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Authors
Navarro, Stefanie J
Trinh, Tuyen
Lucas, Charlotte A
et al.

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The C57BL/6J Mouse Strain Background Modifies the Effect of a Mutation in Bcl2l2

Stefanie J. Navarro, Tuyen Trinh, Charlotte A. Lucas, Andrea J. Ross, Katrina G. Waymire, and Grant R. MacGregor1

Department of Developmental and Cell Biology, School of Biological Sciences, and Center for Molecular and Mitochondrial Medicine and Genetics, University of California Irvine, Irvine, California 92697-2300

ABSTRACT Bcl2l2 encodes BCL-W, an antiapoptotic member of the BCL-2 family of proteins. Intercross of Bcl2l2 +/- mice on a mixed C57BL/6J, 129S5 background produces Bcl2l2 –/- animals with the expected frequency. In contrast, intercross of Bcl2l2 +/- mice on a congenic C57BL/6J background produces relatively few live-born Bcl2l2 –/- animals. Genetic modifiers alter the effect of a mutation. C57BL/6J mice (Mus musculus) have a mutant allele of nicotinamide nucleotide transhydrogenase (Nnt) that can act as a modifier. Loss of NNT decreases the concentration of reduced nicotinamide adenine dinucleotide phosphate within the mitochondrial matrix. Nicotinamide adenine dinucleotide phosphate is a cofactor for glutathione reductase, which regenerates reduced glutathione, an important antioxidant. Thus, loss of NNT activity is associated with increased mitochondrial oxidative damage and cellular stress. To determine whether loss of Bcl2l2 –/- mice on the C57BL/6J background was mediated by the Nnt mutation, we intercrossed Bcl2l2 congenic C57BL/6J (Nnt –/-) mice with the closely related C57BL/6JEiJ (Nnt +/-) strain to produce Bcl2l2 +/- ; Nnt +/- and Bcl2l2 +/- ; Nnt –/- animals. Intercross of Bcl2l2 +/- ; Nnt +/- mice produced Bcl2l2 –/- with the expected frequency, whereas intercross of Bcl2l2 +/- ; Nnt –/- animals did not. This finding indicates the C57BL/6J strain background, and possibly the Nnt mutation, modifies the Bcl2l2 mutant phenotype. This and previous reports highlight the importance of knowing the genetic composition of mouse strains used in research studies as well as the accurate reporting of mouse strains in the scientific literature.

Inbred C57BL/6J mice are widely used in biological and biomedical research and for this reason the strain was selected to be the first mouse genome sequenced by a public consortium (Waterston et al. 2002). The use of inbred mouse strains that are presumed to be genetically homogenous at all loci reduces variability during analysis of a defined genetic modification, increases experimental reproducibility between different laboratories, and facilitates genetic mapping of strain-specific effects. Genetic drift is a concern in maintaining inbred species, and programs have recently been developed to monitor the genetic status of commonly used inbred mouse strains at commercial breeding facilities (Taft et al. 2006). Before such monitoring, C57BL/6J mice in some production facilities developed significant genetic alterations. For example, both the Snca gene, which encodes alpha-synuclein, and the adjacent multimerin1 locus were mutated by a 365-kb deletion that arose spontaneously in the C57BL/6JolaHsd strain in England some time before 1999 (Spector and Schoepfer 2001). Similarly, the C57BL/6J substrain of C57BL/6 has a deletion of 17.8 kb of the Nnt gene, which encodes nicotinamide nucleotide transhydrogenase (Huang et al. 2006). The NntC57BL/6J mutant allele arose spontaneously at The Jackson Laboratory in Bar Harbor, Maine, between 1976 and 1984. The allele has an in-frame deletion of exons 7–11 and a missense (M35T) mutation in the mitochondrial leader peptide sequence that results in reduced expression of Nnt mRNA and no functional NNT protein (Huang et al. 2006).

NNT is located in the inner mitochondrial membrane, where it functions as a redox-dependent proton pump that uses the proton gradient across the inner mitochondrial membrane to catalyze interconversion of nicotinamide adenine dinucleotide phosphate and glutathione reductase, which regenerates reduced glutathione, an important antioxidant. Thus, loss of NNT activity is associated with increased mitochondrial oxidative damage and cellular stress.

