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Exprese a funkce buněčného prionového proteinu na krevních buňkách

Expression and function of cellular prion protein in blood cells

Disertační práce

Vedoucí disertační práce: Ing. Karel Holada, Ph.D.

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This work is dedicated to the memory of my mother,

Helena Glierová

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CONTENT

ABSTRAKT

Buněčný prionový protein (PrPc) je nezbytný pro patogenezi neurodegenerativních prionových onemocnění. V současné době jsou známy čtyři případy přenosu variantní Creutzfeldt-Jakobovy choroby krevní transfuzí. Objasnění vlastností a množství PrPc na krevních buňkách je předpokladem pro vývoj testu pro detekci prionových chorob v krvi. Průtoková cytometrie představuje potenciální detekční metodu, nicméně výsledky studií zabývajících se množstvím PrPc na lidských krevních buňkách jsou rozporuplné.

V této práci jsme ukázali, že většina PrPc v klidových destičkách se nachází intracelulárně a je přítomna v α-granulích. Lidské destičky a červené krvinky nesou významné množství PrPc a mohou tak hrát roli při přenosu prionů krevní transfuzí. Naše výsledky naznačují, že PrPc na červených krvinkách je modifikován. Podobná modifikace patologického prionového proteinu by mohla ovlivnit detekci prionových chorob v krvi. Dále jsme ukázali, že skladování krve před vlastní analýzou a výběr prionových protilátek výrazně ovlivňuje detekci PrPc metodou průtokové cytometrie, a že destičkový satelitismus ovlivňuje detekci PrPc na krevních buňkách. Prokázali jsme, že část PrPc přítomná na leukocytech *cynomolgus monkey (Macaca fascicularis)* je s buňkami volně asociovaná a uvolní se během izolace buněk. Dále jsme studovali možnou funkci PrPc v erytropoéze. Dokázali jsme, že povrchová exprese PrPc na myších erytroidních prekurzorech v kostní dřeni a slezině je regulována v průběhu zrání buněk. Podobně je regulován PrPc během *in vitro* diferenciace MEL buněk. To naznačuje úlohu PrPc v časných stádiích erytroidní diferenciace. Exprese PrPc navíc vedla k efektivnější erytropoéze po experimentálně vyvolané anémii u inbredních myší.

Výsledky této studie přispívají k objasnění vlastností PrPc a jeho distribuce v krvi. Tyto poznatky jsou důležité pro vývoj testu pro rutinní detekci prionových chorob v krvi.

Klíčová slova:

buněčný prionový protein, průtoková cytometrie, erytroidní diferenciace, fyziologická role

ABSTRACT

The cellular prion protein (PrPc) is essential for pathogenesis of fatal neurodegenerative prion diseases. Recently reported four cases of vCJD transmission by blood transfusion raise concerns about the safety of blood products. Proper understanding of PrPc in blood is necessary for development of currently unavailable blood screening tests for prion diseases. Flow cytometry is an attractive method for prion detection, however, the reports on the quantity of PrPc on human blood cells are contradictory.

We showed that the majority of PrPc in resting platelets is present in the intracellular pool and is localized in α-granules. We demostrated that both, human platelets and red blood cells (RBC) express significant amount of PrPc and thus may play an important role in the transmission of prions by blood transfusion. Our results suggest a unique modification of PrPc on human RBC. Such modification of pathological prion protein could distort the results of blood screening tests for prions. Further we showed that the storage of blood prior to analysis and the choice of anti-prion antibody greatly affect the detection of PrPc by flow cytometry and we identified platelet satellitism as a factor contributing to the heterogeneity of PrPc detection in blood cells. Moreover, we demonstrated existence of washable pool of PrPc on the leukocytes of cynomolgus monkeys. Next we studied the role of PrPc in erythropoiesis. We demonstrated that surface expression of PrPc on mouse erythroid precursors in bone marrow and spleen follows similar pattern during the cells' maturation. PrPc expression in differentiating MEL cells mimics the pattern seen *in vivo* suggesting PrPc importance in early stages of erythroid differentiation. Moreover, PrPc expression accelerated recovery from stress erythropoiesis in inbred mice.

In conclusion, this study contributes to knowledge about the PrPc distribution and function in blood cells which is important for development of blood screening test for prion disease.

Key words:

cellular prion protein, flow cytometry, erythroid differentiation, physiological role

ABBREVIATIONS

ADP	adenosine diphosphate
AHSP	alpha-hemoglobin stabilizing protein
Aβ	amyloid beta
Bax	BCL2- associated X protein
Bcl-2	B-cell lymphoma 2 protein
BFU-E	Burst forming units- erythroid
BM	bone marrow
BMP4	bone morphogenic protein 4
BSE	bovine spongiform encephalopathy
cAMP	cyclic adenosine mono phosphate
CD15	cluster of differentiation 15, carbohydrate adhesion molecule
CD25	cluster of differentiation 25, alpha chain of the IL-2 receptor
CD3	cluster of differentiation 3, T cell co-receptor
CD34	cluster of differentiation 34, cell-cell adhesion factor
CD4	cluster of differentiation 4, T cell co-receptor,
CD41	cluster of differentiation 41, integrin alpha chain 2b,
CD43	cluster of differentiation 43, leukosialin
CD45RA	cluster of differentiation 45, leukocyte common antigen isoform
CD45RO	cluster of differentiation 45, leukocyte common antigen isoform
CD56	cluster of differentiation 56, Neural Cell Adhesion Molecule isoform
CD62	cluster of differentiation 62, P-selectin
CD71	cluster of differentiation 71, transferrin receptor
CD8	cluster of differentiation 8, T cell co-receptor
CDI	conformational dependent immunoassay
CFU-E	colony forming units- erythroid
CMP	common myeloid progenitor
CNS	central nervous system
Con A	concanavalin A
CWD	chronic wasting disease
DC	dendritic cells
DELFIA	dissociation-enhanced fluoroimmunoassay
DNA	deoxyribonucleic acid
EDRF	erythroid differentiation related factor

EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalogram
ELISA	enzyme-linked immunosorbent assay
Epo	erythropoietin
EpoR	erythropoietin receptor
Eraf	erythroid associated factor
FCM	flow cytometry
FDC	follicular dendritic cells
Fe-Tf	transferrin-bound iron
FFI	fatal familial insomnia
GALT	gut associated lymphoid tissue
GPI	glycosylphosphatidylinositol
GSS	Gerstmann-Straussler-Scheinker syndrome
Hba	hemoglobin
HIF	hypoxia inducible factor
HIF-1	hypoxia inducible factor-1
HMBA	hexamethylene bisacetamide
HSC	haematopoietic stem cells
iCJD	iatrogenic CJD
IgG	immunolgobulin G
LR	laminin receptor
LRP	laminin receptor precursor
LT-HSC	long-term hematopoietic stem cells
M	methionin, aminoacid
mAb	monoclonal antibody
MEL	murine erythroleukemia cells
MEP	megakaryocyte-erythroid progenitors
MPP	multipotent progenitors
MRI	magnetic resonance imaging
mRNA	messenger RNA
Mw	molecular weight
N-CAM	neural cell adhesion molecule
NK cells	natural killer cells
PCR	polymerase chain reaction
PIPLC	phosphatidylinositol-specific phospholipase C

PK	proteinase K
PLA	Proximity ligation assay
PLT	platelets
PMCA	protein misfolding cyclic amplification
PPS	pentosan polysulphate
Prnp	cellular prion protein gene
PrPc	cellular prion protein
PrPtse	pathological prion protein
qRT-PCR	quantitative real time PCR
RBC	red blood cells
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RT	room temperature
RT-QUIC	real-time quaking-induced conversion assay
SCF	stem cell factor
SCF-R	stem cell factor receptor
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
SOD	superoxide dismutase
Tf	transferrin
TfR	transferrin receptor
TRAP	thrombin receptor-activating peptide
TSE	transmissible spongiform encephalopathies
V	valin, aminoacid
vCJD	sporadic Creutzfeldt-Jakob disease
vCJD	variant Creutzfeldt-Jakob disease
WB	western blot
WBC	white blood cells, leukocytes
WT	wild type
TME	transmissible mink encephalopathy
FSE	feline spongiform encephalopathy

1. INTRODUCTION

1.1. TSE

Prion diseases, also called Transmissible spongiform encephalopathies (TSE) are progressive and invariably fatal neurodegenerative conditions associated with deposition of pathological prion protein (PrPtse) in the central nervous system. TSE have occurred in a wide range of mammalian species including human (Table 1). Human prion diseases can arise sporadically, be hereditary or be acquired. Sporadic human prion diseases include sporadic Creutzfeldt-Jakob disease (sCJD) which has an unknown cause, and is the most prevalent among the other CJD (85% of cases). Hereditary prion diseases (or genetic CJD) are caused by autosomal dominantly inherited mutations in the Prnp gene encoding for PrPc and include familial CJD, fatal familial insomnia (FFI) and Gerstmann-Straussler-Scheinker syndrome (GSS). Acquired prion diseases account for only 5% of cases of human prion disease. They include kuru which occured following cannibalism among aborigines in Papua New Guinea, iatrogenic CJD (iCJD) caused by medical or surgical treatment, such as transplantation of dura mater (Liscic et al., 1999) or the use of pituitary hormones from CJD infected patients (Caboclo et al., 2002). Nowadays, the most important acquired human prion disease is the new variant form of CJD (vCJD) that was transmitted to humans from bovine spongiform encephalopathy (BSE) affected cattle via meat consumption (Bruce et al., 2002). Importantly, there is a possibility of ongoing person to person spread of vCJD as seen with four cases of transfusion associated vCJD infection to date; the individuals received blood from donors who were later diagnosed with clinical vCJD.

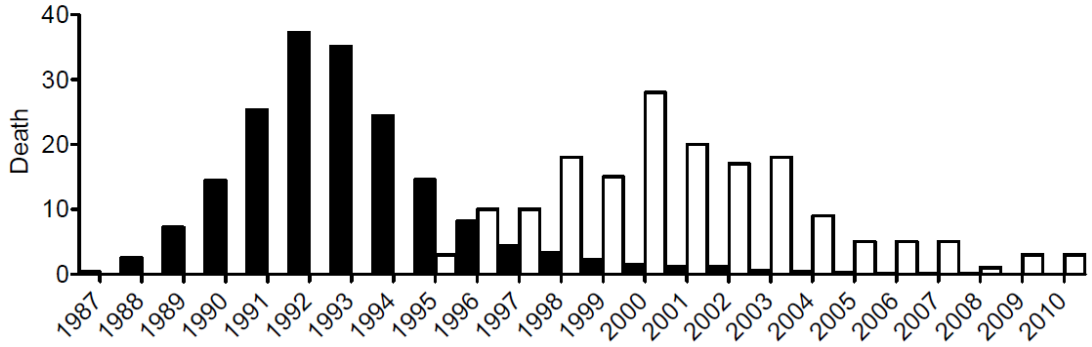
Table 1: Etiology of prion diseases

Disease	Host	Etiology
Kuru	Human	Ritualistic Cannibalism
sCJD	Human	Spontaneous PrPc to PrPSc conversion or somatic mutation
fCJD	Human	Mutations in Prnp gene
GSS	Human	Mutations in Prnp gene
iCJD	Human	Infection with Prions of human origin by cadaveric corneal grafts, h human growth hormone or dura mater
FFI	Human	Human Prnp haplotype 178N-129M
vCJD	Human	Infection with Prions of BSE origin
sFI	Human	Spontaneous PrPc to PrPSc conversion or somatic mutation
VPSPr	Human	Spontaneous PrPc to PrPSc conversion or somatic mutation

Scrapie	Sheep, Goats	Unknown origin
TME	Mink	Prions of either sheep or cattle origin
CWD	Cervids	Unknown origin
BSE	Cattle	Unknown origin
FSE	Cats	Prions of BSE origin

The first case of BSE was reported in 1985 in the United Kingdom, soon evolved to epidemic proportions and peaked in 1992. Subsequently vCJD emerged in 1996 in the United Kingdom (Will et al., 1996). The demographic and clinical features of vCJD and the time of onset correlating with BSE epidemic indicate a cause-effect relationship between the two diseases (Fig. 1). Moreover, experiments on transgenic mice expressing bovine prion protein revealed that the mice were able to propagate both BSE and vCJD prions and mice infected ether with BSE or vCJD had identical symptoms and incubation period, differing from mice propagating scrapie prions (Scott et al., 1999). Beside BSE, other relevant animal TSE are: scrapie, which is known since 1732 and has occurred in sheep, goats and mufflons (Jeffrey and Gonzalez, 2007) and chronic wasting disease (CWD) affecting deers, elk and moose in USA and Canada. Epidemiological and experimental data have provided evidence of efficient horizontal transmission of CWD by contact with affected animals or through environmental exposure (Sigurdson, 2008; Mathiason et al., 2009; Imran and Mahmood, 2011). However, neither scrapie nor CWD have been transmitted to human so far.

Figure 1: Numbers of BSE and vCJD in United Kingdom



The numbers of BSE (■) are reported in thousands, numbers of vCJD (□) are in units. The data are adapted from World Organization for Animal Health (OIE, http://www.oie.int) and The National Creutzfeldt-Jakob Disease Research & Surveillance Unit (NCJDRSU, http://www.cjd.ed.ac.uk). The total number of vCJD cases (since January 1, 1990) in United Kingdom as reffered in May 7, 2012 was 176 comparing to 1329 of sCJD cases recorded during the same period. The number of deaths from definite vCJD, which were neuropathologically confirmed, is 122 and the remaining 54 deaths are from probable vCJD (without neuropathological confirmation). The total number of reported BSE cases in UK is 184 619 as reffered in April 16, 2012.

sCJD and vCJD

All TSE share common traits: spongiform degeneration of brain, accumulation of pathogenic prion protein (PrPtse) in central nervous system (CNS) and lack of systemic immunological response (Prusiner, 1998). However, the two most important human CJD forms, sCJD and vCJD, have certain clinical and pathological differences. Variant CJD affects younger individuals with the median age of onset of 28 (range12-74) years, whereas sCJD usually affects middle-aged and elderly individuals with the average age of 60. However, this difference is not absolute. The duration of vCJD is usually a year or more with the median of 14 month (range 6-40), and the duration of sCJD is typically a few months with the median of 4 months, and, in a few cases, a few weeks. The symptoms of sCJD are clearly neurological with a rapid progression, whereas vCJD often initially presents with psychiatric or behavioural symptoms and it may not be clear that the individual has neurological illness until several months after the onset. Moreover, vCJD also differs from sCJD in the distribution of prions in the tissues as sCJD prions are predominantly found in the CNS whereas vCJD prions are present also in spleen, appendix, tonsils and lymph nodes.

In CJD patients, polymorphism in the Prnp gene at codon 129 encoding either valin (V) or methionin (M) was proved to correlate with age at disease onset and the duration of illness. Heterozygosity M/V appears to associate with a lower risk and/or prolonged incubation time of sCJD (Palmer et al., 1991). To date, all primary vCJD cases have shown M/M homozygosity at the codon 129 of the Prnp gene. However, a case of preclinical secondary vCJD in a patient with M/V heterozygosity at codon 129 who received a blood transfusion from a donor who subsequently developed vCJD was reported (Peden et al., 2004).

1.2. Pathogenesis of TSE

The infectious agent is called prion which comes from proteinaceous infectious particle. Initially it was thought to be a slow virus (Cho, 1976), which was suggested to consist of an agent-specific nucleic acid enveloped in a host specific protein. Nowadays, the modified protein PrPtse (or PrPsc) is considered to be a major, if not the only, component of the infectious agent. PrPtse co-purifies with infectivity in diseased tissues (Bolton et al., 1982), however, the precise nature of the TSE agent is uncertain. Host cellular prion protein (PrPc) is required for TSE pathogenesis, as mice knockouts for PrPc gene are resistant to prion disease (Bueler et al., 1993; Sailer et al., 1994). According to the protein only hypothesis (Prusiner, 1998), an interaction between the pathogenic prion and endogenous PrPc is sufficient to cause the template-driven formation of prions, where the PrPc serves

as a template for PrPtse formation. However, the exact mechanism of PrPc conversion to PrPtse is not known and so is unclear whether a third interaction partner is needed.

Peripheral pathogenesis of TSE

The infectious agent in the case of acquired forms of TSE such as BSE or vCJD enters the body by oral route. The exact mechanism is not known. It has been proposed that after crossing intestinal epithelium, prions are adsorbed through Payers patches and enteric nervous system serves as an entry into the central nervous system (CNS) (Chiocchetti et al., 2008). Prior to neuroinvasion along peripheral nerves, prions are propagated in secondary lymphoid organs. In a murine model, mature follicular dendritic cells (FDC) are required for the replication and accumulation of PrPtse and for neuroinvasion (Brown et al., 1999b). PrPtse was shown to accumulate in FDC in the gut associated lymphoid tissue (GALT) (Mabbott and Bruce, 2001) and within the germinal centres in vCJD (Hill et al., 1999) and sheep scrapie (van Keulen et al., 1996)

The connection between complement receptors on FDC and the spread of prions was suggested, as mice genetically engineered to lack complement factors (Klein et al., 2001) and C3 complement component (Mabbott et al., 2001) exhibited enhanced resistance to peripheral prion inoculation. Recently, it has been demonstrated that PrPc expression only on FDC is sufficient to sustain prion replication in the spleen. Further, prion replication is blocked in the spleen when PrPc expression is specifically ablated only on FDC (McCulloch et al., 2011).

The exact mechanism of neuroinvasion is not clear, and involvement of other cell compartments is suggested. Splenic dendritic cells (DC) from scrapie-infected mice can carry high levels of infectivity and were sufficient for neuroinvasion in RAG-$1^{0/0}$ recipients lacking mature B and T cells (Aucouturier et al., 2001). A possible role of circulating DC in the pathogenesis of prion disease has been suggested, as DC can acquire and transport PrPtse from the gut through lymphatics to lymphoid tissues (Huang et al., 2002). Moreover, bone marrow-derived DC rapidly uptake PrPtse after exposure to infected brain homogenate *in vitro* (Langevin et al., 2011). The significance of B lymphocytes in TSE pathogenesis was shown based on the transgenic mice studies and it was suggested that B lymphocytes may provide signals for the maturation and maintenance of other cell types needed for prion transmission (Kosco-Vilbois et al., 1997). A recent study reported the role of B lymphocytes in the transport of prions to secondary lymphoid organs, including spleen, via the blood and lymph thus mediating the propagation of prions to peripheral tissues (Mok et al., 2012). An alternative route of prions to CNS may involve blood components, however, it remains unclear whether prions cross the blood-brain barrier.

CNS pathology of TSE

It is not clear how PrPtse cause CNS pathology. It is assumed that PrPtse which is present in high amounts in the brains of affected individuals may block axonal transport, interfere with synaptic function, or trigger apoptotic pathways. Another hypothesis presumes that the cause of neurodegeneration in TSE is the loss of PrPc function and/or its decreased availability upon its conversion to PrPtse. Alternatively, interaction with PrPtse may lead to switch of normal neuroprotective function of PrPc which then becomes a transducer of neurotoxic signals (Biasini et al., 2012). Recently it has been suggested that prions are not neurotoxic but catalyse the formation of toxic species from PrPc. Production of such neurotoxic species is triggered when prion propagation saturates (Sandberg et al., 2011).

1.3. Diagnosis of TSE

At present, no diagnostic test exists for the detection of prion diseases in live animals or humans. TSE have been diagnosed post-mortem by their histological hallmarks such as spongiform changes, astrocytic gliosis and amyloid plaques. Due to BSE epidemic, the system of fast and feasible tests for BSE detection in brain samples was validated. The most widely used diagnostic tests are based on the relative protease resistance of PrPtse allowing to discriminate between PrPc and PrPtse, in combination with immunological detection of the proteinase K-resistant part of PrPtse (PrP27–30) by western blot or ELISA using anti-prion antibodies. In these methods, sample preparation is crucial and influences the diagnostic sensitivity and specificity; even slightly incomplete digestion of PrPc can lead to false-positive results. Another aproved method for TSE diagnosis is conformational dependent immunoassay (CDI) based on distinct binding of 3F4 antibody to native and denatured PrPtse. The detection antibody recognizes a conformation-dependent epitope that, while always exposed in the non-infectious PrPc, only becomes exposed in the infectious PrPtse upon denaturation. Thus comparison of signals between native and denatured sample allows detection of PrPtse (Safar et al., 2005). However, none of the above mentioned tests aproved for BSE is validated for diagnosis of human prion diseases.

Human prion diseases are diagnosed clinically and confirmed by post-mortem histopathological analysis of brain tissue (Kordek, 2000; Ingrosso et al., 2002). The clinical diagnosis of sCJD is supported by a variety of clinical tests, including detection of 14-3-3 protein in cerebrospinal fluid, the patterns of electroencephalogram (EEG) and neuro-imaging technologies such as computer tomography and magnetic resonance imaging (MRI). Importantly, EEG and MRI allow distinguish sCJD from vCJD. Moreover, MRI has been recently identified as a robust investigation in sCJD diagnosis (Zerr et al., 2009).

Based on these tests, together with the clinical symptoms, possible or probable sCJD or suspected vCJD may be diagnosed. However, the definite diagnosis of sCJD and vCJD can be made only on brain and tonsil biopsy, respectively. Thus post-mortem neuropathological examination of animal or human brain tissue has remained the 'gold standard' of TSE diagnosis.

1.4. Therapy of human TSE

At present there is no available therapy for human TSE. There are however many potential treatments in development or under consideration. Animal and *in vitro* studies have demonstrated that a number of therapeutic compounds have the potential for interfering with the underlying disease process. To date, various compounds have been tested, including sulphated polyanions such as dextran sulphate (Farquhar and Dickinson, 1986; Kimberlin and Walker, 1986), amphotericin B derivatives (Pocchiari et al., 1987), tetracyclic compounds (Forloni et al., 2002), Congo red (Caughey et al., 1993), tetrapyrroles (Caughey et al., 1998), branched polyamines (Supattapone et al., 1999), and ß-sheet breakers derived from PrP peptides (Soto et al., 2000). Nevertheless, none of them was effective when administered during the clinical phase of disease. The promising compounds are quinacrine and chlorpromazine (Korth et al., 2001; Barret et al., 2003) partly due to their ability to cross blood-brain barrier (Dohgu et al., 2004). Most of these compounds were tested in cell culture models and results differed upon type of cells, however studies *in vivo* did not show any benefit from quinacrine (Collins et al., 2002; Barret et al., 2003). Another promissing therapy represents pentosan polysulphate (PPS). PPS does not cross the blood-brain barrier therefore, intraventricular administration is necessary. PPS has been shown to significantly prolong the incubation period in TSE infected mice (Doh-ura et al., 2004). Although PPS treatment did not significantly improve clinical features in CJD patients (Todd et al., 2005; Whittle et al., 2006), the possibility of extended survival in some patients receiving long-term PPS was suggested (Tsuboi et al., 2009). Further, one study (Otto et al., 2004) reported certain beneficial effects on cognitive function in CJD patients treated with flupirtine (centrally acting non-opioid analgesic) but there is no evidence for increased survival with the treatment.

1.5. Promissing methods for detection of TSE (PrPtse)

Prion diseases are difficult to diagnose using conventional methods such as PCR, serology or cell culture assays because the infectious particle contains no genetic information and

does not provoke any immunological response in the infected organism. Moreover, the only known specific marker of prion disease is PrPtse which accumulates in CNS, however, its levels in peripheral tissues and easily accesible body fluids such as blood or urine are very low. Since emergence of vCJD transmission by blood transfusion, the need for a pre-symptomatic diagnosis of TSE was emphasized. Currently, new diagnostic techniques aimed at increasing sensitivity and specificity of PrPtse detection in body fluids and at identifying novel surrogate markers are under development. Recently, decreased transferrin in cerebrospinal fluid of sCJD patiens has been reported and may be useful for potential pre-mortem diagonstic tets for prions (Singh et al., 2011).

The profound lymphotropism of some prion strains, such as vCJD, natural scrapie and CWD has led to studies using immunohistochemistry for PrPtse detection on lymphoid tissues in the preclinical diagnosis of TSE. PrPtse has been detected in lymphoid tissues as early as 42 days following oral exposure of deer to CWD (Sigurdson et al., 1999). PrPtse is present in tonsils and appendix of vCJD patients (Schreuder et al., 1996; Hill et al., 1997; Hilton et al., 1998) and in peripheral tissue of sCJD patients, such as skeletal muscle, spleen and olfactory epithelium (Glatzel et al., 2003; Zanusso et al., 2003) indicating that a lymphoid tissue biopsy might be useful for diagnosis of pre-symptomatic individuals. Moreover, PrPtse detection in urine was reported in TSE infected animals (Shaked et al., 2001; Haley et al., 2009; Rubenstein et al., 2011) and humans (Dabaghian et al., 2008).

Concerning the accesibility, blood is the most suitable material for screening tests, however, the prions in blood are still poorly characterized. Importantly, decreased TSE infectivity after PK treatment of blood suggesting at least partial sensitivity of blood associated PrPtse to PK digestion has been reported (Yakovleva et al., 2004). Based on that, PrPtse detection using PK treatment is problematic as it may further reduce already low levels of TSE infectivity in blood. Thus new methods for PrPtse detection have been sought and PrPtse conformational specific antibodies are being developed.

PrPtse conformation-specific antibodies could allow PrPtse detection irrespective of PrPc amount present in the sample. First such an antibody, 15B3, has been shown to precipitate bovine, murine, and human PrPtse from brain homogenates, but not PrPc (Korth et al., 1997), however, its utilization failed due to its low afinity. Antibodies directed against the prion protein repeat motif, tyrosine-tyrosine-arginine, recognize PrPtse but not PrPc, on subpopulation of sheep lymph node cells using flow cytometry (Paramithiotis et al., 2003). Recently prepared antibody P1:1 has selectively immunoprecipitated full-length PrPtse from the brains of CJD patients but did not recognize PrPc from normal brains (Jones et al., 2009). Another antibody, V5B2,

recognizes a C-terminally truncated form of PrPtse ending with the residue Y226 (Kosmac et al., 2011). V5B2 binds to PrPtse in CJD brains but not to normal samples or to recombinant prion protein (Curin Serbec et al., 2004). Lately produced monoclonal antibody 6H10 was shown to neutralize prion infectivity by binding to its C-terminus (Horiuchi et al., 2009). Recently, anti-prion mAbs derived from mice immunized with native prion-coated microbeads were shown to specifically detect oligomers/multimers of PrPtse in vitro (Tayebi et al., 2011).

Flow cytometry (FCM) in conjunction with PrPtse-specific antibodies may represent a promissing tool for detection of PrPtse on blood cells, especially if PrPtse is concentrated on a specific cell population to the level allowing its detection. Unfortunately, none of the PrPtse specific mAbs is readily available so the FCM detection of PrPtse in blood has not been fully exploited. The FCM studies till now generally focused on mapping the differences in PrPc expression in infected subjects. In sCJD patients, a significantly lower binding of antibody 3F4 to PrPc on lymphocytes has been reported compared to control patients with other neurological diseases; however, no difference has been found on platelets (Ratzka et al., 2003). On the other hand, in BSE-infected macaques, the expression of PrPc on blood lymphocytes increases long before the onset of clinical signs. However, this increase was detectable with mAb 12F10 but not with mAb 3F4 (Holznagel et al., 2010). Increased binding of mAbs to the N-terminal and C-terminal regions of PrPc and decreased binding to the central part of the molecule was reported in sheep experimentally infected with scrapie (Thackray et al., 2005). Despite these encouraging reports, flow cytometry still seems far from being developed into robust blood test for prion diseases.

Protein misfolding cyclic amplification (PMCA) is one of the promising methods that could overcome the problem of very low quantity of PrPtse in the sample (Saborio et al., 2001; Soto et al., 2002). PMCA is *in vitro* method based on the conversion of large amounts of PrPc into protease-resistant PrPtse-like molecules upon contact with undetectable quantities of PrPtse in tested material. Resulting PrPtse aggregates are sonicated to generate multiple smaller units, which serve as a seeds for formation of further PrPtse. After numerous cycles, PrPtse is amplified to detectable levels and might be efficiently detected by western blot (Saa et al., 2005). PMCA has been first used to detect PrPtse in hamster blood showing 6,600-fold increase in sensitivity over standard detection methods (Castilla et al., 2005). PMCA has allowed pre-symptomatic detection of PrPtse in hamster brains (Soto et al., 2005). Furthermore, blood platelets were shown to serve as a substrate for amplification of vCJD prions demonstrating that platelet PrPc can be

transformed to pathological prion (Jones et al., 2007). Later, prions were detected in blood of TSE infected sheep (Thorne and Terry, 2008) and mice (Tattum et al., 2010). However, PMCA is not evaluated for clinical use and has not yet been optimized for PrPtse detection in human blood. Moreover, it has been shown that PrPtse can be generated de novo and stochastically by PMCA in the absence of pre-existing prions which raises the concerns about the specificity of this method (Deleault et al., 2007).

Proximity ligation assay (PLA) is another potential method for detection of prion disease. In this method, coordinated and proximal binding of a target protein by two DNA aptamers promotes ligation of oligonucleotides linked to each aptamer affinity probe. The ligation of the two proximity probes gives rise to an amplifiable DNA sequence that reflects the identity and amount of the target protein (Fredriksson et al., 2002). The advantages of PLA are high sensitivity and specificity without washes or separations. However, optimalization for CJD has not yet been reached.

Quantitative real-time quaking-induced conversion assay (RT-QUIC) has been recently developed. It is a new PrPtse amplification assay which involves prion-seeded conversion of the alpha helix-rich form of bacterially expressed recombinant PrPc to a beta sheet-rich amyloid fibrillar form followed with thioflavin T fluorescent readout allowing quantification of PrPtse. This approach allows the detection of sub-femtogram amounts of PrPtse in diluted CJD brain homogenates in animal models (Wilham et al., 2010). A non-quantitative RT-QUIC has been reported to detect PrPtse in cerebrospinal fluid of sCJD patients with sensitivity over 80% s and 100% specificity. These findings indicate the promising enhanced diagnostic capacity of RT-QUIC in the ante-mortem evaluation of suspected CJD (Atarashi et al., 2011).

Recently, a **solid-state binding matrix** to capture and concentrate disease-associated prion proteins was developed and coupled to direct immunodetection of surface-bound material. The authors analyzed a masked panel of 190 whole blood samples from 21 patients with vCJD, 27 with sCJD, 42 with other neurological diseases, and 100 normal controls. From this panel, they positively identified 15 samples, all from patients with vCJD showing 71% sensitivity and 100% specificity of vCJD detection in blood (Edgeworth et al., 2011).

1.6. PrPc and PrPtse

PrPc is glycosylphosphatidylinositol (GPI) anchored membrane glycoprotein (Stahl et al., 1987) encoded by Prnp gene located on chromosome 20 in humans. Post-translational modifications of PrPc take place in the endoplasmic reticulum and Golgi apparatus and include cleavage of N-terminal 1-23 signal sequence of 22 aminoacids, N-glycosylation at

positions N181 and N197, dissulfide bond between C179 and C214 (Rudd et al., 2001; Ermonval et al., 2003) and exchange of C-terminal sequence at the position S231 for GPI anchor. Resulting PrPc of molecule mass (Mw) of 30-35 kDa contains 208 aminoacids (Fig. 2). Mw of PrPc varies due to the type of oligosaccharide bound in positions N181 and N197. So far, more than 50 different oligosacharide structures linked to PrPc were detected. Three major glyco-forms of PrPc mono-, di-, and unglycosylated form are recognized. N-terminus is flexible formed structure, whereas C-terminus forms stable globular structure containing three α-helices. Heterogeneity of PrPc and the presence of different glyco- and truncated forms may contribute to existence of various PrPtse strains with different organotropism and incubation time (Pan et al., 2002).

Secondary structure of PrPc is 40% α-helical, less then 10% is in β-sheet conformation. Change in secondary structure of PrPc with increase in β-sheet structure to approximately 40% leads to formation of PrPtse, the pathogenic conformer (Riek et al., 1996; Zahn et al., 2000). Due to conformational change, PrPtse becomes more resistant to digestion by proteolytic enzymes, such as Proteinase K (PK). The cell localization of the conversion is unknown, since PrPc cycles between the cell surface and early endocytic compartment (Shyng et al., 1993) in association with clathrin-coated pits (Shyng et al., 1994) or migrates to late endosomes and lysosomes via caveolae-containing endocytic structures (Vey et al., 1996; Peters et al., 2003). In the cells depleted of clathrin by RNA interference the PrPc endocytosis was shown to depended neither on clathrin nor on caveolin-pathway suggesting that PrPc might be internalized via multiple pathways (Kang et al., 2009).

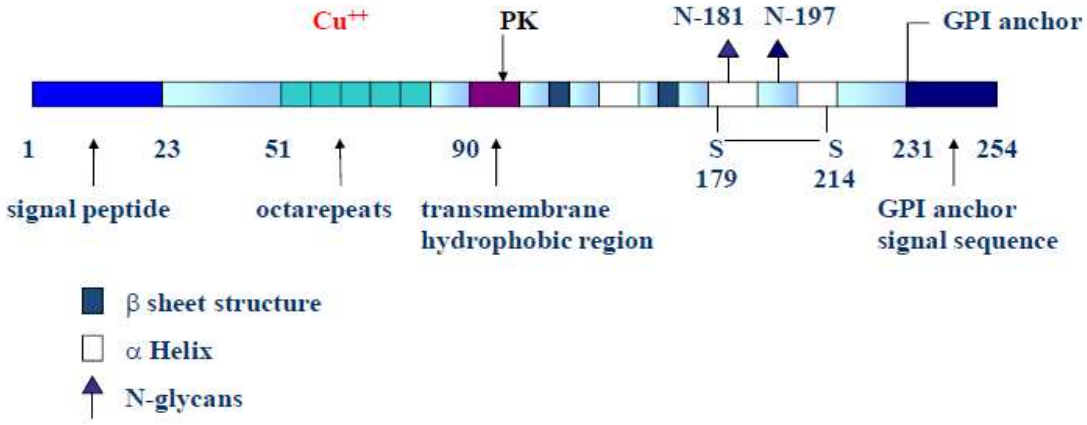


Figure 2: **Schematic structure of PrPc.** Structure of PrPc before post-translational modifications including cleavage of N-terminal 1-23 signal sequence, exchange of C-terminal 231- 254 signal peptide for GPI-anchor, N-glycosylation at positions N181 and N197 and a disulfide bond between the positions C179 and C214. Final product has Mw of 30-35 kDa slightly varying due to the heterogeneity of oligosaccharides in positions N181 and N197. Stable globular C-terminus contains three α-helices, whereas N-terminus is flexibly formed structure.

1.7. Physiological function of PrPc

Although PrPc is highly conserved protein found in all vertebrates and expressed by numerous cell types including blood cells, its physiological function remains unclear. The highest PrPc expression was found in neuronal tissue, nevertheless, PrPc is expressed by many other cells and tissues, such as follicular dendritic cells, circulating dendritic cells, monocytes, haematopoietic progenitors, red blood cells, megakaryocytes, endothelial cells, etc. (Ford et al., 2002). Mice with deleted Prnp gene (Prnp-/-) obviously do not suffer from lack of PrPc (Bueler et al., 1992; Manson et al., 1994). In contrary, Prnp-/- mice are resistant to TSE infection and do not propagate PrPtse (Bueler et al., 1993). PrPc seems to be important for CNS (Collinge et al., 1994) as the phenotype of Prnp-/- mice includes modified circadian rhythm and sleep disturbance (Tobler et al., 1996), impaired motor coordination (Nazor et al., 2007) and cognitive deficits (Criado et al., 2005).

As the Prnp-/- mice have not elucidated the role of PrPc, possible PrPc functions mostly result from *in vitro* studies and are usually derived from physiological functions of molecules interacting with PrPc (Nicolas et al., 2009).

Copper binding activity

The presence of a copper binding site in the N-terminal octapeptide repeat region of PrPc led to assumption that PrPc is a copper binding protein (Hornshaw et al., 1995; Brown et al., 1997). It was also proposed that PrPc may participate in regulation of copper release at synapses (Herms et al., 1999). The role of PrPc in copper uptake was suggested due to correlation between PrPc expression levels and the copper content in cells and tissues observed in mice (Brown, 2003). However, in an earlier study there was no correlation between PrPc level and the amount of copper in brains of mice (Waggoner et al., 2000). Recently, elevated levels of PrPc in rat neuron-like cells upon copper deficiency leading to enhanced copper uptake by the cells were reported suggesting PrPc role in the cell adaptation to copper deficiency (Urso et al., 2012).

Protection against copper mediated oxidative stress

Several studies suggested PrPc may function in the cellular resistance to oxidative stress.

PrPc was shown to have a superoxide dismutase (SOD) activity and to be capable to eliminate reactive oxygen species (ROS) so it may protect against the oxidative stress (Brown et al., 1997; Brown et al., 1999a; Milhavet and Lehmann, 2002; Rachidi et al., 2003; Brown, 2004) or it may just regulate SOD activity (Waggoner et al., 2000). However, the SOD activity of PrPc was not confirmed *in vivo* (Hutter et al., 2003). Another study reported that octapeptide repeat region and N-terminal part of hydrophobic region of PrPc mediate activation of superoxide dismutase and anti-apoptotic function. A direct role for the N-terminal part of PrPc

in protecting cells from ROS induced by serum deprivation has been recently proposed (Haigh et al., 2009).

PrPc in signaling cascades

PrPc may take a part in cell signaling due to its localization within lipid rafts. Those are microdomains in cytoplasmic membrane which are known to serve as a molecular scaffold for signal transduction. PrPc may be involved in monocyte signaling, as PrPc stimulation led to tyrosine phosphorylation of RK1,2 and Akt kinase (Krebs et al., 2006). Moreover, PrPc coupling with tyrosine kinase Fyn was reported in neuroectodermal cell line 1C11 (Mouillet-Richard et al., 2000). Stress-inducible protein 1, a cytoplasmic co-chaperone molecule, binds PrPc *in vitro* and mediates neuroprotective signals through cAMP/protein-kinase dependent pathway which protects cells from apoptosis (Zanata et al., 2002).

