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Research Article

Use of anti-inhibin monoclonal antibody for increasing the litter size of mouse strains and its application to *in vivo*-genome editing technology†

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Abstract

The litter size of mouse strains is determined by the number of oocytes naturally ovulated. Many attempts have been made to increase litter sizes by conventional superovulation regimens (e.g., using equine or human gonadotropins, eCG/hCG but had limited success because of unexpected decreases in the numbers of embryos surviving to term. Here, we examined whether rat-derived anti-inhibin monoclonal antibodies (AIMAs) could be used for this purpose. When C57BL/6 female mice were treated with an AIMA and mated, the number of healthy offspring per mouse increased by 1.4-fold (11.9 vs. 8.6 in controls). By contrast, treatment with eCG/hCG or anti-inhibin serum resulted in fewer offspring than in nontreated controls. The overall efficiency of production based on all females treated (including nonpregnant ones) was improved 2.4 times with AIMA compared with nontreated controls. The AIMA treatment was also effective in ICR mice, increasing the litter size from 15.3 to 21.2 pups. We then applied this technique to an in vivo genome-editing method (improved genome-editing via oviductal nucleic acid delivery, *i*-GONAD) to produce C57BL/6 mice deficient for tyrosinase. The mean litter size following *i*-GONAD increased from 4.8 to 7.3 after the AIMA treatment and genetic modifications were confirmed in 80/88 (91%) of the offspring. Thus, AIMA treatment is a promising method for increasing the litter size of mice and may be applied for the easy proliferation of mouse colonies as well as in vivo genetic manipulation, especially when the mouse strains are sensitive to handling.

Summary Sentence
Treatment of female mice with an anti-inhibin monoclonal antibody increased the litter size after natural mating and could be applied to an in vivo genome-editing method (improved genome-editing via oviductal nucleic acid delivery) for the efficient generation of knockout mice.

Keywords: anti-inhibin monoclonal antibody, genome-editing, *i*-GONAD, inhibin, litter size, mouse, superovulation

Introduction

One of the strategies for improving the reproductive performance of female mammals is by increasing the litter size in multiparous species. The most typical case is the pig, which has long been the subject of selective breeding based on the number of piglets per litter. It is now very common that a single mother gives birth to >10 piglets. In the case of laboratory mice, the Swiss Webster outbred strain was bred for high fecundity and its family strains were dispersed worldwide for a variety of research uses. One of the most common Swiss Webster strains—ICR—has been used broadly in biomedical research because of its large litter size (10–15 pups per mouse) and high reproductive performance [1, 2]. Female ICR mice are also known to be very efficient recipients in embryo transfer experiments because they can accept many embryos (nearly 20 per female) from different strains. By contrast, the litter size of other commonly used inbred mouse strains, such as C57BL/6, is relatively small, at four to nine pups per litter [2–4]. Therefore, it is desirable that in these strains, larger numbers of offspring should be born by natural mating, leading to enhanced conventional breeding as well as efficient establishment of new gene-modified strains.

The number of follicles that develop during the estrus cycle in eutherian mammals is largely determined by the balance between the levels of follicle-stimulating hormone (FSH) secreted from the pituitary gland and inhibin secreted from granulosa cells to inhibit FSH release [5]. To increase the number of oocytes ovulated, sequential treatments with

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equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) have been used routinely in mice and rats [6, 7]. Indeed, eCG/hCG treatment induces ovulation of a large number of mature oocytes, which can be used readily for experiments, such as in vitro fertilization (IVF), intracytoplasmic sperm injection, and nuclear transfer. However, such exogenous administrations of eCG/hCG or even FSH combined with luteinizing hormone often adversely affect fertilization in vivo and subsequent embryo development, leading to poor embryo quality [8, 9] and low rates of implantation and pregnancy [10–13]. Although the exact causes of these superovulation-induced negative effects in vivo are unclear, it is probable that the systemic or local endocrinological milieu is changed [14–18]. Therefore, at present, there is no reliable protocol to increase the litter size of pups following natural mating and pregnancy in mice.

The genome-editing via oviductal nucleic acid delivery method, an in vivo genome technology, has been developed for mice [19]. In this method, the oviducts of females following mating with males are exposed surgically and injected with clustered regularly interspaced short palindromic repeats (CRISPR) guide RNA (gRNA) and mRNA for CRISPR associated protein 9 (Cas9), which is followed by electric pulse application to the zygote-containing part of the oviduct (the ampulla) using forceps-like electrodes. This method is superior to the other conventional genome-editing techniques in that it does not require any handling of embryos in vitro. The method has now been upgraded to the improved genome-editing via oviductal nucleic acid delivery (*i*-GONAD) method, which utilizes Cas9 protein instead of Cas9 mRNA, to increase the efficiency of genome-editing in mice [20–22] and rats [23, 24]. This method is most effective when applied to animals in which the handling of embryos in vitro is extremely difficult such as golden hamsters. With the *i*-GONAD method, we have successfully generated genome-edited hamsters deficient for acrosin [25] and Mov10l1 [26], reporting new functions that have not been identified in mice. However, the *i*-GONAD method still has an inherent drawback—especially in mice—in that the production of a large batch of offspring following *i*-GONAD treatment is often difficult [27] because of an inability to increase the number of surviving fetuses by conventional superovulation treatments, as described above.

We have recently developed a superovulation method that increases the endogenous FSH level by administration of anti-inhibin serum (AIS). This strategy was found to be effective for a number of laboratory mouse strains as well as wild-derived strains [28–31]. The AIS was originally developed for inducing multiple ovulations in several species such as hamsters [32], cows [33, 34], mares [35], and goats [36]. Furthermore, several studies have shown that oocytes superovulated with immunization against inhibin have the ability to develop normally [34, 37, 38], suggesting that inhibin immunization could be applicable to a wide range of animal species [39]. Here, we examined whether the number of in vivo-derived offspring per litter could be increased by using different concentrations of AIS. We also examined the feasibility of anti-inhibin monoclonal antibodies (AIMAs) for the same purpose. We have developed AIMAs by screening a number of clones based on their effectiveness in superovulation of mice. Finally, we examined whether the efficiency of the *i*-GONAD method could be improved by the new superovulation protocol with an AIMA in terms of genome-edited pups born from one female by *i*-GONAD.

Materials and methods

Animals

For superovulation and natural mating experiments, C57BL/6JJcl (B6) and ICR (CLEA Japan Inc., Tokyo, Japan) mice were used as representative inbred and outbred strains, respectively. At the time of these experiments, the female mice were 8–24 weeks old and the male mice were 2.5–17 months old. For embryo transfer experiments, ICR female mice at 10–20 weeks of age and vasectomized ICR male mice at 3–12 months of age were used. At the time of collection of oocytes or spermatozoa or at cesarian section, animals were euthanized by cervical dislocation. All mice were maintained under specific-pathogen-free conditions, provided with water and commercial laboratory mouse chow ad libitum, and housed under controlled lighting conditions (daily light period, 07:00–21:00). All animal experiments except for the preparation of AIMAs described below were approved by the Animal Experimentation Committee at the RIKEN Tsukuba Institute (T2020-Jitsu004 [29 June 2020] and T2021-Jitsu004 [29 June 2021]) or Tokai University (204,019 [31 March 2020]) and were performed in accordance with the Committee's guiding principles.

Preparation of AIMAs

Mature female rats (Wistar; CLEA Japan Inc.) were immunized by injections of peptide antigen (27 amino acids) of the porcine inhibin A subunit [40], and hybridomas were generated from their spleen cells by fusing with myeloma cells (P3U1). Each monoclonal antibody in the supernatant of these cells was assessed with an enzyme-linked immunosorbent assay using the peptide antigen and superovulation tests in B6 female mice. After these assessments, selected clone cells were proliferated and cryopreserved. Frozen–thawed 5×10^6 clone cells/ml in physiological saline were intraperitoneally (i.p.) injected to nude mice (BALB/cAJcl-nu; CLEA Japan) and their ascitic fluid was collected 1–2 months later. Each monoclonal antibody was purified using a Protein G Sepharose column (Cytiva, Tokyo, Japan) and was used for subsequent experiments as an AIMA (5.0 mg/ml). The animal experiments for the preparation of AIMAs described above were approved by the Animal Experimentation Committee at CLEA Japan Inc. (2057-051 [31 August 2020]) and were performed in accordance with the committee's guiding principles. These AIMAs developed by RIKEN and CLEA Japan Inc. are commercially available (https://www.clea-japan.com/inquiry.html).

