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Cleavage Speed and Blastomere Number in DBA/2J Compared with C57BL/6J Mouse Embryos

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DBA/2J mice are among the oldest and most important inbred strains still used in many research fields. However, this strain has reproductive problems, which may consume considerable time and effort during experiments requiring a large population. Because the quality of DBA/2J embryos has not yet been described in detail, we compared DBA/2J mice with the reproductively efficient C57BL/6J strain. Compared with C57BL/6J embryos, DBA/2J embryos had a slower cleavage speed (mean ± 1 SD; first cleavage: C57BL/6J, 16.87 ± 1.32 ; DBA/2J, 19.64 ± 0.96 h; $P < 0.01$; second cleavage: C57BL/6J, 41.12 ± 2.02 h; DBA/2J, 46.20 ± 2.68 h, $P < 0.01$) and lower cell counts at the morula and blastocyst stages (morula stage: C57BL/6J, 15 ± 3 cells per embryo; DBA/2J, 9 ± 5 cells per embryo; $P < 0.05$; blastocyst stage: C57BL/6J, 52 ± 6 cells per embryo; DBA/2J, 35 ± 14 cells per embryo; $P < 0.05$). In addition, the results of reciprocal in vitro fertilization and male–female reciprocal crosses revealed that these phenotypes were not affected by the sperm genome and were recessively inherited. These findings likely will facilitate the production of DBA/2J mice and genetically modified mice with their background. Our results also suggest that, due to their slow cleavage speed, DBA/2J mice can serve as a new model for human infertility.

Abbreviations: IVF, in vitro fertilization

The inbred DBA/2J mouse strain is widely used in various research fields, including mutagenesis,[9,14] autoimmune epilepsy and abnormal behavior,[13] glaucoma,[23] and sensorimotor gating.[12] Furthermore, studies comparing DBA/2 strain with other inbred strains have been performed to understand the quantitative traits related to human genetic predisposition, such as alcoholism,[25] learning and memory,[2] and susceptibility to diabetes.[1] However, DBA/2J mice are characterized by an extremely poor conception rate, a common trait in inbred strains.[8,16] DBA/2J mice exhibit reduced productivity and often experience nonproductive mating, making it difficult to use DBA/2J mice for systematic reproductive studies. Strain-specific embryonic genome modifications exist between C57BL/6J and DBA/2J mice, and specific loci (*Egm1* and *Egm2*) have been identified as a cause of the poor reproduction phenotype.[19,20] However, many aspects of *Egm* remain unknown, including its detailed locus, and the cellular mechanisms underlying the downregulation of fertility and developmental competence in early-implantation embryos of DBA/2J mice remain poorly understood.

Assessing the quality and developmental capacity of embryos likely will be helpful in facilitating the production of DBA/2J mice by using reproductive technologies. The morphology of oocytes and embryos and the number of blastomeres are considered to be indicators of the developmental competence of an embryo.[7,11] In humans, the speed of embryonic cleavage is correlated with embryo viability.[3-5,21] Therefore, given their aforementioned phenotypes, DBA/2J mice might be useful as a disease model for human infertility due to poor-quality embryos and fertilized eggs.

In this study, we investigated embryo quality—particularly embryonic cleavage speed and cell number at the morula and blastocyst stages—and the underlying genetic basis of these parameters to determine the cause of poor fertility in DBA/2J mice compared with C57BL/6J mice. Our results likely will help to increase the production efficiency of DBA/2J mice.

Materials and Methods

Mice, husbandry, and animal welfare. C57BL/6J and DBA/2J male mice (age, 8 to 20 wk) and female mice (age, 4 to 16 wk) were purchased from CLEA Japan (Tokyo, Japan). In preliminary experiments, the developmental rate of DBA/2J embryos did not differ between donor mice that were younger than 8 wk, 8 to 12 wk, or 13 to 20 wk of age (mean ± 1 SD; younger than 8 wk, 14.62% ± 8.66%; 8 to 12 wk, 27.05% ± 20.05%; 13 to 20 wk, 30.64% ± 12.49%; $P < 0.05$) In this study, we used 2 F1 populations (B6D2F1 and D2B6F1 mice) that were derived from C57BL/6J and DBA/2J progenitor mice: B6D2F1 mice were the offspring of C57BL/6J female and DBA/2J male mice, whereas D2B6F1 mice were the progeny of DBA/2J female and C57BL/6J male mice. Animals were maintained under a 12:12-h light:dark cycle at 22 ± 2 °C and a relative humidity of 40% to 60% and had unrestricted access to standard mouse chow (CE2, CLEA Japan) and water. Animal cages were purchased from NKsystems (Osaka, Japan), and the number of mice per unit area was determined according to recommendations in the *Guide for the Care and Use of Laboratory Animals*.[15] Paper-chip cage bedding was purchased from Japan SLC (Shizuoka, Japan). All animals were cared for in accordance with the rules and regulations in the Bylaws for the Care and Use of Laboratory Animals of the University of Toyama. The research

