De novo inbred heterozygous Zeb2/Sip1 mutant mice uniquely generated by germ-line conditional knockout exhibit craniofacial, callosal and behavioral defects associated with Mowat–Wilson syndrome

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Abstract

Mowat–Wilson syndrome (MOWS) is caused by de novo heterozygous mutation at ZEB2 (SIP1, ZFHX1B) gene, and exhibit moderate to severe intellectual disability (ID), a characteristic facial appearance, epilepsy and other congenital anomalies. Establishing a murine MOWS model is important, not only for investigating the pathogenesis of this disease, but also for identifying compounds that may improve the symptoms. However, because the heterozygous Zeb2 knockout mouse could not be maintained as a mouse line with the inbred C57BL/6 background, it was difficult to use those mice for the study of MOWS. Here, we systematically generated de novo Zeb2 Δex7/+ mice by inducing the Zeb2 mutation in the germ cells using conditional recombination system. The de novo Zeb2 Δex7/+ mice with C57BL/6 background developed multiple defects relevant to MOWS, including craniofacial abnormalities, defective corpus callosum formation and the decreased number of parvalbumin interneurons in the cortex. In behavioral analyses, these mice showed reduced motor activity, increased anxiety and impaired sociability. Notably, during the Barnes maze test, immobile Zeb2 mutant mice were observed over repeated trials. In contrast, neither the mouse line nor the de novo Zeb2 Δex7/+ mice with the closed colony ICR background showed cranial abnormalities or reduced motor activities. These results demonstrate the advantages of using de novo Zeb2 Δex7/+ mice with the C57BL/6 background as the MOWS model. To our knowledge, this is the first time an inducible de novo mutation system has been applied to murine germline cells to produce an animal model of a human congenital disease.

Introduction

Mowat–Wilson syndrome (MOWS [OMIM 235730]) is diagnosed in approximately 1 infant per 50 000–70 000 live births and is caused by de novo heterozygous mutations in the ZEB2 (also known as SIP1, ZFHX1B) gene, which encodes zinc-finger and homeodomain-like sequence-containing transcription factor. MOWS results in severe intellectual disability (ID), characteristic facial appearance and other symptoms such as microcephaly, epilepsy, Hirschsprung’s disease, agenesis of the corpus callosum (ACC) and congenital heart disease (1–3). To understand ZEB2 gene function and its relationship to the MOWS phenotype, extensive functional analyses of the Zeb2 gene have been performed in the last decade by investigating Zeb2 knockout (KO) mice: specifically, this has been performed in a tissue-specific manner by combining...
Zeb2 floxed mice and the appropriate tissue-specific Cre recombinase-expressing mice (4–11). In neural crest cell-specific Zeb2 KO mutant mice, craniofacial and enteric nervous defects were observed, which could be related to MOWS’s characteristic facial morphology and Hirschsprung’s disease, thereby suggesting that dysfunctional Zeb2 in the neural crest cells contributes to the symptoms of MOWS (4). Analysis of the dorsal telencephalon-specific removal of Zeb2 revealed its requirement for both hippocampus and corpus callosum formation (5). Zeb2 is also involved in the precise regulation of cortical neurogenesis, particularly in deep and upper neuron layer formation, but also for gliogenesis, via a non-cell autonomous feedback mechanism between postmitotic prospective glutamategic neurons and progenitor cells (6,7). Zeb2 also regulates the axonal growth rate during the formation of the cortical axonal tract, including the corpus callosum (8). Meanwhile, a study on the ventral telencephalon-specific removal of Zeb2 elucidated that Zeb2 contributes to the fate of cortical versus striatal GABAergic interneurons (9,10), and regulates guided migration of GE (ganglionic eminence)-born cortical interneurons. Additionally, Zeb2 is required for oligodendrocytes to myelinate the axon in the embryonic and early postnatal central nervous system (11). Although its function in adult Schwann cells for myelination is yet to be studied, these findings suggest that MOWS patients might have the myelination defects. Thus, all these previous studies have provided valuable insights into the symptoms of MOWS. Nevertheless, the tissue-specific homozygous mutations mentioned above are totally different from the actual de novo heterozygous ZEB2 mutations that cause MOWS in humans. To more precisely understand the pathogenesis of MOWS, analysis using heterozygous Zeb2 KO mice is indispensable.

Despite the availability of heterozygous Zeb2 KO mice (i.e. Zeb2lox7/+ mice, whose exon 7 on the KO allele is deleted to be non-functional) (12), studies on these mice have been delayed. One of the reasons is the technical problems associated with the genetic background. After first establishing Zeb2lox7/+ mice with a mixed genetic background (12), backcrossings with the inbred B6SJL/J strain as well as closed colony ICR strain were performed. Backcrossing with ICR strain was successful, while backcrossing with C57BL/6 was difficult due to the gradual appearance of growth retardation after several backcrossings (Y.H., unpublished findings). Accordingly, analysis of the Zeb2lox7/+ mice with the inbred C57BL/6 background could not be performed. Recently, it was reported that Zeb2lox7/+ mice with the CD1 background, which is thought to be the same as ICR background (13), demonstrate decreased thermal pain responses (14–15), thereby suggesting the role of Zeb2 in regulating pain sensitivity. However, these mice do not demonstrate the other obvious defects related to MOWS symptoms, except infrequent defects in corpus callosum formation (14) (N.W., unpublished). Furthermore, these mice did not show any abnormal behaviors (15). These results imply that the Zeb2lox7/+ mice with the ICR background may not be the optimal murine MOWS model. It is possible that during backcross with the closed colony ICR strain, the mildly affected and therefore relatively healthier Zeb2lox7/+ mice were preferentially selected. In addition, because MOWS is caused by de novo mutation, the model mouse should preferably have de novo Zeb2 gene mutations.

