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The role of cellular prion protein in erythroid differentiation

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Abstrakt

Buněčný prionový protein (PrP^C) je evolučně konzervovaný protein, exprimovaný na povrchu buněk různého původu. Přestože PrP^C hraje zásadní roli v patogenezi neurodegenerativních chorob, jeho fyziologická funkce zůstává neznáma. Prionové choroby jsou charakteristické dlouhou dobou latence, během které nejsou diagnostikovatelné žádnou konvenční metodou. Krev by mohla být ideálním materiálem pro vývoj takových testů, bohužel vlastnosti PrP^C na krevních buňkách a jeho funkce není dosud spolehlivě vysvětlena. Naše práce ukázala, že jednotlivé lidské červené krvinky exprimují pouze malé množství PrP^C, ale vzhledem k počtu erytrocytů v krvi představuje tento protein většinu PrP^C vázaného na krevní buňky. Na základě našich dat usuzujeme, že PrP^C na povrchu erytrocytů je unikátně modifikován. Podobná modifikace by v případě patologického PrP mohla znesnadňovat diagnostiku prionových chorob z krve. Je prokázáno, že v průběhu prionových onemocnění dochází k deregulaci transkripce erytroidních genů a že $PrP^{-/-}$ myši mají oslabenou odpověď vůči experimentálně navozené anémii. Pro objasnění úlohy PrP^C v erytropoéze jsme proto sledovali jeho expresi u myších erytroidních prekursorů *in vitro* i *in vivo*. Prokázali jsme, že v průběhu diferenciace buněk dochází k regulaci povrchové exprese PrP^C na erytroidních prekursorech z myší sleziny a kostní dřeně. Závislost exprese PrP^C na průběhu erytroidní diferenciace jsme potvrdili i na modelu myší erytroleukemické buněčné linie (MEL). Pomocí RNA interference (RNAi) jsme vytvořili erytroleukemické buněčné linie se stabilně sníženou expresí PrP^C, u kterých jsme ukázali, že za normálních podmínek je diferenciace MEL buněk nezávislá na expresi PrP^C. Metodu RNAi jsme dále použili pro studium důležitosti exprese PrP^C při propagaci prionů v neuronální buněčné linii CAD5.

Abstract

The cellular prion protein (PrP^C) is evolutionary conserved protein expressed in cells of various origins. Although PrP^C plays a basic role in the pathogenesis of the fatal neurodegenerative prion disorders, its physiological role remains enigmatic. Prion diseases are characteristic by long latency period during which they are not identifiable by any conventional methods. Although the blood is an ideal material for developing of screening tests, little is known about traits of PrP^C and its role in blood cells. We showed that human erythrocytes express low amounts of PrP^C per cell, but due to the high numbers of erythrocytes, they are major contributors to the pool of blood cell-associated PrP^C. Based on our biochemical characterization we propose that PrP^C on human erythrocytes is uniquely modified. Such a modification in abnormal prion protein may complicate screening tests for prion diseases in blood. It was reported that prion diseases deregulate the transcription of erythroid genes, and $PrP^{-/-}$ mice demonstrate a defective response to experimental anemia. To investigate the role of the PrP^C in erythropoiesis, we studied the protein's expression on mouse erythroid precursors *in vivo* and *in vitro*. We showed that surface expression of PrP^C on erythroid precursors in bone marrow and spleen follows similar pattern as the cells mature. We demonstrated that the regulation of PrP^C expression in differentiating murine erythroleukemia cells (MEL) cells resembles its regulation seen *in vivo*. Using RNA interference (RNAi) we created MEL lines with stably silenced expression of PrP^C, which showed that under normal conditions PrP^C seems dispensable for erythroid differentiation of MEL cells. We further used RNAi methodology to study the effect of PrP^C silencing on the propagation of prion infection and its influence on neuronal CAD5 cell culture.

1. Introduction

The *Prnp* gene codes for the PrP^C, which is expressed in various tissues with the highest level of expression in neurons. Together with its pathological isoform (PrP^{TSE}) plays a crucial role in prion diseases also known as Transmissible Spongiform Encephalopathies (TSE) [1]. Until recently, in modern societies, the only documented mode of human-to-human transmission was spread of the disease via contaminated neurosurgical instruments, tissue grafts or hormones [2]. However, since the year 2003, four cases of probable transmission of vCJD by blood transfusion were described in United Kingdom [3]. Although it seems that annual number of vCJD cases decreases, due to the long and asymptomatic subclinical phase, number of infected humans may be significantly higher. These cases could constitute threat of iatrogenic transmission. The situation requires development of sensitive and non-invasive detection method. Yet, the effort for a development of such test is complicated by the absence of the molecular marker other than PrP^{TSE} itself. Blood is an ideal source for diagnostic screening tests, but detection of PrP^{TSE} in blood is problematic. Properties of blood PrP^{TSE} are not known. Moreover, its detection is complicated by the significant amount of poorly characterized PrP^C, which could interfere with PrP^{TSE} detection on blood cells. In humans, PrP^C was found as early as on hematopoietic stem cells and its expression is regulated during differentiation to diverse blood cell lineages [4][5]. Particularly, the expression of PrP^C on erythrocytes seems to be a critical, due to the high proportion of erythrocytes on blood cell count. Presence of PrP^C on erythrocytes was already proven, but opinions about its amount were inconsistent [5][6][7][8]. Analysis of published data suggested that the difference could be caused by the use of different antibodies for PrP^C detection. To clarify this issue was one of the goals of this study.

