclinical treatment of TSE patients with powerful antiprion drugs.

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Key issues

- Approximately 200 people worldwide have died or suffer from variant Creutzfeldt–Jakob disease (CJD) most likely caused by bovine spongiform encephalopathy.
- The incidences of sporadic and familial CJD are 1.5–2 per million/year and one per 10 million/year, respectively, in Europe.
- No therapeutic is on the market for the treatment of human transmissible spongiform encephalopathies.
- Most of the therapeutics target prion protein $(PrP)^{c}$ and/or PrP^{Sc}.
- Pentosan polysulfate (SP54) is the only drug that cured the mouse strains VM and CBA from scrapie strains 22A and ME7. There are controversial results with SP54 in clinical studies.
- The prion receptor 37/64-kDa laminin receptor (LRP/LR) is targeted by antibodies, polysulfated glycanes, siRNAs directed against LRP mRNA and transdominant negative mutants.
- No vaccination against prion disorders is currently on the market. Most antiprion vaccinations induce a response to PrP.
- A symptomatic therapy exists for CJD-typical myoclonus that responds well to clonazepam or valproate.
- Case reports were conducted with acyclovir, amantadine, amphotericin B, interferon, pentosane, quinacrine and vidarabin.
- Controlled clinical trials were performed with flupiritine only.
- Clinical diagnosis at early stages of CJD is possible with cerebrospinal fluid tests and MRI. Both techniques help to differentiate CJD from other neurodegenerative and inflammatory diseases.
- Preclinical screening tests are required to develop an effective therapeutic strategy, which can be initiated at the preclinical phase.

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Chapter VI

LRP/LR as an alternative promising target in therapy of prion diseases

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LRP/LR as an alternative promising target in therapy of prion diseases

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Abstract: The 37 kDa/67 kDa laminin receptor (LRP/LR) represents a key player for cell adhesion, is associated with the metastatic potential of solid tumors and is required for maintenance of cell viability by preventing apoptosis. LRP/LR acts as a receptor for viruses such as Sindbis, Venezuelean Equine Encephalitis (VEE) virus, Adeno-associated-viruses (AAV) and Dengue Virus, the latter causing 50 to 100 million infections in humans per year. LRP/LR acts further as a receptor for prions and represents a multifunctional protein subcellularly located to the nucleus, the cytoplasm and the cell surface. The receptor represents an alternative target for therapy of viral infections, cancer and prion disorders and might play additional roles in further neurodegenerative diseases such as Alzheimer's disease. The species barrier in prion disorders might be at least in part determined by the presence of LRP/LR in enterocytes of the intestinal epithelium. Anti-LRP/LR antibodies, siRNAs directed against LRP mRNA, polysulfated glycanes such as pentosan polysulfate and heparan mimetics and LRP decoy mutants are promising tools for blocking or downregulating the receptor and may represent alternative therapeutics for the treatment of prion disorders.

Key words: 37kDa/67kDa laminin receptor LRP/LR, prion protein PrP, neurodegenerative disease, HSPG, pentosan polysulfate, single chain antibody scFv, siRNA, species barrier, zoonotic disease, CWD, scrapie, BSE, therapy, cancer, Alzheimer's Disease

1. The 37kDa/ 67kDa laminin receptor LRP/LR

In a yeast two-hybrid screen, the 37 kDa laminin receptor precursor (LRP) was identified as an interaction partner for the prion protein [1]. Coinfection and cotransfection studies in insect and mammalian cells confirmed the interaction, and led to the assumption that LRP might act as the receptor for the cellular PrP [1]. Further *in vitro* studies on neuronal and non-neuronal cells validated the hypothesis and showed that the laminin receptor LRP/LR act as the receptor for the cellular prion protein [2]. Using the yeast two-hybrid system domains on PrP and LRP have been identified to be involved in the PrP-LRP interaction. Two binding domains for LRP on PrP were discovered: a direct binding domain (PrPLRPbd1, aa144-179) and an indirect one (PrPLRPbd2, aa53-93), which depends on the presence of heparan sulfate proteoglycans (HSPGs) that fuction as co-factors or co-receptors for the binding of PrP^c to the LRP/ LR [3]. Furthermore, the yeast two-hybrid system localized the direct PrP-binding domain on LRP between amino acid residues 161 and 180 [3] (Fig. **1**). A second HSPG-dependent binding site, which has not been identified so far, might be located between amino acid 180 and 285. HSPGs are multifunctional macromolecules characterized by a core polypeptide to which glycosaminoglycans (GAGs) are covalently attached and have also been shown to act as initial attachment receptors for several viruses (Tab. **1**) and are associated with Aβ deposits in Alzheimer's disease (AD) [4].

The 37 kDa LRP is thought to be the precursor of the 67 kDa high-affinity laminin receptor (LR), which was first isolated from melanoma cells due to its high binding capacity to laminin [5]. Although the LRP has a transmembrane domain (amino acid residue 86-101, [3, 6]) (Fig. **1**), it is abundantly localized in the cytoplasm [7]. In

mammalian cells, it has been demonstrated that both the 37 kDa LRP and the 67 kDa LR are present in plasma membrane fractions [2]. The exact mechanism by which the 37 kDa precursor forms the mature 67 kDa isoform is up to now still unclear. Data from a yeast two-hybrid analysis and a size exclusion chromatography on recombinant LRP showed that LRP failed to interact with itself [3], which is an argument against the hypothesis of a direct homodimerization. Analysis of the membrane-bound 67 kDa LR indicated, that acylation of LRP might be involved in the processing of the receptor [8]. Additional studies suggested that the 67 kDa LR might be a heterodimer stabilized by fatty acid-mediated interactions [9]. Mammalian genomes contain multiple copies of the LRP gene, particularly 6 copies in the mouse and 26 copies in the human genome [10]. Sequencing revealed that over 50% of the 37 kDa LRP gene copies were pseudogenes most probably generated by retrotranspositional events. The finding of multiple pseudogenes for the 37 kDa LRP might suggest that the accumulation of several copies of this gene might have given a survival advantage to the cell in the course of evolution [11].

Interestingly, the 37kDa LRP appears to be a multifunctional protein involved in the translational machinery [12] and has also been identified as p40 ribosome-associated protein [13]. LRP has also been found in the nucleus, where it is tightly associated with nuclear structures [14]. The 37kDa/67kDa LRP/LR has been described to act as a receptor for laminin, elastin and carbohydrates [15] as well as a receptor for Venezuelan equine encephalitis virus [16], Adeno-associated Virus [17], Sindbis virus [18] and dengue virus [19] (Tab. **1**). Recent studies investigated the role of LRP/LR in the maintainance of cell viability and it has been shown that knockout of the laminin receptor via RNA interference induced apoptosis [20]. Due to the co-localization of LRP/LR and

PrP on the surface of mammalian cells, a possible role of LRP/LR for PrP binding and internalization was assumed. Using a cell-binding assay with recombinant PrP a LRP/LR dependent binding of PrP has been shown [2]. The strict LRP/LR specificity for the PrP binding could be confirmed in competition assays with different anti-LRP antibodies. Furthermore, it has been demonstrated, that the PrP internalization process represents an active receptor-mediated event [2]. Due to the identification of various LRP/LR isoforms, additional studies have been performed to detect the isoforms that are present in the central nervous system and bind PrP. Therefore, mouse brain fractions enriched in the laminin receptor were purified and overlay assays with recombinant PrP were performed [21]. Several LRP/LR isoforms corresponding to different maturation states of the receptor were identified, including a 44 kDa, 60 kDa, 67 kDa and a 220 kDa form. Furthermore, it could be demonstrated, that all of these isoforms were able to bind PrP, supporting a physiological role for the laminin receptor/PrP interaction in the brain [21]. A closer insight into the fine cellular distribution of LRP/LR in the central nervous system was reached by using immunohistochemistry in adult rat brain [22]. It has been shown, that the 67 kDa LR is the major receptor form, which is expressed within the cytoplasm and at the plasma membrane in most neurons and in a subset of glia cells [22]. In contrast, the 37 kDa LRP is much less abundant in adult than in postnatal central nervous system and its expression is restricted to a subclass of cortical interneurons known to be particularly sensitive to abnormal prion accumulation and rapidly degenerate during early stages of Creutzfeldt-Jakob Disease (CJD) [23]. In addition, recent studies showed, that LRP/LR is not only involved in the PrP^c metabolism, but fullfills also a crucial role in prion propagation. Using antisense LRP RNA or small interfering (si)

RNAs specific for LRP mRNA, PrP^{Sc} levels in scrapie-infected neuronal cells were reduced indicating a necessity for the laminin receptor LRP/LR for PrP^{Sc} propagation in cultured cells [24].

Due to the facts, that a (natural) infection with prions mostly occur via an oral route and that LRP/LR act as receptor for prions [2, 25] potential binding sites for PrP in the intestinal mucosa were examined. Tissue expression studies of the LR in human duodenal and jejunal biopsy samples led to the discovery, that this receptor is expressed in the apical brush border of small intestinal epithelial cells. Employing immunohistochemistry LR expression has also been observed in the perinuclear/Golgi apparatus region and in the Paneth cell secretory granules [26]. A colocalization with PrP^{c} in the perinuclear compartment has recently been proven [27]. These findings suggest an involvement of LR in both secretory and endocytotic functions of human small intestinal epithelium. Moreover, it was speculated that the major implication of intestinal expression of the 67 kDa LR may be an increased susceptibility to an oral infection with prions [26]. It has been demonstrated, that the oral transmission of infectious prion particles led to a rapid accumulation of PrP^{Sc} in Peyer's patches [28]. PrP^{Sc} has also been detected in enterocytes of the villous epithelium of the small intestine of primates after oral exposure to prions [29]. Enterocytes represent the major cell population of the intestinal epithelium [30] and are known to actively participate in endocytosis. Since expression of PrP^{c} was demonstrated to be necessary for prion replication, expression of the cellular prion protein in the gastrointestinal tract has been analyzed and indeed it has been shown that PrP^{c} is present in human enterocytes [31]. These results led to the hypothesis that enterocytes might play an important role for the uptake of infectious prion particles.

Previously, it has been demonstrated that bovine PrP^{Sc} is internalized by human enterocytes via an LRP/LR-mediated endocytosis [32]. For a role in the uptake of pathogens from the gut, the 37 kDa/ 67 kDa LRP/LR must be internalized after ligand binding. In terms of internalization, it has been shown that the 67 kDa LR functiones as the major receptor for virus entry into mammalian cells [18]. Association of the laminin receptor with glycolipid-enriched microdomains in the cell membrane might lead to a clustering with other proteins in this region to provide a mechanism for internalization [33]. In summary, an important role of the 37 kDa/ 67 kDa LRP/LR in mediating binding and internalization of the prion protein and its involvement in pathological mechanisms was demonstrated.

2. Role of LRP/LR as a target in prion diseases

2.1. Antibodies as therapeutic tools

Antibodies gained increasing attention in the development of therapeutics for human disease. Especially monoclonal antibodies are used in clinical trials and many of them already obtained the approval from the U.S. Food and Drug Administration (FDA) for therapy e.g. in cancer. Several anti-prion antibodies harboring different formats have already been developed antagonizing prion infection [34-38]. However, none of them achieved striking results, which render them suitable for application in human transmissible spongiform encephalopathies (TSEs).

The 37kDa/67kDa laminin receptor represents the receptor for the cellular prion protein PrP^{c} [2] and a receptor for the infectious PrP^{27-30} [25], implicating that LRP/LR might represent a valuable and alternative target for antibody development in prion disease

therapy [35, 39, 40]. Therefore, different antibody formats (Tab. **2**, Fig. **2**) directed against the LRP/LR have been developed to block or prevent binding and internalization of the prion protein and therefore a possible infection of cells.

A polyclonal anti-LRP antibody termed W3 (Fig. **2**) reduced the PrP^{Sc} propagation in cell culture [24], hampered the binding of BSE prions to human enterocytes [41] and prevented the binding of PrP27-30 to mammalian cells [25]. Passive immunotransfer of W3 into scrapie infected mice significantly reduced peripheral PrP^{Sc} propagation by 66% and prolonged the survival of scrapie infected mice 1.8-fold [42]. Since the amount of the polyclonal antibody W3 is limited, single chain Fv antibodies (scFv) have been selected via phage display on recombinant LRP [43]. Due to a smaller size (approx. 30 kDa) they display a better tissue penetration, might pass the blood brain barrier, and might therefore reach the brain, where prions replicate predominantly. Application of the anti-LRP scFv antibody termed S18 via passive immunotransfer into scrapie infected mice reduced PrP^{Sc} levels in the spleen by approx. 40%, indicating that the peripheral propagation is impaired (Fig. **2**). The fact that incubation times and survival were not prolonged might be explained with the low stability and short half life (approx. 12 hours in blood) of the scFvs. To circumvent these problems, we developed a permanent delivery system based on recombinant Adeno-associated viral vectors (rAAV). rAAV mediated gene delivery is currently investigated in clinical trials for human therapy. A recent study reported the administration of anti-PrP antibodies into scrapie infected mice via rAAV serotype 2, which resulted in a delayed onset of disease [44]. Intracerebral injection of rAAVs encoding for the anti-LRP/LR scFv antibodies S18 and N3 into scrapie infected mice efficiently reduced the peripheral PrP^{Sc} propagation by approx. 60% and 32%,

respectively [45]. This remarkable finding can be explained by the fact that trafficking of intracerebrally administered rAAV to the spleen occurred, resulting in a direct scFv expression and secretion [45].

Although scFvs provide better tissue penetration and probably pass the blood brain barrier, a full length IgG format is much more stable in the organism and displays a longer half life up to 21 days. In contrast to the small scFvs, only the polyclonal anti-LRP/LR antibody W3 prolonged survival in scrapie infected mice. Using an improved version of the scFv S18 (iS18), as a template for the antigen-binding regions, a full length IgG1-iS18 antibody has been engineered [46] and will be applied for prophylaxis and treatment of scrapie infected mice expecting a more pronounced effect regarding survival.

The mode of action of the anti-LRP antibodies as a prion disease therapeutic, remains to be further investigated. Although an epitope mapping of the anti-LRP antibodies scFv S18 and full length IgG1-iS18 exhibits recognition at the very C-terminal part of LRP (Fig. **1**) and not at the direct PrP binding site (aa161-180) [43, 46], the antibodies might act through sterical hindrance preventing the binding of infectious PrP^{Sc} to the receptor. A full length IgG format reveals higher stability *in vivo* and provides a higher affinity to the laminin receptor resulting in a probable more permanent prevention of the LRP/LR-PrP^{Sc} interaction.

2.2. Pentosan polysulfate

Pentosan polysulfate (PPS) is a large polysulfated glycane with weak heparin-like activity and has been shown to prevent PrP^{Sc} propagation in cell culture models [47].

Recently, it has been proposed that PPS functions via inhibition of the binding of PrP^{Sc} to LRP/LR [25] (Fig. **2**). In mouse models, PPS is able to prolong the survival time of scrapie infected animals or even cures of the mouse strains VM and CBA from 22A and ME7 prions, respectively [48]. Furthermore, survival time of scrapie-infected mice was increased using a combined treatment of PPS and Fe(III)meso-tetra(4-sulfonatophenyl)porphine [49]. Orally or intraperitoneally administered PPS, however, is thought to be not strinkingly effective for the treatment of TSEs due to the assumed inability of the drug to cross the blood-brain-barrier [50]. Several case studies for the treatment of vCJD in humans were performed showing that delivery of PPS by chronic intraventricular infusion resulted in no definite clinical benefits [51, 52]. However, continuous intraventricular treatment of a 22-year-old vCJD patient with PPS over a period of 31 month resulted in a prolonged survival [53]. In addition, very recently the effect of continuous intraventricular infusion of PPS was investigated in seven UK patients suffering from human prion diseases. In this report, it has been demonstrated that a pentosan polysulfate therapy during 6 months extended the mean survival of all patients [54]. Nevertheless, further *in vivo* animal experiments and clinical studies, respectively, are required to assess the efficacy of PPS administration in variant Ceutzfeldt-Jakob Disease (vCJD) and other prion diseases.

2.3. RNA interference approaches

Since the 37kDa/67kDa laminin receptor (LRP/LR) has been identified to act as the cell-surface receptor for the cellular prion protein [2] it was further discovered that limited expression of LRP inhibits PrP^{Sc} accumulation in scrapie infected neuronal cells (ScN2a

and ScGT1) [24]. Transfection of ScN2a and ScGT1 cells with vectors encoding for antisense LRP mRNA led to elimination of LRP expression concomitant with an absence of PrP^{Sc} propagation [24] (Fig. **2**). In addition, transgenic mice [tgN(NSEasLRP)] were generated showing a reduced LRP/LR level in hippocampal and cerebellar brain regions and no abnormal behavior compared to control mice [55]. Another approach to knock down LRP expression deals with small interfering (si)RNAs directed against the LRP mRNA. Transfection of ScN2a cells resulted in a strong downregulation of LRP correlating with a complete abolishment of PrP^{Sc} propagation 72 hours post transfection [24] (Fig. **2**).

These findings lead to the conclusion that post-transcriptional gene silencing of LRP by RNAi is a promising approach to delay PrP^{Sc} propagation. Further *in vivo* studies will reveal if permanent expression of siRNAs directed against the LRP mRNA might prolong incubation time and/or survival during scrapie infection.

2.4. Transdominant negative laminin receptor mutant LRP102-295::FLAG

The expression of cellular PrP (PrP^{C}) is a major requirement of scrapie infection [56] and therefore represents a promising therapeutic target. However, identification of the 37kDa/67kDa laminin receptor LRP/LR as the receptor for PrP^{C} [2] and PrP^{Sc} [25, 41] led to an alternative target for the development of TSE therapeutics. Recently, it has been shown that an LRP mutant encompassing only the extracellular domain of LRP/LR (LRP102-295::FLAG) is secreted into the extracellular space and therefore might act in a trans-dominant negative manner as a decoy by trapping PrP molecules [57]. Baby hamster kidney (BHK) cells expressing and secreting the laminin receptor mutant show a

reduced binding of PrP 27-30, and in scrapie-infected neuronal cells the mutant is able to reduce the PrP^{Sc} accumulation [57] (Fig. **2**). Thus, the transdominant negative LRP-mutant might have potential for the development of a TSE therapy. An *in vivo* study with transgenic animals expressing the LRP mutant will reveal if the decoy mutant efficiently influences incubation time and/or survival during scrapie infection.

3. Role of the 37 kDa/67 kDa laminin receptor LRP/LR in zoonotic diseases

The phenomenon that the transmission of prion diseases from one species to another results in prolonged incubation times and survival compared to those of intraspecies transmission is called the "species barrier" [58]. This barrier is due to the variety of prion strains encompassing various infectious potential [59]. In natural animal populations the intraspecies transmission of prion diseases is much more efficient than an interspecies transmission [41], although these efficiencies can vary depending on the animal species, the used infectious agent and the route of infection: BSE and sheep Scrapie have a low intraspecies transmission efficiency [60, 61] whereas CWD is rapidly transmitted in captive deer populations [62] (Fig. **3**). Until today, mechanisms underlying an intraspecies transmission remain unclear. But prions from one species are often less infectious to other species. This is thought to depend on differences in host prion protein sequences [63] (Fig. **4**) but also may result from sequence differences of the 37 kDa/67 kDa LRP/LR (Fig. **5**). The species barrier of an interspecies transmission is sometimes so strong that peripheral injection, oral transmission and sometimes even an intracerebral inoculation with the agent causing a transmissible spongiform encephalopathy (TSE) fails to develop neurodegenerative signs. Nevertheless, susceptible hosts show clinical signs

when they are inoculated with brain homogenates of resistant species [63]. Bovine spongiform encephalopathy (BSE) is the only known TSE agent that was transmitted to humans causing the zoonotic disease vCJD [64]. Due to the fact that a natural infection with prions mostly occurs via oral uptake, like the transmission of BSE to human, further analyses were performed to study the initial prion uptake. Enterocytes represent the major cell population in the intestinal epithelium and express the 37 kDa/67 kDa LRP/LR on their cell surface. It was hypothesized that both enterocytes and the LRP/LR may play a major role in oral uptake of infectious prions and the species barrier. Shmakov and colleagues demonstrated that the 67 kDa LR is expressed in the apical brush border of the small intestine and in the perinuclear region of Paneth cell secretory granules[26]. These findings suggest that LRP/LR might play a crucial role in secretory and endocytic functions in the human small intestine epithelium and that the consequence of the expression of the 37 kDa/67 kDa LRP/LR may result in an increased susceptibility to oral infection with prions by LRP/LR mediated uptake by human enterocytes [26]. Studies of Morel et al. showed that bovine prions were specifically internalized by human enterocytes (Caco-2/TC7 cells) via the LRP/LR, whereas mouse-adapted scrapie prions were not endocytozed [41]. It has been demonstrated that BSE prion uptake was significantly reduced after preincubating the cells with the anti-LRP/LR antibody W3 demonstrating that the 37 kDa/67 kDa laminin receptor is required for BSE prion internalization [41]. Other studies on oral and parenteral interspecies transmission in animals showed that BSE could be transmitted orally to sheep and goat [65], mink [66], mouse [67], but not to poultry [65]. Also pigs failed to be susceptible to oral BSE transmission [65], whereas a parenteral inoculation was successful [68] (Fig. **6**). It still

remains unclear whether BSE is transmissible to humans and the mechanism of interspecies transmission is not elucidated, so far. But some interspecies interaction profiles of prions have already been elaborated employing yeast two-hybrid and conversion assays in vitro and transmission studies in vivo (Tab. **4**). Further binding studies with prions of different species on bovine, porcine, cervid, ovine and human enterocytes, respectively, will help to unravel the species barrier in prion disorders and will prove whether different animal prions such as CWD or sheep Scrapie might cause further zoonotic diseases.

4. LRP/LR as a drug target in cancer

Both, the 37kDa LRP and the 67kDa LR play an important role in cancer. The 67 kDa isoform was first isolated from tumor cells [5] and is termed the high affinity receptor due to its strong binding capacity to laminin. Interaction of the LRP/LR with laminin, a major cell adhesion substrate, is crucial for the invasion of tumorigenic cells. There are at least two binding sites for laminin on LRP, one stretching from aa 205-229 and a second so called Peptide G sequence encompassing aa 161-180 [6] (Fig. **1**) and comprising a heparin dependent laminin binding site [69-71], which is equivalent to the direct PrP binding site. The non-integrin laminin receptor LRP/LR is involved in the metastatic behavior of neoplastic cells compromising laminin mediated basement membrane attachment, accompanied by local degradation and cell movement. Overexpression of the 67kDa LR has been detected in several cancer tissues (Tab. **3**) including gastric cancer [72], colon carcinoma [73], colorectal carcinoma [74], cervical [75], breast [76], lung [77], ovary [78], pancreatic [79] and prostate cancer [80]. In addition to a proposed

prognostic role in the metastatic tumor progression [81, 82], LRP/LR provides a suitable target for therapy in cancer. In a murine model it has been demonstrated that human fibrosarcoma cells pretreated with an anti-LRP antibody display less lung metastases compared to those injected with untreated cells [83]. Furthermore a suppressed LRP expression was followed by a reduced lung cancer cell proliferation and *in vivo* tumor formation [84].

All mentioned tools targeting the LRP/LR for prion disease therapy, are also suitable for a tumor intervening therapy. Blocking or downregulating LRP/LR, significantly reduced the invasive potential of tumorigenic human fibrosarcoma cells (HT1080) [46]. Anti-LRP/LR scFv antibodies, the above mentionend full length format IgG, as well as heparan mimetics and pentosan polysulfate efficiently hamper the invasion through the perturbance of the laminin-LRP interaction on the cell surface [46]. These data suggest that tools targeting LRP/LR might represent effective alternative instruments to interfere with metastasis in cancer. Very recently, a critical role of LRP/LR for the maintainance of cell viability has been reported [20]. The fact that neoplastic cells such as HT1080 reveal an increased LRP/LR level compared to non-tumorigenic cells [85] together with the finding that LRP/LR prevents apoptotic processes [20] suggests that the receptor has multiple effects in cancer development by supporting metastasis and inhibiting apoptosis.

5. Role of LRP/LR in other neurodegenerative and infectious diseases

5.1. Alzheimer's Disease (AD)

Alzheimer´s and prion diseases belong to the group of fatal neurological disorders. Both have in common to form amyloid plaques on neuronal tissues. In the case of Alzheimer´s

disease (AD) the cleavage of the amyloid β precursor protein (APP) is thought to be the crucial process causing AD by producing the Aβ-peptide (Aβ) [86]. In the non-amyloidogenic pathway APP is cleaved by the α- and γ-secretase and the emerging soluble peptides are shedded into the extracellular matrix (Fig. **7**). However, if the proteolytic processing of APP is performed by β- and γ-secretase, the emerging β-peptide (Aβ) which is secreted into the extracellular matrix, accumulates in amyloid plaques on neurons, accompanied by neurotoxic effects and neurodegeneration. Tau-fibres may additionally contribute to the development of the Alzheimer disease phenotype [87].

In prion diseases, misfolding of cellular prion protein (PrP^{c}) to a proteinase K resistent and infectious isoform PrP^{Sc} is thought to be the causative pathogenic mechanism of TSEs. PrP^{c} passes through the secretory pathway and is then anchored to the cell surface by a glycosyl phosphatidyl inositol (GPI) anchor.

It has been shown that cellular PrP has a regulatory effect on the β-secretase cleavage of APP [88]. Overexpression of PrP^{c} inhibited β-secretase cleavage and reduced Aβ formation in neuronal cells [88]. Vice versa downregulation of PrP^{c} by RNAi leads to the secretion of increased amounts of Aβ into the cell culture medium [88]. Additionally, PrP is suggested to inhibit β-secretase (BACE1, β-site APP cleaving enzyme) activity involving interaction with glycosaminoglycanes [88].

The 37kDa/67kDa LRP/LR has been shown to act as a receptor for both, PrP^{C} [2] and the pathogenic isoform PrP^{Sc} [25, 41]. LRP/LR interacts with PrP via an indirect binding domain depending on the presence of heparan sulfate proteoglycans (HSPGs) [25] which belong to the group of GAGs. LRP/LR and APP share the same subcellular localization on the cell surface. Therefore, it is conceivable that LRP/LR might be involved in

secretase mediated cleavage of App and shedding of APPsα, APPsβ and the β-peptide.

5.2. Viral diseases

As an initial step in viral diseases, most viruses enter the host cell by receptor-mediated endocytosis. Several protein receptors have been identified as initial virus attachment receptors including human immunodeficiency virus (HIV) which use the chemokine co-receptors CCR5 and CXCR4 in addition to the CD4 receptor [89] and the Epstein-Barr virus that is associated with infectious mononucleosis and the development of cancer, respectively, and use the complement receptor CR2 [90]. For alphaviruses, e.g. Sindbis Virus, the high-affinity laminin receptor has been described as major receptor in mammalian cells [18] (Tab. **1**). Hamster cells, that overexpress the laminin receptor at the cell surface were more susceptible to a Sindbis virus infection compared to cells transfected with the antisense gene. Moreover, it has been demonstrated that the 67kDa laminin receptor also functions as the Sindbis virus entry receptor in mosquitos [18]. In addition, the 37kDa/67kDa laminin receptor has been identified as a receptor for (i) dengue virus, which is the causative agent of dengue fever and dengue hemorrhagic fever, in human liver cells [91], porcine kidney cells [19] and mosquito cells [92], respectively, and (ii) for several AAV subtypes [17] (Tab. **1**). Thus, the identification of the 37kDa/67kDa LRP/LR as an initial entry receptor for mosquito-borne viral diseases opened up new vistas to establish a specific receptor-based antiviral prophylaxis and/or therapy.