Superovulation treatments and collection of metaphase II oocytes

As a conventional superovulation method, 5 IU eCG (Peamex, Sankyo Co., Tokyo, Japan) was administered i.p. in the evening (18:00–20:00), which was followed by injection with 5 IU hCG (Gonatropin, Sankyo Co.) 48 h later. For alternative superovulation methods, 2 mg (0.08 ml per mouse) of progesterone (P4; Progehormon; Mochida Pharmaceutical, Tokyo, Japan) was administered subcutaneously in the evening (18:00–20:00) once a day for 2 days (designated Days 1 and 2) to synchronize the estrous cycle [30, 41]. In the evening (18:00–20:00) of Day 4, AIS (diluted to 1/1, 1/2, 1/4, or 1/8 concentration, 0.1 ml per female), or AIMAs (0.5 mg/0.1 ml per female), or saline (control) were injected i.p. to the P4-primed females followed by hCG injection 48 h later. At 16–17 h after this, mature metaphase II (MII) oocytes were collected from the oviducts of these superovulated

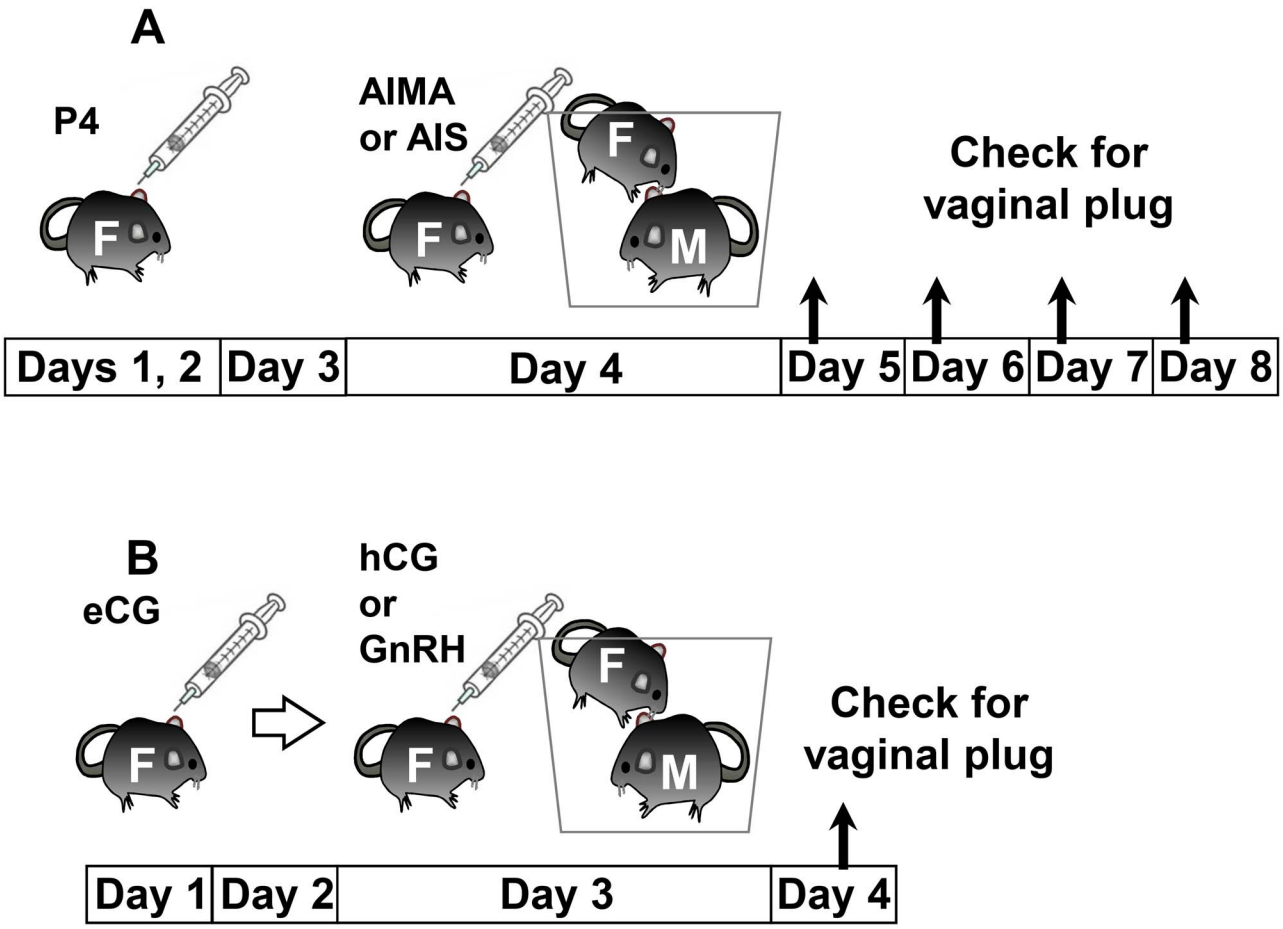

Figure 1. Schematic representation of superovulation treatments and mating tests. (A) AIMA or AIS treatment following estrous synchronization by progesterone (P4) injections. (B) Conventional eCG/hCG or eCG/GnRH treatment. The treated females were paired with male mice on Days 4 (A) and 3 (B). F, female; M, male.

females by puncturing the ampulla region of the oviducts with a 26-G needle and were placed in an 80-μl drop of modified human tubal fluid medium [42]. Morphologically normal and abnormal oocytes were counted under a dissecting microscope.

Mating tests after superovulation treatments

For the mating tests using the AIS and AIMA injection groups (Figure 1A), mice were treated with P4 to synchronize the estrous cycle and treated as above. To determine the day of mating with a male mouse, the female mice were monitored in the morning for the following 4 days until a vaginal plug was found [41]. For mating tests using the conventional superovulation group (Figure 1B), female mice were injected i.p. with 5 IU of eCG in the evening (18:00–20:00) followed by an i.p. injection of 1 or 5 IU of hCG or 0.6 mg of gonadotropin-releasing hormone (GnRH) agonist (buserelin acetate; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) 48 h later. Females injected with hCG were each put into a cage with a sexually mature and experienced male mouse. As a control nontreatment group, the external appearance of the vagina of B6 females was examined, and females showing a gaping vagina and swollen, reddish-pink tissues around the vagina were considered to be at the proestrus stage [43]. They were paired with male mice (1:1) overnight and the presence or absence of a vaginal plug was observed the next morning (Day 1).

After mating, females that became pregnant as assessed by their appearance and body weight gain were injected subcutaneously with 2 mg of P4 in the evening of Days 18 and 19 to prevent natural delivery. In the morning (09:00–12:00) of Day 20, these females were euthanized and examined for the presence of implantation sites and live fetuses by cesarean section. Among the fetuses that were alive at this point, those that started normal respiration after stimulation were defined as "surviving" pups. The body weights of all live and dead fetuses were measured before the live ones were housed with lactating foster mothers.

In vitro fertilization

We prepared IVF-derived embryos for embryo transfer to determine the maximum number of fetuses that could develop in one female. These embryos were prepared as described [30, 41]. In brief, B6 female mice were superovulated using eCG or 2P4-AIS injections followed by hCG injection, as described above. At 16–17 h after hCG injection, MII oocytes were collected from the oviducts of females and transferred into drops of HTF medium containing hypotaurine (0.11 mg/ml, Merck Millipore, Darmstadt, Germany), 0.3% bovine serum albumin (BSA, Calbiochem, Merck Millipore), and 1.25 mM of reduced glutathione [44, 45]. Spermatozoa extruded from

the cauda epididymides of B6 male mice were suspended in HTF medium containing 0.4 mM of methyl-beta-cyclodextrin [46, 47] and 1 mg/ml of polyvinyl alcohol instead of BSA and were incubated at 37°C under 5% CO_2 in humidified air for approximately 60 min. At insemination, preincubated spermatozoa were transferred into 80-μl droplets of fertilization medium containing cumulus-enclosed oocytes at a final concentration of 200–400 sperm/μl. After 3–4 h, the oocytes were transferred into a small drop of CZB culture medium [48] and were incubated overnight. On the next day, two-cell-stage embryos were counted and cryopreserved by vitrification (see below). Cryopreservation of two-cell stage embryos enabled us to perform embryo transfer experiments on any days when recipient females became available.

