The role of prion protein in stem cell regulation

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Abstract

Cellular prion protein (PrP\(^C\)) has been well described as an essential partner of prion diseases due to the existence of a pathological conformation (PrP\(^Sc\)). Recently, it has also been demonstrated that PrP\(^C\) is an important element of the pluripotency and self-renewal matrix, with an increasing amount of evidence pointing in this direction. Here, we review the data that demonstrate its role in the transcriptional regulation of pluripotency, in the differentiation of stem cells into different lineages (e.g. muscle and neurons), in embryonic development, and its involvement in reproductive cells. Also highlighted are recent results from our laboratory that describe an important regulation by PrP\(^C\) of the major pluripotency gene Nanog. Together, these data support the appearance of new strategies to control stemness, which could represent an important advance in the field of regenerative medicine.


Introduction

The cellular prion protein (PrP\(^C\)) is a normal cell surface glycoprotein highly conserved through evolution and throughout mammalian species (Rivera-Milla \textit{et al.} 2006). It is initially expressed during early embryogenesis, while in the adult it is mainly located in brain neurons and spinal cord (Manson \textit{et al.} 1992, Harris \textit{et al.} 1993). It can also be found, but at lower levels, in neuroglia, the reproductive tract, spleen, lymph nodes, and immune cells (Brown \textit{et al.} 2000, Ford \textit{et al.} 2002). Its role in prion diseases has been well described, wherein a conformational isoform of this protein (PrP\(^Sc\)) could lead to the appearance of typical pathognomonic symptoms (Prusiner 1982, Castilla \textit{et al.} 2003). The first knockout (KO) mouse models were generated in the 1990s (Bueller \textit{et al.} 1992, Manson \textit{et al.} 1994), which helped to demonstrate that PrP\(^C\) is essential in these neurodegenerative diseases. However, even though strong efforts have been made to determine its normal function, there is still no unequivocal evidence of its contribution to the physiology of both the cell and the organism. The absence of a remarkable phenotype in these PrP\(^C\)-KO animals (Bueller \textit{et al.} 1992) and the discovery of other members of the prion family, Doppel and Shadoo, are in the top of the list of difficulties that confront functionality studies. In fact, controversies about the particular impact of these proteins on embryo development have arisen. In the case of Shadoo, a recent study failed to observe a clear phenotype in the mouse Shadoo-PrP\(^C\) double-KO embryos (Daude \textit{et al.} 2012) while a previous report described a lethal embryonic phenotype (Young \textit{et al.} 2009). However, the inactivation of PrP\(^C\) in zebrafish induced embryonic lethality (Malaga-Trillo \& Sempou 2009), highlighting the lack of a clear model that could connect the observations. Recently, it has been reported that this lethal consequence is due to the function of the prion protein family in controlling trophoblastic cell lineage maintenance and differentiation (Passet \textit{et al.} 2012).