Interestingly, despite its medical importance, the physiological role of PrP^C remains puzzling. Mice lacking PrP^C surprisingly do not suffer from a loss of PrP^C, but in addition are resistant to TSE infection and do not propagate PrP^{TSE} [9][10]. However, experiments in cell cultures suggested that PrP^C is involved e.g. in the prevention of apoptosis, copper metabolism linked to oxidative stress, iron metabolism, signalization and differentiation [11][12][13]. Several recent reports indicated the role of the PrP^C also *in vivo*. Impaired erythropoiesis in $PrP^{-/-}$ mice after experimental induction of anemia demonstrated Zivny et al. [14]. Singh with colleagues observed systemic iron

deficiency in $PrP^{-/-}$ mice affecting also erythropoiesis [15]. Apparently, the expression of PrP^C seems generally important in hematopoiesis as PrP^C is present in murine long-term hematopoietic stem cells and was shown to support their self-renewal [16]. Investigation of the PrP^C importance for erythropoiesis was second goal of my thesis.

We employed a widely used model for the study of erythroid differentiation *in vitro* presented by murine erythroleukemia cells (MEL), which is cell line blocked at the proliferative proerythroblast stage. The MEL cells are interesting by its ability to re-enter differentiation program upon treatment with polar substances, e.g. hexamethylene bisacetamide (HMBA) [17]. Process resembles natural erythroid differentiation [18][19]. Interestingly, transcriptional activation of the *Prnp* gene has been already demonstrated in growth-arrested MEL cells [20].

2. Aims and hypothesis

The objective of our studies was to contribute to the understanding of the importance of PrP^C in physiology of blood cells, and its possible involvement in TSE pathogenesis in blood system. The knowledge of the PrP^C biology of blood origin can be subsequently applied in a development of non-invasive detection methods that are not still introduced in general use.

Goals of my study:

1. To characterize the biochemical properties of the PrP^C on red blood cells and to reveal the reason for disproportions in published data concerning quantity of erythrocyte-associated PrP^C.

2. To study the importance of PrP^C expression for erythroid differentiation utilizing murine models.
   - To describe regulation of PrP^C on erythroid precursors during their differentiation *in vivo* and *in vitro*.
   - To introduce and optimize methodology of RNAi into our laboratory.
   - To describe the effect of PrP^C silencing on erythroid differentiation using the model system of MEL cells.

3. To study the effect of prion infection on erythroleukemic MEL and neuronal CAD5 cell cultures with silenced transcription of *Prnp*.

3. Material and methods

In current study we worked with human and murine material. Human blood and brain samples were used for comparison of PrP^C properties among platelets, erythrocytes, and brain. Laboratory mice were used for isolation of erythroid precursors from bone marrow and spleen to evaluate expression of PrP^C during precursor maturation. For *in vitro* experiments we used following cell cultures: MEL 707, NIH3T3, CAD5 cells, HEK293 GP2, and Phoenix cells.

To study the function of PrP^C in differentiating erythroleukemic MEL cells, we optimized delivery of retrovector coding short anti-*Prnp* RNAs into target cells by transfection or transduction. Retrovirus produced by HEK293 GP2 packaging cells was subsequently delivered to target cells by spinfection or co-cultivation. After selection with puromycin, we obtained cells expressing LP1, LP2 shRNA targeting PrP mRNA and control-nonsilencing LN shRNA. Retroviral delivery by spinfection was used also to silence PrP^C expression in neuronal CAD5 cells.

To examine the effect of prion infection on MEL and CAD5 cells with silenced expression of *Prnp* we infected them with brain homogenate containing RML prion strain.

Experiments were analysed on various hierarchical levels- cells, proteins and nucleic acids. Basic characteristics of cells e.g. cell viability and cell counts were monitored using cell counter after Trypan Blue exclusion assay.

Fluorescence-activated cell sorting (FACS) was used to analyse the cell populations according to scattergram and the presence of specific proteins. The number of molecules per cell was calculated [21].

Expression of proteins was detected by western blot upon separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis or by cell blot assay and immunodection with specific antibodies. Hemoglobin concentrations in differentiating MEL cells were measured by the tetramethylbenzidine assay [22]. Uptake of radiolabeled ^{59}Fe-transferrin by MEL cells was analyzed by Tesla NA 3601 gamma counter after exposure of cells to ^{59}Fe labeled transferrin.

Transcription of genes was examined by quantitative real time PCR (qRT-PCR). Relative fold expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [23] normalized to the reference *Gapdh* gene.