Embryo cryopreservation by vitrification

We cryopreserved two-cell embryos using a high osmolality vitrification solution, as described [49]. Briefly, about 20–50 embryos were placed on the surface of 50 μl of equilibrium solution (5% dimethyl sulfoxide and 5% ethylene glycol in PB1 [50]) using a sterile glass capillary (100–120 μm diameter) with a small amount of medium at room temperature. After 3 min, embryos that had settled on the bottom were picked up and transferred into a cryotube (MS-4501; Sumitomo Bakelite Co. Ltd., Tokyo, Japan) containing 50 μl of the vitrification solution: 42.5% (v/v) ethylene glycol, 17.3% (w/v) Ficoll, and 1.0 M sucrose in PB1. One minute later, the cryotube was plunged directly into liquid nitrogen (LN_2) at −196°C. On the day of embryo transfer, the cryotubes were retrieved and the cap was removed quickly to allow LN_2 to evaporate. After the tubes were left at room temperature for 2 min, 850 μl of 0.75 M sucrose–PB1 at room temperature was added gently. After 4 min, the solution in the tubes was mixed by gentle pipetting for five times. The entire volume of the solution was then transferred to an empty plastic dish. After 1–3 min, the embryos were transferred to a 0.25-M sucrose–PB1 droplet. After equilibration for another 1–3 min, the recovered embryos were washed in two more droplets of 0.25-M sucrose–PB1 and were then transferred to a droplet of CZB medium in a culture dish. Embryos with normal morphology (i.e., intact plasma membrane and clear cytoplasm) were considered to be viable. The surviving embryos were incubated under 5% CO_2 in humidified air at 37°C until embryo transfer (<3 h).

Embryo transfer

Fourteen or seven vitrified–thawed two-cell embryos were transferred into each oviduct of Day 1 pseudopregnant B6 females after sterile mating with vasectomized ICR males. All recipients were injected subcutaneously with 2 mg of P4 in the evening on Days 18 and 19 to prevent natural delivery. On the morning (09:00–12:00) of Day 20, the recipient females were examined for the presence of live fetuses by cesarean section.

Production of gene knockout mice by *i*-GONAD

i-GONAD gene knockout (KO) experiments were performed as described [20, 27] with slight modifications. Females were randomly selected regardless of the estrous cycle stage and injected with P4 in the evening (18:00) of two sequential days (designated Days 1 and 2), and an AIMA on the evening of Day 4 (18:00). Each female was paired with a male mouse for up to four consecutive days until a vaginal plug was observed. For the *i*-GONAD method, the solution of a CRISPR gene-editing cocktail containing 1 μg/μl Cas9 protein (#1081059; Integrated DNA Technologies [IDT], Inc., Skokie, IL, USA) and 30 μM gRNA (Alt-R crRNA annealed with tracrRNA, IDT 1072534) was prepared freshly in Opti-MEM medium (#31985062; Thermo Fisher Scientific Inc., Waltham, MA, USA). The gRNA was designed to recognize the target site of exon 1 of the mouse tyrosinase (*Tyr*) gene that matches 20 bp of the wild-type DNA sequence (Tyr-wild crRNA: AACTTCATGGGTTTCAACTG) [27]. The ovaries and oviducts of anesthetized females (at around 16:00 of the next day after mating) were exposed and approximately 1.5 μl of cocktail was injected into the lumen of the oviduct upstream of the ampulla region using a fine glass pipette connected to a mouthpiece. After injection, the oviduct was covered with a piece of Kimwipe wetted with phosphate-buffered saline and then pinched by a forceps-type electrode (#LF650P3; BEX Co. Ltd., Tokyo, Japan). Electroporation was performed using a CUY21Edit II electroporator (BEX Co. Ltd.). The electroporation parameters were as described [27]; square (mA), (+/−), Pd V: 80 V, Pd A: 100 mA, Pd on 5 ms Pd off 50 ms, Pd N: 3, decay: 10% decay type: Log. After electroporation, oviducts were returned to their original position. The dorsal skin was closed using a surgical stapler. On Day 19 of pregnancy, offspring were obtained by cesarean section or allowed to deliver naturally and examined for CRISPR-Cas9-induced mutations at the target sites. Pigmentation of the eyes of offspring was assessed under a dissecting microscope. Genomic DNA was extracted from small pieces of ear tissues of offspring showing pigmentation. Polymerase chain reaction (PCR) amplification of a sequence corresponding to *Tyr* was performed in a volume of 10 μl containing 5 μl of 2× GC buffer (Takara Bio Inc., Shiga, Japan), 0.2 mM deoxyribonucleotides mix, 1 μl of crude lysate, 0.5 μM primer pairs (M463: TCC TTC TGT CCA GTG CAC CAT/M999: ATG GGT GTT GAC CCA TTG TT) [19, 42], and 0.125 U TaKaRa Taq (Takara Bio Inc.) under PCR cycling conditions of denaturation at 95°C for 5 min; amplification with 30 cycles of 95°C for 45 s, 58°C for 30 s, and 72°C for 1 min; and extension at 72°C for 5 min. Amplification products were separated by 1% agarose gel electrophoresis. Direct sequencing of the PCR products was performed to confirm the genotypes.

Statistical analysis

We performed statistical analyses to compare the control data with the treatment groups. The numbers of oocytes, litters, implantation sites, and surviving pups were analyzed using the Mann–Whitney nonparametric *U* test using Excel software (Statcel v.2; Microsoft Corp., Edmond, WA, USA). The rates of pregnancy, implantation, and surviving pups were analyzed using Fisher exact probability test; $P < 0.05$ was considered as statistically significant.

Results

AIMA production and screening by superovulation tests

Cell supernatants extracted from each of the 40 clones of hybridoma were each administered to two female mice, except for sample #40 for which the estrous cycle was synchronized by administration of P4 for 2 days (Supplementary Figure S1). Based on the results of the first round of screening, we

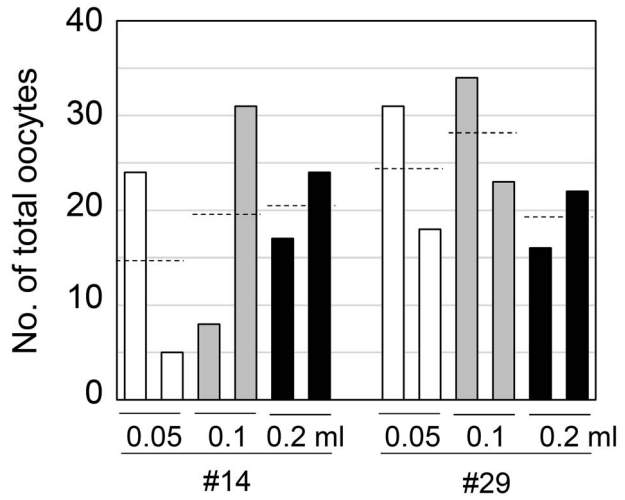

Figure 2. Comparison of the superovulation efficiency of two AIMAs (#14 and #29) at different volumes injected. Each bar represents a result from a single female. The dotted lines indicate the mean value for each group.

conducted another series of superovulation tests using hybridoma cells #14 and #29, which produced more superovulated oocytes. Ascitic fluid obtained from nude mice injected with hybridoma cells from these clones was used at 0.05, 0.1, or 0.2 ml per female. Use of the ascitic fluid from hybridoma cell line #29, but not #14, resulted in consistent superovulation efficiencies at both the 0.05-ml and 0.1-ml doses (24.5 and 28.5 oocytes per mouse, respectively). Therefore, in subsequent experiments, we used ascitic fluid from clone #29 at 0.1 ml per female as the standard AIMA treatment (Figure 2).