Several approaches have been employed in this search, from newly designed KO models to cell culture, where PrP\(^C\) appeared to be involved in oxidative stress (McLennan \textit{et al.} 2004), neuroprotection (Li \& Harris 2005), copper metabolism (Brown \textit{et al.} 1997), synaptic and nervous transmission (Bremer \textit{et al.} 2010), and cellular adhesion (Malaga-Trillo \textit{et al.} 2009). Interestingly, the latest studies pointed to an important role for PrP\(^C\) in pluripotency and differentiation of embryonic stem cells (ESC), and development. PrP\(^C\) transcription is 1.5 times higher in ESC than in somatic cells (Grskovic \textit{et al.} 2007). In fact, results from our laboratory described the first non-redundant role of this protein in the control of early differentiation and the complexity of the interactions that PrP\(^C\) carried out in this process (Miranda \textit{et al.} 2011a, 2011b). Intriguingly, there were several other works that found a relation of PrP\(^C\) with muscle cell regeneration (Stella \textit{et al.} 2010) and its upregulation in the reprogramming of somatic cells into induced pluripotent stem cells (Mikkelsen \textit{et al.} 2008). An increased PrP\(^C\) expression in breast, prostate, and gastric cancers was also reported, which is related to long-term proliferation (Pan \textit{et al.} 2006, Mehrpour \& Codogno 2009). Additionally, PrP\(^C\) was described as being overexpressed by between 1.4 to 6 times during the first 2 weeks of spontaneous mouse ES cell differentiation.
Reproduction was localized at the gray matter (Sales et al. 2008). This expression pattern was conserved throughout the marginal regions of the CNS but was absent from the heart, lung, and intestine. A first peak of PrPC expression occurred between E7.5 and E8.5 mouse embryonic days. This expression of PrPC is restricted to the ventricular zone, particularly in those cells that have undergone neural differentiation. In contrast, neural progenitors did not express the protein (Tremblay et al. 2007). At E11.5, PrPC expression extends to motor neurons of the ventral neural tube and at E13.5 to non-neural tissues, including tooth, kidney, extra-embryonic and amniotic membranes, and intestines (Manson et al. 1992, Hajj et al. 2009). Thus, PrPC expression may be associated with neural differentiation. This hypothesis is supported by in vitro experiments in which mouse PrPC expression was positively correlated with the differentiation of multipotent neural precursors and ESC into neurons (Steele et al. 2006, Novitskaya et al. 2007). Furthermore, treatment of rat embryonic hippocampal neurons with recombinant PrPC induces neurite outgrowth (Kanaani et al. 2005). All this evidence points to a role for PrPC in the cell signaling pathways necessary for self-perpetuation and differentiation of ESC (Lopes & Santos 2012).

In cattle, Prnp mRNA levels correspond to those of the protein immunolabeling during gestation (Peralta et al. 2012). These authors reported expression of PrPC in the nervous system of day 27, 32, and 39 bovine embryos, mainly in brain, spinal cord, ganglia, and peripheral nerves (Peralta et al. 2012). As occurred in mice, PrPC was highly expressed in the differentiated neural cells of the marginal regions of the CNS but was absent from the mitotically active progenitors of periventricular areas. This expression pattern was conserved throughout development until the adult stage, where most PrPC was localized at the gray matter (Sales et al. 2002). In bovine embryos, the peripheral expression of PrPC on days 27, 32, and 39 was slightly different from mice, which was marked only in kidney and liver. Moreover, the authors could not detect the di-glycosylated isoform of PrPC predominant in several adult tissues, suggesting that this isoform arises at later stages of development (Peralta et al. 2012). Concurrent with this latter result, the enzymes involved in the addition of N-glycans to PrP C have been demonstrated to be upregulated during the maturation of the CNS (Goh et al. 2007).

Prnp is phylogenetically conserved in zebrafish since human-related prion protein genes PrP-1 and PrP-2, as well as their transcripts, have been described and a less human-related isoform PrP3 has been recently found (Cotto et al. 2005). RNA deep sequencing of transcripts in early development showed that only PrP-2 persisted as maternal RNA until early gastrula (5.3 hpf), the other isoforms were not detected at this stage. Furthermore, several studies in which these genes were deleted demonstrated their essential role in embryo survival, contrary to what happens in mice (Malaga-Trillo & Sempou 2009, Nourizadeh-Lilabadi et al. 2010). Indeed, PrP-1 KO zebrafish embryos arrested in gastrulation due to a marked decrease in tissue integrity and cell–cell adhesion, and defective brain development was found at 24 hpf in the absence of PrP-2 (Malaga-Trillo & Sempou 2009). Curiously, the PrP-1 knockdown phenotype can be reversed by the extrinsic introduction of zebrafish PrP-2 mRNA or mouse Prnp mRNA, suggesting that Prnp genes of both species share functionality (Malaga-Trillo & Sempou 2009). Together, these data again pointed to an important role for PrPC during early embryo development, which may also be conserved among species.