Here, we induced mutations in the germ cells in order to obtain de novo Zeb2lox7/+ mice with the C57BL/6 inbred genetic background. Using this procedure, we obtained a substantial number of adult Zeb2lox7/+ mice with the C57BL/6 background and evaluated if the resulting mice could be used as a MOWS model.

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**Results**

**Generation of the de novo Zeb2lox7/+ mice**

We used Protamine-Cre transgenic mice (Prm-Cre Tg), which specifically express Cre recombinase in the male germ cells (16), to generate de novo Zeb2lox7/+ mice, based on the observations that the de novo mutations analyzed in human mostly arose from the paternal genome (17–20). By mating Zeb2flox/+; Prm-Cre Tg(+) male mice with wild-type C57BL/6 female mice, we were able to systematically generate de novo Zeb2lox7/+ mice as the progeny (Table 1). Because Prm-Cre recombinase is expressed and shared in connected spermatids via syncytial bridges (16), and because recombination with the Zeb2flox allele does not occur with 100% efficiency, the presence or absence of Prm-Cre Tg does not necessarily coincide with the deletion or non-deletion of the Zeb2flox allele. Thus, the Zeb2lox7/+ and Zeb2flox/+ alleles were observed regardless of the presence of Prm-Cre Tg (Table 1).

Although embryonic lethality was not observed from this mating (Table 1), a substantial number of de novo Zeb2lox7/+ mice died a few days after birth or during nursing; only 64% of all de novo Zeb2lox7/+ mice with the C57BL/6 background survived to 4 weeks (P28 live, Table 1). The occasional early-stage death of a C57BL/6 mouse carrying the Zeb2lox7/+ allele might have brought about the previously observed difficulty in maintaining the Zeb2lox7/+ mice with the C57BL/6 background. Based on our results with this genotype, as well as the phenotype analyses described below, we hereafter used Zeb2lox7/+ (wild-type) and Zeb2flox/+ mice with or without Prm-Cre Tg as the control mice, and Zeb2lox7/+ mice with or without Prm-Cre Tg were used as the mutant mice, unless otherwise specifically mentioned.

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**Table 1. Progeny distribution according to genotype produced by the inducible de novo mutation system**

<table>
<thead>
<tr>
<th>Zeb2 allele</th>
<th>+/+</th>
<th>flox/+</th>
<th>lox7/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prm-Cre Tg</td>
<td>(+)</td>
<td>(−)</td>
<td>(−)</td>
</tr>
</tbody>
</table>

Embryonic stage

| E16.5 | 22 | 28 |
| E18.5 | 12 | 11 |
| Subtotal | 34 | 40 |

Postnatal stage

<table>
<thead>
<tr>
<th>Postnatal stage</th>
<th>P0 dead</th>
<th>P1 dead</th>
<th>P2-27 dead</th>
<th>P28 live</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>257</td>
<td>222</td>
<td>104</td>
<td>78</td>
</tr>
</tbody>
</table>

| Total | 376   |

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*By comparing these values, we concluded no lethality in the embryonic stage because there were no obvious losses in h in comparison to g and f. In contrast, i, the corresponding number in the postnatal stage, is lower than expected.

**The survival rate of the Zeb2lox7/+ mice was calculated as 64% as follows: [(55 + 52)/167] × 100 = 64%, although this survival rate may have been lower due to an unknown loss of pups by muricide as described in the next footnote below.

According to the principles of Mendelism, the values of the number of the +/+ mice and the summed number of thelox7/+ and flox/+ mice were expected to be nearly equal based on our crossing strategy. However, the value of i was about 100 less than that of h (376 versus 479), indicating that some animals could not be counted due to muricide loss during postnatal development.
Facial abnormalities in de novo Zeb2 Δex7/+ mice with the C57BL/6 background

We first examined facial morphology because facial appearance is a valuable indicator for diagnosing MOWS (1). During nursing, the facial appearance of the de novo Zeb2 Δex7/+ mice with the C57BL/6 background could not be distinguished from the controls. But, toward the adult stage, its button-shaped nose became progressively apparent (Fig. 1A). Indeed, the nasal bone of the de novo Zeb2 Δex7/+ mice was shorter (Fig. 1B and C) and the skull was broader (Fig. 1B and D). The ratio of nasal bone length to skull width also confirmed this significant difference (Fig. 1E). It was also reported previously that neural crest cell-specific homozygous Zeb2 KO (Zeb2 flox/flox; Wnt1-Cre Tg(+)) mice also demonstrate shortened nasal bones at the newborn stage (4). Therefore, we examined if short nasal bones can be detected in newborns. Different than the Zeb2 flox/flox; Wnt1-Cre Tg(+) mice, de novo Zeb2 Δex7/+ mice did not demonstrate a shortened nasal bone at this stage (Fig. 1F and G). The differential onset of these defects might be related to the type of Zeb2 mutation, which can be heterozygous or tissue-specific homozygous.

Malformation of the corpus callosum in de novo Zeb2 Δex7/+ mice with the C57BL/6 background

Agenesis of the corpus callosum is a major symptom often observed in MOWS patients (1). Because the fundamental structure of corpus callosum is formed by E17.5, we examined corpus callosum formation in the de novo Zeb2 Δex7/+ mice with the C57BL/6 background.

