Backcrossing to an appropriate genetic background improves the birth rate of carbohydrate sulfotransferase 14 gene-deleted mice

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Abstract: Ehlers–Danlos syndromes (EDSs) are heterogeneous group of heritable connective tissue disorders characterized by joint and skin hyperextensibility as well as fragility of various organs. Recently, we described a new type of EDS, musculocontractual EDS (mcEDS-CHST14), caused by pathogenic variants of the carbohydrate sulfotransferase 14 (CHST14) gene mutation. B6;129S5-Chst14tm1Lex/Mmucd (B6;129-Chst14 KO) mice are expected to be an animal model of mcEDS-CHST14. However, >90% of B6;129-Chst14 KO homozygous (B6;129-Chst14−/−) mice show perinatal lethality. Therefore, improvement of the birth rate of Chst14−/− mice is needed to clarify the pathophysiology of mcEDS-CHST14 using this animal model. Some B6;129-Chst14−/− embryos had survived at embryonic day 18.5 in utero, suggesting that problems with delivery and/or childcare may cause perinatal lethality. However, in vitro fertilization and egg transfer did not improve the birth rate of the mice. A recent report showed that backcrossing to C57BL/6 strain induces perinatal death of all Chst14−/− mice, suggesting that genetic background influences the birthrate of these mice. In the present study, we performed backcrossing of B6;129-Chst14 KO mice to a BALB/c strain, an inbred strain that shows lower risks of litter loss than C57BL/6 strain. Upon backcrossing 1 to 12 times, the birth rate of Chst14−/− mice was improved with a birth rate of 6.12–18.64%. These results suggest that the genetic background influences the birth rate of Chst14−/− mice. BALB/c congenic Chst14−/− (BALB-Chst14−/−) mice may facilitate investigation of mcEDS-CHST14. Furthermore, backcrossing to an appropriate strain may contribute to optimizing animal experiments.

Key words: backcrossing, birth rate, carbohydrate sulfotransferase 14 (CHST14), Ehlers-Danlos syndrome, genetic background
**Introduction**

Ehlers–Danlos syndromes (EDSs) are a heterogeneous group of heritable connective tissue disorders characterized by joint and skin hyperextensibility as well as fragility of various organs [3–5, 9, 10, 15, 19]. Recently, a new type of EDS was found, which is caused by carbohydrate sulfotransferase 14 (CHST14) gene mutations and named musculocontractural EDS (mcEDS-CHST14) [7, 11]. The pathophysiological mechanisms and therapies of mcEDS-CHST14 are not yet established. Therefore, development of disease model animals is indispensable.

The B6;129-Chst14 KO mouse generated by Bian et al. [2] is expected to be a model of the pathophysiology of mcEDS-CHST14. However, perinatal lethality of Chst14−/− mice has made investigations difficult. A previous study showed that the genotypes of Chst14−/− embryos follow an approximate Mendelian distribution (25%) until embryonic day (E) 16.5 [1]. On E18.5 just before delivery, some embryos survive (14.8%) in utero [18]. However, only a limited number of adult B6;129-Chst14−/− mice were produced (1.3%) [18]. These reports suggest that problems with delivery and/or childcare may affect the reproductive efficiency.

To improve the birth rate, in vitro fertilization (IVF)-embryo transfer (ET) is widely used for mouse strains with a poor birth rate [6]. The birth rate is different among some inbred mouse strains [12]. Akyuz et al. reported that backcrossing B6;129-Chst14 KO mice several times to the C57BL/6 strain results in perinatal death of all Chst14−/− mice [1]. The BALB/c strain, a common inbred mouse strain that is widely used for animal experiments, shows lower risks of litter loss than the C57BL/6 strain [17]. Therefore, we considered that the birth rate of Chst14−/− mice might be influenced by the genetic background.

Inbred strain mice have almost the same genetic background. Generally, more than 12 backcrosses are necessary to generate a congenic strain. It is known that the repeat number of microsatellites is different among mouse strains, which is used to identify the genetic background. A database of mouse microsatellites has been provided by the National Institute of Genetics (Shizuoka, Japan) [14].