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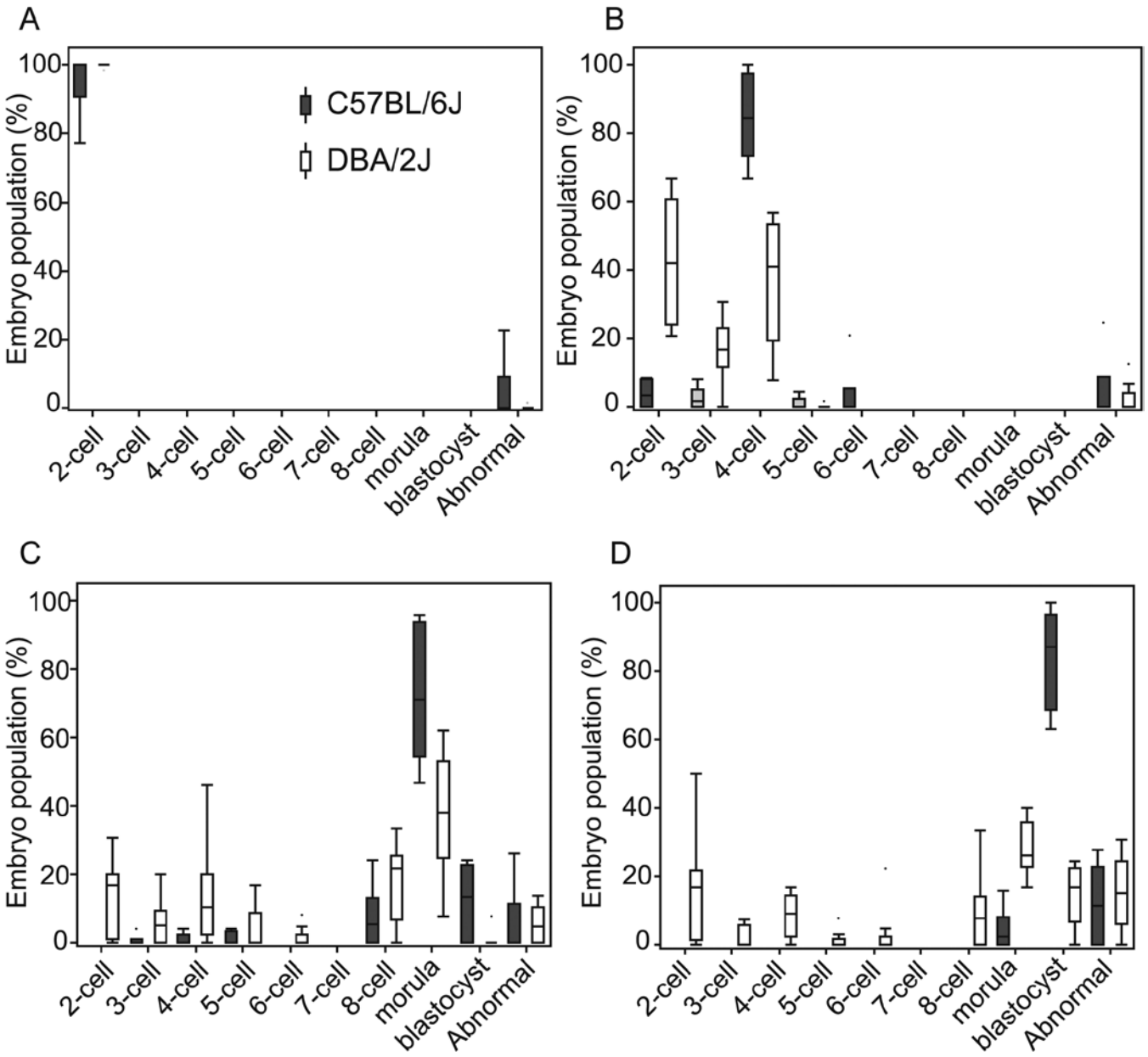

Figure 1. Distribution of developmental stages among C57BL/6J embryos (gray boxes, $n = 6$) and DBA/2J embryos (white boxes, $n = 9$) at (A) 24 h, (B) 48 h, (C) 72 h, and (D) 96 h after insemination. The 2 horizontal edges of the boxes indicate the upper and lower quartile values, and the line inside the box shows the median value. The whiskers extending from the ends of the boxes represent the smallest and largest values of each population.

protocol was approved by the Animal Care Committee of the University of Toyama.