PrPc in cell adhesion

PrPc was described to bind to Laminin receptor precursor (LRP)/Laminin receptor (LR) (Rieger et al., 1997; Gauczynski et al., 2001). Laminin is required for cell growth, differentiation, and migration. PrPc blockade by antibodies resulted in abolishment of adherence of pheochromocytoma PC-12 cells to laminin and inhibition of laminin-mediated differentiation (Graner et al., 2000). Other study suggested PrPc role in synaptic transport (Collinge et al., 1994). PrPc interacts with neural cell adhesion molekule (N-CAM) (Schmitt-Ulms et al., 2001) which upon PrPc binding is recruited into membrane lipid rafts, producing activation of Fyn kinase and mediating cell adhesion and neurit outgrowth (Santuccione et al., 2005). PrPc importance for axonal growth is also suggested by its interaction with vitronectin (Hajj et al., 2007). PrP-1 isoform of PrPc in zebrafish was shown to modulate cell adhesion by influencing delivery of E-cadherin to the plasma membrane during early embryogenesis (Malaga-Trillo et al., 2009). Recent studies showed that PrPc plays a crucial role in regulating self-renewal and differentiation of embryonic stem cells (Miranda et al., 2011; Peralta et al., 2011) and that treatment with normal prion protein delays differentiation and helps to maintain high proliferative activity in human embryonic stem cells (Lee and Baskakov, 2010).

Antiapoptotic function of PrPc

The anti-apoptotic activity of PrPc has been suggested due to the similarity of its octarepeat domain to a BH2 domain of Bcl-2 protein which inhibits apoptosis through interaction with cytosolic pro-apoptotic protein Bax. It was demonstrated that cells derived from $PrP^{-/-}$ mice were more sensitive to serum withdrawal than cells derived from $PrP^{+/+}$ mice (Kuwahara et al., 1999). Since then, several studies reported PrPc role in preventing apoptosis *in vitro* (Roucou et al., 2004). Moreover, PrPc inhibited apoptosis after viral

infection *in vivo* (Nasu-Nishimura et al., 2008). Interestingly, PrP-/- mice are more sensitive to various stress conditions which cause increased neuronal cell death (McLennan et al., 2004; Weise et al., 2004; Weise et al., 2006; Rangel et al., 2007). However, transfection with *Prnp* transgene did not alter levels of Bax and Bcl-2 in neuronal cell lines (Kim et al., 2004). Recently, neuroprotective function of PrPc in a mouse model of amyotrophic lateral sclerosis, likely by influencing factors involved in antioxidative defense of neuronal cells, has been reported (Steinacker et al., 2010).

PrPc in Alzheimer`s disease

Considerable evidence indicates that oligomeric assemblies of Aβ are the key toxic species in Alzheimer`s disease and that they cause neurodegenerative changes by delivering a toxic signal at synapses (Walsh and Selkoe, 2007). It has been recently suggested that PrPc is a high-affinity cell-surface receptor for Aβ oligomers on neurons and mediates their synaptotoxic effects thus playing a role in the pathophysiological process of Alzheimer`s disease (Lauren et al., 2009).

1.8. PrPc role in erythropoiesis

Down-regulated gene expression of erythroid differentiation-related factor (EDRF, also known as alpha-haemoglobin stabilising protein, AHSP) during prion disease (Miele et al., 2001) suggested first link between prions and erythropoiesis. AHSP is specific for erythroid cells and its physiological function is binding of α-globin subunits thus preventing their precipitation. Free α-globin subunits are toxic for the cell and mice lacking AHSP suffer from reticulocytosis and exhibit abnormal morfology of erythrocytes (Kihm et al., 2002; Feng et al., 2004).

PrPc is expressed on human CD34+ hematopoietic stem cells (HSC) and its expression is strongly downregulated during HSC differentiation to the granulocyte lineage, whereas in lymphoid and monocytic lineage is maintained (Dodelet and Cashman, 1998; Li et al., 2001). Further, PrPc seems to be important for self-renewal of long-term repopulating HSC as HSC from bone marrow of Prnp-/- mice are not able to expand after transplantation to irradiated mice. Interestingly, 40% of bone marrow cells in adult mice harbor PrPc, from these more than 80% are erythroid cells (Zhang et al., 2006).

A connection between PrPc and stress erythropoiesis was demonstrated in FVB Prnp-/- mice after experimental induction of hemolytic anemia. Treatment with phenylhydrazine led to a similar decrease in hematocrit in FVB Prnp-/- and WT mice, however, the recovery of Prnp-/- mice was lacking behind the WT mice and correlated with lower levels of reticulocytes and reduced plasma levels of erythropoietin and its message in kidneys.

Interestingly, the number of Ter119+ erythroid precursors was similar in Prnp-/- and WT mice, and enhanced apoptosis of erythroid cells was detected in Prnp-/- mice. (Zivny et al., 2008). The regulation of PrPc expression during the differentiation of cultured human erythroblasts indicates the role of PrPc in the differentiation of erythroid precursors (Griffiths et al., 2007).

Recently it has been demonstrated that PrPc mediates iron uptake and transport in human neuroblastoma cells (Singh et al., 2009c) and is necessary for iron homeostasis (Singh et al., 2009a). FVB Prnp-/- mice show relative iron deficiency with decrease in hemoglobin, serum iron and ferritin (Singh et al., 2009b). In addition, PrPc was shown to colocalize with the transferrin receptor in human cultured erythroblasts (Griffiths et al., 2007). Developing red blood cells require high amount of iron thus PrPc may affect erythropoiesis. However, the nature of PrPc involvement in erythropoiesis is not clarified.

1.9. Erythropoiesis and its regulation

Erythropoiesis is a complex process involving differentiation of hematopoietic stem cells (HSC) to mature erythrocytes with the aim to maintain the steady stade level of red blood cells (RBC). Erythropoiesis in adult humans takes place in bone marrow, whereas in mice also in the spleen, especially the stress erythropoiesis which is a response to the tissue hypoxia (Paulson et al., 2011). Erythropoiesis begins from selfrenewing and multipotent HSC from which all blood cell lineages arise. HSC give rise to multipotent progenitor (MPP) cells, which lose selfrenewal capacity and develop into common myeloid progenitor (CMP) followed by megakaryocyte-erythroid progenitors (MEP). After MEP, the first erythroid lineage-committed cells - early erythroid precursors BFU-E (Burst-Forming Units-Erythroid) - may be observed (Iscove et al., 1974). Subsequently, late erythroid precursors CFU-E (Colony-Forming Units- Erythroid) are formed (Stephenson et al., 1971). BFU-E produces approximately 500 erythrocytes during 6-10 days *in vitro* and CFU-E produces a colony of 8-32 erythrocytes in 2-3 days. In the next phase, erythroblasts mature and go through several morphologically distinctive erythroid precursor stages: proerythroblasts (ProE), basophilic erythroblasts (BasoE), polychromatophilic erythroblasts (PolyE) and orthochromatic erythroblasts (OrthoE) (Fig.3). This phase is characterized by hemoglobin accumulation, erythroblasts expansion, reduction in cell size and chromatin condensation eventually leading to loss of nucleus. For erythroid maturation, the cell-cell interaction between macrophage and erythroblasts creating a stromal microenvironment called “erythroblast island” is essential. Macrophages supply the erythroblasts with iron and bring individual erythroblasts to physical proximity so they

can interact with each other. Finally macrophages engulf the nuclei extruded from erythroblasts (Chasis and Mohandas, 2008). The last phase of erythroid maturation involves reticulocytes release to circulation and maturation. Human reticulocytes mature into RBC within 24-48 hours after their entry to circulation. During the reticulocyte maturation, the proteosynthesis is aborted and internal organelles are degraded by autophagy. Cytoskeleton rearrangement leads to a characteristic biconcave discoid shape of the erythrocytes. RBC in circulation survive approximately 120 days and after that are recognized and engulfed by spleen macrophages (Ingley et al., 2004). Basal erythropoiesis in healthy individuals at the sea level equals the loss of RBC due to senescence which represents 1% of all RBC per day. As a result of stress due to tissue hypoxia, erythropoiesis may increase 10 folds (Socolovsky, 2007).

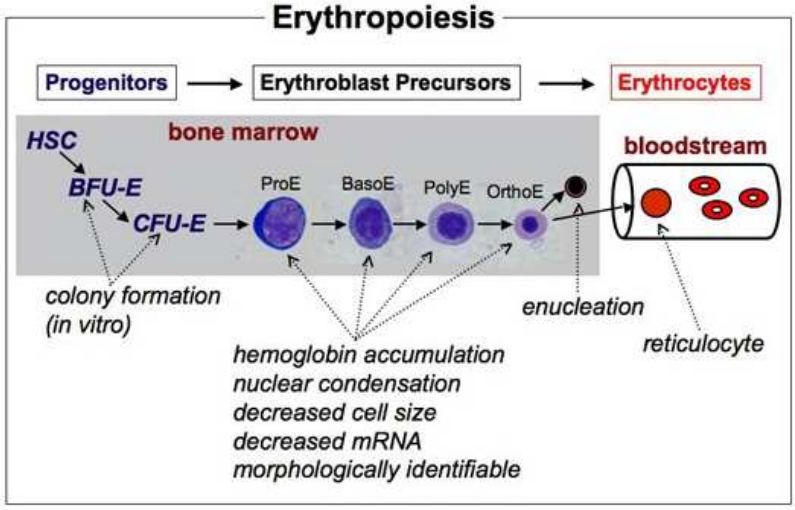


Figure3: **Human erythropoiesis.**The maturation of erythroid lineage involves formation of committed progenitors (BFU-E, CFU-E) from HSC in bone marrow, maturation of erythroblast precursors (ProE, BasoE, PolyE, OrthoE) with characteristic morphological changes and release of reticulocytes to bloodstream and maturation to RBC.

Regulation of erythropoiesis

The main erythroid growth factor is erythropoietin (Epo), a 30-34 kDa glycoprotein, and its receptor (EpoR) is expressed on the surface of erythroid progenitors (D'Andrea et al., 1989). The importance of Epo and EpoR is documented by the fact that Epo-/- or EpoR-/- mice die during the embryogenesis (Wu et al., 1995). In adult mammals, Epo is primarily produced in kidney peritubular interstitial fibroblasts and its production is controlled by oxygen tension in tissues through the transcription factor HIF-1 (hypoxia-inducible factor-1). Severe tissue hypoxia may lead to 1000-fold increase in Epo level (Ebert and Bunn, 1999). High erythropoietin levels together with other factors, such as stem-cell factor (SCF) (Broudy et al., 1996), glucocorticoids (von Lindern et al., 1999) and (Bauer et

al., 1999) and bone morphogenic protein 4 (BMP4) (Lenox et al., 2005) stimulate increased erythropoiesis. Interestingly, Epo is not required for BFU-E development *in vivo*. Epo probably supports growth, survival and differentiation of the cells which have already entered erythroid differentiation. This is supported by EpoR which is during normal physiological development first expressed on late BFU-E and becomes indispensable on CFU-E and proerythroblasts (Wu et al., 1995). Epo function during erythroid differentiation is probably restricted to proerythroblasts and basophilic erythroblasts while late erythroblasts are Epo independent (Socolovsky, 2007). EpoR activation leads to signal transduction through EpoR/Jak2/Stat5 signal pathway (Jelkmann, 2004).

Stem cell factor (SCF) and its receptor (SCF-R), also known as c-kit, are necessary for early hematopoietic cells and high levels of c-kit are maintained till BFU-E and CFU-E. It is important for regulation of proliferation, survival and promoting immature state. During erythroid differentiation, expression of c-kit gradually decreases and it disappears in polychromatophilic and orthochromatic erythroblasts (Tamir et al., 2000; Ronnstrand, 2004; Zhang and Lodish, 2008). Thus erythroid proliferation is dependant on Epo and SCF, whereas erythroid differentiation requires only Epo.

Erythropoiesis is tightly connected to iron metabolism and cellular heme. The main source of iron for erythroid precursors is transferrin-bound iron (Fe-Tf) in plasma. Low iron supply leads to limited erythropoiesis and anemia. Heme biosynthesis is substantially increased during hypoxia and stress erythropoiesis. Heme regulates transcription factors for globin and non-globin genes. HIF influences iron transport by regulation of transferrin and its receptor. Moreover, it increases iron absorption from intestine and mobilization of recycled iron from macrophages.

1.10. MEL cells as a model of erythroid differentiation

A suitable *in vitro* model for study of erythroid differentiation represents murine erythroleukemia cells (MEL). This cell line is blocked at the proliferative proerythroblast stage and the cells are able to re-enter differentiation upon treatment with polar substances, such as hexamethylene bisacetamide (HMBA) (Fibach et al., 1977; Marks et al., 1989). The differentiation of MEL cells in culture resembles the *in vivo* process, with similar maturation stages: proerythroblast-like, erythroblasts-like and, occasionally, reticulocyte-like (Friend et al., 1971; Hyman et al., 2001). Interestingly, transcriptional activation of the *Prnp* gene has been demonstrated during inducer-mediated differentiation of MEL cells (Gougoumas et al., 2001).

1.11. PrPc in blood

In human blood, PrPc has been found in plasma as well as on blood cells. Plasma PrPc is likely to arise from PrPc on blood cell and from endothelial cells, since they were shown to release PrPc positive particles upon apoptosis (Simak et al., 2002; Starke et al., 2002).

Although PrPc expression on human blood cells has been studied by several groups, considerably different levels of PrPc have been reported. Moreover, comparative analyses of PrPc expression on blood cells of various animal species revealed that patterns of PrPc expression differ from that seen in human cells (Holada and Vostal, 2000; MacGregor and Drummond, 2001; Barclay et al., 2002). Even in species relatively close to humans, such as human and non-human primates, significant differences in PrPc levels on platelets, RBC and leukocytes were observed (Holada et al., 2007). RBC and platelets of cynomolgus monkeys and lemurs lack PrPc while chimpanzees, rhesus macaques and squirrel monkeys express high levels of PrPc on RBC. Moreover, mice which are the most common model for prion diseases, do not express any detectable PrPc on platelets (Holada and Vostal, 2000). Thus care should be taken when extrapolating results from animal studies to humans.

1.12. PLT PrPc

A considerable amount of cell-associated PrPc in human blood resides in platelets (Holada et al., 1998; Barclay et al., 1999; MacGregor and Drummond, 2001; Starke et al., 2002), making these a possible target for binding of intravenously introduced prions. Interestingly, similarly to PrPtse (Stahl et al., 1987), PLT PrPc is resistant to phosphatidylinositol-specific phospholipase C (PIPLC), an enzyme able to cleave GPI-anchored proteins.

The expression of PrPc increases with differentiation and polyploidization in the megakaryocyte lineage and PrPc appears to be evenly distributed throughout the megakaryocyte cytoplasm (Starke et al., 2005). Resting human platelets express approximately 600 to 800 molecules of PrPc per cell as detected with mAb 6H4 (Holada and Vostal, 2000; Panigaj et al., 2010). PrPc has been found on the platelet membrane and in intracellular compartments. Activation of human platelets leads to a more than doubling of PrPc molecules on the platelet membrane (Holada et al., 1998; Barclay et al., 1999). This might be of physiological importance as activation dependent up- or down-regulation of other platelet glycoproteins have important physiological functions. For example, surface expression of α-granule P-selectin mediates platelet-leukocyte interactions (Abrams and Shattil, 1991). Platelets contain number of organelles including different

secretory granules. The most abundant and the largest are α- granules which contain a variety of adhesive molecules, coagulation factors and growth factors (such as Platelet-derived growth factor, Platelet factor 4, Thrombospondin, Fibrinogen, fibronectin etc.) The membrane of α-granules contains a number of adhesive receptors which are expressed on the platelet surface after activation (Wencel-Drake et al., 1986), such as P-selectin (CD62).

The correlation of PrPc and P-selectin surface up-regulation after platelet activation led to a hypothesis, that PrPc is associated with platelet granules (Holada et al., 1998). PrPc in α-granules was first detected using immunoelectron microscopy (Starke et al., 2005). In a recent study, PrPc association with platelet lipid rafts linked to the platelet cytoskeleton was revealed. Moreover, the amount of PrPc associated with the cytoskeleton significantly increased after platelet activation (Brouckova and Holada, 2009). Platelet activation also leads to release of surface PrPc on both microvesicles and exosomes (Robertson et al., 2006). Thus PrPc released from platelets may be source of plasma PrPc, as exosomes may persist in blood plasma (Caby et al., 2005). Interestingly, based on the *in vitro* study in cell lines, exosomes were proposed to take part in a cell-to-cell transport of PrPtse (Fevrier et al., 2004). It is not yet known whether the exosomes present in plasma and expressing PrPc might have a similar function *in vivo*.

1.13. RBC PrPc

Although numerous studies on PrPc distribution in human blood were performed, the results on PrPc levels expressed by red blood cells (RBC) significantly differ. Most of the published studies reported that majority of the cellular PrPc in blood resides in platelets (PLT) and white blood cells (WBC) whereas RBC levels of PrPc have been reported as negligible (Barclay et al., 1999; MacGregor et al., 1999). Absence of PrPc expression on RBC was reported using flow cytometry with antibody 3F4 (Dodelet and Cashman, 1998), or 3F4 and FH11 (Barclay et al., 1999). No PrPc expression was reported also in flow cytometry studies utilizing several different PrPc antibodies (8G8, 3B5, 8H4) (Antoine et al., 2000; Li et al., 2001). Time-resolved dissociation-enhanced fluoroimmunoassay (DELFIA) with antibodies FH11 as a capture antibody and 3F4 as a detecting antibody estimated that 84% of PrPc associate with PLT, 10% with WBC and only 6% with RBC (MacGregor et al., 1999). This observation corresponds with the results reported by the above mentioned flow cytometry studies. In contrast to these negative results Holada et al. demonstrated that human RBC express low, but significant level of PrPc which make up as much as 50% of blood cell associated PrPc (Holada and Vostal, 2000). In later study, Barclay et al. utilized panel of 12 monoclonal antibodies to compare the expression of PrPc

on blood cells by flow cytometry (Barclay et al., 2002). PrPc on human RBC was consistently detected only with three antibodies (6H4, 4F2 and 8G8), suggesting that the choice of antibody is critical for PrPc detection on RBC.

1.14. WBC PrPc

Several authors lately discussed possible role of PrPc in immune system (Mabbott and Turner, 2005; Isaacs et al., 2006; Hu et al., 2007; Aguzzi et al., 2008), however, its exact role is unknown. Immune system seems to be important for pathogenesis of prion disease although the PrPtse does not elicit a clear immune response. In contrary, the immune system actually contributes to TSE pathogenesis by amplifying prions in lymphoid tissue thus facilitating efficient prion neuroinvasion (Aguzzi, 2003). This process is dependent at least partly on PrPc expression by the cells of the immune system (Brown et al., 1999b).

PrPc has been detected on human T and B lymphocytes, natural killer (NK) cells, monocytes, dendritic cells (DC) and follicular dendritic cells (FDC) (Dodelet and Cashman, 1998; Barclay et al., 1999; Antoine et al., 2000; Durig et al., 2000; Politopoulou et al., 2000; Burthem et al., 2001; Li et al., 2001; Thielen et al., 2001; Isaacs et al., 2008). In contrary, granulocytes express little to no PrPc (Cashman et al., 1990; Barclay et al., 1999).

PrPc is regulated during ontogenesis of the immune system cells. Human CD34+ hematopoietic stem cells (HSC) express PrPc which is down-regulated upon CD15+ granulocyte differentiation (Dodelet and Cashman, 1998). Similarly, CD43+ Gr-1+ granulocyte precursors in murine bone marrow express PrPc but mature neutrophils do not (Liu et al., 2001). In contrary, maturation of human lymphocytes, monocytes and DC leads to PrPc up-regulation (Durig et al., 2000; Burthem et al., 2001; Li et al., 2001; Ballerini et al., 2006) and the PrPc levels on lymphocytes are further increasing with ageing (Politopoulou et al., 2000). On the other hand, studies in mice have shown down-regulation of PrPc with B and T lymphocytes maturation (Kubosaki et al., 2001; Liu et al., 2001). In humans, PrPc expression is higher in peripheral blood T lymphocytes than in B lymphocytes, and CD8+ cells express more PrPc than CD4+ cells (Durig et al., 2000; Politopoulou et al., 2000). PrPc expression is also higher in CD45RO+ memory compared to CD45RA+ naive T lymphocytes (Li et al., 2001). Moreover, PrPc expression increases during human NK cell differentiation with particularly high levels on CD56+ CD3+ NK T cells (Durig et al., 2000). PrPc levels are increased in murine CD4+ CD25+ regulatory T lymphocytes compared to CD4+ CD25- cells. However, Prnp-/- mice do not show

altered regulatory T lymphocyte numbers or impairment of their function *in vitro* (Isaacs et al., 2008). Interestingly, PrPc up-regulation during mitogenic activation of human T cells isolated from peripheral blood has been reported. Moreover, PrPc crosslinking by specific antibodies regulates mitogenic activation of these cells (Cashman et al., 1990). In murine splenic lymphocytes, PrPc expression is significantly enhanced following *in vitro* stimulation with the non-specific T-cell mitogen concanavalin A (Con A). Moreover, Con A-induced proliferation of lymphocytes from genetically engineered mice with an inactive PrPc gene was significantly reduced to ~50-80% that of wild-type mice suggesting that PrPc is a lymphocyte surface molecule participating in T-cell activation (Mabbott et al., 1997). In contrast to T cells, treatment with lipopolysaccharide does not increase PrPc expression on B cells (Kubosaki et al., 2003).

FDC express high levels of PrPc, nevertheless the role of PrPc on FDC is not clear (Brown et al., 1999b). Importantly, PrPc expression only in FDC is sufficient to sustain prion replication in the spleen (McCulloch et al., 2011). Thus FDC expressing PrPc are essential for the replication and accumulation of PrPtse in lymphoid tissue.

PrPc role in macrophages was suggested by *in vitro* observation that macrophages from PrPc-/- mice had higher rates of phagocytosis than those from PrPc+/+ mice. This finding was confirmed by *in vivo* study showing more efficient phagocytosis of zymosan particles by macrophages in knockout mice than in wild-type mice (de Almeida et al., 2005). However, later study reported decreased phagocytic activity in PrPc-/- macrophage cell lines (Uraki et al., 2010).

Large interspecies variation in PrPc levels expressed by peripheral blood leukocytes further complicate the speculations about physiologic function of PrPc in these cells (Holada and Vostal, 2000). Thus, modelling the function of PrPc in murine lymphoid cells may underestimate the effects of its disruption in humans.

1.15. Prions in blood

Presence of TSE infectivity in blood of vCJD patiens was proved by four cases of transmission of the disease by blood transfusion (Llewelyn et al., 2004; Peden et al., 2004). The patients were transfused with nonleukoreduced red blood cells from healthy donors who later developed vCJD. Recently, vCJD PrPtse was found in a spleen of UK hemophilia patient who died from reasons not related to TSE. The patient had been treated with factor VIII produced from pooled plasma to which a donor who subsequently died from vCJD had contributed (Peden et al., 2010). While the annual number of vCJD victims remains low, the number of asymptomatic infected individuals may be significantly higher due to a long

preclinical phase of the disease. These subclinical cases of CJD may represent a serious threat of iatrogenic transmission of vCJD, in regard to a broad organotropism of vCJD.

As vCJD patients have got significant depostition of PrPtse in lymphoreticular tissues and leukocytes are known to recirculate between lymphoreticular tissues and blood, these cells can probably carry the prions and harbor the TSE infectivity. The distribution of infectivity in blood has been studied on various animal models. In BSE infected primates, buffy coat rich in leukocytes effectively transmitted prions by intracerebral inoculation (Bons et al., 2002). After intracerebral inoculation of vCJD brain homogenate into healthy mice, most of the infectivity was present in buffy coat and plasma, lower levels were in platelets and no infectivity was found in RBC (Cervenakova et al., 2003). Another study reported that blood platelets from hamsters in terminal stage of scrapie contain only low infectivity dose and are not efficient in transmission of the disease after intracerebral inoculation to healthy hamster whereas mononuclear cells contain higher infectivity dose in accordance with the studies on buffy coat infectivity (Holada et al., 2002). Nevertheless, rodents differ from humans in PrPc expression on blood cells (Holada and Vostal, 2000). Since prion distribution may depend on PrPc expression the infectivity results from rodents may not be fully applicable to humans. A recent study reported that blood platelets and B cells from chronic wasting disease (CWD) sufferring deer harbor infectivity and are able to transmit the disease to healthy deer and mice (Mathiason et al., 2010). Recently, prion infectivity was detected in circulating B lymphocytes, peripheral blood mononuclear cells, platelet-rich plasma and platelets of sheep infected with scrapie (Dassanayake et al., 2011; Lacroux et al., 2012). Importantly, all blood components of blood from BSE infected animals in pre-clinical phase of the disease were able to cause the disease following a single transfusion in sheep (McCutcheon et al., 2011). Furthemore, transmission of scrapie by whole blood from infected goats was reported recently (Dassanayake et al., 2012). PrPtse was previously detected in the blood of scrapie-infected hamsters during the presymptomatic phase of the disease (Saa et al., 2006) and on peripheral blood mononuclear cells from scrapie- and BSE-infected sheep (Terry et al., 2009). Subpopulation of B cells in scrapie-infected sheep is likely to sequester PrPtse during both the preclinical and clinical phase of the disease (Edwards et al., 2010).

2. AIMS OF THE STUDY

The overall objective of this work was to enhance the understanding of the cellular prion protein (PrPc) in blood cells and its possible involvement in TSE pathogenesis regarding PrPc amount and localization in blood cells and its physiological function. The knowledge of the PrPc levels expresed by blood cells and its distribution in blood is essential for a development of non-invasive detection methods for prion disease which are currently not available for general use.

The work comprises two main topics which encompass particular aims:

1. Analysis of PrPc levels on blood cells and its localization

   - Assessment of the intracellular pool of PrPc in human platelets and analysis of its localization.
   - Characterization of PrPc on human red blood cells and revelation of the reason for disproportions in published data concerning quantity of PrPc associated with human red blood cells.
   - Clarification of the origin of the discrepancies in PrPc levels on human peripheral blood leukocytes reported in the literature.
   - Verification of the PrPc levels expressed on leukocytes of cynomolgus monkeys used as a model of human prion disease.

2. Study of PrPc expression and function in erythroid differentiation

   - Analysis of PrPc expression regulation on murine erythroid precursors *in vivo* and *in vitro.*
   - Description of the effect of PrPc silencing on erythroid differentiation *in vitro* in model system of MEL cells.
   - Analysis of PrPc relevance for normal and stress-induced erythropoiesis in mice.

3. MATERIAL AND METHODS

A full description is presented in published articles enclosed as Appendices.

Isolation of platelets by gel filtration

Simultaneous isolation of platelets and red blood cell ghosts from whole blood

Isolation of mouse bone marrow and spleen erythroid precursors

Isolation of lymphocytes and monocytes from peripheral blood using Ficoll

Biotin-labeling of mouse RBC *in vivo*

Platelet activation (TRAP)

Proteinase K protection assay for quantification of platelet PrPc intracellular pool

SDS-PAGE and western blot of platelet and red blood cell PrPc

Densitometry

ELISA

Flow cytometry (BD FACS Canto II and BD FACScan flow cytometers):

- Flow cytometry (FCM) of resting and activated platelets
- FCM of transferrin receptor-positive (CD71+) erythroid cells in cord blood
- Flow cytometry of reticulocytes and biotinilated RBC

Quantitative flow cytometry (qFCM):

- Four-color qFCM of PrPc levels on human leukocytes
- Three-color qFCM of PrPc/PrPtse levels on leukocytes of cynomolgus monkeys
- Five-color qFCM of murine bone marrow and spleen erythroid precursors
- Three-color qFCM of MEL cell cultures

Quantification of PrPc molecules expressed per cell using Quantum RP-MESF beads

qRT-PCR (performed by Martin Panigaj)

RNAi (performed by Martin Panigaj)

Cell cultures (performed by Martin Panigaj)

Laboratory mouse handling and procedures – *i.p.* application of phenylhydrazine, *i.v.* application of biotin, blood collection (cardial puncture, venipuncture), organ isolation

Statistics - performed in SigmaStat v3.5 and GraphPad v5.03 software using paired and unpaired student's t-test, respectively, or two-way anova followed with Bonferoni test

4. RESULTS

A full description of results including figures is presented in published articles enclosed as Appendices.

4.1. PrPc levels on blood cells and its localization

Intracellular localization of PrPc in platelets

First we focused on the study of platelet PrPc. Several studies assessed the PrPc levels on resting and activated platelets, however, none of these studies addressed the size of the platelet intracellular PrPc pool and its localization within platelets. Therefore we aimed our initial studies at elucidation of these unknowns.

In the proteinase K protection assay applied to resting human platelets we demonstrated that the majority of PrPc (69%) is localized in intracellular pool. Platelet activation led to translocation of majority of intracellular PrPc onto cell membrane. Moreover, using flow cytometry to follow the up-regulation of the α-granular marker P-selectin and PrPc on platelets from patiens with defect either in α-granules (Hermansky-Pudlak syndrome) or dense granules (grey platelet syndrome) we demonstrated that PrPc within platelets is localized in α-granules. Further evaluation of the exact PrPc localization within platelets and its characterization is subject of another PhD thesis.

Characterization of PrPc on human red blood cells

During our studies we noticed different PrPc levels detected on human platelets and RBC with various prion monoclonal antibodies using flow cytometry. From our previous studies with monoclonal antibody (mAb) 6H4 we knew that RBC express low levels of PrPc, however, no PrPc on RBC was generally reported in literature. Most of the negative studies used traditional mAb 3F4.

To clarify the discrepancies in the PrPc expression on human RBC we performed the quantitative flow cytometric study with both, mAb 3F4 and 6H4 which demonstrated that RBC expressed around 300 molecules of PrPc per cell, assuming equimolar binding of mAb 6H4 to PrPc. Antibody 3F4 bound to RBC significantly less than 6H4. In contrast, the binding of 3F4 and 6H4 to PLT in identical samples was roughly equivalent and we detected around 600 molecules of PrPc per cell. Taking into account the intracellular pool

of PLT PrPc (Holada et al., 2006), one PLT express approximately 6 times more PrPc than one RBC.

The weak binding of mAb 3F4 to RBC PrPc was not caused by PrPc conformation, truncation, or glycosylation. This suggests a covalent modification, likely glycation, of the 3F4 epitope. This modification is apparently introduced after maturation and release of RBC to circulation as CD71+ (transferrin receptor positive) erythroid precursors in cord blood bound antibodies 3F4 and 6H4 equally well as we demonstrated by flow cytometry. The biochemical characterization of PrPc expressed on RBC is subject of another PhD thesis.

Using western blot, we estimated the level of PrPc per one RBC to be four to six times lower than in one PLT. Due to high RBC count ($5x10^9$/ml), which is about 20-fold higher than PLT count ($2.5x10^8$/ml), human RBC carry at least twice as much PrPc than PLT.

PrPc expression on human leukocytes

Although PrPc levels on leukocytes were demonstrated to be altered during prion disease in both, humans and animals, the use of PrPc in diagnosis is limited as the reports on PrPc levels on leukocytes considerably differ, propably due to use of various mAbs for PrPc detection and differences in blood processing preceding the analyses. To evaluate the impact of these variables, we have analyzed the PrPc levels on leukocyte subsets with two different prion antibodies in fresh blood and the effect of different storage conditions on PrPc detection.

The mAb AG4 (PrP 31-51), which recognizes the N-terminal part of PrPc, generally detected lower levels than the C-terminal-recognizing mAb AH6 (PrP 159-175) and the PrPc levels were cell population-specific. The highest numbers of PrPc molecules were detected on NK cells followed by T cells, monocytes, and DC, whereas B cells expressed low levels of PrPc. The pattern of PrPc expression was not affected by the choice of anticoagulant with exception of monocytes on which we detected significantly decreased levels of PrPc in fresh citrate anticoagulated blood. Blood storage preceding analysis led to a cell type-specific decrease in PrPc detection on most leukocytes. The storage temperature itself had moderate effects that reached significance only for DC and monocytes, in which decreased levels of PrPc were observed following storage at RT.

An important finding of our study was the identification of platelet satellitism. $CD41^{pos}$ (PLT-associated) monocytes and granulocytes in fresh blood exhibited significantly increased levels of PrPc compared with $CD41^{neg}$ cells. The storage of blood usually led to a further increase of PrPc levels on $CD41^{pos}$ cells but with large individual differences

among donors. Interestingly, we detected significantly increased levels of PrPc on $CD41^{pos}$ lymphocytes in which the existence of platelet satellitism is currently disregarded. Our results indicate that the increased PrPc levels detected on monocytes and granulocytes following storage were at least in part due to platelet-expressed PrPc.

PrPc expression on leukocytes of cynomolgus monkeys

Cynomolgus monkeys have been used as a model for study of human prion diseases; partly due to similar levels of PrPc expressed on WBC of both human and cynomolgus monkeys. In contrary to previous studies reporting only negligible PrPc levels on granulocytes (Barclay et al., 1999; Holada and Vostal, 2000; Holada et al., 2007), high levels of PrPc on granulocytes of cynomolgus monkeys have been recently reported (Holznagel et al., 2010). To explain these discrepancies, we have undertaken a study to assess the levels of PrPc on WBC of cynomolgus monkeys. We confirmed previously detected negligible levels of PrPc on granulocytes using three different prion antibodies (AG4, AH6, and 3F4). Importantly, we identified presence of washable pool of PrPc on lymphocytes as demostrated by decreased binding of prion antibodies 3F4 and AG4 after Ficoll separation of the cells. The nature of this pool, the manner in which it associates with the cells, and its influence on masking or creating differences in studies of PrPc expression between prion infected and healthy subjects are unknown and require further elucidation.

4.2. PrPc expression and function in erythroid differentiation

PrPc expression during erythropoiesis in vivo and in vitro

Human and mouse RBCs express comparable amount of PrPc molecules per cell, suggesting a similar regulation of its expression in the erythroid lineage in both species. We studied PrPc expression during differentiation of erythroid cells both *in vivo* and *in vitro*. We demonstrated that the surface expression of PrPc on erythroid precursors in the mouse bone marrow and spleen is regulated during the cell maturation and follows a similar pattern. The highest PrPc levels (~16000 molecules per cell) were detected on basophilic erythroblasts and decreased with the maturation of the cells.The most mature small erythroid precursors expressed only around 500 PrPc molecules per cell.

MEL cells were used as a model of erythroid differentiation *in vitro*, because their differentiation in culture resembles the erythroid differentiation *in vivo* and involves similar maturation stages: proerythroblast-like, erythroblast-like and, occasionally, reticulocyte-like. In our study, the uninduced MEL cells expressed approximately six times

more PrPc than proerythroblasts in the mouse bone marrow or spleen. After the induction of differentiation with HMBA, PrPc levels on MEL cells followed the pattern seen *in vivo*.
To learn more about the importance of PrPc in the process of MEL cells' differentiation, the influence of PrPc inhibition by RNAi (performed by Martin Panigaj) was evaluated.
The efficient (> 80%) inhibition of the protein's expression at both the mRNA and protein levels was maintained throughout the MEL cells' differentiation. The downregulation of PrPc did not affect cell growth, viability, hemoglobin production, nor the transcription of selected differentiation markers.

PrPc relevance for normal and stress erythropoiesis in mice

To explore the involvement of PrPc in erythropoiesis, we studied challenged erythropoiesis after induction of acute anemia in mice with manipulated levels of PrPc as well as physiologic erythropoiesis. Induction of anemia in inbred mice resulted in lower hematocrit and higher levels of erythropoietin in plasma of Prnp-/- mice. Reintroduction of Prnp gene in Tga20 mice rescued the animals from severe anemia. WT mice exhibited enhanced turnover of red blood cells in circulation under physiologic conditions compared to Prnp-/- mice. In more stringent models of outbred mice, the effect of PrPc on recovery from acute anemia was less pronounced or even it appeared to be compensated. The physiologic erythropoiesis in outbred mice was not significantly affected by PrPc expression. Nevertheless, we observed slightly lower relative numbers of erythroid precursors in the bone marrow of outbred Prnp-/- mice while in the spleen they were slightly elevated in comparison to Prnp+/+ mice. Our results suggest PrPc role in stress erythropoiesis.

5. DISCUSSION

Since PrPc serves as a template for PrPtse propagation during pathogenesis of prion disease, the knowledge of PrPc distribution on blood cells and its physiological function is important for the development of both diagnostic test and effective prevention of the disease transmission by blood transfusion. However, the levels of PrPc expressed by individual cells reported in literature significantly vary rendering the assessment of the possible role of blood cells in propagation of PrPtse difficult. Moreover, the nature of PrPc expressed by blood cells is still not well characterized and questions regarding its physiological function and involvement in prion disease remain. This work contributes to current knowledge about PrPc and its possible physiologic role.

5.1. PrPc levels on blood cells and its localization

Intracellular localization of PrPc in platelets

We demonstrated that the majority of PrPc in resting platelets is present in the intracellular pool. Platelet activation led to translocation of most but not all of intracellular PrPc on the cell membrane. This is in agreement with results of the previous study revealing the incomplete translocation of PrPc from the organelle fraction to the membrane fraction in activated platelets (Holada et al., 1998). Moreover, our results indicate that intracellular PrPc is localized in platelet α-granules. Similar intracellular distribution of platelet PrPc, determined by immunoelectron microscopy, was reported in other studies (Starke et al., 2005; Robertson et al., 2006). Importantly, a recent study has demonstrated that platelets from deer suffering from chronic wasting disease (CWD), which similarly to human platelets express PrPc, are able to transmit the disease (Mathiason et al., 2010). Hypothetically, PrPtse could be uptaken by platelets and delivered to α-granules where it could propagate without being detected in blood. Thus our results provide background for further studies on the role of platelet PrPc in the transmission of prion diseases by blood transfusion.