In the present study, to improve the birth rate of Chst14−/− mice, we performed IVF-ET and backcrossing to the BALB/c strain and checked the genetic background of the mice by microsatellite markers.

**Materials and Methods**

**Animals**

Chst14 KO mice were obtained from the Mutant Mouse Regional Resource Center (https://www.mmrrc.org) and inbred for more than 12 generations [18]. Mice were housed under SPF conditions with a constant temperature (23 ± 2°C), relative humidity (45–70%), and 12-h light/dark cycle. Animals had free access to tap water and standard mouse chow (Oriental Yeast Co., Ltd., Tokyo, Japan).

Based on the national regulations and guidelines, all experimental procedures were reviewed by the Committee for Animal Experiments of Shinshu University and finally approved by the President of Shinshu University (Approval numbers 280116, 280039, and 280038). All experimental procedures were carried out in accordance with the Regulations for Animal Experimentation of Shinshu University.

**IVF-ET**

Female mice with heterozygous Chst14 gene deletion (Chst14+/−) were superovulated by intraperitoneal injections of 7.5 IU pregnant mare serum gonadotropin (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) followed by 7.5 IU human chorionic gonadotropin (hCG; Nippon Zenyaku Kogyo Co., Ltd.) after 48 h. At 13–15 h after injection of hCG, female Chst14+/− mice were sacrificed humanely. Then, cumulus oocyte complexes were collected and placed in human tubal fluid (HTF) medium (Ark Resource, Co., Ltd., Kumamoto, Japan) under mineral oil (Sigma-Aldrich Japan, Tokyo, Japan). Sperm was collected from the cauda epididymis of male Chst14+/− mice and incubated for 1 h in HTF medium. The cumulus oocyte complexes and sperm were cocultured at 37°C with 5% CO2 for 5–6 h. Fertilized ova were placed in modified human tubal fluid (mHTF) medium (Kyudo Co., Ltd., Saga, Japan) and checked for cleavage the next morning. Embryos (2-cell) were then transferred to a fresh drop of mHTF medium and transplanted into pseudopregnant ICR mice. To generate pseudopregnant recipients, unstimulated ICR female mice (Japan SLC, Shizuoka, Japan) were mated with vasectomized ICR male mice (Japan SLC) overnight. The female mice were then checked for vaginal plugs. Days when plugs were found were designated as E0.5 of pseudopregnancy. At E0.5 of pseudopregnancy, the embryos (2-cell) were transplanted into fallopian tubes (10 embryos/oviduct). The genotypes of fetus (E18.5) and offspring were checked by polymerase chain reaction (PCR). A schema of the method for IVF-ET is shown in Supplementary Fig. 1A.
Backcrossing

B6;129-Chst14 KO mice were backcrossed to the BALB/cAJcl (BALB/cA) strain (CLEA Japan, Inc. Shizuoka, Japan) 12 times. First, female Chst14+/− and male BALB/cA mice were mated. The progeny (F1) were genotyped to select Chst14+/− mice, and then Chst14+/− F1 mice were used for backcrossing again with BALB/cA mice (Supplementary Fig. 1B). The F1 female and male Chst14+/− mice were mated to examine the birth rate of Chst14−/− mice (Supplementary Fig. 1C). This procedure was repeated 12 times to generate the BALB/c congenic Chst14 KO (BALB.Chst14 KO) strain. To replace the sex (Y) chromosome and mitochondrial DNA, males and females were inverted and mated at the seventh generation. (Generally, Y chromosome and mitochondrial DNA are a paternal and maternal inheritance, respectively. The Y chromosome was replaced by mating with inbred male (BALB/cA) and female respectively. The Y chromosome was replaced by mating with inbred male (BALB/cA) and female Chst14+/− mice. The mitochondrial DNA was replaced by mating with inbred female and male Chst14+/− mice.) Schemas of the method for backcrossing and birth rate analyses are shown in Supplementary Fig. 1B and C.