In this study, we investigated whether the C57BL/6J strain background modifies the effect of a mutation in the Nnt gene by intercrossing congenic C57BL/6J and C57BL/6JEiJ mice. Our results indicate that the C57BL/6J strain background, and possibly the Nnt mutation, modifies the Bcl2l2 mutant phenotype. This and previous reports highlight the importance of knowing the genetic composition of mouse strains used in research studies as well as the accurate reporting of mouse strains in the scientific literature.

KEYWORDS -Nnt mutation, genetic modifier, BCL-W, apoptosis

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1Corresponding author: Department of Developmental and Cell Biology, 4213 McLaugh Hall, University of California Irvine, Irvine, CA 92697-2300. E-mail: gmacg@uci.edu

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Table 1 Genotype of Bcl2l2 mice recovered from intercross of Bcl2l2\textsuperscript{GROS4A41Sor} +/- on N12Fx C57BL/6J congenic (Nnt -/-) and [C57BL/6J x C57BL/6JEiJ] F2 (Nnt +/+ or Nnt -/-) strain background

| Strain Background | +/- | +/- | -/- | Total |
|-------------------|-----|-----|-----|-------|
| N12FxC57BL/6J.129S55 (Nnt -/-) | 115 (30.3%) | 208 (54.9%) | 56 (14.8%) | 379 (100%) |
| N12FxC57BL/6J.129S55 (Nnt -/-) | 41 (21.4%) | 66 (33.8%) | 63 (31.7%) | 170 (100%) |
| [C57BL/6J x C57BL/6JEiJ] F3 (Nnt +/+ or -/-) | 29 (26.4%) | 53 (48.2%) | 28 (25.5%) | 110 (100%) |
| [C57BL/6J x C57BL/6JEiJ] F3 (Nnt -/-) | 25 (34.3%) | 43 (58.9%) | 5 (6.8%) | 73 (100%) |

\( \chi^2 = 21.98; 2 \text{ df}; P = 6.35 \times 10^{-08} \)
\( \chi^2 = 14.19; 2 \text{ df}; P = 0.00083 \)

\( P \) values for Fisher's exact test, two-tailed, were calculated by generating a 2x2 contingency table using the genotype of Nnt (+/+) and Nnt (+/+) combined with +/- or -/-.

**Materials and Methods**

**Mice**

C57BL/6J (Nnt -/-; cat. no. 000664) were purchased from The Jackson Laboratory (Bar Harbor, ME) in 1993 and have been maintained in our laboratory since then as a closed colony. The C57BL/6JEiJ (Nnt +/+, cat. no. 000924) strain was established by Dr. Eva Eicher in 1976 at F129. Male C57BL/6JEiJ mice were purchased from The Jackson Laboratory in 2007 at F129+75. The ROSA41 strain has a mutant allele of Bcl2l2, encoding BCL-W (Ross et al. 1998). The mutation is generated by an insertion of the ROSA \( \beta \)-gal gene trap vector (Friedrich and Soriano 1991). The mutant allele is null for Bcl2l2 function (Ross et al. 1998). The ROSA41 mutation was initially produced on a mixed 129SS, C57BL/6J strain background (Friedrich and Soriano 1991; Ross et al. 1998), but has since (as mentioned in the section Crosses) been backcrossed to a congenic C57BL/6J background. Mice on this strain background are available from the Mutant Mouse Regional Resource Center (UC Davis). Mice were provided Purina 5020 and nonacidified tap water ad libitum. Mice were housed in Techniplast ventilated cages with Bed O'Cobs bedding (The Andersons, Inc., Maumee, OH) and cotton nestlets for enrichment, with a light cycle of 13-hr on, 11-hr off, with lights on at 0630. Experiments involving animals were approved by the UCI Institutional Animal Care and Use Committee.

**Crosses**

B6129-Bcl2l2\textsuperscript{GROS4A41Sor} +/- were backcrossed for 12 generations with C57BL/6J mice. To ensure that the Y chromosome and mitochondrial DNA were of C57BL/6J origin, at least one backcross was performed with a male or female C57BL/6J mouse respectively. N12FxC mice were backcrossed to generate the data in Table 1.