Statistical significance was determined by two-tailed *t*-test (paired or unpaired test). A p value of < 0.05 was considered significant. Error bars are represented by mean ± standard deviation.

4. Results

4.1. Characterization of human PrP^C associated with human red blood cells

Using quantitative FACS, we observed notable differences in the binding of prion antibodies FH11, 3F4, and 6H4 to human erythrocytes. In assumption that one IgG molecule binds one PrP^C molecule, the highest number of PrP^C molecules (~ 300) per one cell was found by mAb 6H4. Both mAbs 3F4 and FH11 bound in much lower numbers. In contrast to erythrocytes, 3F4 and 6H4 bound to platelets in identical samples roughly equivalently, suggesting that the difference in binding is caused by features of erytrocyte PrP^C (Ery-PrP^C). This led us to the analysis of biochemical properties of Ery-PrP^C. We found that 3F4 bound to serially diluted brain PrP^C, but minimaly or not at all to PrP^C in serially diluted erythrocyte ghost samples (Fig. 1B). At the same time, 6H4 detected brain and Ery-PrP^C with similar sensitivity (Fig. 1A).

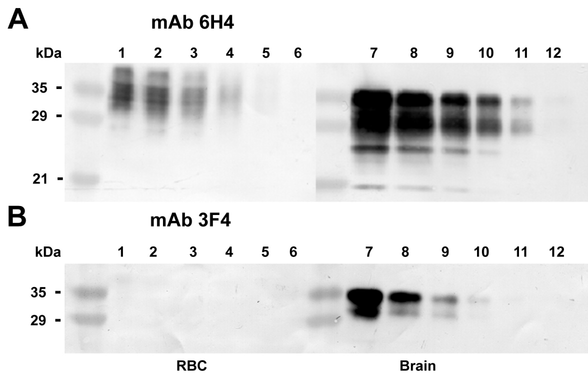

Figure 1: Comparison of PrP^C detection using mAbs 6H4 and 3F4. Western blot of serially twofold diluted samples of 1x 10^{10}/mL human erythrocyte ghosts (1-6) and 10% normal human brain homogenate (7-12) were developed with mAb 6H4 (**A**) or 3F4 (**B**). Denaturation of proteins after boiling with sample buffer, demonstrate that poor binding of 3F4 to human red blood cells is not caused by occlusion of its epitope by conformational change or the interaction of PrP^C with putative binding partner on the surface of cells.

Ery-PrP^C is present mainly in its diglycosylated form with a molecular weight (MW) slightly higher than brain PrP^C. Deglycosylation of PrP^C with PNGase F led to the detection of a single band with a MW similar to deglycosylated brain PrP^C. No fragments suggesting a significant presence of truncated forms of Ery-PrP^C were detected. Denaturation of the sample by boiling with SDS or its deglycosylation did not improve the binding of 3F4 to Ery-PrP^C. The incubation of red blood cell ghosts with increasing concentrations of proteinase K led to

gradual and complete cleavage of PrP^C, demonstrating that Ery-PrP^C is similarly as sensitive to proteinase K as PrP^C in equally treated samples of platelets or brain homogenate. Further confirming that the conformation of Ery-PrP^C is not the cause of diminished 3F4 binding. The remaining explanation of this effect is postranslational modification of 3F4 epitope.

4.2. *In vitro* modification of brain PrP^C causes the low binding of mAb 3F4

We chemically treated brain samples to test if modification of PrP^C may prevent binding of 3F4. Oxidation of brain PrP^C with increasing concentrations of H_2O_2 had little effect on the binding of mAb 3F4 or 6H4. In contrast, modification of lysine residues by incubation with glyoxylic acid inhibited 3F4 binding, while the binding of 6H4 was not affected. Similar situation was observed using Sulfo-NHS-biotin (Fig. 2). Contrary to peripheral erythrocytes, equal binding of 3F4 and 6H4 to $CD71^+$ erythroid precursors in cord blood was recorded, suggesting that the modification of the 3F4 epitope occurs during the 120 days long life of erythrocytes in circulation, possibly by glycation.

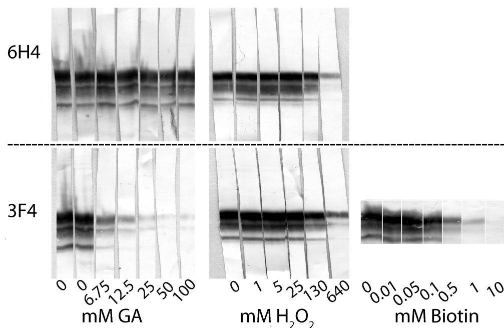

Figure 2: Glycation of PrP^C could be the reason for diminished binding of 3F4 to human erythrocytes. *In vitro* treatment of western blots of normal brain homogenate with increasing concentrations of glyoxylic acid (GA), H_2O_2, and Sulpho-NHS-Biotin. Modification of lysine residues by GA and Biotin in contrast to oxidation of methionines by H_2O_2 mimics the discrepancy in the binding of mAbs 3F4 and 6H4 found *in vivo* in human Ery-PrP^C.