Figure 1. Craniofacial formation in de novo Zeb2 Δex7/+ mice with the C57BL/6 inbred background. (A) Facial appearances of 8-month-old Zeb2 +/- (left, control) and Zeb2 Δex7/+ (right, mutant) mice with the C57BL/6 background. Arrows indicate the button-shaped nose. The horizontal lines show the width of the nose. (B) Dorsal view of the skulls of 5-month-old Zeb2 flox/+ (left, control) and Zeb2 Δex7/+ (right, mutant) mice with the C57BL/6 background. Arrows indicate the nasal bone and their lengths are shown by the vertical white lines. Red bars represent accurate length of the nasal bone. Quantification and comparison of (C) the length of nasal bone, (D) the width of skull, and (E) their ratio. (F) Side view of the skulls of Zeb2 flox/+ (left, control) and Zeb2 Δex7/+ mice (right, mutant) with the C57BL/6 background at E18.5 and (G) quantitative comparison of the length of the nasal bone (red bar in F) indicates no difference between the control and mutant mice. Data are expressed as the mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 according to the student t-test. Scale bars: A and B, 5 mm; F, black, 1 mm. (n = 6-7) for C-E, (n = 4) for G.
callosum formation at E18.5 and found that one of the two de novo Zeb2 Δex7/+ embryos with the C57BL/6 background demonstrated ACC (Fig. 2A). We also examined the axonal tract of the corpus callosum using the anti-L1 antibody at E16.5, or 1 day before the completion of the fundamental structure of corpus callosum. Obvious defects or gaps at the midline crossing of the L1-positive axon fiber were observed in 4 of 25 de novo Zeb2 Δex7/+ embryos (Fig. 2B). In addition, the area of the V-shaped L1-positive axons, which reflects the amount of axonal fiber present, was reduced in de novo Zeb2 Δex7/+ mice (Fig. 2C). We next examined ACC in adult de novo Zeb2 Δex7/+ mice. Contrary to the findings at E18.5, ACC was not observed in the adult de novo Zeb2 Δex7/+ mice with the C57BL/6 background (Supplementary Material, Table S1), while it is infrequently seen in the adult Zeb2 Δex7/+ mouse line with the ICR background (14) (N.W., unpublished). These results might imply that de novo Zeb2 Δex7/+ mice with the C57BL/6 background harboring ACC could die during the perinatal stage. However, it is also possible that the ACC-affected embryos could recover when the CC axons grew to cross over the midline, indicating developmental compensation rather than arrested midline development, as described in the recent report (8).

For the corpus callosum to form properly, reciprocal interactions between the medial neocortex and midline meninges are important and occur via morphogenetic signaling molecules, including BMP (21). Because high Zeb2 expression is observed in the cortex (22) and cranial neural crest cells (23), which give rise to the meninges, we examined whether the heterogeneous Zeb2 deletion specific for the respective tissues causes defects in the corpus callosum or not. Although neural crest-specific heterogeneous Zeb2 mutation did not generate ACC-like defects in Wnt1-Cre Tg mice (Fig. 2D and E) (24), dorsal cortex-specific heterogeneous Zeb2 mutations in Emx1-CreKI mice (25) caused ACC-like L1-patterned defects similar to de novo Zeb2 Δex7/+ mice (Fig. 2F and G). These data suggest that defects in corpus callosum formation in de novo Zeb2 Δex7/+ mice with the C57BL/6 background could be caused by Zeb2 deficiency in the neocortex, not in the meninges. These findings agree well with results of the recent study (8).

Reduced number of the parvalbumin-positive interneurons in de novo Zeb2 Δex7/+ mice with the C57BL/6 background

Because epilepsy is often observed in MOWS patients, we thought de novo Zeb2 Δex7/+ mice would demonstrate defective interneurons, such as a low cell number or altered function. At first, we examined the sensitivity of the de novo Zeb2 Δex7/+ mice against a convulsant drug—pentylentetrazol (PTZ)—but could not find any apparent differences with the control mice (T.T., unpublished). Next, we evaluated the number of parvalbumin (PV) interneurons—a major subtype of GABAergic interneurons—that are derived from medial ganglionic eminence (MGE)—and found that the number of PV interneurons in the cortex was reduced by about 30% in adult de novo Zeb2 Δex7/+ mice in comparison to control mice (Fig. 3A and B). The numbers of somatostatin (SST) interneurons (another subtype of interneuron derived from MGE) (Fig. 3C and D) and calretinin (CR) interneurons (which are derived from caudal ganglionic eminence [CGE]) (Fig. 3E and F) did not change in de novo Zeb2 Δex7/+ mice. The formation of PV interneurons in the hippocampus was not affected in either the rostral or caudal regions in de novo Zeb2 Δex7/+ mice (Fig. 3G). These results suggest that the reduction in PV interneurons in the cortex could be related to epilepsy in MOWS patients.

Since ZEB2 is now known to play a crucial role in migration and/or differentiation of the GABAergic interneurons (9,10), the reduction of PV interneurons observed in de novo Zeb2 Δex7/+ mice might suggest that even heterozygous deletion of the ZEB2 gene could affect partly the interneuron development. To address this possibility, we examined the expression patterns of the two interneuron maker genes, Lhx6 and SST by in situ hybridization at two different embryonic stages along with different regions from the rostral to caudal GEs. There was no gross change of the two maker gene expression patterns in de novo Zeb2 Δex7/+ mice at those stages and regions compared with the control mice (Supplementary Material, Fig. S1), implying that over all interneuron development was not affected, but somehow at least a part of the mature PV interneurons in cortex could be affected and showed the reduced number at the adult stage by the heterozygous conditions. A detailed analysis will be required for explanation of this observation.