The wild-type allele of Nnt was introduced by outcrossing Bcl2l2\textsuperscript{GROS4A41Sor} +/-; Nnt C57BL/6J -/- females on a C57BL/6J congenic background with male C57BL/6JEiJ mice. F1 animals were genotyped for both Bcl2l2 and Nnt, and Bcl2l2\textsuperscript{GROS4A41Sor} +/-; Nnt +/- animals were intercrossed. F2 animals were genotyped as before and Bcl2l2\textsuperscript{GROS4A41Sor} +/-; Nnt +/-, and Bcl2l2\textsuperscript{GROS4A41Sor} +/-; Nnt -/- animals were selected to establish breeding pairs for intercross. Genotypes in Table 1 were recorded from F3 or F4 crosses. Bcl2l2 (chromosome 14) and Nnt (chromosome 13) segregate independently.

**Genotyping by semiquantitative polymerase chain reaction analysis of Nnt or Bcl2l2 locus**

C57BL/6J and C57BL/6JEiJ strains were genotyped for their respective Nnt alleles by the use of a three-primer, two-allele polymerase chain reaction (PCR) assay.
reaction assay that discriminates between the wild-type allele of Nnt in C57BL/6JEiJ and the mutant allele lacking exons 7–11 in C57BL/6J mice as described (Nicholson et al. 2010). Primers were designed by use of the publically available mouse genome sequence of the Nnt locus (Ensembl). The “COM” primer (5’-GTAGGGCCCAACTGTTCCTGAGTA-3’) participates in amplification of both the wild-type and NntC57BL/6J mutant alleles, whereas the “WT” (5’-GGGCTATGAAGGAAATACACGGTG-3’) and “MUT” (5’-GTGGAATTCGCGAGAATCTC-3’) primers are specific to the wild-type and NntC57BL/6J mutant alleles, respectively. The amplification products are 579 bp for the wild-type allele and 743 bp for the mutant allele. Use of the primers with heterozygous mutant NntC57BL/6J template produces an additional faint product of approximately 900 bp that assists assignment of genotype. Amplification was performed in 25 μL with the use of 1x Go-Taq Flexi (Promega, Madison, WI) buffer with final concentration of 2.5 mM MgCl2, 1.0 μM Nt-Com, 0.33 μM Nnt-WT, 0.67 μM Nnt-MUT, 0.67 mM dNTPs, and 1.25 units Go-Taq Flexi enzyme (Promega). Amplification conditions used were initial melt 95°C, 5 min; then 35 cycles of 95°C, 45 sec; 58°C, 30 sec, 72°C, 45 sec; followed by a final extension of 5 min at 72°C. Mice containing the Bcl2l2GtROSA41Sor mutant allele were genotyped as described (Ross et al. 1998).

Statistical analysis

Differences between the expected and observed frequencies of animals of different genotypes were analyzed with a χ2 goodness-of-fit-test, and two-tailed Fisher’s exact test via the method of summing small P values (Graph Pad Software, La Jolla, CA). Significance was defined as P ≤ 0.05.

RESULTS AND DISCUSSION

Intercross of Bcl2l2GtROSA41Sor +/− on a mixed C57BL/6, 129S5 strain background produced Bcl2l2 −/− mice with the expected frequency (Ross et al. 1998). An independent laboratory reported the same result from intercross of Bcl2l2 +/− mice on a mixed 129S1, C57BL/6, FVB strain background (Printt et al. 1998). In contrast, after backcross to a congenic C57BL/6 background, homozygous mutant Bcl2l2GtROSA41Sor mice were recovered at a significantly reduced frequency (Table 1). Genotyping of dead animals found among newborn offspring revealed a significant increase in homozygous mutant animals (Table 1). Visual inspection, necropsy, and histology of dead animals provided no obvious insight into the cause of death of Bcl2l2 −/− animals (data not shown).