Genotyping PCR

The method for PCR was reported in our previous study [18]. Pups were weaned and underwent ear punching (individual identification) at 3–4 weeks of age. The genotype of fetuses was analyzed on perinatal period (E18.5) with tail samples. Genomic DNA was extracted from the tail and ear pieces of the mice using MightyPrep reagent for DNA (Takara Bio Inc., Shiga, Japan). Primer sequences for wildtype genotyping of exon 1 in the Chst14 gene were 5′-GGACCCACCGCAGTGACTT-3′ and 5′-ACAGGCACTCCAATGCTCATCT-3′. Primer sequences for knockout genotyping were 5′-TGCTCTCTACTCAAGCGTATT-3′ and 5′-GTTCTCCTGAGCAGCGTATT-3′. PCR was carried out using TaKaRa Taq™ HS Perfect Mix (TaKaRa Bio Inc.). The PCR conditions were 94°C for 1 min and then 35 cycles of 94°C for 5 s and 65°C for 15 s. The PCR products were analyzed by 1% agarose gel electrophoresis.

Confirmation of the genetic background

Microsatellite markers were selected from the Mouse Microsatellite Database of Japan [14] and used to analyze the genetic background of B6;129-Chst14 KO (N0) mice and to confirm replacement of the genetic background from B6;129 to the BALB/c strain. The sizes of the PCR products of the markers were set to be different by >10 bp between the strains (Supplementary Tables 1 and 2). On the same chromosome, the intergenic distance of the microsatellite markers was set to be >10 centimorgans. When it was difficult to set different PCR-product size of each of the three strains, the markers were set between two strains on the intergenic distance (D4Mit254, D8Mit121, and D16Mit182 were used for the BALB/c vs. C57BL/6 comparison; D4Mit225, D8Mit52, and D16Mit33 were used for the BALB/c vs. 129 comparison). The primer sequences are listed in Supplementary Tables 1 and 2. PCR of the microsatellite markers was carried out using the GoTaq® PCR Core System I (Promega K.K., Tokyo, Japan). The PCR conditions were carried out according to the protocol of the Mouse Microsatellite Database of Japan [14]. PCR products were visualized by electrophoresis with 2% agarose gels.

Statistical analysis

The rate of Chst14−/− was analyzed by the Chi-squared test using SPSS (IBM Corp., Armonk, NY, USA). Differences with a probability value (P) of 0.05 or less were considered statistically significant.

Results

Male and female B6;129-Chst14+/− mice (N0) were mated, and then the genotypes of the pups were checked. The overall rates of wildtype (WT), B6;129-Chst14+/−, and B6;129-Chst14−/− pups were 27.71%, 70.35%, and 1.94%, respectively. Then, B6;129-Chst14 KO mice were backcrossed to the BALB/c strain. The birth rates of Chst14−/− on N1–4, 9, and 12 were significantly improved compared with N0 (13.51%, 6.12%, 7.32%, 18.64%, 6.52%, and 8.05%, respectively; Table 1).

IVF-ET was considered as a method to improve the birth rate of B6;129-Chst14−/− mice. The 2-cell embryos of B6;129-Chst14 KO mice were transplanted into female ICR mice, and then the genotypes of the pups were checked. The overall rates of WT, B6;129-Chst14+/−, and B6;129-Chst14−/− pups were 37.50%, 62.50%, and 0.00%, respectively (Table 2). Therefore, IVF-ET did not improve the birth rate of B6;129-Chst14−/− mice.

Furthermore, the number of Chst14−/− fetuses at the perinatal period (E18.5; the day just before delivery) was analyzed. The percentage of B6;129-Chst14−/− and BALB.Chst14−/− fetuses with natural mating was 14.77% and 19.05%, respectively. The percentage of B6;129-Chst14−/− and BALB.Chst14−/− fetuses with IVF-ET was 17.95% and 15.79%, respectively. The number of the fetuses did not show significant difference between the two strains (Table 3).

Microsatellite markers were checked to confirm the genetic background of the mice that were backcrossed
to the BALB/c strain 12 times. All 56 markers were replaced with those of the BALB/c strain (Table 4 and Supplementary Fig. 2).