As mentioned above, de novo Zeb2 Δex7/+ mice with the C57BL/6 background demonstrated defects in the formation of the craniofacial characteristics, corpus callosum and PV interneurons. However, the enteric neurons in the colon, which are affected by Hirschsprung’s disease and often MOWS, seems to be normal according to the endogenous acetylcholinesterase (AChE) staining patterns (Supplementary Material, Fig. S2). Tooth development could be another important process for Zeb2 function because the tooth bud mesenchyme cells express ZEB2 (4) and the BMP signaling is known to be involved in its development (26). Furthermore, some MOWS have problems in dentication (27). We examined the dentition in the de novo Zeb2 Δex7/+ mice at 5 months old, but there were no malformation in their teeth (Supplementary Material, Fig. S3). Thus, the de novo Zeb2 Δex7/+ mice with the C57BL/6 background do not show all of the MOWS-related defects.

Abnormal behavior in de novo Zeb2 Δex7/+ mice with the C57BL/6 background

Because ID is observed in all MOWS patients (28,29), we investigated such characteristics in de novo Zeb2 Δex7/+ mice. On the open field test—which is one of the behavioral tests for the motor activity and measurement of the anxiety level under the novel open field environment—de novo Zeb2 Δex7/+ mice demonstrated lower motor activities during the 1-h test, including total path length (Fig. 4A) and frequency of rearing (Fig. 4B). The reduction of rearing frequency is thought to reflect reduction of exploring the side of the wall.

The elevated plus maze was used to examine anxiety. In comparison with the controls, the de novo Zeb2 Δex7/+ mice spent less time in the open arms (Fig. 4C), thereby suggesting that the de novo Zeb2 Δex7/+ mice were more sensitive to anxiety.

The three-chamber test was used to examine social behavior. During the habituation phase without a stranger mouse, neither the control nor the de novo Zeb2 Δex7/+ mice exhibited preferences for the chamber (Fig. 4D). On the main test with a stranger mouse, while the control mice showed the staying preferences for the right chamber harboring a stranger mouse, the de novo Zeb2 Δex7/+ mice did not (Fig. 4E). These data suggest the lower sociality of the de novo Zeb2 Δex7/+ mice.

On the rotarod test, the de novo Zeb2 Δex7/+ mice demonstrated comparable performance to the control mice (Fig. 4F), thereby implying that the lower activity of mutant mice in open field test was not caused by the defect of motor function, and also showing that learning ability via the cerebellum and motor cortex of the mutant mice was normal.
Figure 2. Corpus callosum formation in de novo Zeb2 Δex7/+ mice with the C57BL/6 inbred background. (A) Nissl-stained coronal sections at E18.5 obtained from Zeb2 flox/+ (left, control) and Zeb2 Δex7/+ (right, mutant) embryos. cc, corpus callosum. (B) L1-immunostained coronal sections of the cortex at E16.5 obtained from Zeb2 +/+ (left, control) and Zeb2 Δex7/+ (right, mutant) embryos and (C) measurement and quantitative comparison with the V-shaped L1-positive areas. The same sets of analyses are shown for (D and E) conditional KO Wnt1-Cre Tg and (F and G) Emx1-Cre KI mice. (D) Cortices from Zeb2 +/+; Wnt1-Cre Tg(+) (left, control) and Zeb2 flox/+; Wnt1-Cre Tg(+) (right, mutant) embryos and (E) quantitative comparisons. (F) Zeb2 +/+; Emx1-Cre KI/+ (left, control) and Zeb2 flox/+; Emx1-Cre KI/+ embryos (right, mutant) and (G) quantitative comparisons. The quantitative data are expressed as the mean ± s.e.m. *P < 0.05, **P < 0.01 according to the student t-test. Scale bars: 300 μm (n = 11–16) for C; (n = 6–7) for E; (n = 11) for F.
To evaluate learning and memory abilities, we performed a spatial learning test: specifically, the Barnes maze test. The reason we chose this system other than Morris water maze was that unwanted stress, fear and anxiety to the animals should be avoided as much as possible because the elevated plus maze test already suggested the higher sensitivity of the de novo Zeb2

Figure 3. Interneuron formation in de novo Zeb2 Δex7/+ mice with the C57BL/6 inbred background. (A) Parvalbumin-positive interneurons in adult cortices from Zeb2 flox/+ (left, control) and Zeb2 Δex7/+ mice (right, mutant), and (B) quantitative comparisons with the number of parvalbumin interneurons in Zeb2 +/+ or flox/+ (i.e. control) and Zeb2 Δex7/+ (i.e. mutant) mice. The same sets of the analyses for (C and D) somatostatin-positive and (E and F) CR-positive interneurons are shown. (G) Cortices from Zeb2 +/+ (left, control) and Zeb2 Δex7/+ mice (right, mutant) and (G) quantitative comparisons. Note that the number of the parvalbumin interneurons was significantly reduced while two other interneurons did not change. (G) Quantitative comparison with the number of parvalbumin interneurons in adult hippocampus obtained from Zeb2 +/+ (i.e. control) and Zeb2 Δex7/+ mice (i.e. mutant) are shown separate from the rostral and caudal regions. No meaningful differences in the number of parvalbumin-positive interneurons in the hippocampus could be detected. The adult mice used for the analysis were all 8 weeks old. Data are expressed as the mean ± s.e.m. **P<0.01 according to the student t-test. Scale bars: 200 μm. (n = 4) for B, D, F and G.
Δex7/+ mice to anxiety, and Morris water maze test, which is performed in water, is thought to bring much more stress than Barns maze test done on dry-land (30). The test was performed as follows (Fig. 5A): day 1 was the habituation phase, and days 2–11 comprised the training phase. Two 5-min trials were performed every day, thereby resulting in 20 trials during the training phase. Day 12 was the probe phase, where the position of the escape box was changed from its original location. To our surprise, the de novo Zeb2 Δex7/+ mice needed multiple trials to enter the escape box (Fig. 5B). Half of the control mice entered the escape box by the second trial, while the de novo Zeb2 Δex7/+ mice required nine trials before half of them entered the box (Fig. 5B). Eventually all control mice could enter the newly moved escape box (Fig. 5B) and again appeared stationary (Fig. 5E). Because of the complex immobility of the de novo Zeb2 Δex7/+ mice in the Barnes maze test, we cannot conclude that the de novo Zeb2 Δex7/+ mice with the C57BL/6 background had learning and memory defects.