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ABBREVIATIONS

LRP/LR	=	laminin receptor precursor/laminin receptor
PrP (PrP^{c}, PrP^{Sc}, PrP27-30)	=	prion protein (cellular form, scrapie form, protease-resiostant core)
GPI	=	glycosylphosphatidylinositol
TSE	=	Transmissible Spongiform Encephalopathy
CJD, vCJD	=	Creutzfeldt-Jakob Disease, variant Creutzfeldt-Jakob Disease
BSE	=	Bovine Spongiform Encephalopathy
CWD	=	Chronic Wasting Disease
HSPG	=	heparan sulfate proteoglycan
GAG	=	glycosaminoglycan
PPS	=	pentosan polysulfate
siRNA	=	small interfering ribonucleic acid
AD	=	Alzheimer's Disease
APP	=	amyloid precursor protein
Aβ	=	amyloid beta protein (proteolytic fragment of APP)
FDA	=	U.S. Food and Drug Administration
AAV	=	Adeno-Associated Virus

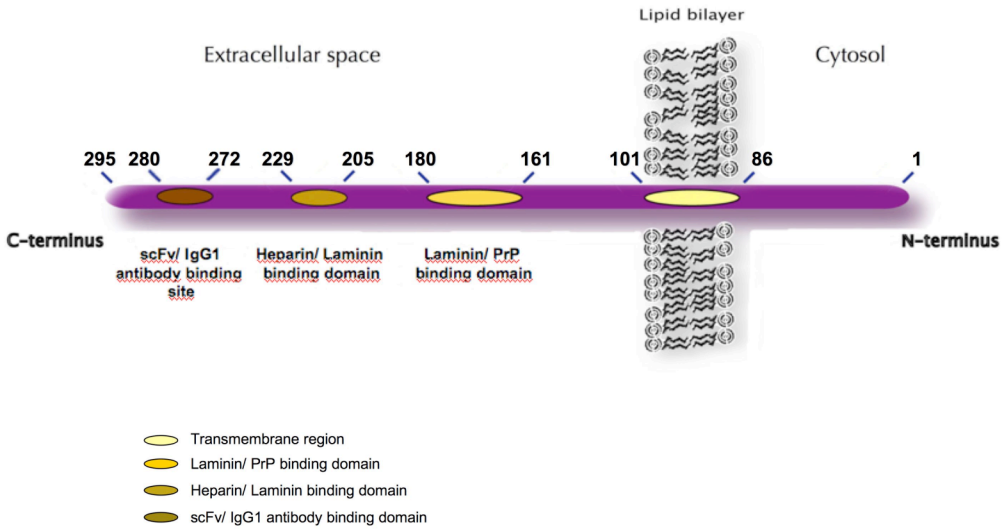

Fig. (1). Functional domains of the 37kDa/ 67kDa laminin receptor LRP/LR.

LRP/LR consist of 295 amino acids (aa) and belong to the group of Type II membrane proteins, spanning the plasma membrane once (aa 86-101) with its C-terminus exposed to the extracellular space. PrP/ Laminin and Heparin/ Laminin binding sites have been characterized at aa positions 161-180 and 205-229, respectively. In addition, the scFv/ IgG1 antibody binding site has been mapped to aa 272-280.

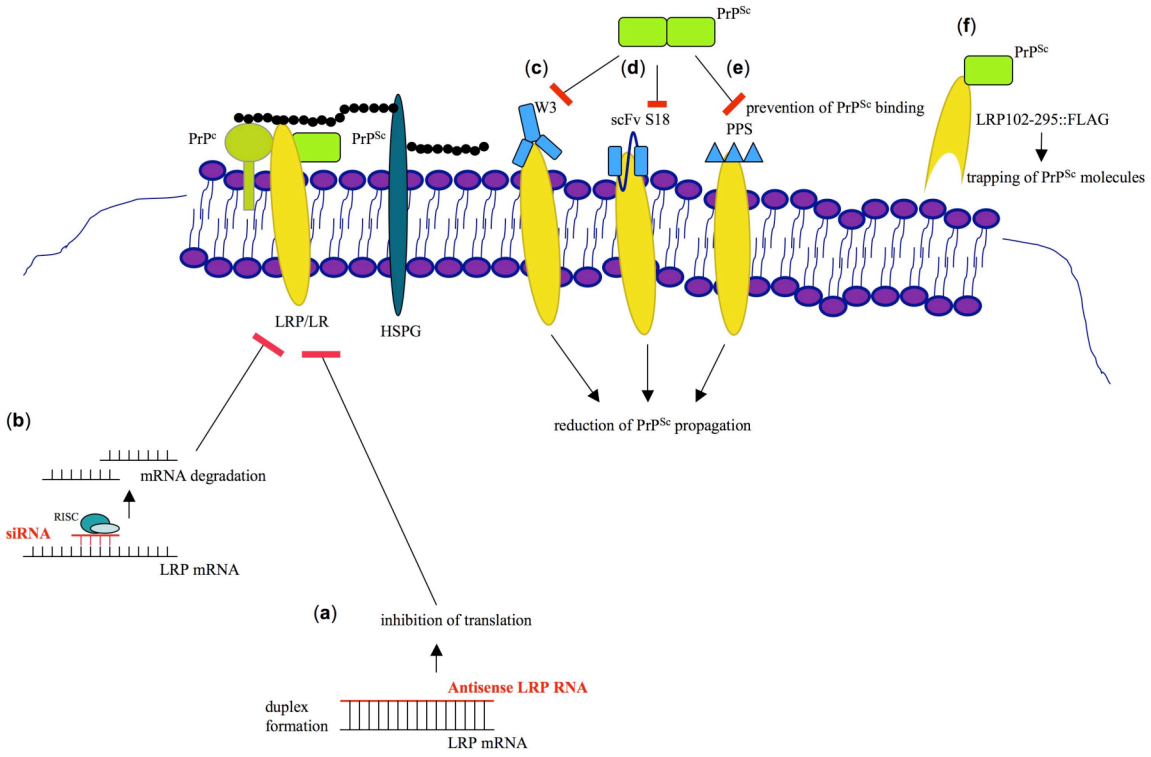

Fig. (2). Therapeutic approaches targeting LRP/LR.

LRP/LR (yellow oval) has been identified as receptor for cellular (dark green circle) and infectious prions (light green square). Several strategies can be employed to interfere with either the expression of LRP/LR (a, b) or binding of PrP^{Sc} (c-f). To ablate LRP/LR expression an antisense RNA (**a**) and a small interfering RNA (**b**) approach have been used resulting in prevention of PrP^{Sc} propagation in scrapie-infected cells [24]. Both the polyclonal anti-LRP/LR antibody W3 (blue immunoglobuline structure) (**c**) and the single chain (scFv) anti-LRP/LR antibody S18 (blue single chain antibody structure) (**d**) have been shown to interfere with the PrP^{Sc} propagation [24, 42, 43]. In addition, drugs such as pentosan polysulfate (PPS) (blue triangles) (**e**) were able to reduce PrP^{Sc} binding in vitro [25] possibly due to an inhibition of the LRP/LR-dependent binding of prions to the cell. As an alternative therapeutic system, a LRP/LR decoy mutant (LRP102-295::FLAG) (yellow semi-oval) (**f**) has been used to interfere with the PrP^{Sc} propagation in scrapie-infected neuroblastoma cells [57].

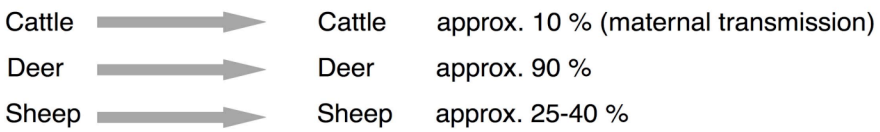

Fig. (3). Intraspecies transmission efficiencies of TSEs.

Experimental transmission of prion diseases have been performed to examine neuropathological profiles, risk factors and incidence rates. A long-term cohort study in cows investigating maternally-associated risk factors for BSE revealed a statistically significant risk of 10% for a transmission to the offspring [60]. For an intraspecies transmission of sheep scrapie, a rate of 25-40% has been calculated [62]. Chronic wasting disease (CWD) is naturally transmitted with remarkably efficacy of approximately 90% that has been studied in a captive mule deer herd [60].

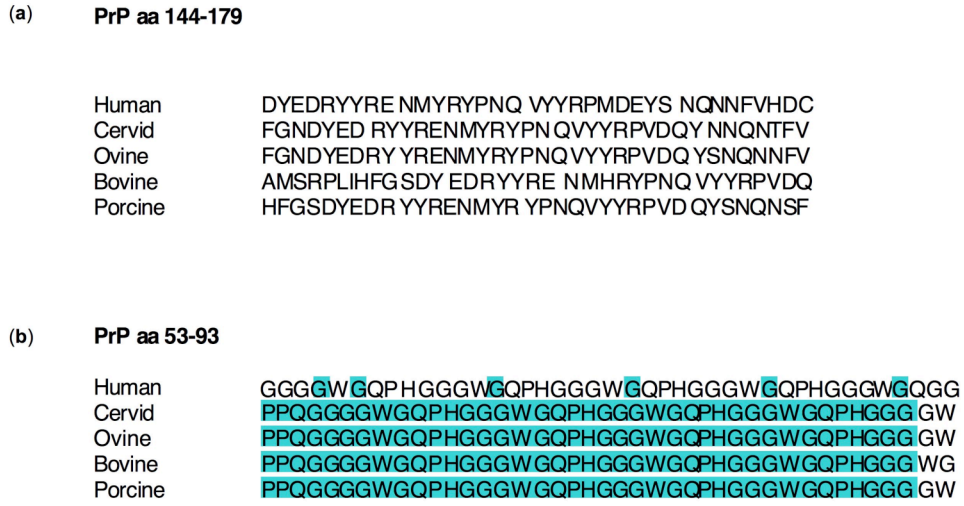

Fig. (4). Comparison of amino acid sequences of the direct and indirect PrP binding sites for LRP/LR in different species.

PrP comprises two binding sites for LRP/LR, a direct (aa 144-179) and an indirect one (aa 53-93), that is medated by HSPGs [3]. (**a**) Sequence alignment for the direct PrP binding site in human, cervid, ovine, bovine and porcine PrP resulted in no sequence homology, whereas the (**b**) alignment for the indirect PrP binding domain for cervid, ovine, bovine and porcine PrP, respectively, revealed approximately 95% sequence identity (green).

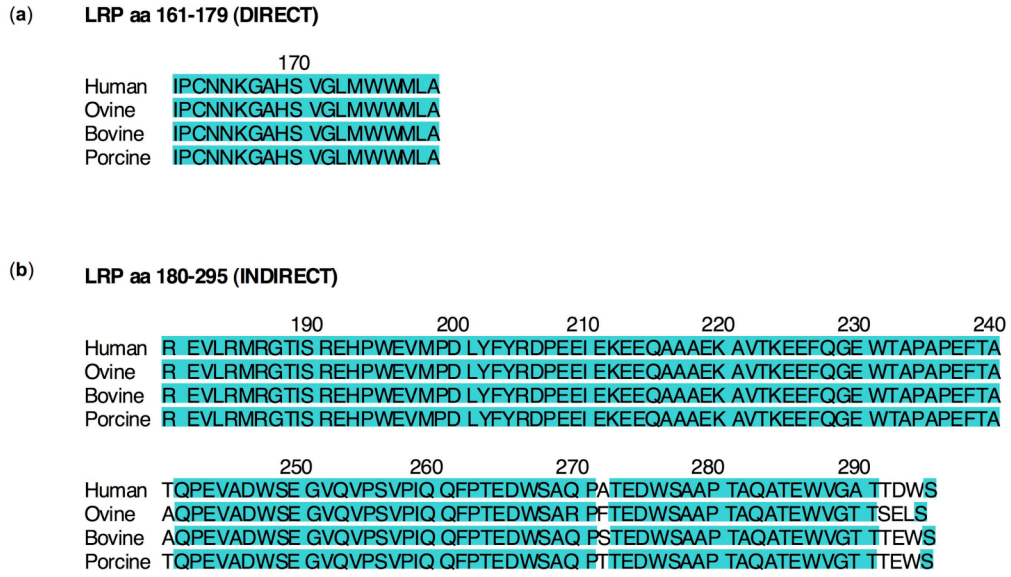

Fig. (5). Comparison of amino acid sequences of the direct and indirect LRP/LR binding sites for PrP in different species.

Binding of LRP/LR to PrP occurs via a direct binding site located between aa 161-179 and an indirect HSPG-dependent domain encompassing aa 180-295 [3]. (**a**) Sequence alignment of the direct LRP/LR binding site for PrP resulted in complete identical sequences for the human, ovine, bovine and porcine proteins. (**b**) Human, ovine, bovine and porcine sequences, respectively, of the indirect LRP/LR binding domain showed approximately 90% homology (green).

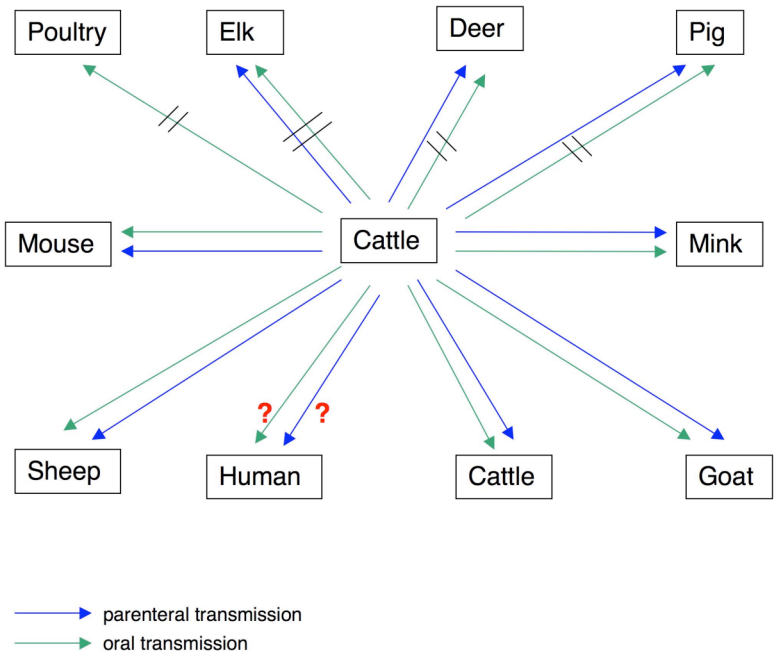

Fig. (6). Oral and parenteral interspecies transmission of BSE.

Obviously, a transmission of BSE to humans can not be tested due to ethical reasons. Oral transmission of the BSE agent (green arrows) has been performed succesfully to sheep and goats [125], minks [66], mice [67] and cows [126], respectively. However, several experiments demonstrated the existence of a species barrier for the oral transmission of TSEs (crossed green arrow), e.g. in elk and deer, pigs [127] and poultry [65], respectively, and parenteral transmission (= circumvention of the intestinal tract, crossed blue arrows) to elk. Interestingly, in pigs, a parenteral BSE transmission (blue arrow) has been reported to be succesful [128]. Furthermore, the BSE agent has been effectively parenteral transmitted to cattle [126], sheep and goats [125], minks [Bradley, Paris Symposium, 1996] and deer [129].

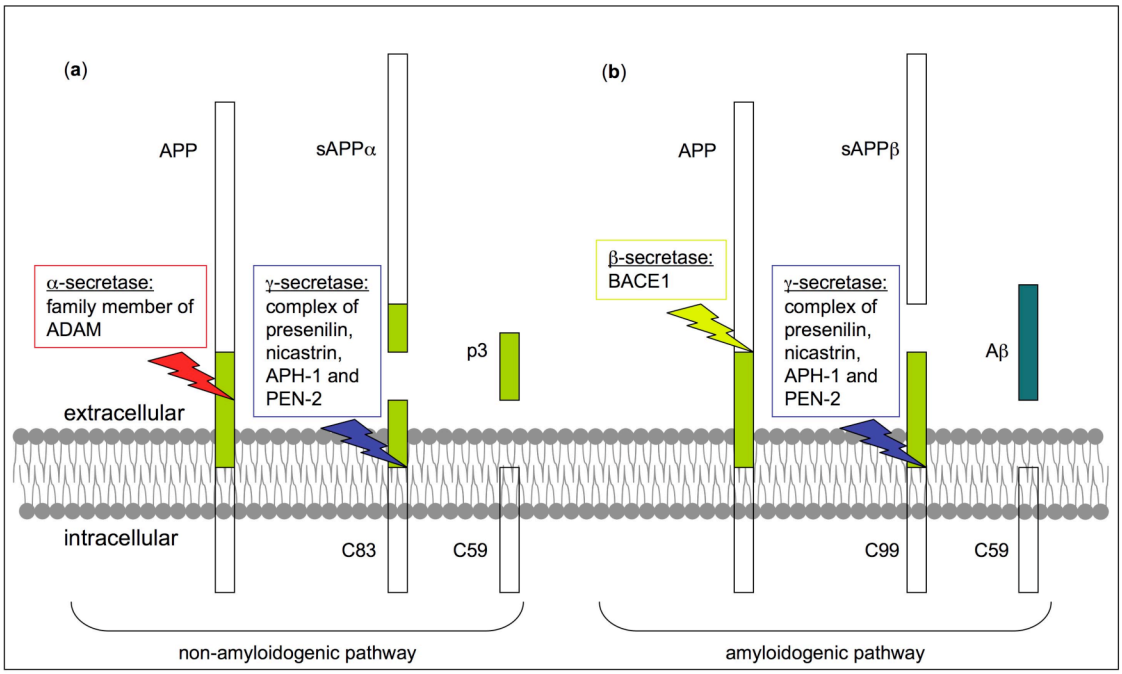

Fig. (7). Overview of the non-amyloidogenic and amyloidogenic APP pathway.

(**a**) In the non-amyloidogenic pathway, APP (Amyloid β precursor protein) is proteolytically cleaved by the α-secretase (red arrow), member of the ADAM (a disintegrin and metalloprotease domain) family. A soluble fragment (sAAPα) is secreted into the extracellular space and the C-terminal domain stays membrane-bound. Afterwards the γ-secretase (blue arrow) cleaves within membrane-bound C-terminus and releases a soluble peptide (p3, light green cuboid) of approximately 3kDa. The γ-secretase minimally consists of 4 individual proteins: the presenilins (aspartyl proteases) form the catalytic subunit, nicastrin, APH-1 (anterior pharynx-defective 1) and PEN-2 (presenilin enhancer). (**b**) In the amyloidogenic pathway, APP is first cleaved by β-secretase (green arrow) (BACE1, β-site APP cleaving enzyme), releasing a soluble fragment (sAPPβ) into the extracellular space, followed by γ-secretase cleavage. Thereby, the amyloid β peptide (Aβ, dark green), which is the primary constituent of Alzheimer's Disease (AD), is shed off the cell surface and aggregate in the brain od AD patients.

Tab. 1. General conspectus of LRP/LR and HSPGs as initial attachment receptors for viruses

Viruses using LRP/LR as receptor	Reference	Viruses using HSPGs as receptors	Reference
Dengue Virus (Serotype 1, 2, 3)	[19]	Adeno-Associated Virus (Serotype 1,6)	[93]
Sindbis Virus	[18, 94]	Adenovirus (Serotype 2, 5)	[95]
Venezuelean Equine Encephalitis Virus	[16]	Human Papilloma Virus	[96]
Adeno-Associated Virus (Serotype 2, 3, 8, 9)	[17]	Hepaptitis B Virus Hepatitis C Virus	[97] [98]
		Human Immunodeficiency Virus	[99]
		Murine Leukaemia Virus	[100]
		Herpes Simplex Virus Type 1	[101]
		Pseudorabies Virus	[102]

Tab. 2. Anti-LRP/LR tools as therapeutics in prion diseases

Anti-LRP/LR tools	**Effect *in vitro***	**Application *in vivo***	**Effect *in vivo***			**Reference**
			Peripheral PrP^{Sc} propagation	**Incubation time**	**survival**	
Antibodies						
Polyclonal antibody W3	blocks PrP^{Sc} propagation and cures scrapie infected cells	passive immunotransfer via i.p. injection	reduced by 66%	no prolongation	1.8 fold prolongation	[42]
Single chain Fv antibody scFv S18	n.d.	passive immunotransfer via i.p. injection	reduced by 40%	no prolongation	no prolongation	[43]
		delivery by i.c. injection of rAAV	reduced by 60%	no prolongation	no prolongation	[45]
Laminin receptor decoy mutant						

LRP102-295::FLAG	Reduces PrP^{Sc} propagation in transfected $N2aSc^{+}$ cells	Generation of transgenic mice	in progress	in progress	in progress	[57]
3 Strategies downregulating LRP/LR expression						
Antisense LRP RNA	Prevents PrP^{Sc} propagation in transfected ScMNB cells	Generation of transgenic mice	n.d.	n.d.	n.d.	[24, 55]
LRP-specific siRNAs	Prevents PrP^{Sc} propagation in transfected $N2aSc^{+}$ cells	i.c. injection of lentiviral particles expressing siRNAs (gene therapy)	in progress	in progress	in progress	[24]

Anti-LRP/LR tools blocking or downregulating the 37kDa67kDa LRP/LR were applied in cell as well as in animal studies to investigate their potential in prion diseases.

i.p. intraperitoneal; i.c. intracerebral, n.d. not determined; $ScN2a^{+}$ Scrapie infected mouse neuroblastoma cells; ScMNB Scrapie mouse neuroblastoma cells;

peripheral PrP^{Sc} propagation was determined analysing the PrP^{Sc} content in the spleen of scrapie infected mice 90 days pot infection; incubation time describes the time span from

the scrapie infection until the mice display the first TSE relevant symptoms (Ataxia of gait, tremor, rigidity in the tail or difficulty righting from a supine position). Survival represents the time span from the day one of the four TSE symptoms occur until the day two of these symptoms are observed for three days.

Tab. 3. Expression of the 67kDa LR has been reported in human tumors

Human tumors with LR expression	**Reference**
Colorectal carcinoma	[74]
Colon cancer	[103]
Cervical preneoplastic and neoplastic squamous epithelial lesions	[75]
Gastric adenocarcinoma	[104]
Breast carcinoma	[76, 105]
Acute myeloid leukemia (AML)	[106]
Human laryngeal sqamous cell carcinoma	[107]
Human small cell lung cancer	[108]
Prostate cancer	[80]
Ovarian carcinoma	[78]
Uterine adenocarcinoma	[109]

Tab. 4. Comparison of interspecies interactions in the Yeast Two-Hybrid System with interconversion studies by *in vitro* Conversion and transmission in transgenic and non-transgenic animals.

PrP species	PrP species	Interspecies interactions by yeast two-hybrid system	Interconversion by in vitro conversion assays	Interspecies transmission in transgenic mice	Interspecies transmission in animals
Human	Cattle	+	+ [110]	+ [64, 111]	+ [112-114]
Sheep	Cattle	+	+ [115]	+ [116, 117]	+ [118, 119]
Sheep	Human	+	+ [110]	+ [116]	n.d.
Hamster	Human	-	-	n.d.	Via guinea pigs [120]
Hamster	Cattle	-	- [115]	n.d.	- [65]
Cervid	Cattle	n.d.	n.d.	- [121]	n.d.
Cervid	Human	n.d.	n.d.	- [121, 122]	n.d.
Cervid	Sheep	n.d.	n.d.	+ [122]	n.d.
Mouse	Human	n.d.	+ [123]	- [124]	n.d.
Mouse	Sheep	n.d.	n.d.	+ [124]	n.d.
Mouse	Hamster	n.d.	n.d.	+ [124]	n.d.

“+” : Interaction in described system

“- “ : No interaction in described system

“n.d.” : not determined

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Chapter VII

Anti-LRP/LR antibody W3 hampers peripheral PrP^{Sc} propagation in scrapie infected mice

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Research Paper

Anti-LRP/LR Antibody W3 Hampers Peripheral PrP^{Sc} Propagation in Scrapie Infected Mice

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KEY WORDS

37kDa/67kDa laminin receptor, LRP/LR, prion, PrP, TSE-therapy

ABBREVIATIONS

CJD	Creutzfeldt-Jakob disease
CWD	chronic wasting disease
FFI	fatal familial insomnia
GSS	Gerstmann-Sträussler-Scheinker
LR	high affinity laminin receptor
LRP	laminin receptor precursor
RML	rocky mountain laboratory
TSE	transmissible spongiform encephalopathy
W3	polyclonal anti-LRP/LR antibody

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See page 212.

ABSTRACT

We identified the 37kDa/67kDa laminin receptor (LRP/LR) as a cell surface receptor for the cellular prion protein (PrP^{c}) and the infectious prion protein (PrP^{Sc}). Recently, we showed that anti-LRP/LR antibody W3 cured scrapie infected N2a cells. Here, we demonstrate that W3 delivered by passive immunotransfer into C57BL/6 mice reduced the PrP^{Sc} content in the spleen significantly by 66%, demonstrating an impairment of the peripheral PrP^{Sc} propagation. In addition, we observed a 1.8-fold increase in survival of anti-LRP/LR antibody W3 treated mice (mean survival of 31 days) compared to preimmune serum treated control animals (mean survival of 17 days). We conclude that the significant effect of anti-LRP/LR antibody W3 on the reduction of peripheral PrP^{Sc} propagation might be due to the blockage of the prion receptor LRP/LR which is required, as previously shown in vitro, for PrP^{Sc} propagation in vivo.

INTRODUCTION

Transmissible spongiform encephalopathies (TSE) are a group of neurodegenerative protein-misfolding diseases, also known as prion diseases affecting both animals including scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease (CWD) in elk and deer as well as humans (e.g., Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS) syndrome and fatal familial insomnia (FFI)) (for review see refs. 1–4). Affected individuals display rapidly progressive symptoms due to various effects such as gliosis, astrocytosis, neuronal loss and spongiosis.

TSEs are associated with an abnormal form of the prion protein, termed PrP^{Sc}. The conversion of the host encoded PrP^{c} into the disease associated isoform (PrP^{Sc}) results in accumulation which is perpetuated by an autocatalytic process.[5,6] Although various therapeutic approaches have already been developed (for review see refs. 7–11), no treatment was available until now, which is able to cure affected individuals. The first successful approach in antibody-based therapies was the passive immunotransfer of a monoclonal anti-PrP antibody which cured rodents peripherally infected with PrP^{Sc}.[12] Besides monoclonal antibodies targeting the prion protein,[13,14] also single chain anti-PrP antibodies are currently investigated for a TSE therapy.[15]

Among many interaction partners identified for PrP^{c},[10,16-18] the non-integrin 37/67 kDa laminin receptor (LRP/LR) has been discovered as a receptor for both the cellular PrP^{c} [19,20] and the disease associated PrP^{Sc}.[21,22] Downregulation of LRP/LR by antisense LRP RNA or siRNAs directed against LRP mRNA abrogates PrP^{Sc} propagation in ScN2a cells.[23] Secretion of a transdominant negative LRP mutant also abolishes PrP^{Sc} propagtion in neuronal cells.[24] A polyclonal anti-LRP/LR antibody termed W3 interferes with PrP27-30 cell binding[21] and internalization of bovine PrP^{Sc} by human enterocytes.[22] Most notably, W3 has been shown to cure PrP^{Sc} propagating cells from scrapie.[23] In order to investigate whether W3 is able to hamper prion propagation in vivo, we delivered W3 into mice by passive immunization. Spleen analysis confirmed a significant reduction in peripheral PrP^{Sc} propagation in W3 treated mice. Moreover, W3 treated mice revealed a 1.8-fold increase in survival (the time span from the day one of the four TSE-relevant symptoms occur until the day mice show two of the four TSE-relevant symptoms over three days[25]) compared to the control group injected with preimmune serum. Our results suggest that LRP/LR plays an important role for PrP^{Sc} propagation in vivo and that targeting LRP/LR is a relevant strategy for therapy in prion diseases.

MATERIALS AND METHODS

Antibodies and preimmune serum. In order to get the polyclonal anti-LRP antibody pAb W3, we immunized albino rabbits [(New Zealand; ZRL:kbl (nzw)br; Charles River Breeding Laboratories, Wilmington, Massachusets)]. One mililiter of a mixture of GST:: LRP fusion protein expressed in *E. coli* system[26] and CWS adjuvant (RIBI adjuvant, Sigma) was subcutaneously injected into rabbits (see ref. 27). After 28 days, animals were boosted and after additional 14 days the animals were immunized a third time. Eleven days later 200 ml of blood were collected and coagulated for one hour at 37°C and incubated over night at 4°C followed by two centrifugation steps ten minutes at 9,000 rpm and 10.500 rpm at 4°C. Purification was done using a protein A sepharose column (Pierce, Rockford, Illinois). W3 was selected from several anti-LRP sera tested for recognition efficiency of LRP/LR by FACS and western analysis.[27] Preimmune serum was obtained from rabbit prior to immunization.

Passive immunotransfer of anti-LRP/LR antibody W3 into mice. Animals were maintained and treated in accordance with ethical guidelines of Bavaria. Experiments were approved by the Regierung von Oberbayern (Munich, Germany, Ar.: 209.1/211-2531-83/04). For infection studies C57BL/6 mice were injected intraperitoneally (i.p.) with a total amount of 1 mg of W3 or preimmune serum. Treatment was performed once per week over a period of 12 weeks. One week after the first antibody injection mice were inoculated i.p. with 100 µl 10% RML Scrapie homogenate. The time span from the day of RML inoculation until one of the four symptoms: ataxia of gait, tremor, difficulty righting from a supine position and rigidity in the tail occured (termed as symptom onset) and survival (the time span from the day one of the four TSE-relevant symptoms occur until the day mice show two of the four TSE-relevant symptoms over three days[25]) were monitored. In all monitoring procedures the investigators were blinded as to the experimental groups individual mice belonged to.