Characterization of PrPc on human red blood cells

Next we investigated the discrepancies in PrPc levels on human red blood cells (RBC) reported in the literature (Dodelet and Cashman, 1998; Barclay et al., 1999; MacGregor et al., 1999; Antoine et al., 2000; Li et al., 2001). Most of the studies reporting no PrPc on RBC employed the prion monoclonal antibody (mAb) 3F4 (Kascsak et al., 1987)

whereas study using mAb 6H4 reported low PrPc levels on RBC (Holada and Vostal, 2000). Our results suggest that the decreased binding of 3F4 to RBC PrPc is caused by posttranslational modification of its epitope by glycation which takes place after release of RBC to circulation. Glycation of other membrane proteins in human RBC during aging in circulation was already reported (Ando et al., 1999). Importantly, glycation of lysine residues on the N-terminal part of PrPtse was demonstrated in brains of TSE infected rodents (Choi et al., 2004). The hypothetical existence of similar modification in human blood PrPtse would prevent its detection by Conformation Dependent Immunoassay, based on mAb 3F4, (Safar et al., 1998), which is already approved for BSE detection. In order to ensure a direct proof of RBC PrPc glycation, we are developing mAb against the modified structures in PrPc (Dvorakova et al., 2011), as discussed elsewhere. Glycation of proteins is believed to take part in pathogenesis of many diseases such as diabetes, atherosclerosis and neurodegenerative disorders including Alzheimer`s disease (Vicente Miranda and Outeiro, 2010). Modification of PrPc on RBC may result in unique properties of the molecule affecting, and possibly enhancing, its interaction with PrPtse present in blood.
Moreover, our results indicate that the use of mAb 3F4 led to underestimation of the quantity of PrPc associated with human RBC in most previous studies. We demonstrate that the surface PrPc levels on PLT are twice as high as in RBC, in accordance with a previous study. The overall quantity of PrPc associated with RBC is at least two times higher than in PLT which makes RBC the main source of cell associated PrPc in human blood. Despite this observation RBC are unlikely source of prions in blood as they are not able to support prion propagation by synthesis of new molecules of PrPc.

PrPc expression on human leukocytes

To clarify the impact of preanalytical variables on the detection of PrPc on human WBC we analyzed the PrPc levels on WBC upon storage of blood under different conditions. We demonstrated that the choice of the antibody greatly affects the detected levels of PrPc on blood cells as the N-terminus-recognizing mAb generally detected lower levels than the C-terminus-recognizing mAb in agreement with our previous study on blood cells of non-human primates (Holada et al., 2007). This may be explained by hypersensitivity of N-terminal part of PrPc to proteolytic digestion (Li et al., 2001) or by reduced accessibility of the PrPc epitope (Leclerc et al., 2003), or its modification which we have already demonstrated for RBC PrPc (Panigaj et al., 2010). The distribution of PrPc among the leukocyte subpopulations corresponded to previous reports (Barclay et al., 1999; Durig et al., 2000). We demonstrated that preanalytical variables such as blood storage, the type

of anticoagulant and storage temperature may significantly affect the levels of PrPc detected on blood leukocytes and may contribute to the heterogeneity of results obtained by flow cytometry. Since the choice of antibody significantly affects the PrPc detection, preliminary studies to assess the suitable mAb for PrPc detection on target cell population should be performed and the impact of preanalytical variables should be minimized when developing flow cytometry based test for PrPc/PrPtse detection.

Importantly, we identified platelet satellitism (Peters et al., 1997; Barnard et al., 2005) as a factor contributing to the heterogeneity of PrPc detection in blood cells. Since we detected significant proportion of platelet associated WBC which further increased after blood storage and platelets express considerable levels of PrPc (Holada and Vostal, 2000; Panigaj et al., 2010) which further increases after platelet activation (Holada et al., 1998; Holada et al., 2006), the increased levels of PrPc detected on WBC are likely to be caused at least in part by platelet-expressed PrPc. This was specifically evident in granulocytes, which are known to express very low to undetectable levels of PrPc (Cashman et al., 1990; Dodelet and Cashman, 1998; Barclay et al., 1999). Recently, platelets were shown to harbor infectivity in deer infected with chronic wasting disease (Mathiason et al., 2010) and sheep infected with scrapie (Lacroux et al., 2012). Therefore, platelet satellitism may also affect the distribution of prion infectivity in the blood.

Binding of prion antibodies to white blood cells of nonhuman primates

We assessed PrPc/PrPtse binding to leukocytes of healthy and BSE-infected cynomolgus monkeys using three mAb aimed at different epitopes of prion protein molecule. The binding of the antibodies to granulocytes was negligible in accordance with a previous study (Holada et al., 2007) and opposing a recent study reporting high PrPc levels on granulocytes (Holznagel et al., 2010). The high PrPc levels on granulocytes are likely to originate in the protocol for cell labeling used in the study. Next, we demonstrated that Ficoll isolation of peripheral blood mononuclear cells led to detection of decreased levels of lymphocyte PrPc by two of three of our mAb in accordance with the results of Holznagel and colleagues who reported that isolation of cells using Ficoll leads to decreased binding of prion antibodies. This could be explained by washing out a loosely associated pool of PrPc from the cell surface, however, its nature needs to be elucidated in further studies. Similar behavior of PrPtse would significantly complicate its detection on blood cells by flow cytometry using PrPtse-specific antibodies.

5.2. PrPc expression and function in erythroid differentiation

PrPc regulation during erythropoiesis *in vivo* in mice

The role of PrPc in erythropoiesis was suggested in several reports Miele et al., 2001b; Brown et al., 2007, Zivny et al., 2008, Singh et al., 2009b).

In this study, we demonstrated that the surface expression of PrPc on erythroid precursors in the mouse bone marrow and spleen is regulated during the cell maturation and follows a similar pattern. The PrPc levels significantly increase with basophilic erythroblasts which express more than double the levels found on proerythroblasts and then decrease with the maturation of the cells. This expression pattern suggests involvement of PrPc in the early stages of erythroid differentiation. As PrPc was shown to bind both, laminin and the laminin receptor necessary for cell adhesion (Graner et al., 2000; Gauczynski et al., 2001), it may be involved in cell-cell contacts within the erythroblastic niche where the cells mature, or with a surrounding extracellular matrix. Downregulation of PrPc then could facilitate the release of reticulocytes from the niche. Hovewer, further studies are needed to elucidate this hypothesis.

PrPc regulation during erythropoiesis *in vitro* in MEL cells

Differentiation of MEL cells in culture resembles the erythroid differentiation *in vivo* with similar maturation stages. (Hyman et al., 2001; Griffiths et al., 2007). In our study, the surface levels of PrPc on uninduced MEL cells were approximately six times higher than that observed on proerythroblasts in the mouse bone marrow and spleen. The induction of MEL cell differentiation with HMBA led to similar regulation of PrPc expression as was seen *in vivo*. However, the decrease of PrPc on differentiated MEL cells did not reach the degree observed in the late erythroblasts and reticulocytes in mice. This finding corresponds with the limited ability of MEL cells to reach this stage of differentiation. Alike the down-regulation of PrPc during the differentiation of highly responsive subclones of MEL cells was reported (Otsuka et al., 2008), however, the initial increase of PrPc expression was not detected, most likely due to the different condition of the cells. In summary, our results demonstrate that the regulation of PrPc levels in differentiating MEL cells mimics its regulation in maturing mouse erythroid precursors *in vivo*.

Characterization of MEL cell lines with silenced PrPc

To learn more about the importance of PrPc in the process of MEL cells' differentiation, we used RNAi to create cell lines with stably inhibitted expression of PrPc. RNAi

introduced by shRNA from a retrovector had previously been succesfully employed to inhibit PrPc expression *in vitro* and *in vivo* (Tilly et al., 2003; Pfeifer et al., 2006). In our study, efficient 80% inhibition of the PrPc expression at both the mRNA and protein levels was maintained during the differentiation of MEL cells. Despite the silencing of PrPc, the regulation of its expression during the differentiation of LP1- and LP2-transduced cell lines followed similar pattern as in unmodified MEL cells, although at suppressed levels. Although PrPc was suggested to have anti-apoptotic effects in various cell cultures (Roucou et al., 2004), we did not observe higher susceptibility of cells with silenced PrPc expression to apoptosis, consistent with a recent study demonstrating only a minimal cytoprotective effect of PrPc *in vitro* (Christensen and Harris, 2008). Our results suggest that PrPc is dispensable for unchallenged erythropoiesis *in vitro.*

PrPc relevance for normal and stress erythropoiesis *in vivo* in mice

Inbred Prnp-/- mice showed deeper anemia with lower hematocrit comparing to WT mice, in accordance with a previous report (Zivny et al., 2008). Prolonged elevation of erythropoietin levels in plasma and its mRNA and retarded recovery to normal hematocrit levels suggest that the absence of PrPc negatively affected erythropoiesis under stress conditions. Physiological erythropoiesis seemed to be more efficient in inbred WT mice with higher turnover of RBC in circulation comparing to Prnp-/- mice. Consistent to this, the relative numbers of erythroid precursors were slightly increased in the spleen compared to bone marrow of outbred Prnp-/- mice. As steady state erythropoiesis at a constant rate occures in mouse bone marrow whereas murine spleen is the site of stress erythropoiesis (Paulson et al., 2011) we imply a compensation mechanism for less efficient basal erythropoiesis in the bone marrow of Prnp-/- mice.

Thus PrPc might be important during stress erythropoiesis in inbred mice whereas its role seems to be compensated during unchallenged erythropoiesis. However, the effect diminished in outbred mice. Thus recognition of the PrPc role in erythropoiesis requires further experimentation.

6. CONCLUSIONS

The present work is focused on the study of PrPc expressed by blood cells, its localization, and distribution. Our results contribute to the knowledge needed for further study of PrPc physiological function as well as the role of PrPc on blood cells in the pathogenesis and transmission of prion diseases. The information about PrPc distribution among blood cells is important for development of blood screening test for prions.

The results are summarized as follows:

1. Two thirds (69%) of platelet PrPc are present in the intracellular pool.
2. PrPc within platelets is located in α-granules.
3. Platelet activation leads to translocation of intracellular PrPc onto the cell membrane, together with an α-granular marker P-selectin.
4. One platelet express six times more PrPc than one red blood cell, however, when total cell number is taken into account red blood cells possess major pool of cell-associated PrPc in human blood.
5. PrPc on human red blood cells undergoes a posttranslational modification in circulation which results in the loss of epitope for widely used prion mAb 3F4.
6. The choice of anti-prion antibody greatly affects the PrPc detection on leukocytes in peripheral blood.
7. Blood storage preceding analysis leads to a decrease in PrPc detection on most leukocytes.
8. Platelet satellitism contributes to the heterogeneity of PrPc detection in blood cells as the platelet association with leukocytes may significantly increase the levels of PrPc detected on leukocyte subsets.
9. Existence of washable pool of PrPc on blood cells, identified on lymphocytes of cynomolgus monkeys, may complicate detection of TSE by flow cytometry.
10. PrPc expression is regulated during erythroid differentiation of murine erythroid precursors *in vivo* and *in vitro*.
11. PrPc is dispensable for erythroid differentiation under unchallenged conditions *in vitro*.
12. PrPc might be important during stress erythropoiesis *in vivo* whereas its absence seems to be compensated during unchallenged erythropoiesis.

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8. APPENDICES

Appendix 1

Holada K, Glierova H, Simak J, Vostal JG. Expression of cellular prion protein on platelets from patients with gray platelet or Hermansky-Pudlak syndrome and the protein's association with alpha-granules. Haematologica. 2006 Aug;91(8):1126-9.

Appendix 2

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Appendix 3

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Appendix 4

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Appendix 5

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Appendix 6 (unpublished article)

Glier H, Panigaj M, Janouskova O, Semberova J, Hudakova A, Holada K. Impairment of erythropoiesis in inbred cellular prion protein deficient mice.

Expression of cellular prion protein on platelets from patients with gray platelet or Hermansky–Pudlak syndrome and the protein's association with α-granules

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The cellular prion protein (PrPc) is a membrane glycoprotein expressed on many human cells including platelets. We investigated the cellular localization of platelet PrPc. In resting platelets most PrPc was localized inside the cells. The correlation of PrPc and P-selectin surface up-regulation after platelet activation suggested its association with α-granules. This was confirmed by normal expression of PrPc on Hermansky-Pudlak syndrome platelets, which lack dense granules, and failure of gray platelet syndrome platelets, which lack α-granules, to up-regulate PrPc. Our results warrant further studies on the role of platelet PrPc in the transmission of prion diseases by blood transfusion.

Key words: prion protein, PrPc, platelets, α granules.

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Two recent cases of probable variant Creutzfeldt-Jacob disease (vCJD) transmission by transfusion of non-leukodepleted packed red cells donated by asymptomatic vCJD infected donors[1,2] emphasize the necessity for detailed understanding of blood-related prion pathogenesis. The central role of cellular prion protein (PrPc) expression in the disease process was demonstrated by the resistance of PrP-negative mice to prion infection.[3] Prions seem to be composed mainly, if not entirely, of a conformationally changed isoform of prion protein (PrPsc) which can, upon physical contact, initiate a similar change in the secondary structure of normal PrPc.[4] Thus, molecules of PrPc on the cell membrane may serve not only as a substrate for conversion, but also as a cellular receptor for PrPsc. The level of PrPc expression by cells may influence the distribution of prions in blood and affect their fate in the organism.[5] Studies in laboratory animals demonstrated the presence of roughly equal amounts of infectivity in the plasma and cellular fraction of blood.[6,7] Cell-associated infectivity seems to be enriched in the buffy coat. Very little infectivity was recovered in purified, washed platelets of scrapie-infected hamsters.[8] However, hamster as well as mouse platelets do not express PrPc.[9,10] In contrast, most cell- associated PrPc in human blood seems to be present in platelets,[11] making these a possible target for binding of intravenously introduced prions. PrPc is expressed by $CD34^+$ hematopoietic cells and its expression has been shown to be higher in megakaryocytes.[12] Activation of human platelets leads to a more than doubling of PrPc molecules on the platelet membrane, demonstrating the existence of a significant intracellular pool of PrPc.[13,14] The aim of the present study was to investigate the intracellular localization of PrPc in human platelets and to confirm these observations through experimentation with platelets from patients with hereditary defects of platelet storage granules: Hermansky-Pudlak syndrome (HPS) in which there is a lack of dense granules) and gray platelet syndrome (GPS) in which α-granules are lacking.[15]

Design and Methods

Subjects

Donor blood samples were obtained from Departments of Transfusion Medicine of the Institute of Hematology and Blood Transfusion in Prague and the National Institutes of Health in Bethesda. In addition, blood samples from two patients with HPS and two with GPS were studied (provided by Dr. Gahl, NICHD, NIH, Bethesda, USA). Type 1 HPS patients were of Puerto Rican origin and had a 16-bp duplication in exon 15 of the *HPS-1* gene.[16] GPS patients were two siblings of Moslem Bedouin origin.[17] Blood was collected at a ratio of 9:1 into 3.8% sodium citrate and processed within 2 hours. The study was conducted in accordance with the Helsinki Declaration. Samples were obtained following informed consent under a protocol approved by the Institutional Review Board of the NICHD, NIH in Bethesda.

Quantification of platelet PrPc intracellular pool by proteinase K protection assay

Donor platelets were isolated by gel filtration into Tyrode's/ HEPES buffer (THB). The platelet suspension was supplemented with 2 mM EDTA and one half was activated with 20 μM thrombin activating peptide (TRAP) at 37°C for 10 minutes. Aliquots of resting and activated platelets were either treated with 250 μg/mL proteinase K on ice for 1 hour or left untreated. The control aliquots of both resting and activated platelets were solubilized with 1% Triton X-100 before proteinase K treatment. The proteinase K treatment was stopped by addition of 5 mM phenylmethylsulfonyl fluoride. Proteins were precipitated by cold methanol at -20°C and sedimented by centrifugation. The supernatant was removed and the pellet was resuspended in sodium dodecyl sulfate sample buffer. Samples of the intact and treated platelets were analyzed by western blotting using a mixture of monoclonal antibodies 6H4 (1:5000, Prionics AG) and AG4 (1:2000, TSE RC). Binding of monoclonal antibodies was visualized by anti-mouse IgG goat $F(ab)_2$ linked to alkaline phosphatase (Biosource International) with a 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium phosphatase substrate and quantified by densitometry.

Flow cytometry evaluation of dose-dependent PrPc up-regulation on platelet membrane

Donor platelets were activated by ADP (1-50 μM) or TRAP (20 μM) for 10 minutes at room temperature and labeled with fluorescein-conjugated monoclonal antibodies against LAMP-3 (CD63, CLBGran/12, Immunotech) and phycoerythrin-conjugated anti P-selectin (CD62P, AC1.2, Becton Dickinson) or biotinylated anti-prion monoclonal antibodies 3F4 (CD230, a gift from Dr. Kascsak) followed by phycoerythrin - streptavidin (Caltag Laboratories). Samples were analyzed by a FACScan flow cytometer (Becton Dickinson) equipped with CELLQuest™ software. The mean fluorescence of resting platelets labeled with each monoclonal antibodies was assigned as 0%, and the mean fluorescence of fully TRAP-activated platelets was 100%. The relative increment of expression of specific glycoproteins on the platelet surface was calculated after activation with different concentrations of ADP.

Flow cytometry study of PrPc expression on patients' platelets

Donor and patient platelet-rich plasma was prepared by layering 0.5 mL of citrate anticoagulated blood on 1 mL of Ficoll-Hypaque (Amersham Biosciences) and sedimenting red blood cells at 1 g. Platelet-rich plasma was diluted 10 times with THB and an aliquot of platelets activated with 20 μM TRAP (10 minutes, room temperature). Resting and activated platelets were labeled with phycoerythrin-conjugated anti P-selectin, or fluorescein-conjugated monoclonal antibodies against LAMP-3 or PrPc (1562, Chemicon International or 6H4, Prionics) and analyzed by flow cytometry.

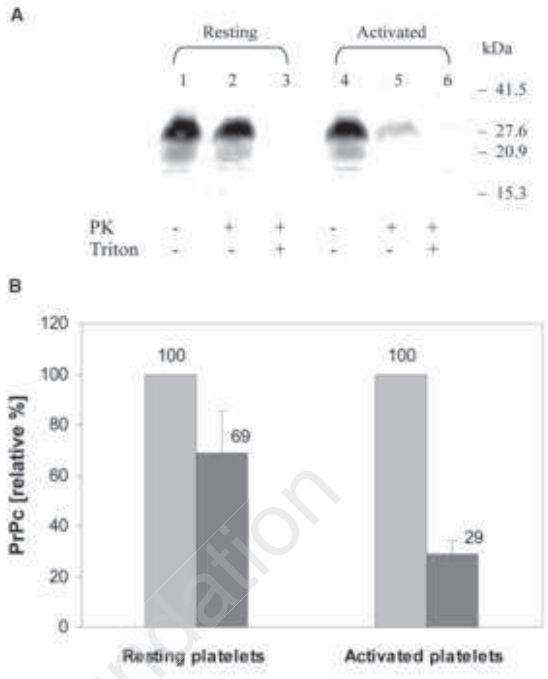

Figure 1. Most platelet PrPc resides in the intracellular pool. Intact resting and TRAP-activated platelets or platelets solubilized by Triton X-100 were treated with proteinase K (PK) to cleave accessible PrPc. The presence of PrPc was analyzed by western blotting using a mixture of monoclonal 6H4 and AG4 (A). Most PrPc in resting platelets was protected against proteolysis (line 2). Platelet activation led to translocation of intracellular PrPc onto the cell membrane and increased the proportion of PrPc accessible to PK (line 5) while solubilization of platelet membranes by Triton X-100 led to complete cleavage of PrPc (lines 3, 6). The blot is representative of five independent experiments used for densitometric quantification of PrPc (B). Light gray bars – non-treated platelets, dark gray bars – PK-treated platelets. Approximately 70% and 30% of platelet PrPc reside in the intracellular pool of resting and activated platelets, respectively.

Results and Discussion

The quantity of PrPc molecules per cell expressed on the platelet membrane was reported to be between 300 and 1800 for resting and between 600 and 4800 for activated platelets.[12-14] However, none of these studies addressed the size of the platelet intracellular PrPc pool. The proteinase K protection assay applied to resting human platelets demonstrated that the majority of PrPc (69%) is not accessible to the protease (Figure 1). This indicates that the amount of PrPc on membranes of resting platelets is substantially smaller than that in the intracellular pool. Treatment of activated platelets with proteinase K led to cleavage of a greater part of platelet PrPc (71%), although 29% of the molecules remained protected against proteolysis. This suggests that not all intracellular PrPc is up-regulated on the platelet surface

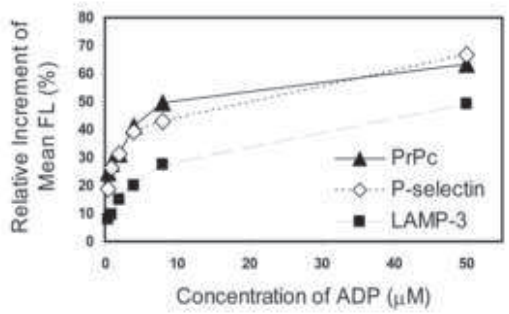

Figure 2. PrPc is up-regulated on the surface of activated platelets together with an α-granular marker P-selectin. Platelets were activated with ADP or TRAP, fixed and analyzed for expression of P-selectin, LAMP-3 and PrPc using flow cytometry. ADP induced co-expression of P-selectin and PrPc on the platelet surface at lower concentrations than those required for expression of LAMP-3, suggesting that PrPc associates with α-granules, but not with lysosomes. Data are presented as the relative increase in comparison to the level of expression on platelets fully activated with TRAP (100 %). Data from three independent experiments are presented in the graph.

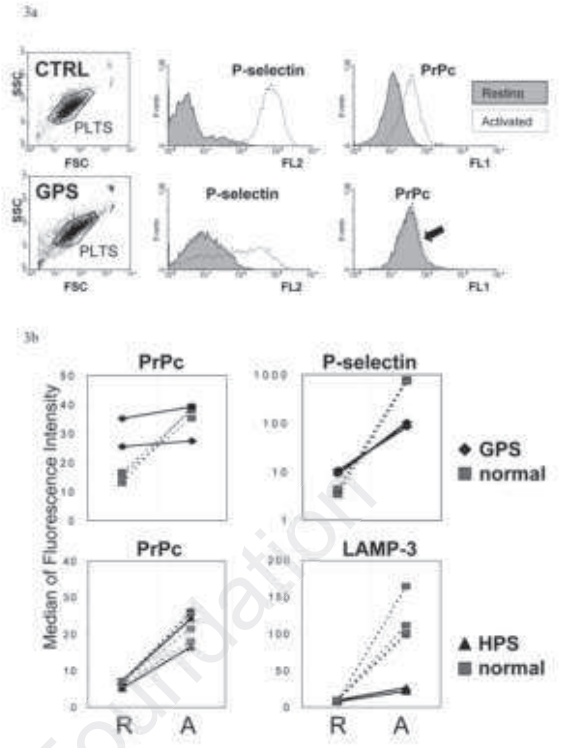

Figure 3. Platelets from patients with gray platelet syndrome (GPS) patients, but not those from Hermansky-Pudlak syndrome (HPS), fail to up-regulate PrPc after activation. The expression of P-selectin, LAMP-3 and PrPc was studied by flow cytometry on resting and TRAP-activated platelets from two GPS patients and three normal donors. Platelets from GPS patients have decreased numbers of α-granules. Resting GPS platelets expressed high levels of α-granular P-selectin and similarly increased levels of PrPc when compared with normal donor platelets (CTRL) (A). Activation of GPS platelets led to an aberrant up-regulation of P-selectin and almost no up-regulation of PrPc expression (arrow) which suggests that PrPc is located in α-granules. Typical scattergrams with gated platelets (PLTS) from CTRL (upper part) and GPS patients (lower part) are shown in 3A. Histograms are representative of P-selectin and PrPc expression on resting (filled peak) and activated (dashed line) platelets. A similar study was performed on platelets from two HPS patients and four normal donors. HPS platelets have decreased numbers of δ granules and this correlated with a decreased expression of LAMP-3 on activation. The expression of PrPc in resting and activated platelets from HPS patients was normal. GPS (upper part), HPS (lower part) and CTRL platelet fluorescence is shown in 3B. Data are presented as medians of fluorescence intensity. Patients: GPS: black diamonds; HPS: black triangles; healthy donors: gray rectangles; R: resting platelets; A: activated platelets.

or shed from platelets[18] after activation. Furthermore, solubilization of the platelet membranes with Triton X-100 led to complete degradation of platelet PrPc by proteinase K, demonstrating that intracellular PrPc is sensitive to proteolysis. This PrPc distribution is in agreement with results of our previous study revealing the incomplete translocation of PrPc from the organelle fraction to the membrane fraction in activated platelets.[13]

In order to elucidate the intracellular localization of platelet PrPc we conducted immunoelectron microscopy studies with a mixture of anti-PrP monoclonal antibodies and a gold-labeled secondary antibody. Gold particles were found to be associated with plasma membranes, membranes of the open canalicular system and α-granules (*KH and Dr. Michael Jarnik, Fox Chase Cancer Center, Philadelphia, unpublished results*). However, the signal was not strong enough to allow conclusive evaluation of the intracellular PrPc distribution. Recently, Starke *et al.*[12] and Robertson *et al.*[18] reported a similar intracellular distribution of platelet PrPc, determined by immunoelectron microscopy with polyclonal anti-PrP antibodies P3 and FL253, respectively.

To learn more about the intracellular localization of PrPc in platelets we used flow cytometry to follow the correlation of an agonist dose-dependent membrane up-regulation of PrPc, P-selectin (α-granular protein) and LAMP-3 (dense granular and lysozomal protein) on platelets from normal donors (Figure 2). The concentrations of ADP necessary to induce co-expression of P-selectin and PrPc on the platelet surface were lower than those required for expression of LAMP-3 (e.g. 5 μM ADP vs. 50 μM ADP to reach 40% of maximal expression). This suggests that PrPc is up-regulated from the same compartment as P-selectin, but from a different compartment than LAMP-3.

To further address the question of the origin of intraplatelet PrPc, we evaluated the expression of platelet PrPc in two patients with HPS and two patients with GPS. HPS platelets have a low number of dense granules, but normal numbers of α-granules.[15] The expression of LAMP-3 was equivalent on resting control and HPS platelets, but was substantially decreased on HPS platelets after full platelet activation (Figure 3B). In comparison, similar levels of PrPc and α-granular P-selectin were expressed on normal and HPS activated platelets demonstrating that a lack of dense granules does not affect PrPc up-regulation (Figure 3B). Platelets

from patients with GPS are deficient in α-granules.[15] Resting GPS platelets demonstrated higher expression of P-selectin and PrPc than normal platelets (Figure 3). This difference may represent a redirection of proteins from insertion into membranes of absent α-granules to platelet cytoplasmic membranes. In contrast to normal platelets, GPS platelets failed to up-regulate P-selectin and PrPc with agonist-induced activation illustrating that intact α-granules are essential for normal up-regulation of these proteins (Figure 3).

Taken together, our data confirm the localization of intracellular PrPc in platelet α-granules. The potential role of platelets and platelet α-granular PrPc in transmission of prion diseases by blood transfusion remains to be investigated. Hypothetically, PrPsc present in infected donor plasma could bind to PrPc on the platelet surface of the transfusion recipient and be delivered into α-granules. This mechanism may prevent PrPsc from reaching cells which would be capable of supporting prion replication. Our results warrant further studies on the interactions of platelets with intravenously introduced prions.

KH designed and performed experiments, analyzed and interpreted the data and wrote the manuscript. HG performed and analyzed the PK protection assay. JS was involved in interpretation of flow cytometry data and revised the manuscript. JGV was involved in the design of experiments and revised the intellectual content of the manuscript.

The views of the authors represent scientific opinion and should not be construed as opinion or policy of the US Food and Drug Administration. Conflict of interest: none.

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Underestimation of the expression of cellular prion protein on human red blood cells

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BACKGROUND: Recent transmissions of variant Creutzfeldt-Jakob disease by blood transfusion emphasize the need for the development of prion screening tests. The detection of prions in blood is complicated by the presence of poorly characterized cellular prion protein (PrP^C) in both plasma and blood cells. According to published studies, most of PrP^C in blood cells resides in platelets (PLTs) and white blood cells.

STUDY DESIGN AND METHODS: To clarify conflicting reports about the quantity of PrP^C associated with human red blood cells (RBCs), quantitative flow cytometry, Western blot (WB), and enzyme-linked immunosorbent assay (ELISA) were used to measure protein levels in healthy donors.

RESULTS: RBCs expressed 290 ± 140 molecules of PrP^C per cell, assuming equimolar binding of monoclonal antibody (MoAb) 6H4 to PrP^C. Binding of alternate PrP^C MoAbs, FH11 and 3F4, was substantially lower. WB estimated the level of PrP^C per cell on RBCs to be just four times lower than in PLTs. A similar level of PrP^C was detected using ELISA. The weak binding of commonly used MoAb 3F4 was not caused by PrP^C conformation, truncation, or glycosylation, suggesting a covalent modification, likely glycation, of the 3F4 epitope.

CONCLUSIONS: Taken together, human RBCs express low but significant amounts of PrP^C/cell, which makes them, due to high RBC numbers, major contributors to the pool of cell-associated PrP^C in blood. Previous reports utilizing MoAb 3F4 may have underestimated the amount of PrP^C in RBCs. Likewise, screening tests for the presence of the abnormal prion protein in blood may be difficult if the abnormal protein is modified similar to RBC PrP^C.

Cellular prion protein (PrP^C) is expressed in various cell types and tissues, including blood cells. The physiological function of PrP^C remains unclear. Together with its conformationally altered form, PrP^{TSE}, it plays a crucial role in prion diseases.[1] Prion diseases, or transmissible spongiform encephalopathies (TSEs), are neurodegenerative disorders affecting a broad spectrum of mammalian species. The common trait of TSEs is a long subclinical period with a final rapid deterioration of cerebral function followed by inevitable death. Until recently, in modern societies, the only documented mode of transmission from human to human was iatrogenic cases in which disease was spread

ABBREVIATIONS: CML = N^{ε}-(carboxymethyl)lysine; GA = glyoxylic acid; PBST = phosphate-buffered saline with Tween 20; PK = proteinase K; PMSF = phenylmethylsulfonyl fluoride; PRP = platelet-rich plasma; PrP^C = cellular prion protein; PWB = platelet wash buffer; RT = room temperature; sulfo-NHS-biotin = *N*-hydroxysulfosuccinimide biotin; TBS = Tris-buffered saline; TSE(s) = transmissible spongiform encephalopathy(-ies); vCJD = variant Creutzfeldt-Jakob disease; WB = Western blot.

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via contaminated neurosurgical instruments, tissue grafts, or hormones.[2] Despite the proven transmissibility of prion diseases via blood transfusion in laboratory animals, such cases in humans remained undetected until recently.[3] As of this year, four cases of variant Creutzfeldt-Jakob disease (vCJD) transmission by blood transfusion, including one asymptomatic vCJD carrier, have been described in the United Kingdom.[4] All of the patients received nonleukoreduced red blood cells (RBCs) from apparently healthy donors who developed vCJD symptoms many months after the donation. Recently, vCJD PrP^{TSE} was found in the spleen of a UK hemophilia patient who died from reasons not related to TSE.[5] While the annual number of vCJD victims remains low, the number of asymptomatically infected individuals may be significantly higher.[6,7] These subclinical cases can pose a serious threat of vCJD iatrogenic transmission. In this situation, the development of a fast, sensitive, and noninvasive donor screening test for prion diseases remains an important and controversial issue.[8,9] Unfortunately, PrP^{TSE} is currently the only known specific molecular marker of the disease, and its detection in blood is challenging.[10] The nature and properties of blood PrP^{TSE} are not known, and the availability of PrP^{TSE}-specific antibodies is limited. Commercial diagnostic tests usually depend on proteinase K (PK) treatment to distinguish partially resistant PrP^{TSE} from sensitive PrP^{C}. Another principle is used in the conformation-dependent immunoassay developed by Safar and colleagues[11] that utilizes the difference in the affinity of monoclonal antibody (MoAb) 3F4 to native and denatured PrP^{TSE}. The 3F4 epitope is buried in the PrP^{TSE} molecule and becomes accessible after denaturation. Thus, the comparison of signals between native and denatured sample allows for the detection of PrP^{TSE}. Human blood contains a substantial amount of poorly characterized PrP^{C}, complicating the detection of PrP^{TSE} by providing heterogeneous background. According to published studies, most of PrP^{C} in the cellular compartment of blood resides in platelets (PLTs) and on white blood cells (WBCs).[12,13] Opinions on the expression of PrP^{C} on RBCs differ significantly. A number of studies have reported no or insignificant expression of PrP^{C}. Dodelet and Cashman[14] have reported the absence of PrP^{C} expression on RBCs using flow cytometry with MoAb 3F4. Similarly, Barclay and coworkers[12] did not detect any PrP^{C} on RBCs using flow cytometry with MoAbs 3F4 and FH11. In addition, no expression was reported in flow cytometry studies of Antoine and colleagues[15] and Li and colleagues[16] using different PrP antibodies (8G8, 3B5, and 8H4). MacGregor and colleagues[13] used time-resolved dissociation-enhanced fluoroimmunoassay with FH11 as the capture antibody and 3F4 as the detecting antibody to measure the amount of PrP^{C} in separated human blood cells. They estimated that 84% of PrP^{C} levels were associated with PLTs, 10% with WBCs, and only 5.7% with RBCs. This observation was in accordance with the negligible PrP^{C} expression reported by the above-mentioned flow cytometry studies. Later, Barclay and colleagues[17] used a panel of 12 MoAbs to compare the expression of PrP^{C} on blood cells using flow cytometry. Out of 12 antibodies, only three (6H4, 4F2, and 8G8) demonstrated weak but clearly distinct binding to human RBCs. We first observed the expression of a minute amount of PrP^{C} on human RBCs using flow cytometry with the 6H4 antibody.[18] Our finding was questioned by some authors, particularly due to the single-antibody approach.[19] In view of the above controversial reports, the question of the presence of PrP^{C} on circulating human RBCs has remained uncertain. This uncertainty has been furthered by the repeated failure of MoAb 3F4, one of the most trusted prion antibodies, to detect a significant level of RBC PrP^{C}.[12-14,17] Here, we provide a conclusive study demonstrating the expression of PrP^{C} on circulating human RBCs using various antibodies and different methods. In addition, we provide an explanation for the weak binding of 3F4 to human RBC PrP^{C} and discuss possible implications of this phenomenon for the development of a PrP^{TSE} detection test.

MATERIALS AND METHODS

Samples

Citrate-anticoagulated blood of healthy volunteers was provided by the Transfusion Department of Institute of Hematology and Blood Transfusion in Prague. Samples of cord blood were provided by the Institute for the Care of Mother and Child in Prague. Normal brain samples (lobus frontalis) were provided by the Institute of Pathological Medicine (1st Faculty of Medicine, Charles University, Prague, Czech Republic). The collection of samples was conducted in accordance with the Helsinki Declaration.

Flow cytometry

Quantitative fluorescence-activated cell sorting (FACS) analysis with fluorescein isothiocyanate (FITC)-labeled MoAbs FH11 (TSE Resource Centre, Compton, UK), 3F4 (MAB1562, Chemicon, Inc., Temecula, CA), and 6H4 (Prionics AG, Zürich, Switzerland) against different epitopes of human PrP^{C}- PrP_{51-55}, $PrP_{106-112}$, and $PrP_{144-152}$ (Fig. 1) was performed on RBCs of eight healthy donors as described previously.[18] Standard beads (Quantum 24, Bangs Laboratories, Inc., Fishers, IN) were used for construction of calibration regression line, and the number of immunoglobulin (Ig)G molecules bound per cell was calculated. Two-color flow cytometry was used for the comparison of MoAb 3F4 and 6H4 binding to transferrin receptor–positive (CD71+) erythroid cells in cord blood. RBCs were sedimented ($1 \times g$, 45 min, room temperature [RT]) on Ficoll-Hypaque. Cells in the supernatant were incubated

Fig. 1. Schematic structure of human PrP^C with the location of epitopes of MoAbs used in the study. MoAbs FH11, 3F4, and 6H4 were used in the flow cytometry study of its expression on human RBCs. MoAbs AG4 and GE8 were used for measurement of PrP^C content in RBCs and PLTs by ELISA and together with AH6 in WB studies. The mixtures of MoAbs AG4 and 6H4 or AG4, AH6, and GE8 were used in attempts to detect truncated forms of RBC PrP^C. The octarepeats region (51-90), two N-glycosylation sites (181, 197), and the disulfide bridge (179-214) are shown.

with saturating concentrations of MoAb 3F4 or 6H4, fixed with 1% paraformaldehyde, and after washing, incubated with FITC-labeled anti-mouse immunoglobulin (Ig)G goat secondary antibody (Beckman Coulter, Inc., Brea, CA) followed by phycoerythrin-conjugated CD71 MoAb (MEM-75, Exbio, Prague, Czech Republic). CD71-positive cells were gated on FL2/forward scatter plot, and their FITC fluorescence was compared.

Preparation of brain homogenate

Brain tissue was homogenized (1:9) in ice-cold Tris-buffered saline (TBS; 20 mmol/L Tris and 145 mmol/L NaCl, pH 7.4) with 1 mmol/L ethylenediaminetetraacetate (EDTA) and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Coarse fragments were removed ($4000 \times g$, 10 min, 4°C), and the supernatant was stored frozen in aliquots at –80°C.

Simultaneous isolation of PLTs and RBC ghosts

Blood was diluted 1:1 with PLT wash buffer (PWB; 12.9 mmol/L citrate, 30 mmol/L glucose, 120 mmol/L NaCl, and 5 mmol/L EDTA, pH 6.5) and centrifuged ($300 \times g$, 15 min, RT). PLT-rich plasma (PRP) was transferred into a new tube, and sedimented cells were resuspended in PWB. This step was repeated three times to remove the majority of PLTs and plasma. The numbers of blood cells in individual stages of separation were monitored using a cell counter (ADVIA 60, Bayer, Leverkusen, Germany) and served as a base for estimation of numbers of contaminating cells in separated blood fractions. RBCs were resuspended in cold phosphate-buffered saline (PBS; pH 7.4) and centrifuged ($1500 \times g$, 10 min, 4°C). The supernatant and the buffy coat containing WBCs were discarded together with the upper quarter of the RBC phase. The middle half of the RBC phase was diluted with the same volume of PBS and RBCs were lysed with 14 volumes of ice-cold lysis buffer (5 mmol/L sodium phosphate and 1 mmol/L EDTA, pH 7.4). The remaining contaminating PLTs and WBCs were removed using centrifugation ($2000 \times g$, 5 min, 4°C). RBC ghosts were sedimented at $20{,}000 \times g$ (40 min, 4°C), washed three times with lysis buffer, resuspended in TBS, and stored frozen at –80°C. PLTs were isolated from PRP combined with washing RBC PLT-containing fractions. PLTs were sedimented in one tube ($1500 \times g$, 10 min, RT) and resuspended in PWB. Contaminating WBCs and RBCs were centrifuged ($300 \times g$, 15 min, RT), and the supernatant containing pure PLTs was transferred to a new tube. The PLTs were washed twice with PWB, resuspended (5×10^9 PLT/mL) in TBS (pH 7.4), and stored frozen at –80°C.