We next tried the shuttle avoidance test, which is another learning and memory test. We performed the test three consecutive times, but differences between the de novo Zeb2 Δex7/+ and control mice were not observed (Fig. 5F). These results suggest that the de novo Zeb2 Δex7/+ mice with the C57BL/6 background do not have obvious defects in associative learning, including contextual fear conditioning.

The genetic background of Zeb2 Δex7/+ mice influences phenotype appearance

In contrast to inbred C57BL/6 mice, Zeb2 Δex7/+ mice that had been backcrossed with the closed colony ICR strain could be maintained as the Zeb2 Δex7/+ mouse line. However, these mice did not show the obvious skull features observed in the de novo Zeb2 Δex7/+ mice with the C57BL/6 background (Supplementary Figure 4). Emotional and social behaviors of de novo Zeb2 Δex7/+ mice with the C57BL/6 background.

(A) Distance moved and (B) total number of rearings during the 5-min intervals of the open field test. (C) Total time spent in the open arms during the elevated plus maze test. (D) Total time spent in the left and right chambers during the three-chamber test without a stranger mouse or (E) with a stranger mouse in the right chamber. (F) Total running time during each trial of the rotarod test. The genotypes of the mice for the control (Zeb2 +/+ or flox/+) and mutant mice (Zeb2 Δex7/+) were used in these tests (A–F). Data are shown as the mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 according to the student t-test. (n = 17) for A and B; (n = 12) for C–E; (n = 6) for F.
To compare inbred and closed colony strain phenotypes of the de novo Zeb2 Δex7/+ mutant mice, we first backcrossed both the Zeb2 flox/+ and Prm-Cre Tg+ mice with the ICR mice, and by following the same mating strategy as in the C57BL/6 case, we obtained the de novo Zeb2 Δex7/+ mice with closed colony ICR background. Contrary to expectations, the de novo Zeb2 Δex7/+ mice with the ICR background did not develop the skull phenotype like the heterozygous Zeb2 KO mouse line with the ICR background (Fig. 6A–D). Furthermore, these de novo Zeb2 Δex7/+ mice with the ICR background did not show the reduced motor activities observed in the de novo Zeb2 Δex7/+ mice with the C57BL/6 background (Fig. 6E). The transition patterns of the rearing number in the de novo Zeb2 Δex7/+ mice with the ICR background were less evident than in the de novo Zeb2 Δex7/+...
mice with the C57BL/6 background (Fig. 6F). These data suggest that the phenotypes of the Zeb2Δex7/+ mice are largely influenced by the genetic background itself, not the de novo mutations or germline. Considering these results, using an inbred strain such as C57BL/6 seems to be more suitable for generating Zeb2 Δex7/+ mice for use as the MOWS model.

Discussion

ZEB2 has now become one of the most important transcription factors for the various tissue developments, especially being essential for neural tissue development from early embryo to adult (4–11). The successful clarification of those ZEB2 functions has been achieved with the conditional KO technique using the appropriate tissue-specific Cre and Zeb2 floxed mice. Simultaneously, those studies brought the valuable insights into understanding of MOWS. From the clinical point of view, however, the MOWS murine model of the simple heterozygous Zeb2 mutation, which is the same genetic conditions as the human case, is indispensable to understand more precisely the pathogenesis of MOWS and to develop the medication for the patients. In this report, we efficiently produced heterozygous Zeb2 KO mice with the inbred C57BL/6 background by applying an inducible de novo mutation system to the germline cells. Examining de novo Zeb2 Δex7/+ mice with the C57BL/6 background confirmed that the mice developed multiple defects that are correlated with the symptoms of MOWS, such as craniofacial defects, ACC and reduced parvalbumin interneurons in the cortex. On the behavioral tests, the mice demonstrated affected anxiety and sociability. While anxiety in MOWS patients has not been reported, lower sociability was observed in MOWS (28). This evidence suggests that de novo Zeb2 Δex7/+ mice with the C57BL/6 background are the most appropriate murine model for MOWS from among the other Zeb2 Δex7/+ mice that have been studied so far. Future detailed analyses on de novo Zeb2 Δex7/+ mice with the C57BL/6 background would contribute to understanding MOWS and could lead to the development of medical methods that ameliorate MOWS.

In the present study, we also confirm that the system used to induce de novo mutations in germ cells is very effective when the model mutant mouse is difficult to maintain because of severe phenotypes and/or growth retardation due to the original causal mutation, which brings about a syndromic phenotype in mouse as well as in the original human disease. To the best of our knowledge, this is the first time a de novo mutation has been induced in germline cells in order to systematically produce a murine model of human congenital disease. This method could also be applied to produce other murine models that are difficult to maintain. In particular, its application would be worthwhile in cases where a substantial number of age-matched mice are required for the analysis, such as behavioral tests. Recently, it was reported that stress events can influence subsequent generations via epigenetic changes and/or the microRNA contents in mice with the C57BL/6 background (Fig. 6F). These data suggest that the phenotypes of the Zeb2 Δex7/+ mice are largely influenced by the genetic background itself, not the de novo mutations or germline. Considering these results, using an inbred strain such as C57BL/6 seems to be more suitable for generating Zeb2 Δex7/+ mice for use as the MOWS model.