Efficiencies of superovulation following different regimens

In our previous study, we found that the best superovulation efficiency was obtained with AIS (0.1 ml per female) and hCG injections following estrous cycle synchronization by sequential injections of P4 (2P4-AIS/hCG) (see Materials and methods). Therefore, we applied the same protocol to AIMA (2P4-AIMA/hCG) to determine whether it could substitute for AIS. After the 2P4-AIMA/hCG treatment, a mean of 24.6 normal oocytes was obtained per female, which was significantly lower than with the 2P4-AIS/hCG treatment (51.8 oocytes, $P < 0.01$; Table 1). The superovulation efficiency with AIMA roughly corresponded to treatment with AIS at the one-fourth concentration (2P4-1/4 AIS/hCG; 25.0 oocytes) but was slightly better than the conventional eCG/hCG treatment (17.1 oocytes; Table 1). Importantly, the AIMA treatment produced only 0.1 abnormal oocytes per female: much less than that of other treatment groups (Table 1). In the estrous cycle synchronization and saline injection group, there was a mean of only 6.2 oocytes: the basal ovulation level (Table 1).

Mating tests of females treated with different superovulation regimens

Next, we examined whether the AIMA treatment might affect the pregnancy rates after mating, as it is known that females superovulated with the conventional eCG/hCG treatment frequently fail to become pregnant even after successful mating [8, 10]. After 2P4-AIMA treatment followed by mating with males for 4 days (Figure 1A), 10/14 (71%) females exhibited a vaginal plug during this period and of them, 8/10 (80%) became pregnant (Figure 3A, Table 2). We also tested AIS for its effect on the mating rates, but it appeared that the original concentration of AIS might cause the production of too many numbers of embryos and fetuses. Therefore, we treated females with 2PA-1/4 AIS or 2PA-1/6 AIS and mated them with males. The rates of vaginal plug formation during Days 5–8 were 7/8 (88%) and 4/7 (57%) and the pregnancy rates were 5/7 (71%) and 4/4 (100%), respectively (Figure 3B and C, Table 2). The vaginal plug was most frequently observed at Day 8 (mating during the night of Day 7) for the AIMA group and at Day 7 (mating during the night of Day 6) for the AIS group (Figure 3). In the eCG/hCG (5 IU/1 IU or 5 IU/5 IU) groups (Figure 1B), the mating rates were 6/10 (60%) and 5/8 (63%) and the pregnancy rates were 4/6 (67%) and 2/5 (40%), respectively (Table 2). To determine whether eCG alone might affect the efficiency of producing offspring, we performed additional superovulation experiments without the hCG treatment (Supplementary Figure S2A). As a result, only half of the females mated successfully with males during the observation period and few embryos developed to term (Supplementary Figure S2B and Supplementary Table S1). These findings indicate that the eCG treatment alone compromises the females' condition and causes inefficient offspring production. In the control experiment, untreated females were selected for mating during proestrus based on the vaginal appearance (see Materials and methods). After overnight pairing with males, 7/22 (32%) females had a vaginal plug on the next day and all of these became pregnant (Table 2).

The litter size and number of pups surviving after superovulation and mating

We next analyzed the number of pups born alive (designated "living") and those recovering respiration and active movement (designated "surviving") following superovulation and mating. In the AIMA group, significantly more embryos were implanted, and significantly more surviving pups were produced (16.3 and 11.9, respectively) compared with the control group ($P < 0.01$; Table 2, Figure 4A and B). All AIMA-derived pups were morphologically indistinguishable from those derived from untreated females (Figure 4C). In a subset of experiments, we halved the dose of AIMA, but the mean numbers of implanted embryos (13.8 sites/litter from 13/15 [pregnant/plugged] females), the living pups (12.5 pups per female), and the surviving pups (12.3 pups per female) were similar to those involving the original dose of AIMA, indicating that the half dose of AIMA might be practicable. The efficiency of mating was high (15/19; 79%). In the AIS groups, the mean numbers of implanted embryos per female (13.8–19.6) were higher than in the control group, but the numbers of surviving pups (7.0–8.0) did not exceed that of the control group (Table 2, Figure 4A and B). These results indicate that AIS compromised the survival of postimplantation embryos. In the eCG/hCG groups, the mean numbers of implanted embryos (5.0–8.0 per female) were smaller than in the control groups and the numbers of pups that survived were further decreased (0.5–5) (Table 2, Figure 4A and B), indicating

Table 1. Results of superovulation in C57BL/6 mice after different superovulation treatments

Treatment	No. (%) of females		Mean number of oocytes (±SEM)		
	Treated	Ovulated	Total	Normal	Abnormal
2P4-AIMA/hCG	10	10 (100)	24.7 ± 1.7**	24.6 ± 1.7**	0.1 ± 0.1
2P4-AIS/hCG	19	19 (100)	53.2 ± 4.0**	51.8 ± 3.9**	1.4 ± 0.4*
2P4-1/2 AIS/hCG	16	16 (100)	53.4 ± 3.4**	51.3 ± 3.9**	2.1 ± 1.7
2P4-1/4 AIS/hCG	6	6 (100)	28.7 ± 5.6**	25.0 ± 6.3**	3.7 ± 3.7
2P4-1/8 AIS/hCG	6	6 (100)	21.3 ± 3.7**	14.3 ± 2.7*	7.0 ± 4.6
eCG/hCG	37	33 (89)	21.7 ± 1.9**	17.1 ± 1.5**	4.6 ± 0.6**
2P4-saline/hCG (control)	10	8 (80)	6.2 ± 1.2	6.2 ± 1.2	0

Data from the experimental groups were compared with that from 2P4-saline/hCG treatment (control). $^{**}P < 0.01$, $^{*}P < 0.05$ versus control within the same column by Mann–Whitney nonparametric *U* test. 2P4, progesterone (twice); SEM, standard error of the mean.

