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The prion or the related Shadoo protein is required for early mouse embryogenesis

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ABSTRACT

The prion protein PrP has a key role in transmissible spongiform encephalopathies but its biological function remains largely unknown. Recently, a related protein, Shadoo, was discovered. Its biological properties and brain distribution partially overlap that of PrP. We report that the Shadoo-encoding gene knockdown in PrP-knockout mouse embryos results in a lethal phenotype, occurring between E8 and E11, not observed on the wild-type genetic background. It reveals that these two proteins play a shared, crucial role in mammalian embryogenesis, explaining the lack of severe phenotype in PrP-knockout mammals, an appreciable step towards deciphering the biological role of this protein family.

1. Introduction

The *Prnp* gene plays a pivotal role in transmissible spongiform encephalopathies, encoding for a protein needed for prion replication and scrapie pathogenesis [1,2], an abnormal conformer of which is the only known constituent of mammalian prions [3]. The cellular prion protein, PrP^C, is a glycosyl-phosphatidyl-inositol (GPI)-anchored cell surface protein with two sites of Asn-linked glycosylation, expressed in a broad range of vertebrate tissues [4]. The physiological function of PrP^C remains unclear although various functions have been suggested [5]. A neuroprotective role has been documented as well as a potential implication in cell proliferation and differentiation. PrP^C temporal regulation highlighted its possible involvement in embryogenesis [6–8]. However, *Prnp*-knockout mice, beside slight circadian rhythm, hippocampal function and behavioural alterations, have no drastic phenotype [9–13], even when PrP^C is invalidated in adult neurons [14]. The lack of PrP^C did not apparently dramatically affect the development or the behaviour of knockout *Prnp* cattle [15] or goat [16]. One advanced reason for such observations is the putative expression by the host of a yet uncharacterised π protein that compensates for allelic and/or induced alteration of PrP expression [17–19].

Recently, a PrP-related protein Shadoo having similarities with a C-terminally truncated, GPI-anchored PrP was discovered [20,21]. The highly conserved encoding gene, *Sprn*, is located on a chromosome different from that of *Prnp*. Its expression was analysed in various species [21–24]. In the central nervous system, Shadoo and PrP^C were shown to have partially overlapping expression patterns. A positive correlation between cerebral and cerebellar cortex expression of these two genes was reported in sheep [25]. Expression of Shadoo was found to be altered in the brains of prion-diseased mice [24] and to be potentially involved in human prion pathobiology [26]. Shadoo shares some neuro-protective properties with PrP^C and thus is a good candidate for being the hypothetical PrP-like protein π and a modulator for the biological actions of normal and misfolded PrP [24,27]. According to mouse EST databases (GenBank), *Sprn* is transcribed in the embryo alongside *Prnp*. This study was aimed to assess if PrP^C and Shadoo share a function related with early embryogenesis.

2. Materials and methods

Intra-perivitellin space injections of the lentivirus solutions were done according to Lois et al. [28] on FVB/N and FVB/N Prnp knockout (FVB.129-Prnp trn^{1Zrch}) [9,29] mouse eggs. All sh-RNA lentiviral vector solutions were purchased from Sigma with infectious titers over 10^9 infectious units/ml (LS1: TRCN0000179960, LS2: TRCN0000184740 and Lfox: TRCN0000086505). Animal

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manipulations were done according to the French "Commission de Génie Génétique" recommendations.

Total RNA was isolated from whole mouse embryos and adult brain using RNA Now (Biogentex Inc.) and 3 μg of purified RNA was reverse transcribed with SuperScript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's protocol.

For RT-PCR analysis, cDNA was transcribed as described above. Sprn was amplified using the primers 5′-CTGGCTAGTGTATAGGTTTC-3′ and 5′-CTGCAATGAGGGAAAAGCCT-3′ with 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 12 s.