Analysis of PrP^{Sc} and total PrP levels in the spleen of RML inoculated mice. Ninety days post RML inoculation six mice per group were sacrificed for analysis of peripheral PrP^{Sc} propagation. Spleens were collected and homogenized in PBS buffer. Adjusting the total protein amount to 200 µg, samples were digested with Proteinase K to a final concentration of 20 µg/ml for 60 minutes at 37°C. Samples were analysed on a 12% SDS PAGE and blotted onto a PVDF membrane. Immunodetection was performed using SAF83 as the primary and anti-mouse-POD conjugate (Jackson Immunoresearch) as the secondary antibody. Blots were developed using an enhanced chemiluminescence system (Perkin Elmer Lifescience) and exposed on Kodak Biomax light films. Quantification of the western blot signals was carried out by densitometric measurements using the Image J software. To determine the total PrP amount, spleen samples were treated as described for the PrP^{Sc} detection but without a Proteinase K treatment. For total PrP detection SAF32 was used as the primary and anti-mouse-IgG-POD as the secondary antibody.

Analysis of PrP^{Sc} and total PrP levels in the brain of terminal mice. Mice were sacrified after two of the four characteristical TSE symptoms[25] were detected for a period of three days. Total brain samples of six mice per group were collected and homogenized in PBS buffer. Protein levels were adjusted to 200 µg per sample and digested with Proteinase K to a final concentration of 20 µg/ml for 60 minutes at 37°C. The PrP^{Sc} content was determined by analysis on a 12% SDS PAGE and blotted onto a PVDF membrane. Immunodetection was performed using SAF83 as the primary and anti-mouse-IgG-POD (Jackson Immunoresearch) as the secondary antibody. Blots were developed using an enhanced chemiluminescence system (Perkin Elmer Lifescience) and exposed on Kodak Biomax light films. Quantification of the western blot signals was carried out by densitometric measurements using the Image J software. To determine the total PrP amount total brain samples were treated as described for PrP^{Sc} detection in the absence of Proteinase K treatment. Detection for total PrP was carried out using SAF32 as the primary and anti-mouse-IgG-POD as the secondary antibody.

Statistical analysis. Statistical analyses were performed employing a Student's t test with two tailed distribution and two-sample unequal variance.

RESULTS

Anti-LRP/LR antibody W3 reduces peripheral PrP^{Sc} propagation. One milligram per week of anti-LRP/LR antibody W3 and preimmune serum was intraperitoneally injected into scrapie infected mice for a period of 12 weeks (Fig. 1). One week after the first injection mice were intraperitoneally inoculated with RML Scrapie homogenate and monitored for symptom onset (incubation times) and survival (Fig. 1). W3 and preimmune serum treated mice were controlled daily and showed normal behavior till they were sacrificed. No side effects were detectable at any time points.

Western blot analysis of the spleen of W3 treated mice 90 days post inoculation revealed a significant reduction of the PrP^{Sc} level by 66% compared to the preimmune serum treated mice (Fig. 2A and B), suggesting that W3 reduces significantly peripheral PrP^{Sc} propagation. The amount of total PrP (PrP^{Sc} plus PrP^{c}) was reduced by approx. 40% (Fig. 2C and D) in W3 treated mice, suggesting that PrP^{c} levels remain unaffected by the antibody treatment.

Anti- LRP/LR antibody W3 prolongs the survival in scrapie infected mice. Anti-LRP/LR antibody W3 treated mice did not show a prolongation of incubation times compared to the preimmune serum treated control group (Fig. 3A and Table 1). However, the anti-LRP/LR antibody W3 treated mice revealed a mean survival of 31 days, which represents a 1.8-fold prolongation of the survival in comparison with the preimmune serum (mean survival 17 days) treated control group (Fig. 3B, C and Table 1).

At the terminal state the PrP^{Sc} level in the brain of W3 treated mice was reduced by 17% compared to preimmune serum treated mice (Fig. 4A and B), whereas W3 and preimmune serum treated mice showed no alteration in the total PrP content (Fig. 4C and D), suggesting that PrP^{c} levels remained unaltered in both experimental groups.

DISCUSSION

At present no therapeutic strategy is available for the treatment of TSEs which cures prion diseases.[8,9,11,28,29] Since the laminin receptor acts as a receptor for PrP^{c},[30] and PrP^{Sc},[21] LRP/LR exhibits a promising target for therapeutic strategies in prion diseases. The anti-laminin receptor polyclonal antibody W3 was well efficient in vitro by curing scrapie propagating cells from PrP^{Sc}.[23] In order

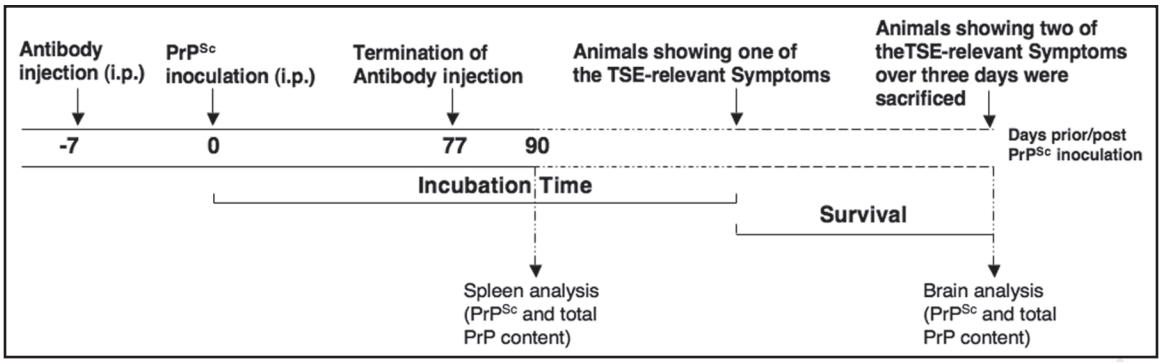

Figure 1. Schematic overview of the passive immunotransfer modalities. One milligram of antibody (either W3 or preimmune serum) per C57BL/6 mouse was intraperitoneally injected seven days prior to intraperitoneal inoculation with 10% RML brain homogenate. Antibodies injections (i.p.) were performed at doses of 1 mg per week for a period of 11 weeks. Treatment was terminated 77 days post inoculation. Ninety days post inoculation, animals were sacrificed for analysis of the PrP^{Sc} and total PrP content of the spleen. Incubation times represent the time span from the day of RML inoculation until one of the four symptoms occur: ataxia of gait, tremor, difficulty righting from a supine position and occurrence of rigidity in the tail. Survival represent the time span from the day one of the four TSE-relevant symptoms occurs until the day mice show two of the four TSE-relevant symptoms over three days.[25] At this time point mice were sacrificed followed by determination of the PrP^{Sc} and total PrP content in the brain.

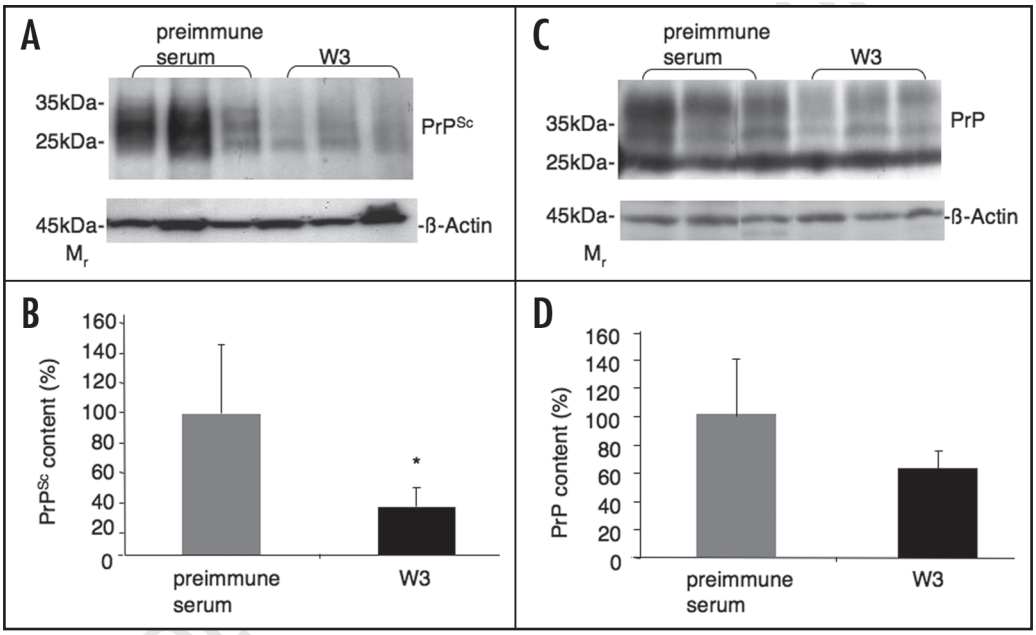

Figure 2. Analysis of total PrP and PrP^{Sc} levels in the spleen of scrapie infected mice treated with polyclonal anti-LRP antibody W3 and preimmune serum 90 days post infection. LRP/LR antibody W3 and preimmune serum was intraperitoneally injected into C57BL/6 mice for 12 weeks. C57BL/6 mice were intraperitoneally inoculated with RML prions (10%) one week after the first antibody/preimmune serum injection. (A) Spleen of C57BL/6 mice were collected 90 days post infection and analyzed for the PrP^{Sc} content after Proteinase K digestion by Western blotting using anti-PrP antibody SAF 83. Western blot analysis of the PrP^{Sc} levels in the spleen of three W3 and three preimmune serum treated mice are shown. β-actin was used as a loading control (detection by an anti-β-actin antibody). (B) Densitometric measurements of Western blots from six spleens per group revealed a significant reduction of the PrP^{Sc} level by 66% in the W3 treated group compared to the preimmune serum treated group, for which the PrP^{Sc} level was set to 100% (*$p < 0.05$). Quantification of PrP^{Sc} signals were normalized by β-actin levels. Quantification of the western blot signals was carried out by densitometric measurements using the Image J software (mean + SD). (C) Spleen samples of C57BL/6 mice (collected 90 days post infection) were analyzed for quantification of the total PrP content by western analysis using anti-PrP antibody SAF 32. Western blot analysis of the PrP levels (in the absence of Proteinase K) in the spleen of three W3 and three preimmune serum treated mice is shown. β-actin was used as a loading control (detection by an anti-β-actin antibody). (D) Densitometric measurements of western blots from six spleens per group revealed a reduction of total PrP content by 39% in the W3 treated group compared to the preimmune serum treated group, for which the total PrP level was set to 100% ($p < 0.2$). Quantification of total PrP signals were normalized by β-actin levels. Quantification of the western blot signals was carried out by densitometric measurements using the Image J software (mean + SD).

Table 1 **Incubation times and survival of scrapie infected mice treated with W3 and preimmune serum**

Experimental Group	Incubation Times, Days (Mean+/- SD)	Survival, Days (mean+/- SD)	Incubation Times + Survival, Days (mean+/- SD)	Number of Mice (Affected/ Inoculated)
preimmune serum	169,5 ± 1,2	17,5 ± 7,2	187,8 ± 10,6	6/6
W3	171 ± 1,78	31,4 ± 16 (1.8-fold increase in survival)	202,2 ± 18,4	5/5

LRP/LR antibody W3 and preimmune serum was intraperitoneally injected into C57BL/6 mice (1 mg per week) for a period of 12 weeks. C57BL/6 mice were intraperitoneally inoculated with RML prions one week after the first antibody/preimmune serum injection. Incubation times represent the time span from the day of RML inoculation until one of the four symptoms occur: ataxia of gait, tremor, difficulty righting from a supine position, and occurrence of rigidity in the tail.[25] Survival represent the time span from the day one of the four TSE-relevant symptoms occurs until the day mice show two of the four TSE-relevant symptoms over three days.[25] At this time point mice were sacrificed.

to prove an in vivo effect of W3 on (1) peripheral PrP^{Sc} propagation and (2) prolongation of survival, we passively transferred W3 into C57BL/6 mice by intraperitoneal injections followed by i.p. RML prion inoculations. W3 treated mice revealed a significant reduction (66%) of the peripheral PrP^{Sc} propagation compared to preimmune serum treated mice as analyzed by determining the PrP^{Sc} levels in the spleen. Total PrP levels (PrP^{Sc} plus PrP^{c}) in the spleen were reduced by approx. 40%, suggesting that W3 treatment has no or only a weak influence on the reduction of PrP^{c} levels in the spleen.

In addition, W3 treated mice revealed a 1.8-fold increase in survival (31 days) compared to the preimmune serum treated control group (17 days), suggesting that W3 also hampers PrP^{Sc} propagation in the central nervous system contributing to prolongation of survival. Moreover, this is of potential interest for treatment of CJD patients because the effects were observed during the clinical stages, when mice had already neurological symptoms (prolongation of survival time from onset of the disease to death). Due to the limited amount of the polyclonal antibody W3, which was raised against LRP in a rabbit,[27] we could not elongate the antibody treatment over the 12 weeks treatment period and could not increase the number of animals and/or the dose of antibody applied (1 mg) which all together might have resulted in an even more obvious effect on the increase in survival. In contrast to survival, incubation times were not affected. We assume that by increasing the antibody amount and the time period of treatment, the incubation time might also be prolonged. Application of a monoclonal anti-PrP antibody in mice with 2 mg of antibody twice a week resulted in a delayed onset of the disease.[12] Many promising anti-prion drugs which are effective in vitro failed to be active also in vivo (reviewed in ref. 9). Among them, are the antimalaria drugs mefloquine[31] and quinacrine.[32] In contrast, W3 is effective both in vitro[23] and in vivo. Currently, the efficacy of doxycyclin in the treatment of CJD is under investigation in observational studies in Milano (Italy) and Göttingen (Germany). First data indicate that administration of doxycyclin might prolong the survival by two-fold. In the German study on 23 patients, the survival was prolonged from four months (median in sCJD) to eight months. Whether the prolongation time is due to a specific prion effect, will be tested in a prospective double blind study. No conclusions can be drawn,

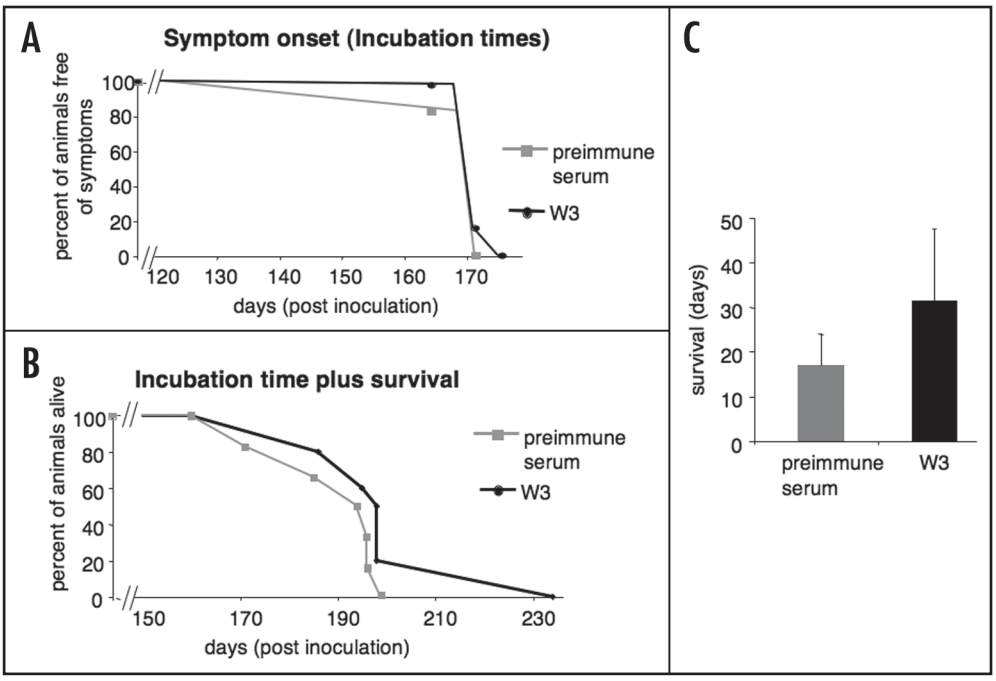

Figure 3. Symptom onset (Incubation times) and survival in scrapie infected C57BL/6 mice treated with polyclonal anti-LRP antibody W3 and preimmune serum. C57BL/6 mice were intraperitoneally inoculated with RML prions one week after the first antibody/preimmune serum injection and monitored for the occurrence of characteristic TSE symptoms. Symptom onset (Incubation times) represents the time span from the day of RML inoculation until the day one of the four symptoms appear: ataxia of gait, tremor, difficulty righting from a supine position and occurance of rigidity in the tail.[25] Survival represents the time span from the day one of the four symptoms occurs until the day mice show two of the four TSE-relevant symptoms over three days.[25] At this time point, mice were sacrificed. (A) Kaplan-Meier curve (symptom onset) showing percent of animals free of symptoms dependent from days post RML inoculation. 20% of the W3 treated animals revealed a prolonged symptom onset compared to the preimmune serum control group ($p < 0.2$). (B) Kaplan-Meier curve (symptom onset plus survival) showing percent of animals alive dependent from days post RML inoculation. (C) Survival (days) of W3 and preimmune serum treated animals. The median revealed a 1.8-fold prolonged survival for the W3 treated group compared to the control group injected with preimmune serum ($p < 0.19$) (mean + SD).

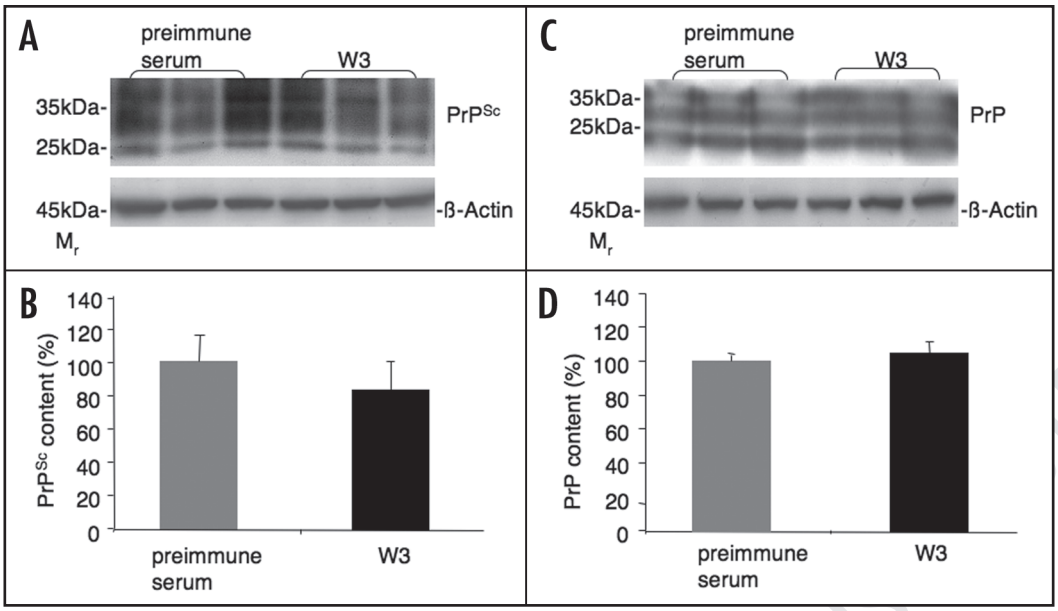

Figure 4. Analysis of total PrP and PrP^{Sc} levels in the brain of scrapie infected mice treated with polyclonal anti-LRP antibody W3 and preimmune serum at the terminal state. LRP/LR antibody W3 and preimmune serum was intraperitoneally injected into C57BL/6 mice for 12 weeks. One week after the first antibody/preimmune serum injection C57BL/6 mice were intraperitoneally inoculated with RML prions (10%). (A) Mice were sacrificed after showing two of the four characteristical TSE symptoms[25] over three days (terminal state) and total brain was analyzed for the PrP^{Sc} content (after Proteinase K digestion) by Western blot analysis using anti-PrP antibody SAF 83. PrP^{Sc} levels in total brain samples of three W3 and three preimmune serum treated mice are shown. β-actin was used as a loading control (detection by an anti-β-actin antibody). (B) Densitometric measurements of Western blots from six brain samples per group revealed a reduction of PrP^{Sc} levels by 17% in the W3 treated group compared to the preimmune serum treated group, for which the PrP^{Sc} level was set to 100% ($p < 0.3$). Quantification of PrP^{Sc} signals were normalized by β-actin levels. Quantification of the Western blot signals was carried out by densitometric measurements using the Image J software (mean + SD). (C) Total brain samples of terminal C57BL/6 scrapie infected mice were analysed for the total PrP content (in the absence of Proteinase K) by Western blot analysis using anti-PrP antibody SAF 32. Total PrP levels in total brain samples of three W3 and three preimmune serum treated mice are shown. β-actin was used as a loading control (detection by an anti-β-actin antibody). (D) Densitometric measurements of western blots from six brains per group revealed approx. equal levels in both groups ($p < 0.2$). Quantification of total PrP signals were normalized by β-actin levels. Quantification of the Western blot signals was carried out by densitometric measurements using the Image J software (mean + SD).

however, regarding a potential prolongation of incubation times in CJD patients by doxycyclin. A combination therapy with doxycyclin and antibodies targeting LRP/LR might have some additive or even synergistic effects.

On the molecular level LRP/LR specific antibody W3 blocks (1) PrP^{c} binding to neuronal cells,[30] (2) PrP27-30 binding to mammalian cells[21] and (3) BSE prion internalization by human enterocytes,[22] suggesting that the antibody interferes with PrP^{c} and PrP^{Sc} internalization processes prohibiting as a consequence PrP^{Sc} propagation, which might occur in compartments of the endocytic pathway rather than on the cell surface (for review see refs. 8 and 10). In scrapie infected mice, we show that W3 is able to interfere efficiently with peripheral PrP^{Sc} propagation, which takes place in organs of the lymphoreticular system such as the spleen. At the time point when the animals were sacrificed (at the day when two TSE-associated symptoms appeared for three days[25]) the detected PrP^{Sc} levels in the brain were not significantly different between W3 treated and preimmune serum-treated mice, suggesting that PrP^{Sc} propagation was not delayed in the brain of W3 treated animals. The total PrP content at the terminal state in the brain of mice treated with W3 and preimmune serum was unchanged, suggesting that also PrP^{c} levels in the brain were not affected by W3 treatment.

Taken together, this pilot study revealed important results regarding an antibody therapy or post-exposure prophylaxis with W3 resulting in a signifcant reduction of peripheral PrP^{Sc} propagation and a slight prolongation of survival. Since we started the antibody treatment seven days prior to PrP^{Sc} inoculation and terminated the treatment 77 days post PrP^{Sc} inoculation (long before first symptoms occur) (Fig. 1), we preformed in this study a post-exposure prophylaxis rather than a therapy, which is usually initiated at the stage when first symptoms appeared.

Since the amount of W3 is limited, we generated in a parallel study single chain antibodies (scFv) directed against LRP/LR by phage display.[33] Passive immunotransfer of the scFv S18 by intraperitoneal injections into scrapie infected mice also resulted in a reduction of the PrP^{Sc} level in the spleen by approx. 40% without a significant prolongation of incubation times and survival.[33] W3 reduced PrP^{Sc} levels in the spleen significantly by 66% and slightly prolonged the survival 1.8-fold. One of the reasons for the slightly better efficacy of W3 compared to scFv S18 might be the longer half-life of full-length IgGs in the blood (approximately 14 days) compared to single chain antibodies (approximately 12 hours). The amount of antibody (1 mg per week) was the same for both studies although the duration of antibody treatment was different (eight weeks for scFv S18 versus 12 weeks for W3). A polyclonal serum raised against a specific antigen (here LRP/LR) contains approximately 5–10% antibodies directed against this antigen.[34] Therefore approximately 50–100 μg of LRP/LR specific antibodies per week were injected in the present study,

i.e. 10 to 20-fold less compared to the scFv S18 trial (1 mg/week), suggesting that full-length IgG molecules are more potent than scFv fragments in passive immunotherapy.

Our results demonstrated that the polyclonal antibody W3 significantly impaired PrP^{Sc} replication in the spleen. These findings are an incentive to pursue with studies of antibodies directed against LRP/LR to obtain more effective results on the neuroinvasion phases of the infection. Passive immunotransfer studies with improved versions of the single chain antibody S18, as well as full-length IgG versions thereof might represent promising regimens for an efficient treatment of prion diseases.

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Chapter VIII

Single chain Fv antibodies directed against the 37 kDa/67 kDa laminin receptor as therapeutic tools in prion diseases

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Single chain Fv antibodies directed against the 37 kDa/67 kDa laminin receptor as therapeutic tools in prion diseases

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Abstract

Transmissible spongiform encephalopathies are a group of neurological disorders associated with the deposition of PrP^{Sc}, an abnormal form of the cellular prion protein PrP^{c}. The 37 kDa/67 kDa laminin receptor (LRP/LR) has been identified as a prion receptor and several lines of evidence strongly suggest that this protein plays a role during prion pathogenesis. Here we report the selection of recombinant single chain antibodies (scFvs) directed against LRP from naïve and synthetic phage scFv libraries for therapeutic application. Western blotting and FACS analysis confirmed a specific LRP/LR recognition pattern of the two selected scFvs S18 and N3. Both scFvs specifically interfered with the PrP/LRP interaction in vitro. High yield production of the scFvs of approx. 1 mg/L of culture medium was achieved in *E. coli*. Passive immunotransfer of the scFv S18 antibody reduced PrP^{Sc} levels by approx. 40% in the spleen of scrapie infected C57BL/6 mice 90 days post scFv injection, suggesting that scFv S18 interferes with peripheral PrP^{Sc} propagation, without a significant prolongation of incubation and survival times.

Keywords: Prion; PrP; 37 kDa/67 kDa laminin receptor; LRP/LR; Single chain antibody; Prion disease therapy

1. Introduction

Prion diseases describe fatal neurodegenerative diseases (for review (Aguzzi and Weissmann, 1998; Lasmézas and Weiss, 2000; Prusiner, 1998; Weissmann, 2004), lacking any effective therapeutic treatment (for review Vana et al., 2007; Weissmann and Aguzzi, 2005; Zuber et al., 2007). This group of infectious disorders includes Creutzfeldt–Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease (CWD) in cervids. The causative agents do not represent classical pathogens such as bacteria or viruses but are infectious proteins. The infectious scrapie prion protein (PrP^{Sc}) represents an abnormally folded isoform of the cellular prion protein (PrP^{c}). A key event in the disease pathology is the conversion of PrP^{c} into PrP^{Sc} that accumulates in the brain (for review: Weissmann, 2004). The conversion process is thought to take place in a compartment of the endocytic pathway after internalization (Arnold et al., 1995) or at the cell surface (Kaneko et al., 1997) where PrP^{c} locates GPI anchored.

We identified the non-integrin 37 kDa/67 kDa laminin receptor (LRP/LR) as the cell surface receptor for PrP^{c} (Gauczynski et al., 2001b) and showed that it is required for PrP^{Sc} propagation in scrapie infected cells (Leucht et al., 2003). Bovine prions are endocytosed by human enterocytes via LRP/LR (Morel et al., 2005) and moPrP27-30 binds LRP/LR dependent to mammalian cells confirming that LRP/LR acts as a receptor for infectious prions (Gauczynski et al., 2006). Molecular tools blocking the LRP/LR represent alternative therapeutic agents for the treatment of TSEs (for review Vana et al., 2007; Zuber et al., 2007). These include a trans-dominant negative LRP mutant (Vana and Weiss, 2006), polysulfated glycanes (Gauczynski et al., 2006), siRNAs directed against LRP mRNA (Leucht et

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al., 2003), which can be delivered by lentiviral vectors (Vana and Weiss, 2006) and antibodies directed against LRP/LR (for review Vana et al., 2007; Zuber et al., 2007). Polyclonal anti-LRP antibodies (Rieger et al., 1997) are able to interfere with (i) PrP^{BSE} internalization (Morel et al., 2005), (ii) PrP27-30 cell binding (Gauczynski et al., 2006), (iii) PrP^{Sc} propagation in cultured neuronal cells (Leucht et al., 2003) and (iv) prolong the survival time in scrapie infected mice 2.3 fold (Zuber et al., submitted). These findings recommend anti-LRP antibodies as promising alternative tools in the prophylaxis and/or therapy of prion diseases. Since polyclonal antibodies are due to their limited amounts and their relative high molecular weight (approx. 150 kDa) inappropriate for long-term therapeutic applications, we selected monoclonal single chain antibodies (scFv) (Bird et al., 1988) directed against LRP, termed N3 and S18, by phage display (Clackson et al., 1991). Both scFvs were produced in high amounts in *E. coli*, interfered with the PrP/LRP interaction process and sc Fv S18 reduced peripheral PrP^{Sc} propagation in scrapie infected mice.