In vitro fertilization and culture protocols. Our previously reported in vitro fertilization (IVF) and culture methods[24] were further modified in the current study. Briefly, inbred female mice were superovulated by using intraperitoneal injection of pregnant mare serum gonadotropin (7.5 IU per mouse) followed by human chorionic gonadotrophin (7.5 IU per mouse) 48 h later. At 16 h after the injection of human chorionic gonadotrophin, the mice were euthanized, and their oviducts were removed. Cumulus–oocyte complexes were placed in a 100-µL drop of HTF medium (ARK Resources, Kumamoto, Japan) covered with paraffin oil (Nacalai Tesque, Kyoto, Japan).

Spermatozoa were obtained from the C57BL/6J and DBA/2J male mice. After these mice were euthanized, the 2 caudae epididymides were removed from each mouse and were placed in a single 200-µL drop of HTF medium for capacitation (1.5 h). The average sperm count was 8000 cells/µL, and 1 µL of the sperm suspension was added to 200 µL of HTF medium HTF containing cumulus–oocyte complexes (final sperm concentration, 200 to 400 cells/µL). We previously showed that the C57BL/6 and DBA/2 strains do not differ in terms of sperm ratio for IVF and that sperm motility is not an effective indicator for IVF.[24] The IVF suspension was placed in a sealed modular incubator chamber containing 5% CO_2 in air and maintained at 37 °C for 5 to 6 h. After incubation, the embryos and oocytes were washed 3 times with mWM medium (ARK Resources) to eliminate excess sperm and cultured.[17,30,31,33] mWM medium is still used for mouse embryo culture and embryo transfer, and we obtain similar results with either KSOM (ARK Resources) or mWM (Developmental rate [%, mean ± 1 SD] in DBA/2J mice: mWM, 19.19% ± 12.12%, KSOM, 23.89% ± 16.01%, $P = 0.574$).

For each strain, groups of 2-cell-stage embryos (n = 30 to 100) were placed in 100-µL drops of mWM covered with paraffin oil and incubated at 37 °C in 5% CO_2. Embryo quality and developmental stages were monitored hourly for 24 h after insemination. Each IVF and culture was replicated at least 5 times by using sperm from different male mice in each experiment. To measure the times of the first and second cleavages, fertilized eggs of C57BL/6J and DBA/2J mice were cultured individually and observed hourly after insemination by using a microscope equipped with a hotplate and temperature sensor.

Counting blastomeres. To determine the numbers of blastomeres, the nuclei of morula- and blastocyst-stage embryos were labeled in HTF medium containing 5 µg/mL Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan) for 30 min at 37 °C with 5% CO_2. After staining, the embryos were washed 3 times in HTF medium and examined under a laser scanning confocal microscope (model TCS-SP5, Leica Microsystems, Wetzlar, Germany).

Statistical analyses. Data are reported are mean ± 1 SD. Data normality was assessed by using the χ^2 test for goodness of fit. Statistical differences between cell numbers were determined by using the Student t test. Cleavage time was compared between C57BL/6J and DBA/2J embryos by using a log-rank test. A P value less than 0.05 was used to define statistical significance, and 2-tailed tests were applied. All analyses were performed by using JMP 11 (SAS Institute, Cary, NC).

Results

Cleavage speeds of DBA/2J and C57BL/6J embryos. To ascertain the embryonic cleavage speed of C57BL/6J and DBA/2J embryos in vitro, we monitored the embryonic stage hourly for 24 h after insemination (Figure 1). Normal growth was defined as a 4-cell stage at 48 h after insemination, a morula stage at 72 h, and a blastocyst stage at 96 h.

The rates of 4-cell embryos at 48 h after insemination and morula-stage embryos at 72 h were significantly different between C57BL/6J and DBA/2J embryos. At 48 h, 84.50% ± 12.60% of C57BL/6J embryos were at the 4-cell stage, compared with 37.56% ± 17.69% of DBA/2J embryos (P = 0.0002). At 72 h, whereas 72.50% ± 19.83% of C57BL/6J embryos were at the morula stage, only 38.00% ± 17.51% of DBA/2J embryos had reached this stage (P = 0.008). For the DBA/2J strain, numerous delayed 2-cell embryos were present at the 48-h time point, with 4-cell embryos at 72 h after insemination. In addition, many DBA/2J embryos consisting of an odd number of blastomeres were observed, such as 3- and 5-cell embryos (Figure 1 B through D). In contrast, some C57BL/6J mouse embryos had achieved the 8-cell stage by 48 h and were early blastocysts by 72 h.