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In the present study, we also confirm that the system used to induce de novo mutations in germ cells is very effective when the model mutant mouse is difficult to maintain because of severe phenotypes and/or growth retardation due to the original causal mutation, which brings about a syndromic phenotype in mouse as well as in the original human disease. To the best of our knowledge, this is the first time a de novo mutation has been induced in germline cells in order to systematically produce a murine model of human congenital disease. This method could also be applied to produce other murine models that are difficult to maintain. In particular, its application would be worthwhile in cases where a substantial number of age-matched mice are required for the analysis, such as behavioral tests. Recently, it was reported that stress events can influence subsequent generations via epigenetic changes and/or the microRNA contents in...
sperm (31,32). Therefore, another advantage of using de novo mutations is avoiding the secondary effects that arise in the previous generations of the mutant mice.

We showed that the genetic background largely influences the appearances of the phenotypes that are correlated with the symptoms of MOWS: while the de novo Zeb2 Δex7/+ mice with the C57BL/6 background developed multiple defects associated with MOWS, both the de novo and Zeb2 Δex7/+ mice with the closed colony background did not (except lower sensitivity to thermal pain and occasional ACC). It was also reported that the phenotype of the heterozygous Cbp KO mutant mouse, which is the model mouse of another syndrome—type ID—Rubinstein–Taybi syndrome 1 (RSTS1 [OMIM 180849])—is influenced by the contributions of different mixed genetic backgrounds (33). Furthermore, heterozygous Cbp KO mutant mice cannot be maintained with an inbred genetic background, such as C57BL/6, very similar to Zeb2 Δex7/+ mice (S. Ishii, personal communication). The PANDER KO mouse, which is a useful model mouse for studying human type 2 diabetes, is also influenced by the genetic background. Enhanced glucose tolerance was observed in PANDER KO mice with the C57BL/6 background (94), but not mice with the mixed genetic background (35). These observations suggest that using an inbred background, such as C57BL/6, could be appropriate for producing some relevant murine models.

One of the surprising results of this study is the distinctive behavioral patterns of the de novo Zeb2 Δex7/+ mice with the C57BL/6 background during the Barnes maze test. First, de novo Zeb2 Δex7/+ mice needed extensive training before entering the escape box. This was largely caused by extended immobility. Second, even though about half of the de novo Zeb2 Δex7/+ mice needed extensive training before entering the escape box—therefore, heterozygous Cbp KO mutant mice cannot be maintained with an inbred genetic background, such as C57BL/6, very similar to Zeb2 Δex7/+ mice (S. Ishii, personal communication), the PANDER KO mouse, which is a useful model mouse for studying human type 2 diabetes, is also influenced by the genetic background. Enhanced glucose tolerance was observed in PANDER KO mice with the C57BL/6 background (94), but not mice with the mixed genetic background (35). These observations suggest that using an inbred background, such as C57BL/6, could be appropriate for producing some relevant murine models.

One of the surprising results of this study is the distinctive behavioral patterns of the de novo Zeb2 Δex7/+ mice with the C57BL/6 background during the Barnes maze test. First, de novo Zeb2 Δex7/+ mice needed extensive training before entering the escape box. This was largely caused by extended immobility. Second, even though about half of the de novo Zeb2 Δex7/+ mice needed extensive training before entering the escape box, the mice were subsequently immobile for long periods of time. This suggests that the immobility could not have been caused only by exposure to a new environment, such as the board of the Barnes maze itself, because the immobility did not appear immediately after placing the mice as reported previously (36). Rather, being left on the uncomfortable maze board for long period of time seems to cause the immobility. We believe there are two possibilities that explain the lack of movement. One is that the de novo Zeb2 Δex7/+ mice stopped moving because of fear or stress after being left alone on the uncomfortable and shiny Barnes maze board; the other is that the de novo Zeb2 Δex7/+ mice might have given up exploration due to the rapid decrease in motivation or loss in curiosity after initially searching. Considering the fact that none of the de novo Zeb2 Δex7/+ mice could enter the escape box during the first probe trial—while most of the de novo Zeb2 Δex7/+ mice had been able to enter the escape box during the previous training trial—the motivation of the de novo Zeb2 Δex7/+ mice seemed to be easily lost, as compared with the control, at least under the non-aversive conditions such as Barnes maze test, because there were no differences between the mutant and control mice in aversive conditions during the shuttle avoidance test. To further understand the behavioral responses of the de novo Zeb2 Δex7/+ mice during the Barnes maze test, it may be worth examining endocrine conditions, such as those that affect the hypothalamic-pituitary-adrenal (HPA) axis in future studies.

Although MOWS patients exhibit ID, obvious defects in learning and memory were not observed in the de novo Zeb2 Δex7/+ mice with the C57BL/6 background whose learning processes seems to mainly depend on the amygdala rather than the hippocampus, at least when studied using contextual fear conditioning tests (37,38). One possible explanation for this result is the selective loss of adult de novo Zeb2 Δex7/+ mice with neuronal defects related to the learning and memory. As previously mentioned, a significant number of de novo Zeb2 Δex7/+ mice with the C57BL/6 background died during the early postnatal and nursing stages, and it is possible that these dead mice might have had profound neuronal defects for learning and memory, thereby resulted in the biased survival of neurologically healthier mice who demonstrated no obvious defects on the shuttle avoidance test. Another possible reason is that a large part of the ID diagnosed in human case of MOWS might be attributable to the defect of motivation, which we think most probable cause for the results of Barnes maze test. On the Barnes maze test, the de novo Zeb2 Δex7/+ mice actually demonstrated abnormal responses: they tended to stop and stay at the periphery of the board and required a long time to search and enter the final goal box, which seemed to be caused by weak motivation for tasks under non-aversive conditions. Thus, we think it possible that an altered motivation or incentive might have affected the process of diagnosis on ID in MOWS patients, although such evidence has not been reported yet.