2. Materials and methods

2.1. Phage display selection ELISA screening

Three rounds of selection were performed on a GST::LRP fusion protein expressed in Baculovirus infected Sf9 cells (Rieger et al., 1997). Approximately 10^{12} phages from each library were incubated with polystyrene immobilized GST::LRP. Unspecifically bound phages were removed by several washing steps. Bound phages were eluted and used for infection of *E. coli* XL1 Blue cells. Cells successfully transduced with phagemids encoding scFvs were selected for ampicillin resistance and subsequently infected with M13K07 helper phage to generate phage progeny displaying scFv for the following in vitro selection. After the third round of selection individual colonies were grown in LB medium (100 μg/mL ampicillin, 20 μg/mL tetracycline) at 30 °C. Cells were harvested by centrifugation and resuspended in 200 mM Tris–HCl, pH 7.5, 20% Sucrose, 1 mM EDTA. During incubation on ice the outer membrane is destroyed so that soluble periplasmic proteins including the scFv are released into the liquid. After removal of cellular debris, crude extracts were tested in ELISA for scFv antibody fragments binding GST::LRP fusion protein.

Detection of scFv bound to immobilized GST::LRP (200 ng/well) was carried out using an anti-His HRP-conjugate (Qiagen, 1 μg/mL). The signal was developed with the tetramethyl benzidine solution (TMB) (KPL) and detected at 450 nm after termination of the reaction with 0.5 M H_2SO_4.

2.2. ScFv expression and purification

The clones S18 and N3 were subcloned as NcoI–NotI restriction fragments into the vector pSKK2 (Le Gall et al., 2004) resulting in the plasmids pSKK2-S18 and pSKK2-N3, respectively. The clone C9 encoding for a scFv antibody directed against preS1 a hepatitis B coat protein (Persing et al., 1987) was subcloned into pSKK2 resulting in pSKK2-C9. The constructs were transformed into *E. coli* RV308 and plated onto 2YT agar containing 100 μg/mL ampicillin and 50 mM glucose. For expression, bacteria were cultured at 26 °C in 2 YT medium supplemented with ampicillin and glucose until an optical density between 0.6 and 0.8 at a wavelength of 600 nm was achieved. After centrifugation bacterial pellets were resuspended in YTBS medium supplemented with 1 M D-sorbitol and 2.5 mM betaine with 0.2 mM IPTG and grown at 21 °C overnight. The cells were resuspended in 50 mM phosphate buffer pH 8, 300 mM NaCl containing 20 mM imidazole, β-mercaptoethanol and protease inhibitors (PMSF, aprotinin, leupeptin). After snap-freezing in liquid nitrogen, the lysate was digested 1 h with 1 mg/mL lysozyme and centrifuged at 4 °C 14,500 rpm for 1 h. The supernatant was incubated with Probond nickel-chelating resin (Invitrogen). Beads were washed with the buffer described and finally eluted with 50 mM phosphate buffer pH 8, 300 mM NaCl and 250 mM imidazole. For their application in animal experiments, scFvs were further purified by size exclusion chromatography using a sephadex S200 column and filter sterilized.

2.3. Expression of LRP::FLAG in the Semliki-Forest-Virus (SFV) system

Expression of mouse and human LRP::FLAG employing the SFV system was described previously (Gauczynski et al., 2001b). Briefly, SFV based vectors carrying mouse or human LRP::FLAG were used. Recombinant SFV-1 RNAs were generated by in vitro transcription and transfected into BHK cells by electroporation.

2.4. FACS analysis (flow cytometry)

Cell suspensions were prepared in PBS, 2% fetal calf serum, 20 mM EDTA, 0.01% sodium azide (FACS buffer). For flow cytometry, cells were incubated with the primary antibody at concentrations of ∼1 μg/10^6 cells for 15 min at room temperature and washed in FACS buffer before incubation with FITC-conjugated anti-myc or anti-rabbit antibodies for 15 min at room temperature. After washing in FACS buffer, data acquisition and analysis were performed with an EPICS XL-MCL (Coulter) flow cytometer. For each condition, 5000 cells were counted. A polyclonal anti-gal-3 antibody (Gauczynski et al., 2001b) and the scFvs N3 and S18 were used as primary antibodies.

2.5. Western blot analysis

Cells were lysed in 10 mM Tris–HCl pH 7.5, 10 mM NaCl, 10 mM EDTA, 0.5% Triton X-100 and 0.5% sodium deoxycholate. Equal amounts of cellular proteins were resuspended in SDS sample buffer and heated to 95 °C for 5 min. Samples were analysed on a 12% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane, blocked with 5% milk and incubated with the primary antibodies N3, S18 (diluted 1:1000 to a final concentration of 1 μg/mL) or anti-LRP 43512 (diluted 1:5000 to a final concentration of 0.5 μg/mL). The scFvs were detected with an anti-c-myc antibody (Santa Cruz 1:1000

to a final concentration of 1 μg/mL), followed by a peroxidase conjugated anti-mouse antibody (Santa Cruz 1:5000 to a final concentration of 0.5 μg/mL). Detection was performed by enhanced chemiluminescence (Perkin-Elmer Life Sciences).

2.6. Epitope mapping

The entire sequence of human LRP was covered by 92 different synthetic peptides of 15 amino acids length. The N-terminus of each peptide was shifted with respect to the previous peptide by three amino acids, leading to an overlap of 12 amino acids. Synthesis was performed on a cellulose membrane (AIMS, Braunschweig) using Fmoc chemistry (PyBop/NMM activation, Trt/tBu/Pbf/Boc side chain protection) according to the SPOT-synthesis method of Frank (1992) using a spotting robot (Syro, MultiSynTech GmbH, Witten, Germany). Detection was performed as described for western blotting.

2.7. K_D estimation by ELISA

Two hundred nanograms of GST::LRP per well in 800 mM $NaHCO_3$ pH 8.6 has been coated on a Nunc immunoplate Maxisorb and incubated over night at 4 °C. After three washing steps with PBS/0.1% Tween 20, 300 μL per well of blocking solution (2% milk in PBS pH 7) was added for 2 h at room temperature. After three washing steps (PBS/0.1% Tween) serial dilutions (1:5) on anti-LRP scFv S18 starting with 100 μg/mL were added, incubated for 1 h at room temperature followed by three washing steps (PBS/0.1% Tween). Detection was performed by adding anti $(His)_5$-HRP (1/1000), followed by four washing steps (PBS/0.1% Tween) and one washing step (PBS). Fifty microlitres per well of solution mix (TMB peroxidase substrate (KPL) and peroxidase substrate solution B (KPL) ration 1:1) was added to start the substrate reaction, which was determined by adding 50 μL 0.5 M H_2SO_4. Equilibrium dissociation constants K_D were estimated by fitting the experimental values (each anti-LRP scFv S18 concentration was added in triplicate) to equation $Y = B_{max}X/(K_d + X)$ for one-site binding (hyperbola) using the software program PRISM (GraphPad Software, San Diego, CA).

2.8. Generation of recombinant proteins

pGEX-4T (Amersham) was used for GST expression and pGEX-2T-huPrP23-230 for GST::huPrP23-230 expression. HuPrP23-230 was cloned into pGEX2T as described for the construction of pGEX2T-haPrP23-231 (Weiss et al., 1995). The cDNA fragment encoding huLRP (aa 1–295) was amplified by PCR and subcloned via BamHI and EcoRI into the expression vector pGEX-4T. GST, GST::huPrP23-230 and GST::huLRP were produced in *E. coli* BL21 cells as described for GST and GST::haPrP (Weiss et al., 1995). These proteins were purified by glutathione-sepharose affinity chromatography (Amersham Biosciences). GST::LRP was digested with 5 units biotinylated thrombin (Thrombin Kit, Novagen). The supernatant was incubated with streptavidin agarose beads to remove the recombinant thrombin according to the manufacturer's instructions.

2.9. Pull-down assay

Four micrograms of LRP were pre-incubated with 100 μg scFv. GST::PrP beads, GST beads and unloaded beads were first saturated with bovine serum albumine for 1 h. Twenty-five microlitres of beads were then added to the reaction in a final volume of 350 μL binding buffer (50 mM Tris–HCl pH 8, 300 mM NaCl, 0.025% NP40) for 1 h at room temperature. The beads were washed four times in the same buffer. Bound proteins were eluted directly in SDS-loading buffer for SDS-PAGE analysis.

2.10. Animal experiments

All animal experiments were approved by the Bavarian Government (Az: 209.1/211-2531-83/04).

2.10.1. Analysis of peripheral PrP^{Sc} accumulation

Three groups of six C57BL/6 female mice were injected intraperitoneally with 100 μL of a 10% RML brain homogenate prepared from the brains of terminally sick mice. The mice were treated intraperitoneally once a week with one mg of antibodies (S18 or C9) diluted in PBS or a total period of 8 weeks, the first treatment being given one day prior to RML prion inoculation. Control mice were treated with PBS. Ninety days after prion inoculation, mice were sacrificed and spleens were homogenized in PBS to 10% (w/v). The homogenates were adjusted to 5 mg/mL and digested with 50 μg/mL of proteinase K (1 h, 37 °C). One hundred and fifty micrograms of total protein were analysed by western blotting using the antibody SAF83. Undigested lysates were used as a loading control. The density bands obtained were analyzed using NIH software.

2.10.2. Analysis of the incubation and survival times

Three groups of C57BL/6 female mice were intraperitoneally injected with S18, C9 and PBS followed by inoculation with RML prions. The same conditions were applied as for the analysis of the peripheral PrP^{Sc} accumulation described above. Incubation times represent the time span from the day of RML inoculation until one of the four symptoms: ataxia of gait, tremor, difficulty righting from a supine position, and rigidity in the tail occurs. Survival times represent the time span from the day one of the four symptoms occurs until the day mice show two of the four TSE-relevant symptoms over 3 days (Sethi et al., 2002). At this time point the mice were sacrificed.

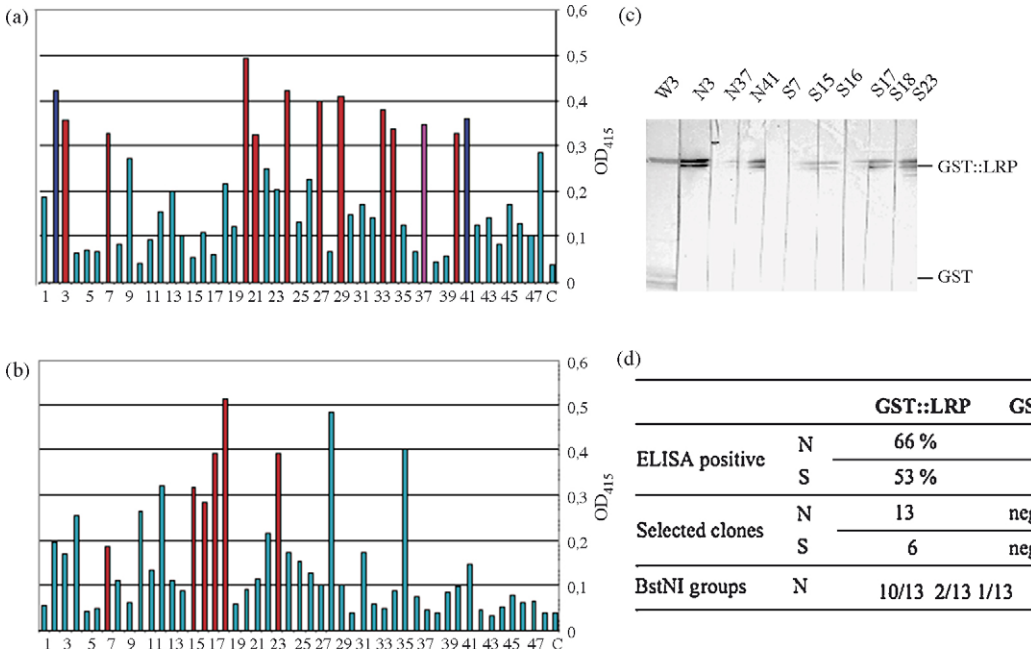
2.10.3. Investigation of side effects caused by scFvs

To investigate side effects of scFv, 2 groups of 6 C57BL/6 mice were injected with PBS or S18 during 8 weeks. The animals were sacrificed 4 and 8 weeks or 120 days after beginning of treatment, blood was collected in EDTA and analysed for immune cell content (Vet-Med-Labor, Munich). Different organs (liver, brain, kidney, spleen) were collected and analysed.

3. Results

3.1. Selection of anti-LRP scFvs by phage display

Specific phage display fragments binding to GST::LRP were selected from a naïve and a synthetic phage scFv library



(d)

		GST::LRP	GST
ELISA positive	N	66 %	
	S	53 %	
Selected clones	N	13	neg
	S	6	neg
BstNI groups	N	10/13 2/13 1/13	

Fig. 1. Selection of scFvs by phage display. Clones of each library ((a) naive library; (b) synthetic library) were analysed for their ability to bind GST::LRP by ELISA. (c) Unique clones were used for the detection of recombinant GST and GST::LRP by western blotting. (d) Selection summary: a BstNI fingerprinting of the DNAs of the 13 clones selected from the naïve library revealed 10 identical clones. One clone was identified twice and another one revealed an individual restriction pattern. All clones tested recognized specifically GST::LRP. Due to their strong antigenic recognition, scFv clones S18 and N3 were selected for further characterization. N: naïve library, S: synthetic library.

(Schwarz et al., 2004). After three rounds of affinity selection on GST::LRP, 47 individual clones from each library were selected to test their ability to recognize GST::LRP by ELISA. 66% of the selected clones from the naïve library and 53% from the synthetic library showed positive signals (Fig. 1a–c).

The selected antibodies clearly recognized GST::LRP but not GST (Fig. 1c), demonstrating that the antibodies specifically recognized the LRP part of the fusion protein, in contrast to the polyclonal antibody W3 which was generated by immunization of animals with the GST::LRP fusion protein. A *Bst*NI fingerprinting of the DNAs of the 13 clones selected from the naïve library showed that 10 clones were identical. One clone was identified twice and another one revealed an individual restriction pattern (Fig. 1d). Due to their strong antigenic recognition, we selected the scFv clones S18 and N3 for further characterization.

3.2. Specific recognition of native and denatured LRP by scFvs S18 and N3

The scFvs S18 and N3 were expressed in *E. coli* and purified by IMAC. Up to 1 mg scFv per L of culture medium was achieved. The purified antibodies migrated with molecular weights of approx. 35 kDa as analysed by SDS-PAGE (Fig. 2a).

Purified S18 and N3 specifically recognized both mouse LRP::FLAG and human LRP::FLAG overexpressed in BHK cells employing the Semliki Forest Virus (SFV) sytem as well as the endogenous LRP (Fig. 2b). In contrast to S18, N3 also recognized the 67 kDa LR form (Fig. 2b), which does not implicate a general inability for S18 to recognize the 67 kDa form on other cell types. The FACS profiles obtained with both scFvs demonstrate a specific recognition of cell surface LRP/LR in LRP overexpressing BHK cells (Fig. 2c), suggesting that both scFvs recognize LRP/LR on the cell surface under native conditions. Taken together, these results indicate that N3 and S18 specifically recognize the denatured and the native form of LRP on the cell surface.

3.3. Epitope mapping of scFvs S18 and K_D estimation

The scFv S18, used for animal experiments due to its better recognition of native moLRP (Fig. 2c), was subjected to an epitope mapping. Three dots were detected for S18 (Fig. 3a), whereas no signals were detected for scFv C9 (data not shown). One intense dot observed for S18 is related to an unspecific signal. Comparing the signal positions with the corresponding peptide sequence, we identified the epitope stretching from amino acid 272–280 of LRP. This epitope locates within the extracellular domain of huLRP (Fig. 3b) (Hundt et al., 2001). In addition a K_D value of 5.1×10^{-9} mol/L for the binding of scFv S18 on GST::LRP has been estimated by ELISA.

3.4. ScFvs S18 and N3 interfere with PrP/LRP interaction

GST::huPrP23-230 interacts with huLRP in vitro (Fig. 4) analyzed by pull-down assay. In contrast to the control antibody C9, both scFv antibodies S18 and N3 after pre-incubation with huLRP blocked the GST::huPrP23-230/huLRP interaction (Fig. 4).

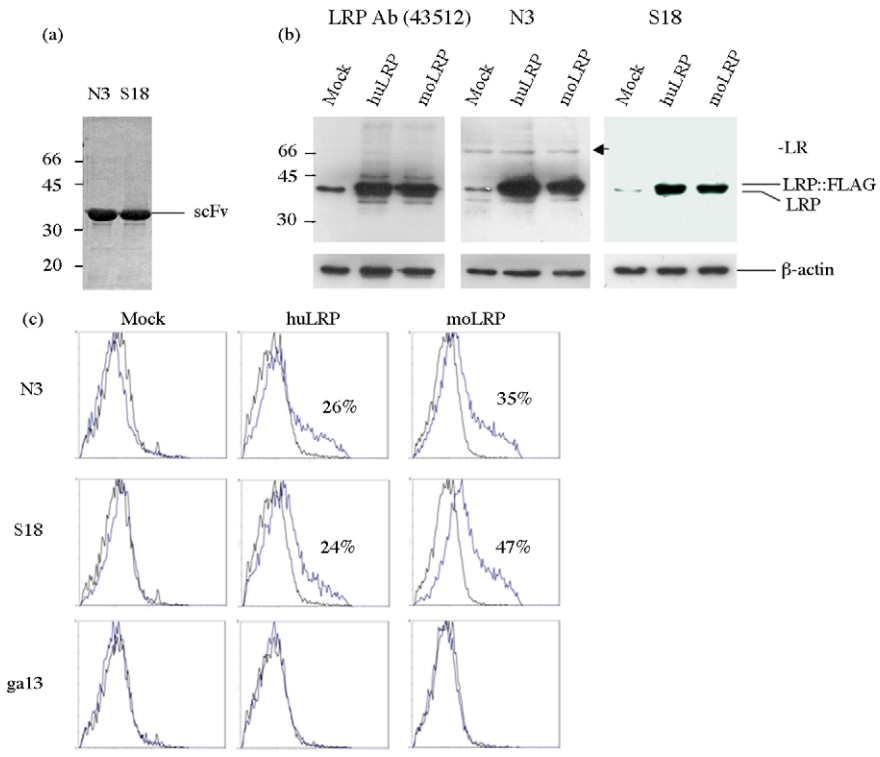


Fig. 2. scFvs N3 and S18 specifically detect denatured and native LRP/LR. (a) SDS-PAGE analysis of purified scFvs expressed in *E. coli*. Proteins were visualized by Coomassie brillant blue staining. (b) BHK cells were transiently transfected with SFV RNA (mock), SFV huLRP::FLAG RNA (huLRP) and SFV moLRP::FLAG RNA (moLRP), respectively. Cell lysates were analysed by western blotting, using N3, S18 or the monoclonal anti-LRP antibody 43512 (control). (c) Non-permeabilized cells transfected as described in (b) were analysed by FACS using scFvs N3, S18 or the anti-gal-3 antibody.

3.5. ScFv S18 reduced PrP^{Sc} accumulation in the spleen in a murine scrapie model by passive immunotransfer

We chose scFv S18 for animal experiments due to its better recognition of moLRP on the cell surface (Fig. 2c). Pretreatment of C57BL/6 mice with scFv S18 confirmed no side effects. We determined the PrP^{Sc} levels in the spleen, 90 days after RML infection, which corresponds to the plateau phase of PrP^{Sc} accumulation. S18 treated C57BL/6 mice revealed a reduction of the

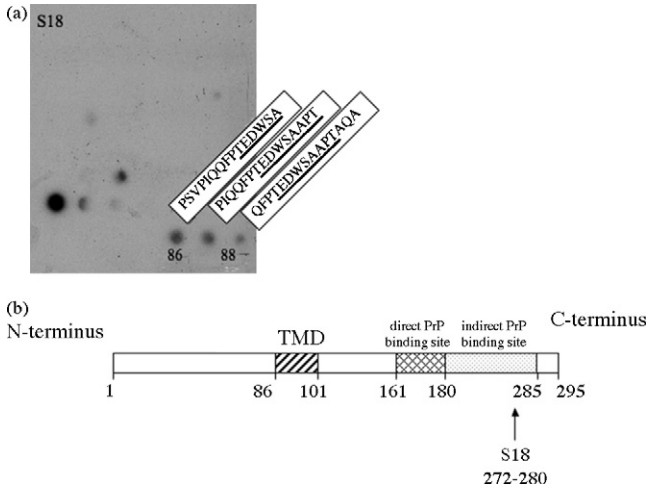


Fig. 3. Epitope mapping of scFv S18. (a) Membranes encompassing the huLRP sequence were hybridized with S18. The sequence of the peptides detected and the amino acid positions are indicated. (b) Schematic representation of LRP. The following binding sites are indicated: direct binding site to PrP (aa 161–179) (Hundt et al., 2001), a suggested indirect HSPG-dependent binding domain for PrP (aa 180–285) (Hundt et al., 2001), scFvS18 recognizes the epitope aa 272–280.

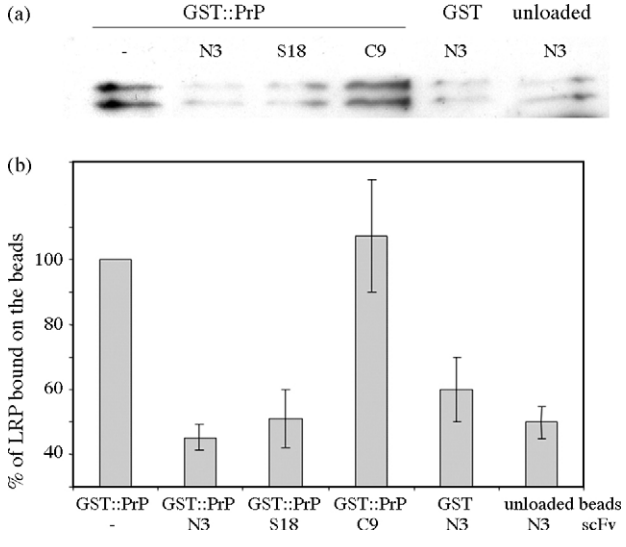


Fig. 4. S18 and N3 block the PrP/LRP interaction in vitro. (a) GST::huPrP immobilized on sepharose glutathione beads was incubated with recombinant LRP in presence or absence of scFvs as indicated. GST immobilized on beads and unloaded beads were employed as negative controls. After elution, LRP bound on beads was analysed by western blotting using N3 for detection. (b) Quantitative analysis of western blots performed by densitometric evaluation of the LRP level in three independent experiments.

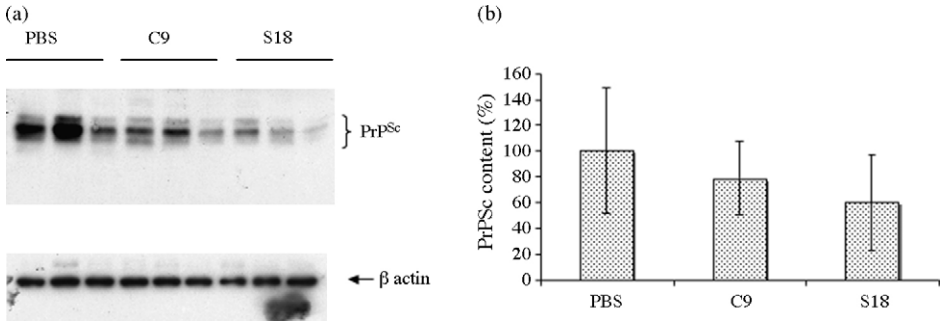


Fig. 5. Spleen analysis of mice inoculated with prions after intraperitoneal treatment with PBS, scFvC9 or scFvS18. Each group consists of six animals. (a) Analysis of PrP^{Sc} levels (after PK digestion) in the spleen has been performed 90 days after scrapie inoculation by western blotting. Shown are the PrP^{Sc} levels of three mice of each group injected with PBS, C9 and S18, respectively. (b) Densitometric measurements of western blots from six spleens per group revealed a reduction of the PrP^{Sc} level by 40% in the scFv S18 treated group compared to the PBS treated group, for which the PrP^{Sc} level was set to 100%. Quantification of the western blot signals was carried out by densitometric measurements using the Image J software.

Table 1
Incubation and survival times of scrapie infected mice treated with scFv C9, scFv S18 and PBS

Experimental group	Incubation times (occurence of first TSE symptoms, days) (mean ± S.D.)	Survival times, days (mean ± S.D.)	Incubation + survival times, days (mean ± S.D.)	Number of mice (affected/inoculated)
PBS	177 ± 0	14.4 ± 9.5	191.4 ± 8.9	10/10
C9	170 ± 2.7	27.6 ± 3.2	197.6 ± 1.3	6/6
S18	182.4 ± 8	17.8 ± 6.2	200.2 ± 14.2	5/5

Incubation times represent the time span from the day of RML inoculation until one of the four symptoms: ataxia of gait, tremor, difficulty righting from a supine position, and rigidity in the tail occurs. Survival times represent the time span from the day one of the four symptoms occurs until the day mice show two of the four TSE-relevant symptoms over three days (Sethi et al., 2002). At this time point the mice were sacrificed.

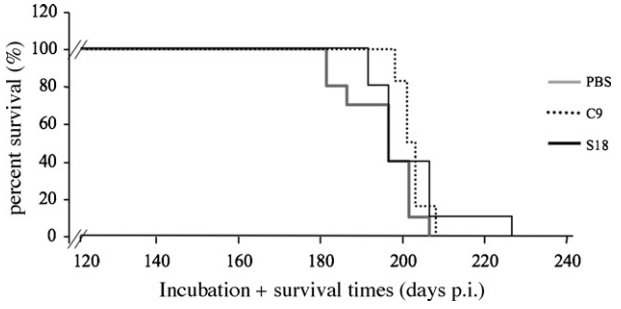


Fig. 6. Incubation plus survival times of scFv treated mice after RML inoculation. PBS treated mice show mean incubation plus survival times of 191.4 (±8.9) days post infection, whereas the S18 and C9 group show incubation plus survival times of 200.2 (±14.2) and 197.6 (±1.3) days, respectively. For definitions of incubation and survival times see footnote of Table 1.

PrP^{Sc} content in the spleen by approx. 40% (Fig. 5b), suggesting that S18 hampers peripheral PrP^{Sc} propagation. However, the reduction of peripheral PrP^{Sc} propagation did not result in a significant prolongation of the incubation and survival times in scrapie infected mice (Fig. 6, Table 1).

4. Discussion

Among the numerous molecules harboring an anti-prion activity (for review Gauczynski et al., 2001a; Vana et al., 2007) antibodies represent promising tools for prion diseases therapy. Antibodies against PrP inhibit PrP^{Sc} propagation in cell culture (Enari et al., 2001; Pankiewicz et al., 2006; Peretz et al., 2001; Perrier et al., 2004) and also in mice (Heppner et al., 2001; Peretz et al., 2001; White et al., 2003). Recently, scFv antibodies directed against specific epitopes of PrP^{c} have been developed (Adamson et al., 2006). However, anti-TSE strategies targeting PrP have been contested, since PrP antibodies injected into the brain of mice led to rapid neuronal apoptosis (Solforosi et al., 2004). Therefore, LRP/LR, acting as a receptor for both PrP^{c} and PrP^{Sc} (Gauczynski et al., 2006; Morel et al., 2005), represents an alternative promising target for therapy in prion diseases.

We selected and characterized two single chain antibodies (scFvs) N3 and S18 directed against LRP/LR. The epitope for S18 on LRP has been located within the extracellular domain of LRP (aa272–aa280) (Hundt et al., 2001), a prerequisite for the interference capacity of the antibody with the extracellular located PrP.

We investigated whether the single chain antibody S18 shows inhibitory effects on prion replication in vivo by passive immunotherapy. S18 reduced PrP^{Sc} levels in the spleen by approx. 40%, demonstrating that the antibody has the capacity to interfere with peripheral prion propagation. However, the S18 mediated reduction of the peripheral PrP^{Sc} propagation was not concomitant with a significant prolongation of the incubation and survival times in scrapie infected mice treated with scFv S18. One reason for that might be the short half life of the antibody, which we calculated with approx. 12 h in the blood of animals implicating a daily scFv delivery. A more frequent application of higher amounts of the antibody for a longer period of time might be required to obtain a certain type of threshold to achieve a regression of the disease reflected in prolonged incubation and/or survival times. Gene transfer resulting in a permanent

in vivo expression of scFv provides an alternative delivery system (Afanasieva et al., 2003; Arafat et al., 2002). A recent work reported that anti-PrP single chain antibodies expressed in mammalian cells exert a paracrine anti-prion activity (Donofrio et al., 2005). Therefore it might be interesting to consider a bi-therapy associating anti-LRP and anti-PrP scFvs to improve anti-prion treatment.

The capacity of the selected scFvs to block the PrP/LRP interaction in vitro (i) and the anti-prion effect on the peripheral PrP^{Sc} accumulation in a murine model (ii) recommend anti-LRP scFvs as efficient tools in therapy of prion disorders.