## Discussion

Model animals are essential to investigate human disease mechanisms and develop therapies. mcEdS-CHST14 is a newly found syndrome that shows serious progressive symptoms in skin and bone (e.g., skin fragility, recurrent dislocations, progressive talipes or spinal deformities, and large subcutaneous hematomas) [7, 11]. Pathophysiological mechanisms and therapies are not yet established for the disease. Several reports show that Chst14 gene deletion induces changes in the spinal cord and nervous system functions [1, 8, 13] as well as an abnormality of blood capillaries in the placenta [18] of mice. However, a model animal that focuses on the skin and bone (tissues related to serious symptoms of mcEdS-CHST14) has not been reported. Although B6;129-Chst14 KO mice are expected to be a model animal of mcEdS-CHST14, adult Chst14−/− mice are rarely produced because of perinatal lethality [18]. Therefore, improvement of the birth rate is essential to establish a disease model mouse of mcEdS-CHST14.

IVF-ET is often carried out to improve the birth rate of a mouse strain that has problems with reproductive performance [5]. Generally, ICR is a strain that shows a

### Table 1. Birth rates of backcrossed mice produced by natural mating

| N   | +/+  | +/−  | −/−  | total |
|-----|------|------|------|-------|
| N0  | 143 (27.71%) | 363 (70.35%) | 10 (1.94%) | 516 (100%) |
| N1  | 18 (24.32%) | 46 (62.16%) | 10 (13.51%)** | 74 (100%) |
| N2  | 33 (33.67%) | 59 (60.20%) | 6 (6.12%)* | 98 (100%) |
| N3  | 3 (7.32%) | 35 (85.37%) | 3 (7.32%)* | 41 (100%) |
| N4  | 5 (8.47%) | 43 (72.88%) | 11 (18.64%)** | 59 (100%) |
| N5  | 10 (20.83%) | 35 (72.92%) | 3 (6.25%) | 48 (100%) |
| N6  | 11 (22.92%) | 34 (70.83%) | 3 (6.25%) | 48 (100%) |
| N7  | 19 (35.85%) | 31 (58.49%) | 3 (5.66%) | 53 (100%) |
| N8  | 22 (37.29%) | 34 (57.63%) | 3 (5.08%) | 59 (100%) |
| N9  | 9 (19.57%) | 34 (73.91%) | 3 (6.52%)* | 46 (100%) |
| N10 | 12 (32.43%) | 23 (62.16%) | 2 (5.41%) | 37 (100%) |
| N11 | 20 (58.82%) | 12 (35.29%) | 2 (5.88%) | 34 (100%) |
| N12 | 106 (30.46%) | 214 (61.49%) | 28 (8.05%)** | 348 (100%) |

Absolute numbers and percentages of wildtype (+/+), heterozygous KO (+/−), and homozygous KO (−/−) offspring among backcrossed heterozygous breeding pairs (mated between the same generations) analyzed at postnatal ages (3–4 weeks). “N” indicates the number of backcrossings.*P<0.05, **P<0.01, compared with N0; Chi-squared test.

### Table 2. Birth rates of B6;129-Chst14 KO mice produced by natural mating and IVF-ET

| N   | +/+  | +/−  | −/−  | total |
|-----|------|------|------|-------|
| N0  | 143 (27.71%) | 363 (70.35%) | 10 (1.94%) | 516 (100%) |
| N12 | 30 (37.50%) | 50 (62.50%) | 0 (0.00%) | 80 (100%) |

Absolute numbers and percentages of wildtype (+/+), heterozygous KO (+/−), and homozygous KO (−/−) mice among offspring produced by natural mating and IVF-ET analyzed at postnatal ages (3–4 weeks). Significant difference was not detected between natural mating and IVF-ET by Chi-squared test.

