Aggregation-promoting factors in neurodegenerative diseases
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CHAPTER VI

Loss of Serf2 in mice results in embryonic lethality with incomplete penetrance

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Work in progress


Abstract

Neurodegenerative disorders such as Parkinson's, Alzheimer's and polyglutamine diseases are commonly characterized by the presence of protein aggregates in specific neurons. How the formation of these proteinaceous inclusions occurs is still not fully understood, nor is their role in pathogenesis. A positive regulator of aggregate formation has been discovered in C. elegans models for aggregation of alpha-synuclein, amyloid-beta and polyglutamine. This protein, known as modifier of aggregation MOAG-4, has two human orthologs, SERF1A and SERF2, which drive polyglutamine aggregation in human cells. This suggests that SERF may drive disease-associated protein aggregation in the mammalian brain. To test this hypothesis, we generated a Serf2 knockout mouse model in a C57BL/6 background. We found that the homozygous null allele is not inherited in Mendelian proportion, but some individuals are able to survive until adulthood. Our results suggest that full body knockout of Serf2 is embryonic lethal with incomplete penetrance. Since such mice will be of limited use to study the function of Serf2 in adult mice, we generated mice harboring a brain-specific knockout for Serf2. We report that the ablation of Serf2 expression from the brain resulted in viable and fertile animals.

We conclude that Serf2 is required for normal mouse development. Mice harboring the Serf2 conditional knockout allele can now be used to study the biological function of Serf2 in mouse development. Additionally, by mating these brain-specific knockout mice with mouse models of neurodegenerative diseases, we can also investigate whether Serf2 is indeed a genetic modifier of protein aggregation.
Introduction

While protein aggregation and toxicity are hallmarks of neurodegenerative diseases such as Parkinson’s (PD), Alzheimer’s (AD) and polyglutamine diseases, their role in these diseases is currently unknown (reviewed in [1, 2]). To better understand the role of protein aggregation in disease, efforts have been made to identify genes and pathways that regulate protein aggregation. The identification of such genes has been aided by genetic screens in *Caenorhabditis elegans* models expressing aggregation-prone proteins, including alpha-synuclein, amyloid-beta and polyglutamine [3-10]. The function of the genetic modifiers identified in these screens has been shown to be conserved in human cell models and in mice [5, 11, 12].

In *C. elegans* models for PD, AD and polyglutamine diseases, we previously identified MOAG-4/SERF as a regulator of age-related proteotoxicity [5]. We showed that MOAG-4 promotes polyglutamine aggregation and toxicity and that its function is evolutionarily conserved in the mammalian orthologues SERF1A and SERF2 (small EDRK rich factor 1A and 2) [5]. In human cell models expressing polyglutamine, overexpression of MOAG-4/SERF enhanced aggregation and cell death [5].

The molecular function of the SERF proteins is unknown. These proteins are ubiquitously expressed and are therefore predicted to have a role in general cellular pathways. SERF1A was first identified in a comparative genomics study, where it was found to be a genetic modifier of spinal muscular atrophy in human patients [13]. A transcriptome analysis later revealed that SERF1A is downregulated in the cerebellum of HD patients [14]. Recently, SERF1A was shown to promote amyloid aggregation *in vitro* [15].

Here we aimed to test the hypothesis that SERF2 drives disease-associated protein aggregation in the mammalian brain. To this end, we generated *Serf2* homozygous null mutant mice. By intercrossing *Serf2*+/- mice, we discovered that the segregation of the knockout allele did not follow Mendelian inheritance, suggesting that the targeted disruption of *Serf2* has fundamental implications for mouse development. To overcome this limitation, we generated brain-
specific Serf2 knockout mice that are viable and fertile. We further investigated the distribution of Serf2 expression and found that Serf2 is transcribed in various tissues, namely brain, heart, intestine, kidney and liver.

Results and Discussion

Deletion of Serf2 results in embryonic lethality with incomplete penetrance

To study the function of Serf2, we generated a mouse strain with an integrated promoter-driven selection cassette (Figure 1A) [16]. Serf2 has 3 exons and spans about 4 kb in chromosome 2. Two loxP sites were inserted around exon 2 of the Serf2 gene and its expression was prevented by the presence of an intronic Neo selection cassette that was surrounded by two loxP sites and two FRT sites (Figure 1A). Hprt Cre-induced recombination excised the Neo gene and exon 2 of Serf2, thereby generating a heterozygous reporter knockout for Serf2, with the lacZ reporter transgene under the control of the Serf2 promoter (Serf2\(^{+/+}\), Figure 1A, B).