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Chapter IX

Delivery of single-chain antibodies scFvs directed against the 37 kDa/67 kDa laminin receptor into mice via recombinant Adeno-associated viral vectors for prion disease gene therapy

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Short Communication

Delivery of single-chain antibodies scFvs directed against the 37/67 kDa laminin receptor into mice via recombinant adeno-associated viral vectors for prion disease gene therapy

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The 37/67 kDa laminin receptor (LRP/LR) acts as a receptor for prions providing a promising target for the treatment of prion diseases. Recently, we selected anti-LRP/LR single-chain antibodies (scFvs) and proved a reduction of the peripheral PrP^{Sc} propagation by passive immunotransfer into scrapie-infected mice. Here, we report the development of an *in vivo* gene delivery system based on adeno-associated virus (AAV) vectors expressing scFvs-S18 and -N3 directed against LRP/LR. Transduction of neuronal and non-neuronal cells with recombinant (r)AAV serotype 2 vectors encoding scFv-S18, -N3 and -C9 verified the efficient secretion of the antibodies. These vectors were administered via stereotactic intracerebral microinjection into the hippocampus of C57BL/6 mice, followed by intracerebral inoculation with 10 % RML at the same site 2 weeks post-injection of rAAV. After 90 days post-infection, scFv-S18 and -N3 expression resulted in the reduction of peripheral PrP^{Sc} propagation by approximately 60 and 32 %, respectively, without a significant prolongation of incubation times and survival. Proof of rAAV vector DNA in spleen samples by real-time PCR strongly suggests a transport or trafficking of rAAV from the brain to the spleen, resulting in rAAV-mediated expression of scFv and thereby reduced PrP^{Sc} levels in the spleen are most likely due to blocking of the prion receptor LRP/LR by scFv.

INTRODUCTION

Prion diseases are fatal lethal neurodegenerative diseases affecting humans and animals (for review see Weissmann, 2004; Zuber *et al.*, 2007a). None of the affected individuals can be treated or cured effectively (Ludewigs *et al.*, 2007; Vana *et al.*, 2007; Weissmann & Aguzzi, 2005). The abnormal form of the prion protein, PrP^{Sc}, is frequently associated with infectivity and propagates mainly in the brain and the lymphoreticular system (LRS). Accumulation of the aggregated PrP^{Sc} leads to neuronal death. PrP^{Sc} is distinct from the host protein PrP^{C} by its biochemical properties such as proteinase K sensitivity and insolubility, but harbours the same amino acid sequence. The generation of PrP^{Sc} from PrP^{C} involves conformational changes accompanied by modifications in the secondary structure of the protein (for review see Aguzzi & Weissmann, 1998; Prusiner, 1998; Weissmann, 2004).

The 37/67 kDa laminin receptor (LRP/LR) is a multi-functional protein (i) playing an important role in cell adhesion, movement and growth of many cell types, (ii)

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acting as a receptor for some subtypes of adeno-associated virus (AAV), alphaviruses and dengue virus, (iii) playing an important role in cancer progression and metastasis (for review see Gauczynski *et al.*, 2001a; Nelson *et al.*, 2008; Rieger *et al.*, 1999) We recently showed that blockage or downregulation of LRP/LR in neoplastic cells prevents invasion of these cells into neighbouring tissues, suggesting that LRP/LR plays a major role in cancer metastasis (Zuber *et al.*, 2008b) and (iv) finally, LRP/LR represents a key player in prion infection (Ludewigs *et al.*, 2007; Vana *et al.*, 2007; Zuber *et al.*, 2007a). LRP has been shown to act both as the PrP^{C} (Gauczynski *et al.*, 2001b) and PrP^{Sc} receptor (Gauczynski *et al.*, 2006) and is responsible for bovine PrP^{Sc} internalization by human enterocytes (Morel *et al.*, 2005). The fact that LRP levels are increased in organs of the LRS and central nervous system (CNS) such as spleen and brain of infected animals (Rieger *et al.*, 1997) strongly suggests that this receptor is not only essential for prion uptake after oral infection but also plays an important role for PrP^{Sc} propagation and prion pathogenesis in the peripheral nervous system, including the LRS and CNS. Additionally, several laminin receptor isoforms have been found in mouse brain all binding to PrP (Simoneau *et al.*, 2003). LRP/LR, plays a key role as a cell surface receptor for prions, was also recently found to interact with PrP in the perinuclear compartment and in part with a mutated PrP lacking the signal sequence in the nucleus (Nikles *et al.*, 2008). LRP/LR attracts more and more attention as a target for therapy in prion diseases and cancer. Multiple strategies on LRP inactivation have been shown to be successful by inhibiting PrP^{Sc} propagation *in vitro*: (i) downregulation of LRP via antisense or siRNA strategies completely blocks PrP^{Sc} propagation (Leucht *et al.*, 2003) and delays the incubation time in scrapie-infected mice (H. Ludewigs and others, unpublished data), (ii) a *trans* dominant-negative LRP mutant interferes with PrP^{Sc} propagation in ScN2a cells (Vana & Weiss, 2006), (iii) polysulfated glycanes block the PrP^{Sc}–LRP/LR interaction and strongly reduce PrP^{Sc} binding (Gauczynski *et al.*, 2006) and (iv) the anti-LRP antibody, W3, abrogates PrP^{Sc} accumulation in scrapie-infected cells (Leucht *et al.*, 2003) and prevents binding and internalization of PrP^{BSE} prions (Morel *et al.*, 2005). W3 reduces peripheral PrP^{Sc} propagation significantly by 66 % and prolongs the survival in scrapie-infected mice by 1.8-fold (Zuber *et al.*, 2007b). Many of these anti-LRP/LR tools particularly antibodies, siRNAs and polysulfated glycanes interfere with the laminin–LRP/LR interaction, which results in a reduced invasive potential of neoplastic cells, recommending these tools as powerful therapeutics in the treatment of cancer, especially metastasis formation (Zuber *et al.*, 2008b).

Monoclonal antibodies are attractive therapeutic agents and at least 21 of them obtained FDA approval for therapeutic use in patients (Reichert *et al.*, 2005; Waldmann, 2003). Nevertheless immunotherapy is limited by the immunogenicity of murine-derived antibodies and the restricted tissue penetration. Single-chain antibodies (scFv) as an alternative system have been developed to circumvent such problems. In contrast to entire immunoglobulins, scFv are much smaller in size, which allows them to penetrate into tissues and lacking the Fc part they do not provoke an immune response (for review see Sanz *et al.*, 2005). We recently described the selection of anti-LRP scFvs termed S18 and N3 from a human antibody library by phage-display (Zuber *et al.*, 2008a). Employing a passive immunotransfer approach, scFv-S18 reduced PrP^{Sc} deposition in the spleen of infected mice by approximately 40 % (Zuber *et al.*, 2008a). However, intraperitoneal injection of the antibody did not significantly prolong the incubation times and survival (Zuber *et al.*, 2008a) was most likely due to the short half-life of scFvs in the blood (approx. 12 h or less) and probably due to insufficient amounts that had been administered (1 mg per week). In addition, due to the low stability, scFvs might have failed to cross the blood–brain barrier and therefore have failed to reach the brain where most of the prion agent propagates. To circumvent these limitations, we exploited a gene therapeutic approach based on the recombinant (r)AAV vector system. Due to its non-inflammatory and non-pathogenic nature, we chose the AAV system for *in vivo* delivery of scFvs-S18 and -N3 to achieve a permanent expression of the antibodies from neuronal cells. Up to now 12 serotypes have been identified named AAV type 1–12 (for review see Wu *et al.*, 2006) (Schmidt *et al.*, 2006), AAV serotype 2 is the best characterized one and is conventionally utilized as a gene therapy vector. This serotype offers a series of advantages including, e.g. transduction of a wide variety of cell types and low immunogenicity after *in vivo* application (Tal, 2000). Furthermore, the vector genome persists for extended periods of time enabling long-term transgene expression.

AAV received increasing attention as a promising candidate for gene therapy and at least 13 gene therapeutic approaches are currently under investigation in clinical trials worldwide (see http://www.clinicaltrials.gov). Applications of AAV to treat Parkinson's disease are actively studied in experimental models (Hayashita-Kinoh *et al.*, 2006; Luo *et al.*, 2002). AAV was efficiently used to target the *Prn*-P gene (Hirata *et al.*, 2004) and PrP^{C} was overexpressed by adenovirus-mediated gene targeting (Shyu *et al.*, 2005).

rAAV2-mediated delivery of PrP^{C}-specific scFvs targeting the prion protein delayed the onset of prion pathogenesis in mice (Wuertzer *et al.*, 2008). Here, we provide the proof of principle for a successful AAV-mediated gene therapy targeting the prion receptor LRP/LR by anti-LRP/LR scFvs.

To examine which AAV serotype is suitable for the transduction of neuronal cells, we determined transduction efficiencies for serotypes 2, 3 and 5 based vectors in two neuronal cell lines, N2a and GT1, using the green fluorescent protein as the marker gene. The highest transduction efficiency was achieved by AAV-2 (data not shown). Consequently, we constructed rAAV-2 vectors

encoding anti-LRP scFv-S18, -N3 and -C9, respectively. The cDNAs encoding anti-LRP scFv-N3 and -S18 and the anti-preS1 (coat protein of the hepatitis B virus) scFv-C9 were subcloned from the expression vector pSKK2-N3, -S18 or -C9 (Le Gall *et al.*, 2004) into the mammalian expression vector pSecTag2B (Invitrogen) to attach the Igκ leader sequence (Coloma *et al.*, 1992) for antibody secretion, a carboxy-terminal myc tag for immunodetection, a polyhistidine tag and a CMV promoter, resulting in the vectors pSecTag2B-N3, -S18 and -C9, respectively. The cDNA sequences were then cloned into the *Xba*I restriction site of the AAV vector plasmid pSub/CEP4 (Wendtner *et al.*, 2002), resulting in the vector plasmids pSub/CEP4-N3, -S18 and -C9, respectively. Transfection of N2a cells with these vector plasmids confirmed that all recombinant scFvs-N3, -S18 and -C9 were expressed and secreted into the medium (Fig. 1a). Detection of scFvs was achieved by using a murine anti-c-myc tag antibody. rAAV-2 vectors coding for scFvs were generated by triple transfection of the vector plasmid pSub/CEP4-N3, -S18 or -C9, respectively, the packaging plasmid pRC (Wendtner *et al.*, 2002) and the adenoviral helper plasmid pXX6 (Xiao *et al.*, 1998). Vector production and purification was performed as described previously (Hacker *et al.*, 2005) and followed by a heparin affinity chromatography step for further purification and concentration of the vector preparation. All three viral plasmid preparations (rAAV-S18, rAAV-N3 and rAAV-C9) used within the animal experiments were analysed for their capability to express the transgene in neuronal (N2a and GT1) and non-neuronal (HeLa) cells. scFv-S18 was released into the medium as depicted in the Western blot of supernatants obtained 3 and 6 days post-transduction from N2a and GT1 cell cultures, respectively (Fig. 1b). Furthermore, expression and secretion of all three scFvs-N3, -S18 and -C9 by rAAV-transduced HeLa cells was verified (Fig. 1c).

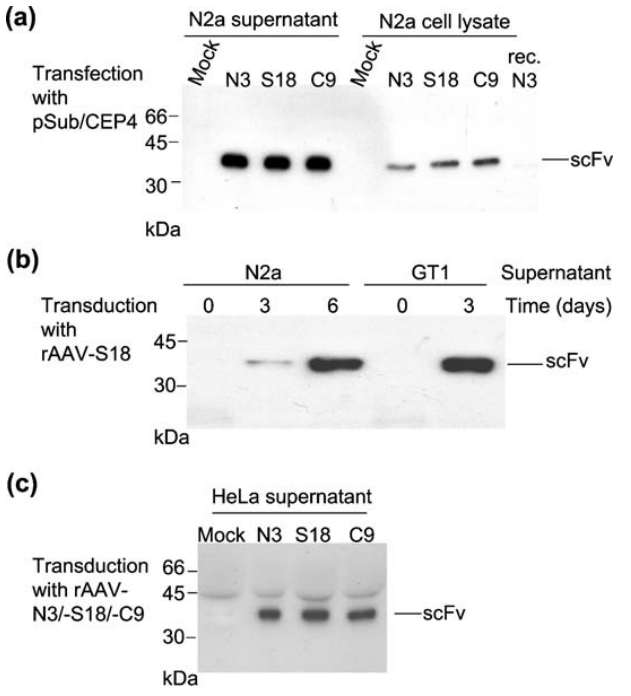

Fig. 1. (a) Expression and secretion of scFvs-N3, -S18 and -C9 in N2a cells after transfection with AAV vector plasmids pSub/CEP4-N3, -S18 or C9, respectively. Mock, transfection with the pSub/CEP4 vector plasmid only. After transfection (48 h), medium and N2a cell lysate were immunoblotted and analysed with an anti-c-myc antibody (Santa Cruz Biotechnology) for the presence of scFvs. (b) Transduction of N2a and GT1 cells with rAAV-S18 results in the secretion of scFv-S18. Depicted are supernatants collected 3 and 6 days post-transduction, respectively. (c) Secretion of scFv-S18, -N3 and -C9 after rAAV-N3, -S18 and -C9, respectively, transduction with the corresponding rAAV vectors from HeLa cells. Supernatants were analysed by immunoblotting 72 h post-transduction. Detection was performed with an anti-c-myc antibody.

To investigate the therapeutic feasibility, scrapie-infected mice were microinjected with rAAV-N3, -S18 and -C9, respectively. PrP^{Sc} accumulates mainly in the CNS and particularly high amounts have been detected in the hippocampus. For that reason, we decided to target this region of the brain by stereotactic microinjection. A volume (5×10^9) of genomic particles was injected intracerebrally (i.c.) into each mouse and the presence of the secreted scFv-N3 was confirmed 30 days post-injection by immobilized metal ion affinity chromatography (IMAC) purification (Fig. 2a). Briefly, homogenates were diluted in 6 M guanidium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 pH 8, sonicated and incubated with 100 µl Ni^{2+} beads (Probond resin; Invitrogen) in the presence of 10 mM imidazole. After extensive washing, beads were eluted in SDS sample buffer and analysed by Western blotting.

A volume of 10 % RML scrapie homogenate was administered into the same area of the brain 2 weeks after rAAV microinjection to allow mice to recover from the application procedure. Neither the intracerebral microinjection of the rAAV nor the viral particles themselves had any obvious effect on the behaviour of the mice. To examine the peripheral PrP^{Sc} propagation, spleen samples were analysed 90 days post-infection (p.i.) by Western blotting. Spleens were homogenized and 200 µg total protein was digested with proteinase K (final concentration of 20 µg ml^{-1}) for 30 min at 37 °C. We observed a reduced PrP^{Sc} content in mice treated with rAAV-N3 by approximately 32 % compared with the control group (rAAV-C9). Furthermore, rAAV-S18-injected mice displayed a significant PrP^{Sc} reduction by approximately 60 % (Fig. 2b, c), suggesting that both antibodies hampered peripheral PrP^{Sc} propagation. In addition, the presence of the scFv-S18 and -C9 encoding DNA sequences in the spleen were verified by real-time PCR (Fig. 2d). The reduced PrP^{Sc} level might therefore be due to the presence of the scFvs encoded by the rAAV-2s, which had trafficked or had been transported from the brain to the spleen. Since

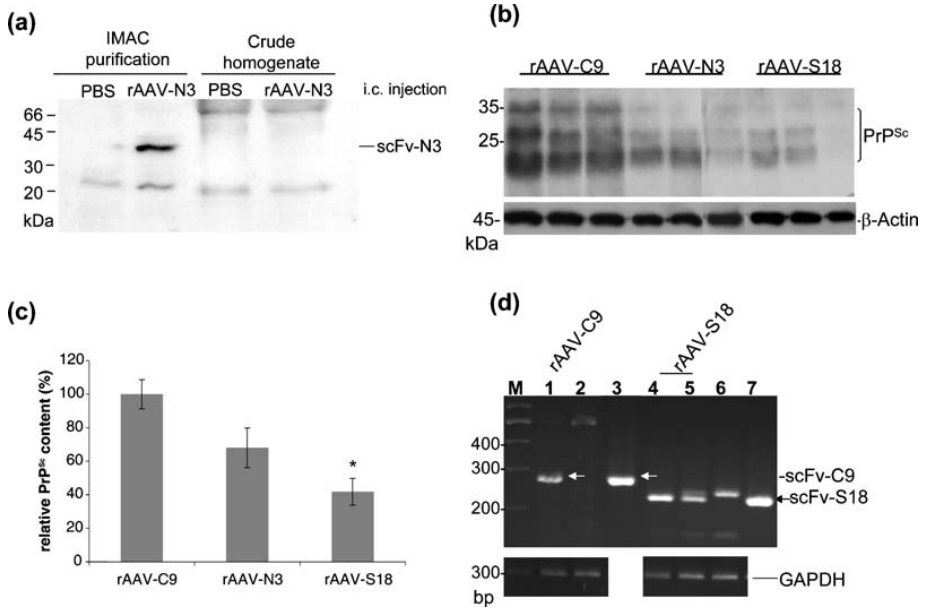

Fig. 2. (a) Detection of scFv-N3 in the brain of C57BL/6 mice 30 days post-stereotactic microinjection of rAAV-N3. Crude brain homogenates were IMAC purified. scFv-N3 expression was detected with an anti-c-myc antibody. (b) Reduction of PrP^{Sc} levels in the spleen of RML-infected C57BL/6 mice 90 days post-infection. Spleen homogenates from six mice per group (rAAV-C9, negative control; rAAV-S18, rAAV-N3) were analysed after proteinase K digestion for determination of PrP^{Sc} levels by Western blotting employing the SAF83 antibody (1 : 5000). Four individual spleen samples from each treated group are shown. A reduced PrP^{Sc} content is observed in the rAAV-S18- and -N3-treated mice. β-Actin was used as a loading control. For this, a corresponding amount of non-proteinase K digested spleen homogenates was analysed by Western blotting using an anti-β-actin antibody. (c) Densitometric quantification of Western blot signals was performed using Image J software. Six individual spleen samples per group (rAAV-C9, negative control; rAAV-S18, rAAV-N3) were analysed by Western blotting and the density of the PrP^{Sc} signals were determined. The mean density of all collected spleen samples (six individual spleen samples per group) was plotted as a histogram. The PrP^{Sc} content of rAAV-C9-injected mice was set to 100 %. PrP^{Sc} values determined from the rAAV-S18-treated group (six individual spleen samples, $n=6$) were compared with the control group (rAAV-C9, $n=6$) using a Student's t-test and revealed significantly reduced PrP^{Sc} levels by approximately 60 % ($P=0.04$). Spleen samples from rAAV-N3-treated mice display a reduction in the PrP^{Sc} level by approximately 32 %. (d) Real-time PCR analyses on spleen DNA extracted 90 days post-infection. To examine the presence of the rAAV-2 in the spleen, primer pairs that amplify a small part within the corresponding scFv encoding DNA sequences were used. A part of the scFv-C9 DNA sequence (approx. 271 bp, white arrow) was amplified from DNA isolated from spleen homogenates of a rAAV-C9-treated C57BL/6 mouse (lane 1). Real-time PCR analysis from DNA from spleen homogenate of an unrelated C57BL/6 mouse (lane 2). The PCR product from the vector plasmid pSub/CEP4-scFv-C9 encoding the respective scFv-DNA sequence is shown as a positive control (lane 3). Spleen DNA from two different rAAV-S18-treated mice (lanes 4 and 5) display positive signals for scFv-S18 DNA sequence (approx. 239 bp). The signal in lane 6 represents an unspecific PCR product of spleen DNA from an unrelated control mouse. The signal in lane 7 describes the positive control for the amplified part of the scFv-S18 encoding DNA sequence of pSub/CEP4-S18. GAPDH-PCRs from DNA from a spleen served as a quantitative standard and displayed no significant differences in the DNA quality used for the specific PCRs.

heparan sulfate proteoglycans (HSPGs) have been reported to act as initial attachment receptors for AAV-2 (Summerford & Samulski, 1998) concomitant with the fact that the spleen exhibits high HSPG levels (Murdoch *et al.*, 1994; Wrenshall & Platt, 1999) we speculate that the administered rAAVs might have crossed the blood–brain barrier and targeted the spleen followed by transgene expression, resulting in the hampering effect on peripheral PrP^{Sc} propagation by the expressed scFvs. In addition to the primary receptor HSPG, the administered AAV-2 might have also used the LRP/LR as a receptor, since it has been reported that LRP/LR can act as a receptor for AAV serotype 2 (Akache *et al.*, 2006).

Although we observed a significant reduction in the PrP^{Sc} level in the spleen of mice after i.c. RML inoculation post-microinjection with rAAV-S18, incubation times and survival were not significantly affected (Table 1). This correlates with an earlier study, describing an unaltered incubation period in splenectomic hamsters intracerebrally infected with 'Chandler' scrapie strain (Kimberlin & Walker, 1977). These hamsters lacking spleens displayed

Table 1. Incubation times and survival of scrapie-infected C57BL/6 mice treated with rAAV-C9, -S18 and -N3

Incubation time represents the time span from the day of RML inoculation until one of the four characteristic TSE symptoms: ataxia of gait, tremor, difficulty righting from a supine position or rigidity in the tail, occurs. Survival represents the time span from the day one of the four symptoms occurs until the day mice showed two of the four TSE-related symptoms over 3 days (Sethi *et al.*, 2002). At this time point mice were sacrificed.

Experimental group	Incubation times (days, mean ± SD)	Survival (days, mean ± SD)	Incubation times plus survival (days, mean ± SD)	No. mice (affected/inoculated)
rAAV-C9	125.3 ± 1.9	27 ± 3.4	152 ± 3.7	6/6
rAAV-S18	128.6 ± 3.7	20.1 ± 14.92	148.6 ± 15.7	7/7
rAAV-N3	126.7 ± 7.4	6.5 ± 1.7	133.2 ± 7.9	4/4

a prolongation in the incubation time only if they were infected intraperitoneally. We conclude therefore, that a reduction in the peripheral PrP^{Sc} propagation observed in the spleen does not necessarily implicate a prolonged survival or incubation time after intracerebral inoculation with RML prions.

Furthermore, the rAAVs might not have reached all the relevant brain cells supporting prion propagation. AAV-2 infects a restricted region near the injection site of the brain, which is believed to result from rapid HSPG-mediated uptake of AAV-2 particles by neurons (Bartlett *et al.*, 1998; Wang *et al.*, 2003). Multiple injection approaches into both hippocampal hemispheres might increase the expression of the scFvs. It is also possible to combine intracerebral treatment with systemic delivery to inhibit PrP^{Sc} invasion in peripheral organs and the CNS simultaneously. In the rAAV study described by Wuertzer *et al.* (2008), which resulted in a delayed onset of the prion disease due to the expression of anti-PrP scFvs, mice were infected intraperitoneally. We might therefore have observed an effect on the incubation time or survival if we would have used this route of infection.

The fact that we observed a reduction in PrP^{Sc} levels in the spleen without a prolongation of incubation times or survival, provides further evidence for the assumption that PrP^{Sc} levels in spleen and brain do not correlate with infectivity. Several reports discuss the absent link between infectivity or disease progression and high titres of proteinase K-resistant PrP^{Sc} (Lasmezas *et al.*, 1997). Manson and colleagues demonstrate in a 101TG mouse model that infectivity is not automatically linked with the presence of PrP^{res} (Manson *et al.*, 1999). Although no or low levels of the disease-associated PrP were found, Gerstmann-Straussler-Scheinker syndrome was followed by the development of clinical transmissible spongiform encephalopathy (TSE) signs. Moreover tissues containing little or no proteinase K-resistant PrP can be infectious with high titres of TSE infectivity (Barron *et al.*, 2007).

Taken together, the load of PrP^{Sc} does not automatically predict disease progression. To examine whether also the infectivity is reduced concomitant with the observed reduction in PrP^{Sc} levels in the spleen of rAAV-treated mice expressing scFvs directed against LRP/LR, a potential infectivity titre of the spleen has to be determined by employing bioassays.

We described here a promising gene therapeutic approach employing rAAVs encoding scFvs targeting LRP/LR. Single microinjections of rAAV carrying scFv sequences directed against LRP/LR into the brain resulted in the expression of the therapeutic antibody followed by a significant reduction by approximately 60 % of the PrP^{Sc} level in the spleen of rAAV-S18-treated mice. This result is in line with our previously reported studies: passive immunotransfer of the polyclonal anti-LRP/LR antibody W3 (Zuber *et al.*, 2007b) and the scFv-S18 (Zuber *et al.*, 2008a) both resulted in a reduction of the PrP^{Sc} content in the spleen, indicating that anti-LRP/LR antibodies reduce peripheral PrP^{Sc} propagation. Despite the significant reduction of the PrP^{Sc} content in the spleen achieved by all three delivery approaches, a prolongation of the survival of scrapie-infected mice was only achieved by the treatment with the polyclonal antibody W3 (Zuber *et al.*, 2007b). This might be explained by the higher stability of full-length IgGs in the organism (half life up to 21 days in blood) compared with scFvs (half life less than 12 h in blood). Both half life and stability of the scFvs have to be improved to achieve an even more pronounced therapeutic effect for the treatment of prion diseases or the availability has to be improved by stable expression from a vector genome.

In summary, the AAV system either used for expression of scFvs directed against PrP (Wuertzer *et al.*, 2008) or LRP/LR represent a powerful delivery system for the treatment of prion disorders targeting the prion protein or its LRP/LR receptor.

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mice were anaesthetized and placed in a stereotaxic apparatus (SR-6N Narishige).

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Chapter X

CWD and sheep Scrapie prions colocalize with the 37kDa/67kDa laminin receptor on human enterocytes

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CWD and sheep Scrapie prions colocalize with the 37kDa/67kDa laminin receptor on human enterocytes

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Abstract

Enterocytes, a major cell population of the intestinal epithelium, represent one of the major entry barriers of prions after oral exposure. We employed enterocytes of human, bovine, porcine, ovine and cervid origin and incubated them with brain homogenates from cervids, sheep and cattle suffering from CWD, Scrapie and BSE, respectively. We confirmed the recently published finding that BSE prions colocalized with the prion receptor 37 kDa/67 kDa LRP/LR on human enterocytes (Caco2 TC-7 cells) supporting the hypothesis that BSE caused the human zoonotic disease vCJD. CWD and sheep scrapie prions also co-localized with LRP/LR on human Caco-2/TC-7 cells suggesting a possible oral transmissibility of CWD and sheep scrapie prions to humans. Further interspecies colocalization studies revealed that CWD might not be transmissible to cattle, pigs and sheep and sheep scrapie might be transmissible to cattle which might have caused BSE but not to cervids and pigs.

Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of lethal neurodegenerative disorders affecting both humans and animals. In contrast to other "protein misfolding diseases" such as Alzheimer's or Huntington's disease, prion diseases are known to be infectious. According to the protein only hypothesis (Griffith, 1967; Prusiner, 1989) an abnormal form of the cellular prion protein (PrP^{c}), the PrP Scrapie (PrP^{Sc}) is thought to be the major infectious constituent (Prusiner, 1982). PrP^{c} is suggested to be converted to PrP^{Sc} which accumulates in the central nervous system (CNS) resulting in neuronal loss and spongiform degeneration in the brain.

TSEs can be transmitted within one animal species (intraspecies transmission) or among different species (interspecies transmission). An oral transmission of prion disease has been widely observed, e.g. BSE (Bovine Spongiform Encephalopathy) has been suggested to originate from the consumption of sheep Scrapie contaminated cattle food (Wilesmith et al., 1988). In addition, it has been demonstrated that the ingestion of BSE-tainted meat resulted in the development of human variant CJD (Creutzfeld-Jacob-disease) (Bruce et al., 1997), showing a transmissibility of BSE prions to humans. Extensive experimental transmission studies of prions to other animal species have been performed (Bruce et al., 1994; Chesebro, 2003), resulting in altered incubation times and survival or in an inablility to transmit the disease (Collinge et al., 1995; Pattison, 1965; Scott et al., 2005; Wells et al., 2003) that is known as "species barrier". Beside the variety of prion strains and their structural differences, the route of infection plays also an important role in the species barrier. In pigs, it has been shown that there are intracerebrally and intraperitoneally infectable with the BSE agent, whereas an oral infection and disease transmission failed (Wells et al., 2003). Currently, BSE and vCJD represent orally acquired TSEs, but little is known about the mechanisms underlying the infection process. After ingestion of prion-contaminated food, the TSE causing agent has to cross the intestinal epithelial cell barrier, where both M-cells (microfold cells)

(Heppner et al., 2001) and enterocytes are proposed to mediate the prion uptake and transport (Okamoto et al., 2003) (Morel et al., 2005). Enterocytes are the major cell population in the intestine (Booth and Potten, 2000) expressing PrP^c on their surface (Morel et al., 2004). It has been reported, that a protease resistant prion protein is transcytosed across human enterocytes independently of endogenous PrP^c expression (Mishra et al., 2004) referring to a receptor-mediated prion uptake. Furthermore, the 37 kDa/67 kDa laminin receptor LRP/LR is expressed on the apical brush border of enterocytes (Shmakov et al., 2000) and has been shown to be responsible for the binding of both PrP^c (Gauczynski et al., 2001) and infectious prions (Gauczynski et al., 2006). Moreover, recent studies on human enterocytes (Caco-2/TC7) revealed an LRP/LR-dependent binding and internalization of bovine prions (PrP^{BSE}), that could be blocked by preincubation with the anti-LRP/LR antibody W3 (Morel et al., 2005), previously reported as a therapeutic tool in a murine Scrapie model (Zuber et al., 2007b).