Detection of sh-RNA in transgenic embryos was realised by RT-PCR analysis. Five micrograms of total RNA was polyadenylated using the Ambion PolyA Polymerase protocol (Ambion, Applied Biosystems). The polyadenylated RNA was reverse transcribed using a RTQ primer (5′-CGAATTCTAGAGCTCGAGGCAGGCGACATGGCTGGCTAGTTAAGCTTGGTACCGAGCTCGGATCCACTAGTCC$(T)_{25}$VN) and SuperScript II Reverse Transcriptase. RT-PCR analysis was performed using a universal reverse primer (5′-CGAATTCTAGAGCTCGAGGCAGG-3′) and a forward primer specific for the sh-RNA sequence (FoxL2: 5′-CGGCATGTTCGAGAAGGGCA-3′). The PCR conditions comprised 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 7 s.

Quantitative real-time PCR analysis was performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems) and SYBR Green (Applied Biosystems). Mouse *Sprn* and β-actin were amplified using the primers 5′-CTGGCTAGTGTATAGGTTTC-3′ and 5′-CTGCAATGAGGGAAAAGCCT-3′ for *Sprn* and 5′-CACCAGTCCGCCATGGATG-3′ and 5′-TCCCCACCATCACACCCTG-3′ for β-actin, respectively. These primers were designed over exon–exon borders. The PCR conditions used comprised 45 cycles at 95 °C for 15 s and 60 °C for 1 min. Each sample was analysed in triplicate and data analysed using the Delta-Delta Ct method.

3. Results and discussion

3.1. In utero knockdown of Sprn can be lethal in the absence of Prnp

Two anti-mouse *Sprn* sh-RNA lentiviral vector solutions, LS1 and LS2, were used to knockdown the expression of murine *Sprn* in both FVB/N and FVB/N *Prnp*-knockout mice. These sh-RNA are under the transcriptional regulation of the ubiquitous U6 promoter and each recognises a different target sequence located within the 3′-UTR of *Sprn*. Viable transgenic mice were obtained with injections of lentivirus LS1 and LS2 on FVB/N and FVB/N *Prnp*-knockout genotypes (Table 1A). These founder animals were analysed by QPCR at 2 months of age and all showed significant cerebellar *Sprn* gene down-regulation, ranging from ~50% to 90%, demonstrating the functionality of the two sh-RNAs (Fig. 1). No correlation could be made between the level of down-regulation and the genetic background of the mouse.

A striking observation was the significantly lower percentage of animals born and the concomitant higher percentage of transgenics obtained following injection of the anti-*Sprn* lentivirus in the FVB/N *Prnp*-knockout versus the wild-type genotype (Table 1A). The diminution of the number of animals born suggests a lethality induced by the expression of the anti-*Sprn* sh-RNA in the absence of PrP. Because two different sh-RNAs were used, this lethality is unlikely to be related to an off-target phenomenon. Furthermore, such an effect should have been detected in both genotypes. Intriguingly, the percentage of transgenics obtained in the FVB/N *Prnp*-knockout genotype was higher, suggesting that the lethal phenotype affects non-transgenic animals more, i.e. injected animals with no integrated lentivirus, which appears contradictory to being a consequence of the *Sprn*-down-regulation. In order to further define the phenotype and to clarify this paradox, we assessed the developmental stage at which the lethality occurs.

3.2. Evidence of an early embryonic lethality in injected FVB/N Prnp-knockout embryos

Since *Prnp* expression is activated between E7 and E9 ([7,8], BQ257016 in GenBank), the percentage of reabsorption was analysed at E10.5 following injection of LS2. In parallel a similar study was performed following injection of anti-mouse *FoxL2* sh-RNA lentiviral vector suspension. This latter lentivirus (Lfox) was used as a control since the knockout of the targeted gene was reported not to be lethal [30]. The obtained results summarised in Table 1B showed (i) a similar rate of reabsorption of the embryos injected with either LS2 or Lfox on a FVB/N genotype and (ii) a significantly higher rate of reabsorption of embryos injected with LS2 on mouse FVB/N *Prnp* knockout eggs, while no statistically significant difference was found between the two genetic backgrounds with Lfox, therefore arguing against a higher sensibility of one genetic background towards a lentiviral infection.

The FVB/N *Prnp*-knockout embryos not reabsorbed following injection of the LS2 lentivirus were further analysed macroscopically. More than 80% of them were en route to being reabsorbed or were smaller (Fig. 2A). Strikingly, the latter embryos had a failure of closure of the cranial neural tube, a feature that was also observed in a proportion of injected FVB/N $Prnp^{+/-}$ but not in FVB/N

Table 1
Effect of sh-RNA-mediated *Sprn* knockdown.