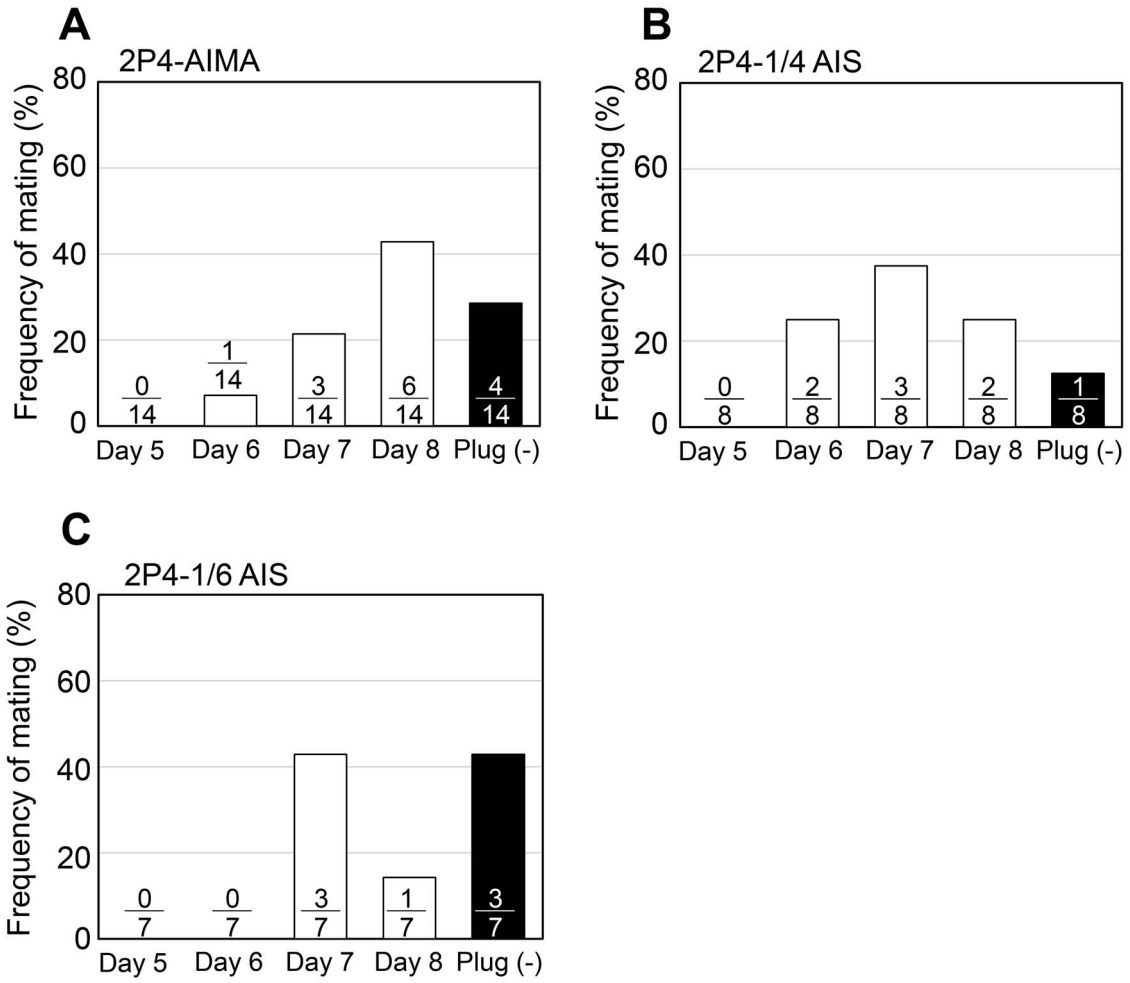

Figure 3. Distribution of days of vaginal plug appearance during continuous pairing with experienced male mice. Before mating, females were treated with progesterone (P4) injections followed by AIMA (A), 1/4 AIS (B), or 1/6 AIS (C). Values within the bars indicate the numbers of females that had a vaginal plug among the total number of pairs.

that eCG/hCG treatments also impaired the postimplantation development of embryos. When GnRH was administered instead of hCG, the survival rate increased to 80%, but the rate of pups surviving after cesarean section was not improved (4.0 per female) (Table 2, Figure 4A and B). Thus, the AIMA treatment is advantageous to other superovulation regimens in increasing the litter size after natural mating and pregnancy. The average numbers of implantation, living pups, and surviving pups of each group are summarized in a multilayered bar graph, as shown in Figure 4D, to show the tendencies for each superovulation treatment: thus, AIS tended to increase the number of implanted embryos, but many of them died before term.

The maximum number of offspring produced by embryo transfer

Although we found that the AIMA treatment significantly increased the litter size after natural mating and pregnancy, we did not know whether this was the maximum in terms of the receptive capacity of B6 females. To identify the maximal

Table 2. Offspring production in C57BL/6 mice after different superovulation treatments

Treatment	No. (%) of females			Implantation sites per female (total no.)	Survival rate after imp. to term (%)	Litter size		Survival rate (%)
	Treated A	With plug B (B/A)	Pregnant C (C/B)			Living D	Surviving E	F (E/D)
2P4-AIMA	14	10 (71)*	8 (80)	16.3 ± 1.6 (130)**	99/130 (76)**	12.4 ± 1.3**	11.9 ± 1.2**	96
2P4-1/4 AIS (0.025 ml)	8	7 (88)*	5 (71)	19.6 ± 3.4 (98)	53/98 (54)**	10.6 ± 1.4	8.0 ± 0.8	75**
2P4-1/6 AIS (0.017 ml)	7	4 (57)	4 (100)	13.8 ± 4.9 (55)	35/55 (64)**	8.8 ± 0.9	7.0 ± 0.4*	80**
eCG/hCG (5 IU–1 IU)	10	6 (60)	4 (67)	5.0 ± 0.6 (15)**	8/15 (53)**	2.0 ± 0.8**	0.5 ± 0.5**	25**
eCG/hCG (5 IU–5 IU)	8	5 (63)	2 (40)*	8.0 ± 2.0 (16)	10/16 (63)**	5.0 ± 3.0	5.0 ± 3.0	100
eCG/GnRH (5 IU-0.1 ml)	7	6 (86)*	4 (67)	6.3 ± 1.5 (25)	20/25 (80)	5.0 ± 1.8	4.0 ± 2.3	80**
Control	22	7 (32)	7 (100)	9.1 ± 0.4 (64)	60/64 (94)	8.6 ± 0.3	8.6 ± 0.3	100

The percentages of females with plug/pregnant and implantation were analyzed by Fisher exact probability test. The numbers of implantation sites per female and litter size (mean ± SEM) were analyzed by the Mann–Whitney nonparametric *U* test. $^{**}P < 0.01$, $^{*}P < 0.05$ versus the corresponding control value.

number of fetuses that can normally develop in utero, we performed embryo transfer experiments using excess embryos. When seven two-cell-stage embryos produced by IVF and vitrification–thawing were transferred to each oviduct (total of 14 embryos), the litter size was 9.6 offspring per female (68% per transferred) and all pups survived (Table 3). When the number was increased to 14 embryos per oviduct (28 embryos in all), the birth rate was decreased to 51% and the final litter size was 13.3 per female (Table 3, Figure 4A, B, and D). The implantation rates were nearly the same between the two groups (84% vs. 82%; Table 3). Because of ethical concerns about animal welfare, we did not test larger numbers of embryos for transfer, but we estimated that around 13 fetuses per female might be the maximal number, considering the decreased survival rate of postimplantation embryos to term. Therefore, we conclude that the AIMA treatment can achieve the near-maximum litter size (11.9) provided that B6 female mice are used for experiments.

Body weights of pups and overall production efficiency

The mean body weights of living pups at birth were significantly lower ($P < 0.01$) in all the experiment groups than in the control group (Figure 5A). There seemed to be no relationship between the number of living pups at term and their body weight. However, there was an apparent relationship between the survival rate of offspring and the body weight at birth; about two-thirds of the offspring weighing <0.8 g failed to survive (Figure 5B). Therefore, we examined the ratios of offspring classified by their body weight in each treatment group. As a result, we found that >20% of the pups in the two AIS groups and the eCG/hCG(1 IU) group weighed ≤0.8 g (Figure 5C), which is consistent with the low survival rates in these groups (Table 2, Figure 4D). Finally, to determine the overall efficiencies of the different superovulation treatments, we calculated the mean numbers of living pups per female by incorporating all the treated females, including nonpregnant ones. There were increases in two AIS groups and the AIMA group compared with the control group, with AIMA being 2.4 times more effective (Figure 6).

Application of AIMA-induced superovulation to *i*-GONAD

We expected that the litter size increase produced by the AIMA treatment could improve the genome-editing experiments using *i*-GONAD, an in vivo-electroporation method, by increasing the chance of birth of offspring carrying the expected genome modifications. We applied our 2P4-AIMA treatment to the *i*-GONAD method, as shown in Figure 7A. When 16 randomly selected females were subjected to estrous cycle synchronization by P4 injections and induced to ovulate with AIMA on Day 4, 81% of them mated during Days 6–8 (2, 6, and 5 females, respectively) and their cumulative pregnancy rate was 92% (Table 4, Figure 7B). At term, 88 fetuses were born from 12 females, with a mean litter size of 7.3 (Figure 7C and D). Thus, the litter size recovered near to the control level (8.6) even after the harsh electroporation treatment to embryos during the *i*-GONAD procedure. This mean litter size was about a 1.5-fold increase from that of the conventional *i*-GONAD, namely, 4.8; 48 pups from 10 females we reported previously (Table 4) [27]. The rate of genome-edited pups following 2P4-AIMA *i*-GONAD treatment was 80/88, which is similar to that of conventional *i*-GONAD (96%; Table 4). Thus, our AIMA-induced superovulation protocol improved the efficiency of *i*-GONAD in B6 mice by increasing the litter size without any noticeable adverse effects. Furthermore, based on the factors involved in the overall efficiency, we can expect that the number of females used for *i*-GONAD experiments can be reduced to 1/9 (Figure 7E).