To determine whether mutation of Nnt, or a closely linked locus, in the C57BL/6 strain caused the reduced frequency of live Bcl2l2 −/− animals, we restored Nnt function by outcrossing congenic C57BL/6 Bcl2l2GtROSA41Sor +/− N12Fx female mice with C57BL/6JEiJ (Nnt +/+ ) males. The C57BL/6JEiJ strain was selected because it is most closely related to C57BL/6 while being wild-type for Nnt (Huang et al. 2006; Petkov et al. 2004). To maintain the mitochondrial genotype of the congenic C57BL/6 Bcl2l2GtROSA41Sor animals, females were crossed with C57BL/6JEiJ males. Live Bcl2l2GtROSA41Sor −/− offspring were recovered from intercross of [C57BL/6 × C57BL/6JEiJ] F2 Bcl2l2GtROSA41Sor +/− ; Nnt +/+ animals at the expected frequency (Table 1). In contrast, Bcl2l2 −/− animals were significantly reduced frequency from intercross of [C57BL/6 × C57BL/6JEiJ] F2 Bcl2l2GtROSA41Sor +/− ; Nnt −/− F2 animals (Table 1). This finding indicates that the C57BL/6 strain background modifies the phenotype of the Bcl2l2GtROSA41Sor mutation.

Our data implicate the Nnt mutation in C57BL/6J mice as the genetic modifier responsible for loss of a proportion of Bcl2l2 −/− animals. Mutation of Nnt increases the sensitivity of an animal to oxidative stress (Arkblad et al. 2005). Loss of BCL-W increases the likelihood that a cell (e.g. neurons, intestinal epithelial cells) will undergo apoptosis in response to cellular damage or oxidative stress (Courchesne et al. 2011; Pazeya-Murphy et al. 2009; Pritchard et al. 2000). Taken together, this suggests a model in which loss of NNT activity reduces the survival of BCL-W-deficient mice by increasing oxidative stress within cells in which BCL-W is normally expressed and that are essential for embryogenesis or early postnatal survival. However, whether the death of Bcl2l2 −/−, Nnt −/− mice is caused by increased apoptosis of a specific cell type remains unknown. At present we cannot exclude the possibility that other loci on mouse chromosome 13 linked to Nnt contribute to this effect. However, on the basis of the aforementioned model, it seems plausible that the Nnt mutation in C57BL/6J mice contributes at least some, if not all, of the modifier effect that enhances the impact of loss of Bcl2l2.

The C57BL/6J strain background also modifies the severity of the phenotype of mice lacking manganese super-oxide dismutase 2 (sod2) (Huang et al. 2006; Kim et al. 2010). Sod2 −/− mice on a DBA/2J background are born at normal frequency but die at approximately postnatal day 8 as the result of severe acidosis. In contrast, most Sod2 −/− on a C57BL/6J background die at approximately embryonic day 15 as the result of dilated cardiomyopathy. A QTL analysis of long-lived Sod2 −/− mice generated from DBA/2J-Sod2 +/− mice back-crossed to C57BL/6J (N7) mapped the modifier locus to a 10-Mb region on distal chromosome 13, which contains Nnt (Huang et al. 2006). Mutation of the Nnt locus in C57BL/6J mice has also been shown to be responsible for glucose intolerance and defective insulin secretion in C57BL/6J mice (Freeman et al. 2006).

These results reemphasize the importance of knowing the genetic composition of mouse strains used in studies, as well as accurately reporting mouse strains in the scientific literature (Stevens et al. 2007; Taft et al. 2006; Wotjak 2003). They also emphasize the particular importance of knowing the strain of C57BL/6 mice used in studies involving analyzing of cellular stress and cell death. The latter point is illustrated by a recent study (Ni et al. 2008), which confirmed a report (Bouillet et al. 2001) of increased severity of a mutant phenotype of Bcl2 −/− mice on a C57BL/6J background. Ni et al. 2008 described the C57BL/6 mice used as being from The Jackson Laboratory, although the authors did not specify which of the six sublines of C57BL/6J mice available at The Jackson Laboratory they used. The absence of such information hinders the present and future scientific community from fully interpreting a published report.

The advent of high-density single nucleotide polymorphism (SNP) genotyping platforms offers a powerful tool to monitor genetic stability within an individual sub-strain in a breeding facility (Petkov et al. 2004; Yang et al. 2009). Recently SNP mapping was used to identify a copy-number variation allele on chromosome 19 of C57BL/6J mice that affects expression of the Ide and Fgfbp3 genes and that is segregating in what were presumed to be inbred C57BL/6J mice (Watkins-Chow and Pavan 2008). Continued improvements in methods and reduced cost of SNP genotyping should ultimately facilitate routine genotyping of mouse strains used in research studies.

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