Lentivirus	LS1[a]			LS2[a]		
Genetic background	wt[b]	KO[c]	*P* value[d]	wt[b]	KO[c]	*P* value[d]
A: On the number and transgenic status of the injected animals						
Transferred eggs	127	83		291	102	
Nb. of animals born (% born/transferred)	33 (26.0)	14 (16.8)	<0.05	54 (18.5)	4 (3.9)	<0.001
Nb. of transgenic animals (% transgenic/born)	6 (18.2)	8 (57.1)	<0.001	17 (31.5)	4 (100)	<0.001
	LS2[a]			Lfox[b]		
	wt[b]	KO[c]	*P* value[d]	wt[b]	KO[c]	*P* value[d]
B: On embryo reabsorption at E10.5						
Implanted	101	95		56	41	
Nb. of reabsorbed embryos (% reabsorbed/implanted)	49 (48.5)	73 (76.8)	<0.001	28 (50.0)	16 (39.0)	NS

Nb.: Number.
[a] *Sprn* sh-RNA (LS2) or *FoxL2* sh-RNA (Lfox) lentivirus vectors.
[b] wt: Wild-type FVB/N mice.
[c] KO: FVB/N PrP-knockout mice.
[d] Significance *P* value (Student *t*-test). NS: Not significant.

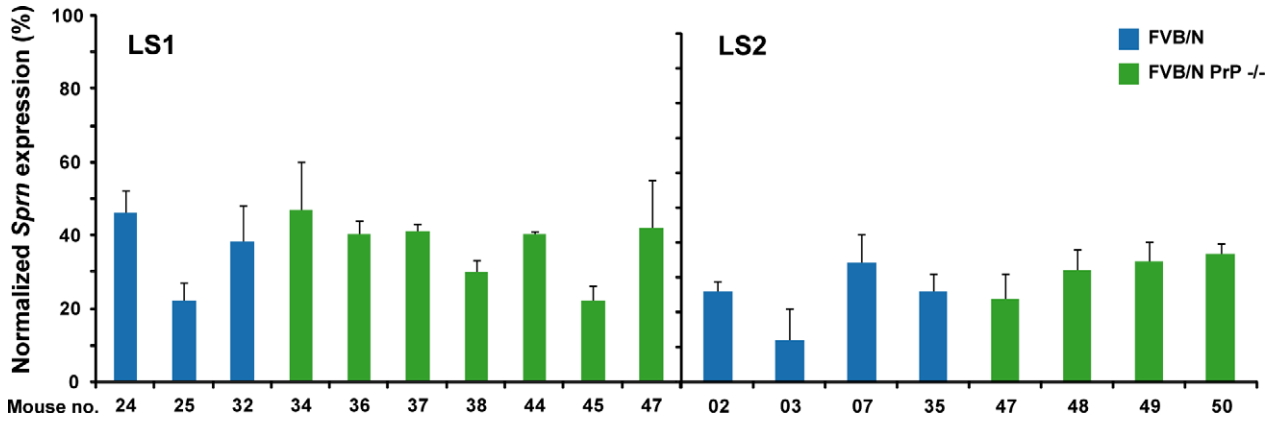

Fig. 1. *Sprn* knockdown in the cerebellum of adult founder LS transgenic mice. QPCR was performed in triplicate using total RNA isolated from the cerebellum of adult founder transgenic mice. The amount of *Sprn* RNA is expressed as an arbitrary value following β-actin normalisation and assuming a 100% level of expression in wild-type FVB/N mice. The injected lentivirus (LS1 or LS2) and the genetic *Prnp* background of the mice are indicated. The number refers to the founder identification.