Furthermore, we cultured 1-cell embryos individually and observed them hourly after insemination under a microscope to measure the time required to reach the 2-cell (first cleavage time) and 4-cell (second cleavage time) stage (Figure 2). The first and second cleavage times differed significantly between C57BL/6J and DBA/2J embryos (first cleavage time: C57BL/6J, 16.87 ± 1.32 h; DBA/2J, 19.64 ± 0.96 h; P < 0.0001; second cleavage time: C57BL/6J, 41.12 ± 2.02 h; DBA/2J, 46.20 ± 2.68 h; P < 0.0001).

Numbers of blastomeres in morula- and blastocyst-stage embryos. We stained the nuclei of C57BL/6J and DBA/2J embryos and counted the cells to investigate whether the strain-associated differences in cleavage speed affected the morula- and blastocyst-stage cell number. The mean blastomere number of DBA/2J morula embryos was 9 ± 5 cells/embryo, which was significantly lower (P = 0.011) than that of C57BL/6 morula embryos (15 ± 3 cells/embryo, Figure 3 A and B). Blastocyst-stage embryos similarly showed a greater number of blastomeres in C57BL/6J embryos than in DBA/2J embryos (C57BL/6J, 52 ± 6 cells/embryo; DBA/2J, 35 ± 14 cells/embryo; P = 0.027; Figure 3 C and D).

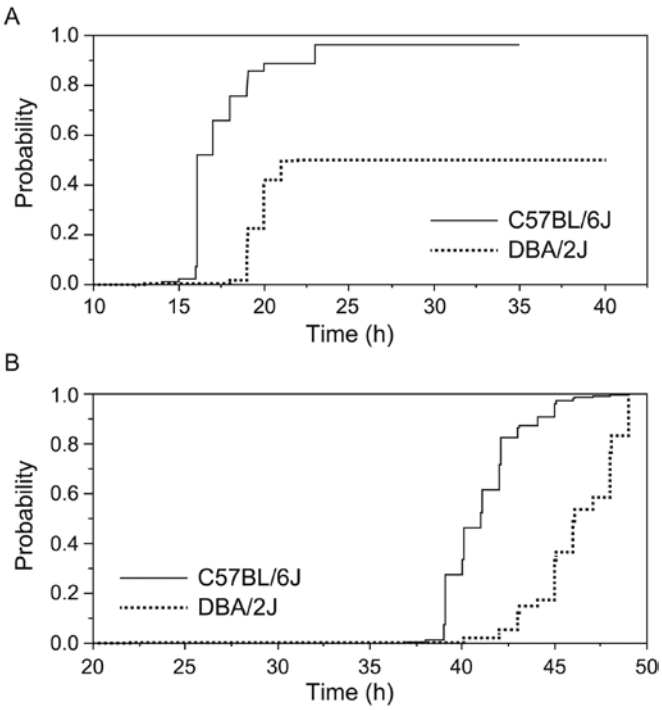

Figure 2. Kaplan–Meier plots of the times for the (A) first and (B) second cleavages of C57BL/6J embryos (solid line) and DBA/2J embryos (dotted line). Values differed significantly (P < 0.0001, log-rank test) between C57BL/6J embryos (n = 229) and DBA/2J embryos (n = 251) during both the first and second cleavages.

Effects of genetic background of sperm on blastomere numbers. Reciprocal IVF was performed by using the oocytes of DBA/2J mice and sperm of C57BL/6J mice (that is, D2 × B6 cross) as well as the oocytes of C57BL/6J mice and sperm of DBA/2J mice (that is, B6 × D2 cross) to examine whether the genetic background of the sperm was associated with the phenotypic differences in cell counts.

According to our results, the mean blastomere number of D2 × B6 morula-stage embryos was 8 ± 3 cells/embryo, which was significantly lower (P = 0.0036) than that of B6 × D2 morulas (16 ± 3 cells/embryo, Figure 4 A and B). In comparison, the number of blastomeres in B6 × D2 embryos (54 ± 11 cells/embryo) was significantly (P < 0.001) higher than that of D2 × B6 embryos (30 ± 8 cells/embryo; Figure 4 C and D). These results indicate that cell counts at the morula and blastocyst stages are unaffected by the genetic background of the sperm but are dependent on oocyte strain.