Effect of AIMA treatment on litter size in ICR strains

We also tested whether our AIMA-induced superovulation could be effective for the ICR strain, one of the major outbred strains with high fecundity, leading to the production of more litters. In the untreated control group, all the living pups survived after cesarean section and the mean number of pups per female ($n = 6$) was 15.3 (Table 5). In the 2P4-AIMA group, most (297/299) living pups survived: an increase of 1.4-fold (21.2 pups/female) (Supplementary Figure S3). No noticeable differences were found in the rates of mating and

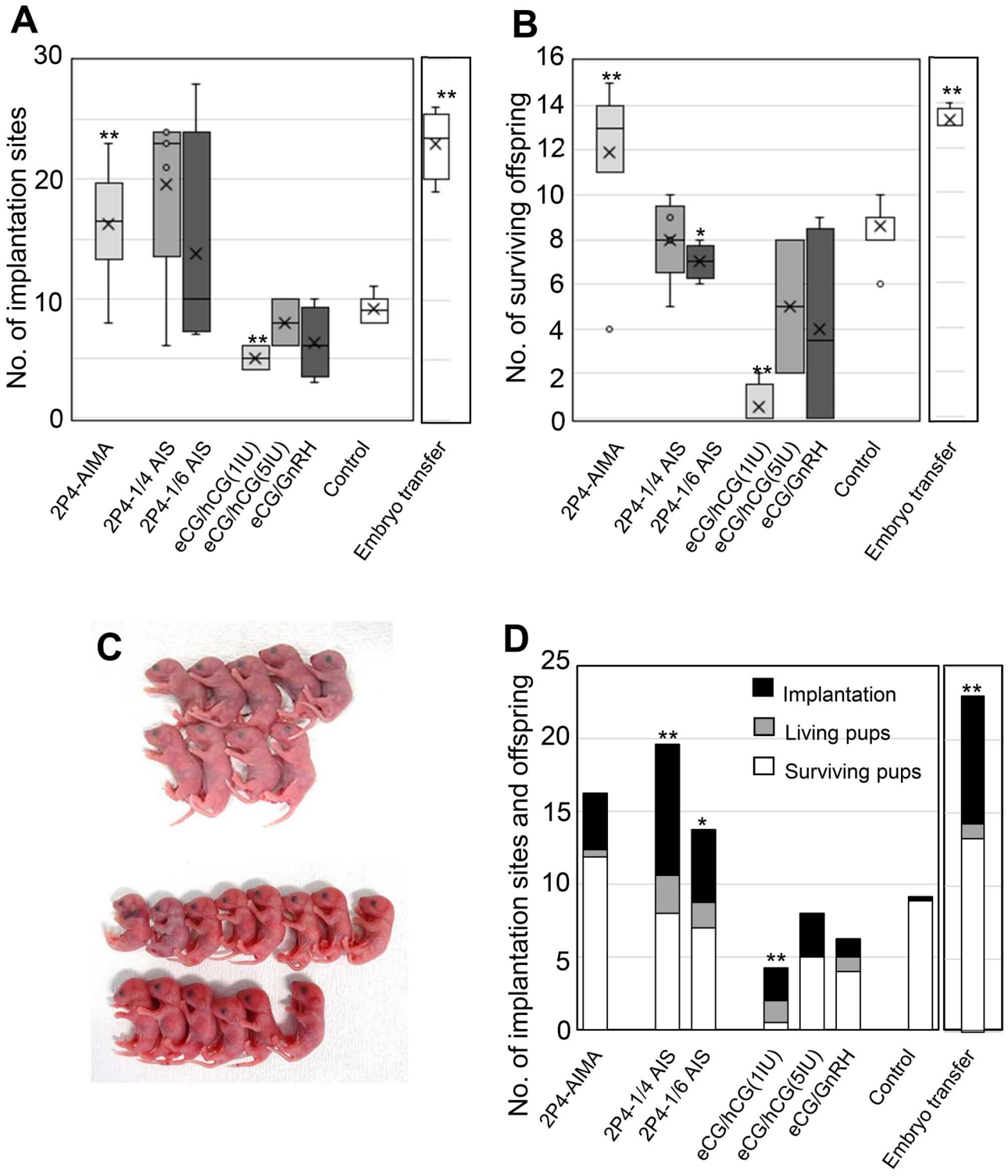

Figure 4. Implantation and births of offspring following superovulation treatments and mating with males. (A) The numbers of implantation sites following different superovulation treatments. (B) The number of surviving pups following different superovulation treatments. (C) Pups born from a nontreated control female (upper) and those from a female following 2PA-AIMA treatment (lower) (D). The mean numbers of implantation sites, living pups, and surviving pups showing the steps of loss of embryos/fetuses. "Embryo transfer" in (A), (B), and (D) indicates the results from the transfer of excessive numbers of embryos compared with control values by the Mann–Whitney nonparametric *U* test.* $P < 0.05$;** $P < 0.01$.

pregnancy between groups (Table 5). The mean weight of pups at birth was significantly lower in the AIMA group than in the control group (1.66 ± 0.02 g vs. 1.49 ± 0.02 g, $P < 0.01$) but was considered to be within the normal range.

Discussion

Here, we aimed to develop superovulation methods to increase the litter size of mice after natural mating and pregnancy. Among the protocols tested in this study, the AIMA-induced superovulation gave the best result in terms

Table 3. Results of embryo transfer using different numbers of embryos per recipient female in the C57BL/6J strain

No. of embryos transferred per recipient	No. of recipient mice	Total no. of embryos transferred	No. (%) of embryos				Litter size		Body weight (g)	
			Implanted	Developed to offspring						
				Living	Surviving	Survival rate (%)	Living	Surviving	Living	Surviving
		A	B (B/A)	C (C/A)	D	D (D/C)				
14	7	98	82 (84)	67 (68)	67	100	9.6 ± 0.9	9.6 ± 0.9	1.17 ± 0.01	1.17 ± 0.01
28	4	112	92 (82)	57 (51)*	53	93**	14.3 ± 0.5**	13.3 ± 0.3*	1.03 ± 0.02**	1.04 ± 0.02**

The rates of implantation and offspring production were compared between groups using Fisher's exact probability test. The litter sizes and body weights (mean ± SEM) were compared between groups using the Mann–Whitney nonparametric *U* test. $^{**}P < 0.01$, $^{*}P < 0.05$.