We next attempted to generate Serf2 null mice (Serf2\(^{-/-}\)) by intercrossing Serf2\(^{+/+}\) mice. The numbers of Serf2\(^{-/-}\) progeny obtained were far lower than those

![Gene targeting strategy for the Serf2 knockout model. (A) Diagram of the Serf2 targeting vector. The Serf2 exon 2 is flanked by two loxP sites. Expression is prevented by an intronic Neo selection cassette surrounded by two loxP sites and two FRT sites. Mating with a Hprt Cre deleter mouse strain removes exon 2, resulting in a reporter knockout whereby the lacZ transgene is expressed under the control of the Serf2 promoter. Diagram adapted from [16]. (B) Genotype of wild type, heterozygous and knockout Serf2 transgenic mice. Primers F3 and R3 were used to distinguish the targeted allele (420 bp) from the untargeted wild type allele (335 bp), identified with primers F4 and R3. (C) Numbers of mice obtained for each genotype from intercrossing Serf2\(^{+/+}\) mice or by mating Serf2\(^{+/+}\) mice with Serf2\(^{-/-}\) mice. Numbers in parentheses represent the expected Mendelian ratio for each genotype.](image-url)
Loss of Serf2 in mice results in embryonic lethality with incomplete penetrance expected according to Mendelian inheritance and only a small proportion was able to survive throughout adulthood, suggesting embryonic lethality with incomplete penetrance (Figure 1B, C). When we attempted to mate Serf2+/− mice with Serf2−/− mice, Serf2−/− offspring of this cross were also underrepresented (Figure 1C). Together, these results demonstrate that Serf2 is essential for mouse development.

**Serf2 is expressed in disease-related brain areas**

To understand the role of Serf2, we next wanted to identify the tissues in which Serf2 is normally expressed in Serf2+/− and Serf2+/+ mice. To do so, primers specific for Serf2 were used to investigate its expression in the brain, heart, intestine, kidney, liver and pancreas by qPCR (Figure 2A). Serf2 was expressed in most the tissues examined in this study. mRNA expression in Serf2+/− mice

![Figure 2. Expression of Serf2 in different tissues. (A) Expression of Serf2 in tissues of 3-month-old mice. Relative mRNA levels were measured by qPCR in wild type (n=3), heterozygous (n=8) and knockout mice (n=1) for Serf2. (B) Protein expression of Serf2 in liver samples obtained from wild type, heterozygous and knockout mice by Western blot. (C) Protein expression of Serf2 in the cerebellum (Cer) and hippocampus (Hip) of wild type and heterozygous mice by Western blot. In both experiments, actin was used as a loading control. (D) Expression of Serf1a in different brain regions of wild type mice (n=5) and mice heterozygous for the Serf2 knockout allele (n=5). (E) Expression of Serf2 in different brain regions of wild type mice (n=5) and mice heterozygous for the Serf2 knockout allele (n=5). Relative mRNA levels were measured in the olfactory bulb, hippocampus, striatum, cortex, cerebellum and brainstem. **** p<0.0001; *** p<0.001; ns is not significant.](image-url)
was significantly lower (up to 50%) than that in control mice (Figure 2A). This was later confirmed by measuring Serf2 protein levels in the brain and liver (Figure 2B, C).

Knowing that Serf2 was expressed in the brain and aiming to use this model to later generate a Serf2 knockout in a neurodegenerative disease background, we asked in which brain areas Serf2 was expressed. We found that Serf2 was expressed in different brain regions, including the brainstem, the olfactory bulb, the cerebellum, the striatum and, importantly, the hippocampus and the cortex, which are frequently affected in neurodegenerative diseases (Figure 2E). We examined 3 and 6-month old mice and confirmed that Serf2+/- mice had reduced expression of Serf2 while Serf1a expression was unaltered (Figure 2D, E). A reduction in expression of Serf2 was not compensated by the expression of Serf1a, supporting the use of this model to study Serf2 function. Our findings show that Serf2 is expressed in different tissues, including the brain. Specifically, Serf2 is expressed in brain areas relevant to disease and loss of Serf2 does not affect Serf1a expression.

**Mice with brain-specific knockout for Serf2 are viable and fertile**

Since the full body knockout of Serf2 resulted in embryonic lethality, we could not take advantage of these mice to study the role of Serf2 in protein aggregation in the brain. To overcome this limitation, we specifically eliminated Serf2 expression in the brain. First, mice harboring the Serf2 construct were crossed with FLP deleter mice to generate a conditional Serf2 allele (Serf2^flox/flox, Figure 3A). Serf2^flox/flox mice express the transgene as wild type and have normal appearance (data not shown). To specifically eliminate Serf2 expression in the brain, Serf2^flox/flox mice were mated with mice that express the Cre recombinase under the control of the Sox1 promoter, which restricts expression of Cre to the central nervous system (Figure 3A). Conditional knockout mice (Cre Sox1+ Serf2^-/-) derived from this cross were viable and fertile (Esther Stroo, personal communication). To demonstrate that Serf2 expression was eliminated only in the brain, we measured expression levels of Serf2 in the brain and compared them with those in other tissues, including the kidney and the liver. When compared with expression in Serf2^flox/flox control mice, expression of Serf2 in Cre Sox1+ Serf2^-/- mice was almost completely abolished.
Loss of Serf2 in mice results in embryonic lethality with incomplete penetrance.

in the brain but unaltered in the kidney and liver (Figure 3C). Together, these results demonstrate the successful knockout of Serf2 in the brain without its expression in other tissues being affected.