4.3. Expression of PrP^C is regulated during murine erythroid differentiation

4.3.1. Expression of PrP^C differs on murine erythroblast subpopulations

The expression of PrP^C on mouse bone marrow and spleen progenitors was evaluated by flow cytometry. Erythroid precursors were gated and divided to subpopulations according their Ter119, CD71 and the FSC signals (Fig. 3A) [24]. PrP^C is upregulated in early erythroblast precursors and then decreases during their maturation to erythrocytes (Fig. 3B). Observed regulation of PrP^C expression suggests its involvement in erythroid differentiation.

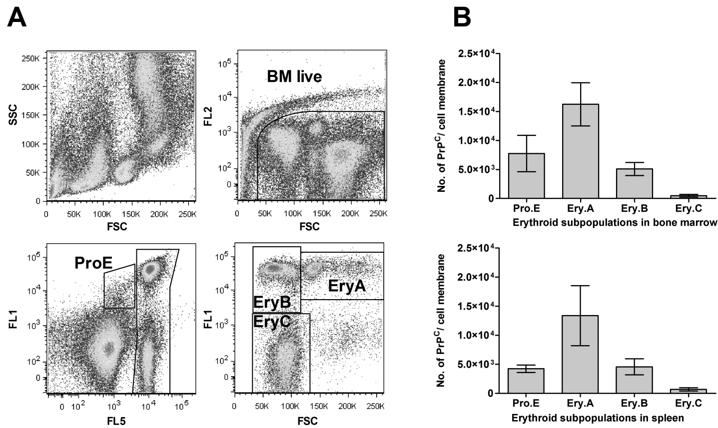

Figure 3: The expression of PrP^C on mouse bone marrow and spleen erythroid precursors is upregulated in early erythroblasts and then decreases with the cells maturation. (**A**) Gating strategy for erythroid precursors: upper left- scattergram of BM cells; upper right- gating of viable 7-AAD negative cells (BM live); lower left- live BM cells labeled with CD71-FITC (FL1) and Ter119-eFluor450 (FL5): ProE- $CD71^+Ter119^{+/-}$; $Ter119^+$ cells were further gated on CD71-FITC (FL1) and FSC plot (lower right): EryA- large $CD71^+$, EryB- small $CD71^+$, EryC- small $CD71^-$. (**B**) Quantitative FACS analysis of PrP^C expression on erythroid precursors in mouse bone marrow and spleen. Pro.E= proerythroblasts; Ery.A= early basophilic erythroblasts; Ery.B= late basophilic and polychromatic erythroblasts; Ery.C= orthochromatic erythroblasts and reticulocytes.

4.3.2. Distinct regulation of PrP^C during inducer mediated differentiation

Next we studied the expression of PrP^C using MEL cell model. The level of *Prnp* mRNA in MEL cells induced to differentiation by 0.5 mM HMBA doubled within 24 h after induction, a similar increase in the uninduced cells was observed after 48 h (Fig. 4AB). Upregulation of PrP^C protein in uninduced cells correlated with the expression of *Prnp* mRNA, reaching a maximum in confluency at 96-120 h (Fig. 4C). In contrast, the expression of the PrP^C protein in differentiating cells peaked at 24-48 h post-induction (Fig. 4D) with a subsequent decrease to almost basal level at 120 h mimicking the regulation of PrP^C expression detected *in vivo* (4.3.1.).

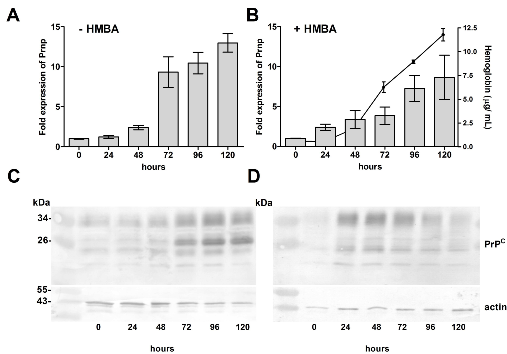

Figure 4: Distinct expression of PrP^C protein in differentiating (+ HMBA) and uninduced (- HMBA) MEL cells. Transcription of *Prnp* mRNA increases in uninduced MEL cells (**A**) slower, but similarly like in cells induced to erythroid differentiation (**B**). Hemoglobin production (B, full line) illustrates progress of differentiation. Increased amount of PrP^C protein correlates with elevated mRNA transcription in growth-arrested uninduced cells (**C**), but not in the differentiated cells, where expression of PrP^C protein reaches its maximum 24-48 hours post induction (**D**).