Phenotypes of B6D2F1 and D2B6F1 mice. We investigated the F1 offspring of the female–male reciprocal crosses (that is, D2B6F1 and B6D2F1 mice) to determine the genetic basis of their phenotypes. To this end, the B6D2F1 mice were crossed with DBA/2J male and C57BL/6J female mice, whereas the D2B6F1 mice were crossed with C57BL/6J male and DBA/2J female mice.

According to our results, there was no significant difference (P = 0.5342) between the mean blastomere number in embryos from B6D2F1 (14 ± 4 cells/embryo) and D2B6F1 (16 ± 6 cells/embryo) mice (Figure 5). Furthermore, morula- and blastocyst-stage cell counts did not differ between the 2 F1 populations, suggesting that the phenotype of low cell counts in the morula and blastocyst stages is recessively inherited in DBA/2J mice.

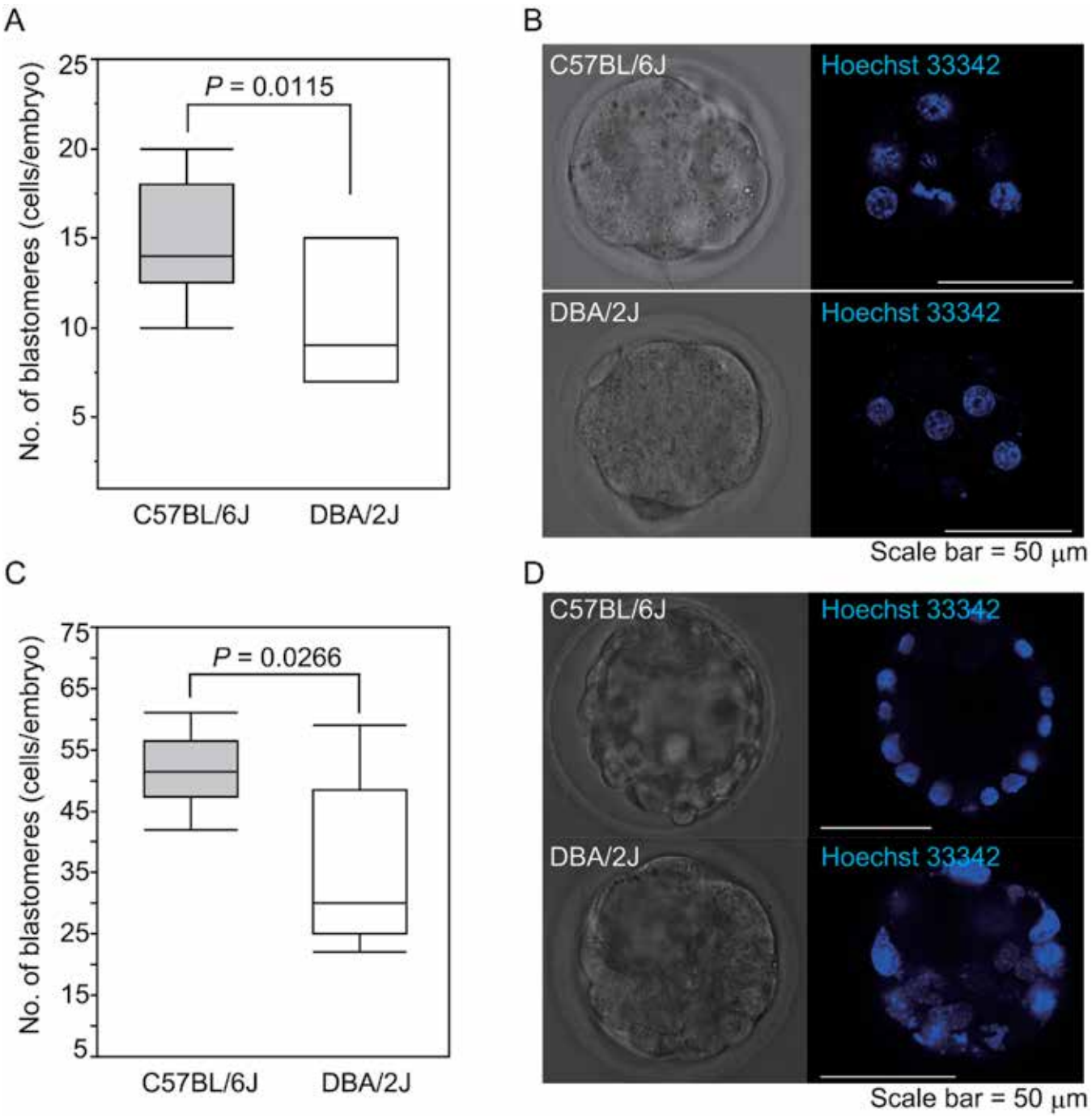

Figure 3. The numbers of blastomeres in C57BL/6J and DBA/2J embryos. (A) Morula stage. The 2 horizontal edges of the boxes indicate the upper and lower quartiles, and the line inside the box shows the median value. The whiskers extending from the end of boxes represent the smallest and largest values of the population. (B) Embryos from C57BL/6J (upper panel) and DBA/2J (lower panel) mice were stained with Hoechst 33342 dye (blue) at the morula stage. (C) Blastocyst stage. (D) Blastocysts from C57BL/6J (upper panel) and DBA/2J (lower panel) strains.

Discussion

Our comparison of embryos from DBA/2J and C57BL/6J mice showed that DBA/2J embryos developed more slowly, with delays during the first and second cleavages. In addition, cell counts at the morula and blastocyst stages were significantly lower in DBA/2J compared with C57BL/6J embryos. Although interstrain differences in the cell count at the blastocyst stage has attracted little attention to date, the differences between DBA/2J and C57BL/6J were dramatic. Moreover, we showed that these phenotypes were not affected by the sperm strain and were recessively inherited.

DBA/2J mice one of the oldest inbred strains and remain widely used in many research fields.[9,12-14,23] However, DBA/2J mice are characterized by a poor conception rate.[8,16] The use of DBA/2J mice for systematic reproductive studies, therefore, remains difficult. In our current study, DBA/2J embryos had a slower cleavage rate than did C57BL/6J embryos (Figures 1 and 2, $P < 0.0001$), and DBA/2J mouse embryos had lower cell counts at the morula and blastocyst stages (Figure 3, $P < 0.05$). In addition, the number of morulae and blastocysts is not dependent on the genetic background of the sperm (Figure 4). One previous study investigated the effect of dietary components, including