The spread of CWD (Chronic Wasting Disease), a prion disease affecting free ranging cervids, represents a severe risk in some regions of North America and parts of Canada due to hunting and consumption of their meat. Although animal transmission experiments propose a species barrier for CWD prions to humans (Kong et al., 2005), oral transmission of CWD to humans via contaminated food cannot be excluded and remains to be examined.

In the present study, we examined a possible oral transmission of prions by culturing different enterocyte species in the presence of brain homogenates originating from prion infected cervids, cattle and sheep. Furthermore, the role of the laminin receptor LRP/LR in prion protein uptake was investigated using confocal immunofluorescence analysis. Interestingly, fluorescence labelling of the CWD prion protein revealed a colocalization with LRP/LR on human enterocytes, providing evidence for a possible oral transmissibility of CWD to humans resulting in a new zoonotic disease. In addition, sheep Scrapie prion proteins was also bound to and colocalized with LRP/LR on human enterocytes suggesting an oral transmission of

sheep Scrapie to humans. Taken together, we provide (i) a cell culture model to study in oral transmission of TSEs and (ii) further evidence for an important role of the 37 kDa/67 kDa LRP/LR in prion uptake on enterocytes.

RESULTS

LRP/LR levels on human and animal enterocytes

Since the LRP/LR has been identified as a receptor for PrP^{c} (Gauczynski et al., 2001) and PrP^{Sc} (Gauczynski et al., 2006), respectively, we examined whether the laminin receptor level might correlate with the ability of prion protein uptake. As a prerequisite, cell surface LRP/LR levels of five different enterocyte species were measured using FACS analysis (Figure 1). A relatively high LRP/LR surface level has been observed on human (72.01 %), ,cervid (66.34 %) and bovine enterocytes (40,78 %), respectively, compared to ovine (3.69 %) and porcine (16.05%) enterocytes (Table I).

PrP^{CWD} is colocalized with LRP/LR on human enterocytes

To investigate whether CWD prions might bind LRP/LR-dependent, human enterocytes (Caco-2/TC7) were cultured in the absence or presence of CWD- infected or non-infected brain homogenate for 2 hours and subsequently stained for LRP/LR and CWD prions (PrP^{CWD}), respectively. Immunofluorescence analysis revealed that CWD brain derived PrP is detectable and colocalizes with LRP/LR on the cell surface of Caco-2/TC7 cells (Figure 2 E-G). In parallel, cervid prion protein from healthy brain can be also found to bind to human enterocytes in a LRP/LR mediated manner (suppl. Figure S2 E-G). Cervid enterocytes were used as a positive control and revealed a clear colocalization of LRP/LR with PrP^{CWD} (Figure 2 A-C) and LRP/LR with cervid prion protein (suppl. Figure S2 A-C). In contrast, on porcine enterocytes no colocalization of LRP/LR and PrP^{CWD} (Figure 2 I-K) and PrP^{c} (suppl. Figure S2 I-K), respectively, was observed. Additionally, both bovine and ovine enterocytes

displayed no surface-colocalization of PrP^{CWD} with LRP/LR (Figure 2 M-O and Q-S) and cervid PrP^{c} (suppl. Figure S2 M-O and Q-S), respectively. Immunofluorescent background signals were estimated by the signal of the secondary antibodies alone (data not shown). To exclude that the PrP signal resulted from any unspecific antibody recognition, cells were stained with 8G8 in the absence of brain homogenates (Figure 2D, H, L, P, T).

Sheep Scrapie PrP^{Sc} is colocalized with LRP/LR on human and bovine enterocytes

We cocultured human and animal enterocytes with ovine brain homogenate from both non-infected and sheep Scrapie-infected animals.. Following staining of the 37 kDa/67 kDa LRP/LR and ovine PrP^{Sc},respectively, we observed a colocalization of PrP^{Sc} with LRP/LR on the cell surface of Caco-2/TC7 cells (Figure 3 E-G). Ovine PrP^{c} was also colocalized with LRP/LR on human, bovine and ovine enterocytes (suppl. Figure S3 E-G), respectively, concluding that both ovine PrP^{Sc} and PrP^{c} bind to human enterocytes in a LRP/LR dependent manner.

Additionally, binding of ovine PrP^{Sc} (Figure 3 M-O) and PrP^{c} (suppl. Figure S3 M-O) on bovine enterocytes might occur via LRP/LR. In contrast, cervid enterocytes displayed no clear colocalization of LRP/LR with ovine PrP^{Sc} (Figure 3 A-C) and PrP^{c} (suppl. Figure S3 A-C). Also porcine enterocytes failed to bind PrP^{Sc} (Figure 3 I-K) as well as ovine PrP^{c} (suppl. Figure S3 I-K) LRP/LR-dependent. As an intraspecies control, ovine enterocytes were used, revealing a cell surface colocalization of LRP/LR and both ovine PrP^{Sc} (Figure 3 Q-S) and PrP^{c} (suppl. Figure 3S Q-S).

PrP^{BSE} is colocalized with LRP/LR on human and bovine enterocytes

Human, cervid, bovine, ovine and porcine enterocytes, respectively, were incubated with BSE infected brain homogenate (PrP^{BSE}) following LRP/LR and PrP^{BSE} staining. Immunofluorescence studies revealed a colocalization of both LRP/LR and PrP^{BSE} on Caco-

2/TC7 cells (Figure 4 E-G). Bovine enterocytes that were used as positive control displayed a clear colocalization of PrP^{BSE} with the laminin receptor on the cell surface (Figure 4 M-O). In contrast, porcine enterocytes (Figure 4 I-K) as well as cervid and ovine enterocytes showed no colocalization of PrP^{BSE} and LRP/LR (Figure 4 M-O and Q-S).

DISCUSSION

In the present manuscript, we established an enterocyte cell culture model to study the oral route of prion infection. Brain homogenates originating from healthy and prion-diseased animals were applied to human and animal enterocytes, respectively, and the LRP/LR dependent PrP binding was studied employing immunofluorescence analysis. Enterocytes represent the model of choice, since this cell type has been demonstrated to bind and internalize BSE prions via the 37 kDa/67 kDa LRP/LR (Morel et al., 2005), suggesting an important implication in the oral transmission of prion diseases. Different enterocyte species were employed to investigate LRP/LR-dependent binding of different prion strains.

Colocalization of BSE prions with LRP/LR on Caco-2/TC7 cells confirmed the LRP/LR mediated endocytosis of bovine prions (Morel et al., 2005), providing evidence for the important role of enterocytes together with LRP/LR in the oral transmission of BSE to humans. In contrast, no colocalization of BSE derived prions with LRP/LR on porcine enterocytes confirms that pigs are not orally infectable with BSE prions (Wells et al., 2003). BSE prions do also not colocalize with LRP/LR on cervid and ovine enterocytes suggesting that cervids and sheep might not or only scarcly orally infectable with BSE.

Colocalization of CWD and sheep scrapie prions with LRP/LR on human enterocytes suggests a potential risk for an oral transmission of CWD and sheep scrapie to humans, which might result in a new zoonotic disease. In contrast, CWD prion proteins failed to colocalize with LRP/LR on bovine and ovine enterocytes, which might suggest that CWD cannot be transmitted orally to cattle and sheep.

Sheep scrapie prions colocalize with LRP/LR on bovine enterocytes confirming the transmission of sheep scrapie to cattle originating in the development of BSE. In contrast, sheep scrapie prions do not colocalize with LRP/LR on cervid and porcine enterocytes, suggesting that cervids and pigs cannot become orally infected with sheep scrapie.

FACS analyses revealed that 72.01 % of human, 66.34% of cervid, 40.78% of bovine, 16.05% of porcine and 3.69% of ovine enterocytes, respectively, express LRP/LR on the cell surface (Table I). However, all enterocytes bind CWD, sheep scrapie and BSE prions (Fig.2, 3 and 4 B, F, J, N, R) but only in a few cases the prions colocalize with LRP/LR (Table 1). We conclude that the expression level of LRP/LR does not determine whether prions of a specific species can target LRP/LR followed by internalization. Although prion proteins and LRP/LR molecules are highly conserved among different mammalian species, defined amino acid alterations within the prion protein and the laminin receptor molecules (sequence comparison from www.expasy.org, data not shown) might determine whether both molecules can specifically interact on the cell surface. We proved in addition, that the anti-PrP specific antibody 8G8 did not recognize endogenous PrP (Fig. 2, 3 and 4, D, H, L, P, T) and is therefore specific for the infectious PrP employed in our studies.

Cellular cervid, sheep and bovine (data not shown) PrP^c reveal the same colocalization pattern as infectious CWD, sheep scrapie and BSE prions on human and animal enterocytes (supplementary Figures S2 and S3, respectively), demonstrating that endogenous PrP show the same binding behaviour to LRP/LR compared to their infectious counterparts.

Although we cannot exclude that prions might use alternative infection pathways besides the LRP/LR mediated PrP internalization into enterocytes such as M-cells (Ghosh, 2002) which might also mediate prion uptake, which further leads to prion propagation in the gut associated lymphoid tissues (GALT) (Glaysher and Mabbott, 2007, review Press et al., 2004), we have strong evidence that enterocytes represent a major cell population in the intestine, responsible for LRP/LR dependent BSE prion binding and internalization (Morel et

al., 2005) and, as shown in this manuscript, for LRP/LR dependent CWD and sheep scrapie binding.

Since CWD and sheep scrapie prions might enter the human body via the oral route and may cause a zoonotic disease, tools targeting LRP/LR such as, polysulfated glycanes, siRNAs or antibodies (Ludewigs et al., 2007; Vana et al., in press; Vana et al., 2007; Zuber et al., 2007a) might provide alternative therapeutics or prophylactics for intervention of orally aquired TSEs.

METHODS

Isolation of primary ovine enterocytes. A female Leine sheep (provided by B.Brenig, > 18 months of age) was euthanized by intravenous injection of T61® and Narcoren®. Since it was a pregnant sheep the duodenum of the fetus was also isolated. A segment of the duodenum was taken, from the fetus and the adult animal, distal to the pylorus within 5 min after euthanasia and rinsed with Krebs-Ringer buffer (containing 25 mM HEPES) to remove feed particles. Duodenum was cut into 1 cm pieces and split longitudinally. Duodenal segments were digested in PBS containing 0.8 U/ml collagenase, 0.8 U/ml dispase and 5 mM $CaCl_2$, 30 min at 37 °C. The mucosa was scrapped off carefully from underlying musculature using a scalpel and digested additionally for 10 min at 37 °C in PBS with 0.8 U/ml dispase and 5 mM $CaCl_2$. Mucosal enterocytes were separated from other cells by sequential filtration through a 1000-μm and a 300-μm sieve. The filtrate was centrifuged at 200 x g for 5 min and washed in PBS with 100 U/ml penicillin/100 μg/ml streptomycin. The resulting pellet was resuspended in Medium 199, with Earle´s salts, 2mM L-Glutamin, 25 mM HEPES, 15 % feltal calf serum (FCS) and 50 μg/ml gentamicin (Gibco Invitrogen). Cell yield and viability was determined using a hemacytometer and 0.4 % trypan blue. 4-6 x 10^5 cells/cm^2 were seeded on 6-cm petri dishes in Medium 199.

Cell culture. Caco-2/TC7 cells (human enterocytes) (provided by M. Rousset) were grown in DMEM, 4500 mg l^{-1} D-glucose, 2 mM Glutamax, 20 % fetal calf serum (FCS), 1 % penicillin-streptomycin and 1 % non-essential amino acids (Gibco Invitrogen). FBJ cells (bovine enterocytes) (provided by R. Riebe) were maintained in DMEM:MEM with Hank´s salts (1:1), 1000 mg l^{-1} D-glucose, 2 mM Glutamax, 10 % FCS, 1 % penicillin-streptomycin, 1 % non-essential amino acids (Gibco Invitrogen) and 500 mg l^{-1} $NaHCO_3$. DWM-R cells (cervid enterocytes, provided by R. Riebe) were cultured in IMDM:F12 Nutrient Mix (1:1), 2 mM Glutamax and 10 % FCS (Gibco Invitrogen). IPEC-J2 cells (porcine enterocytes, provided by B. Brenig) were cultured in DMEM:F12 Nutrient Mix (1:1), 1000 mg l^{-1} D-

glucose, 2 mM Glutamax, 5 % fetal calf serum, 1 % penicillin-streptomycin, 1 % non-essential amino acids, 0.1 % Insulin-Transferrin-Selenium and 5 ng ml^{-1} Epidermal growth factor (Gibco Invitrogen). After two days growing in Medium 199 fetal ovine enterocytes were grown in MEM with Earle´s salts, 25 mM HEPES, 10 % FCS, 50 μg/ml gentamicin and 2 mM L-glutamin, whereas adult enterocytes were cultured in IMDM:F12 Nutrient Mix (1:1), 2 mM Glutamax and 10 % FCS (Gibco Invitrogen). All cell types were grown at 37 °C with 5 % CO_2.

Brain homogenate preparation. Brains samples from infected cattle (BSE, provided by B.Brenig), white tailed deer (CWD) and sheep (Scrapie) (both provided by J. Richt) were homogenized to 20% (w/v) at 4°C in PBS.

FACS analysis. Cultured cells were detached with 1 mM PBS/EDTA, centrifuged at 1200 rpm at 4 °C for 10 min and fixed in 4 % paraformaldehyde (4 °C). The cell surface LRP/LR levels of each cell type were stained by the single chain anti-LRP/LR antibody scFv S18 (Zuber et al., 2008). scFv C9, an antibody against hepatitis B surface protein, was used as negative control. Primary antibodies were diluted 1:50 in FACS buffer (0.01 % sodium azide, 20 mM EDTA, 2 % FCS in 1x PBS) and incubated for 1 h at 4 °C. After three washing steps cells were incubated with the secondary antibody c-myc-FITC (1:50, Santa Cruz) and resuspended in FACS buffer for analysis.

Immunofluorescence. Cells were fixed with 4 % paraformaldehyde, and permeabilized with 0.3 % Triton X-100. The primary antibody (8G8) and the secondary antibodies (Alexa Fluor® 488 goat anti-mouse IgG (H+L), Alexa Fluor® 633 goat anti-rabbit IgG (H+L), Molecular Probes, Invitrogen) were diluted in PBS/0.3 % Triton X-100. Cells were incubated overnight at 4 °C with primary antibodies (1:150) followed by incubation with secondary antibodies (1:300). Immunofluorescence backgrounds were estimated by the signal obtained in cells incubated with non-infectious brain homogenates or by the fluorescence of infected cells

when secondary antibodies were applied alone. Examination was done by confocal fluorescence microscopy (Zeiss LSM 510).

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Supplementary information is available at EMBO reports online

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FIGURES

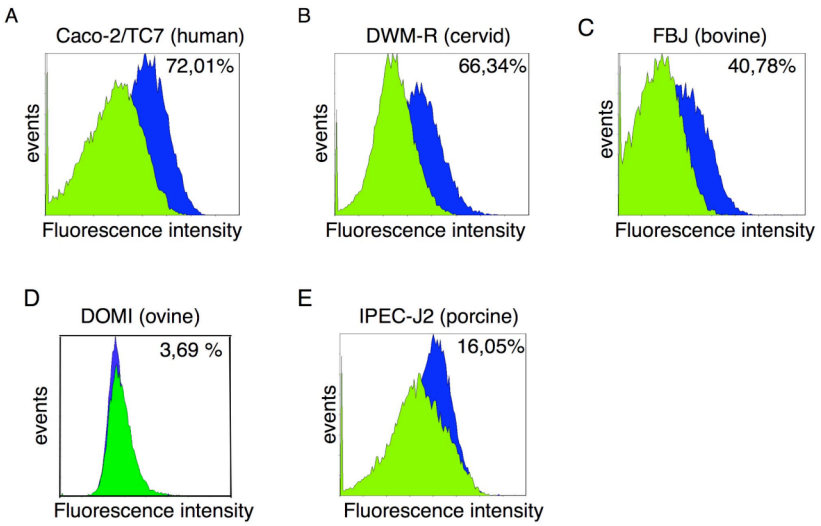

Figure 1: Detection of cell surface LRP/LR levels on human and animal enterocytes by FACS analysis. (A) Human (Caco-2/TC7), (B) cervid (DWM-R), (C) ovine (DOMI-1), (D) bovine (FBJ) and (E) porcine (IPEC-J2) enterocytes were analyzed for LRP/LR surface level. While cervid and human enterocytes revealed a high LRP/LR level, porcine enterocytes showed low levels of the laminin receptor on the surface. Almost undetectable LRP/LR levels were observed in ovine enterocytes. LRP/LR levels were detected using the anti-LRP/LR scFv S18 antibody (blue curve). As negative control the anti-CD19 Hd37 antibody was employed (green curve). 20,000 cells were counted per single experiment. One representative experiment out of five is shown for each cell line.

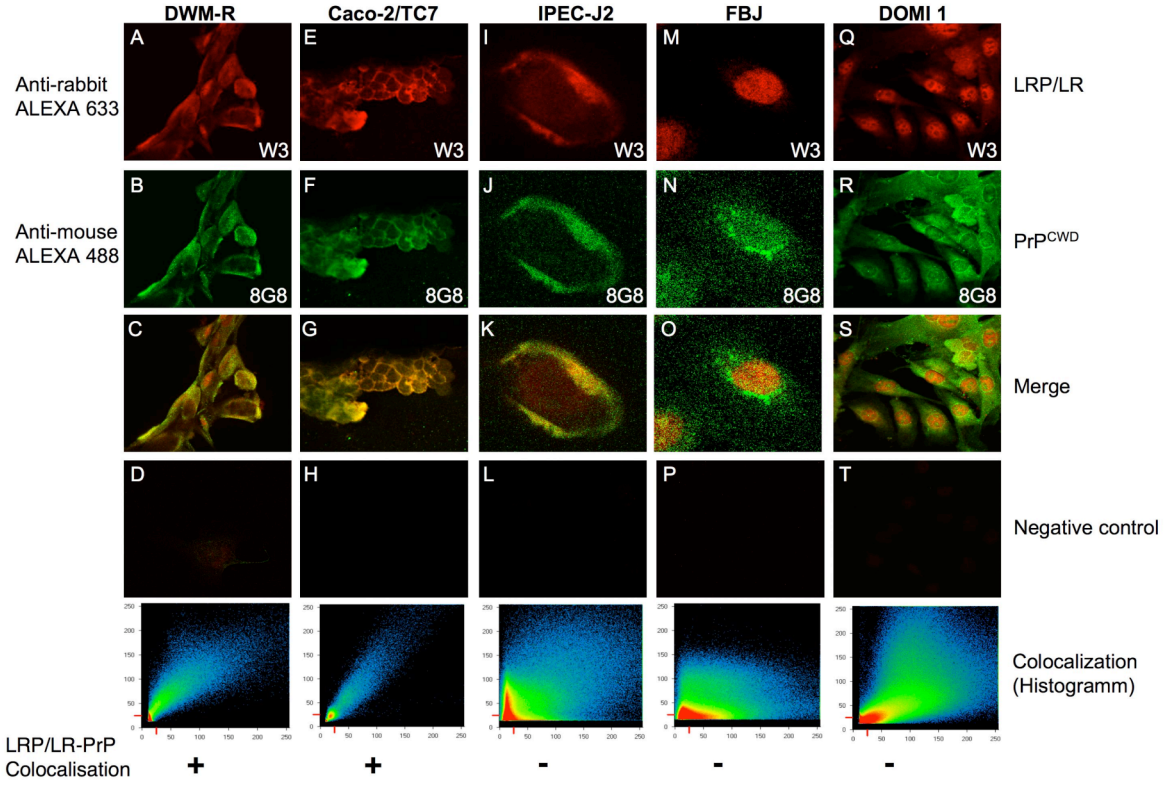

Figure 2: Binding and colocalization of CWD prions with LRP/LR on human and animal enterocytes. Staining of the 37kDa/67 kDa LRP/LR on cervid (A), human (E), porcine (I), bovine (M) and ovine (Q) enterocytes with the anti-LRP/LR antibody W3. A localization of the receptor on the cell surface and inside the cells can be observed. Staining of cervid prions (PrP^{CWD}) on cervid (B), human (F), porcine (J), bovine (L) and ovine (R) enterocytes with the anti-PrP antibody 8G8. The merge of both stainings shows a colocalization of LRP/LR and PrP^{CWD} on cervid (C) and human (G) enterocytes but not on porcine (K), bovine (O) and ovine (S) enterocytes, respectively. As a control of the specificity of the PrP staining was carried out by incubation of cells with 8G8 in the absence of infected and noninfected brain homogenates (D, H, L, P, T). All cells were incubated with 50 μg protein

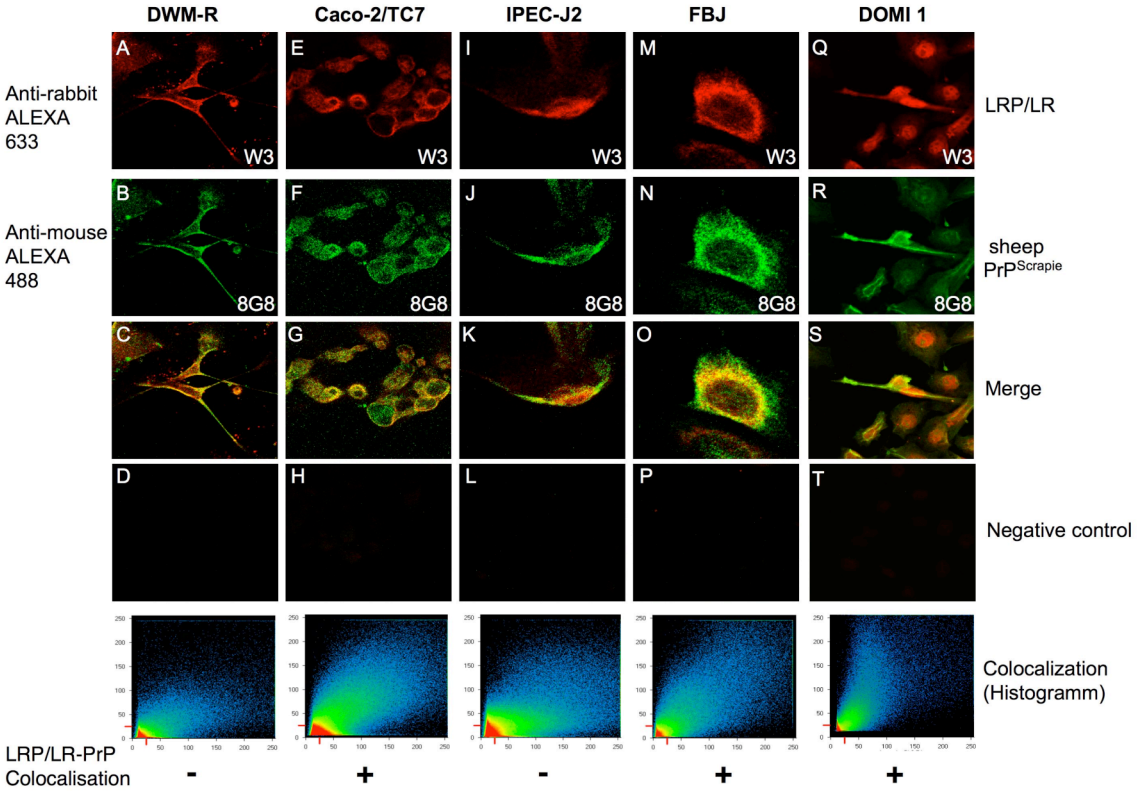

Figure 3: Binding and colocalization of sheep Scrapie prions with LRP/LR on ovine, human and bovine enterocytes. The 37kDa/67 kDa LRP/LR on cervid (A), human (E), porcine (I), bovine (M) and ovine (Q) enterocytes was stained with the anti-LRP/LR antibody W3. Localization of sheep PrP^{Sc} was detected using the anti-PrP antibody 8G8. The merge of both pictures shows a colocalization of LRP/LR and $shPrP^{Sc}$ on human (G), bovine (O) and ovine (R) enterocytes. No colocalization was observed on cervid (C) and porcine enterocytes (K). All cells were incubated with 50 μg protein. As a control for a specific PrP staining cells were stained with 8G8 in the absence of infected and noninfected brain homogenates (D, H, L, P, T).

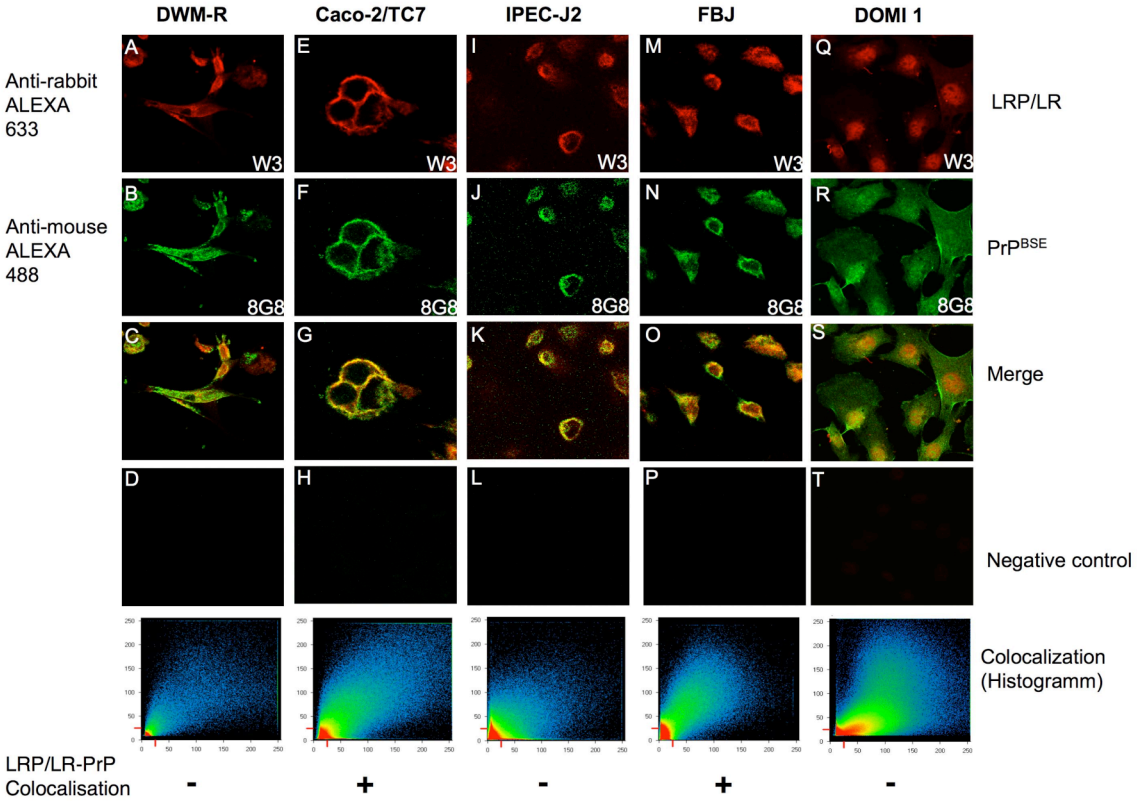

Figure 4: Binding and colocalization of BSE prions with LRP/LR on human and bovine enterocytes. Cervid (A-D), human (E-H), porcine (I-L), bovine (M-P) and ovine (Q-T) enterocytes were incubated with BSE infected brain homogenate. LRP/LR was detected with the W3 antibody and BSE prion were stained using the 8G8 antibody. A colocalization of the laminin receptor and BSE prions on the cell surface is observed on human (G) and bovine (O) enterocytes in contrast to cervid (C), porcine (K) and ovine (S) enterocytes. All cells were incubated with 50 μg protein.

Table I Colocalization of cellular and infectious prion proteins of different species with LRP/LR on the cell surface of human and animal enterocytes.