4.2.3. Treatment with inhibitor of histone deacetylases upregulated PrP^C

We evaluated the dependence of *Prnp* gene transcription on methylation status of its promoter sequence and chromatin status in its locus by treatment of MEL cells with Aza-deoxycytidine (AzaC) an inhibitor of methyltransferases and trichostatin A (TSA) an inhibitor of histone deacetylases. Incubation of uninduced MEL cells with TSA upregulated *Prnp* transcription 10-fold higher than in untreated cells. No difference was seen in cells with AzaC, suggesting that *Prnp* transcription in MEL cells is activated by chromatin relaxation.

4.2.4. Inhibitor of differentiation does not prevent expression of PrP^C

To get closer insight to PrP^C expression during erythroid differentiation, we treated MEL cells with dexamethasone (DEX), a suppressor of the commitment to terminal differentiation. The presence of HMBA during first 24 h of culture leads to increased levels of PrP^C irrespective of DEX presence, suggesting that the effect of HMBA is superior to DEX. In contrast, DEX decreases the level of PrP^C in uninduced cells after 120 h to the level seen in differentiated cells, demonstrating that the activation of the glucocorticoid receptor can interfere with the transcriptional activation of the *Prnp* gene.

4.3. Introduction of RNAi methodology and optimization of gene delivery methods in effort to study of PrP^C role

4.3.1. Optimization of retrovector delivery to MEL cells

Great effort was spent on the optimization of RNAi methodology. Extensive analysis of stably transfected MEL cells with the vectors coding anti-

Prnp shRNAs suggested that vector was recombined during its insertion to genomic DNA. Irreproducibility of previous nonviral gene delivery system urged us to employ approach based on retroviral transduction. Using virus producing cell line Phoenix, we succeeded to infect NIH3T3 cells and inhibit its expression of *Prnp*. However we were unable to repeat it in MEL cells and it forced us to use HEK293 GP2 packaging cells capable to produce pantropic virus.

4.3.2. RNAi by shRNAmiRs led to stable and efficient PrP^C silencing

Final transduction of MEL cells with anti-*Prnp* LP1 and LP2 shRNAmiR led to the efficient silencing of PrP^C on mRNA and protein level (Fig. 5AB).

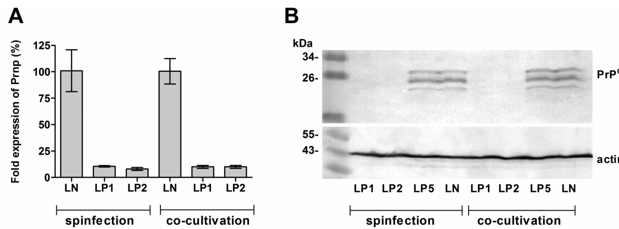

Figure 5: Downregulation of PrP^C expression by RNAi in MEL cells after selection with puromycin. (**A**) Both methods of retroviral delivery led to ~ 90% silencing of *Prnp* mRNA in both lines expressing anti-*Prnp* shRNAmiR (LP1 and LP2) when compared to the cells expressing nonsilencing shRNAmiR (LN). (**B**) Confirmation of PrP^C downregulation at the protein level. No silencing was observed in LP5 cell line.

Silencing of PrP^C was stable during the entire differentiation. In comparison with MEL LN line, PrP^C expression in LP1 and LP2 cells was inhibited on average down to ~ 20 and 5% at 0 or 120 h, respectivelly (Fig. 6).

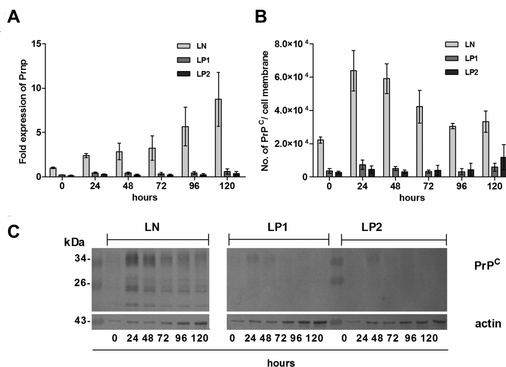

Figure 6: Expression of PrP^C is stably repressed during the differentiation of MEL cells. (**A**) Level of *Prnp* mRNA is downregulated in LP1 and LP2 lines stably expressing anti-*Prnp* shRNAmiR in comparison with control (LN) cells during the course of cell differentiation. Confirmation of stable PrP^C silencing on protein level by quantitative FACS (**B**) and western blot (**C**).

4.4. Silencing of *Prnp* gene by RNAi suggests that PrP^C is dispensable for erythroid differentiation *in vitro*

To evaluate the effect of PrP^C silencing on differentiation of MEL cells we investigated number of markers. Transcription of *c-myb* was downregulated similarly in all lines (LN, LP1 and LP2), demonstrating similar loss of proliferative capacity after HMBA induction. As well, cell counts during differentiation did not show effect of PrP^C silencing on proliferation and viability of MEL cells. This was supported also by similar levels of proapoptotic *Bax* transcription. Similar quantity and pattern of transferrin receptor (TfR) expression, production of hemoglobin, and expression of selected erythroid specific genes- *Eraf*, *Hba*, and *GATA1* during the differentiation was observed. Uptake of ^{59}Fe-Tf in intervals up to 60 min was increased in LN line over the rest of the lines. However, variability in uptakes of ^{59}Fe-Tf among control MEL lines with non-silenced PrP^C expression prevented us to assign this finding as significant. Similar expression of TfR was found in all lines after iron chelation by desferral.