It is interesting to note that Prnp is transcriptionally activated during a period of embryogenesis when there is a shift in the type of bioenergy production utilized from anaerobic to an aerobic metabolism. Aerobic metabolism carries the concomitant production of potentially damaging reactive oxygen species (ROS), such as the superoxide radical. Cu/Zn superoxide dismutase (SOD) is an antioxidant enzyme whose activity is impaired in brains from PrPC-KO mice, while its activity is elevated in brains of transgenic mice overexpressing PrPC (Brown et al. 1997). This might result from PrPC being a cuproprotein (Stockel et al. 1998) and observations show that Cu/Zn SOD receives copper from PrPC. Therefore, a gene dosage effect of PrPC on Cu/Zn SOD activity is clear. Additionally, it was previously suggested that PrPC itself may possess SOD activity (Brown et al. 1999); thus, a potential role for PrPC in the cellular response to oxidative stress seems likely. Miele et al. (2003) proposed that PrPC is activated during the mouse embryonic E8.5–E9 period as part of an antioxidant mechanism required to deal with elevated levels of ROS production, which occurs as aerobic metabolism begins. It is known that copper induces rapid endocytosis of PrPC and, therefore, the role of this protein in the antioxidant defense might be via the provision of copper to the cytosolic Cu/Zn SOD. Alternatively, increased levels of Prnp mRNA in response
to exposure to superoxide radicals may indicate that PrP<sup>C</sup> itself has more direct antioxidant capabilities. Indeed, evidence suggesting that both recombinant and immunoprecipitated PrP<sup>C</sup> possess SOD activity supports this view (Brown et al. 1999).

**PrP<sup>C</sup> in ESC pluripotency and differentiation**

PrP<sup>C</sup> is highly conserved among mammals and it is widely distributed in different tissues (Linden et al. 2008). Taking this assessment as a starting point, the functionality of PrP<sup>C</sup> was thought to be easily described from the development of the first PrP<sup>C</sup>-KO models (Bueler et al. 1992, Manson et al. 1994). However, several difficulties appeared as PrP<sup>C</sup> is not just a single protein but a family of at least two other proteins (Doppel and Shado) (Fig. 1; Watts & Westaway 2007) that could have redundant roles and could compensate for its absence (Behrens 2003, Young et al. 2009). Regarding this compensative mechanism, previous results from our laboratory demonstrated that the time at which these studies are performed has to be carefully selected (Miranda et al. 2011b). Several transcriptional changes in PrP<sup>C</sup>-KO mouse ES cell differentiation were observed early in the process, but not at later stages. This included downregulation of an important gene involved in self-renewal, Nanog, and a high pluripotency degree in a percentage of the embryoid bodies (EBs) developed in these cultures (Fig. 2).

In the same way, several other studies point to a major role for PrP<sup>C</sup> in the behavior of ES cells. Peralta et al. (2011) demonstrated how PrP<sup>C</sup> had a clear influence on the transcription of the ectodermal marker Nestin and, as a consequence, in the differentiation of mouse ESC into neural progenitor cells. Remarkably, this is not just an in vitro effect. As PrP<sup>C</sup> is highly expressed in the CNS, most interest has been focused on defining the events that it executes in neurons. Using overexpressing PrP<sup>C</sup> (OE), PrP<sup>C</sup> wild-type (WT), and PrP<sup>C</sup>-KO mice, it has been shown that the subventricular zone and the dentate gyrus of brains from OE mice have more proliferating cells than PrP<sup>C</sup>-KO mice (Steele et al. 2006). Moreover, after inducing differentiation of E13.5 multipotent neural precursors, ~26% of OE cells were MAP-2-positive neurons, in comparison with 18 and 14% from the WT and PrP<sup>C</sup>-KO cells respectively. Importantly, although in a lower proportion, KO cells still formed new neurons that translated into the absence of differences in the gross morphology of the CNS and in the net final number of neurons in comparison with WT or OE mice (Steele et al. 2006). Hence, these authors suggested a clear influence by PrP<sup>C</sup> in neuronal differentiation but not an essential involvement, being part of the transition from multipotent precursors to mature neurons. These results, as has already been mentioned in this review, were supported by the fact that differentiated neurons, but not their mitotically active progenitors, express PrP<sup>C</sup> during mouse and cattle development (Tremblay et al. 2007, Peralta et al. 2012). Concerning other proteins that could help the prion protein in this process, Santos et al. (2011) recently described that the complex formed by PrP<sup>C</sup> and the heat-shock protein STI1 was involved in nervous system development, as the formation of neurospheres from the fetal forebrain of PrP<sup>C</sup>-KO mice was significantly reduced when compared to WT counterparts.