Because Zeb2 is a Smad interacting protein, impairment in BMP signaling could be associated with MOWS. Juvenile polyposis syndrome (JPS [OMIM 174900]) is caused by mutations in the genes involved in the BMP signaling, such as MADH4 (also known as SMAD4) (39,40) and BMPRIA (41,42). JPS exhibits multiple congenital abnormalities, some of which are common in MOWS patients including hypertelorism and ID (43,44). Bmpr1a is also expressed in the neural crest cells and brain that also express Zeb2 (45,46). Indeed, transgenic mice that express the dominant negative form of BMPRIA in neural crest cells-derived tissues demonstrate craniofacial abnormalities that are very similar to de novo Zeb2 Δex7/+ mice (47). Therefore, some of the symptoms of MOWS and JPS may be caused by impaired BMP signaling.

Materials and Methods

Mice

Zeb2 flox/+ mice (12) were backcrossed with C57BL/6N wild-type mice >10 times, or backcrossed with the ICR strain 5 times. Pro
tamine-Cre mice (16) were backcrossed 5 times with C57BL/6N or ICR mice. To produce de novo Zeb2 Δex7/+ mice, the wild-type C57BL/6N females were mated with Zeb2 flox/+, Prm-Cre Tg(+) male mice. Emx1-Cre Kl/Kl mice (25) with the C57BL/6J background were provided by RIKEN BRC via the National Bio-
Resource Project of MEXT, Japan. To obtain Zeb2 flox/+, Emx1-Cre Kl/+ embryos, Zeb2 flox/+ females were mated with Emx1-Cre Kl/Kl male mice. To obtain the Zeb2 flox/+, Wnt1-Cre Tg(+) embryos, Zeb2 flox/+ females were mated with Wnt1-Cre Tg(+) male mice with the C57BL/6N background (24). A line of Zeb2 Δex7/+ mice with the ICR background was produced after backcrossing with ICR 6 times. All mice were housed in the animal facility of Insti-
tute for Developmental Research (IDR), Aichi Human Service Center (Aichi, Japan) and used in accordance with the protocols and guidelines approved by IDR.

Immunostaining

To immunostain the cryosections, brains at E16.5 were fixed in 4% paraformaldehyde and PBS overnight at 4°C, treated in 30% sucrose in PBS at 4°C and embedded in a plastic dish using Surgi
tissue FSC22 (Leica, Nussloch, Germany) at ~80°C with 2-methyl-
butane. The cryosections were cut to 10 μm using a cryostat (model CM1950; Leica, Nussloch, Germany), blocked with 3% BSA PBS containing 0.05% Tween-20 and incubated with rat
anti-L1 antibody (1:1000; MAB5272, Millipore). The Alexa Fluor 488-labeled secondary antibody was used to detect anti-L1. Fluorescent images were obtained as previously described (22). The L1-immunostained samples were examined at 80-μm intervals, and the largest V-shaped region of each L1 stain was used in subsequent analyses. The L1-stained area was measured using imageJ (48).

To immunostain the paraffin sections, adult mice were perfused with 4% PFA in PBS, and the brain was dissected, post-fixed and processed for paraffin embedding. The paraffin sections were cut to a thickness of 7 μm using a microtome (model HM360E; Thermo Scientific, Waltham, MA, USA), deparaffinized and hydrated. The sections were treated for antigen retrieval in 10 mM sodium citrate (for parvalbumin, the sections were treated at 60°C for 1 h; for CR, the sections were treated with autoclaving for 20 min). After blocking, the sections were incubated with anti-parvalbumin antibody (1:1000; MAB1572, Millipore) or anti-CR (1:500; ab92341, Abcam) at room temperature overnight. Primary antibodies were detected using a peroxidase-anti-peroxidase (PAP)-soluble immune complex (Jackson ImmunoResearch Laboratories), according to the instructions of the manufacturer. Enzymatic staining using peroxidase with diaminobenzidine (DAB) was performed at room temperature for 1 h.

**In situ hybridization**

In situ hybridization was performed using a previously described method (49). Briefly, 7-μm-thick paraffin sections were deparaffinized and hydrated. The sections were treated with 10 μM/mL proteinase K at 37°C for 15 min, fixed in 4% paraformaldehyde in PBS and acetylated. Prehybridization was performed at room temperature for 4 h using a hybridization solution that contained 5× SSC, 50% formamide, 5× Denhardt’s, 100 μg/mL salmon sperm DNA and 375 μg/mL yeast tRNA. The templates for the DIG-labeled RNA probe were amplified using primers as following (somatostatin: forward 5′-GGAGACGCTACCGAAGCCGTC-3′, reverse 5′-TCATAATCTCACCATATTTAATTTGTAT-3′, Lhx6: forward 5′-CAGCCCAAGAGGGGGGCTGGTCACCTT-3′, reverse 5′-TGGGGGGGCTGTGTAACCTGAGACCTTTC-3′). Hybridization was performed overnight at 72°C using 300 ng/mL RNA probes in the hybridization solution. After washing in 5× SSC, 0.2× SSC at 60°C for 1 h, and 0.2× SSC at room temperature, the sections were fused with 4% PFA in PBS, and the brain was dissected, post-fixed and processed for paraffin embedding. The paraffin sections were cut to a thickness of 7 μm using a microtome (model HM360E; Thermo Scientific, Waltham, MA, USA), deparaffinized and hydrated. The sections were treated for antigen retrieval in 10 mM sodium citrate (for parvalbumin, the sections were treated at 60°C for 1 h; for CR, the sections were treated with autoclaving for 20 min). After blocking, the sections were incubated with anti-parvalbumin antibody (1:1000; MAB1572, Millipore) or anti-CR (1:500; ab92341, Abcam) at room temperature overnight. Primary antibodies were detected using a peroxidase-anti-peroxidase (PAP)-soluble immune complex (Jackson ImmunoResearch Laboratories), according to the instructions of the manufacturer. Enzymatic staining using peroxidase with diaminobenzidine (DAB) was performed at room temperature for 1 h.