4.5. Utilization of RNAi in study of infectious prion in cell culture

4.5.1. Attempts to establish MEL cell line propagating abnormal PrP

To establishe *in vitro* model for the study of influence of prion infection on erythroid differentiation, we infected MEL707, LN, LP1, and LP2 with RML prion strain. However, we detected PrP^{TSE} even in MEL cells with minimal expression of PrP^C. Decreased presence of RML signal in LP1 and LP2 lines after washing of cells suggested that we detected inoculum.

4.5.2. RNAi leads to efficient silencing of *Prnp* gene in CAD5 cells

Transduction of CAD5 cells by LP1 and LP2 retrovectors led to downregulation of *Prnp* mRNA for ~90%, in comparison to LN line. Similar administration of LP1 and LP2 retrovectors to infected, RML prions propagating CAD5 cells silenced *Prnp* transcription for ~80%. Interestingly, expression of stress response *Eif2ak2* gene in LP1 and LP2 lines was significantly lower than expression in cells with normal PrP^C level. In contrast, expression of *Eif2ak2* in LP1 and LP2 cells incubated with RML inoculum was significantly higher in comparison with *de novo* RML infected LN cells. This would suggest the role of PrP^C in cell stress.

4.5.3. Downregulation of PrP^{C} expression reduce PrP^{TSE} formation

Silencing of PrP^{C} expression prior to infection wih RML prion strain prevents *de novo* PrP^{TSE} formation in LP1 and LP2 cells contrary to control lines, which propagated PrP^{TSE}. This observation was supported by the presence of infectious prions in conditioned media and cell lysates from infected LMP or LN cells, but not from LP1 and LP2 cells. Silencing of PrP^{C} expression in already prion infected CAD5 cells reduced, but did not prevent propagation of PrP^{TSE}, suggesting limited ability of RNAi to cure cells from TSE.

4.5.4. Expression of endogenous retroviruses (ERVs) in CAD5 cells

To study possible involvement of ERVs in prion infection, we screened transcription of selected ERVs in CAD5 cells in dependence of PrP^{C} expression and presence of PrP^{TSE}. Expression of three ERVs was detected at significant level: *IAP-1*, *MLV* and *VL30-2*. In uninfected cells expression of *IAP-1* and *VL30-2* in LP1 and LP2 cells was significantly lower than in LN (Fig. 7A). On the other side, incubation of cells with RML prions led to significantly higher expression of *IAP-1* and *MLV* in LP1 and LP2 cells than in LN cells (Fig. 7B).

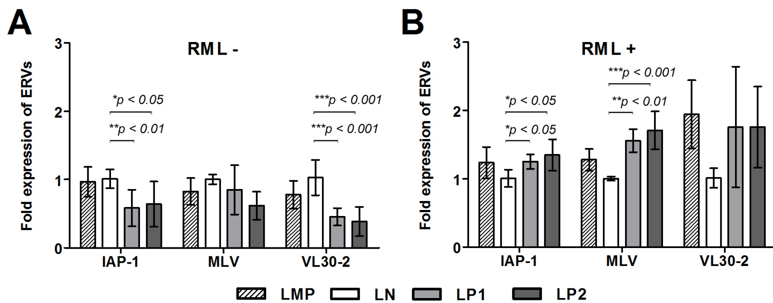

Figure 7: Transcription of ERVs tends to decrease in uninfected LP1 and LP2 cells (**A**) contrary to RML treated cells (**B**), where transcription of ERVs has ascending tendency.