Interestingly, PrP<sup>C</sup> has also been described as an important marker in mouse hematopoietic stem cells (HSCs; Liu et al. 2001, Zhang et al. 2006). In these cells, Palmqvist et al. (2007) revealed that NUP98-HOX fusions, which were known to promote primitive hematopoietic cell expansion, blocked in vitro differentiation and leukemic transformation (Pineault et al. 2004, Ohta et al. 2007) and could induce the expression of several genes mainly involved in cell development, cell proliferation, and signal transduction. Remarkably, Prnp was identified among them and was upregulated between 2.1 and 2.3 times, suggesting a role in the processes analyzed (Palmqvist et al. 2007).

In contrast, PrP<sup>C</sup>-KO HSC showed a lack of long-term HSC activity, one of the hallmarks of these cells, when they were introduced into lethally irradiated bone marrow (BM) recipient mice (Zhang et al. 2006). Several months after transplant, in contrast to what normally happened with WT mice, the authors could not find

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**Figure 1** Schematic diagram of the domain structure similarities of the prion protein family members. Doppel and PrP<sup>C</sup> have an analogous globular domain, which consists of three α-helices and two β-sheets. Disulphide bridges i-S-S-i and glycosylation sites (CHO) are also present. Shadoo shares with PrP<sup>C</sup> a highly conserved central hydrophobic tract and a repeated amino acid sequence zone consisting in tetrarepeats in the former and octarepeats in the latter. All the members possess a GPI site to anchor themselves to the membrane.
PrP<sup>C</sup>-KO blood peripheral cells derived from PrP<sup>C</sup>-KO HSC. Furthermore, the absence of PrP<sup>C</sup> revealed a diminished reconstitution ability of peripheral blood in response to the stress of successive serial transplants. The most notable difference was obtained in tertiary transplantation, where again no mice survived in the case of using PrPC-KO BM. Curiously, this deficiency was partially rescued when PrPC-KO BM cells extracted from the secondary transplantation were genetically modified to express PrP<sup>C</sup>, demonstrating that PrP<sup>C</sup> sustained hematopoietic engraftment during long-term transplantation (Zhang et al. 2006). Similarly, in the mammary gland, only those cells expressing PrP<sup>C</sup> were able to form mammospheres and regenerate the mammary gland when implanted into the mammary fat pad (Liao et al. 2007). Indeed, small molecules that modulate PrPC expression can be used to enhance their proliferation and extend their life span in culture, while still retaining their ability to differentiate and engraft to BM (Mohanty et al. 2012).

Recently, some other experiments were performed in human ES cells (hESCs). Lee & Baskakov (2010) demonstrated that the introduction of ectopical PrP<sup>C</sup> delayed spontaneous differentiation. Interestingly, if PrP<sup>C</sup> was overexpressed or inhibited in these hESCs, long-term proliferation was promoted or ectodermal differentiation was suppressed respectively (Lee & Baskakov 2013). Moreover, the hESC G<sub>1</sub> phase is characteristically very short and, as a consequence, a low proportion of G<sub>1</sub> cells are found in culture under self-renewal conditions. When PrP<sup>C</sup> was artificially expressed, the percentage of G<sub>1</sub> phase cells was significantly increased at the expense of S phase cells, which triggered differentiation (Lee & Baskakov 2013). Furthermore, the overexpression or inhibition of PrP<sup>C</sup> during hESC spontaneous differentiation downregulated all three germ layer markers in the first condition or just ectodermal markers in the latter (Lee & Baskakov 2013). These data suggest that PrP<sup>C</sup> influences not only pluripotency but also cell fate and the cell cycle, providing a mechanistic insight into its involvement in hESC.