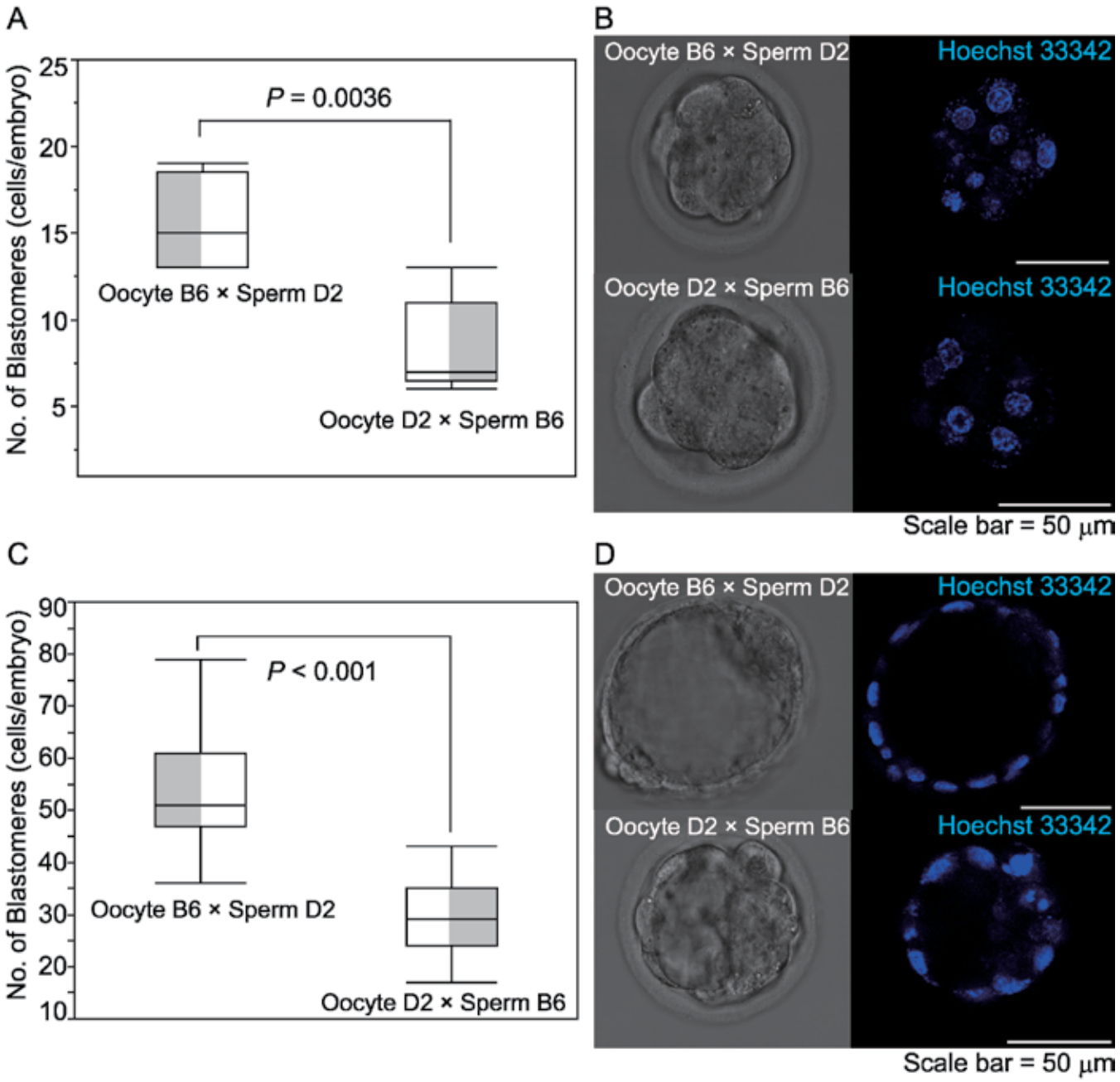

Figure 4. In vitro fertilization with reciprocal crosses between C57BL/6J (B6) and DBA/2J (D2) strains. (A) The number of blastomeres in morula-stage embryos containing the B6 maternal and D2 paternal genomes (B6 oocyte × D2 sperm) or the D2 maternal and B6 paternal genomes (D2 oocyte × B6 sperm). (B) Morula-stage embryos of B6 oocyte × D2 sperm (upper panel) and D2 oocyte × B6 sperm (lower panel) crosses were stained with Hoechst 33342 dye (blue). (C) Numbers of blastomeres at the blastocyst stage in B6 oocyte × D2 sperm embryos and D2 oocyte × B6 sperm embryos. (D) Numbers of blastocysts in B6 oocyte × D2 sperm (upper panel) embryos and D2 oocyte × B6 sperm embryos (lower panel).

phytoestrogens, on embryogenesis.[26] In the current study, we used the CE-2 diet, which contains soy, for both mouse strains. In the future, we aim to study the effects of soybean-free diets on the developmental rate of DBA/2J embryos.

The results of reciprocal IVF experiments demonstrated that embryo cell counts were not influenced by the genetic background of the sperm; however, they were dependent on oocyte strain or maternal factors inside the oocyte. Furthermore, using the 2 F1 populations in male–female reciprocal crosses revealed the B6-like phenotypes of embryos from both the B6D2F1 and D2B6F1 populations, suggesting that the DBA/2J phenotypes of low morula- and blastocyst-stage cell counts and delayed cleavage rate are recessively inherited (Figure 5).

Therefore, developmental indicators such as cleavage speed[3-6,10,22,27,28,34] and cell counts at the morula and blastocyst stages[7,11] can be used as quality biomarkers in human oocytes and embryos. In particular, one study reported a decreased rate of high-quality human embryos within the slow-cleavage group.[21] Therefore, DBA/2J mouse embryos are deemed to be poor quality embryos, with a low cleavage speed. These results show that embryo quality assessment followed by the selection of individual high-quality embryos is possible for DBA/2J mice, which might benefit from systematic reproduction by using IVF and embryo-transfer methods. In the present study, fertilized DBA/2J eggs with poor embryonic development had a low cleavage speed; this insight is consistent with the low