5. Discussion

5.1. Characterization of human erythrocyte-associated PrP^C

Considering the importance of PrP^C expression in prion pathogenesis, our study was aimed at the elucidation of controversies surrounding its expression on human erythrocytes. Previous work detected that most of the membrane bound PrP^C resides on platelets and erythrocytes do not express PrP^C or at low level [5] [8]. These studies utilized probably the best characterized prion mAb 3F4 [25]. Our quantitative FACS analysis using mAbs FH11 and 3F4, like in published negative reports, confirmed that they bound to erythrocytes substantially less than 6H4. Interestingly, 3F4 bound to platelets equivalently to 6H4. The lower binding of 3F4 could be caused by a proteolytic loss of its epitope similarly as was shown for FH11 [26]. The presence of PrP^C C1 fragment lacking the 3F4 epitope has been described in the normal brain tissue [27], but we did not find similar fragments in human erythrocyte ghosts, demonstrating that truncation was not the cause of the decreased 3F4 binding. The Ery-PrP^C band had slightly lower electrophoretic mobility and was more diffuse than PrP^C in the brain. This could be caused by its different glycosylation pattern, which in theory may contribute to the inaccessibility of the 3F4 epitope. However, enzymatic deglycosylation of Ery-PrP^C did not restore its recognition by 3F4. This, together with the fact that low binding of 3F4 was also recorded after denaturation of proteins by boiling in SDS suggests that the low binding is not caused by its conformation or by the shielding effect of an unknown binding partner of Ery-PrP^C. Our results suggest that the reason for decreased binding of 3F4 to Ery-PrP^C is posttranslational modification of its epitope, which occurs after the release of erythrocytes into circulation, as $CD71^+$ erythroid precursors in cord blood bound antibodies 3F4 and 6H4 equally well. The importance of methionine and lysine residues in the 3F4 epitope for binding of the antibody has been already demonstrated [28][29]. It seems that modification of lysine residues and not oxidation of PrP^C is the cause of the low binding of 3F4 as showed treatment with H_2O_2 or sulfo-NHS-biotin. Glycation is one of the physiologically occurring modifications of lysine residues. The treatment of PrP^C with glyoxylic acid, which modifies lysine residues to the major advanced glycation end products (AGEs) led to a dose-dependent decrease of 3F4 binding, while the binding of 6H4 was unaffected. The time dependent modification of membrane proteins on human erythrocytes during their aging in circulation has been reported [30]. This corresponds with our observation that the epitope of 3F4 in

erythroid precursors in cord blood as well as in platelets in circulation is not modified. Indeed, erythrocytes with their life span reaching 120 days compared to the 10 days of platelets are more likely to be modified. Interestingly, glycation of PrP^{TSE} has already been demonstrated in brains of TSE-infected rodents [31]. Despite the lack of direct proof, our data suggest that the diminished binding of 3F4 to Ery-PrP^C may be caused by glycation. In effort to prove it directly, we are developing monoclonal antibodies targeting AGEs on PrP^C (Dvorakova et al., 2011). In addition to the explanation of negative reports about Ery-PrP^C, we demonstrate that the difference in the level of PrP^C between one platelet and one erythrocyte is less than 10-fold, while the normal amount of erythrocytes is about 20-fold higher than the amount of platelets. This implies that erythrocytes should carry at least twice as much PrP^C than platelets, which makes erythrocytes the main source of cell-associated PrP^C in human blood and corrects previous reports in the literature.

5.2. Expression of PrP^C is regulated during murine erythroid differentiation

Downregulation of several erythroid genes during prion infection was the first observation that peripheral pathogenesis of prion diseases is linked to erythropoiesis [32][33]. 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. In agreement with observations *in vivo,* we found that initial upregulation of PrP^C in differentiating MEL cell culture was followed by a gradual downregulation of PrP^C along with the progression of differentiation, 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 [34] 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^C protein and mRNA levels in neuronal cells [35]. Transcription of *Prnp* gene in proliferating cells may be repressed by inaccessibility of its promoter for transcription factors. In agreement with previous study [36] *Prnp* transcription is probably activated by chromatin relaxation as we show here by treatment of MEL cells with TSA, an inhibitor of histone deacetylases. Stimulation of the glucocorticoid receptor by DEX induces the proliferation of erythroid progenitors and delays the terminal differentiation of erythrocytes [37]. In our hands DEX did not prevent the HMBA-induced initial upregulation of PrP^C in MEL cells suggesting that it precedes the effect of DEX which is known to suppress the HMBA-mediated

commitment to terminal cell division at a relatively late step in this process [38]. However, DEX prevented the increase of PrP^C protein levels in confluent MEL cells after 120 h of culture demonstrating that the activation of glucocorticoid receptor can interfere with the transcriptional activation of the *Prnp* gene mediated by cell-cycle arrest. The mechanism of DEX's action on the prevention PrP^C protein upregulation in confluent MEL cells is unknown at present.

5.2.1. Establishment of cell lines with stable silenced *Prnp* expression

To study the importance of PrP^C in the differentiation of MEL cells, we created cell lines with stably silenced *Prnp* expression by RNAi. To overcome possible problems with virus entry to target cells we used HEK293 GP2 cells. This cell line allows producing G glycoprotein of the vesicular stomatitis virus, which mediates the fusion of viral and cellular membranes independently to receptor [39]. Second improvement of our system was founded by the shRNA expressed in context of endogenous miRNA. Such shRNA (shRNAmiR) enters the RNAi pathway at the beginning, raising thus effectivity of silencing [40]. Sequences designed here as LP1 and LP2 targeting *Prnp* gene were adopted from "RNAi codex" database [41].