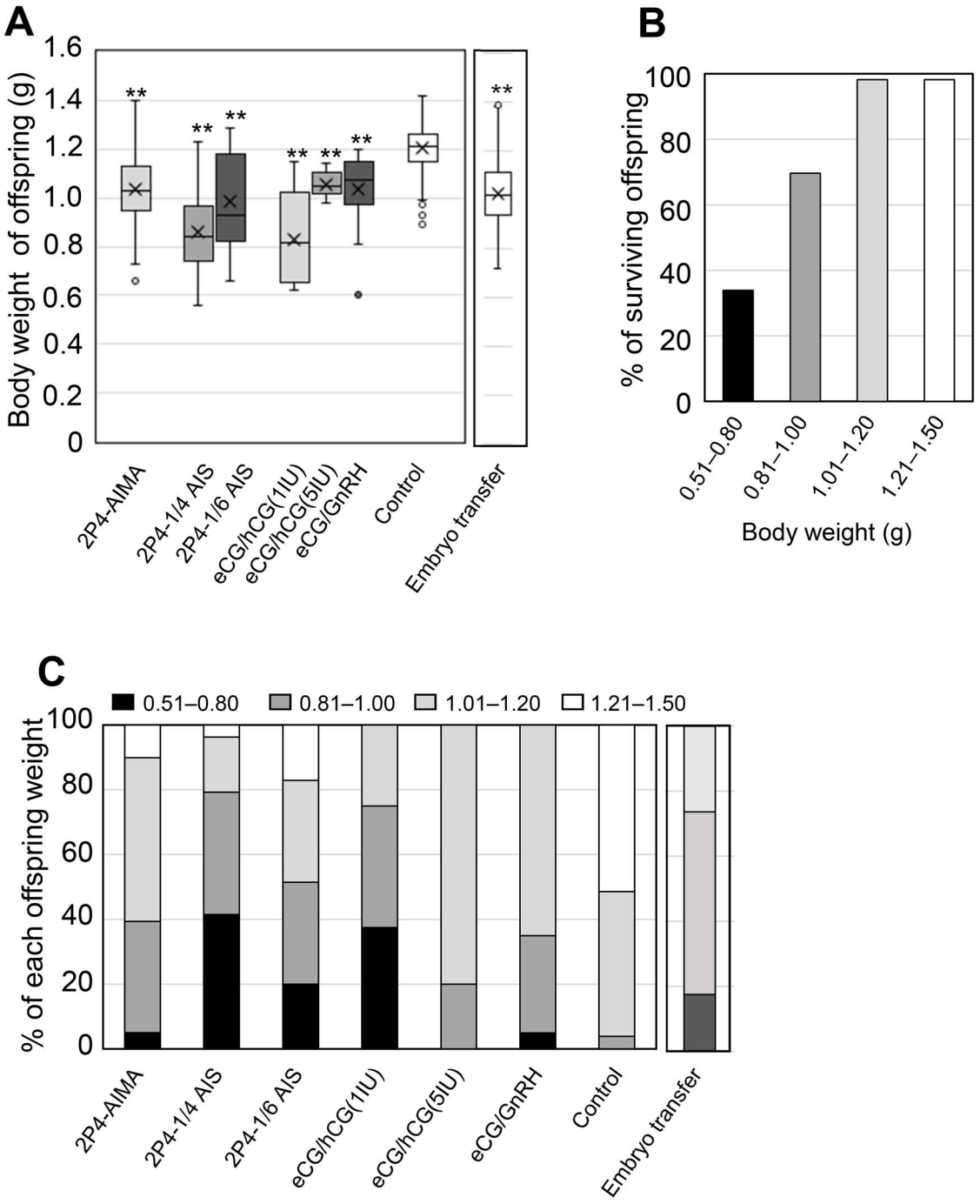

Figure 5. The status of pups born following different superovulation treatments. (A) Mean body weights of offspring (in g) at cesarean section in different superovulation treatment groups compared with control value using the Mann–Whitney nonparametric *U* test.$^{*}P < 0.05$;$^{**}P < 0.01$. (B) Percentages of surviving pups grouped by body weight range at birth. (C) Ratios of pups classified by the body weight range at birth. "Embryo transfer" indicates the result from transfer of excessive numbers of embryos.

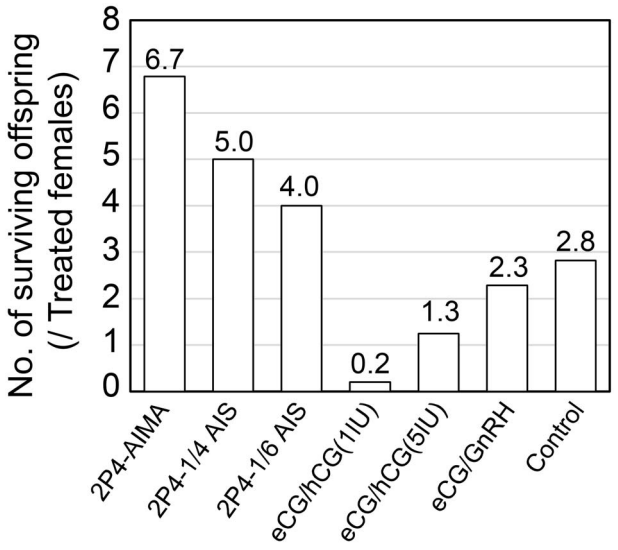

Figure 6. Total yields of surviving offspring per treated female following different superovulation treatments.

of the mean litter size per female that became pregnant (Figure 4B) and also per female used for experiments (Figure 6). There have been several superovulation protocols that can consistently induce superovulation of MII oocytes, such as eCG/hCG and AIS/hCG treatments. The MII oocytes thus collected can develop into normal mice at a practical rate following IVF and embryo transfer. However, here, we confirmed that these treatments were not always reliable to obtain in vivo-derived offspring. Through our detailed analyses, we identified the developmental steps that decreased the survival of in vivo-derived embryos/fetuses specifically for each superovulation protocol (Figure 5C). Importantly, the AIMA-induced superovulation protocol in this study can effectively overcome these limiting steps—from mating with males to the survival of offspring—leading to the best efficiency in producing in vivo-derived offspring. Our embryo transfer experiments using excess numbers revealed that the AIMA protocol produces littermates at the near-maximal level that B6 females can deliver. These findings indicate that we could select the best-qualified AIMA among the many candidate AIMAs generated in our experiments. It would be interesting to examine if this AIMA is also effective for other strains of mice or other laboratory species such as hamsters and rabbits. Possibly, AIMA could also be used for breeding strategies to improve the reproductive performance of wild animals under captive conditions.

As mentioned above, the eCG/hCG treatment conventionally used for the collection of MII oocytes is not effective in producing offspring by natural mating and pregnancy. This indicates that injections of eCG and/or hCG may result in unexpected systemic conditions of females that are not suitable for natural conception. Indeed, it is known that the combination of eCG and hCG reduces implantation and fetal development rates [8, 9, 13, 15] and significantly decreases the mean size of trophoblastic outgrowths [8]. Even administration of hCG alone is known to disrupt the steroid hormone environment, inhibit uterine receptivity and implantation [13, 17], and disrupt the expression of angiogenic factors in the endometrium during pregnancy [18]. Here, we found that the eCG-based protocols suffer from low pregnancy rates after mating (40–67%; Table 2), leading to poor efficiencies of offspring production even lower than that of the nontreated control (Figure 6). As far as we know, the only successful outcome for the eCG/hCG treatment was from the CFW strain, increasing the mean numbers of fetuses at Day 18 from 10.9 (control) to 12.9–17.3 [51]. However, this may be an exceptional case depending on this strain, which was derived from high-fecundity Swiss Webster mice.

The adverse effects of eCG/hCG treatment on the reproductive performance of females are understandable because these hormones originate from different species (mares and humans, respectively) and are administered exogenously. However, at present, we do not know why AIMA is superior to AIS, as both induce the secretion of endogenous FSH from the pituitary gland. The AIS treatment is often associated with embryonic lethality after implantation and with the deaths of neonates (Figure 4D). The survival rates of pups were consistently high with AIMA, reaching 96% in total (Table 2). Anti-inhibin monoclonal antibody may have merit in its milder effect on FSH secretion than AIS. In this study, smaller amounts of AIS, at least the lot we used, did not reproduce the AIMA results in terms of the efficient production of healthy offspring (Table 2). However, it is possible that some other lots of AIS might act similarly to AIMA with appropriate dilutions. Alternatively, perhaps unidentified contents in AIS (goat serum) might cause unexpected effects on the reproductive system of mice, although this could be hard to ascertain. Nevertheless, the effect of AIMAs is expected to be reproducible because they are monoclonal antibodies produced from hybridoma lines. This is one of the advantages of AIMAs compared with AIS, which always needs quality checking for every new batch. Furthermore, AIMAs can be easily transported internationally because they do not contain animal serum, which often requires quarantining.

The *i*-GONAD method enables gene-editing with just two steps: the delivery of a genome-editing mixture into embryos in the oviduct and subsequent electroporation. Using *i*-GONAD, we can skip all the steps of in vitro embryo handling, such as embryo collection or IVF, electroporation, culture, and transfer to recipient females, all of which need special technical skills. However, even with *i*-GONAD, damage to embryos by electric stimuli and genetic modification itself is unavoidable, leading to significant decreases in the birth rates of pups [27]. Here, we found that this drawback associated with *i*-GONAD could be circumvented by AIMA-induced superovulation. The mean numbers of pups born following AIMA treatment and the *i*-GONAD method were similar to that in nontreated natural pregnancies without diminishing our genome-editing efficiency. The original i-GONAD method enabled us to reuse females because the method is based on natural mating and natural delivery and then for oocyte collection or embryo transfer experiments [52]. Therefore, by combining *i*-GONAD with AIMA treatment, we can minimize the number of females used for experiments, which leads to a reduction in costs and efforts and fulfills the "3R" principles of animal experimentation ("Reduction, Replacement, and Refinement"). We also expect that this improved *i*-GONAD protocol could be applied to other laboratory species such as rats and hamsters. As these rodents can deliver more than 10 pups per litter and nurse them