### Table 3. Genotype of fetuses

| N   | +/+  | +/−  | −/−  | total |
|-----|------|------|------|-------|
| A) Natural mating N0 | 40 (26.85%) | 87 (58.39%) | 22 (14.77%) | 149 (100%) |
| N12 | 12 (28.57%) | 22 (52.38%) | 8 (19.05%) | 42 (100%) |
| B) IVF-ET N0 | 13 (33.33%) | 19 (48.71%) | 7 (17.95%) | 39 (100%) |
| N12 | 9 (23.68%) | 23 (60.53%) | 6 (15.79%) | 38 (100%) |

Absolute numbers and percentages of wildtype (+/+), heterozygous KO (+/−), and homozygous KO (−/−) fetuses among non-backcrossed (N0) and backcrossed (N12) heterozygous pairs analyzed at perinatal period (E18.5). The fetuses produced by natural mating (A) and IVF-ET (B). “N” indicates the number of backcrossings. Significant difference was not detected between N0 and N12 by Chi-squared test.
good nurturing ability and is used as the recipient of ET. We found that most B6;129-Chst14−/− mice did not survive at delivery, although some embryos had survived on E18.5 (the day just before delivery) [1, 18]. Therefore, IVF-ET was performed for the strain. However, the birth rate of B6;129-Chst14−/− mice was not improved (Table 2). These results suggest that a critical cause for the poor birth rate was not only the mother mouse (e.g., problems with delivery and/or their nurturing ability), but also other factors.

A recent report showed that backcrossing of B6;129-Chst14 KO mice to the C57BL/6 strain results in perinatal death of all Chst14−/− mice [1]. This observation suggests that the genetic background affects the birth rate of Chst14−/− mice. In B6;129-Chst14 KO (N0) mice, about 80% of microsatellite markers showed the same PCR-product size as the C57BL/6 strain (Supplementary Fig. 3). In the present study, we performed backcrossing to the BALB/c strain, an inbred strain that shows lower risks of litter loss than the C57BL/6 strain [17]. The backcrossing was performed 1 to 12 times, which improved the birth rate of Chst14−/− mice (Table 1).

There are few reports about the reason why the reproductive performance is different among mouse strains [17]. The number of Chst14−/− fetuses at perinatal period (E18.5; the day just before delivery) did not show a significant difference between B6;129-Chst14 KO and BALB.Chst14 KO, suggesting that the influence of genetic background on the intrauterine environment and viability of the fetuses was restrictive before delivery (Table 3). These results suggest that tolerance for stress and viability of the pups on delivery were different in the genetic background.

B6;129-Chst14−/− and BALB.Chst14−/− mice both showed lower body weight than WT (data not shown), suggesting Chst14 affected growth of the mice regardless of the genetic background. Performing a detailed analysis of the phenotype of the mice is the agenda from now on.

In the present study, the birth rate and number of backcrossings were not correlated (Table 1). Especially, one to four backcrossings significantly improved the birth rate. Heterosis of hybrid mice may be the cause of this birth rate improvement. However, it is known that inbred strain show better reproducibility than hybrid strains [16]. Thus, congenic mice may be valuable for experiments. Because the genetic background can influence the results of animal experiments, confirmation of the genetic background is important to improve reproducibility. Generally, 12 backcrossings are needed to generate a congenic strain. In the present study, the birth rate