Collectively, these data point to an important role for PrP<sup>C</sup> in terms of pluripotency and self-renewal, and present important evidence of its role before the establishment of compensatory mechanisms.

**Prion protein in brain and muscle stem cell regeneration**

As the first hESC was isolated in 1998 (Thomson et al. 1998), the idea of inoculating ES cells in an infarcted heart or damaged brain to recover its functionality has motivated intensive scientific research. As mentioned previously, the presence of PrP<sup>C</sup> not only in the CNS but also in other organs led us to propose that this protein is a conserved regulator of the differentiation and/or regeneration of many tissues. In neurogenesis, in addition to what has already been described in this review, it has
been reported that PrP<sup>C</sup> is a saturable, specific, high- 
affinity receptor for laminin (LN). Keeping in mind that 
LN plays a major role in neural differentiation, migration, 
and survival, it has been confirmed through a WT/null 
strategy that the PrP<sup>C</sup>-LN interaction is involved in 
neuritogenesis induced by neural growth factor. Furthermore, 
the authors reported that the C-terminal binding 
site stimulated neurite outgrowth, modulated the elec-
trical activity of neocortical pyramidal neurons, and was 
highly expressed in rat neocortical and hippocampal 
neurons (Kibbey et al. 1993, Hager et al. 1998, Koch et al. 
1999). The participation of PrP<sup>C</sup> in neurite outgrowth by 
LN also supports an important role for the cellular prion 
protein in neuronal differentiation (Graner et al. 2000).

Furthermore, there are some studies that provide 
evidence of a role for this protein in brain function. In 
PrP<sup>C</sup>-KO mice, several minor alterations have been 
described (Weissmann & Flechsig 2003, Criado et al. 
2007, Prestori et al. 2008), although major physiological 
disturbances were expected due to its ubiquitous 
location and conserved structure in nature. Perhaps, 
the most important advance in this field was the 
demonstration of a clear PrP<sup>C</sup> involvement in nervous 
impulse transmission, maintaining and generating the 
peripheral myelin (Bremer et al. 2010). Interestingly, it 
has been described that PrP<sup>C</sup> mediates neuronal iron 
uptake and transport, which is important to keep the 
brain in working order (Singh et al. 2009). It was also 
reported that PrP<sup>C</sup> has a high expression at synapses and 
that it was involved in the homeostasis of Ca<sup>2+</sup> of 
cerebellar granule neurons (Lazzari et al. 2011). It can 
bind copper as well for an antioxidant activity, which, in 
turn, might have vital implications for synaptic homeo-
stasis (Brown 2001).

Tissue regeneration can have similarities with embry-
ogenesis in terms of physiological parameters. That is the 
case in muscle, in which regeneration after injury seems to 
resemble muscle development. In a study of in vitro 
myocyte differentiation, full cell accumulation of PrP<sup>C</sup> 
was described to be after around 4 days from the 
beginning of cell growth in culture, a feature that 
perfectly matched the timeline of myocyte maturation 
(Massimino et al. 2006). The expression and cellular 
processing of PrP<sup>C</sup> changed substantially during myogen-
esis as well. Metabolism of PrP<sup>C</sup> also varied in skeletal 

Figure 3 Putative pluripotency pathways under the control of the cellular prion protein. PrP<sup>C</sup> regulated the expression of Nanog, with a peak of both mRNAs at day 5 of spontaneous mouse stem cell differentiation. Furthermore, Stat3 mRNA also showed a progressive overexpression from that point, highlighting the pro-pluripotent properties of PrP<sup>C</sup>. The cross-link between both routes could lead to the expression pattern observed in our work for the Bmp pathway genes (arrows, activation; dash-verticle, inhibition; P, phosphorylation).
Prion protein in reproductive cells