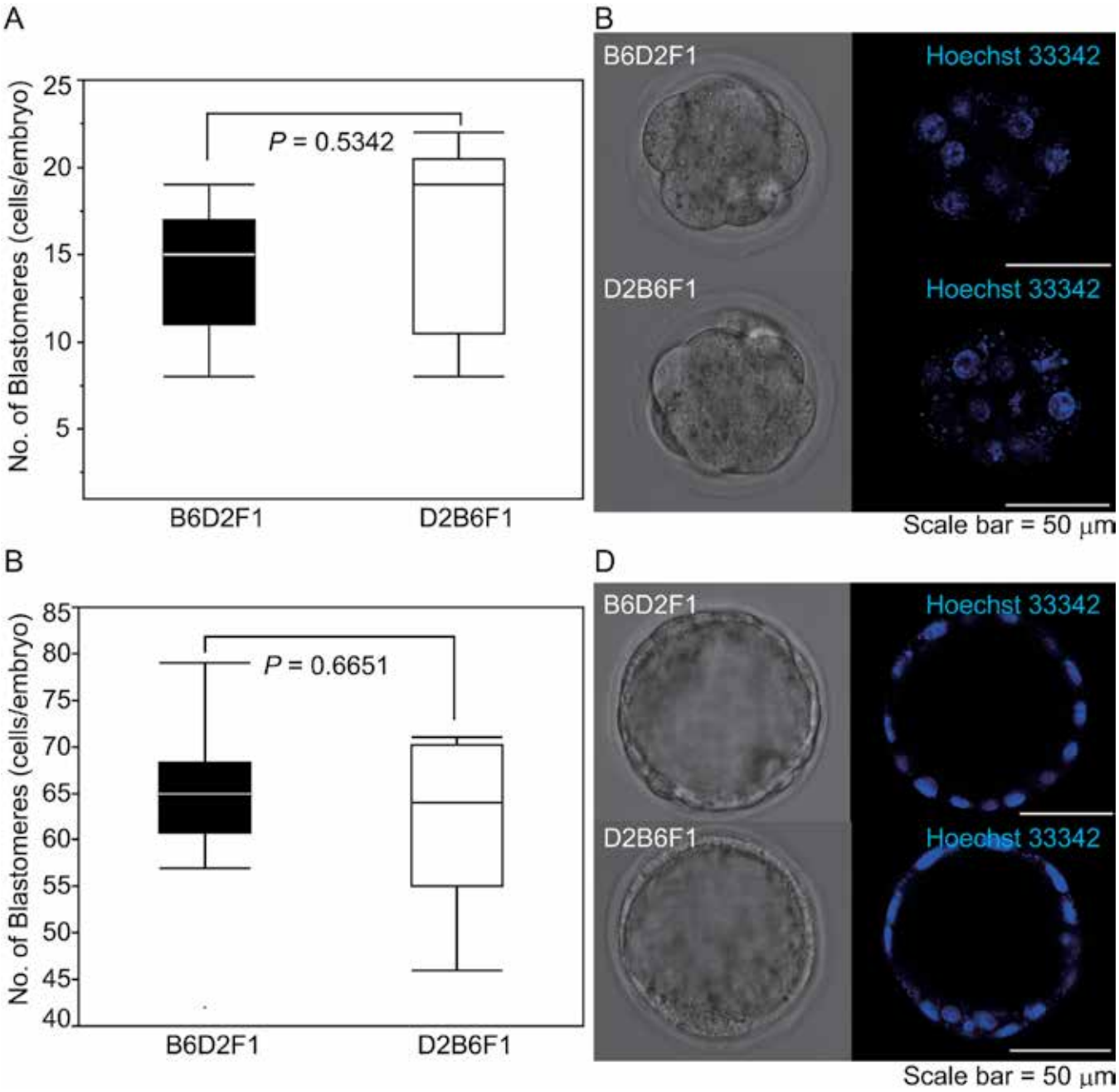

Figure 5. The numbers of blastomeres per morula and blastocyst in embryos from B6D2F1 and D2B6F1 mice. (A) Morula stage. The 2 horizontal edges of boxes indicate upper and lower quartiles of values, whereas the line inside the box shows the median value. The whiskers extending from the end of boxes represent the smallest and largest values of the population. (B) Embryos from B6D2F1 (upper panel) and D2B6F1 (lower panel) mice were stained with Hoechst 33342 dye (blue) at morula stage. (C) Blastocyst stage. (D) Blastocyst from B6D2F1 (upper panel) and D2B6F1 (lower panel) mice.

fertility of embryos with a low division rate during human IVF.[3-6,10,21,22,27,28,34] Therefore, noninvasively measuring the rate of division might provide a tool for quality assessment of human embryos; for example, a miniaturized instrument that includes a live-imaging system and a CO_2 gas-control chamber might aid this process.

Several existing mouse lines fail to produce viable embryos, namely infertile model mice with no capacity for developing embryos, such as *Esco2* knockout[32] and *Hormad1*-deficient mice.[18,29] However, no lines producing poor-quality (but not embryos that fail to develop) have been described to date. Our results are useful not only for systematic production of DBA/2J mice but also indicate that DBA/2 mouse embryos are valuable tools for studying oocyte and embryo quality.

Taking together all of the findings, our current study demonstrates the possibility of systematic production of DBA/2J mice by individually selecting good-quality embryos and the usefulness of DBA/2J mice as a model for human infertility due to poor-quality eggs.

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