5.2.2. Influence of *Prnp* silencing on erythroid differentiation *in vitro*

To the best of our knowledge, our model is the first murine cell line of non-neuronal origin with stably silenced PrP^C expression. Growth curve of LP1, LP2 and control LN line after the induction of differentiation was comparable. Similarly, the level of the proto-oncogene *c-myb*, expression of which is characteristic of the proliferative state and its constitutive expression has been demonstrated to block MEL cells' differentiation [42][43], decreased upon induction similarly in all created cell lines. All cell lines demonstrated similar dynamics and level of hemoglobinization and regulation of the transferrin receptor on their cell membranes even under the iron deprivation. Furthermore, we did not observe reliable difference in uptake of transferrin coupled with radiolabeled iron. 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 uptake was described *in vitro* and *in vivo* [15][44]. Monitoring of selected erythroid genes (*Eraf*- erythroid associated factor, *Hba*- hemoglobin α, and *GATA1*) on the transcriptional level also did not reveal significant differences among LP1, LP2 and LN cell lines. In many cell cultures, the overexpression of PrP^C was proposed to facilitate cytoprotective effects [45].

However, overexpression of exogenously delivered PrP^C in MEL cells did not protect the cells against apoptosis initiated by serum withdrawal [46]. 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. This result is concurrent with Christensen and Harris, who revaluated 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* [47]. We can speculate that PrP^C, which was shown to bind both laminin and the laminin receptor, can be involved in cell-cell contacts in the maturating erythroblasts, or with a surrounding extracellular matrix [48][49]. Such a role for PrP^C is unlikely to be detected by the MEL cell model. Other explanations could be that PrP^C exerts its function only under stress conditions, the effect of PrP^C silencing was compensated for by an unknown pathway, or that the remaining expression is sufficient to sustain its role.

5.3. Effect of *Prnp* downregulation on propagation of infectious prion protein in cell culture and expression of endogenous retroviruses

We hypothesized that erythroid differentiation of MEL cells could provide an *in vitro* model for the confirmation of previously observed influence of prion disease on transcription of erythroid genes [32][33]. However, our first insight shows that MEL cells resist to propagate PrP^{TSE}, which could be explained by several factors, e.g. relative low amount of PrP^C, blocking effect by composition of growth medium [50], and cell division halving the amount of PrP^{TSE} at each cell-cycle [51]. However, we showed that infectious RML inoculum resists in cell culture sufficient time for possible interaction with erythroid differentiation. On the other side, we introduced *de novo* prion infection in susceptible CAD5 lines, but not in cells with silenced PrP^C. Besides the study of prion propagation in cell culture, we planned to describe the influence of PrP^C silencing together with the PrP^{TSE} propagation on expression of endogenous retroviruses (ERVs). We observed differential transcription of *IAP-1*, *VL30-2*, and *MLV* ERVs in LP1 and LP2 cells in comparison to LN. Albeit our findings suggest involvement of PrP^C in regulation of ERVs transcription, it remains to be elucidated if observed differences are also biologically meaningful. Interestingly, transcription of *Eif2αk2* one of the prominent stress-response genes correlated with expression of ERVs. Althoug dsRNA, which may also arise during ERVs biogenesis [52], is one of the main triggers of *Eif2αk2* known as double-stranded RNA-activated protein kinase, the implication of such regulation is unclear.

6. Conclusion

1. Our data show that erythrocytes are the main source of cell-associated PrP^C in human blood. Furthermore, we emphasize that PrP^C on human erythrocytes may differ from PrP^C on other blood cells by its postranslational modification and as such might play significant role in prion pathogenesis.

2. We found out that expression of PrP^C is regulated during erythroid differentiation *in vivo* and *in vitro*. Using RNAi, we created murine *in vitro* cell culture model for erythroid differentiation with downregulated PrP^C expression. To the best of our knowledge, our model is the first murine cell line of non-neuronal origin with stably silenced PrP^C transcription. We showed that under unchallenged growth condition PrP^C is dispensable for erythroid differentiation *in vitro*.

3. MEL cells seem to be resistant to propagation of the infectious prion protein. However, RML inoculum is sustained in cell culture at sufficient time required for erythroid differentiation, suggesting suitability of such model for the study of PrP^{TSE} influence on erythroid differentiation. *De novo* propagation of PrP^{TSE} in neuronal CAD5 cells with downregulated PrP^C is effectively abrogated. However, downregulation of PrP^C post PrP^{TSE} infection does not eliminated prion infection.

4. We found differential regulation of several endogenous retroviruses in CAD5 cells in dependence on the level of PrP^C expression and the presence of prion infection. However, biological relevance of our findings remains for future clarification.

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8. Publications

Present work is based on the following articles:

1. Panigaj M., Brouckova A., Glierova H., Dvorakova E., Simak J., Vostal J.G., Holada K., Underestimation of the expression of cellular prion protein on human red blood cells, *Transfusion* 51, 1012-1021.
   IF= 2.982

2. Panigaj M., Glierova H. and Holada K., Expression of prion protein in mouse erythroid progenitors and differentiating murine erythroleukemia cells, (submitted).

Articles related to present study:

3. Dvorakova E., Prouza M., Janouskova O., Panigaj M. and Holada K., Development of monoclonal antibodies specific for glycated prion protein. J Toxicol Environ Health A, (accepted).
   IF= 1.724

4. Glierova H., Simak J., Panigaj M., Vostal J.G. and Holada K., The post transfusion recovery and survival of red blood cells in mice is affected by the expression of cellular prion protein, (submitted).