### Table 4. Confirmation of the genetic background by microsatellite markers

| Locus Name | N12 | B6J | 129 | BALB |
|------------|-----|-----|-----|------|
| D1Mit294   | 217 | 194 | 209 | 217  |
| D1Mit18    | 172 | 158 | 158 | 172  |
| D1Mit33    | 120 | 97  | 97  | 120  |
| D2Mit42    | 150 | 134 | 126 | 150  |
| D2Mit51    | 143 | 130 | 130 | 143  |
| D3Mit17    | 156 | 176 | 176 | 156  |
| D3Mit64    | 143 | 131 | 131 | 143  |
| D3Mit29    | 201 | 147 | 147 | 201  |
| D4Mit214   | 148 | 124 | 124 | 148  |
| D4Mit15    | 349 | 290 | 331 | 349  |
| D4Mit254   | 111 | 134 | 111 | 111  |
| D4Mit225   | 155 | 155 | 139 | 155  |
| D5Mit257   | 97  | 144 | 144 | 97   |
| D5Mit20    | 127 | 154 | 144 | 127  |
| D5Mit101   | 116 | 129 | 127 | 116  |
| D6Mit83    | 129 | 149 | 149 | 129  |
| D6Mit223   | 102 | 124 | 124 | 102  |
| D6Mit326   | 114 | 92  | 92  | 114  |
| D7Mit246   | 171 | 135 | 152 | 171  |
| D7Mit330   | 138 | 124 | 124 | 138  |
| D7Mit362   | 102 | 83  | 83  | 102  |
| D8Mit155   | 137 | 148 | 95  | 137  |
| D8Mit4     | 200 | 161 | 161 | 200  |
| D8Mit46    | 201 | 229 | 229 | 201  |
| D8Mit121   | 224 | 252 | 224 | 224  |
| D8Mit52    | 383 | 383 | 400 | 383  |
| D9Mit162   | 122 | 138 | 138 | 122  |
| D9Mit273   | 233 | 122 | 122 | 145  |
| D9Mit16    | 241 | 254 | 224 | 224  |
| D10Mit189  | 128 | 107 | 105 | 128  |
| D10Mit170  | 147 | 134 | 134 | 147  |
| D10Mit135  | 174 | 150 | 150 | 174  |
| D11Mit259  | 356 | 113 | 113 | 356  |
| D11Mit338  | 165 | 153 | 153 | 165  |
| D12Mit12   | 170 | 144 | 154 | 170  |
| D12Mit201  | 200 | 214 | 224 | 200  |
| D12Mit7    | 303 | 283 | 283 | 303  |
| D13Mit17   | 155 | 172 | 167 | 155  |
| D13Mit88   | 180 | 168 | 166 | 180  |
| D13Mit159  | 159 | 137 | 137 | 159  |
| D14Mit37   | 91  | 136 | 134 | 91   |
| D14Mit69   | 129 | 147 | 147 | 129  |
| D14Mit266  | 172 | 149 | 149 | 172  |
| D15Mit270  | 180 | 201 | 201 | 180  |
| D15Mit261  | 139 | 123 | 144 | 139  |
| D16Mit182  | 183 | 218 | 183 | 183  |
| D16Mit33   | 124 | 124 | 148 | 124  |
| D16Mit12   | 180 | 192 | 157 | 180  |
| D17Mit49   | 221 | 249 | 229 | 221  |
| D17Mit217  | 72  | 119 | 115 | 72   |
| D18Mit64   | 175 | 155 | 155 | 172  |
| D18Mit209  | 82  | 122 | 119 | 82   |
| D19Mit68   | 118 | 133 | 133 | 118  |
| D19Mit46   | 130 | 116 | 116 | 130  |
| D19Mit10   | 187 | 151 | 151 | 187  |
| DXM1t1     | 81  | 97  | 97  | 81   |

Results of electrophoresis of PCR for microsatellite markers. N12, B6J, 129, and BALB indicate a backcrossed mouse (BALB.Chst14 KO), C57BL/6J strain, 129X1/SvJ strain, and BALB/c strain, respectively. The electrophoresis patterns of PCR products are presented in Supplementary Fig. 2.
was also improved by 12 backcrossings to the BALB/c strain (Table 1). The significant difference found on N9 may be an influence in the middle of the replacement of the genetic background (Table 1). Finally, 56 microsatellite markers were checked and confirmed to be replaced by those of the BALB/c strain in mice backcrossed 12 times (Table 4). Therefore, BALB.Chst14 KO mice were established.

In the present study, we found that backcrossing to the BALB/c strain improved the birth rate of $\text{Chst14}^{-/-}$ mice. BALB.Chst14 $^{-/-}$ mice are expected to greatly facilitate investigation of disease mechanisms and development of therapies for mcEDS-CHST14. Establishment of a model animal for mcEDS-CHST14 using BALB. Chst14 $^{-/-}$ is the next important research task. In addition, this study suggests that backcrossing to an appropriate genetic background facilitates efficient animal experimentation and breeding.

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