Electrophoresis and Western blot

Proteins were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a 0.2-µm nitrocellulose membrane. The membrane was blocked with TBS with 0.05 % Tween 20, pH 7.4, followed by TBST with 5% dry nonfat milk and incubated with appropriate MoAb (0.5 µg/mL 6H4, 1 µg/mL AG4, or 0.5 µg/mL 3F4) diluted in TBSTM. After being washed, the membrane was incubated with anti-mouse IgG goat F(ab)2 linked to alkaline phosphatase (Biosource International, Camarillo, CA) and bands were visualized with a 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium phosphatase substrate.

Comparison of the amount of PrP^C on PLTs and RBCs using Western blot

Cells were isolated as described above. Care was taken to avoid contamination of RBC ghosts and PLTs by other cells. The comparison was performed by comparing the intensity of PrP^C bands on blots between samples prepared from 1×10^{10} RBCs/mL and serial dilutions of sample containing 5×10^9 PLTs/mL. PrP^C was detected using MoAb 6H4 or a mixture of MoAbs AG4, AH6, and GE8 (epitopes $PrP_{37\text{-}50}$, $PrP_{159\text{-}174}$, and $PrP_{183\text{-}191}$, respectively; 1 µg/mL of each, TSE Resource Centre, Compton, UK). In the first round of experiments, the comparison was performed between homologous PLTs and RBCs and repeated three times on cells from three donors. The final experiment was performed with homologous PLTs and RBC ghosts isolated from five individual donors. The density of bands on blots was quantified using densitometer software (MiniLumi, DNR Bio-Imaging Systems Ltd, Jerusalem, Israel).

Production of recombinant human prion protein

The pRSET A plasmid with the full-length human prion protein sequence 23-230 (hrPrP) was provided by Prof.

Wüthrich (Institute of Molecular Biology and Biophysics, ETH Zurich, Switzerland). Transformed bacteria (*Escherichia coli* BL21 [DE3]) were grown at 37°C in Luria-Bertani medium until the cell density was approximately 0.6 OD_{600} units. hrPrP expression was then induced using 1 mmol/L isopropyl β-D-1-thiogalactopyranoside and the cultures were grown for an additional 4 hours. The cells were harvested using centrifugation and sonicated in PBS with 1 mmol/L PMSF. Inclusion bodies were solubilized in 8 mol/L urea and the prion protein was purified on metal affinity resin (TALON, Clontech, Saint-Germain-en-Laye, France) and oxidized to form intramolecular disulfide bonds, as previously described.[20]

Comparison of the amount of PrP^C on PLTs and RBCs using enzyme-linked immunosorbent assay

Homologous PLTs and RBC ghosts of healthy donors (n = 5) were isolated as described above. A microtitration plate (MaxiSorp, Nunc, Rosklide, Denmark) was coated overnight with MoAb AG4 (1 μg/mL) in carbonate buffer, pH 9.6, at 4°C. The plate was washed with PBS with 0.05% Tween 20 (PBST) and blocked for 1 hour at RT in 5% nonfat milk in PBST. Samples of PLTs, RBC ghosts, and hrPrP were diluted in PBS with 1% Triton X-100 and incubated overnight at 4°C (50 μL/well). The plate was washed and incubated for 2 hours at 37°C with custom biotinylated MoAb GE8. After being washed with PBST, streptavidin-horseradish peroxidase (Invitrogen) diluted 1:10,000 was added to wells and incubated for 1 hour at 37°C. The plate was washed and developed with TMB substrate for 4 minutes. The reaction was stopped with the addition of 1 mol/L H_2SO_4, and absorbance was measured at 405 nm.

Deglycosylation

RBC ghosts and brain samples were deglycosylated using PNGase F (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. The samples were denatured, supplemented with complete protease inhibitor (Roche, Mannheim, Germany), Triton X-100, incubated overnight at 37°C with 100 U/mL PNGase F, and analyzed using immunoblotting. Fresh untreated RBC ghosts and brain samples and their aliquots incubated overnight without PNGase F served as controls.

PK resistance assay on intact cells

Nine volumes of PRP were mixed with 1 volume of whole blood to obtain similar numbers of PLTs and RBCs in the sample. Cells were diluted 10-fold in *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid–bovine serum albumin (HEPES-BSA; 4 mmol/L HEPES, 137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L $MgCl_2$, 5.5 mmol/L glucose, 3 mmol/L NaH_2PO_4 with 0.1% BSA, pH 7.4) and treated for 15 minutes with increasing concentrations (0, 1, 5, 10, and 50 μg/mL) of PK at 30°C. Proteolysis was terminated using 2 mmol/L PMSF and aliquots of samples were labeled with FITC-conjugated MoAbs 3F4 or 6H4. After incubation, the cells were sedimented ($2000 \times g$, 5 min), resuspended in HEPES-BSA, and analyzed using FACS.

PK resistance assay on cell lysates

Homologous RBC ghosts and PLTs and normal brain homogenate were lysed using 1% Triton X-100 and treated for 30 minutes on ice with 0, 2.5, 5, 50, and 100 μg/mL PK. The incubation was terminated using 2 mmol/L PMSF, and samples were analyzed using immunoblotting with MoAbs 6H4 and AG4.

In vitro modification of brain PrP^C by hydrogen peroxide, *N*-hydroxysulfosuccinimide biotin, and glyoxylic acid

Samples of normal brain homogenate were separated using SDS-PAGE and blotted, and the membrane was cut into strips. Individual strips were treated with increasing concentrations of hydrogen peroxide (0, 1, 5, 25, 130, and 640 mmol/L) in PBS for 30 minutes at RT or with increasing concentrations of *N*-hydroxysulfosuccinimide biotin (sulfo-NHS-biotin, Thermo Fischer Scientific, Rockford, IL; 0, 0.01, 0.05, 0.1, 0.5, 1, and 10 mmol/L) in PBS (1 hr, 37°C). Incubation with increasing concentrations (0, 6.75, 12.5, 25, 50, and 100 mmol/L) of glyoxylic acid (GA) was performed in 0.2 mol/L phosphate buffer (pH 7.4) containing 150 mmol/L sodium cyanoborohydride for 24 hours at 37°C.[21] After incubation, the strips were washed three times with PBS and then developed with MoAb 3F4 or 6H4 as described above.

In vitro modification of recombinant human prion protein by GA

Oxidized hrPrP was diluted to 1 μg/mL in carbonate buffer (15 mmol/L Na_2CO_3 and 35 mmol/L $NaHCO_3$, pH 9.6) and used for coating an enzyme-linked immunosorbent assay (ELISA; MaxiSorp, Nunc) plate (3 hr, 37°C). After being washed with PBST, the wells were incubated overnight at 4°C with increasing concentrations of GA (0, 6.75, 12.5, 25, 50, and 100 mmol/L) in 0.2 mol/L phosphate buffer (pH 7.4) containing 150 mmol/L sodium cyanoborohydride. The plate was washed, blocked, and developed with MoAb 3F4 or AG4 (0.1 μg/mL in PBST) as described above.

RESULTS

Quantitative FACS analysis of PrP^C expression on RBCs

Notable differences in the binding of antibodies to human RBCs were found. One RBC bound 290 ± 140

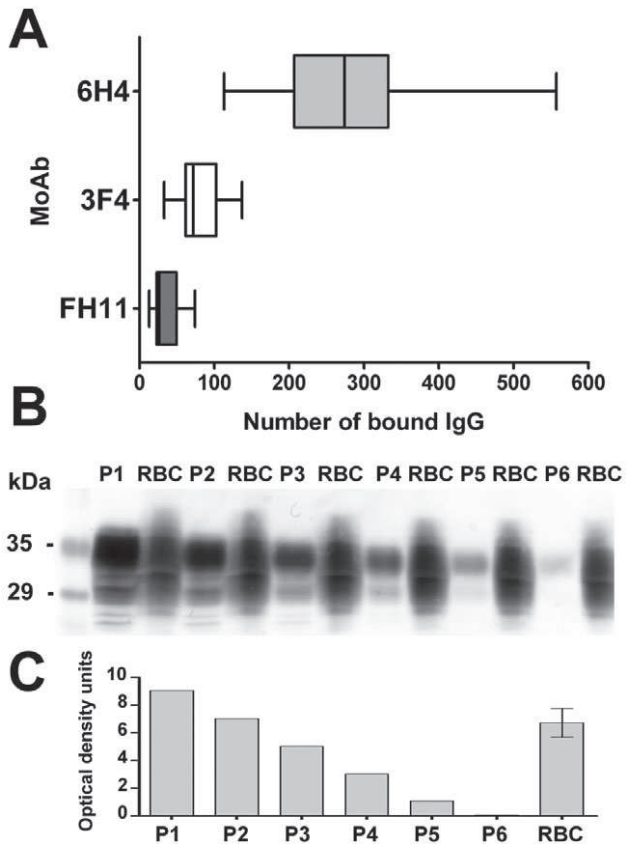

Fig. 2. Quantification of PrP^C expression on human RBCs using flow cytometry and WB. (A) Binding of MoAbs to RBCs of healthy donors measured using quantitative flow cytometry (n = 8). The range of the data, 50% percentile (box), and median are shown. (B) WB comparison of PrP^C levels in representative standardized samples of RBC ghosts (1×10^{10} RBC/mL) and PLTs (P1-P6 = 5×10^9, 2.5×10^9, 1.25×10^9, 6.25×10^8, 3.13×10^8, and 1.56×10^8 PLT/mL) developed with MoAb 6H4. (C) Densitometry of bands on blot (B) demonstrates that RBCs contain four times less PrP^C levels than an equal number of PLTs.

(median ± SD) IgG molecules of 6H4. The binding of 3F4 and FH11 was much lower: 80 ± 35 and 35 ± 20 (median ± SD) IgG molecules per cell, respectively (Fig. 2A). In contrast, the binding of 3F4 and 6H4 to PLTs in identical samples was roughly equivalent: 630 ± 150 and 635 ± 280 (median ± SD) IgG molecules per cell, respectively (not shown).

Purity of homologous simultaneously isolated PLTs and RBC ghosts

The isolation procedure led to the preparation of pure cell fractions. Isolated PLTs contained a mean of 1 WBC per 680 ± 270 (mean ± SD, n = 6) PLTs, and contamination with RBCs in this fraction was below the limit of the cell counter. Isolated RBCs before hypotonic lysis contained 1 WBC per 1360 ± 280 (mean ± SD, n = 6) RBCs, and the PLT count was below the limit of the cell counter. In addition, the subsequent centrifugation step after the lysis of RBCs removed virtually all remaining nonlysed cells, leaving RBC ghosts free of cell contamination.

Western blot comparison of PrP^C amount in RBCs and PLTs

In the first set of Western blot (WB) experiments in three separate donors, one PLT contained two- to fourfold more PrP^C than one RBC ghost when measured with MoAb 6H4 and four- to eightfold more when measured with a mixture of PrP MoAbs GE8, AG4, and AH6 (not shown). In the second set of experiments, PLTs and RBC ghosts were prepared from the blood of five separate donors and then pooled to obtain representative mixed PLT and RBC ghost samples. Densitometry analysis of the blot developed with 6H4 confirmed that one PLT contained approximately four times more PrP^C than one RBC ghost (Figs. 2B and 2C).

ELISA comparison of PrP^C amount in RBCs and PLTs

Recombinant human prion protein was used for the construction of the calibration curve in a sandwich ELISA using MoAbs AG4 and GE8. The assay gave a linear response in the range of 2 to 25 ng hrPrP/mL (not shown). Isolated homologous PLTs and RBC ghosts of five separate donors were assayed in triplicate in serial dilutions containing 1×10^9, 5×10^8, 2.5×10^8, and 1.25×10^8 cells/mL. The mean level of PrP^C per 1×10^9 PLTs was 8.2 ± 1.6 ng (mean ± SD, n = 5) and 2.9 ± 0.6 ng (mean ± SD, n = 5) per 1×10^9 RBC ghosts. This result estimates the mean amount of PrP^C per PLT to be approximately three times higher than one RBC ghost.

Characterization of RBC PrP^C using WB

Little to no 3F4 bound to PrP^C in serially diluted RBC ghost samples after blotting, while 3F4 binding to brain PrP^C was readily detected (Fig. 3B). At the same time, 6H4 detected RBC PrP^C on blots with similar sensitivity to brain PrP^C (Fig. 3A). WB analysis revealed that RBC PrP^C was present mainly in its diglycosylated form, detected as a diffuse band with a molecular weight slightly higher (35-38 kDa) than brain PrP^C (Fig. 4A). Deglycosylation of PrP^C with PNGase F led to the detection of a single band with a molecular weight similar to deglycosylated brain PrP^C. No major fragments suggesting a significant presence of truncated forms of PrP^C in human RBCs were detected. Denaturation of the sample by boiling with SDS or its deglycosylation did not improve the binding of MoAb 3F4 to RBC PrP^C (Fig. 4B).

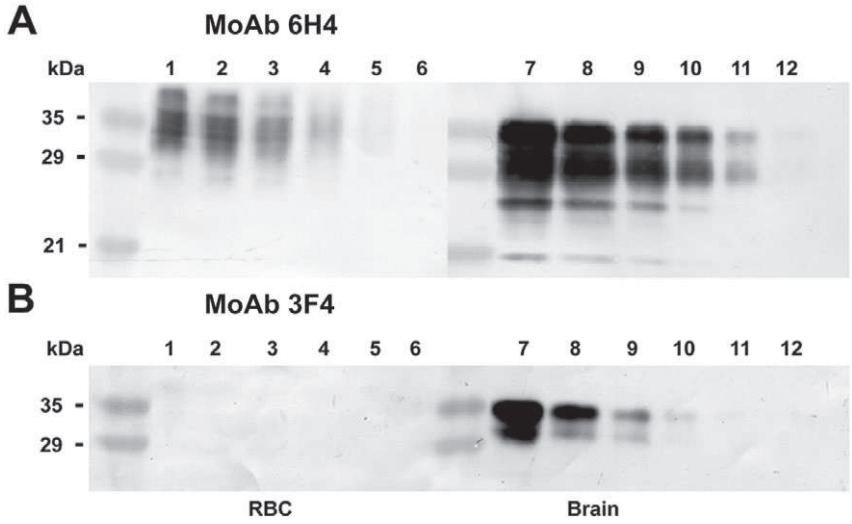

Fig. 3. WB comparison of PrP^C detection using MoAbs 6H4 and 3F4. WBs of serially twofold diluted samples of 1×10^{10}/mL human RBC ghosts (Lanes 1-6) and 10% normal human brain homogenate (Lanes 7-12) were developed with MoAb 6H4 (A) or 3F4 (B). Binding of 3F4 to RBC PrP^C is not improved by denaturation of proteins after boiling with electrophoretic sample buffer, demonstrating that poor binding of 3F4 to human RBCs is not caused by occlusion of its epitope by conformational change or the interaction of PrP^C with its binding partner on the surface of cells. A plausible explanation of poor 3F4 binding is covalent modification of its epitope in RBC PrP^C.

Sensitivity of RBC PrP^C to PK

The incubation of solubilized RBC ghosts with increasing concentrations of PK led to gradual and complete cleavage of PrP^C, demonstrating that RBC PrP^C is similarly as sensitive to PK as PrP^C in equally treated samples of PLTs (not shown) or brain homogenate (Fig. 4C). A similar result was obtained after PK treatment of intact RBCs and PLTs with subsequent analysis of the presence of PrP^C using flow cytometry (not shown).

FACS analysis of 3F4 and 6H4 binding to CD71+ erythroid cells in cord blood

In contrast to peripheral RBCs, equal binding of 3F4 and 6H4 to transferrin receptor–positive (CD71+) erythroid precursors in cord blood was recorded (Fig. 5A).

The influence of in vitro modification of brain PrP^C or recombinant human PrP on the binding of MoAb 3F4

The low binding of 3F4 to RBC PrP^C can be caused by modification of its epitope (KTNMKHM). Oxidation of brain PrP^C with increasing concentrations of hydrogen peroxide up to 130 mmol/L had little effect on the binding of 3F4. At 640 mmol/L, the binding of both 3F4 and 6H4 was diminished (Fig. 5B). In contrast, modification of lysine residues with increasing concentrations of Sulfo-NHS-biotin led to a gradual loss of 3F4 binding to PrP^C (not shown). Similarly, the modification of lysine residues to N^{ε}-(carboxymethyl)lysine (CML) by incubation with increasing concentrations of GA caused complete inhibition of 3F4 binding, while the binding of 6H4 was not affected (Fig. 5B). Similar results were obtained after GA treatment of hrPrP adsorbed on ELISA plates. Concentrations of GA above 50 mmol/L led to the complete loss of 3F4 binding, while the binding of AG4 was not affected (not shown).

DISCUSSION

The presence of TSE infectivity in blood has repeatedly been demonstrated in various species, including mouse, hamster, sheep, deer, and nonhuman primates.[22,23] In addition, the presence of infectivity in the blood of donors incubating vCJD was revealed by the transmission of the disease to three transfusion recipients.[4] All of them received nonleukoreduced RBCs. The nature of cells associated with TSE infectivity in blood remains unclear. In rodents, infectivity seems to be concentrated in WBCs, while both PLTs and RBCs are devoid of significant infectivity.[24,25] Interestingly, PLTs harbor infectivity in the blood of infected deer,[23] suggesting that association of prions with blood cells could be species and prion strain dependent. The expression of PrP^C may play a role in this discrepancy because rodent PLTs, in contrast to deer PLTs, do not express the protein.[17,18] Considering the importance of PrP^C expression in many aspects of prion pathogenesis, our study was aimed at the elucidation of controversies surrounding its expression in human RBCs. PrP^C is present on CD34+ marrow stem cells, and its expression is regulated in different cell lineages.[14,26] Griffiths and colleagues[27] demonstrated that PrP^C expression in cultured human erythroblasts was mostly intracellular, with faint membrane staining, and it was down regulated during their in vitro differentiation to more mature erythroid cells. This finding is compatible with the low expression of the protein on the membrane of circulating RBCs detected in our previous study.[18] However, our finding was contradicted by several studies reporting no expression of PrP^C on human RBCs.[12-16] Most of those studies utilized probably the best characterized prion MoAb 3F4.[28] The antibody was developed against hamster scrapie–associated fibrils; however, it also crossreacts with human PrP^C. The epitope of the 3F4

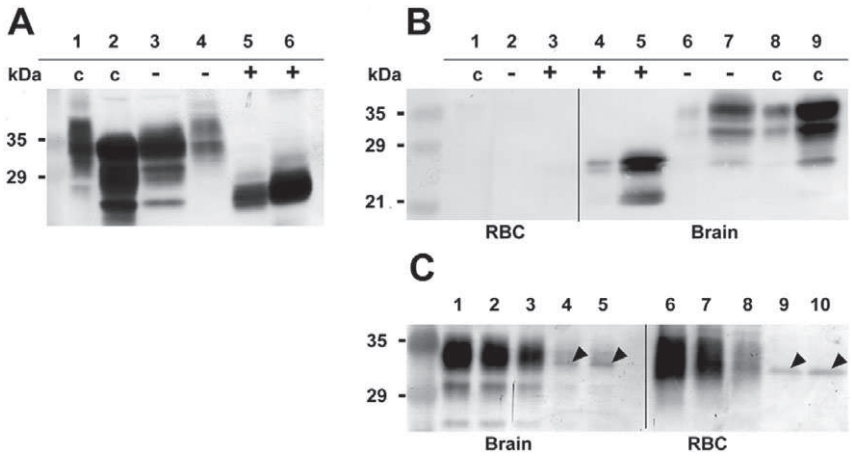

Fig. 4. Characterization of basic features of PrP^C on human RBCs. (A) WB comparison of RBCs (Lanes 1, 4, and 5) and brain (Lanes 2, 3, and 6) PrP^C demonstrate that RBC PrP^C is not significantly truncated. (c) Fresh untreated samples, (+) samples deglycosylated using incubation with PNGase F, and (–) samples incubated without PNGase F. The blot was developed with a mixture of MoAbs 6H4 and AG4. (B) Deglycosylation of RBC PrP^C does not lead to the restoration of 3F4 binding. RBC (Lanes 1-3) and brain (Lanes 4-9) samples were (c) fresh untreated, (+) treated with PNGase F, or (–) incubated without PNGase F. Samples 4, 6, and 8 are 10-fold dilutions of Samples 5, 7, and 9, respectively. The blot was developed with MoAb 3F4. (C) Brain and RBC PrP^C displayed similar sensitivity to proteolysis by increasing concentrations of PK (0, 2.5, 5, 50, and 100 μg/mL). Lanes 1 through 5 = brain samples; Lanes 6 through 10 = RBC samples. Sharp bands (~33 kDa) in Lanes 4, 5, 9, and 10 represent a nonspecific signal of PK (arrowhead). The blot was developed with MoAb 6H4.

(KTNMKHM) is located in the central part of the molecule ($PrP_{106-112}$),[29] which is known to undergo conformational change into PrP^{TSE}, resulting in epitope inaccessibility.[11] To elucidate the controversies surrounding the expression of PrP^C on human RBCs, we first repeated the quantitative flow cytometry study utilizing MoAb 6H4 and two MoAbs that were used in previous negative reports: FH11 and 3F4. Indeed, both antibodies bound to RBCs substantially less than 6H4: 8.3-fold lower for FH11 and 3.6-fold lower for 3F4. This result was puzzling, especially for 3F4, as its binding to PLTs in the same tube was equivalent to 6H4. The overall lower binding of FH11 to a variety of blood cells has been previously described[30] and can be caused by a proteolytic loss of its epitope (PrP_{51-55}) located on the sensitive N-terminus of PrP^C.[31] A similar mechanism could also be responsible for the lower binding of 3F4, although its epitope ($PrP_{106-112}$) is located close to the center of the molecule. The presence of a substantial amount of the stable C1 fragment of PrP^c lacking the 3F4 epitope has been described in the normal brain tissue of man and other mammalian species.[32,33] In contrast, we did not find similar fragments in blots of human RBC ghosts, demonstrating that truncation was not the cause of the decreased 3F4 binding. The RBC PrP^C band had slightly lower electrophoretic mobility and was more diffuse than PrP^C in the brain. This could be caused by a different glycosylation pattern of RBC PrP^C, which may in theory contribute to the inaccessibility of the 3F4 epitope. However, enzymatic deglycosylation of RBC PrP^C did not restore its recognition by 3F4. Considering that the binding of 3F4 is prevented by the change in the conformation of protease-resistant PrP^{TSE},[11] we tested the sensitivity of PrP^C on RBCs to proteolysis. However, we did not detect any protease-resistant PrP^C on RBCs. This, together with the fact that low binding of 3F4 was also recorded on blots after denaturation of proteins by boiling with SDS suggests that the low binding is not caused by the conformation of the protein. On the same basis, the hypothesis that the low binding of 3F4 is caused by the shielding effect of an unknown binding partner of PrP^C on the membrane of RBCs can be rejected. Taken together, our results suggest that the reason for decreased binding of 3F4 to RBC PrP^C is posttranslational modification of its epitope KTNMKHM. This modification is apparently introduced after the maturation and release of RBCs into circulation as CD71+ erythroid precursors in cord blood bound antibodies 3F4 and 6H4 equally well. The modification does not significantly change the electrophoretic mobility of RBC PrP^C because after its deglycosylation, it has similar mobility to deglycosylated brain PrP^C. The importance of both methionine and lysine residues in the 3F4 epitope for binding of the antibody has been previously demonstrated.[29,34] Methionine residues in the 3F4 epitope have been shown to be sensitive to oxidation and may serve as internal antioxidants, protecting the PrP^C molecule from oxidative damage.[35] However, in our hands, oxidation of PrP^C with H_2O_2 did not diminish binding of 3F4 relative to 6H4, suggesting that oxidation is not the cause of the low binding of 3F4 to RBC PrP^C. In contrast, modification of lysine residues by sulfo-NHS-biotin readily led to the loss of 3F4 binding, confirming a previous report by Bolton and coworkers.[36] One physiologically occurring modification of lysine residues in proteins is glycation. The treatment of prion protein with GA, which modifies lysine residues to the major advanced glycation endproducts constituent CML, led to a dose-dependent decrease of 3F4 binding, while the binding of 6H4 was unaffected. The modification of membrane proteins by CML in human RBCs during their aging in circulation has been previously reported.[21] This is in agreement with our observation that the epitope of 3F4 in erythroid precursors in cord blood as well as in PLTs in circulation is not modified. Obviously, time is needed for

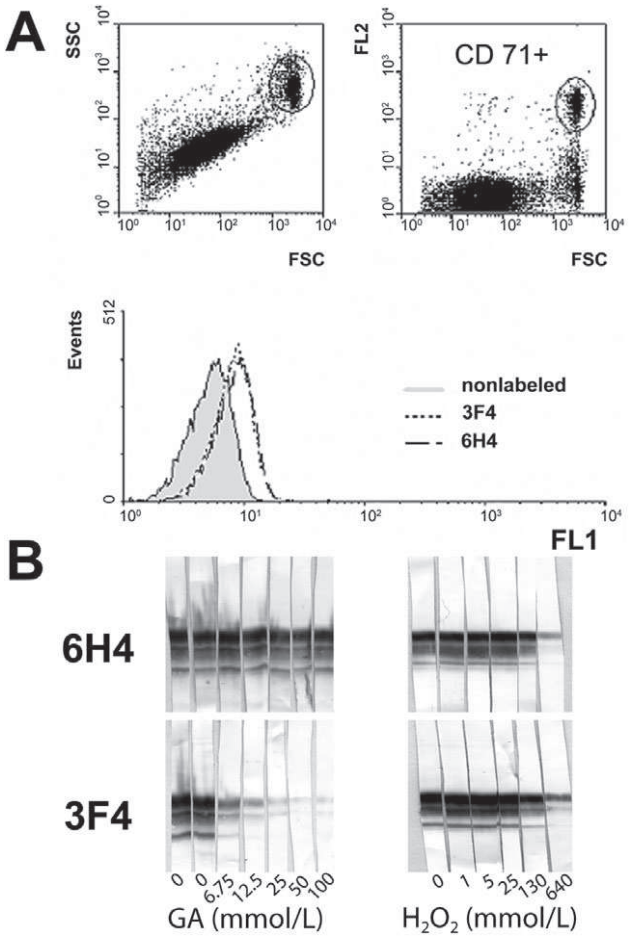

Fig. 5. Glycation of PrP^C could be the reason for diminished binding of 3F4 to human RBCs. (A) MoAbs 3F4 and 6H4 bind equally well to transferrin receptor–positive (CD71+) erythroid precursors in human cord blood. This suggests that the modification of the 3F4 epitope (KTNMKHM) occurs after the release of RBCs into circulation. Normal cord blood (n = 3) samples were analyzed using two-color flow cytometry. The logarithmic side scatter/forward scatter (SSC/FSC) plot shows the scatter properties of cord blood and the position of the RBC gate (top left plot). CD71+ cells were gated on a FL2/FSC logarithmic plot (top right plot) and their 3F4 and 6H4 fluorescence compared (bottom histogram overlay). (B) In vitro treatment of WBs of normal brain homogenate with increasing concentrations of GA or hydrogen peroxide (H_2O_2). Blots were developed with 6H4 (top) or 3F4 (bottom). Modification of lysine residues by GA to carboxymethyl lysine in contrast to oxidation of methionines by H_2O_2 readily mimics the discrepancy in the binding of MoAbs 3F4 and 6H4 found in vivo in human RBC PrP^C, suggesting that glycation may be the cause of the modification of its 3F4 epitope.

the modification to occur, and RBCs, with their life span reaching 120 days compared to the 10 days of PLTs, are in a better position to be modified. Interestingly, glycation of lysine residues on the N-terminal of PrP^{TSE} has already been demonstrated in brains of TSE-infected rodents.[37] Despite the lack of direct proof, our data suggest that the diminished binding of 3F4 to PrP^C on RBCs may be caused by glycation. The hypothetical existence of a similar modification of blood PrP^{TSE} would prevent its detection using the conformation-dependent immunoassay[11] and other assays that use 3F4. In addition, it is likely that the use of the 3F4 antibody was the reason for the underestimation of the overall amount of the PrP^C pool associated with human RBCs in some previous studies. All three methods employed in our study to estimate the amount of PrP^C in RBCs relative to PLTs gave convergent results. Using quantitative flow cytometry, approximately 300 IgG molecules of antibody 6H4 bound to one RBC, while the binding to resting PLTs in identical samples was only twice as high. We have previously estimated that approximately 70% of PrP^C is localized inside of resting PLTs.[38] This suggests that the amount of PrP^C per PLT, including its intracellular pool, is roughly sevenfold higher than in one RBC. Conformation-independent WB analysis of PrP^C levels in carefully isolated PLTs and RBC ghosts indicated that the amount of PrP^C per PLT is just fourfold higher than in one RBC. An even lower difference was detected using sandwich ELISA. Taken together, our data demonstrate that the difference in the level of PrP^C between one PLT and one RBC is less than 10-fold, while the normal amount of RBCs (5×10^9/mL) in man is about 20-fold higher than the amount of PLTs (2.5×10^8/mL). This result implies that RBCs should carry at least twice as much PrP^C than PLTs, which makes RBCs the main source of cell-associated PrP^C in human blood. Previous studies have described the release of up to 45% of PLT PrP^C after in vitro activation of PLTs by agonists or during the 5-day storage of PLT concentrates.[39,40] Although we deem it unlikely, we cannot exclude that some release of PrP^C also occurred during the isolation of PLTs used in our WB and ELISA studies. However, even if we underestimated the physiological amount of PLT PrP^C by approximately 50%, the overall amount of PrP^C associated with RBCs would still surpass the PrP^C PLT pool.

Despite this observation, RBCs remain an unlikely source of prions in blood because they do not have the ability to supplement their propagation with new molecules of PrP^C. However, the existence of posttranslational modification in the critical part of the molecule may give RBC PrP^C unique properties and favor their binding of prions released to blood by other cells. Further studies aimed on the molecular characterization of this modification and its influence on the interaction of PrP^C with prion particles should clarify if our current view neglects the role of RBCs in the blood pathogenesis of prion diseases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research paper

Blood storage affects the detection of cellular prion protein on peripheral blood leukocytes and circulating dendritic cells in part by promoting platelet satellitism

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ABSTRACT

Background: Flow cytometry represents an attractive approach for developing currently unavailable screening tests for prion diseases. Several studies have reported significant differences in the binding of antibodies directed against cellular prion protein (PrP^C) to blood cells of prion-infected subjects compared with healthy controls. However, flow cytometry data usually show large individual variations in detected PrP^C levels in both infected and control groups, rendering the interpretation of individual patient data difficult.
Objectives: To determine how pre-analytical variables, such as the choice of anticoagulant, whether or not the blood was stored, and the storage temperature, affect the detection of PrP^C in blood cells.
Methods: Blood from healthy donors was collected in EDTA or citrate anticoagulant and processed either immediately or after storage overnight at room temperature or at 4 °C. The expression of PrP^C by T cells, B cells, NK cells, monocytes and circulating dendritic cells was evaluated using quantitative flow cytometry with the PrP^C monoclonal antibodies AG4 and AH6.
Results: The anticoagulation of blood with citrate resulted in decreased levels of PrP^C on monocytes but not the other cell types. The storage of blood prior to analysis led to a significant decrease in the levels of PrP^C on the cells studied, although there were substantial differences between the cell populations. This decrease was more pronounced when using mAb AG4, which targets the N-terminal portion of the PrP^C molecule, or following storage at room temperature. Moreover, we identified platelet satellitism on leukocytes, especially on monocytes and granulocytes, as an additional factor contributing to the heterogeneity of PrP^C detection in stored blood.
Conclusions: Our study demonstrates that the storage of blood prior to analysis greatly affects the detection of PrP^C by flow cytometry. To limit the inclusion of storage-generated artifacts, we recommend the processing of blood samples immediately after their collection.

1. Introduction

Cellular prion protein (PrP^C) is crucial for the pathogenesis of prion diseases (Brandner et al., 1996), and its conversion to the abnormal form PrP^{TSE} is an essential feature of those diseases (Prusiner, 1998). Human prion diseases are fatal, neurodegenerative disorders for which no routine screening test or treatment is yet available. PrP^{TSE} is likely the only constituent of the infectious prion particle and is the only known specific marker for prion diseases.

Several recent studies have demonstrated the presence of prion infectivity or PrP^{TSE} in the blood cells of prion-infected subjects (Brown, 2005; Saa et al., 2006; Edwards et al., 2010; Mathiason et al., 2010). In addition, four blood transfusion-

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related infections of variant Creutzfeldt–Jakob disease were reported in the United Kingdom (Chohan et al., 2010). Flow cytometry, with its vast analytical potential, represents a promising approach for the development of mechanisms for detecting PrP^{TSE} in the blood but is hindered by limited knowledge about the nature of PrP^{TSE} in the blood and the unavailability of reliable PrP^{TSE}-specific antibodies. Therefore, flow cytometry studies to date have generally focused on mapping PrP^{C} expression. PrP^{C} has been found in many blood cells, including T and B lymphocytes, NK cells, monocytes, dendritic cells (Barclay et al., 1999; Li et al., 2001), and platelets (Holada et al., 1998; Barclay et al., 1999). Lower levels of PrP^{C} have been detected in red blood cells (Holada and Vostal, 2000; Panigaj et al., 2011), whereas granulocytes express little to no PrP^{C} (Cashman et al., 1990; Barclay et al., 1999). Interestingly, several studies utilizing antibodies recognizing PrP^{C} reported differences in their binding to blood cells of prion-infected individuals. In patients with sporadic Creutzfeldt–Jakob disease, a significantly lower level of binding of the monoclonal antibody (mAb) 3F4 to lymphocytes compared with control patients with other neurological diseases has been reported (Ratzka et al., 2003). In infected macaques, the level of binding of mAb 12F10 to blood lymphocytes increases long before the onset of clinical symptoms (Holznagel et al., 2010). In addition, an increase in the binding of mAbs to the N- and C-terminal regions of PrP^{C} and a decrease in binding to the central part of the molecule was reported in the leukocytes of sheep that were experimentally infected with scrapie (Thackray et al., 2005). Despite these encouraging reports, flow cytometry remains far from being developed into a screening blood test for prion diseases. Flow cytometry data usually demonstrate relatively large variations in PrP^{C} levels within a particular cell type, even in cohorts of healthy controls that overlap with values obtained from patient groups. The source of this variation, aside from natural, individual variations in PrP^{C} expression, may originate in the pre-analytical procedures, which include the choice of anticoagulant and the storage of blood samples. Moreover, blood processing that precedes the analysis may affect platelets, which are known to associate with monocytes and neutrophils resulting in a phenomenon called platelet satellitism. The platelet satellitism may be caused by different mechanisms, such as the presence of platelet autoantibodies or by platelets activation with their subsequent adhesion to leukocytes via P-selectin (Peters et al., 1997; Barnard et al., 2005). As human platelets express substantial levels of PrP^{C}, their interaction with leukocytes may contribute to the PrP^{C} levels detected on the latter cells.

The evaluation of pre-analytical procedures is a necessary step towards the development of flow cytometry-based detection tests for prion diseases (Green et al., 2010). The present study was undertaken to identify the effects of basic pre-analytical factors on the detection of PrP^{C} by flow cytometry on diverse populations of white blood cells.

2. Materials and methods

2.1. Subjects and blood samples

Blood samples from 10 healthy adults (26–47 years old, median age 31 years old) were provided by the Transfusion Department of the General Teaching Hospital in Prague after informed consent in accordance with the guidelines of The Declaration of Helsinki was obtained. Peripheral blood was drawn by venipuncture into vacutainers either containing 5.4 mg of K_2EDTA (BD, San Jose, CA, USA) or 0.129 M sodium citrate (BD). One-milliliter aliquots from each tube were transferred under sterile conditions into two empty vacutainers (BD). One aliquot was stored overnight at room temperature (RT) and another at 4 °C. The remaining blood was kept at RT and processed within 2 h following collection.

2.2. Antibodies

2.2.1. Anti-prion monoclonal antibodies

The detection of PrP^{C} on the surface of cells was accomplished using mouse anti-prion monoclonal antibodies (mAbs) directed against different epitopes of the prion protein: AG4 (IgG2a, PrP 31–51, final concentration 5 μg/mL) and AH6 (IgG2b, PrP 159–175, final concentration 6 μg/mL), both from TSE Resource Centre, Compton, England (catalog nos. RC058 and RC059, respectively). Both mAbs were custom-conjugated to phycoerythrin (PE) (Exbio, Prague, Czech Republic).

2.2.2. Isotype controls

The isotype controls IgG2a-PE (clone PPV-04; Exbio, catalog no. 1P-458-C100), and IgG2b-PE (clone PLRV219; Exbio, catalog no. 1P-801-C100) were used at a final concentration of 5 μg/mL for the evaluation of non-specific binding of anti-prion antibodies.

2.2.3. Antibodies used for lymphocyte (LY) identification

For the identification of LY subsets, the following antibodies were used: anti-CD3-PerCP (clone SK7; BD, catalog no. 345766), anti-CD19-APC (clone LT19; Exbio, catalog no. 1F-305-T100), and anti-CD56-FITC (clone MEM-188, Exbio, catalog no. 1F-231-T100); all were used at a 1/20 dilution.

2.2.4. Antibodies used for dendritic cell (DC) identification

The identification of circulating DCs was based on a negative-gating strategy using a cocktail of antibodies against leukocyte lineage markers (all custom prepared by Exbio, 5× concentrated and used at 1/50 dilution): anti-CD3-FITC (clone MEM-57; Exbio, catalog no. 1F-202-T100), anti-CD14-FITC (clone MEM-15; Exbio, catalog no. 1F-293-T100), anti-CD16-FITC (clone LNK16; Exbio, catalog no. 1F-399-T100), anti-CD19-FITC (clone LT19; Exbio, catalog no. 1F-305-T100), anti-CD20-FITC (clone LT20; Exbio, catalog no. 1F-414-T100), anti-CD56-FITC (clone MEM-188; Exbio, catalog no. 1F-231-T100), and anti-HLA-DR-PE-Dy647 (clone MEM-12; Exbio, catalog no. 1Y-474-T100, used at a 1/10 dilution).