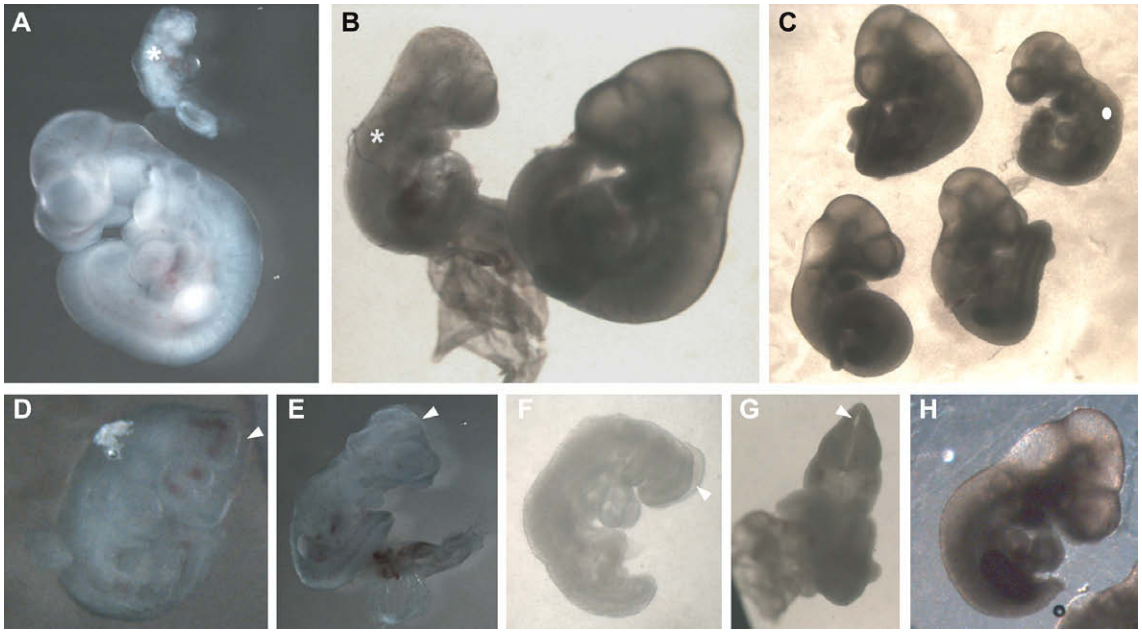

Fig. 2. Abnormal neural development of FVB/N *Prnp*-knockout E10.5 embryos injected with *Sprn* sh-RNA lentivirus (LS2). (A, D and E) Macroscopic examination of injected FVB/N *Prnp*-knockout embryos. Among the embryos that were not already reabsorbed at this stage (see Table 1B), 9 out of the 11 analysed were smaller or partially reabsorbed (star in A). The smaller embryos had an abnormal developmental phenotype with a failure of closure of the cranial neural tube, leaving a region of open neural folds (arrowhead in D and E). (B, F and G) Injected FVB/N $Prnp^{+/-}$ embryos. Three out of the 16 implanted embryos were smaller (star in B) and presented the same defect of neural tube closure (arrowhead in F and G) as observed in PrP-knockout embryos. At a later developmental stage, E13, only normal embryos could be observed. (C and H) Injected FVB/N embryos. Although some embryos (2 out of 14) were found to be slightly smaller (closed circle in C), none presented an abnormal developmental phenotype.

animals (Fig. 2). Similar observations were made using the LS1 lentivirus albeit the number of abnormal embryos was reduced, consistent with the seemingly lower efficiency of this vector (Table 1A; Fig. 1). It suggests that a genotype with hypomorphic *Prnp* and *Sprn* alleles can result in a neuronal defect, probably transient since it was not observed in the born pups.

Our results indicate that the *Sprn*-knockdown-induced lethality occurred at an early embryonic stage, after implantation and before E11 in FVB/N *Prnp*-knockout embryos. Since it is specific to this genotype, this window can be further theoretically reduced according to the published developmental regulation of *Prnp* ([7,8] and BQ257016 in GenBank) and to that observed for *Sprn* (AI550081 in GenBank and Fig. 3A) to E8 to E11. The absence of such a phenotype on FVB/N genetic background also suggests that, as was observed for *Prnp*, absence of *Sprn* expression alone does not affect normal embryonic development.