	Human (Caco-2/TC7)	Bovine (FBJ)	Cervid (DWM-R)	Porcine (IPEC-J2)	Ovine (DOMI 1)
CWD / cervid PrP^c	+	-	+	-	-
shScrapie / ovine PrP^c	+	+	-	-	+
BSE / bovine PrP^c	+	+	-	-	-
Cell surface LRP/LR levels	72.01 %	40.78 %	66.34 %	16.05 %	3.69 %

Supplementary Figures

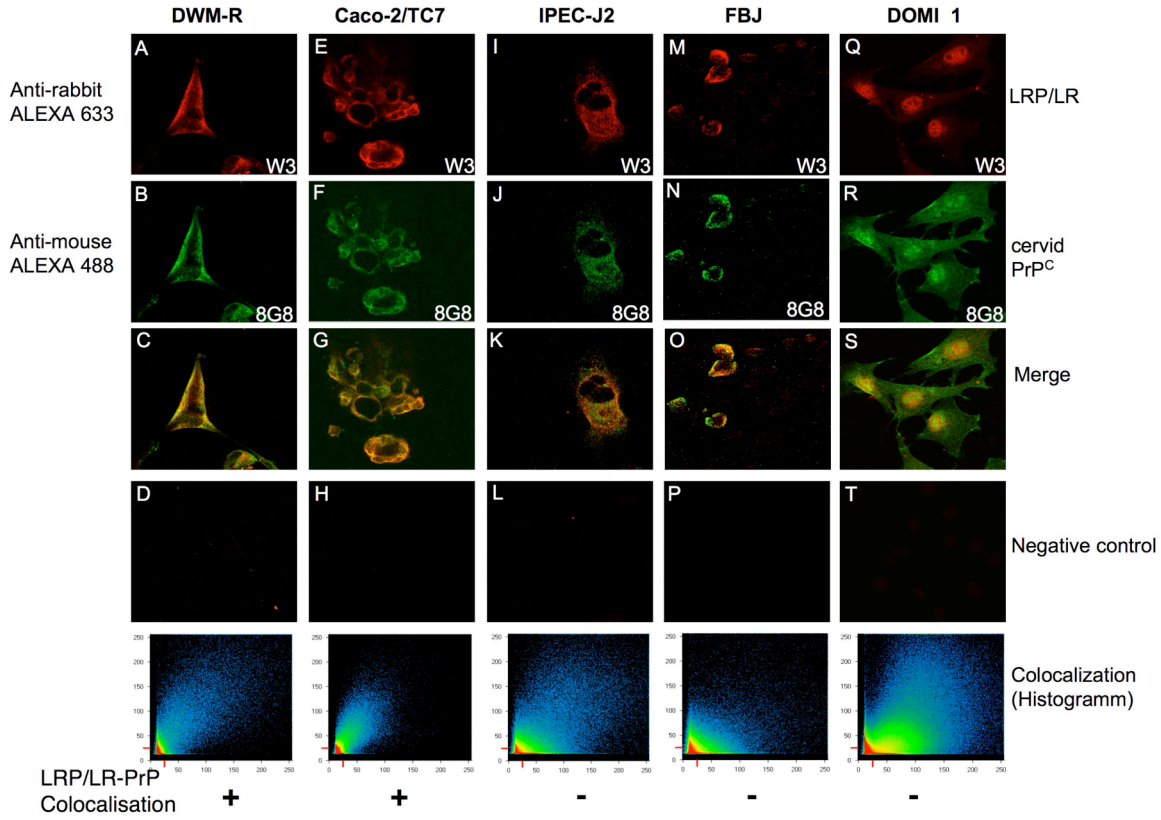

Figure S2. Binding of cervid prion protein on cervid and human enterocytes. Intracellular and cell surface staining of the 37kDa/67 kDa LRP/LR on cervid (DWM-R) (A), human (Caco-2/TC7) (E), porcine (IPEC-J2) (I), bovine (FBJ) (M) and ovine (DOMI 1) (Q) enterocytes with the anti-LRP/LR antibody W3. Staining of cervid prion protein (cervid PrP^C) on cervid (B), human (F), porcine (J), bovine (N) and ovine (R) enterocytes was carried out with the anti-PrP antibody 8G8. The merge of both stainings shows a colocalization of LRP/LR and cervid PrP^C on cervid (C) and human (G) enterocytes but not on porcine (K), bovine (O) and ovine (S) enterocytes. Cervid enterocytes were used as internal positive control for the binding of cervid prion protein to enterocytes. All cells were incubated with 50 μg protein. Incubation of all samples with the secondary antibody only leads to negative signals (D, H, L, P, T).

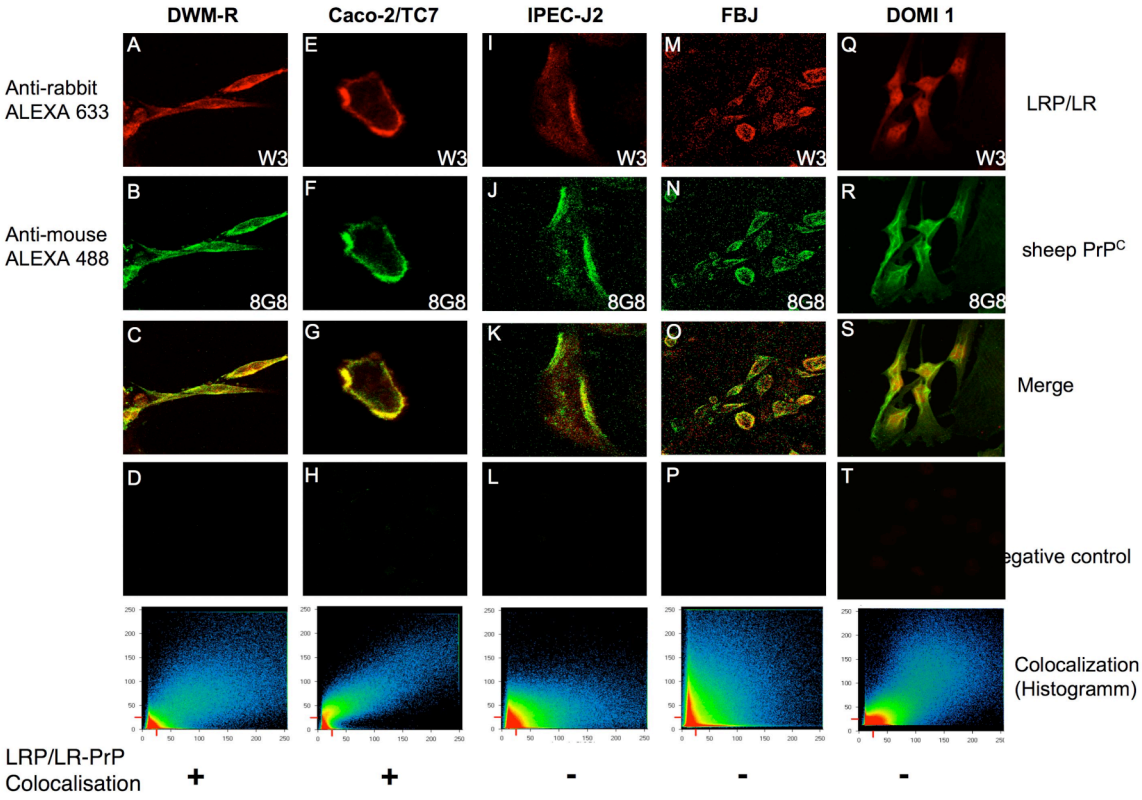

Figure S3. Binding of ovine prion protein on ovine, human and bovine enterocytes. Cervid (A-D), human (E-H), porcine (I-L), bovine (M-P) and ovine (Q-T) enterocytes were incubated with non-infected ovine brain homogenates. Staining for the 37 kDa/67 kDa LRP/LR was performed using the anti-LRP/LR antibody W3. Detection of ovine prion protein was done using the anti-PrP antibody 8G8. In contrast to cervid (C) and porcine enterocytes (K) a colocalization of ovine prion protein and LRP/LR was detected on human (G), bovine (O) and ovine (S) enterocytes. Ovine primary enterocytes were used as internal positive control for the binding of cervid prion protein to enterocytes. All cells were incubated with 50 μg protein. Incubation of all samples with the secondary antibody only leads to negative signals (D, H, L, P, T).

Chapter XI

Invasion of tumorigenic HT1080 cells is impeded by blocking or downregulating the 37-kDa/67-kDa laminin receptor

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Invasion of Tumorigenic HT1080 Cells Is Impeded by Blocking or Downregulating the 37-kDa/67-kDa Laminin Receptor

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The 37-kDa/67-kDa laminin receptor precursor/laminin receptor (LRP/LR) acting as a receptor for prions and viruses is overexpressed in various cancer cell lines, and their metastatic potential correlates with LRP/LR levels. We analyzed the tumorigenic fibrosarcoma cell line HT1080 regarding 37-kDa/67-kDa LRP/LR levels and its invasive potential. Compared to the less invasive embryonic fibroblast cell line NIH3T3, the tumorigenic HT1080 cells display approximately 1.6-fold higher cell-surface levels of LRP/LR. We show that anti-LRP/LR tools interfere with the invasive potential of HT1080 cells. Anti-LRP/LR single-chain variable fragment antibody (scFv) iS18 generated by chain shuffling from parental scFv S18 and its full-length version immunoglobulin G1-iS18 reduced the invasive potential of HT1080 cells significantly by 37% and 38%, respectively. HT1080 cells transfected with lentiviral plasmids expressing small interfering RNAs directed against LRP mRNA showed reduced LRP levels by approximately 44%, concomitant with a significant decrease in the invasive potential by approximately 37%. The polysulfated glycans HM2602 and pentosan polysulfate (SP-54), both capable of blocking LRP/LR, reduced the invasive potential by 20% and 35%, respectively. Adhesion of HT1080 cells to laminin-1 was significantly impeded by scFv iS18 and immunoglobulin G1-iS18 by 60% and 68%, respectively, and by SP-54 and HM2602 by 80%, suggesting that the reduced invasive capacity achieved by these tools is due to the perturbation of the LRP/LR–laminin interaction on the cell surface. Our *in vitro* data suggest that reagents directed against LRP/LR or LRP mRNA such as antibodies, polysulfated glycans, or small interfering RNAs, previously shown to encompass an anti-prion activity by blocking or downregulating the prion receptor LRP/LR, might also be potential cancer therapeutics blocking metastasis by interfering with the LRP/LR–laminin interaction in neoplastic tissues.

Keywords: laminin receptor; prion; cancer; metastasis; therapy

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Abbreviations used: CMD, carboxymethylated dextran; LRP/LR, laminin receptor precursor/laminin receptor; GAG, glycosaminoglycan; IgG1, immunoglobulin G1; scFv, single-chain variable fragment antibody; HEK, human embryonic kidney; PBS, phosphate-buffered saline; HSPG, heparan sulfate proteoglycan; FACS, fluorescence-activated cell sorting; GST, glutathione *S*-transferase; MMP, matrix metalloproteinase; DMEM, Dulbecco's modified Eagle's medium; HRP, horseradish peroxidase; EDTA, ethylenediaminetetraacetic acid.

Introduction

The 37-kDa/67-kDa laminin receptor precursor/laminin receptor (LRP/LR), a nonintegrin cell-surface protein, has been reported to correlate with the aggressiveness of cancer cells, particularly with their invasive potential and metastatic potential, which have been investigated intensively in various studies.[1,2] Overexpression of the 67-kDa LR form is correlated with the metastatic potential, suggesting that the receptor might play an important role in the development of the metastatic phenotype.[3]

Besides several relevant physiological functions such as adhesion or angiogenesis,[4,5] LRP/LR acts as the receptor for the cellular prion protein,[6] infectious prions,[7] and several viruses such as the dengue virus,[8] the Venezuelan equine encephalitis virus,[9] the Sindbis virus,[10] and the adeno-associated virus serotypes 2, 3, 8 and 9[11] (for a review, see Refs. 12–14). Interestingly, the Sindbis virus is known to infect mammalian cells via the 67-kDa LR. A recent gene therapeutic strategy, using this virus, has raised considerable interest since it is known to attack and kill selectively metastatic cells without adverse effects.[15]

Several isoforms of the nonintegrin LR have been identified,[16] whereas the 37-kDa LRP and the 67-kDa LR are the dominant isoforms present in most cell types. The mechanism by which the 67-kDa LR evolves from the 37-kDa LRP form is still not clarified. Although mRNA and protein expression levels have been examined, the relationship between LRP and LR and the molecular nature of the 67-kDa LR remains unclear. The full-length gene encoding the 37-kDa precursor has been isolated, encoding for the 295-amino-acid LRP.[17] However, the composition of the 67-kDa high-affinity LR remains unsolved.

The 67-kDa LR reveals a high affinity to laminin,[18] a major glycoprotein of the basement membrane, which is involved in the attachment, spreading, migration, and differentiation of normal and neoplastic cells. Two binding sites are present on the LRP for laminin. A sequence termed peptide G (LMWWML, amino acids 161–180) on LRP/LR represents the laminin binding site and might induce conformational changes mimicking the effect of the entire receptor.[19,20] The resulting proteolytic cleavage of laminin-1 promotes tumor cell migration.[3] The second binding site stretches from amino acid 205 to 229 at the carboxy terminus of LRP and binds the β1 chain of laminin.[2] The interaction between laminin and the tumorigenic cell is a prerequisite for basement membrane invasion and metastasis.[18,21] Laminin also mediates the interaction of malignant cells and the immune system.[22] The laminin–67-kDa LR interaction (i) enhances the ability of tumorigenic cells to invade, (ii) leads to an increased expression of the 67-kDa form,[23] and (iii) results in an activation of proteolytic enzymes and their regulators.[24]

Here, we examined the potential of anti-LRP/LR tools to influence the invasion and metastatic potential of the tumorigenic human fibrosarcoma cells HT1080. These cells revealed a higher expression of the 37-kDa LRP on the cell surface compared to the nontumorigenic mouse embryonic fibroblast cell line NIH3T3, suggesting that LRP/LR contributes to the invasive potential of tumorigenic cells. Furthermore, HT1080 cells represent the model cell system of choice for invasion assays. Anti-LRP/LR-specific antibodies such as the parental single-chain variable fragment antibody (scFv) S18,[25] the polyclonal W3,[26,27] and the polysulfated glycans[7] are known to block LRP/LR acting as a prion receptor. Anti-LRP/LR antibodies such as the single-chain anti-LRP/LR antibody scFv iS18, its full-length version immunoglobulin G1 (IgG1)-iS18, and the polysulfated glycans HM2602 and pentosan polysulfate (SP-54) significantly inhibit the invasion of HT1080 cells. HT1080 cells transfected with recombinant lentiviral plasmids expressing small interfering RNAs (siRNAs) directed against LRP mRNA revealed reduced levels of LRP, concomitant with a significantly reduced invasive behavior. These data suggest the important role of LRP/LR for the invasive potential of the tumorigenic HT1080 cells. The reduction of the invasive potential of HT1080 cells by our anti-LRP/LR tools obviously results from inhibition of the laminin–LRP/LR interaction on the cell surface. Anti-LRP tools may therefore act as alternative potential agents for an antimetastatic therapy.

Results

HT1080 cells reveal increased cell-surface LRP/LR expression levels compared to the nontumorigenic cell line NIH3T3

To investigate the correlation of the LRP/LR expression profile and the invasive potential of HT1080 cells, we compared the LRP/LR levels on HT1080 and NIH3T3 cells displaying a less invasive behavior. For detection of LRP/LR, we used the single-chain antibody termed scFv S18, recently characterized for recognition of the 37-kDa LRP on mouse neuroblastoma cells (N2a).[25] Fluorescence-activated cell sorting (FACS) analysis revealed an approximately 1.6-fold higher LRP/LR level on the surface of HT1080 cells (63%) compared to NIH3T3 cells (39%) (Fig. 1a). Although scFv S18 recognized the 37-kDa LRP form by Western blotting only (Fig. 1c), the fact that the antibody recognizes the 67-kDa LR form by FACS cannot be excluded. Total LRP/LR levels determined by FACS analysis in permeabilized cells displayed a similar LRP/LR level in both cell lines (Fig. 1b). This finding correlates with the results obtained by Western blot analysis. Cell lysates of HT1080 and NIH3T3 revealed no significant difference in the total 37-kDa LRP content (Fig. 1c).

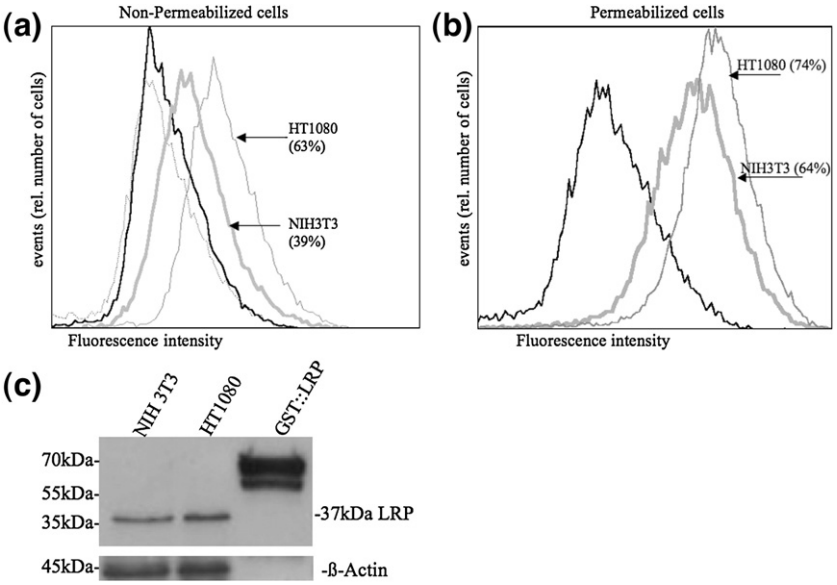

Fig. 1. Expression of the 37-kDa/67-kDa LR on HT1080 and NIH3T3 cells. (a) FACS analysis was carried out using S18, an anti-LRP scFv recognizing the 37-kDa LRP on the cell surface. HT1080 cells displayed higher LRP levels on the surface (63%, thin gray curve) compared to the nontumorigenic cell line NIH3T3 (39%, thick gray curve). Cells were stained with scFv HD37 as a negative control (black curve) as well as with the secondary antibody only to detect background binding (dotted gray line). Data are representative of three independent experiments comprising cells from different cultures. (b) FACS analysis of LRP on permeabilized cells using the anti-LRP scFv S18. HT1080 and NIH3T3 cells displayed nearly similar total LRP levels (black curve: HD37 staining, negative control; thin gray curve: HT1080, 74%; thick gray curve: NIH3T3, 64%). (c) Western blot analysis of HT1080 and NIH3T3 cell lysates revealed similar total LRP levels. The anti-LRP antibody scFv S18 was used for detection of the 37-kDa LRP. The blot is representative of at least three independent analyses including cell lysates from different cell cultures. β-Actin was used as a quantitative loading control.

Anti-LRP/LR antibodies (scFv iS18) generated by chain shuffling from parental scFv S18 and its full-length version IgG1-iS18 significantly reduce the invasive potential of HT1080 cells

We examined the invasion of HT1080 cells in the presence of antibodies directed against LRP/LR. From S18, a previously described single-chain anti-LRP antibody,[25] we generated an improved version termed iS18 by chain shuffling, which revealed an approximately 10-fold lower K_d (5.6×10^{-10} M) compared to the parental S18 ($K_d = 5.1\times10^{-9}$ M)[25] for binding to glutathione *S*-transferase (GST): :LRP. scFv iS18 has been expressed in *Escherichia coli* in high yields (approximately 1 mg/l culture medium) and purified to homogeneity (Fig. 2a). IgG1-iS18 has been expressed in human embryonic kidney (HEK) 293 cells in high yields (5 mg/l culture medium) and also purified to homogeneity (Fig. 2b). Epitope mapping of IgG1-iS18 identified the epitope stretching from amino acid 272 to 280 of LRP (Fig. 2c), demonstrating that the improved IgG1-iS18 recognizes the same epitope as the parental scFv S18.[25] The epitope (amino acids 272–280) does not match with the binding site for laminin-1 to LRP, which stretches from amino acid 161 to 180[19,20] (Fig. 2d).

Preincubation of HT1080 cells with 0.2 μg/μl of scFv iS18 resulted in a significant reduction in the number of invasive cells by 37% ($p = 0.01$) (Fig. 3) compared to HD37, which had an even adverse effect, which, however, is not significant. Both scFv HD37 directed against the human B-cell-surface receptor CD19 and scFv C9 directed against the hepatitis B surface protein preS1 failed to significantly decrease the number of invasive HT1080 cells (Fig. 3). Treatment of cells with full-length IgG1-iS18 revealed a significant reduction in the number of invading cells by 38% ($p = 0.03$) compared to non-treated cells (Fig. 3). We did not observe significant effects on the reduction of invasion by antibody concentrations below 0.2 μg/μl (scFv iS18) and 0.5 μg/μl (IgG1-iS18).

HT1080 cells transfected with recombinant lentiviral plasmids expressing anti-LRP siRNAs reveal reduced LRP/LR levels and display reduced invasive potential

Transfection of HT1080 cells with recombinant lentiviral plasmids pENTR-siRNA-LRP 4, -LRP 7, and -LRP 9 reduced LRP levels in these cells by 53%, 36%, and 41%, respectively (Fig. 4a), concomitant with a significant reduction by 36%, 39%, and 37%, respectively ($p = 0.014$), of the number of invading cells (Fig. 4b). Note that this value is comparable to the invasion of the nontumorigenic cell line NIH3T3. Transfection of HT1080 cells with pENTR-siRNA-lamin A/C revealed no effect on the LRP expression level and its invasive potential (Fig. 4). pENTR-siRNA-lamin A/C-transfected cells show the same invasive behavior compared to nontransfected cells (data not shown).

The heparan mimetic HM2602 and the pentosan polysulfate SP-54 reduce the invasive potential of HT1080 cells

Heparan sulfate proteoglycans (HSPGs) play a critical role in cancer growth and tumor metastasis[28] and act as coreceptors for PrP^c binding to LRP/LR.[6] Since the polysulfated glycans such as the heparan mimetic HM2602 and the pentosan polysulfate SP-54 interfere with the LRP/LR-dependent binding of infectious prions, presumably by blocking LRP/LR,[7]

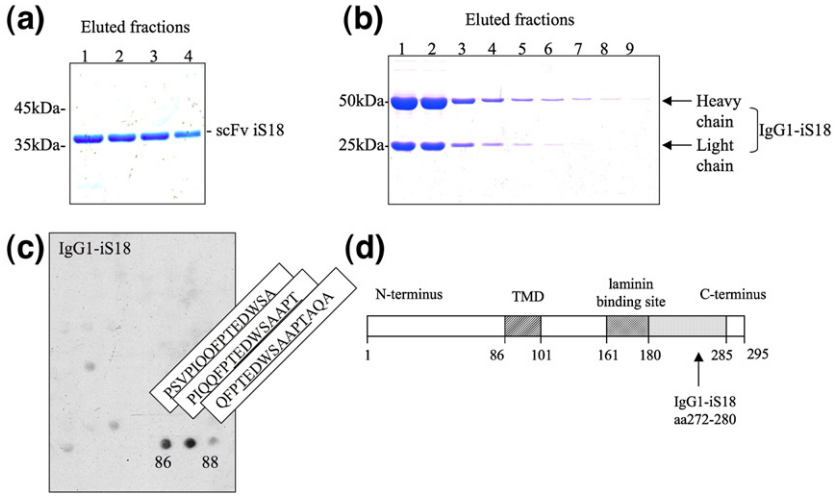

Fig. 2. Overexpression of anti-LRP/LR-specific antibodies. (a) ScFv iS18 overexpressed in *E. coli* RV308 through IPTG induction and subsequently purified via His affinity purification according to the production of scFv S18.[25] Lanes 1–4, eluted fractions after incubation with nickel-chelating resin (Invitrogen) reveal purified scFv iS18 at approximately 35 kDa by Coomassie staining. (b) IgG1-iS18 overexpressed in HEK293 cells (EBNA) and secreted into the medium was purified via protein A Sepharose. Lanes 1–9, eluted fractions from protein A Sepharose reveal IgG1-iS18 (heavy and light chain at approximately 55 and 25 kDa, respectively) by Coomassie staining. (c) Epitope mapping of the recognition of IgG1-iS18 on human LRP. The anti-LRP/LR antibody IgG1-iS18 recognizes the peptide TEDWSAAPT stretching from amino acid 272 to 280 on the human LRP sequence. This correlates with the recognition pattern of scFv S18 on LRP.[25] Dots numbered 86, 87, and 88 indicate the spotted peptide sequence of huLRP (amino acids 263–283). (d) Schematic representation of the human 37-kDa LRP. The laminin binding site is indicated (161–180); the binding epitope of IgG1-iS18 (amino acids 272–280) corresponds to the epitope recognized by scFv S18/scFv iS18. TMD, transmembrane domain.

we investigated whether these carbohydrates might influence the invasive potential of tumorigenic cells. SP-54-treated cells displayed a significant reduction in the number of cells that invaded the matrigel by 35% ($p = 0.0001$) (Fig. 5). We did not observe significant effects on the reduction of invasion by SP-54 concentrations below 0.2 μg/μl. HM2602 reduced the invasive potential of HT1080 cells by 20%. An unspecific carboxymethylated dextran (CMD) had no effect on the invasive potential of HT1080 cells (Fig. 5).

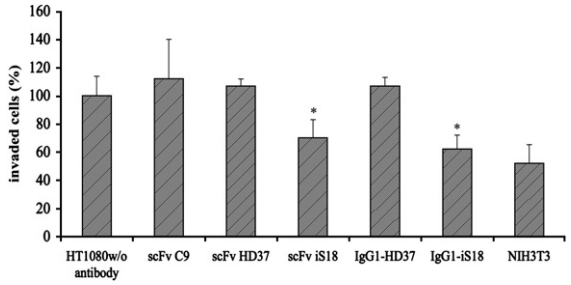

Fig. 3. Effect of anti-LRP single-chain antibodies and IgG antibodies on the invasion of HT1080 cells. HT1080 cells were preincubated with anti-LRP scFv iS18, scFv C9, and, as a negative control, HD37 at concentrations of 0.2 μg/μl or with IgG1-iS18 and IgG1-HD37 (negative control) at concentrations of 0.5 μg/μl prior to application onto the matrigel chamber. Invasive cells were stained with toluidine blue, and after extraction of the dye, absorbance was measured at 620 nm. NIH3T3 cells displayed a less invasive behavior (51%) since they are nontumorigenic. The percentage of untreated HT1080 cells (w/o antibody) that invaded the matrigel was set to 100%. Treatment with scFv iS18 reduced the invasion of HT1080 cells by 37% (*$p = 0.01$) compared to cells treated with scFv HD37. Cells treated with IgG1-iS18 displayed a significantly reduced invasion by 38% (*$p = 0.03$) compared to cells treated without antibody. Experiments were performed in triplicate; data are representative of three independent experiments. One experiment included six wells per experimental series.

HT1080 cell adhesion to laminin-1 is blocked by anti-LRP/LR tools

To elucidate the molecular mechanism by which the anti-LRP/LR reagents interfere with the invasion of HT1080 cells, we analyzed their potential to block the binding of HT1080 cells to laminin-1. Laminin-1-mediated adhesion was significantly blocked after preincubation of HT1080 cells with anti-LRP scFv iS18 by approximately 60% ($p = 0.00001$) and full-length IgG1-iS18 by 68% ($p = 0.001$) (Fig. 6a and c). In addition, pretreatment of cells with SP-54 or the heparan mimetic HM2602 significantly decreased the laminin-1-dependent attachment by 79% and 81% ($p = 0.0002$), respectively, compared to nontreated cells (Fig. 6d). CMD, which had no effect on the invasion of HT1080 cells, did not significantly influence the adhesion of the cells to laminin-1 as well (Fig. 6d).