2.2.5. Antibodies used to evaluate platelet satellitism

The proportion of leukocytes with bound platelets and the expression of PrP^{C} on the cells were evaluated using anti-CD45-PE-Dy647 (clone MEM-28; Exbio, catalog no. 1Y-222-T100, used at 1/10 dilution), anti-CD41-PE (clone P2; Immunotech, catalog no. IM1416U, used at a 1/10 dilution), and AH6-FITC (TSE Resource Centre; custom conjugated to FITC using the Fluorescein Labeling Kit (Roche Diagnostics,

Mannheim, Germany, catalog no.11386093001), used at a final concentration of 6 μg/mL).

2.2.6. Antibodies used for compensation

Single-color compensation controls were stained with the following mAbs: anti-CD8-FITC (clone HIT8a; BD, catalog no. 555634), anti-CD8-PE (clone HIT8a; BD, catalog no.555635), anti-CD19-APC (clone LT19; Exbio, catalog no. 1F-305-T100), and anti-HLA-DR-PE-Dy647 (clone MEM-12; Exbio, catalog no. 1Y-474-T100) for the DC panel and anti-CD3-PerCP (clone SK7; BD, catalog no. 345766) for the LY panel. All mAbs were used at a 1/10 dilution.

2.3. Flow cytometry

The staining of whole blood was performed for 15 min at RT in a final volume of 50 μL for the LY panel and 100 μL for the DC panel. All mAbs were used at saturating concentrations as estimated by previous titration (not shown). After staining, the erythrocytes were lysed using 1 mL BD FACS™ Lysing solution (BD, catalog no. 349202) according to the manufacturer's instructions, and the samples were washed with 2 mL PBS containing 0.1% BSA (450 g, 5 min, RT). Cells were resuspended in PBS containing 0.1% BSA and immediately analyzed using the FACS Canto II flow cytometer (BD) equipped with BD FACSDiva v6.0 software (BD). The instrument was equipped with three lasers, and the optical configuration was as supplied by manufacturer. The instrument was calibrated before every measurement using BD™ Cytometer Setup and Tracking Beads (BD, catalog no. 641319). Unstained cells and single fluorochrome-stained cells were used for software compensation prior to the sample analysis. Five hundred events in the DC gate (DC panel) created in a lineage cocktail-FITC vs. HLA-DR-PE-Dy647 plot were acquired, and 50,000 events in the leukocyte gate (LY panel) were acquired using a protocol with a FSC vs. SSC plot in linear mode excluding debris.

Quantum™ R-PE MESF beads (Bangs Laboratories, Fishers, IN, USA, catalog no 827), which were used to quantify the measured fluorescence signals, were run each day as a separate tube before and after the acquisition of the samples.

2.4. Gating strategy

Our gating strategy to identify DCs is shown in Fig. 1A. After gating on target cell populations in the FSC vs. SSC plot, DCs were identified as lineage cocktail-negative ($CD3^{neg}$ $C14^{neg}$ $CD16^{neg}$ $CD19^{neg}$ $CD20^{neg}$ $CD56^{neg}$) and $HLA\text{-}DR^{high}$ cells. Lymphocyte subtypes were identified in the FSC vs. SSC plot after gating on lymphocytes (LY), monocytes (M), and granulocytes (G) (Fig. 1B). Cells in the lymphocyte gate were identified as B cells ($CD19^{pos}$ $CD3^{neg}$), T cells ($CD3^{pos}$ $CD19^{neg}$), and NK cells ($CD3^{neg}$ $CD56^{pos}$).

The gating strategy to identify $CD41^{pos}$ cells in the leukocyte subsets is shown in Fig. 3A. After gating the leukocytes in the CD45 vs. SSC plot, lymphocytes ($CD45^{high}$ SSC^{low}), monocytes ($CD45^{pos}SSC^{med}$), and granulocytes ($CD45^{low}SSC^{high}$) were identified. Platelets bound to leukocytes were recognized in the CD45 vs. CD41 plot as $CD41^{pos}$ cells in each leukocyte subset. The levels of PrP^C expression on $CD41^{pos}$ and $CD41^{neg}$ cells were evaluated by comparing the medians of fluorescent intensities of the peaks obtained after the labeling of blood with mAb AH6-FITC.

2.5. Quantification of PrP^C molecules expressed per cell

The quantification of cell-bound anti-prion monoclonal antibodies was performed using Quantum™ R-PE MESF beads (Bangs Laboratories, catalog no. 827) as described previously (Holada and Vostal, 2000). Briefly, a calibration regression line was constructed using molecules of equivalent soluble fluorochrome (MESF) values of Quantum™ R-PE MESF beads and the median of their measured fluorescence intensity. The MESF value for each sample was calculated using the equation of the calibration line and the median fluorescence of the cells.

The effective PE/IgG ratio was assessed for each anti-prion mAb using Quantum™ Simply Cellular® anti-Mouse IgG kit (Bangs Laboratories, catalog no. 815) and Quantum™ R-PE MESF beads (Bangs Laboratories) as described previously (Holada and Vostal, 2000). The PrP^C expression per cell was derived from the numbers of anti-prion mAb molecules bound per cell, assuming that one molecule of the antibody binds to one PrP^C molecule.

2.6. Statistics

Statistical data analyses were performed using SigmaStat v3.5 software (Systat Software Inc., San Jose, CA, USA). The data were analyzed for significant differences between the groups of samples using the paired student's t-test, and a P value of 0.05 was considered significant.

3. Results

3.1. Detection of PrP^C in fresh blood

Assuming that one molecule of IgG bound to one molecule of PrP^C, the number of PrP^C molecules per cell varied from 1000 on B cells to more than 5000 on NK cells (Table 1). In fresh blood, we detected significantly higher levels of PrP^C on NK cells and DCs in EDTA and on NK cells, B cells, and DC in citrate using the mAb AH6 versus AG4 (Fig. 2). In monocytes, we detected higher levels of PrP^C with AG4, but the difference was not significant. The type of anticoagulant used had little effect on the detection of PrP^C in fresh blood, as there were no significant differences between the EDTA and citrate samples for T cells, B cells, NK cells, or DCs. However, for monocytes we detected significantly lower levels of PrP^C in citrate-anticoagulated compared to EDTA-anticoagulated samples (Fig. 2).

3.2. Expression of PrP^C on analyzed cell populations following the 24-hour storage of blood

Storage generally led to a decrease in the detection of PrP^C; however, large differences among cell types were recorded (Fig. 2), and the decrease was more profound using the mAb AG4. B cells displayed an almost complete loss of PrP^C expression as detected by AG4, with only 190 ± 190 (mean ± SD) PrP^C molecules per cell in samples stored for 24 h in EDTA compared with 850 ± 410 (mean ± SD)

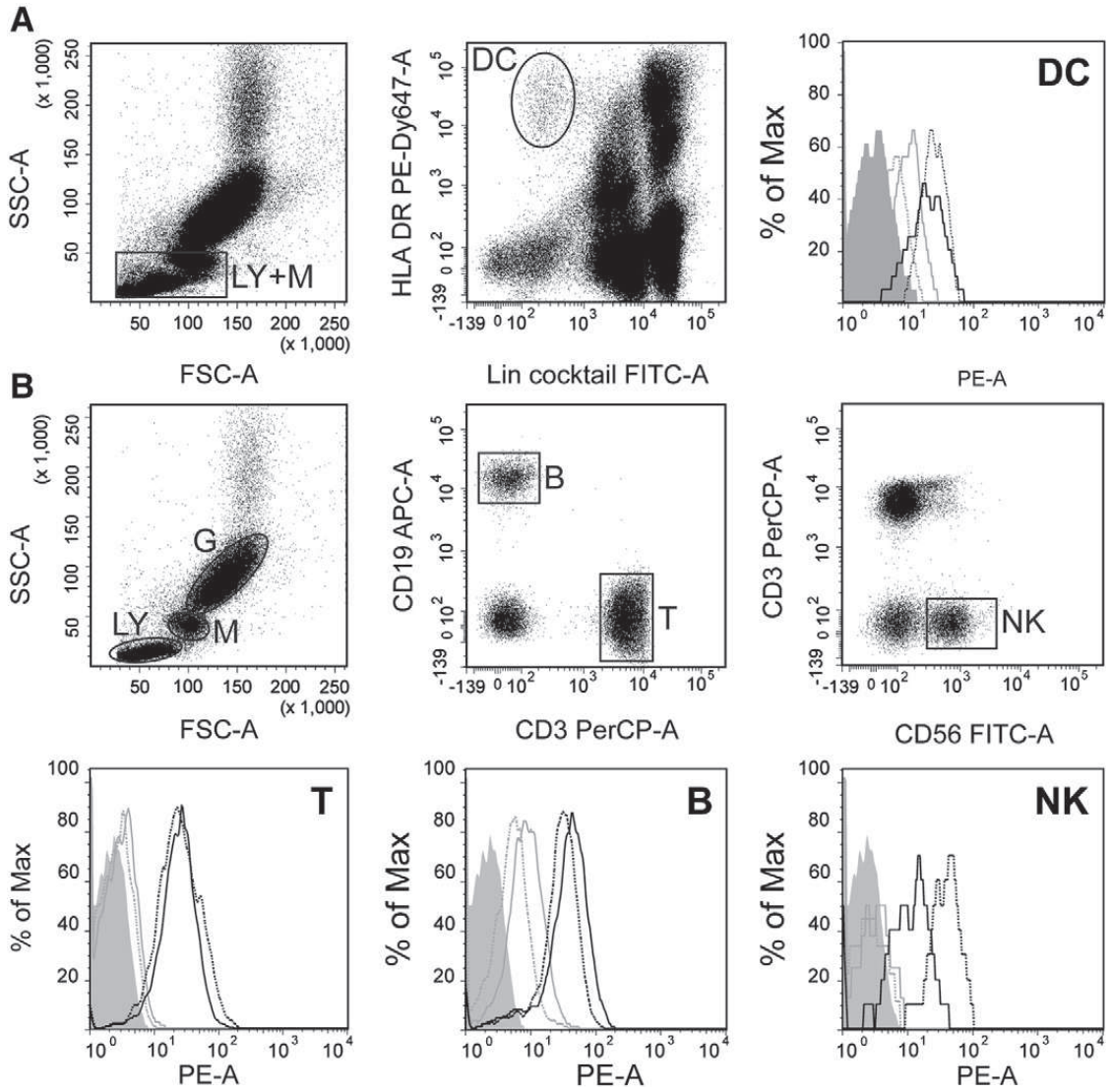

Fig. 1. Gating for DCs and lymphocytes and representative PrP^C expression by analyzed cell populations. (A) After pre-selecting target populations (lymphocytes and monocytes) in the FSC vs. SSC plot, DCs were gated as lineage cocktail-negative and $HLA\text{-}DR^{high}$. The flow cytometry histogram plot shows the levels of PrP^C expressed by DCs. The solid black line represents mAb AG4, the dotted black line represents mAb AH6, and the solid gray and dotted gray lines represent the isotype controls IgG2b and IgG2a, respectively. The background fluorescence of non-labeled cells is shown in the gray-filled histogram. (B) Lymphocytes (LY), monocytes (M), and granulocytes (G) were identified in the FSC vs. SSC plot. Next, T cells were gated as $CD3^{pos}$ $CD19^{neg}$, B cells as $CD19^{pos}$ $CD3^{neg}$, and NK cells as $CD3^{neg}$ $CD56^{pos}$. Representative levels of PrP^C as expressed by T cells, B cells, and NK cells are shown in the flow cytometry histogram plots. The solid black and dotted black lines represent mAb AG4 and AH6, respectively. The solid gray and dotted gray lines represent the isotype controls IgG2b and IgG2a, respectively. The gray-filled histogram shows the background fluorescence of non-labeled cells.

molecules per cell in fresh samples ($P=0.0003$). A similar decrease was observed for citrate-stored samples. However, no significant changes in B cell PrP^C levels were detected with mAb AH6. In NK cells, storage led to a significant decrease in PrP^C levels as detected by AH6 in citrate and in both citrate and EDTA using AG4 (Fig. 2 and Supplementary data Table 1). For T cells, we detected moderately decreased levels of PrP^C after storage; however, a significant decrease was recorded only using AG4 in EDTA samples. DCs displayed significantly decreased levels of PrP^C in citrate-stored samples using AH6 and in both EDTA- and citrate-stored samples using AG4. The PrP^C levels in monocytes were significantly decreased in EDTA-stored samples using AG4; however, we recorded a significant increase in PrP^C detection in citrate-stored samples using AH6. We detected 2150 ± 450 (mean $\pm$ SD) PrP^C molecules in fresh blood in citrate using AH6 and 3100 ± 900 (mean $\pm$ SD) in stored samples ($P=0.0147$) (Fig. 2 and Supplementary data Table 1). Increases in PrP^C detection in stored samples using AH6 were also detected for granulocytes (Supplementary data Table 1).

3.3. Storage temperature

We generally detected lower levels of PrP^C with AG4 in samples that had been stored at RT compared with samples stored at 4 °C; however, the difference reached significance only for DCs stored in EDTA. Significant decreases in PrP^C levels with AH6 were detected only for DCs and monocytes from samples stored in citrate at RT. (Fig. 2 and Supplementary data Table 1).

Table 1
PrP^C levels expressed by $CD41^{neg}$ and $CD41^{pos}$ leukocyte subsets. Shown are the levels of PrP^C expression as the median fluorescence intensity of the anti-prion mAb AH6 per cell on $CD41^{neg}$ and $CD41^{pos}$ lymphocytes, monocytes, and granulocytes. Data are the mean ± SD from 6 healthy donors. Paired student's t-test was used for statistical analyses.

AH6 antibody median fluorescence per cell (Mean ± SD)						
	EDTA 2 h	EDTA 24 h (4 °C)	EDTA 24 h (RT)	Citrate 2 h	Citrate 24 h (4 °C)	Citrate 24 h (RT)
Lymphocytes						
$CD41^{neg}$	190 ± 40	150 ± 70	170 ± 90	200 ± 10	110 ± 70	100 ± 50
$CD41^{pos}$	210 ± 40	160 ± 90	160 ± 70	**220 ± 20	150 ± 90	*140 ± 70
Monocytes						
$CD41^{neg}$	250 ± 10	210 ± 60	260 ± 60	220 ± 10	220 ± 90	210 ± 60
$CD41^{pos}$	**280 ± 20	270 ± 120	310 ± 100	**240 ± 10	*260 ± 100	*240 ± 70
Granulocytes						
$CD41^{neg}$	150 ± 10	260 ± 260	190 ± 50	160 ± 10	190 ± 110	190 ± 120
$CD41^{pos}$	**170 ± 20	370 ± 280	340 ± 160	**180 ± 10	*320 ± 180	300 ± 320

*$P<0.05$ and **$P<0.005$ ($CD41^{pos}$ vs. $CD41^{neg}$).

3.4. Anticoagulant type in stored samples

The type of anticoagulant did not appear to influence the detection of PrP^C in neither the samples stored at RT nor in samples stored at 4 °C. The only exception was in the case of B cells, in which we detected significantly higher levels of PrP^C with AH6 in citrate-stored compared with EDTA-stored samples at RT (Fig. 2 and Supplementary data Table 1).

3.5. Platelet satellitism

We detected a remarkable portion of $CD41^{pos}$ (platelet-associated) leukocytes, and this portion was further increased following storage. The effect was more obvious in citrate-stored samples, especially in the cases of monocytes and granulocytes (Fig. 3B). The degree of satellitism was different for individual donors. In fresh blood, we detected 25–29% $CD41^{pos}$ lymphocytes, monocytes, and granulocytes in EDTA samples. The percentage of $CD41^{pos}$ lymphocytes in citrate samples was similar; however, the percentage of $CD41^{pos}$ monocytes and granulocytes was increased, reaching 46% ± 16% and 37% ± 6% (mean ± SD), respectively. Following the storage of the blood samples, the percentage of $CD41^{pos}$ lymphocytes was changed minimally relative to storage conditions. The percentage of $CD41^{pos}$ monocytes in EDTA was also changed minimally after storage (at 4 °C or RT). However, in citrate samples, we detected 77% ± 18% and 79% ± 12% (mean ± SD) of $CD41^{pos}$ monocytes in samples stored at 4 °C and RT, respectively. The percentage of $CD41^{pos}$ granulocytes was moderately increased to 51% ± 24% (mean ± SD) in EDTA samples stored at 4 °C, whereas in citrate samples stored at 4 °C and RT, we detected 69% ± 20% and 50% ± 12% (mean ± SD) $CD41^{pos}$ granulocytes, respectively (Fig. 3B).

3.6. PrP^C levels in $CD41^{pos}$ platelet-associated leukocytes

A comparison of PrP^C expression in $CD41^{neg}$ and $CD41^{pos}$ lymphocytes, monocytes, and granulocytes of individual donors revealed increased levels of PrP^C in $CD41^{pos}$ cells (Table 1). The difference was significant for all cell populations in citrate fresh blood and for monocytes and granulocytes in EDTA. Storage in citrate, but not EDTA, led to a significant increase in PrP^C levels in $CD41^{pos}$ monocytes and granulocytes stored at 4 °C and in $CD41^{pos}$ lymphocytes and monocytes stored at RT. Importantly, great variations in the levels of PrP^C on $CD41^{pos}$ leukocytes were detected among individual donors (Fig. 4).

4. Discussion

The choice of antibody may greatly affect the detected levels of PrP^C on blood cells. In our study, the mAb AG4 (epitope 31–51 aa), which recognizes the N-terminal part of PrP^C, generally detected lower levels than the C-terminal-recognizing mAb AH6 (epitope 159–175 aa). This finding is in agreement with our previous study of PrP^C expression on blood cells of non-human primates, in which we also detected lower levels with the N-terminal mAb FH11 (epitope 51–55 aa) compared to the C-terminal mAb 6 H4 (epitope 144–152 aa) (Holada et al., 2007). In peripheral blood mononuclear cells, the N-terminal part of PrP^C was shown to be hypersensitive to proteolytic digestion (Li et al., 2001), offering a plausible explanation of these discrepancies. Alternatively, the discrepancies may originate from differences in the availability of separate PrP^C epitopes (Leclerc et al., 2003) due to the conformational heterogeneity of PrP^C or due to their modification, which we recently demonstrated for red blood cell PrP^C (Panigaj et al., 2010). The differences in the levels of PrP^C as detected by AG4 and AH6 were cell population-specific, suggesting the diverse processing of PrP^C in individual subsets of leukocytes. For example, AG4 and AH6 detected very similar and very different levels of PrP^C in T cells and NK cells, respectively. In accordance with previous studies by Barclay et al. (1999) and Durig et al. (2000), the highest numbers of PrP^C molecules were detected on NK cells followed by T cells, monocytes, and DCs, whereas B cells expressed low levels of PrP^C. This pattern was not affected by the choice of anticoagulant with exception of monocytes. Surprisingly, both AG4 and AH6 detected significantly decreased levels of PrP^C on monocytes in fresh blood anticoagulated with citrate. The reason for this phenomenon is unclear, but it could not be explained by the lower binding of PrP^C-expressing platelets to the cells, as the proportion of $CD41^{pos}$ monocytes was higher in citrate compared to EDTA. The overnight storage of blood had profound and cell type-specific effects on the detected levels of PrP^C. Previous

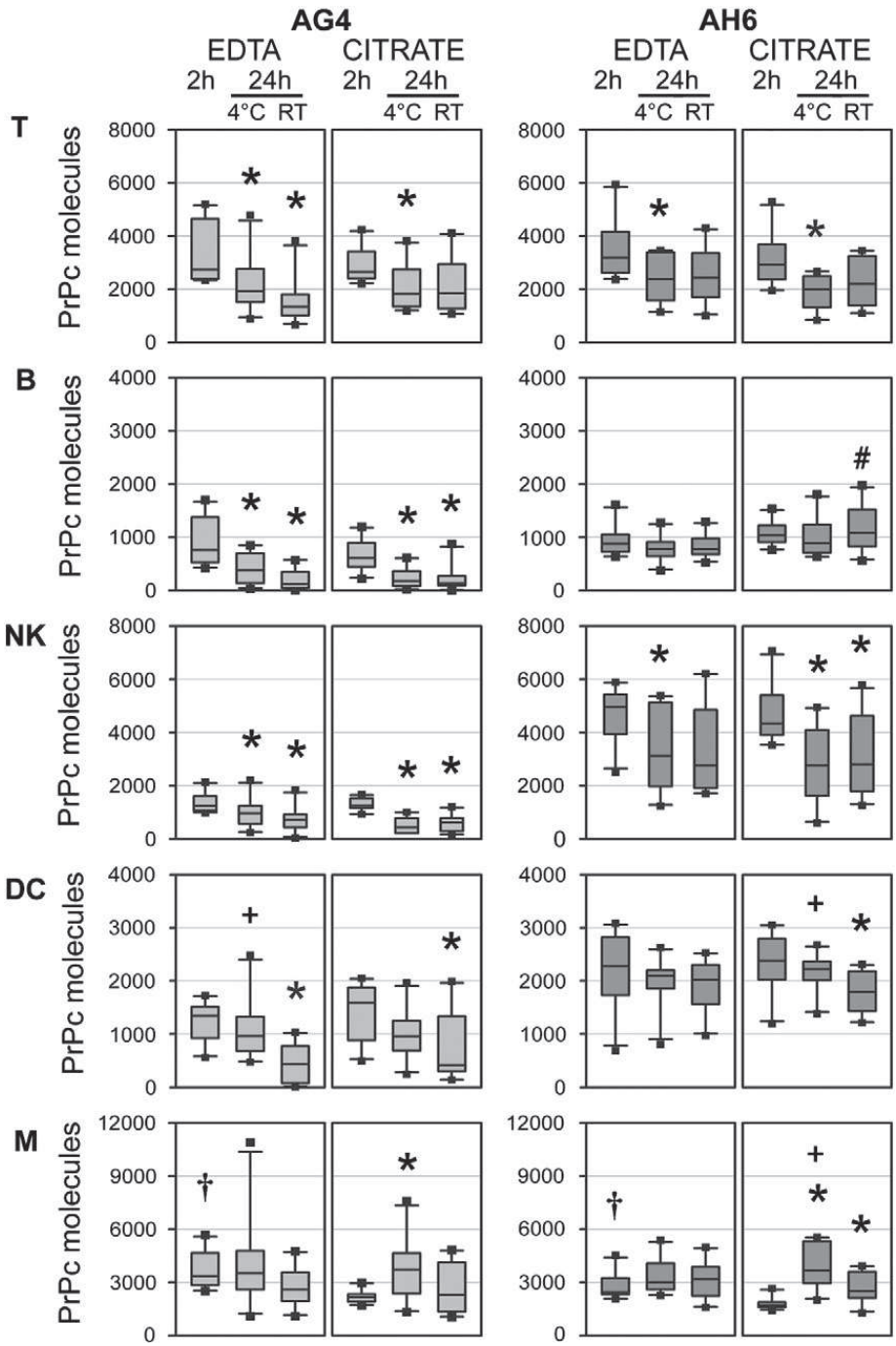

Fig. 2. The levels of PrP^C expression on T cells, B cells, NK cells, DCs, and monocytes in fresh blood and following overnight storage under different conditions. The data presented are the medians of the number of IgG molecules of anti-prion mAb bound per cell. Assuming an equimolar binding of mAb to PrP^C molecules, these data represent the number of PrP^C molecules expressed by a single cell. The box-and-whisker plot delineates the quartiles and median. Here, we present data from 10 healthy donors. $^*P<0.05$ vs. samples processed within 2 h after blood collection (2 h). $^+P<0.05$ vs. samples stored 24 h at RT. $^\#P<0.05$ vs. EDTA samples stored 24 h. $^\dagger P<0.05$ vs. EDTA samples processed within 2 h after blood collection (2 h).

studies evaluating the effect of storage on the expression of other cell surface molecules detected by flow cytometry have provided contradictory results. Ekong et al. (1992) observed a significant decrease in both CD3 and CD4 and a significant increase in CD8 in EDTA blood samples that were stored for 24–96 h at 4 °C. However, for samples stored at RT, CD8 remained stable while CD3 and CD4 increased significantly over time. Naranbhai et al. (2011) reported a progressive downregulation of the chemokine receptors CCR4 and CCR7 in NK cells after a 24-hour storage of blood at RT, and Schmidtke et al. (1999) reported unchanged levels of CD19 and CD45 after a 3-day storage of blood at 4 °C and RT but decreased surface immunoglobulins after storage at RT. We hypothesized that the type of anticoagulant might affect PrP^C detection due to the superior ability of EDTA to chelate bivalent ions, resulting in a decrease in metalloprotease activity during storage. For example, the metallopeptidase ADAM 10 has been suggested to play a role in the shedding of PrP^C from the cell surface (Taylor et al., 2009). However, the only significant difference in PrP^C levels between EDTA- and

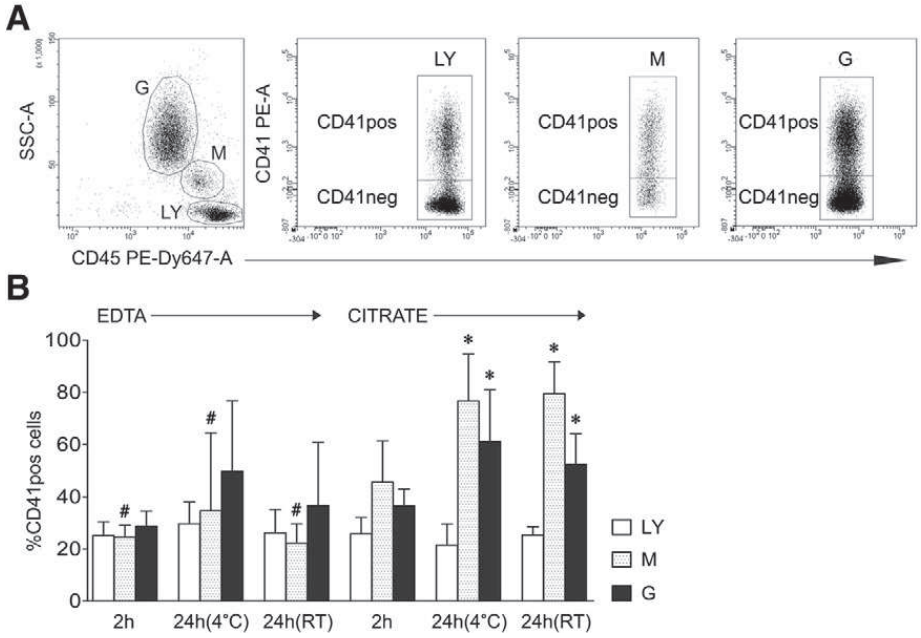

Fig. 3. Evaluation of platelet satellitism by leukocyte populations. (A) Lymphocytes (LY), monocytes (M), and granulocytes (G) were gated in the FSC vs. SSC plot, and the CD41^{pos} and CD41^{neg} subpopulations were discriminated. (B) The percentages of CD41^{pos} (platelet-associated) lymphocytes, monocytes, and granulocytes were assessed for samples stored under different conditions. Data are the mean ± SD from 6 donors. $^{*}P<0.05$ vs. samples processed within 2 h after blood collection (2 h). $^{\#}P<0.05$ vs. citrate anticoagulated samples.

citrate-stored samples was recorded for monocytes in fresh blood. Similarly, the storage temperature itself had moderate effects that reached significance only for DCs and monocytes, in which decreased levels of PrP^{C} were observed following storage at RT. This suggests that storage-dependent changes in PrP^{C} detection could not be prevented by the use of EDTA and the cooling of blood samples. An important finding of our study was the identification of platelet satellitism (Peters et al., 1997; Barnard et al., 2005) as a factor contributing to the heterogeneity of PrP^{C} detection in blood cells. We previously demonstrated that resting human platelets express approximately 600 to 800 molecules of PrP^{C} per cell (Holada and Vostal, 2000; Panigaj et al., 2010) and that PrP^{C} is present within lipid rafts that are associated with the platelet cytoskeleton (Brouckova and Holada, 2009). In addition, platelet activation results in the rapid up-regulation of PrP^{C} onto the cell membrane (Holada et al., 1998, 2006). Thus, the platelet association with leukocytes may significantly increase the levels of PrP^{C} detected on leukocyte subsets. Indeed, CD41^{pos} monocytes and granulocytes in fresh blood

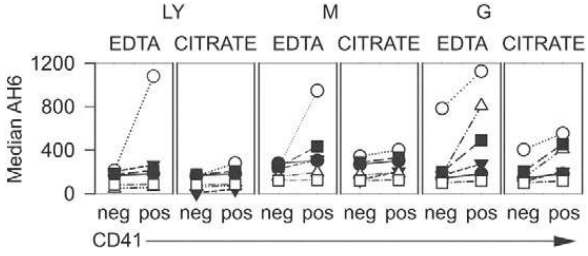

Fig. 4. Platelet binding to leukocytes increases the levels of PrP^{C} detected on subsets of leukocytes. PrP^{C} levels are displayed for CD41^{neg} and CD41^{pos} (platelet-associated) lymphocytes, monocytes, and granulocytes stored in EDTA and citrate for 24 h at 4 °C. Data displayed are the median fluorescence of the AH6 antibody for individual donors (n = 6) before and after storage.

exhibited significantly increased levels of PrP^{C} compared with their CD41^{neg} counterparts. The storage of blood usually led to a further increase of PrP^{C} levels on CD41^{pos} cells but with large individual differences among donors. Interestingly, we also detected significantly increased levels of PrP^{C} on CD41^{pos} lymphocytes in citrate, suggesting that platelet satellitism may also affect the analysis of PrP^{C} levels in those cells where its existence is currently disregarded. In contrast, the percentage of CD41^{pos} lymphocytes in EDTA-stored samples did not significantly increase following blood storage nor did the levels of PrP^{C} on the cells. The large variability recorded in our study between PrP^{C} levels on CD41^{pos} and CD41^{neg} cells among individual donors demonstrates the ability of platelet satellitism to generate spuriously irregular data. Our results suggest that the increases in PrP^{C} levels detected on monocytes and granulocytes following storage were caused, at least in part, by platelet-expressed PrP^{C}. This was clearly evident in granulocytes, which are known to express very low to undetectable levels of PrP^{C} (Cashman et al., 1990; Dodelet and Cashman, 1998; Barclay et al., 1999). However, platelet satellitism may have also other implications. Recently, platelets were shown to harbor infectivity in deer that were infected with chronic wasting disease (Mathiason et al., 2010) and sheep infected with scrapie (Lacroux et al., 2012). Therefore, platelet satellitism may also affect the distribution of prion infectivity in the blood.

5. Conclusions

The choice of the antibody considerably affects the detection of PrP^{C} on blood cells; hence preliminary studies should be carried out to identify adequate antibodies for PrP^{C} detection on blood cells of interest. Our study demonstrates that pre-analytical variables also have the potential to

significantly affect the levels of PrP^C detected on blood leukocytes and may contribute to the heterogeneity of results obtained by flow cytometry. The storage of blood prior to analysis was the most influential factor. To prevent the inclusion of storage-generated artifacts, we recommend the processing of blood immediately after collection. Finally, our study identified platelet satellitism as a currently unappreciated factor that affects PrP^C detection on blood leukocytes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jim.2012.04.002.

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Binding of prion antibodies to white blood cells of nonhuman primates and the existence of washable pool of cellular prion protein associated with lymphocytes in peripheral blood

To the Editor:

In their article, Holznagel and colleagues[1] have used flow cytometry and a whole blood, no-wash protocol for the study of cellular prion protein (PrPc) association with peripheral lymphocytes of bovine spongiform encephalopathy (BSE)-infected cynomolgus monkeys. The authors have found that one of the antibodies (12F10) used in the study produced significantly higher fluorescence in BSE-infected primates when compared to uninfected controls. If the difference in the fluorescence is sufficiently robust, then this finding would be of considerable interest because it could be used as a foundation for the development of a currently absent preclinical test for prion diseases. However, the presentation of the data makes such conclusion difficult. The authors have chosen to assess the area under the curve obtained by plotting the mean fluorescence intensity ratio (MFIR) against time rather than using a direct assessment of the MFIR of cells of infected and healthy animals. While the difference in the area under the curve between repeated measurements was significant when distinguishing infected and healthy animals early in the incubation period, it is not clear if the difference in the MFIR of cells is large enough to distinguish the experimental groups at any time point. Closer observation of the individual MFIR plots reveals the existence of local MFIR peaks that seem to occur simultaneously in all studied animals. This may be explained either by a physiologic variation of PrPc expression after some external influence (e.g., seasonal variation) or by a systematic error of the labeling protocol employed in the study. The authors have developed a labor-saving, indirect, whole blood, no-wash protocol of cell labeling that not only produced very high fluorescent signals, but also allowed them to handle large quantities of samples at once. The protocol was extensively validated to prove its specificity. However, the data that were obtained by its use noticeably differ from data in the literature. This can be best illustrated by results obtained for human and macaque granulocytes. Previous studies have demonstrated that, in man, the expression of PrPc is down regulated upon differentiation along the granulocyte lineage.[2] As a result, human granulocytes express very little, if any, PrPc.[3,4] In contrast, the protocol used by Holznagel and colleagues suggests that all human granulocytes express high levels of PrPc (MFIR ~40). Similarly in macaques, a previous quantitative flow cytometry study[5] using a standard protocol with directly labeled fluorescein isothiocyanate–conjugated prion

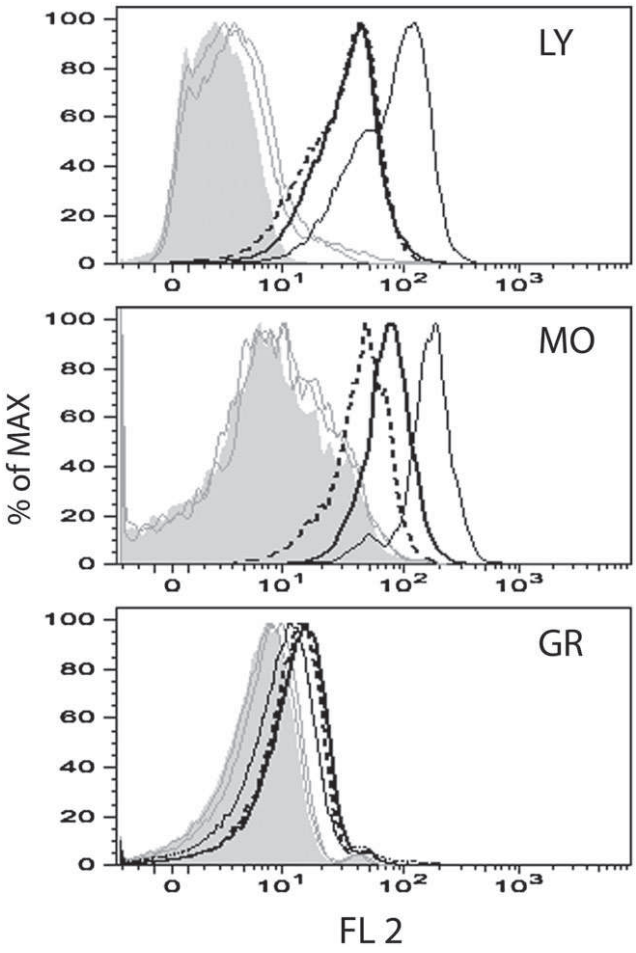

Fig. 1. The binding of prion antibodies to granulocytes of healthy cynomolgus monkeys is negligible. Peripheral blood WBCs of healthy cynomolgus monkeys (n = 15) were labeled using standard protocol with PE-conjugated prion MoAbs AG4, 3F4, and AH6. Aliquots of ethylenediaminetetraacetate-anticoagulated whole blood were incubated with saturating concentration of antibodies on ice. Subsequently, red blood cells were lysed and cells were fixed by solution (FACSlyse, BD Biosciences, Le-Pont-de-Claix, France). Cells were spun down, washed once with buffer, and analyzed using a flow cytometer (FACScan, BD Biosciences). Lymphocytes (LY), monocytes (MO), and granulocytes (GR) were gated according to their characteristic light scattering properties on FSC/SSC plot. Representative fluorescence histograms of gated cells of a single monkey are shown. AG4 = thick dashed black line; 3F4 = thin black line; AH6 = thick black line; IgG2a and IgG2b isotypic control = thin gray lines; fluorescence of unlabeled cells = gray filled peak. Both lymphocytes and monocytes express modest levels of PrPc while its expression on macaque granulocytes is close to none.