3.3. Early embryonic Sprn down-regulation can be observed in the absence of sh-RNA lentivirus integration

The observation that the lethal phenotype occurs in early embryogenesis led us to assess if the persistence of transcriptionally active non-integrated lentivirus and/or siRNA could explain the abovementioned paradox, i.e. that it also affects "non-transgenic" individuals, with no integrated lentivirus. It is established that non-integrated lentivirus can persist in infected cells and be transcriptionally active [31–33]. Lentivirus-derived Lfox siRNA was detected by RT-PCR in 10 out of the 10 E10.5 injected embryos analysed (Fig. 3C), a result indicating that it was actually independent from the retrovirus integration since only 36% of pups genotyped were transgenic for the lentivirus (data not shown). Furthermore, *Sprn* down-regulation was detected in LS2 E9 embryos, as compared to the non-injected controls (Fig. 3B). Finally,

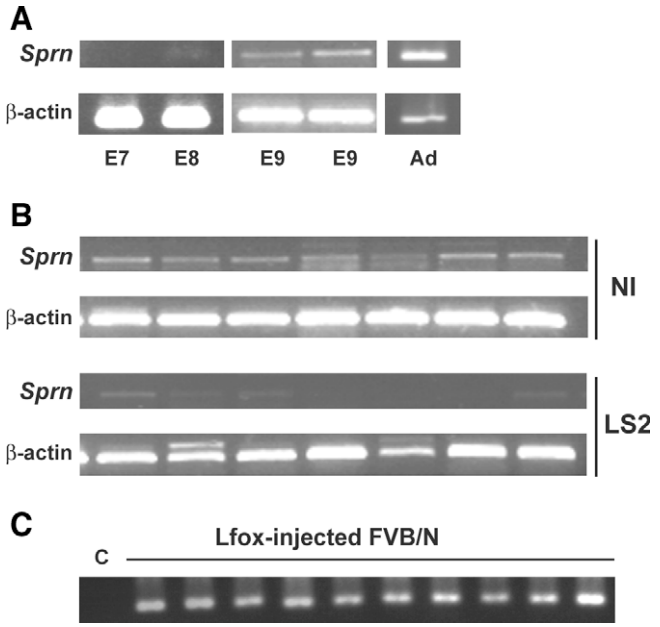

Fig. 3. *Sprn* expression in embryos and its siRNA-induced knockdown. (A) Expression profile of *Sprn* during embryonic development. Detection of *Sprn* mRNA by RT-PCR using total RNA from E7 to E9 embryos. Ad: adult cerebellum. (B) Detection of *Sprn* mRNA from E10.5 FVB/N *Prnp*-knockout embryos non-injected (NI) or injected with the LS2 lentivirus suspension (LS2). (C) Detection of the Lfox-encoded siRNA by RT-PCR using total RNA isolated from whole E10.5 embryos. The siRNA was detected in 10 out of 10 analysed embryos, implying that non-integrated lentiviral vector DNA is transcribed. C: No RNA template.

the observed increase in the percentage of transgenic pups when LS1 or LS2 were injected on the FVB/N *Prnp*-knockout genotype might be explained by a lower susceptibility to sh-RNA-mediated down-regulation of transgenic embryos, as a result of silencing mechanisms known to frequently occur with lentiviral vectors upon integration [34].

Overall, our findings provide strong evidence for a crucial role for prion family proteins in early embryogenesis with the need for either *Prnp* or *Sprn* expression between E8 and E11. They lend support to the view that both proteins share biological functions involved in the development of a normal embryo and that one can compensate for the absence of the other. It thus gives a mechanistic explanation for the otherwise surprisingly healthy-looking PrP-knockout mammals.

The detected transient abnormal embryonic phenotype found in some of the surviving E10.5 embryos appears to involve a default of closure of the cranial neural tube (Fig. 2). Based on the PrP and Shadoo neuro-protective properties and the developmental timescale, it is tempting to speculate that *Prnp* and *Sprn* are involved in mammalian neurulation [35], for which a role for PrP has already being suspected [36]. Incidentally, it mimics the potential role recently attributed to the Zebrafish *PrP-2* gene during embryonic development (quoted in [37]).

Finally, our findings also highlight the need for careful examination of animals resulting from the injection of lentiviral vectors that encode for genes affecting early embryonic events, regardless of their transgenic status.

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