The role of PrP<sup>C</sup> in reproduction has been tackled in several studies since its expression was observed in both male and female reproductive tracts (Bendheim et al. 1992, Tanji et al. 1995). In male gonads, Prnp expression is restricted to spermatogonia, spermatocytes, and round spermatids. Curiously, two different PrP<sup>C</sup> transcripts have been found, a major 2.2 kb fragment, which is present in testis at all ages, and a minor 1.1 kb product, which has only been found in mice older than 2 weeks (Fujisawa et al. 2004). This minor band may be a mature transcript produced by an alternative polyadenylation, as this mechanism has been previously described in peripheral tissues in rodents (Robakis et al. 1986). However, the role of these different transcripts remains to be clarified.

Prnp is also transcribed throughout the epididymis and PrP<sup>C</sup> is secreted under different isoforms by the epididymal epithelium (Gatti et al. 2002). PrP<sup>C</sup> isoforms differ between reproductive fluid and sperm and are compartmentalized within the male reproductive tract. In fact, PrP<sup>C</sup> in fluid was found both in hydrophobic membrane vesicles, named epididymosomes, and in soluble form (Ecroyd et al. 2004), while in sperm it is localized in the membrane raft domains (Shaked et al. 1999, Gatti et al. 2002, Peoc'h et al. 2002, Ecroyd et al. 2004). However, it remains to be established whether certain isoforms may be processed during the epididymal maturation and may be transferred from the fluid to the sperm via the epididymosomes.

Nevertheless, Prnp is not essential for gametogenesis as both male and female PrP<sup>C</sup>-KO mice are fertile and no noticeable defects in testis histology have been observed in its absence (Sakaguchi et al. 1996). However, PrP<sup>C</sup> may possibly have a protective role in specific stress situations related to copper toxicity, as it has been demonstrated that sperm cells from PrP<sup>C</sup>-KO mice were more susceptible to high copper concentrations than sperm from WT mice (Shaked et al. 1999). In contrast, it has been reported that the PrP<sup>C</sup> family protein Doppel, mostly expressed in the testis, has an important antioxidant function necessary for sperm integrity, and its deficiency resulted in male infertility (Behrens et al. 2002, Paisley et al. 2004). Interestingly, in a recent study performed in our laboratory, Doppel, as well as the other PrP<sup>C</sup> family protein Shadoo, was not able to compensate for the absence of PrP<sup>C</sup> in a KO cell culture model (Miranda et al. 2011b; Fig. 2). Both genes were found to be highly downregulated in PrP<sup>C</sup>-KO ESCs and expressed more frequently during early differentiation than in their WT counterparts. In fact, after 5 days of differentiation, a morphologically different subpopulation of PrP<sup>C</sup>-KO ESbs appeared. This particular transcriptional behavior led to a highly active system in terms of glucose and oxidative metabolism, which maintained a high pluripotency level and expressed several PGC markers until late periods of spontaneous differentiation (Miranda et al. 2011b; Fig. 2). These results highlight the necessity of further studies to demonstrate more thoroughly the possible relationship between PrP<sup>C</sup> and the germline.

Conclusions

Data from different laboratories have identified a crucial function for PrP<sup>C</sup> during early embryogenesis and adult regeneration, suggesting a putative mechanistic explanation of the pathogenesis of prion diseases. In fact, the absence of a PrP<sup>C</sup>-mediated protection due to its transformation into the pathogenic form could let harmful agents produce an uncontrolled degeneration. Furthermore, the importance of PrP<sup>C</sup> for the expansion of stem cells in culture, and how some of them lose its expression with serial passage, is now recognized. As a consequence, we can now begin to understand the mechanism of action through which PrP<sup>C</sup> mediates its effects at this important cell stage, both in relation to ROS protection and the control of stem cell fate, which translates into the control of pluripotency and early differentiation (Fig. 3). Thus, it may be possible to manipulate the life span and function of stem cells by modulating PrP<sup>C</sup> expression, which could represent the appearance of a new strategy for regeneration therapies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.
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