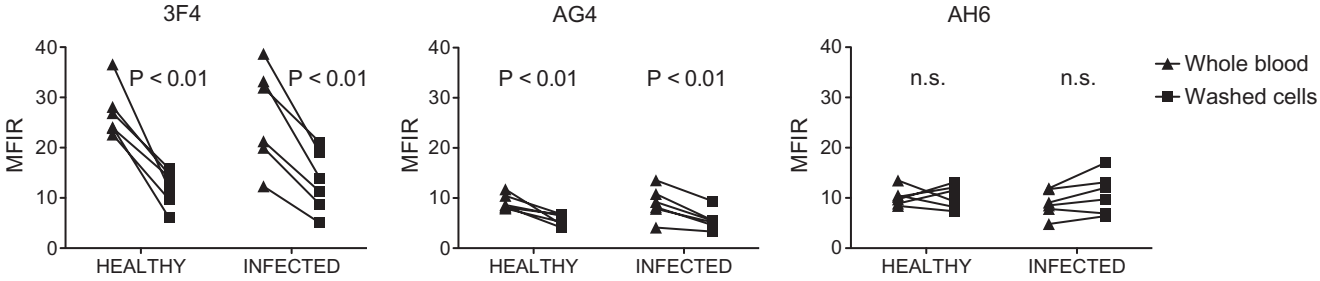

Fig. 2. Isolation of lymphocytes using Ficoll leads to decreased binding of prion antibodies to the cells. Healthy (n = 6) and BSE-infected (n = 6) cynomolgus macaques were analyzed for the binding of PE-conjugated prion MoAbs (AG4, 3F4, and AH6) to lymphocytes in whole blood (▲) or after their isolation using the standard Ficoll procedure (■). Isolated cells were washed twice with buffer before labeling with antibodies. Labeling was done as described in the legend to Fig. 1. MFIR was calculated as median fluorescence of prion antibody divided by median fluorescence of appropriate isotypic control. Both AG4 and 3F4 detected significantly less PrPc on washed lymphocytes ($p < 0.01$), suggesting the existence of a loosely bound pool of PrPc, which can be removed from the cell surface by washing. No significant difference was detected with AH6 (n.s.).

antibodies (3F4 and 6H4) had estimated the number of PrPc molecules on macaque granulocytes to be less than 250 per cell. We have recently repeated the study of PrPc expression on peripheral white blood cells (WBCs) in a larger cohort of cynomolgus macaques (n = 15) using fresh blood and a different set of directly labeled phycoerythrin (PE)-conjugated prion antibodies (AG4, 3F4, and AH6). Representative fluorescence histograms of main WBC subsets are shown in Fig. 1. While both lymphocytes and monocytes expressed moderate levels of PrPc, the binding of prion antibodies to granulocytes was again negligible (MFIR range for AG4, 0.98-1.64 [median, 1.43]; 3F4, 1.09-1.91 [median, 1.24]; and AH6, 1.24-1.91 [median, 1.45]). This very low fluorescence contrasts with the high fluorescence of macaque granulocytes (MFIR ~20) obtained by the indirect, no-wash protocol. A possible explanation for this disparity could lie in the nature of the no-wash protocol. Sequential addition of primary and secondary antibody to whole blood may lead to the formation of highly fluorescent immune complexes that can bind to cells contributing to their unusually high fluorescence. Formation of the complexes could be facilitated by plasma PrPc (soluble or microparticle associated) and contribute to a high variation of results in time. Having this in mind, we decided to verify the interesting finding of Holznagel and colleagues that isolation of cells using Ficoll leads to decreased binding of prion antibodies. This could be explained either by washing out a loosely associated pool of PrPc from the cell surface, as suggested by the authors, or by reduced formation of fluorescent immune complexes due to the absence of plasma PrPc. In accordance with the results of Holznagel and colleagues, two of three of our monoclonal antibodies (MoAbs) have detected substantial decreases of lymphocyte fluorescence (Fig. 2). Therefore, despite providing artificially high fluorescence, the indirect, no-wash protocol rightly detects the presence of a washable pool of lymphocyte PrPc. The nature of this pool, the manner in which it associates with cells, and its influence on masking or creating differences in studies of PrPc expression between prion-infected and healthy subjects are unknown and await elucidation in future studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Three different PrP fractions detectable on simian white blood cells

In reply:

In their letter, Holada and coworkers used monoclonal antibodies (MoAbs) directly conjugated to fluorochromes to analyze PrP^c fluorescence on simian blood cells in ethylenediaminetetraacetate-anticoagulated whole blood and gradient-purified peripheral blood mononuclear cells (PBMNCs). They confirmed our data that a washable PrP pool exists on blood cells,[1] but speculated that an indirect method may be less suitable due to a possible formation of immune complexes. To support their assumption that nonspecific staining of granulocytes of simian blood samples in our article was due to the indirect staining method, they cited data from a study showing that expression of PrP^c is down regulated in human granulocytes. Moreover, they stated that our data obtained by indirect staining differed from data in the literature. Second, they recommended the use of quantitative flow cytometry, that is, the calculation of the number of PrP molecules per cell rather than the calculation of mean fluorescence intensity ratios (MFIRs).[1] Third, they discussed that the calculation of area under curve was less suitable as a statistical method to determine significant differences between bovine spongiform encephalopathy (BSE)-infected macaques and control animals. However, Holada and coworkers did not show any comparative histograms of indirectly and directly staining blood samples in their letter. Moreover, they used only MoAb 3F4.

Holada and coworkers interpreted their results as an argument against the indirect method without taking into account that the 1) indirect staining method is a technique used to amplify signals at least twice as much as the direct method,[2] 2) the calculation of the MFIRs corrected unspecific staining, 3) the highest fluorescence signals were obtained by MoAb 12F10 and the lowest by MoAb 3F4,[1] and 4) granulocytes generally tend to display unspecific staining, since their cell membranes are less stable than that of PBMNCs.

First of all, the signal-to-noise data (MFIRs) shown in our article are in accordance with data published in the literature showing for instance that granulocytes had the lowest fluorescence of all three main white blood cell (WBC) subsets (see dot blot data of Fig. 6 of Holznagel et al.).[1] Furthermore, it is obvious that the absolute signals were somewhat higher in our experiments compared to previously published data, since we used a modified method. Moreover, two experimentally derived data did not support the suggestion of Holada and coworkers that immune complexes contributed to the relatively high fluorescence of simian granulocytes: 1) the concentration of soluble PrP in simian plasma obtained by $1 \times g$ sedimentation is below the detection limit of DELFIA-based enzyme-linked immunosorbent assay systems (in contrast to human plasma samples) assuming that the possible formation of immune complexes in simian whole blood may be negligible and 2) incubation of PBMNCs from healthy donor macaques with centrifuged plasma from infected macaques showing increased concentrations of soluble PrP did not increase fluorescence of donor granulocytes after indirect staining. Moreover, we did not postulate that simian granulocytes expressed PrP molecules, but stated that there was a detectable fluorescence. Last but not least, we used different statistical methods to analyze raw MFIR data, for instance, the F test (see Table 1 of Holznagel et al.[1]).

In our article published in the February issue of **TRANSFUSION**, we analyzed data from intracerebrally BSE-infected macaques and control animals showing an increase in PrP fluorescence intensity on blood cells, which reflected the increase detected in lymphoid and nonlymphoid tissues by Western immunoblot of non–proteinase K-digested samples (data not shown). In the meantime, a second BSE study could be finalized. Here, blood samples were collected and examined from 17 orally BSE-infected monkeys and 12 age- and sex-matched controls. We not only analyzed the PrP fluorescence, but additionally examined CD- and non-CD markers by multicolor analyses. Blood cell mRNA samples were also collected and analyzed by quantitative polymerase chain reaction. Preliminary data from this study confirm our data published in **TRANSFUSION** and additionally demonstrate the existence of a pool of PrP molecules that are bound to a cellular receptor. This PrP^c receptor is present on all main WBC subsets (although levels differed significantly among the WBC subsets), thereby explaining specific PrP fluorescence on simian granulocytes. Interestingly, immune activation enhanced and aging decreased its expression.

In conclusion, there are at least three different PrP fractions on blood cells: one fraction represents endogenously expressed and glycosylphosphatidylinositol-anchored molecules, the second fraction is bound to a

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Expression of Prion Protein in Mouse Erythroid Progenitors and Differentiating Murine Erythroleukemia Cells

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Abstract

Prion diseases have been observed to deregulate the transcription of erythroid genes, and prion protein knockout mice have demonstrated a diminished response to experimental anemia. To investigate the role of the cellular prion protein (PrP^C) in erythropoiesis, we studied the protein's expression on mouse erythroid precursors *in vivo* and utilized an *in vitro* model of the erythroid differentiation of murine erythroleukemia cells (MEL) to evaluate the effect of silencing PrP^C through RNA interference. The expression of PrP^C and selected differentiation markers was analyzed by quantitative multicolor flow cytometry, western blot analysis and quantitative RT-PCR. The silencing of PrP^C expression in MEL cells was achieved by expression of shRNAmir from an integrated retroviral vector genome. The initial upregulation of PrP^C expression in differentiating erythroid precursors was detected both *in vivo* and *in vitro*, suggesting PrP^C's importance to the early stages of differentiation. The upregulation was highest on early erythroblasts (16200±3700 PrP^C / cell) and was followed by the gradual decrease of PrP^C level with the precursor's maturation reaching 470±230 PrP^C / cell on most mature $CD71^-Ter119^+$ small precursors. Interestingly, the downregulation of PrP^C protein with maturation of MEL cells was not accompanied by the decrease of PrP mRNA. The stable expression of anti-Prnp shRNAmir in MEL cells led to the efficient (>80%) silencing of PrP^C levels. Cell growth, viability, hemoglobin production and the transcription of selected differentiation markers were not affected by the downregulation of PrP^C. In conclusion, the regulation of PrP^C expression in differentiating MEL cells mimics the pattern detected on mouse erythroid precursors *in vivo*. Decrease of PrP^C protein expression during MEL cell maturation is not regulated on transcriptional level. The efficient silencing of PrP^C levels, despite not affecting MEL cell differentiation, enables created MEL lines to be used for studies of PrP^C cellular function.

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Introduction

The cellular prion protein (PrP^C) is expressed in cells of various origins. It is conserved through the whole vertebrae class, suggesting its importance in cellular physiology[1]. However, its role in physiological processes remains enigmatic although PrP^C plays a basic role in the pathogenesis of the fatal neurodegenerative disorders known as Transmissible Spongiform Encephalopathies. Observation of PrP^C deficient mice ($PrP^{-/-}$) did not reveal significant health problems. On the other hand, experiments in cell cultures suggested that PrP^C is linked to such processes as the prevention of apoptosis, copper metabolism linked to oxidative stress, iron metabolism, signalization and differentiation[2,3,4]. A connection between prion pathogenesis and erythropoiesis was suggested by the downregulation of the α-hemoglobin stabilizing protein (AHSP) mRNA during prion disease[5]. A later study indicated that the disease progression affected the transcription of several other murine erythroid genes, e.g., Kell, GPA, band 3 and ankyrin[6]. A link between PrP^C expression and erythropoiesis was also demonstrated in $PrP^{-/-}$ mice after the experimental induction of hemolytic anemia. Upon treatment with phenylhydrazine, $PrP^{-/-}$ mice produced fewer reticulocytes than did $PrP^{+/+}$ mice. This result was probably due to the reduced level of erythropoietin production and higher percentage of apoptotic erythroid precursors[7]. Apparently, the expression of PrP^C is generally important in hematopoiesis. PrP^C is present on the surface of mouse long-term hematopoietic stem cells (LT-HSCs) and supports their self-renewal and engraftment during serial transplantation. Quantitatively, 40% of mouse bone marrow (BM) cells express PrP^C, and, from this population of cells, 80% are erythroid cells[8]. $CD34^+$ human bone marrow stem cells also express PrP^C[9,10], and circulating human and mouse red blood cells have similarly low levels of PrP^C on their cellular membranes[11,12]. A widely used model for the study of erythroid differentiation *in vitro* is presented by murine erythroleukemia (MEL) cells. MEL cells are blocked at the proliferative proerythroblast stage, and, after the addition of polar substances, e.g., hexamethylene bisacetamide (HMBA), they lose their proliferative capacity and enter cell-cycle arrest. This process is characterized by structural (decreased cell volume and nuclear condensation) and biochemical changes (activation

of erythroid genes, hemoglobin accumulation), which resemble those exhibited by natural erythroid differentiation[13]. Gougoumas and colleagues demonstrated transcriptional activation at the mRNA level of the PrP gene in growth-arrested MEL cells[14]. Our study extends their observations by demonstrating divergences in the regulation of PrP^C at the protein and mRNA levels during inducer-mediated erythroid differentiation and cell-growth arrest caused by confluency. In addition, we exploited MEL cell lines with stably downregulated levels of PrP^C to study its importance in the differentiation of MEL cells.

Results

Regulation of PrP^Cexpression on mouse bone marrow and spleen erythroid precursors

Erythroid precursors were gated according their Ter119 and CD71 signals and the forward scatter (FSC) signals to the proE, EryA, EryB and EryC subpopulations (Fig. 1 A). $CD71^+Ter119^{+/-}$ bone marrow proerythroblasts (proE) expressed 7800 ± 3100 PrP^C molecules / cell, assuming that one molecule of mAb AH6 binds one molecule of PrP^C. The expression of $CD71^+Ter119^+$ basophilic erythroblasts (EryA) was elevated to 16200 ± 3700 PrP^C / cell and decreased in late basophilic and polychromatic erythroblasts (EryB) to 5100 ± 1100 PrP^C / cell and was also diminished in late $CD71^-Ter119^+$ small precursors (EryC) (470 ± 230 PrP^C / cell). Corresponding erythroid precursors in the spleen expressed 4200 ± 600, 13400 ± 5200, 4600 ± 1400 and 680 ± 280 PrP^C / cell, respectively (Fig. 1 B).

Regulation of PrP^C expression during the erythroid differentiation of MEL cells

MEL cells were grown for five days in the absence or presence of 5 mM HMBA. The cells increased their expression of Prnp mRNA, reaching 13 ± 1.2 -fold and 8.7 ± 2.8-fold relative expression after 120 hours in uninduced and differentiating cells, respectively (Fig. 2 A and B). While the level of PrP mRNA in the differentiating cells more than doubled within 24 h after induction, a similar increase in the uninduced cells was observed after 48 h in culture when cells were reaching confluency. At the protein level, the uninduced cells increased their PrP^C, reaching a maximum expression in confluent culture at 96–120 h (Fig. 2C) which correlated with the expression of Prnp mRNA. In contrast, the expression of the PrP^C protein in differentiating cells peaked at 24–48 h post-induction (Fig. 2D) with a subsequent decrease to almost its basal level at 120 h, as demonstrated by densitometry (Fig. 2F). The increased density of the PrP^C band on the WB was already visible within 6 h post-induction (not shown). These results were confirmed by quantitative FACS analysis, which demonstrated approximately twofold increase of PrP^C membrane expression after 24 h of differentiation, with a subsequent return to the basal level after 96 h (Fig. 2E).

Dexamethasone treatment of MEL cells lowers their expression of PrP^C in confluent culture irrespective of production of hemoglobin

Induction of MEL cell differentiation by incubation with HMBA for 24 h with their subsequent incubation in the media

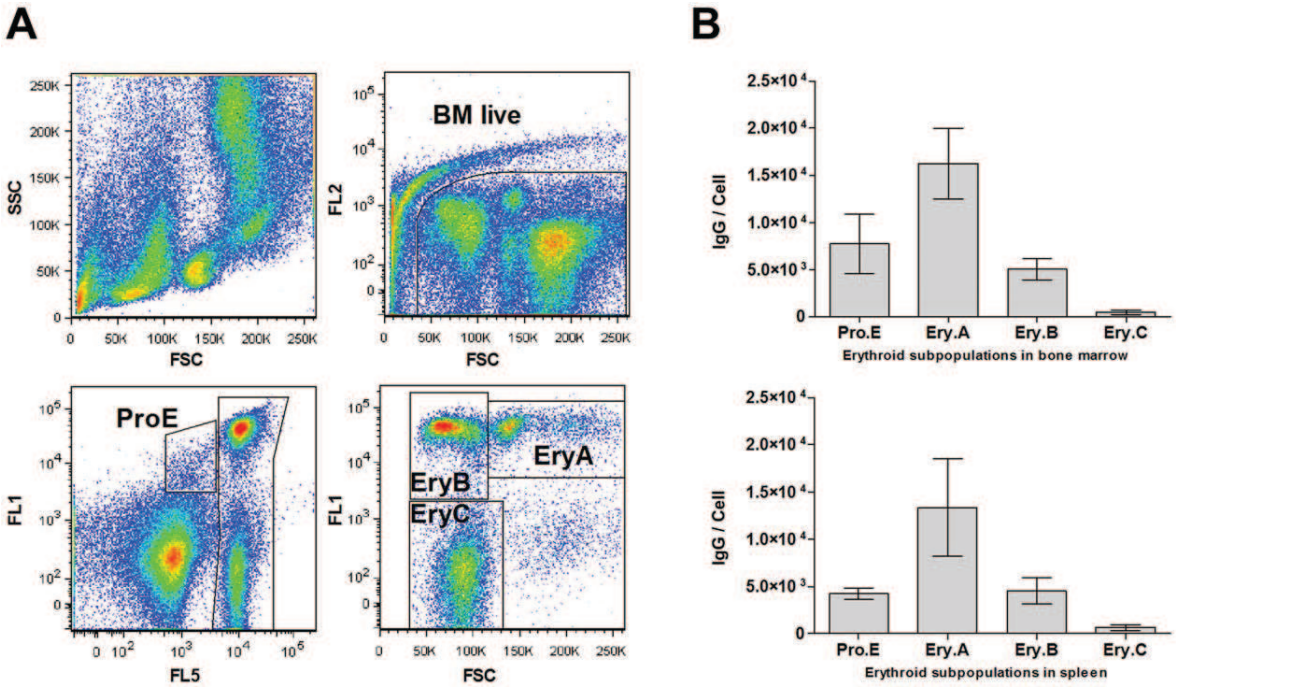

Figure 1. The expression of PrP^C on mouse bone marrow (BM) and spleen erythroid precursors is upregulated in early erythroblasts and, then, decreases with the cells' maturation. (A) Gating strategy for erythroid precursors: upper left - scattergram of BM cells; upper right – gating of viable 7-AAD negative cells (BM live); lower left – live BM cells labeled with CD71-FITC (FL1) and Ter119-eFluor450 (FL5): ProE - $CD71^+Ter119^{+/-}$ proerythroblasts; $Ter119^+$ cells were further gated on CD71-FITC (FL1) and FSC plot (lower right): EryA - large $CD71^+$ early basophilic erythroblasts, EryB - small $CD71^+$ late basophilic and polychromatic erythroblasts, EryC - small $CD71^-$ orthochromatic erythroblasts and reticulocytes. (B) Quantitative FACS analysis of PrP^C expression on erythroid precursors in mouse bone marrow and spleen. Expression of PrP^C on early basophilic erythroblasts (EryA) is significantly higher ($p>0.005$, $n=5$) in comparison with proerythroblasts (ProE), both in BM (upper) and spleen (lower). The initial increase of expression is followed by its decrease on late basophilic and polychromatic erythroblasts (EryB), and most mature small precursors (EryC) express a low number of PrP^C. Quantification is based on assumption that one IgG molecule of MAb AH6 binds to one molecule of PrP^C.
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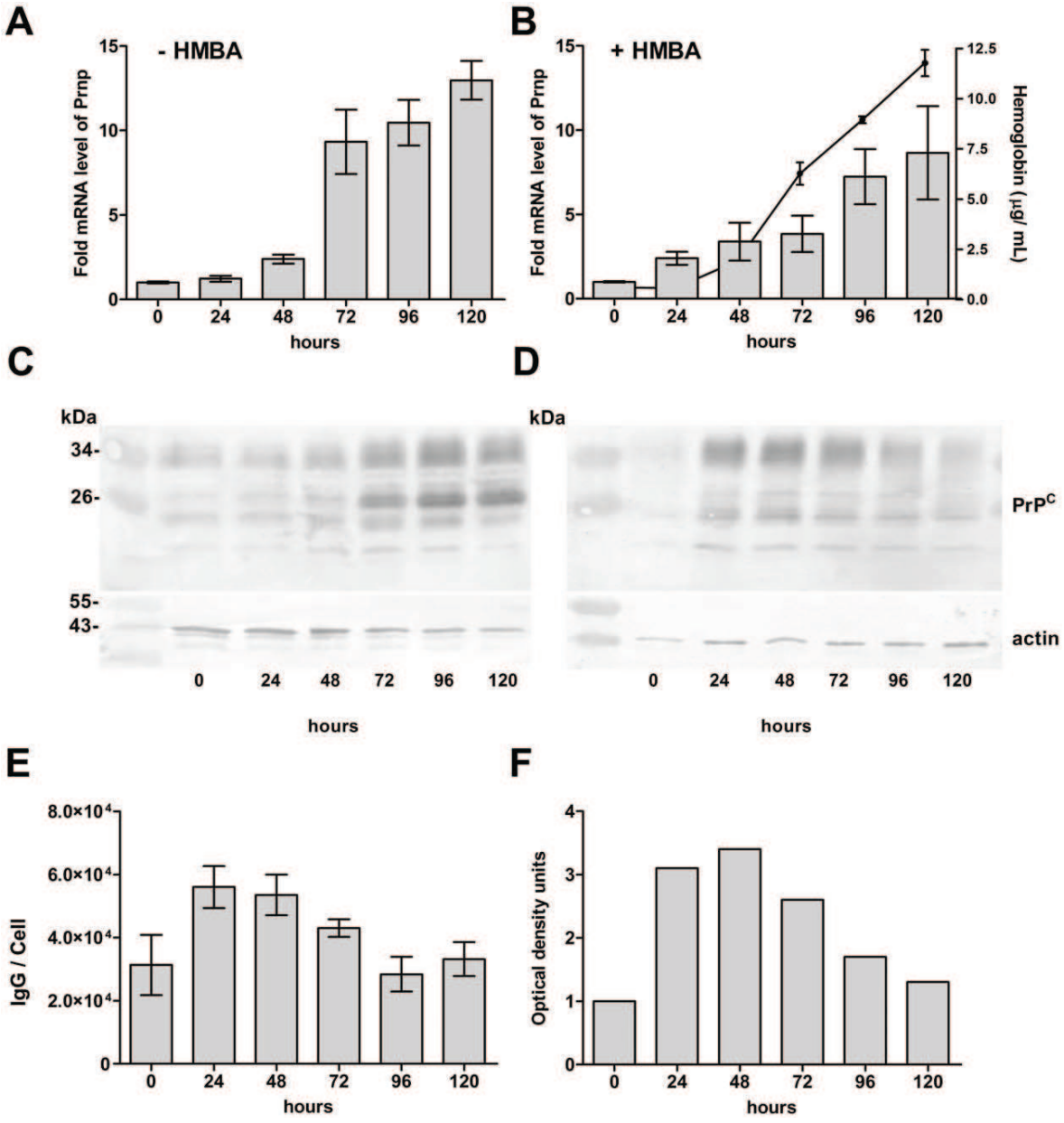

Figure 2. Initial increase of PrP^C protein expression in differentiating MEL cells is followed by its downregulation. (A) Transcriptional activation of the Prnp gene in uninduced MEL cells (- HMBA) correlates with growth arrest in confluent culture at 72 h, as demonstrated by qRT-PCR. A similar, but lower, increase of PrP mRNA is seen in cells induced to erythroid differentiation (+ HMBA) (B). Progress of cell differentiation is monitored by overall hemoglobin level (full line, n = 2, mean ± SD). The amount of PrP^C protein on western blots (WB) in uninduced cells (C) correlates with the level of mRNA (A). In contrast, the amount of PrP^C protein in differentiating cells (D) is highest 24–48 h after induction and, then, is downregulated, as shown by densitometry (F), despite the level of PrP mRNA continues to rise (B). Blots were developed with a mix of PrP^C mAbs (AH6, AG4, 6H4) and actin antibody was used as a loading control. Surface expression of PrP^C on HMBA induced differentiating MEL cells (number of mAb AH6 IgG molecules / cell) measured by quantitative flow cytometry (E) correlates with the levels detected by WB (F) (n = 2, means ± range).
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without HMBA (H/M), or the inclusion of 4 µM dexamethasone in the medium with HMBA (HD/HD), both led to a comparable (~60%) reduction of MEL cells hemoglobinization after 120 h of culture (Fig. 3 A). The H/M treatment was connected with the significant increase of PrP^C level (~220%) at 120 h comparable to the increase recorded in non-differentiated confluent cells (NT) (Fig. 3 B, C). At the other hand, HD/HD treatment led to low PrP^C level (~80%), lower than in differentiated fully hemoglobinized MEL cells (H/H). Treatment of MEL cells with up to 40 µM dexamethasone did not influence the initial up-regulation of PrP^C in HMBA stimulated cells 24 h post induction, but generally led to lower PrP^C levels in comparison with NT cells after 120 h in culture (Fig. 3 B, C). Interestingly, induction of PrP^C expression in growth-arrested confluent culture led to a different PrP western blot pattern with predominant ~26 kDa band which is much less present in both, fully differentiated or actively dividing

MEL cells (Fig. 3 B). The detected changes in the levels of PrP^C were not caused by differences in gel loading, as demonstrated by actin labeling.

Retroviral vector delivery of anti-Prnp shRNAmir leads to stable and efficient PrP^C silencing

Both spinfection and co-cultivation methods led to efficient (~90%) silencing of PrP^C at the mRNA (Fig. 4 A) and protein levels (Fig. 4 B) by LP1 and LP2 constructs in comparison with control construct LN. The LP5 construct did not produce any downregulation of PrP^C levels (Fig. 4 B). Retroviral infection of cells did not induce cell-protective effects, as demonstrated by qRT-PCR analysis of chosen interferon-stimulating genes *Oas1a*, *Rnase1* and *Eif2ak2* (not shown). The silencing of PrP^C in LP1 and LP2 lines was stable during the course of HMBA-induced cell differentiation (Fig 5). In comparison with LN, LP1- and LP2-transduced cells exhibited 79% and 84% inhibition of Prnp mRNA expression at the beginning of differentiation and 93% and 96% after 120 h of differentiation, respectively (Fig. 5 A). The levels of Prnp mRNA and protein during differentiation of the control LN-transduced cells (Fig. 5 A, C) was upregulated similarly as in non-manipulated MEL cells (Fig. 2 B, D). The stability of PrP^C silencing was confirmed by quantitative FACS analysis, which demonstrated downregulation of PrP^C at the cell membrane ranging from 17% (LP1) and 12% (LP2) to 8% (LP1) and 5% (LP2) of its expression (100%) in LN-transduced cells at the beginning of differentiation and after 48 h, respectively (Fig. 5 B). These results were confirmed by western blot analysis in which we could faintly detect PrP^C in LP1 and LP2 cells only after 24 hours, while the protein in LN-transduced cells was readily detected (Fig. 5 C).

Silencing of the Prnp gene by RNAi does not affect HMBA-induced differentiation of MEL cells

Cell numbers in differentiating LN and LP1 lines increased similarly, reaching 2.4×10^6 cells/mL at 72 h. The LP2-transduced cell line produced slightly more cells, with a maximum of 3.0×10^6 cells/mL at 72 h (Fig. 6 A). In a Trypan Blue exclusion assay, all cell lines demonstrated a similar viability (~94%) over a 72-h post-induction period and, also, a similar slight decrease of viability after 96 and 120 h (Fig. 6 B). We did not detect any differences in the transcriptional levels of proapoptotic Bax (not shown). The dynamic of hemoglobin production was comparable in all cell lines, irrespective of the level of PrP^C (Fig. 6 C). Similarly, surface expression of the transferrin receptor (CD71) was similarly regulated in all cell lines (Fig. 6 D). Also, no difference was demonstrated in the levels of c-myb mRNA, which were downregulated approximately eightfold after 24 h post-induction in all lines. After 48 h, c-myb mRNA was downregulated to ~2% of the starting level and remained silenced until the end of the experiment (not shown). Similarly, monitoring of the erythroid markers Eraf and Hba (Fig. 7A and B) and GATA-1 (not shown) during the differentiation of cells at the transcriptional level indicated analogous expression in all lines, irrespective of PrP^C levels.

Discussion

The role of PrP^C in cellular physiology has been proposed for a variety of processes, but with no prevailing consensus to date[1]. The downregulation of erythroid genes during prion infection has established a link between the peripheral pathogenesis of prion diseases and erythropoiesis. However, it is not clear if the effect is caused by direct interaction of prion particles with erythroid cells or if it is triggered by some yet unknown humoral response to the infection. The expression of PrP^C on circulating red blood cells (RBCs) of closely related nonhuman primates varies from several thousand per cell to zero[15], implying that its function on mature erythrocytes is not conserved. Griffiths et al. demonstrated the regulation of PrP^C expression during the differentiation of cultured human erythroblasts *in vitro*, indicating that it may play a role in the differentiation of erythroid precursors. PrP^C was found mostly in the perinuclear region of proerythroblasts, and the amount of PrP^C declined as erythroid cells matured[16]. Human and mouse RBCs express approximately 200 PrP^C molecules per cell[11], suggesting a similar regulation of its expression in the erythroid lineage in both species. In this study, we demonstrated that the surface expression of PrP^C on erythroid precursors in the mouse bone marrow and spleen follows a similar pattern as the cells mature. The protein's levels first increase with basophilic erythroblasts, expressing more than twice as much PrP^C as proerythroblasts, and then declines significantly in late basophilic and polychromatic erythroblasts, with most mature small erythroid precursors expressing only around 500 PrP^C molecules per cell. This pattern of expression is suggestive of PrP^C's involvement in the early stages of erythroid differentiation. The differentiation of MEL cells in culture is known to resemble the *in vivo* process, with similar maturation stages: proerythroblast-like, erythroblasts-like and, occasionally, reticulocyte-like[16,17]. The surface expression of PrP^C on uninduced MEL cells in the exponential phase of growth was approximately six times higher than that observed on proerythroblasts in the mouse bone marrow or spleen. However, the induction of differentiation led to nearly doubling of the PrP^C number on MEL cell membranes within 24 h, which resembled a similar increase of PrP^C expression on basophilic erythroblasts *in vivo*. In agreement with previous observations *in vivo*, we found that this initial upregulation was followed by a gradual downregulation of PrP^C surface levels along with the progression of MEL cells' differentiation. This decrease did not reach the degree observed in small $CD71^-Ter119^+$ erythroid precursors *in vivo*, which is in line with the limited ability of differentiating MEL cells to reach this stage of maturation. The differentiation of MEL cells is composed of two separate events, growth arrest and terminal differentiation. Both processes are characterized by the activation of early (cell-cycle control) and late (morphological changes) genes[17]. The first period lasts approximately 12 hours, after which the first committed cells materialize. The majority of cells are terminally differentiated between 24 and 50 h[18]. In agreement with previous studies by Gougomas et al.[14], we found that the Prnp gene in MEL cells is transcriptionally activated both during inducer-mediated differentiation and in confluent cells undergoing cell-cycle arrest. In addition, we were able to demonstrate the regulation of PrP^C at the protein level by western blot. Interestingly and in accordance with the flow cytometry results, in differentiating cells, the expression of the protein was downregulated after an initial increase, even though the level of PrP mRNA continued to rise. This result suggests that MEL cells' differentiation leads to a translational regulation of PrP^C levels[19] not seen in uninduced cells undergoing cell-cycle arrest. Alternatively, more differentiated cells could degrade PrP^C at an increased rate, as has been proposed to explain the disparity between PrP protein and mRNA levels in different types of neuronal cells[20]. The downregulation of PrP^C protein during the differentiation of highly responsive subclones of MEL cells was recently reported by Otsuka et al.[21]; however, their study did not detect an initial increase of PrP^C expression, most likely due to the different status of cells at the point of induction. Stimulation of the glucocorticoid receptor by

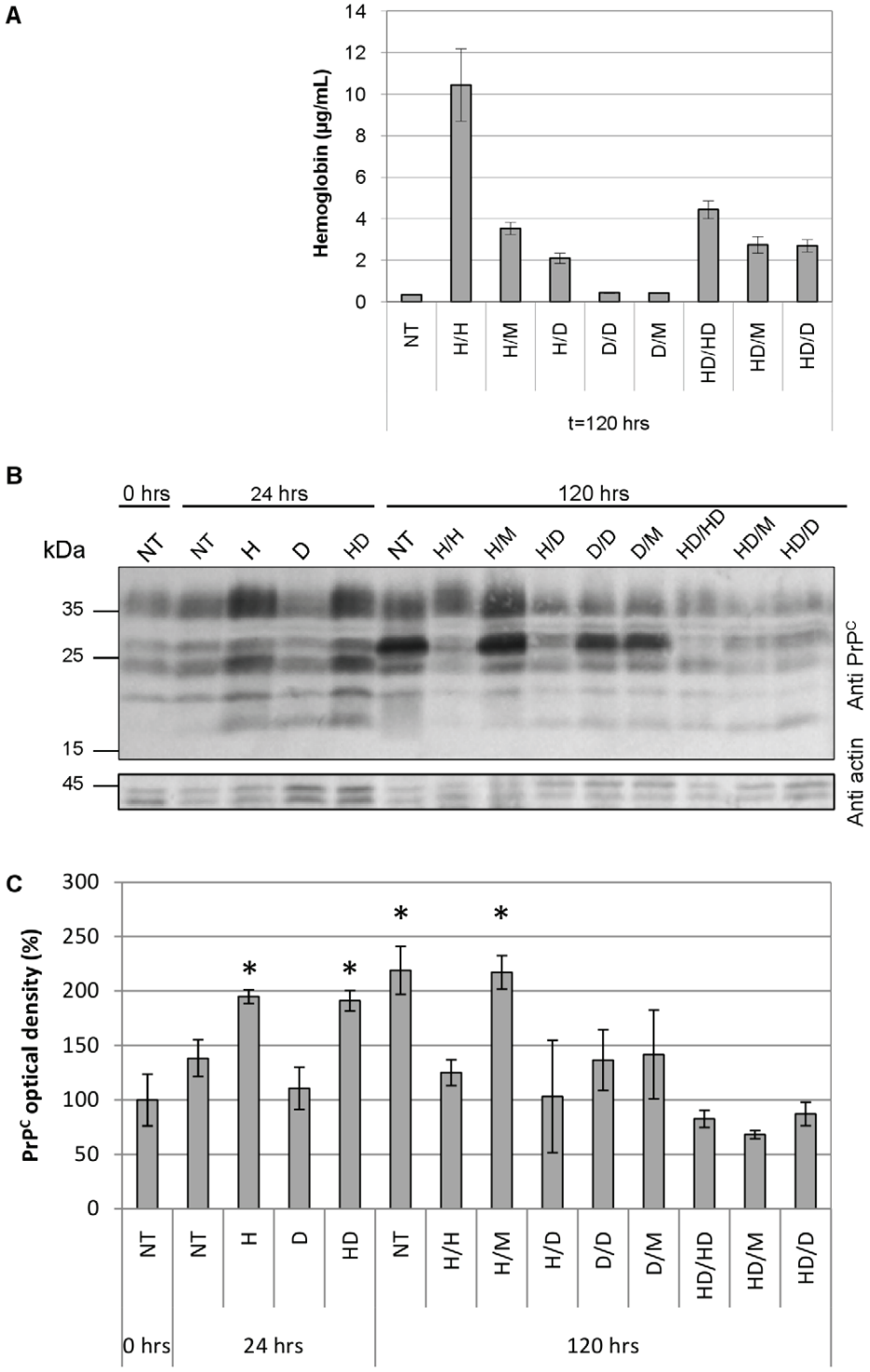

Figure 3. The initial upregulation of PrP^C expression after the induction of MEL cells' differentiation by HMBA is not affected by dexamethasone. (A) Hemoglobin levels 120 hrs post induction of MEL cells differentiation. Cells were treated with HMBA (H), Dexamethasone (D) or the combination thereof (HD). After 24 hrs media were exchanged and the treatment continued, or was changed for different one as shown on x-axis labels (M stands for DMEM medium only). Non-treated (NT) control represents cells kept in DMEM only. Cell lysates were normalized to the total protein content (measured by BCA assay) and hemoglobin was quantified by TMB assay. (B) Western blot analysis of PrP^C expression. Samples were harvested after 24 and 120 hrs treatments described above. NT control at 0 hrs represents the initial conditions of MEL culture before treatment. The gel loading was normalized to the total protein content (measured by BCA assay) and controlled by actin staining. The blot is a representative of three independent experiments. (C) Quantification of the PrP^C level on western blots by densitometry. Graph columns represent the average density of PrP^C bands and error bars represent the standard deviations; PrP^C expression level of NT control at 0 hrs was set as 100% (*$p<0.05$, $n=3$).
doi:10.1371/journal.pone.0024599.g003

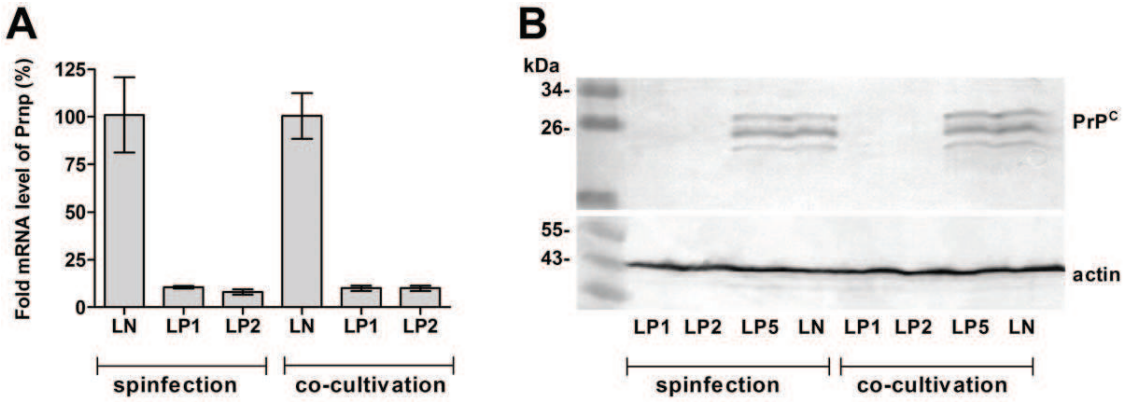

Figure 4. Downregulation of PrP^C expression by RNAi in MEL cells after selection with puromycin. (**A**) Both methods of retroviral vector delivery led to ~90% silencing of Prnp mRNA in both lines expressing anti-Prnp shRNAmir (LP1 and LP2) when compared with the MEL line expressing nonsilencing shRNAmir (LN), as depicted by qRT-PCR. (**B**) Confirmation of PrP^C downregulation in LP1- and LP2-transduced cells at the protein level by western blot. PrP^C was detected by mAb AH6. No silencing was observed in LP5 cell line. Actin was used as a loading control.
doi:10.1371/journal.pone.0024599.g004

dexamethasone induces the proliferation and expansion of erythroid progenitors and delays the terminal differentiation of erythrocytes[22]. In our hands, dexamethasone did not prevent the HMBA-induced initial upregulation of PrP^C in MEL cells, suggesting that it precedes the effect of dexamethasone, which is known to suppress the HMBA-mediated commitment to terminal cell division at a relatively late step in this process[23]. However, dexamethasone prevented the increase of PrP^C protein levels in confluent MEL cells after 120 h of culture, demonstrating that the activation of the glucocorticoid receptor can interfere with the transcriptional activation of the Prnp gene mediated by cell-cycle arrest. The mechanism of dexamethasone's action on the prevention PrP^C protein upregulation in confluent MEL cells is unknown at present. Dexamethasone has been shown to induce cell-cycle arrest in number of various cell lines[24], but not in MEL cells, in which it increases cell viability[25], both in induced and uninduced culture. In summary, our results demonstrate that the regulation of PrP^C levels in differentiating MEL cells resembles, at least in part, its regulation in maturing mouse erythroid precursors *in vivo*. To learn more about the importance of PrP^C in the process of MEL cells' differentiation, we created cell lines using RNAi to stably inhibit expression of the protein. RNAi