Anti-LRP/LR antibodies are noncytotoxic on HT1080 and NIH3T3 cells

Cell viability in the presence of anti-LRP antibodies and cell count were assessed by trypan blue

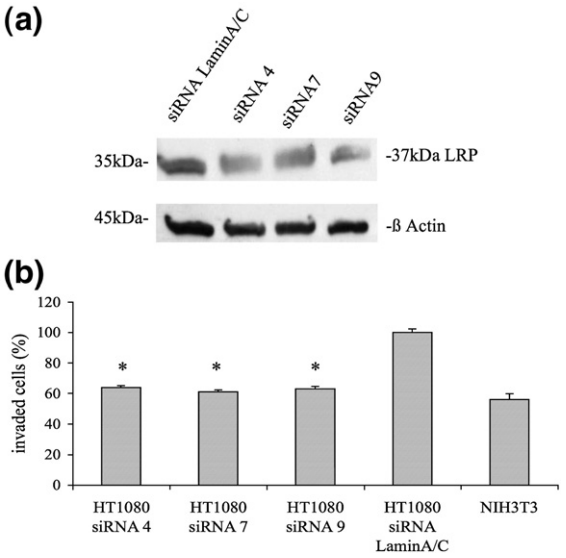

Fig. 4. HT1080 cells transfected with siRNA expressing lentiviral plasmids downregulating LRP display a reduced invasive behavior. (a) Western blot analysis of LRP level in HT1080 cells transfected with siRNA expression vectors pENTR-siRNA-LRP 4, -LRP 7, and -LRP 9, respectively. The 37-kDa LRP was detected 24 h after transfection by anti-LRP antibody S18. Compared to cells transfected with the control vector pENTR lamin A/C (lane 1), cells transfected with pENTR-siRNA-LRP 4, -LRP 7, and -LRP 9 show reduced LRP levels (lanes 2–4) by 53% (siRNA 4), 36% (siRNA 7), and 41% (siRNA 9), respectively. β-Actin was used as a quantitative loading control. (b) HT1080 cells transfected with pENTR-siRNA-LRP 4, -LRP 7, and -LRP 9, respectively, downregulating LRP, exhibit a decreased invasion rate analyzed 24 h after transfection with the corresponding lentiviral plasmids by 36%, 39%, and 37%, respectively (*$p = 0.014$), which is comparable with the less invasive NIH3T3 cells (56%). HT1080 cells transfected with pENTR-siRNA-lamin A/C were set to 100% invasion rate. One experimental series was investigated sixfold.

staining (data not shown), which revealed no toxic effect of the antibodies used up to a concentration of 0.2 μg/μl. After 24 h of incubation time, the antibodies had no effect on proliferation or cell viability, confirming that neither scFv iS18 nor IgG1-iS18 is toxic to HT1080 and NIH3T3 cells.

Discussion

The metastatic potential of the 37-kDa/67-kDa LRP/LR has been recently investigated in various studies, indicating its important role in cancer progression. For dissemination and de novo blood vessel formation, invasive and metastatic cells have to cross basement membranes. Overexpressed in tumorigenic cell lines, the 67-kDa LR acts through the proteolytic cleavage of laminin-1, which is a relevant event in basement membrane degradation[3] and within the migration process. The 67-kDa LR[29] and the 37-kDa LRP[30] mediate laminin-induced cell attachment. An important role for the 37-kDa LRP in tumor progression has been described in the treatment of mice with lung cancer cells displaying a reduced 37-kDa LRP level due to an introduction of antisense LRP/p40 mRNA. Mice subcutaneously inoculated with these cells displayed a prolonged survival,[30] suggesting that the 37-kDa LRP might influence cell adhesion, tumor progression, and the metastatic potential.

In some other cancers, αVβ3, α6β4 and other integrins are clearly overexpressed, and their suppression significantly reduces metastasis, as shown recently for breast cancer metastasis.[31] These integrins may interact, for example, with laminin-5 or with fibronectin and other basement membrane components. As mentioned above, LRP/LR plays an important role in laminin-1 cleavage, resulting in basement membrane degradation[3]; another very important process of extracellular matrix degradation favoring tissue invasion is related to the action of equally overexpressed matrix metalloproteinases (MMPs), which are turned on through integrins. HT1080 cells, used in this article for laminin-1–LRP/LR adhesion studies and invasion assays, overexpress MMP-2 and MMP-9 via an integrin–fibronectin interaction.[32]

In this article, we investigated the effect of tools directed against the 37-kDa LRP in invasion assays and laminin-1-mediated cell adhesion. To investigate whether tumorigenic fibrosarcoma HT1080 cells reveal increased LRP/LR levels compared to the nontumorigenic embryonic fibroblast cell line NIH3T3, we performed Western blot and FACS analyses. HT1080 cells revealed an approximately 1.6-fold higher LRP/LR cell-surface expression level

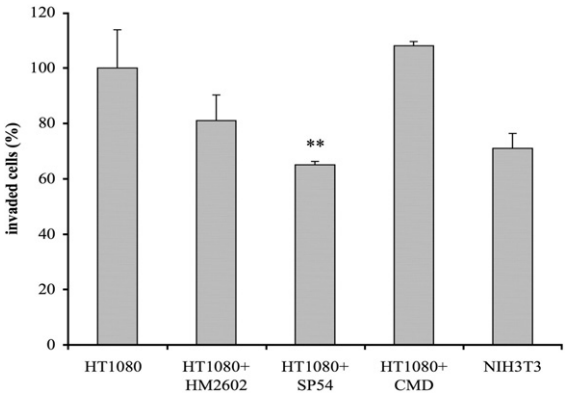

Fig. 5. HT1080 cells treated with the heparan mimetic HM2602 and SP-54 revealed a less invasive potential. HT1080 cells were preincubated with HM2602, SP-54, and CMD as a negative control at concentrations of 0.2 μg/μl. Cells were applied onto a matrigel-filled transwell, and invasion was determined by measurement of toluidine-blue-stained cells at 620 nm. SP-54 reduces the invasion by 35%, significantly (**$p = 0.0001$) comparable to the less invasive NIH3T3 cells. HM2602 decreased also the invasive potential of HT1080 cells by 20%. The percentage of untreated HT1080 cells that invaded the matrigel was set to 100%. One experiment included six wells per experimental series. Three independent experiments were performed.

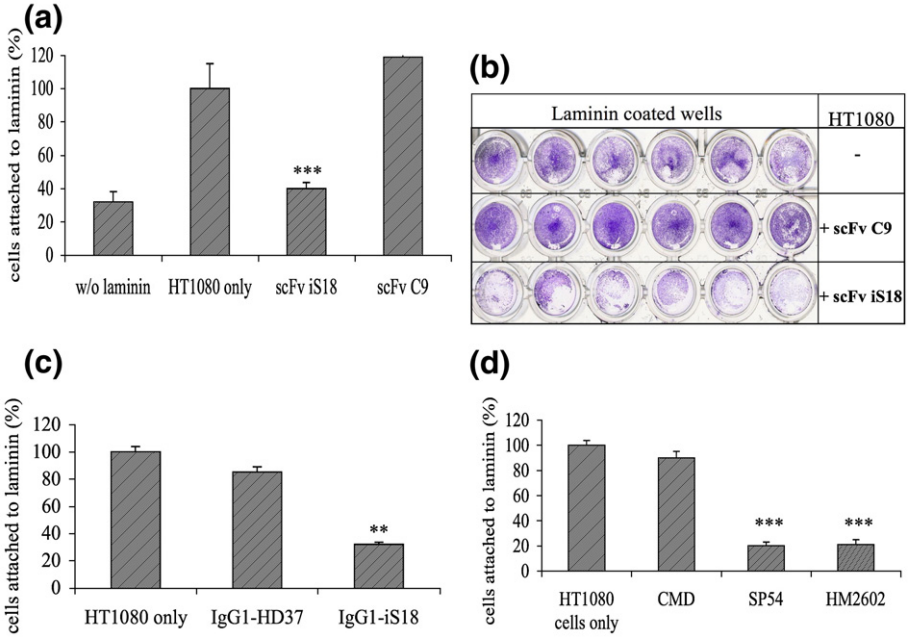

Fig. 6. Cell attachment to laminin-1 is inhibited by anti-LRP/LR tools. (a) HT1080 cells were incubated with anti-LRP scFv iS18 and scFv C9 as a negative control. Cells were applied onto a 96-well plate, either coated with laminin-1 or uncoated (w/o laminin-1), for 1 h at 37 °C. After the attached cells were extensively washed with PBS, they were stained with 0.1% crystal violet and absorbance was measured at 570 nm. Untreated cells (HT1080 only) attached to laminin-1 were set to 100%. Cells attached to the uncoated plate (w/o laminin-1) represent the background binding. Preincubation with scFv iS18 (0.2 μg/μl) resulted in a significantly reduced adhesion of cells to laminin-1 by 60% compared to scFv C9-treated control cells (***$p = 0.00001$). (b) Representative photograph of laminin-attached HT1080 cells stained with 0.1% crystal violet. This image was taken prior to extraction of the color and measurement of the absorbance at 570 nm. The staining correlates with the percentage of laminin-attached cells. Row 1 represents HT1080 cells only; rows 2 and 3 display cells preincubated with scFv C9 and scFv iS18, respectively, before application to the laminin-1-coated well. (c) HT1080 cells were incubated with IgG1-iS18 and IgG1-HD37, respectively (each 0.2 μg/μl), before they were applied to the laminin-1-coated plate. Untreated cells attached to laminin-1 were set to 100%. IgG1-iS18-treated cells revealed a reduced adhesion to the plate by 68% (**$p = 0.001$). (d) HT1080 cells preincubated with 0.2 μg/μl of HM2602 and SP-54, respectively, and subsequently added to the laminin-1-coated well. Attachment of cells is reduced by 79% and 81%, respectively (***$p = 0.0002$). One experiment included 6 wells per experimental series. Three independent experiments were performed.

compared to NIH3T3 cells. Since this nontumorigenic cell line displayed a reduced invasive behavior observed in the applied invasion studies, we suggest a relationship between the lower 37-kDa LRP/LR level on the cell surface and the invasive potential. We want to point out that in all invasion studies performed, the NIH3T3 cells reveal a lower invasive potential compared to the tumorigenic HT1080 cells. Some of our anti-LRP/LR tools (e.g., anti-LRP antibodies scFv iS18 and IgG1-iS18 and pentosan polysulfate) reduced the invasive potential of HT1080 cells even below the invasion rate of the nontumorigenic NIH3T3 cells. The relatively high invasion rate (50–80%) observed in the reported nontumorigenic NIH3T3 cell line might be due to a transformation that has been reported to occur spontaneously in cell culture.[33,34]

Although cell-surface staining by FACS demonstrates a higher LRP/LR level for HT1080 cells, the total LRP/LR content did not differ significantly as analyzed by Western blotting, which might be due to the high intracellular LRP levels localized in the translational machinery[35] and the nucleus.[36] However, since the laminin–LRP/LR interaction takes place on the cell surface, only the cell-surface level of LRP/LR is thought to be crucial for the tumorigenic potential of cells. Although scFv S18 mainly recognizes LRP by Western blotting, FACS analysis employed for cell-surface detection of the receptor cannot reveal which of the LRP/LR isoforms are detected on the cell surface.

Anti-LRP/LR antibodies scFv iS18 and IgG1-iS18 significantly reduced the invasive potential of HT1080 cells by 37% and 38%, respectively, and were shown to significantly inhibit laminin-1-mediated cell attachment by 60% and 68%, respectively, suggesting that the inhibition of LRP/LR–laminin-1 binding on the cell surface contributes to the reduction of the invasion. Recently, we showed that transfection of siRNAs directed against LRP mRNA efficiently downregulates LRP/LR expression, resulting in a reduction of PrP^{Sc} propagation in neuronal cells.[26] In order to investigate whether reduced LRP/LR levels in tumorigenic cells also diminish the invasive potential, we transfected HT1080 cells with recombinant lentiviral plasmids expressing siRNAs directed against LRP mRNA. Indeed, transfection of HT1080 cells with lentiviral

plasmids pENTR-siRNA-LRP 4, -LRP 7, and -LRP 9 expressing siRNAs directed against LRP mRNA resulted in downregulation of the 37-kDa LRP level by approximately 44%, concomitant with a significant reduction by 37% of HT1080 cells invading the matrigel.

Since the downregulation of the invasiveness by pENTR-siRNA-LRP 4, -LRP 7, and -LRP 9 (approximately 37%) is similar to the effect of anti-LRP/LR antibodies scFv iS18 and IgG1-iS18 (reduction by 37% and 38%, respectively) and given the fact that LRP expression is reduced by approximately 44%, these data suggest that transfection may primarily affect cell-surface expression of LRP/LR. Our data suggest that reduction of the LRP/LR levels in tumorigenic cells by an siRNA approach might represent an alternative strategy for reducing the metastatic potential of tumor cells.

HSPGs are sulfated polyanions, either secreted or membrane bound, which consist of a protein moiety to which various sulfated glucosaminoglycans (GAGs) are attached. HSPGs play a critical role in the regulation of tumor progression, invasion, and metastasis[28] and contribute to cell adhesion.[37] Depending on the protein core, GAGs, GAG-associated molecules, or tumor-cell-specific factors may reveal tumor-promoting or -suppressing activities.[38] Therefore, various treatment strategies targeting HSPGs have been developed.[38,39] HSPGs act as coreceptors for PrP^c,[40] and polysulfated glycans such as heparan mimetics and pentosan polysulfate (SP-54) are able to inhibit the LRP-mediated prion uptake, most likely by blocking LRP/LR on the cell surface.[7] Therefore, we tested the influence of polysulfated glycans on the LRP/LR-mediated invasion and the LRP/LR–laminin-1 binding on HT1080 cells. The pentosan polysulfate SP-54 and the heparan mimetic HM2602 significantly reduced the invasion of HT1080 cells by 35% and 20%, respectively. SP-54 and HM2602 significantly decreased the binding of HT1080 cells to laminin-1 by approximately 80%, suggesting that the reduction of the invasive potential of HT1080 cells by these polysulfated glycans is due to the blockage of the laminin-1–LRP/LR interaction on the cell surface. It is also conceivable that anti-LRP/LR-tool-treated HT1080 cells may migrate slower through the matrigel or adhere more slowly to laminin-1. The slightly better effect of SP-54 (81%) on the inhibition of the laminin–HT1080 adhesion compared to HM2602 (78%) can hardly explain the fact that SP-54 reduces HT1080 invasion by 35% compared to the reduction of invasion (20%) by HM2602.

Since it has been reported that laminin interaction with the 67-kDa LR promotes invasion,[24] we analyzed, employing an adhesion assay, whether our anti-LRP/LR reagents, for example, anti-LRP/LR-specific antibodies and polysulfated glycans, block the binding of cells to laminin-1-coated plates. We show that our anti-LRP/LR-specific antibodies scFv iS18 and IgG1-iS18 block the adhesion of HT1080 cells to laminin-1 by 60% and 68%, respectively. The polysulfated glycanes HM2602 and SP-54 interfere with the laminin-1–HT1080 adherence by even 80%. If one would deduce the background binding of HT1080 cells to noncoated plates, which represents 30%, anti-LRP/LR antibodies and polysulfated glycanes would almost completely block laminin-1-specific binding. This correlates with the finding that a higher LRP/LR expression level corresponds to a higher adhesion to laminin.[29] Our data suggest that the reduction of invasiveness of HT1080 cells achieved by our anti-LRP/LR tools might be due to the interference of the laminin-1–LRP/LR interaction on the cell surface. The exact mechanism by which our anti-LRP tools such as antibodies and polysulfated glycans function, for example, influencing the MAPK signaling cascade induced through laminin, sterical blockage of the laminin binding site, or directly occupying the binding site for laminin, has yet to be examined. For cancer therapy, maybe a combination therapy targeting (i) LRP/LR, (ii) the interaction of $\alpha V\beta 3$ or $\alpha 6\beta 4$ with laminin-5 or with fibronectin,[31] and (iii) the integrin–fibronectin interaction leading to overexpression of metalloproteinases[32] might be, at least for some types of cancer, very effective.

The significant reduction in the invasive potential of HT1080 cells due to the blockage/inhibition of the 37-kDa/67-kDa LRP/LR or the downregulation of LRP suggests an alternative strategy in cancer treatment. A previous study confirmed that passive delivery of scFvs into mice revealed no side effects[25] and that a polysulfated glycane (HM PI88[41]) is currently being tested in a clinical phase III trial. (i) Transfer of anti-LRP/LR antibodies by passive immunization, (ii) application of polysulfated glycanes such as SP-54, and (iii) gene delivery of siRNAs directed against LRP mRNA by appropriate gene delivery systems such as lentiviral vectors into tumorigenic animals will demonstrate whether our anti-LRP/LR tools might be potential therapeutics for the treatment of cancer.

Materials and Methods

Cell culture and conditions

Human fibrosarcoma cells (HT1080) and mouse embryonic fibroblast cells (NIH3T3) were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose (4.5 g/l) (Invitrogen Gibco) supplemented with 10% fetal calf serum, nonessential amino acids, and penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

Reagents and antibodies

Matrigel™ originating from the mouse Engelbreth–Holm–Swarm tumor was obtained from Sigma (Munich, Germany). For cell adhesion assays, laminin-1 was purchased from Sigma. The anti-LRP/LR antibody scFv S18 and C9 (scFv directed against the hepatitis B cell-surface protein preS1) were overexpressed in *E. coli* RV308 according to Zuber *et al.*[25]

Generation of scFv iS18

An improved version of the scFv S18,[25] termed scFv iS18, was generated by permutations of residues in the CDR3 of the light chain. An scFv phage display library of 8×10^7 different clones with retained S18 V_H and permutated light chains was cloned. The light chains contained complementarity determining region 3 regions with stretches of four to seven random amino acids [CNSR $(N)_{4-7}$ VLFG]. Four rounds of selection were performed using biotinylated GST: :LRP fusion protein expressed in baculovirus-infected Sf9 cells[42] as a target. To increase the stringency, we reduced by 10-fold the antigen concentration in every panning round, starting with a concentration of 10 nM in the first round. Prior to every selection round, the library was consecutively preadsorbed to polystyrene-coated GST and streptavidin-coated paramagnetic beads (Dynal, Oslo, Norway) in order to remove binders to these proteins. The streptavidin-coated beads were used to capture the biotinylated antigen and bound phage. After extensive washing, bound phage particles were used for infection of *E. coli* XL1-Blue cells without preceding elution. Cells successfully transduced with phagemids encoding scFvs were selected for ampicillin resistance and subsequently infected with M13K07 helper phage to generate phage progeny displaying scFv for the following selection round. After the fourth panning round, individual scFvs were expressed and tested for their binding ability as described.[25] scFv iS18, revealing the highest affinity, was subcloned into an expression vector, expressed, and purified as described for scFv S18.[25] The K_d of iS18 was estimated by ELISA according to Zuber *et al.*[25] as 5.6×10^{-10} M (parental scFv S18: $K_d=5.1\times10^{-9}$ M[25]).

Generation of full-length IgG1-iS18 and IgG1-HD37

The full-length antibody IgG1-iS18 was constructed as follows. The variable domains of the light chain and heavy chain were amplified individually by PCR and cloned separately into two different mammalian expression vectors containing the respective human constant domains. The vectors pEU1.2 and pEU4.2 were kindly provided by Cambridge Antibody Technology,[43] representing an integrated vector expression system for the eukaryotic expression of antibodies or their fragments after selection from phage display libraries.[43] IgG1-iS18 was transiently expressed in HEK293 cells. Antibodies were purified from the supernatant by protein A Sepharose. Approximately 5 mg of IgG1-iS18 was synthesized per 1 l of culture medium.

IgG1-HD37, directed against the human B-cell-surface receptor CD19, was used as a negative control. IgG1-HD37 was expressed in HEK293 cells and purified according to IgG1-iS18. Approximately 8 mg of protein was produced per 1 l of culture medium.

For cell attachment studies and invasion assays, the heparan mimetic HM2602 and pentosan polysulfate (SP-54)[7] were used in concentrations of 0.2 μg/μl (20 μg per 100 μl cell suspension containing 10^4 cells). CMD[7] was used as a negative control.

Epitope mapping

To determine the binding site of IgG1-iS18 to LRP, the entire amino acid sequence of human LRP was spotted onto a membrane and immunodetection with IgG1-iS18 was performed as described for Western blotting. For detection of the bound IgG1-iS18, a horseradish peroxidase (HRP)-coupled secondary anti-human antibody (1:10,000) was used. The membrane encompasses 92 different synthetic peptides with a length of 15 amino acids. The N terminus of each peptide was shifted with respect to the previous peptide by 3 amino acids, leading to an overlap of 12 amino acids. Synthesis was performed on a cellulose membrane (AIMS, Braunschweig) using Fmoc chemistry (PyBop/NMM activation, Trt/tBu/Pbf/Boc side-chain protection) according to the SPOT-synthesis method of Frank[44] using a spotting robot (Syro, MultiSynTech GmbH, Witten, Germany).

FACS analysis

For determination of LRP/LR expression, cells were detached in phosphate-buffered saline (PBS)/1 mM ethylenediaminetetraacetic acid (EDTA) to prevent trypsinization of LRP/LR on the cell surface, fixed with 4% paraformaldehyde, and resuspended in FACS buffer containing 0.01% sodium azide, 20 mM EDTA, and 2% fetal calf serum in 1× PBS. For cell-surface staining of LRP/LR, cells were incubated with the first antibody scFv S18 (dilution 1:50 in FACS buffer) or scFv HD37 as a negative control, on ice, for 1 h followed by three washing steps and additional incubation with a fluorescein-isothiocyanate-coupled anti-c-*myc* antibody (Santa Cruz) (dilution 1:50 in FACS buffer) for 1 h on ice to detect the c-*myc* tag coupled to the scFvs (see Ref. 25). For detection of total LRP/LR, cells were permeabilized in 100% methanol for 20 min at room temperature and treated further as for detection of LRP/LR surface staining. Measurements and evaluation were performed using a Beckman Coulter EPICS flow cytometer and software. Data were collected from three independent experiments comprising cells from different cultures.

Western blotting

For determination of total LRP/LR levels, cells were detached in PBS/1 mM EDTA and resuspended in lysis buffer (10 mM Tris/HCl, pH 7.5, 10 mM EDTA, 100 mM NaCl, 0.5% Nonidet-P40, and 0.5% desoxycholate). Thirty micrograms of total protein was subjected to SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. After the membrane was blocked in 10% horse serum in PBS containing 0.1% Tween for 1 h, it was incubated with S18 as the primary antibody for LRP/LR recognition. After three washing steps in PBS/Tween, the membrane was incubated with the secondary anti-c-*myc* (to detect the c-*myc* tag on the scFvs) and a third anti-mouse-HRP-coupled antibody for 1 h. For quantitative loading control, the membranes were incubated in parallel with an anti-β-actin antibody (1:5000, Sigma) followed by an HRP-coupled anti-mouse antibody (1:10,000, Jackson ImmunoResearch). The membranes were developed by enhanced chemiluminescence reagents (Perkin Elmer Life Sciences).

Transfection of HT1080 cells with recombinant lentiviral plasmids expressing siRNA directed against LRP mRNA

Cells were seeded into six-well plates the day prior to transfection. Transfection with plasmids encoding for

siRNA directed against LRP mRNA pENTR-siRNA-LRP 4, -LRP 7, and -LRP 9 (Ludewigs, H. *et al.*, unpublished data) was done using gene porter reagent 2 (Peqlab) according to the manufacturer's instructions. As a negative control, cells were transfected with pENTR-siRNA-lamin A/C directed against lamin A/C mRNA (control) (Invitrogen).

Invasion assay

This assay, also termed chemoinvasion assay, is used to detect and quantify the capacity of the cells to invade a matrigel-coated transwell chamber. Culture inserts (Nunc, 8 μm pore size) were filled with Matrigel™ diluted in DMEM (Gibco) and set into a 24-well plate filled with serum-containing medium (DMEM). Confluent cells were adjusted to 10^6 cells per 100 μl of cell suspension, resuspended in serum-free media, and applied onto the matrigel-filled transwell chamber. After 18 h incubation, noninvasive cells were scraped off with a cotton swab and invasive cells were fixed and stained with toluidine blue. The dye was solubilized by incubation at 37 °C in a 1% SDS solution for 1 h, and samples were measured at 620 nm using an ELISA reader. The fraction of invasive cells was calculated as follows: % invasion = number of invasive cells × 100/total number of cells. One experiment comprised 6 wells per experimental series. Each experiment was repeated at least in triplicate, which includes different plates containing cells from another culture.

Cell adhesion assay to laminin-1

The adhesion assay was performed on 96-well plates coated with laminin-1 (0.2 μg/μl) (Sigma) at 4 °C overnight. To ensure that cells can only bind to laminin-1, we used plates without precoating (for suspension cell culture). In addition, to detect potential unspecific binding of cells, we used plates without laminin-1 precoating in parallel. Surfaces were blocked with 1% bovine serum albumin in PBS for 1 h at 37 °C. HT1080 cells in serum-free medium were added to wells (10^4 cells/well) followed by incubation for 1 h at 37 °C. To examine the influence of the anti-LRP/LR antibody, we preincubated cells for 10 min with scFv iS18 (0.2 μg/μl) and scFv C9 (0.2 μg/μl, as a negative control) or with IgG1-iS18 (0.5 μg/μl) and IgG1-HD37 (0.5 μg/μl, as a negative control). In case of heparan studies, CMD was used as a control. After the attached cells were extensively washed with PBS, they were fixed in 3.7% formaldehyde and stained with 0.1% crystal violet. The pigment was extracted with 33% acetic acid for 5 min, and the absorbance was measured at 570 nm as an indicator of cells attached to laminin-1. One experiment comprised 6 wells per experimental series. Each experiment was repeated at least in triplicate including different plates and cells from another culture.

Evaluation of *in vitro* cytotoxicity of anti-LRP/LR antibodies and siRNAs directed against LRP mRNA

HT1080 cells were cultured in the presence of anti-LRP antibodies for 2 and 24 h. The maximum concentration of the antibodies used was 0.2 μg/μl (20 μg per 10^4 cells). After the viable cells were stained with trypan blue, they were counted and compared to cells treated without antibody. HT1080 cells transfected with pENTR-siRNA-LRP 4, -LRP 7, and -LRP 9 displayed normal growth behavior at least 1 week after transient transfection.

Data analyses and statistics

Statistical analyses were performed using a Student's *t* test. *p*-Values of less than 0.05 were considered significant. Values were expressed as mean + SD.

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ABBREVIATIONS

1-character code for amino acids

A	alanine
C	cytseine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophane
Y	tyrosine

%	percentage
37 kDa LRP	37 kDa laminin receptor precursor
67 kDa LR	67 kDa laminin receptor
aa	amino acid
AAV	adeno associated virus
approx.	approximately
APP	amyloid precursor protein
BBB	blood brain barrier
BSE	bovine spongiform encephalopathie
Caco-2/TC7	human enterocytes
cDNA	complementary deoxyribonucleic acid
CHO	chinese ovary hamster cells

CJD (sCJD, vCJD, fCJD, iCJD)	Creutzfeldt-Jakob disease (sporadic, variant, familial, iatrogenic form of CJD)
CLD	caveolae-like domains
CNS	central nervous system
C-terminal	carboxyterminal
Cu^{2+}	copper (II) ion
CWD	Chronic wasting disease
DNA	desoxyribonucleic acid
Dpl	doppel
DWM-R	cervid enterocytes
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ER	endoplasmatic reticulum
EU	European Union
EUE	Exotic ungulate encephalopathy
FBJ	fetal bovine enterocytes
Fab	antigen-binding Fragment
Fc	constant region of Ig
FFI	Fatal familial insomnia
FSE	Feline spongiform encephalopathy
GAG	Glycosaminoglycan
GPI	glycosyl-phosphatidyl-inositol
GSS	Gerstmann-Sträussler-Scheinker Syndrome
h	hour
hGH	human growth hormone
hGNH	human gonadotropin
HM	Heparan sulfate mimetic
Hsp	heat shock protein
HSPG	Heparan sulfate proteoglycan
HT1080	human fibrosarcoma cells
Ig	Immunoglobulin
IPEC-J2	duodenal jejunum piglet cells
kb	kilobase
kDa	kilodalton
M	Molar
MCX-7	human breast carcinoma cells
µg	microgram

mg	milligram
ml	milliliter
mRNA	messenger RNA
N2a	mouse neuroblastoma cells
NIH3T3	mouse fibroblast cells
NMR	nuclear magnetic resonance
N-terminal	amino-terminal
PCV	polymerase chain reaction
PK	proteinase K
PRNP	prion protein gene
PrP^{c}	cellular Prion protein
PrP^{Sc}	prion protein scrapie
$PrP^{0/0}$	prion protein knock out
PrP^{res}	protease resistant form
$^{Ctm}PrP/^{Ntm}PrP$	transmembrane forms of the prion protein
PS	pentosan polysulfate
rER	rough endoplasmatic reticulum
RNA	ribonucleic acid
ROS	reactive oxygen species
SAF	scrapie associated fibrils
scFv	single chain fragment variable
ScN2a	scrapie infected mouse neuroblastoma cells
SDS	sodium dodecylsulfate
SDS-PAGE	SDS polyacrylamide electrophoresis
sFI	sporadic familial insomnia
Sho	shadow of the prion protein, shadoo
siRNA	small interfering RNA
SOD	superoxid dismutase
ST-1	stress inducible protein
Tg	transgeneic/transgene
TME	Transmissible mink encephalopathy
TSE	transmissible spongiform encephalopathy
UK	United Kingdom
US/USA	United States of America
V_H	variable heavy chain of Ig
V_L	variable light chain of Ig
VEE	Venezuelan enquine encephalitis virus

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04/2008	Gene Center Annual Retreat, Wildbad Kreuth
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