To investigate whether the knockout of Serf2 affected Serf1a expression, we also measured Serf1a in the brain. We detected a statistically significant increase of Serf1a in the brain of Cre Sox1+ Serf2−/− mice, which suggests a compensation mechanism by Serf1a in the absence of Serf2 (Figure 3B).
Conclusion
In summary, Serf2 is essential for mouse development. We describe the generation of brain-specific Serf2 knockout mice that can be used not only to further study the role of Serf2 in mouse development but also to determine Serf2 involvement in proteotoxicity by mating these knockout mice with mouse models of neurodegenerative diseases.

Methods

Expression construct and generation of transgenic mice
The Serf2 targeting vector was generated by the International Knockout Mouse Consortium (IKMC, Figure 1A) [16, 17]. Briefly, the exon 2 of Serf2 is flanked by two loxP sites its expression is prevented by an intronic Neo selection cassette, which is flanked by two loxP sites and two FRT sites. The construct was injected into C57BL/6N embryonic stem cells [18]. Two founder lines (B11 and G9) were positive for the construct and subsequently expanded for this study. To generate a full body knockout of Serf2, both lines were crossed with a Hprt Cre deleter mouse strain to remove the PGK-Neo cassette and Serf2 floxed exon. Confirmation of correct recombination was performed by PCR genotyping analysis of ear biopsy. All mice were heterozygous with respect to the construct. Animals were backcrossed into the C57Bl/6J background (Charles River) six times.

Genotyping
DNA was prepared from ear biopsy and processed with prepGEM Tissue (#PTI0500, ZyGEM Corporation Ltd). PCR reactions contained three primers, one sense primer specific for the Serf2 transgene F3 (5’-CCGGTCGCTACCATTACCAG-3’); a second sense primer specific for genomic Serf2 F4 (5’-GATGATGGGCTTTCTGCTGC-3’); and one antisense primer present in the transgene and mouse Serf2 R3 (5’-CTTGATATGGAAGCCCTGTCG-3’). The knockout allele was identified with pair F3 and R3, generating a 420 bp product; and the wild type allele was identified with pair F4 and R3, generating a 335 bp product. Cycling conditions were 2 min at 94°C; 35 cycles of 30 sec at 94°C; 30 sec at 60°C; 1 min at 72°C; and 7 min at 72°C.
Quantitative PCR
Total RNA was extracted using TRIzol Reagent (Life Technologies) according to the manufacturer’s description. Total RNA quality and concentration were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific/Isogen Life Science). cDNA was made from 1.5 ug (tissues) or 2 ug (brain regions) total RNA with a RevertAid H Minus First Strand cDNA Synthesis kit (Life Technologies) using random hexamer primers.

Quantitative real-time PCR was performed using a Roche LightCycler 480 Instrument II (Roche Diagnostics) with SYBR green dye (Bio-Rad Laboratories) to detect DNA amplification. Relative transcript levels were quantitated using a standard curve of pooled cDNA solutions. Expression levels were normalized to 18S mRNA levels. The primers for RT-PCR used were Serf1 F2 (5’-TGGCCCGTGGAATCAAAGAGAAA-3’); Serf1 R2 (5’-TGCATGATCTCTGAATCCCTCTGCT-3’); Serf2 F2 (5’-CCGCGGTAAACCAGCGAGACG-3’); Serf2 R2 (5’-TCCGAGTCCCTCTGCTTGCG-3’); 18S F1 (5’-CGGACAGGATTGACAGATTG-3’); 18S R1 (5’-CAAATCGCTCCACCAACTAA-3’).

Western Blot
Frozen tissue was homogenized in RIPA buffer (10mM Tris-Cl pH 8.0; 1mM EDTA; 0.5mM EGTA; 1% Triton X-100; 0.1% sodium deoxycholate; 0.1% SDS; 140mM NaCl). Samples were homogenized with a tissue grinder pestle and incubated on ice for 30 min, followed by centrifugation at high speed for 30 min at 4ºC. The supernatant was transferred to a new tube and protein was quantified using the Pierce BCA Protein Assay kit (#23225, Life Technologies). Approximately 130 ug (cerebellum and hippocampus) or 40 ug (liver) of protein were loaded onto 15% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes and blocked with 5% BSA in PBS-T. Membranes were incubated with primary antibodies for SERF2 at 1:1500 dilution overnight (#11691-1-AP, Proteintech) or actin at 1:10.000 dilution overnight (#3134S, Cell Signaling Technology). Washes were performed with PBS-T. Incubation with secondary anti-rabbit for SERF2 or anti-mouse for actin was done at 1:10.000 dilution for 1 hour at room temperature. Antibody binding was visualized with an ECL kit (Amersham).
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Author contributions
O. S., W. H. and E. A. A. N. designed the experiments. O. S. and W. H. performed the experiments. O. S. and E. A. A. N. wrote the manuscript.
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