**Behavior analysis**

Behavior analyses were performed using age-matched male mice that were 3-6 months old. Mice were kept in group housing and moved to the experimental room 1 h before starting the experiment.

**Open field testing**

Open field tests were performed in a square box measuring 28 cm wide × 28 cm deep × 22 cm tall (MED Associate, Inc., St. Albans, VT, USA). Each open field trial was performed for 1 h. Data analyses were performed using active-monitoring software (SOF-911; MED Associate, Inc.).

**Elevated plus maze testing**

The elevated plus maze test included arms measuring 40 cm long × 10 cm wide × 50 cm tall (O’hara & Co., Tokyo, Japan). Closed-arm boxes measuring 20 cm tall were wrapped with black drawing paper along the walls. Each trial was performed for 10 min, and the open arms were illuminated using 100-lux light. Elevated plus maze test trials analyzed using a video-tracking system (EthoVision XT version 10.1; Noldus, Wageningen, The Netherlands). A mouse was considered inside the open arms when the center of mouse’s body entered inside.

**Three-chamber test**

The three-chamber test was performed using a chamber box measuring 60 cm wide × 40 cm deep × 20 cm tall, which was made using an acrylic plate (BrainScience Idea Co., Osaka, Japan). Inverted transparent pencil cups with an 85-mm diameter were placed in the left and right chambers. All trials were conducted as follows: (1) the mouse was placed in the center chamber and allowed to freely move for 10 min; (2) after removing the trial mouse, a wild-type C57BL/6 male mouse that was unfamiliar with the trial mouse was placed in the pencil cup in the right chamber; (3) the trial mouse was returned to the center chamber and allowed to freely move for 10 min. The results of the three-chamber tests were analyzed using EthoVision XT (Noldus).

**Rotarod test**

The rotarod test was performed using a single-lane apparatus (model MK-6308; Muromachi, Tokyo, Japan). Before starting the rotarod trials, all mice were trained for 1 min under 4-rpm conditions. The rotarod trial was performed daily for three consecutive days using a progressive acceleration setting from 4 to 40 rpm over a 5 min period.

**Barnes maze test**

The Barnes maze was made from white polyvinyl chloride and measured 91 cm in diameter × 5 mm thick × 90 cm tall (BrainScience Idea Co., Osaka, Japan). The Barnes maze had twenty 65-diameter holes located 30 mm from the edge. The luminous intensity of the Barnes maze was 200 lux. The escape box, which was 50 mm deep and had a volume of 465 mm³, was colored black. The Barnes maze was cleaned with 0.012% sodium hypochlorite before every trial. Before starting the daily trials, all mice were individually transferred to new cages. In the room that contained the Barnes maze test, the room supplies (including the shelves) were covered with white cloth, except for the maze table, and four large objects were displayed on the cloth as spatial cues. On day 1 of habituation, the mouse was placed directly in the escape box and set under the hole of the Barnes maze at random positions for 3 min, and this was repeated three times in order to allow the mouse to habituate to the target box. Next, the mouse was placed at the center of the Barnes maze and covered with a 2-litter beaker. After 30 s, the mouse was moved to the target hole region by dragging the beaker to cover the entire target hole and allowing 3 min for the mouse to enter the escape box. If the mouse did not autonomously enter the escape box, we made the mouse enter the escape box using a 300-mL beaker and left for 3 min. During the training and probe trials, the mouse was placed in an open dark-colored polyvinyl chloride cylinder measuring 10 cm in diameter × 9 cm tall. After 30 s, the trials were started by pulling up the cylinder, and then the experimenter left the room. All trials were performed using six mice per series, and trials were conducted twice per day and separated by about 90 min. Each trial lasted 5 min. When the mouse could not
enter the escape box, we introduced the mouse to the escape box using a beaker and left the room for 1 min. To enhance autonomous movement among the de novo Zeb2 Δex7/+ mice that exhibited extremely prolonged immobility, we left these mice on the board for an additional 5 min if they had not entered the escape box during the first 5 min. In the probe trial, the position of the target hole was changed to the opposite location. The trials were recorded using a commercial web camera placed on the ceiling. All Barnes maze trails were analyzed using EthoVision XT (Noldus). Movement was determined when the mice moved 2 cm/second. The entry zone was defined as the area within 4 cm of the edge of the target hole.

Shuttle avoidance test
The shuttle avoidance test was performed using a box apparatus measuring 42 cm wide × 16 cm deep × 21 cm tall that contained two compartments (model SG-65102; MED Associate, Inc., St. Albans, VT, USA). Before starting the session, the mouse was allowed to freely move within the compartment for 5 min. The shuttle avoidance sessions (50 trials total) delivered an unconditional stimulus (US), which consisted of an electric shock at the termination of the conditioned stimulus (CS) (i.e. tone and light for 5 s), into the compartment where the mouse was staying. Shuttle avoidance sessions were performed over 3 consecutive days. Data analysis was performed using active-monitoring software (SOF-735; MED Associate, Inc., St. Albans, VT, USA).

Supplementary Material
Supplementary Material is available at HMG online.

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