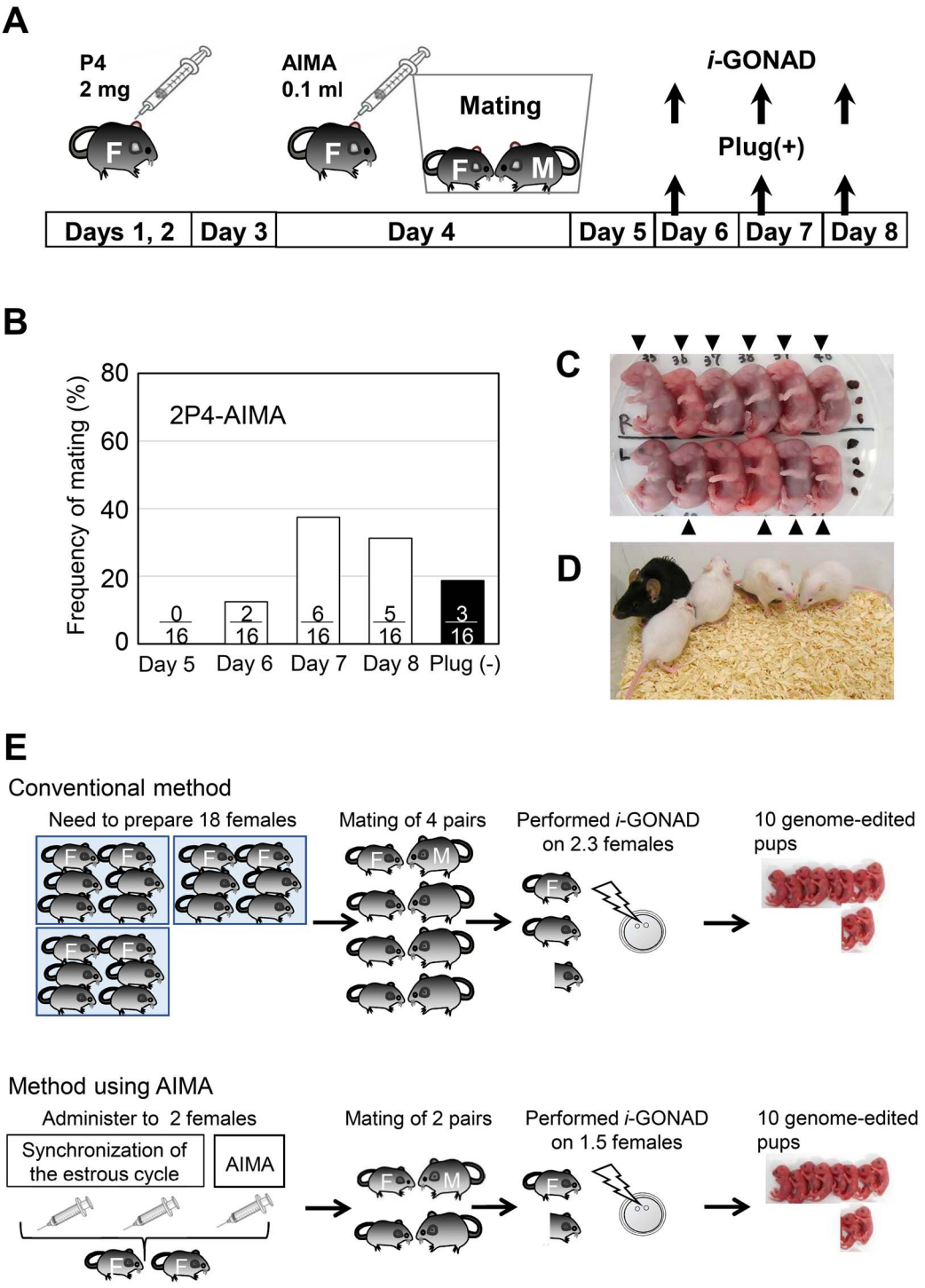

Figure 7. Application of AIMA-induced superovulation to the *i*-GONAD method. (A) Schematic representation of the procedure for superovulation with AIMA. The *i*-GONAD experiment was performed on the day of vaginal plug formation during Days 6–8 (mating during Days 5–7). (B) Distribution of the day of vaginal plug formation following continuous pairing with experienced males. Values within the bars indicate the numbers of females that had a vaginal plug among the total number of treated females. (C) A littermate obtained from an *i*-GONAD-treated female after AIMA administration. Ten out of 12 pups had eyes without pigmentation, indicating that they carried homozygous gene KOs (arrowheads). (D) Four-week-old offspring born after the *i*-GONAD treatment with their mother (black coat). Thus, albino C57BL/6 pups could be produced by tyrosinase gene KO via *i*-GONAD from AIMA-treated females. (E) Estimated numbers of females necessary for the production of 10 genome-edited pups by the *i*-GONAD method in two different protocols with the conventional method (upper) and when using AIMA (lower). Calculations have been based on the results shown in Table 4. When the AIMA treatment was employed for superovulation, the number of females necessary for the production of 10 genome-edited pups could be reduced from 18 to 2 females (1/9) compared with the conventional method.

Table 4. Results of the *i*-GONAD method with or without AIMA treatments

Treatment			No. (%) of females			No. (%) of offspring			
AIMA	*i*-GONAD		Treated A	With plug B (B/A)	Pregnant	Total	Litter size (living fetuses)	Without eye pigmentation	With indel mutation
–[a]	–		22[b]	7 (32)	7/7 (100)	60	8.6 ± 0.3	–	–
–[a]	+		72[c]	43 (60)	10/10 (100)	48	4.8 ± 0.8**	43 (90)	46 (96)
+	+		16	13 (81)	12/13 (92)	88	7.3 ± 1.0	71 (81)	80 (91)
		1st	8	6 (75)	5/6 (83)	36	7.2 ± 0.9	28 (78)	30 (83)
		2nd	8	7 (88)	7/7 (100)	52	7.4 ± 1.4	43 (83)	50 (96)

[a]For the AIMA (−) groups, females for mating trials were selected using vaginal smears or the external appearance of the vagina. [b]Data for the control females (first line) were as in Table 2. [c]Data of the AIMA (−)/*i*-GONAD females (second line) are cited from our previous study [27]. Litter size was analyzed using the Mann–Whitney nonparametric *U* test. For other parameters, data were compared from pairs of groups using Fisher's exact probability test. $^{**}P < 0.01$.

Table 5. Offspring production after AIMA treatments in ICR mice

Treatment	No. (%) of females			Implantation		Litter size		
	Treated A	With plug B (B/A)	Pregnant	No. of females	Sites (total no.)	Living D	Surviving E	Survival rate (%) F (E/D)
Control	557	381 (68)	242/282 (86)[a]	6	16.3 ± 1.5 (98)	15.3 ± 1.4	15.3 ± 1.4	100
2P4-AIMA	28	20 (71)	19/20 (95)	14	24.1 ± 1.6 (337)**	21.4 ± 1.4*	21.2 ± 1.4*	99

[a]Of the 381 females, 282 were checked for pregnancy. The percentages of females with vaginal plugs or who were pregnant were compared between groups using Fisher exact probability test. The numbers of implantation sites and litter size (mean ± SEM) were compared using the Mann–Whitney nonparametric *U* test. $^{**}P < 0.01$,$^{*}P < 0.05$ versus the corresponding control value. 2P4, progesterone (two injections).

until weaning, we expect that the chance of obtaining gene-edited animals in these species will be increased by the use of AIMA.

Conclusions

Anti-inhibin monoclonal antibody-induced superovulation was highly effective in increasing the litter size of in vivo-derived offspring in mice. This superovulation protocol improves the efficiency of *i*-GONAD by increasing the number of pups born following experimental treatments without affecting genome-editing efficiency. It will be necessary to examine how broadly AIMAs can be applied to different animal species for improving their reproductive performance.

Authors' contributions

A.H., K.M., M.O., and A.O. conceived the project, designed the experiments, and wrote the manuscript. A.H., K.M., A.N., R.M., M.O., and M.H. performed the experiments.

Supplementary material

Supplementary material is available at *BIOLRE* online.

Conflict of interest

The authors have declared that no conflict of interest exists.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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