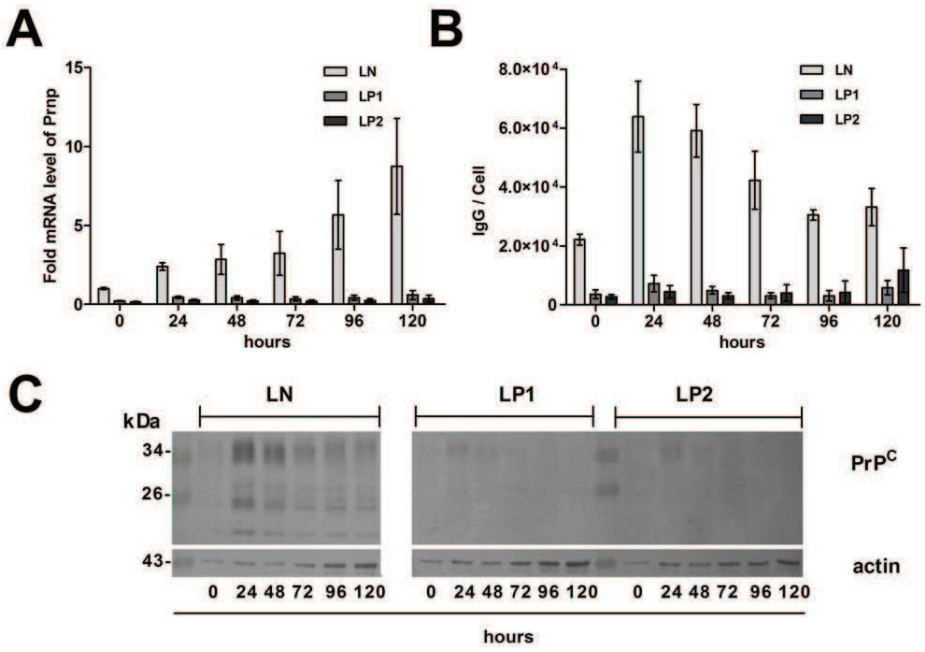

Figure 5. Expression of PrP^C is stably repressed during the differentiation of MEL cells. (**A**) Level of PrP mRNA measured with qRT-PCR in cell lines stably expressing anti-Prnp shRNAmir (LP1 and LP2) is downregulated in comparison with control (LN) during the course of cell differentiation (2 experiments, mean ± SD). (**B**) The effect of silencing on the level of PrP^C on cell membrane estimated by quantitative flow cytometry (four experiments, mean ± SD. (**C**) Confirmation of stable PrP^C silencing on the protein level by western blot. PrP^C was detected by mAb mix AH6, AG4 and 6H4. Actin was used as a loading control.
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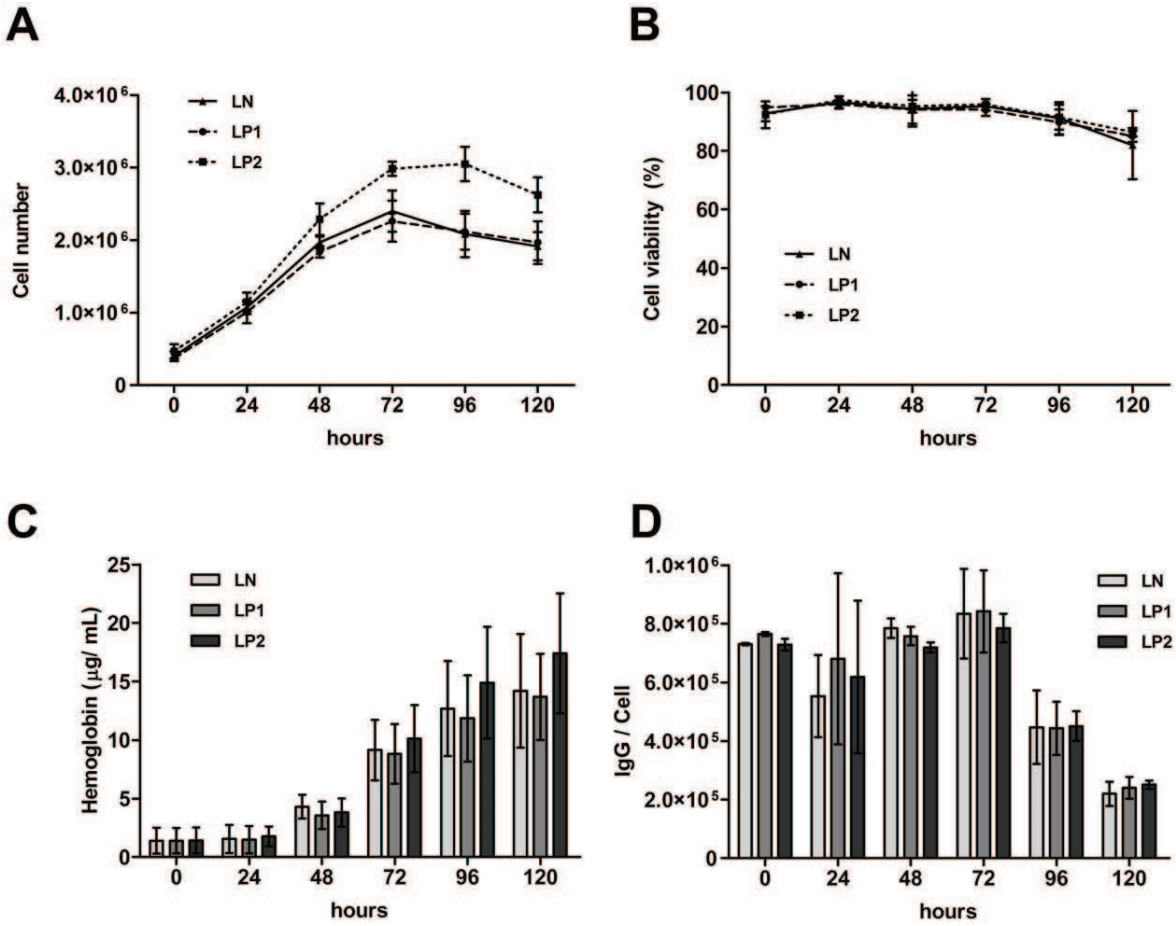

Figure 6. Differentiating MEL cells display similar characteristics irrespective to the level of PrP^C expression. (**A**) Growth curves of LP1, LP2 and control LN lines during erythroid differentiation induced by HMBA. (**B**) Cell viability based on a Trypan Blue exclusion assay. (**C**) Concentration of total hemoglobin in the cells per 100 μg/mL of total cell proteins. (**D**) Number of CD71 (transferrin receptor) molecules per cell, analyzed by quantitative flow cytometry, based on assumption that one anti- CD71-PE mAb binds one molecule of transferrin receptor.
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administered by shRNA from a retrovector had previously been employed efficiently to inhibit PrP^C expression *in vitro* and *in vivo*[26,27]. The main objective for using RNAi to suppress PrP^C was to study its therapeutic potential in preventing propagation of infectious prions. To the best of our knowledge, our model is the first murine cell line of non-neuronal origin with stably silenced PrP^C expression. Inhibition of the protein's expression at both the mRNA and protein levels was efficiently maintained during the differentiation of MEL cells, although it varied between 75 and 95% in individual time points. Despite the silencing, the induction of differentiation led to a detectable increase of PrP^C signal on blots after 24 h, suggesting that the regulation of the protein's expression in LP1- and LP2-transduced cell lines follows similar pattern as in unmodified MEL cells, although at a suppressed level. Growth curve and viability of LP1-, LP2- and control LN-transduced cell lines after the induction of differentiation was similar, although the LP2-transduced cell line exhibited a higher proliferation capacity. Since the LP1-transduced cell line did not differ from control LN-transduced cell line, we could not assign the LP2-transduced cell line's divergence solely to PrP^C silencing. All cell lines observed here demonstrated similar dynamics and level of hemoglobinization and regulation of the transferrin receptor on their cell membranes. This finding suggested that silencing of PrP^C in MEL cells does not lead to gross perturbation of iron homeostasis, although the involvement of PrP^C in iron-cell uptake was described recently[28,29]. Similarly, the level of the proto-oncogene c-myb, expression of which is characteristic of the proliferative state and has been demonstrated to block MEL cells' differentiation[30,31], decreased upon induction similarly in all created cell lines and remained low during the entire course of the experiment. Monitoring of selected erythroid markers (AHSP, Hba and GATA-1) on the transcriptional level also did not reveal significant differences among LP1-transduced, LP2-transduced and LN-transduced cell lines, confirmingthat PrP^C silencing does not appear disturb the differentiation of MEL cells. In many cell cultures, the enhanced expression of PrP^C was proposed to facilitate cytoprotective effects[32]. However, overexpression of exogenously delivered PrP^C in MEL cells did not protect the cells against apoptosis initiated by serum withdrawal[33]. We also found that silencing of PrP^C did not seem to sensitize cells to apoptosis during differentiation, as demonstrated by a Trypan Blue exclusion assay and by monitoring of Bax expression by qRT-PCR. This result is concurrent with Christensen and Harris, who reevaluated former assays reporting a protective activity of PrP^C and suggested that the presence of PrP^C has only a modest effect in cytoprotection *in vitro*[34].

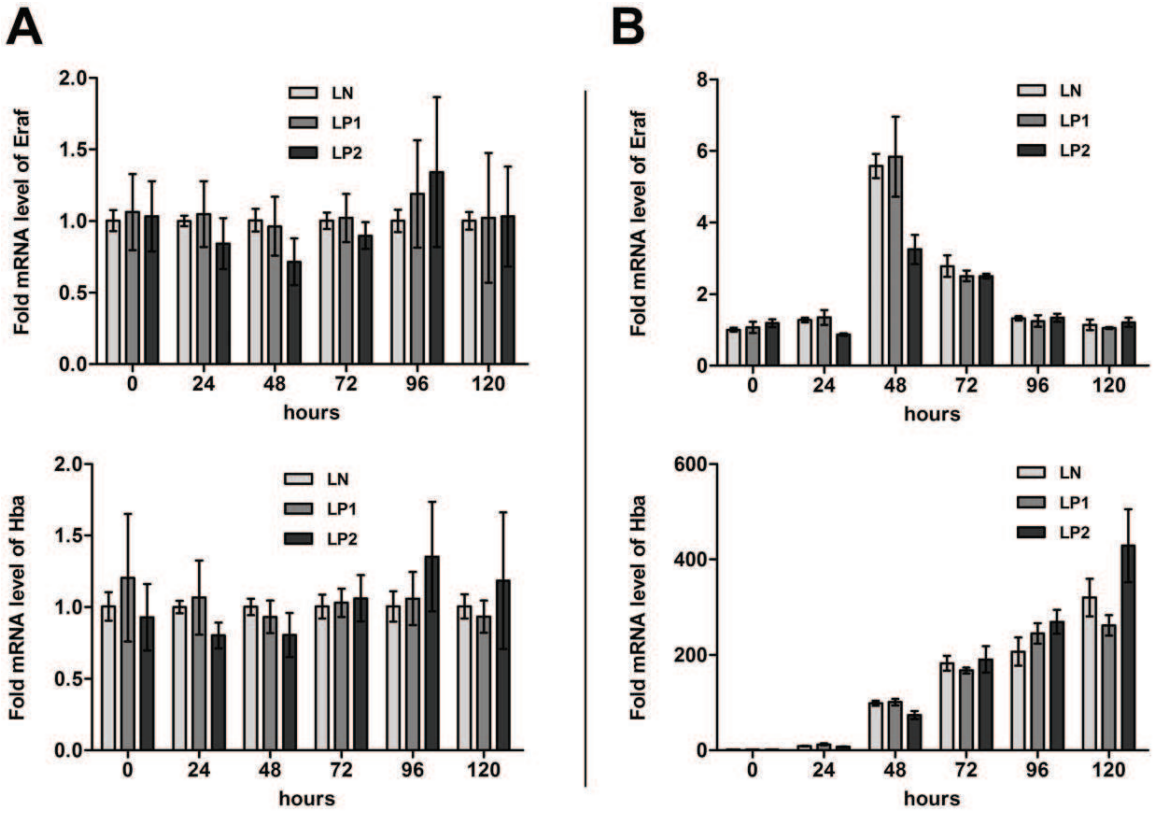

Figure 7. Transcriptional levels of selected erythroid markers in differentiating MEL cells indicate a similar pattern, irrespective to PrP silencing. (A) Expression of Eraf (α-hemoglobin stabilizing protein) and Hba (Hemoglobin α) in LP1- and LP2-transduced cells was compared with the level in the control LN-transduced cell line on individual days, as measured by qRT-PCR (n = 4, mean ± SD). (B) Representative pattern of Eraf and Hba expression during differentiation. The level of expression in individual days was normalized to the level in the LN-transduced cell line in day 0.
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Taken together, our results imply that, under normal conditions, PrP^C seems dispensable for the erythroid differentiation of MEL cells. The pattern of PrP^C regulation on erythroid precursors *in vivo* suggests that PrP^C may play a role in the maturation of erythroblasts in erythroblastic islands. We can speculate that PrP^C, which was shown to bind both laminin and the laminin receptor[35,36], can be involved in cell-cell contacts in an erythroblastic niche, or with a surrounding extracellular matrix. Downregulation of PrP^C, then, could play a role in the dissociation of matured reticulocytes from the erythroblastic niche. Such a role for PrP^C is unlikely to be detected by the MEL cell model. Another explanation could be that PrP^C exerts its function only under stress conditions. This hypothesis is consistent with the documented poor recovery of $PrP^{-/-}$ mice from experimental anemia[7]. Finally, it is possible that the effect of PrP^C silencing was compensated for by an unknown pathway or that the remaining expression is sufficient to sustain its role.

In conclusion, we created model cell lines with efficiently silenced expression of PrP^C, which are the first of their kind and may serve as a valuable tool in subsequent searches for the function of PrP^C beyond the erythroid differentiation.

Materials and Methods

Flow cytometry of mouse bone marrow and spleen erythroid precursors

The study was approved by the Committee on the Ethics of Animal Experiments of the First Faculty of Medicine, Charles University in Prague (Permit Number: 217/07). Bone marrow (BM) cells were isolated from the femurs of $PrP^{+/+}$ mice (C57BL/6×129/SvxCD1 mixed background) by washing with PBS, 1% BSA and 2 mM EDTA, pH 7.4 (PBS-BE). Spleen and BM cells were passed through a 40-μm cell strainer (Becton Dickinson, San Diego, CA, USA) to eliminate stroma and debris. The cells were labeled (30 min, 4°C) with saturating concentration of monoclonal antibodies (mAbs): anti-mouse Ter-119 eFluor 450, anti-mouse CD71-FITC (both eBioscience, San Diego, CA, USA) and AH6 (TSE Resource Center, Roslin, UK), custom conjugated to PE (Exbio, Vestec, Czech Republic). Labeled cells were washed and resuspended in PBS-BE, and their fluorescence was analyzed on a BD FACSCanto II flow cytometer equipped with BD FACSDiva Software v6.0 (Becton Dickinson). Dead cells were excluded from the analysis by 7-aminoactinomycin D (7-AAD) (Molecular Probes, Eugene, OR, USA) labeling, and the fluorescence of live cells (10^5) was analyzed. Erythroblast subpopulations were resolved according Ter119, CD71 and forward-scatter (FSC) signals. Proerythroblasts (proE) were identified as $Ter119^{medium}$ $CD71^+$ cells. $Ter119^{high}$ cells were divided using both the CD71 and FSC parameters into three populations, labeled EryA, EryB and EryC, as described previously[37], and the binding of the prion mAb AH6 was quantified using Standard Quantum R-PE MESF beads (Bangs Laboratories, Fishers, IN, USA)[11,15].

Flow cytometry of cell cultures

Cells were washed with 1% BSA-PBS, pH 7.4 (PBS-B) and labeled for 20 min at RT with saturating concentrations of the

AH6-PE or anti-mouse CD71-PE (eBioscience) mAbs. Next, washed cells were resuspended in PBS-B and analyzed using a flow cytometer to standardize the fluorescence readings and to allow for the quantification of PrP^C and CD71 expression Standard Quantum R-PE MESF beads were run as a separate sample. Viable cells were identified as a 7-AAD-negative population.

Cell cultures

The MEL cell line (clone 707, ECACC, Salisbury, UK) and packaging cell line HEK293 GP2 (Clontech, Mountain View, CA USA) were maintained in DMEM growth medium containing high glucose (4.5 g/L), L- glutamine, sodium pyruvate, 10% fetal bovine serum and puromycin/streptamycin (all reagents from PAA, Pasching, Austria). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. For all experiments, MEL cells between the second and fourth passages were seeded at a density of 10^5 cells/mL 24 h before induction of differentiation by 5 mM HMBA (final concentration). In some instances, 4 μM dexamethasone (Sigma-Aldrich, Prague, Czech Republic) was used to block the commitment of MEL cells. The cells were incubated with either HMBA, HMBA and dexamethasone or dexamethasone alone for 24 h, and then the medium was changed, and the cells were grown in the presence of either HMBA, dexamethasone, HMBA and dexamethasone, or in medium only.

Cloning

For the silencing of cellular prion expression, we used shRNAmir sequences HP_285770 (LP1) and HP_288208 (LP2), which are available in the RNAi codex database (http://cancan.cshl.edu/cgi-bin/Codex/Codex.cgi). The shRNAs, together with the control nonsilencing shRNAmir (LN), were ordered in the pSM2 retro vectors V2MM_66187, V2MM_63696 and RHS1707 (Open Biosystems, Huntsville, AL, USA)[38]. The third anti- Prnp mRNA sequence (LP5), adopted from Pfeifer et al., was purchased as an oligonucleotide[27]. All sequences were cloned into the LMP retrovector MSCV/LTRmiR30-PIG (Open Biosystems). Constructs created in this study were verified by sequencing.

RNAi of Prnp expression

Packaging HEK293 GP2 cells were seeded at a density of approximately 1.5×10^5 cells/well (24-well plate). After 24 h, the cells were transfected with a mixture of the VSV-G plasmid, coding the envelope protein of the vesicular stomatitis virus (Clontech), and the appropriate LMP retrovector (LN, LP1, LP2 or LP5). Plasmids were delivered in a ratio of 9:1 with the Arrest-in transfection agent (Open Biosystems). Three days after transfection, media containing retroviral particles were centrifuged (400 g, 4°C) to pellet eventual cell contaminants, and the infectious supernatant, containing 4 μg/mL Polybrene (Sigma-Aldrich), was added to MEL cells in a 1:1 ratio. Subsequently, transduced MEL cells were spun down at 450 g for 90 min at RT (a method known as spinfection). In a second approach, the MEL cells were infected by co-cultivation with a packaging cell line. In this method, medium in HEK293 GP2 cells was aspirated 24 h postinfection, and 2×10^4 MEL cells/well were added inside the 0.4-μm-pore translucent polyphthalate insert (Becton Dickinson) in a 0.7 mL total volume of fresh media containing 4 μg/mL Polybrene. After 48 (co-cultivation) or after 72 (spinfection) hours of incubation, the cells were diluted with fresh medium, and selection started the next day through the addition of puromycin (Sigma) at a final concentration of 0.5 μg/mL. The percentage of cells with an integrated retrovector genome was monitored by FACS utilizing eGFP positivity. Cell lines prepared by both methods were mixed after three weeks after reaching >95% eGFP positivity and were frozen in aliquots.

Western blot and densitometry

Cells were washed in PBS and lysed with 1% TX-100, 0.5% sodium deoxycholate and 0.1% SDS in 150 mM NaCl, 2 mM $MgCl_2$ and 50 mM Tris, pH 8.0, supplemented with 12 units/μL benzonase (Sigma) and the EDTA-free protease inhibitor Complete (Roche Diagnostics, Basel, Switzerland). Protein concentration was measured by BCA assay (Thermo Fisher Scientific, Rockford, IL, USA), and 20 μg proteins/lane were loaded for SDS-PAGE analysis. Separated proteins were blotted to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) and PrP^C detected with a mix of the mAbs AH6, AG4 (both 1 μg/mL, TSE resource center) and 6H4 (0.05 μg/mL, Prionics AG, Zurich, Switzerland). The anti-actin polyclonal Ab I-19 (Santa Cruz, Santa Cruz, CA, USA) (0.5 μg/mL) was used as a loading control. Secondary antibodies were goat anti mouse IgG $F(ab)_2$ alkaline phosphatase (Biosource) and goat anti rabbit IgG $F(ab)_2$ alkaline phosphatase (Caltag, Buckhingham, UK), both at a dilution of 1: 4000. BCIP/NBT (Caltag) was used as a chromogen. The density of bands on blots was quantified using MiniLumi densitometer software (DNR Bio-Imaging Systems Ltd., Israel).

Reverse transcription and quantitative PCR

RNA was isolated using the RNA Blue solution according to the manufacturer's manual (Top-Bio, Prague, Czech Republic). Contaminating genomic DNA was degraded by treatment with TURBO DNase (Ambion, Austin, TX, USA). RNA integrity was evaluated by electrophoresis, and 0.5 μg RNA was reverse-transcribed with the RevertAid first-strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada) according to the manufacturer's manual. Complementary DNA was tenfold diluted, and 2 μL were used for qRT-PCR performed in an ABI 7300 PCR System using TaqMan primers, probes and the Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). We monitored the expression of the following genes: glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), the prion protein (*Prnp*), the hemoglobin alpha adult chain 1 (*Hba-a1*), the myeloblastosis oncogene (*Myb*), the α-hemoglobin stabilizing protein (*Eraf*), the GATA-binding protein 1 (*GATA-1*), 2′-5′ oligoadenylate synthetase 1A (*Oas1a*), ribonuclease L (*Rnase1*), the eukaryotic translation initiation factor 2-alpha kinase 2 (*Eif2ak2*) and the BCL2-associated X protein (*Bax*). Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method[39] and normalized to the reference Gapdh gene. Expression quantities were normalized as described in results.

Spectrophotometric determination of hemoglobin content

The hemoglobin concentration in MEL cell lysates was measured with the TMB assay as described previously, with adjustments for 96-well plates[40]. The cell lysates were diluted to contain 100 μg of protein per mL, and their absorbance at 660 nm was measured using a $VICTOR^2$ D fluorometer (PerkinElmer, Waltham, Massachusetts, USA). The amount of hemoglobin was subtracted from the calibration curve composed of serial dilutions of purified hemoglobin (Sigma).

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Author Contributions

Conceived and designed the experiments: KH MP HG MW. Performed the experiments: MP HG MW. Analyzed the data: KH MP HG MW. Wrote the paper: MP KH.

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Impairment of Erythropoiesis in Inbred Cellular Prion Protein Deficient Mice

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Abstract

Cellular prion protein (PrPc) is necessary for pathogenesis of prion diseases, however, its physiologic role remains unclear. PrPc is expressed by hematopoietic stem cells and erythroid precursors and seems to be involved in stress erythropoiesis. To explore the involvement of PrPc in erythropoiesis, we studied challenged erythropoiesis after induction of acute anemia in mice with manipulated levels of PrPc as well as their physiologic erythropoiesis. Moreover, we investigated relative numbers of erythroid precursors in bone marrow and spleen of the mice.

The anemia was induced with phenylhydrazine (PHZ) and the recovery was monitored by analyses of hematocrit, plasma levels of erythropoietin (ELISA), Epo mRNA expression in kidneys (quantitative RT-PCR) and reticulocyte counts (flow cytometry). Dynamics of unchallenged erythropoiesis *in vivo* was examined by flow cytometry. The numbers of erythroid precursors were analyzed by multicolor flow cytometry employing selected differentiation markers.

Induction of anemia in wild type (WT) and Prnp-/- (KO) mice resulted in lower HCT and enhanced erythropoietin response in latter. Reintroduction of Prnp gene in Tga20 mice rescued the animals from severe anemia. WT mice exhibited enhanced turnover of red blood cells in circulation under physiologic conditions compared to KO mice. In more stringent model of B6KO mice, the effect of PrPc on PHZ anemia recovery was less pronounced whereas in CD1KO mice it appeared to be compensated. The physiologic erythropoiesis in B6KO and CD1KO mice was not significantly affected by PrPc expression. Nevertheless, relative numbers of erythroid precursors in bone marrow of B6KO Prnp-/- mice were slightly lower whereas in spleen were slightly elevated in comparison to Prnp+/+ mice.

In conclusion, PrPc expression accelerates recovery from stress erythropoiesis in inbred mice. The effect diminished in outbred mice. PrPc expression seems to be irrelevant or its deficiency compensated during normal erythropoiesis.

Introduction

Prion protein (PrPc) is GPI-linked cell surface glycoprotein necessary for pathogenesis of fatal neurodegenerative diseases, also called transmissible spongiform encephalopathies (TSEs). The essential event in the pathophysiology of TSEs is conversion of cellular PrPc to its conformational isoform PrPres [1]. PrPres is resistant to proteolysis and its accumulation in CNS is associated with spongiform degeneration and mortality [2].

Although PrPc is ubiquitously expressed and conserved throughout the species its physiologic function remains unclear. PrPc is important for normal CNS function as Prnp-/- mice show altered circadian rhythm, increased susceptibility to neuronal damage by oxidative stress and motor and cognitive abnormalities [10,11]. It was suggested that PrPc can act as copper binding protein, antioxidant [3-5], signal transductor [6] and receptor for various molecules, such as Bax-2, neural cell adhesion molecule (NCAM), stress inducible protein and plasminogen as reviewed in [7]. Moreover, it was reported its neuroprotective and anti-apoptotic activity (reviewed in [8,9]). Recent studies showed that PrPc plays a crucial role in regulating self-renewal and differentiation of embryonic stem cells [12,13] and that treatment with normal prion protein delays differentiation and helps to maintain high proliferation activity in human embryonic stem cells [14]. Recently published findings demonstrate importance of PrPc in hematopoiesis. It was demonstrated that PrPc is expressed by murine long-term hematopoietic stem cells (LT-HSC) [15] and is necessary for their self-renewal as HSC of Prnp-/- mice are not able to colonize after transplantation to irradiated mice [16]. Human bone marrow CD34+ stem cells also express PrPc [17,18] and PrPc expression is regulated during white blood cell differentiation with down-regulation along granulocytic lineage [15]. A connection between PrPc and erythropoiesis was demonstrated in Prnp-/- mice. We have previously shown that FVB Prnp-/- mice have prolonged recovery from experimental anemia probably due to reduced erythropoietin (Epo) response and enhanced apoptosis of erythroid precursors [19]. Recently it has been demonstrated that PrPc mediates iron uptake and transport in human neuroblastoma cells [20] and is necessary for iron homeostasis [21]. FVB Prnp-/- mice show relative iron deficiency with decrease in hemoglobin, serum iron and ferritin [22]. Developing red blood cells require high amount of iron thus PrPc may affect erythropoiesis. However, the nature of PrPc involvement in erythropoiesis is still not clarified. This study extends our previous observations by exploiting different mouse models with manipulated levels of PrPc expression to elucidate the role of PrPc in stress erythropoiesis. Moreover, we investigated PrPc role in unchallenged erythropoiesis. In addition, we analyzed proportion of erythroid precursors in various stages

of maturation in bone marrow and spleen of mice with manipulated levels of PrPc. Our results show that PrPc is important for stress erythropoiesis but lack of PrPc is compensated during normal erythropoiesis.

Results

PHZ anemia in C57BL/6 (WT) and C57BL/6 x129/Sv Prnp-/- (KO) mice

KO mice exhibited substantially lower HCT and corresponding higher Epo levels in plasma and Epo expression in kidneys during 120 h after PHZ administration than WT mice. As shown in Figure 1A, PHZ caused a rapid decrease in HCT for both WT and KO mice from approximately 49.8% ± 3.1 (mean ± SD) and 52.5% ± 1.8 (mean ± SD) to 42.0% ± 3.7 (mean ± SD) and 38% ± 1.2 (mean ± SD), respectively, 24 hours after induction of anemia and the decrease was significant in KO mice ($p < 0.05$). HCT was significantly lower for KO compared to WT at 72 h, 96 h and 120 h ($p < 0.01$).

Epo levels in plasma were comparable in WT and KO at 0 h (33 vs. 48 pg/mL) as shown in Figure 1B, however in WT mice peaked at 24 h (1810 pg/mL) whereas in KO mice at 48 h (2250 pg/mL) and remained elevated comparing to WT till 120 h after PHZ administration (478 vs. 108 pg/mL). Epo mRNA expression in kidneys of WT mice increased approximately 30-fold 24 hours after PHZ administration and declined after 72 h whereas in KO mice increased slowly with maximal 30-fold increase at 96 h Figure 1C . Relative expression of Epo mRNA was normalized within each group to 0 h.

Rescue effect of PrPc overexpression in Tga20 mice during PHZ anemia

After PHZ administration, the decrease in HCT was milder in Tga20 comparing to KO mice ($p < 0.05$) corresponding with less elevated RTC counts, plasma levels of Epo and Epo mRNA expression in kidneys (Figure 2A-D). HCT for Tga20 and KO mice decreased from 54.0% ± 1.7 (mean ± SD) and 56.7% ± 0.6 (mean ± SD) respectively, to 41.7% ± 5.0 (mean ± D) and 33.0% ± 7.2 (mean ± SD), respectively, 24 h after PHZ administration and the decrease was significant in KO mice ($p = 0.033$). HCT remained higher in Tga20 mice and the difference reached significance at 96 h as shown in Figure 2A.

Epo levels in plasma were lower in KO than Tga20 mice at 0 h (87 vs. 150 pg/mL) as shown in Figure 2B. At 24 h we detected a rapid increase in plasma Epo in both, KO and Tga20 mice to 2650 and. 2350 pg/mL, respectively, with decline at 96 h. Epo mRNA expression in kidneys of KO mice increased approximately 7-fold at 24 h after PHZ administration and still was 5-fold increased by 96 h whereas in Tga20 mice increased 5-fold by 24 h and

declined to almost normal levels at 96 h, as shown in Figure 2C. Relative expression of Epo was normalized within each group to 0 h.

Basal RTC counts were lower in Tga20 comparing to KO mice and reached 4.5% ± 0.2 (mean ± SD) in Tga20 vs. 6.0% ± 0.6 (mean ± SD) in KO mice ($p < 0.05$), as shown in Figure 2D. RTC increased to 8.1% ± 2.2 (mean ± SD) and 9.4% ± 0.9 (mean ± SD) in Tga and KO mice, respectively, 96 h after PHZ administration.

Phenylhydrazine dose response in Tga20 and Prnp-/- (KO) mice

24 h after PHZ administration had KO mice lower HCT than Tga20 in all groups treated with various concentrations of PHZ (Fig.3A) and the difference reached significance between groups which received PHZ 60 mg/kg mouse body weight ($p < 0.05$). Expression of Epo mRNA was strongly upregulated with increasing PHZ dose in KO mice, but not in Tga20 mice (Fig.3B). Epo levels in plasma increased more in KO mice in response to increasing dose of PHZ. Interestingly, Epo and Epo mRNA increased more rapidly in Tga20 mice than in KO mice when using PHZ concentration up to 60 mg/kg mouse weight. However, when PHZ reached concentration 80 mg/kg mouse weight we could see a switch in Epo response with higher levels of Epo mRNA and Epo in KO mice (Fig.3C). KO mice had smaller spleen than Tga20 mice under physiologic conditions, however, after PHZ administration the size of spleen in KO mice doubled (Fig.3D).

PHZ anemia in B6KO and CD1KO mice

B6KO Prnp-/- mice had significantly higher basal levels of HCT than Prnp+/+ and Prnp+/- mice (53.2% ± 2.3 (mean ± SD) vs. 49.0% ± 2.9 (mean ± SD) and 50.0% ± 2.1 (mean ± SD) ($p < 0.05$). After PHZ administration, we observed similar decrease in HCT in the three experimental groups with minimum at day 3 (Prnp-/- 32.3% ± 2.5 (mean ± SD) vs. Prnp+/+ 29.0% ± 2.2 (mean ± SD) and Prnp+/- 31.4% ± 1.8 (mean ± SD). However, the recovery to normal HCT was significantly retarded in Prnp-/- mice (Fig.4A). RTC counts rose more rapidly in Prnp+/+ B6KO after PHZ administration and declined to basal levels faster than in Prnp-/- and Prnp+/- Fig.4B) with significant differences from Prnp-/- mice at days 1, 2, 3 and 4 ($p < 0.05$). RTC counts culminated at day 7 reaching 23.3% ± 5.5 (mean ± SD) in Prnp+/+ vs. 19.8% ± 4.3 (mean ± SD) in Prnp-/- and. 23.4% ± 6.8 (mean ± SD) in Prnp+/- mice, respectively, and declined to almost basal levels at day 15.

In CD1KO mice, normal HCT levels were similar in Prnp+/+, Prnp+/- and Prnp-/- mice (56.6% ± 2.3 (mean ± SD) vs. 54.1% ± 6.4 (mean ± SD) vs. 54.5% ± 3.6 (mean ± SD) and

decreased equally after PHZ administration (Fig.4C) to minimum at day 3; 37.3% ± 3.8 (mean ± SD) in Prnp+/+ vs. 36.3% ± 3.5 (mean ± SD) in Prnp+/- and 36.7% ± 2.2 (mean ± SD) in Prnp-/-). Since day 5, HCT were slowly and equally recovering to almost normal levels at day 16 (51.8% ± 1.7 (mean ± SD) vs. 51.3% ± 4.8 (mean ± SD) vs. 52.3% ± 3.2 (mean ± SD) in Prnp+/+, Prnp+/- and Prnp-/- mice, respectively). Basal RTC counts were equal in all three groups (3.1% ± 0.1 (mean ± SD) vs. 3.3% ± 0.3 (mean ± SD) vs. 3.4% ± 0.5 (mean ± SD) for Prnp+/+, Prnp+/- and Prnp-/- mice) and substantially increased after PHZ administration (Fig.4D) with maximum at day 5 (17.5% ± 6.1 (mean ± SD) vs. 15.1% ± 4.7 (mean ± SD) vs. 18.2% ± 6.6 (mean ± SD) for Prnp+/+, Prnp+/- and Prnp-/- mice) and then slowly declined to basal levels at day 16 (4.0% ± 0.8 (mean ± SD) vs. 3.3% ± 1.7 (mean ± SD) vs. 3.2% ± 1.6 (mean ± SD) in Prnp+/+, Prnp+/- and Prnp-/- mice). No significant differences were found either in HCT or in RTC counts of CD1KO mice.

Dynamics of physiological erythropoiesis

Percentage of newly produced RBC released to peripheral blood after labeling of circulating RBC with NHS-biotin (described in Material and methods section) was estimated.

WT mice had significantly higher counts of newly produced RBC in comparison with KO and Tga20 mice during 46 days of chase (Fig.5C). The half life of RBC (T50) in peripheral blood was significantly shorter in WT mice and reached 23.0 ± 1.9 days (mean ± SD) compared to 30.7 ± 1.3 days (mean ± SD) in KO and 28.8 ± 1.1 days (mean ± SD) in Tga20 mice ($p < 0.05$).

In B6KO mice, the rate of RBC production was comparable in all three groups (Prnp-/-, Prnp+/+ and Prnp+/-) with no significant differences in numbers of newly produced RBC at analyzed time points during 57 days of chase (Fig.5D). However, the half life of RBC in circulation was slightly shorter in Prnp-/- mice reaching 26.2 ± 2.5 (mean ± SD) days compared to 28 ± 2.1 (mean ± SD) days and 28.1 ± 2.1 (mean ± SD) days in Prnp+/+ and Prnp+/- mice, respectively.

Similarly, in CD1KO mice we did not see significant differences in numbers of newly produced RBC released to circulation at investigated time points during 50 days of chase in Prnp+/+, Prnp+/- and Prnp-/- mice, respectively (Fig.5E). Again, the half life of RBC in circulation was slightly shorter in Prnp-/- mice reaching 24.5 ± 2.7 days (mean ± SD) compared to 26.5 ± 2.8 days (mean ± SD) in Prnp+/+ and 27.2 ± 2.5 days (mean ± SD) in Prnp+/- mice.

Erythroid precursors in bone marrow and spleen of B6KO and CD1KO mice

According to the expression of differentiation markers Ter119 and CD71 and the cell size (FSC), four erythroid precursor populations were identified, as described by Socolovsky et al. [23]: $CD71^{pos}Ter119^{dim}$ proerythroblasts (ProE), $CD71^{pos}Ter119^{pos}FSC^{high}$ basophilic erythroblasts (EryA), $CD71^{pos}Ter119^{pos}FSC^{low}$ late basophilic and polychromatic erythroblasts (EryB) and $CD71^{neg}Ter119^{pos}FSC^{low}$ orthochromatic erythroblasts and reticulocytes (EryC) (Fig.6A). Percentages of erythroid precursor populations and cell viability were analyzed in bone marrow (BM) and spleen (SP) of Prnp+/+, Prnp-/- and Prnp+/- B6KO and CD1KO mice.

The viability of BM cells was similar in B6KO Prnp-/-, Prnp+/+ and Prnp +/- and reached 87.7% ± 1.6 (mean ± SD), 88.3% ± 1.4 (mean ± SD) and 88.2% ± 1.8 (mean ± SD), respectively. We recorded slightly lower numbers of $Ter119^{pos}$ erythroid precursors in BM of B6KO Prnp-/- mice (51.1% ± 8.4) (mean ± SD) compared to Prnp+/+ (61.1% ± 9.8) (mean ± SD) and Prnp +/- (62.8% ± 12.5) mice (mean ± SD). However, the differences were not significant. SP cell viability was slightly lower in Prnp-/- (68.4% ± 6.3) (mean ± SD) than in Prnp+/+ and Prnp +/- (71.2% ± 6.4 and 71.9% ± 2.5, respectively) (mean ± SD). Interestingly, Prnp-/- mice had slightly higher numbers of $Ter119^{pos}$ precursors in SP (42.0% ± 9.4) (mean ± SD) compared to Prnp+/+ (36.8% ± 5.8) (mean ± SD) and Prnp +/- (34.3% ± 9.5) (mean ± SD), opposed to BM. Relative numbers of BM erythroid progenitors ProE were slightly higher in Prnp-/- mice, whereas percentages of EryA, EryB and EryC were lower in Prnp-/- compared to Prnp+/+ and Prnp +/- B6KO mice (Fig.6B) upper left panel). Interestingly, in SP we detected higher relative numbers of erythroid progenitors in Prnp-/- compared to Prnp+/+ mice (Fig.6B) upper right panel), althought the difference was not significant.

In CD1KO mice, BM cell viability was equal in Prnp-/- (95.0% ± 2.3) (mean ± SD), Prnp+/+ (95.0% ± 2.3) (mean ± SD), and Prnp +/- (94.1% ± 2.2) mice (mean ± SD). Numbers of $Ter119^{pos}$ precursor cells in BM were slightly higher in Prnp+/+ mice (50.4% ± 10.7) (mean ± SD) compared to Prnp-/- (45.2% ± 9.8) (mean ± SD) and Prnp +/- mice (44.2% ± 4.0) (mean ± SD). Numbers of erythroid precursors ProE, EryA, EryB and EryC were similar without significant differences (Fig.6B) lower left panel). SP cell viability was equal, reaching 89.4% ± 2.8 (mean ± SD) in Prnp-/- and 89.6% ± 3.2 (mean ± SD) and 90.1% ± 2 (mean ± SD) in Prnp+/+ and Prnp +/- mice, respectively. Percentages of $Ter119^{pos}$ precursors in SP of Prnp-/, Prnp+/+ and Prnp +/- mice were similar, reaching 33.3% ± 5.7,

31.6% ± 13.1 and 29.1% ± 6.0, respectively (mean ± SD). Relative numbers of ProE, EryA, EryB and EryC in SP were similar in Prnp+/+ and Prnp-/- mice (Fig.6B) lower right panel).
Expression of EpoR and GATA1 by BM cells of KOCD1 mice revealed slightly higher levels in Prnp+/+ mice, however, the difference was not significant.
PrPc expression on erythroid precursors was regulated during their maturation. Initial PrPc levels on ProE almost doubled on EryA and decreased in EryB and EryC, as we described previously [24].

Discussion

In this report, we demonstrate that PrPc may be important for efficient stress erythropoiesis in mice. We employed mice with various genetic backgrounds to investigate the role of PrPc in erythropoiesis *in vivo*. We followed stress erythropoiesis after acute hemolytic anemia caused by PHZ injection and basal erythropoiesis under physiological conditions. Moreover, we analyzed relative numbers of erythroid precursors in BM and SP of the mice.
We first used WT, KO and Tga20 mice. After PHZ administration, KO mice showed deeper anemia with lower HCT and retarded recovery to baseline comparing to WT mice, in accordance with our previous results in FVB mice [19]. Plasma Epo and its mRNA peaked later in KO than in WT mice and remained elevated suggesting delayed Epo response in KO mice. It is assumed that as anemia progresses, tissue hypoxia increases and leads to the induction of Epo expression in the kidney [25] and the increase in plasma Epo concentration drives the expansion and differentiation of bone marrow erythroid progenitors [26,27].
We could see only less than two fold higher increase in Epo mRNA in WT compared to KO mice 72h after PHZ anemia induction whereas in our previous results we reported approximately 3 fold higher elevation of plasma Epo and Epo mRNA at day 3 in FVB Prnp+/+ mice comparing to FVB Prnp-/- mice [19]. The discrepancy might be caused by different genetic background of mice used in the studies. Reintroduction of Prnp gene in Tga20 mice led to improved recovery from PHZ anemia. These data suggest that the absence of prion protein negatively affected erythropoiesis under stress conditions. Moreover, relative to PrPc overexpressing Tga20 mice of the same genetic background, PrP KO mice exhibited increased basal RTC numbers. This is in accordance with findings of Singh et al. who reported higher RTC counts in FVB Prnp-/- than in FVB Prnp+/+ mice [22]. Administration of increasing dose of PHZ (20 – 100 mg/kg mouse weight) to KO and Tga20 mice resulted in lower HCT in KO mice irrelevant of PHZ dose. Epo mRNA and plasma Epo rose more in KO than Tga20 mice after application of higher dose (≥ 80 mg/kg) of PHZ

indicating enhanced Epo response to higher stress in KO mice. Spleen size almost doubled in KO mice suggesting higher rate of senescence of KO RBC upon PHZ induced-anemia.

Observation of unchallenged physiological erythropoiesis in WT, KO and Tga20 mice revealed significantly higher numbers of newly produced RBC released to circulation in WT comparing to KO mice suggesting a higher rate of erythropoiesis in WT mice. However the numbers of newly produced RBC in Tga20 mice were only slightly higher than in KO mice. The results obtained in Tga20 mice did not seem physiological as we expected higher impact of PrPc overexpression by Tga20 mice on analyzed parameters and possible higher effect on erythropoiesis. These discrepancies could be due to different genetic background of mice used in experiments, as WT mice had C57BL/6 genetic background whilst KO and Tga20 mice had mixed C57BL/6x129/Sv background.

To elucidate the influence of genetic background we decided to construct more stringent mouse model by crossing inbred C57Bl/6 (B6) wild type mice with C57BL/6x129/Sv Prnp-/- (KO) mice. We obtained F2 generation offsprings with identical background and various PrPc expression (B6KO Prnp+/+, Prnp+/- and Prnp-/-). In this model we could see deeper decrease in HCT in Prnp+/+ mice during first days of PHZ anemia followed by elevated numbers of RTC compared to Prnp-/- mice. However, Prnp+/+ mice recovered faster to normal HCT levels. This suggests a more efficient erythropoiesis as a response to acute anemia in Prnp+/+ mice. Mice heterozygous for PrPc (Prnp+/-) constantly had HCT levels between Prnp+/+ and Prnp-/- demonstrating the importance of PrPc expression for erythropoiesis. However when studying unchallenged erythropoiesis we did not detect significant differences in numbers of newly produced RBC released to circulation in Prnp+/+ and Prnp-/- mice suggesting more complex mechanism of erythropoiesis regulation or possible compensation of PrPc deficit. Analysis of Epo levels in plasma and Epo mRNA levels in kidneys was done only the last day of PHZ anemia experiment due to limited numbers of mice available and no significant differences were recorded. As mice were almost recovered to normal at this time, the result is in accordance with normal erythropoiesis.

Next we constructed outbred control line by crossing outbred CD1 (wild type) mice with inbred KO mice. In F2 generation we obtained mice with different PrPc levels and the same genetic background (CD1KO Prnp+/+, Prnp+/- and Prnp-/-). In this model we detected equal decrease in HCT after PHZ anemia with similar numbers of RTC in all three PrPc genotypes. Similarly we did not find substantial differences in numbers of newly produced RBC released to circulation during unchallenged erythropoiesis. This could be partly due to relatively high

variability among the individual mice in the experimental groups. Probably the mice with outbred genotype can compensate the lack of PrPc to higher extend that inbred mice.

When we investigated the numbers of erythroid precursors in bone marrow during normal erythropoiesis we detected slightly but not significantly lower relative numbers of basophilic erythroblasts (EryA), late basophilic and polychromatic erythroblasts (EryB) and orthochromatic erythroblasts and reticulocytes (EryC) in BM of Prnp-/- B6KO mice suggesting less effective erythropoiesis. On the other hand, relative numbers of these erythroid precursors were slightly increased in SP of Prnp-/- B6KO. Steady state erythropoiesis producing new erythrocytes at a constant rate occures in mouse bone marrow whereas murine spleen is the site of stress erythropoiesis [28]. Thus the increased numbers of erythroblasts in the spleen of Prnp-/- B6KO suggest a compensation mechanism for less efficient basal erythropoiesis in bone marrow of the mice. In CD1KO mice the effect seems to be compensated due to outbred genotype of the mice. These findings are consistent with our results on unchallenged erythropoiesis and support our hypothesis that PrPc is important during stress erythropoiesis whereas its role might be compensated during unchallenged erythropoiesis.

As we report we could see significant effect of PrPc expression on stress erythropoiesis when comparing WT, KO and Tga20 mice. Nevertheless this effect was less pronounced in B6KO crossbreeds and diminished in CD1KO mice. WT mice had significantly enhanced RBC turnover in periphery compared to KO and Tga20 mice. However, we did not record significant differencies in unchallenged erythropoiesis in B6KO and CD1KO mice.

To sum up our data suggest that PrPc plays a role in stress erythropoiesis. Moreover, our data indicate that the genetic background is also important for the efficacy of erythropoiesis. However, elucidation of the mechanism regulating the interaction between PrPc and erythropoietin and the exact role of PrPc in erythropoiesis requires further experimentation.

Materials and methods

Animals

The study was approved by the Committee on the Ethics of Animal Experiments of the First Faculty of Medicine, Charles University in Prague (Permit Number: 217/07).

Inbred Prnp+/+ (WT) C57BL/6 (B6) and $Prnp^{0/0}$ (KO) Zurich line on mixed genetic background C57BL/6x129/Sv and outbred Prnp+/+ CD1 mice were purchased from Anlab (Prague, Czech Republic). PrPc overexpressing Tga20 mice (C57BL/6x129/Sv $Prnp^{0/0}$ with

randomly reintroduced Prnp gene) were purchased from European Mouse Mutant Archive (EMMA).

Generation of crossbreeds – more stringent models

C57BL/6 (B6) and C57BL/6x129/Sv $Prnp^{0/0}$ (KO) mice or CD1 and C57BL/6x129/Sv $Prnp^{0/0}$ (KO) mice, respectively, were crossed to obtain F2 generation designated B6KO and CD1KO, respectively. Genotype of the individuals was assessed by PCR [29] and animals were divided into 3 groups according to PrPc expression: Prnp+/+, Prnp+/- and Prnp-/- mice.

Phenylhydrazine induced anemia

First we performed experiments in WT, KO and Tga20 mice and later in B6KO and CD1KO crossbreeds with slight modifications as follows.

In WT, KO and Tga20 mice, acute hemolytic anemia was induced in 7-11-week-old males (median age WT 7 weeks, Tga20 11 weeks, KO 10 weeks) by an intra peritoneal (i.p.) administration of phenylhydrazine (PHZ; phenylhydrazine hydrochloride, Sigma-Aldrich, Prague, Czech Republic) in phosphate buffered saline (PBS) at the dose of 80 mg/kg mouse body weight. At defined time points 3 animals from each group were euthanized by diethylether (Penta, Prague, Czech Republic) inhalation and blood was collected by cardiac puncture through a heparinized needle. Hematocrits (HCT) were determined for each animal by cell counter Advia 60 (Bayer Health Care, Leverkusen, Germany) and by centrifugation of whole blood in 1 ml glass capillaries (2000xg 20 min). Plasma was prepared by centrifugation of whole blood at 2000x g for 5 min and frozen at -80ºC. Kidneys were immediately after extraction placed in RNA later (Ambion, Austin, USA) and stored at 4ºC until RT-PCR analysis.

B6KO and CD1KO mice, respectively, were grouped according to PrPc expression (Prnp+/+, Prnp+/-, Prnp-/-), 4-5 animals in each group. Anemia was induced in 25-27-week-old B6KO males (median age 26 weeks) and 13-15-week-old CD1KO males (median age 15 weeks) by an i.p. administration of PHZ in PBS at the dose of 60 mg/kg mouse body weight. Due to restricted numbers of animals, mice had been kept alive and blood was collected from tail vein at each time point and HCT was determined in CritSpin microhematocrit system (StatSpin, Norwood, MA, USA). For flow cytometry analysis of reticulocytes (RTC), 2 μl of whole blood were collected into 200 μl of PBS supplemented with 1% bovine serum albumin (Sigma-Aldrich) and 2 mM EDTA (Sigma-Aldrich) (PBS-BE).

Phenylhydrazine dose response study

Anemia was induced in KO (13-21 weeks old, median age 18 weeks) and Tga20 (9-14 weeks old, median age 12 weeks) males by an i.p. administration of PHZ in PBS at the dose range 20-100 mg/kg mouse body weight. Control group (n=4) received only PBS. Groups of KO and Tga20 mice were created (n=3) as though to reduce the difference in age between KO and Tga20 mice receiving the same PHZ dose. Animals were euthanized 24 hours after PHZ administration and heparinized blood was collected along with kidneys as described above. Blood of individual mice was analysed on cell counter Advia 60 (Bayer Health Care) and HCT was determined for each animal by centrifugation of whole blood in 1 ml glass capillaries (2000xg 20 min). Epo levels in plasma and Epo mRNA expression in kidneys were determined for each mice as described below.

ELISA determination of plasma erythropoietin

Plasma isolated from individual mice in a group was pooled and levels of erythropoietin (Epo) were analyzed by sandwich ELISA as described previously [19]. Briefly, mouse plasma was diluted 1:2 and incubated in a 96-well plate pre-coated with rat anti-mEpo (IgG1, BD) (50 µL of 4 µg/mL solution in PBS) overnight at 4°C. Bound Epo was detected by polyclonal rabbit anti-hEpo antibody (R&D Systems, Inc., Minneapolis, MN, USA) (10 µg/mL in blocking buffer, incubated 1 h at RT) followed by 50 µL of HRP-conjugated $F(ab')_2$ fragment of goat anti-rabbit IgG (H+L) antibody (Jackson Immunoresearch, West Grove, PA, USA) (1:3000 in blocking buffer, 50 µL per well, 1 h at RT). After addition of substrate (TMB) and stop solution (H_2SO_4**)**, the absorbance at 450 nm was measured using TECAN Sunrise microplate reader and Epo concentration was calculated from the calibration curve of Epo standards (Sigma Aldrich, Prague, Czech Republic).

Quantitative RT-PCR (qRT-PCR)

Kidneys were homogenized in RNA Blue (TopBio, Prague, Czech Republic) and RNA was isolated according to the manufacturer's protocol. RNA from individual mice was pooled in each group and treated with DNase (Turbo DNA-free, Ambion, Austin, TX, USA). Reverse transcription was performed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada) according to the manufacturer's manual. Erythropoietin was detected by qRT-PCR using Mm00433126_m1 probe (Applied Biosystems, Carlsbad, CA, USA) and 18S rRNA (Applied Biosystems,) was used as a reference gene. Quantitative RT-PCR was run with TaqMan Fast Universal PCR Master Mix, No AmpErase UNG

(Applied Biosystems) in ABI 7300 PCR System. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [30] and normalized to the reference 18S rRNA gene. Expression levels were normalized as described in results.

Biotinilation of erythrocytes in vivo

Erythrocytes (RBC) in peripheral circulation were labeled with 100 µl of Sulfo-NHS-biotin (*N* –Hydroxysulfosuccinimide-biotin) (Pierce, Rockford, USA) at 4 mg/ml in PBS by injection into tail vein. Only mice with at least 95% biotinilated RBC were included in the experiment. At defined time points 2 µl of blood were collected from tail vein and percentage of newly produced RBC released to circulation was assessed for each mouse by flow cytometry. Gating for RBC is showed in Fig.5A and example of increasing numbers of new nonlabeled RBC in circulation is shown in histogram plots in Fig.5B.

Flow cytometry of reticulocytes and RBC

Cell samples were analysed by BD FACS Canto II flow cytometer (BD, San Diego, CA, USA) with 405, 488 and 633 nm lasers and detectors in standard configuration.

The reticulocyte (RTC) counts were measured using thiazole orange (TO) (Sigma-Aldrich) at 0.1 µg/ml final concentration in PBS-BE (5µl of 1:10 diluted whole blood in 1 ml of buffer with TO or buffer alone for negative control were incubated for 1 hour at RT). RBC gate was established using forward (FSC) vs. side scatter (SSC). 200000 events in RBC gate were acquired and TO positive cells were counted as reticulocytes.

Biotinilated RBC were diluted 1:100 in PBS-BE and incubated with streptavidin (1:300) conjugated to Alexa488 (Invitrogen, Prague, Czech Republic) for 20 min at RT and washed with PBS-BE (450x g 5 min). 30000 events in RBC gate were acquired and percentage of non-labeled RBC was determined.

Flow cytometry of erythroid precursors in bone marrow (BM) of B6KO and CD1KO mice

BM cells were collected the last day of the PHZ experiment by washing femur with ice cold PBS-BE. Cells (~1 million) were labeled for 30 min on ice in dark with a mixture of monoclonal antibodies (mAbs) in saturating concentrations: anti-mouse Ter-119 eFluor® 450 (eBioscience, San Diego, CA, USA, clone Ter119), anti-mouse CD71 FITC (eBioscience, clone 17217) and anti-prion mAb AH6 PE (TSE Resource Centre, The Roslin Institute, University of Edinburgh, Roslin, United Kingdom; custom conjugated to PE by Exbio, Vestec, Czech Republic). Non-labeled cells were used to evaluate autofluorescence. After

labeling, cells were washed and kept on ice until FACS analysis. Dead cells were excluded using 7AAD (7-amino actinomycin D, Invitrogen). 100000 events in "Live" cells gate (7AAD negative) were acquired. Erythroid precursor subpopulations were gated as described previously [23]. Percentage of erythroid precursors and their PrPc expression were analyzed.

Statistical analysis

Statistical analyses were performed using GraphPad Prism v5 (GraphPad Software, Inc., La Jolla, CA, USA). T-test and two-way anova followed with Bonferoni post test were applied.

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Figure Legends

Figure 1

Course of acute anemia was significantly deeper in Prnp-/- (KO) mice than in WT mice.

Anemia was induced by phenylhydrazine (PHZ) (80 mg/kg). At defined time poins (0-120 h) 3 animals from each group were euthanized and blood was collected by cardial puncture. **(A)** PHZ administration resulted in deeper anemia with significantly lower hematocrit (HCT) in KO mice (*$p < 0.05$) with slower recovery to normal levels. HCT was determined for each animal. Values are mean ± SD (n=3). **(B)** Maximum plasmatic levels of erythropoietin (Epo) after PHZ administration were higher in KO mice. Samples of individual mice (n=3) were pooled. **(C)** Epo mRNA levels in kidneys rose 30-fold 24 h after PHZ administration in WT and 96 h after PHZ in KO mice. Samples of individual mice (n=3) were pooled and analyzed in duplicates, data presented are mean ± range.

Figure 2

Reintroduction of Prnp gene in Tga20 mice rescued the animals from severe anemia.

After induction of anemia by PHZ (80 mg/kg), 3 animals from each group were euthanized at defined time points (0, 24, 96 h). (**A**) After PHZ administration KO mice had significantly lower HCT levels than Tga20 (*$p < 0.05$). Values are mean ± SD (n=3). (**B**) Plasma Epo levels rose substantially 24 h after PHZ administration with only mild differences between KO and Tga20 mice. Samples of individual mice (n=3) were pooled. (**C)** Epo mRNA levels in kidneys increased apparently 24 h after PHZ administration and were higher in KO than Tga20 mice. Samples of individual mice (n=3) were pooled and measured in duplicates, values are mean ± range. (**D**) Reticulocyte counts were higher in KO at 0 h (*$p < 0.05$). Values are mean ± SD (n=3).

Figure 3

Phenylhydrazine dose response in Prnp-/- (KO) and Tga20 mice

Mice were euthanized 24 h after PHZ administration. (**A**) KO mice had lower HCT than Tga20 in all groups treated with various concentration of PHZ and the difference reached significance between groups which received PHZ 60 mg/kg mouse body weight (*p < 0.05). Values are mean ± SD (n=3). (**B**) Expression of Epo mRNA was strongly upregulated with increasing PHZ dose in KO mice but not in Tga20 mice. Samples of individual mice (n=3) were pooled and measured in duplicates. (**C**) Epo levels in plasma increased more in KO mice

in response to increasing dose of PHZ. Samples of individual mice (n=3) were pooled. (**D**) KO mice had smaller spleen than Tga20 mice under normal conditions, however, after administration of higher dose of PHZ the size of spleen in KO mice doubled suggesting higher rate of senescence of KO RBC. Values are mean ± SD (n=3).

Figure 4

PrPc influence on acute anemia recovery was mild in B6KO and diminishef in CD1KO mice. Acute anemia was induced by PHZ (60mg/kg). Mice were kept alive and blood was collected from tail vein at defined time points. Two independent experiments were performed. (**A**) B6KO Prnp-/- mice show significantly less efficient HCT recovery to normal levels comparing to Prnp+/+ mice ($*p < 0.05$) although the initial decrease in HCT was deeper in Prnp+/+ compared to Prnp-/- ($*p < 0.05$) and Prnp+/- ($^{\#}p < 0.05$) mice. Prnp+/- mice had faster HCT recovery to normal levels than Prnp-/- mice although significant only in day 7 ($+p < 0.05$). HCT was determined for each animal (n=5). Values are mean ± SD. (**B**) Higher relative numbers of reticulocytes in B6KO Prnp+/+ mice compared to Prnp-/- ($*p < 0.05$) and Prnp+/- ($^{\#} p < 0.05$) mice correspond with deeper course of experimental anemia and faster recovery of Prnp+/+ mice. (**C**) CD1KO mice with different levels of PrPc expression did not differ in the HCT after PHZ administration or in percentage of reticulocytes (**D**).

Figure 5

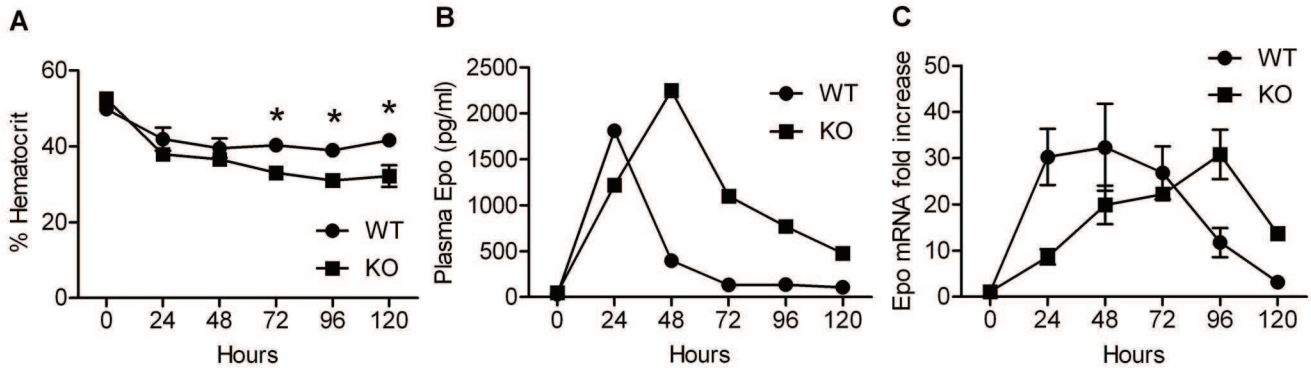
Dynamics of unchallenged erythropoiesis is significantly enhanced in WT compared to KO and Tga20 mice. RBC in peripheral blood were labeled in vivo with Sulfo-NHS-biotin. Blood was collected from tail vein and percentage of new nonlabeled RBC released to circulation was assessed by flow cytometry. (**A**) RBC were gated according to their FSC/SSC characteristics. (**B**) Example of gradual increase in nonlabeled RBC in circulation with time. (**C**) Numbers of new nonlabeled RBC were higher in WT compared to KO ($*p < 0.05$) and Tga20 ($^{\#}p < 0.05$) mice at all time points suggesting higher rate of erythropoiesis in WT mice. Values are mean ± SD (n=5). (**D**) Dynamics of erythropoiesis was similar in B6KO mice and (E) KOCD1 mice with different levels of PrPc. Values are mean ± SD (n=5).

Figure 6

Flow cytometric analysis of erythroid precursors in mouse bone marrow (BM) and spleen (SP)

(**A**) Gating strategy for erythroid precursors; upper left – FSC/SSC plot of BM cells, upper right – exclusion of dead cells using 7-AAD, lower left – live cells labeled with CD71/Ter119: ProE - $CD71^{pos}Ter119^{dim}$ proerythroblasts, Ter119+ cells were further gated on CD71 vs. FSC plot showed in lower right: EryA - $CD71^{pos}Ter119^{pos}FSC^{high}$ basophilic erythroblasts, EryB - $CD71^{pos}Ter119^{pos}FSC^{low}$ late basophilic and polychromatic erythroblasts and EryC - $CD71^{neg}Ter119^{pos}FSC^{low}$ orthochromatic erythroblasts and reticulocytes. (**B**) Relative numbers of erythroid precursors in mouse BM and SP. The percentage of ProE, EryA and EryB were approximately two times higher in BM (left panels) than in SP (right panels) of B6KO nad CD1KO mice. Prnp-/- B6KO mice display slightly lower percentages of erythroblasts in BM (upper left) with slight elevation in SP (upper right). Percentages of erythroblasts in BM and SP of CD1KO mice (lower left and lower right, respectively) do not differ between mice expressing and lacking PrPc.

Figure 1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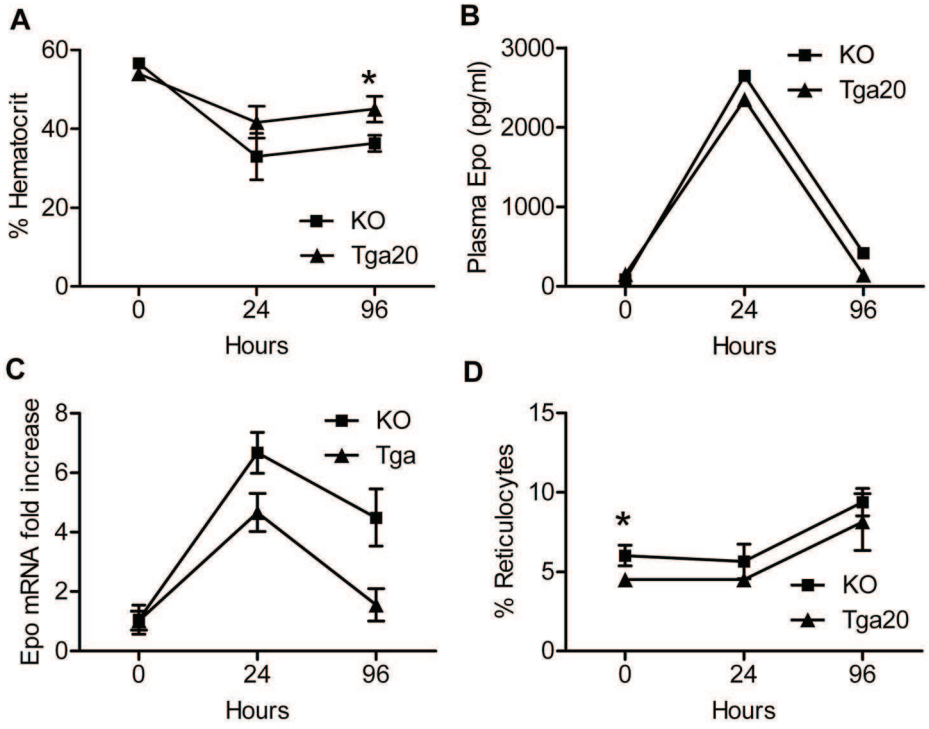
% Hematocrit
60
40
20
0
0 24 48 72 96 120
Hours
*
*
*
WT
KO
B
Plasma Epo (pg/ml)
2500
2000
1500
1000
500
0
0 24 48 72 96 120
Hours
WT
KO
C
Epo mRNA fold increase
50
40
30
20
10
0
0 24 48 72 96 120
Hours
WT
KO

Figure 2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% Hematocrit
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KO
Tga20
0
24
96
Hours
B
Plasma Epo (pg/ml)
3000
2000
1000
0
KO
Tga20
0
24
96
Hours
C
Epo mRNA fold increase
8
6
4
2
0
KO
Tga
0
24
96
Hours
D
% Reticulocytes
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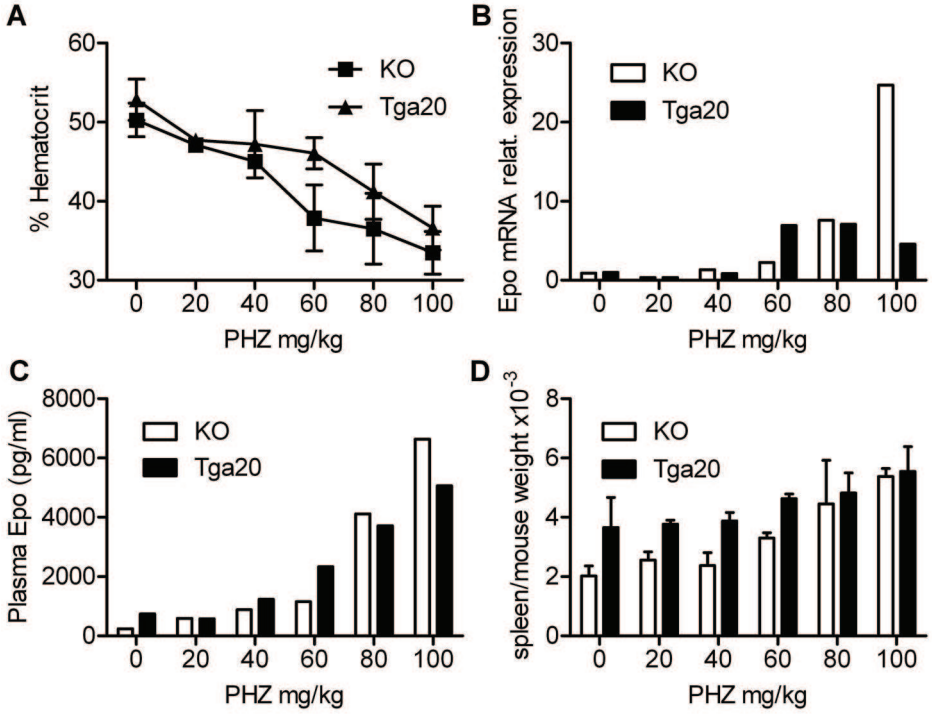
10
5
0
*
KO
Tga20
0
24
96
Hours

Figure 3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50
40
30
KO
Tga20
0
20
40
60
80
100
PHZ mg/kg
B
Epo mRNA relat. expression
30
20
10
0
KO
Tga20
0
20
40
60
80
100
PHZ mg/kg
C
Plasma Epo (pg/ml)
8000
6000
4000
2000
0
KO
Tga20
0
20
40
60
80
100
PHZ mg/kg
D
spleen/mouse weight x10-3
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6
4
2
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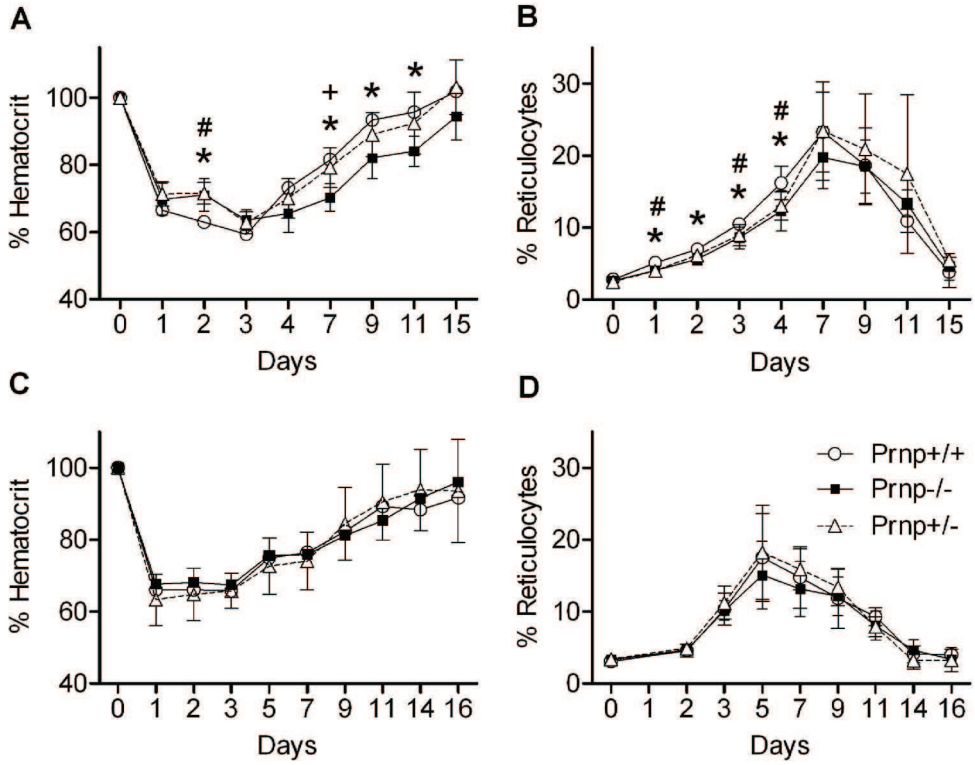
KO
Tga20
0
20
40
60
80
100
PHZ mg/kg

Figure 4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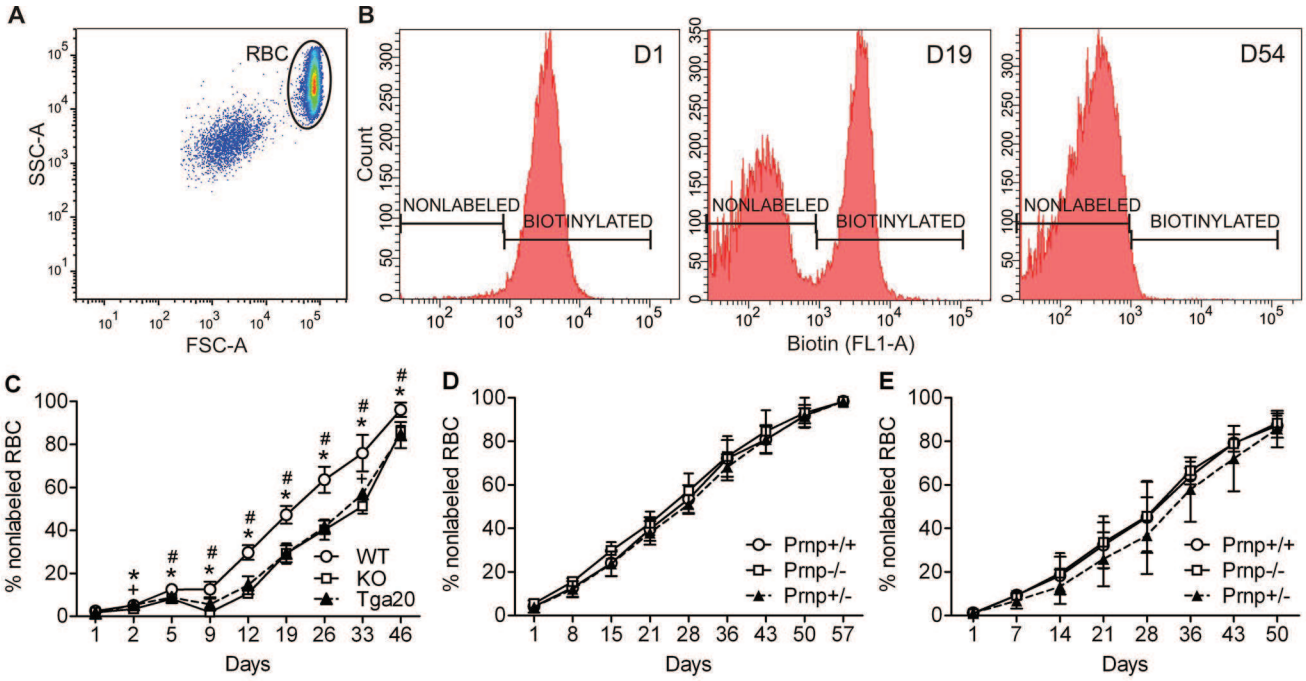
100
80
60
40
0 1 2 3 4 7 9 11 15
Days
B
% Reticulocytes
30
20
10
0
0 1 2 3 4 7 9 11 15
Days
C
% Hematocrit
100
80
60
40
0 1 2 3 5 7 9 11 14 16
Days
D
% Reticulocytes
30
20
10
0
0 1 2 3 5 7 9 11 14 16
Days
Prnp+/+
Prnp-/-
Prnp+/-

Figure 5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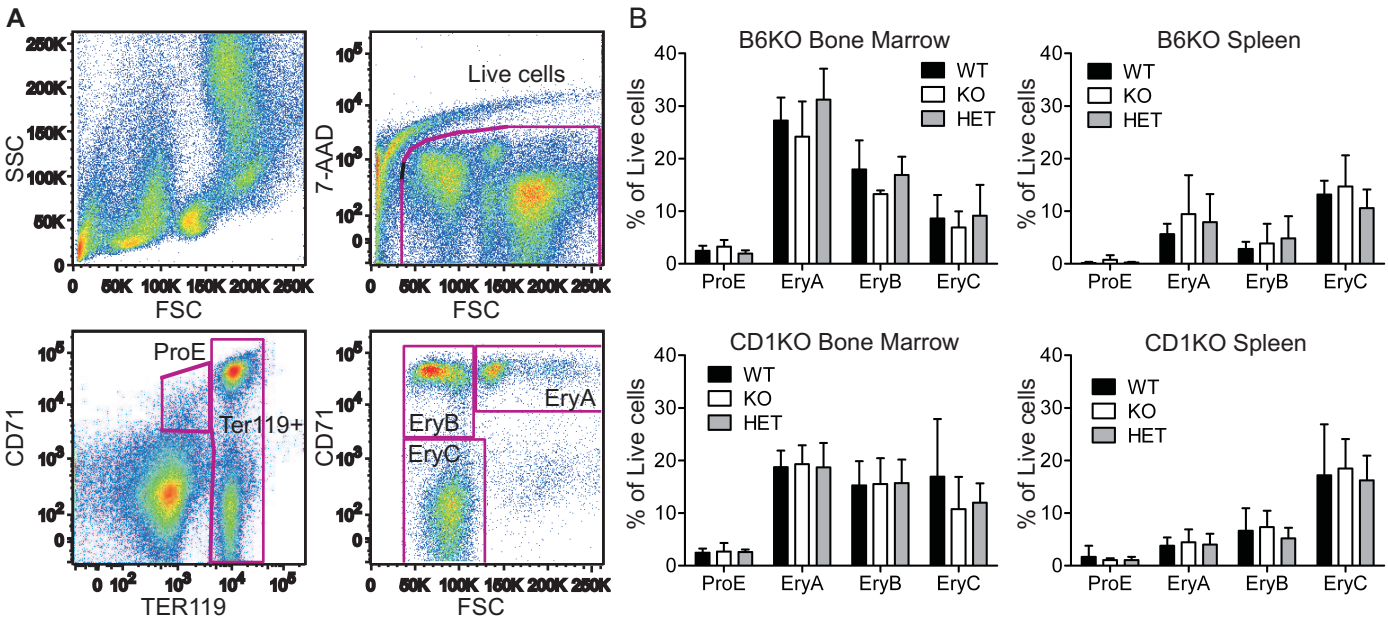
RBC
SSC-A
FSC-A
B
D1
D19
D54
Count
NONLABELED
BIOTINYLATED
Biotin (FL1-A)
C
% nonlabeled RBC
Days
WT
KO
Tga20
D
Prnp+/+
Prnp-/-
Prnp+/-
E

Figure 6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SSC
FSC
Live cells
7-AAD
ProE
Ter119+
CD71
TER119
EryA
EryB
EryC
B
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B6KO Spleen
CD1KO Bone Marrow
CD1KO Spleen
WT
KO
HET
% of Live cells
ProE
EryA
EryB
EryC