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Mild hypothermia promotes the viability of *in vitro*-produced bovine blastocysts and their transcriptional expression of the cold-inducible transcription factor *Rbm3* during *in vitro* culture

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Abstract. In this study, we evaluated the effects of holding *in vitro*-produced bovine blastocysts under mild hypothermia (33°C or 35°C), by examining viability and hatching rates of day 7 blastocysts (day 0: *in vitro* fertilization) cultured for 6 days and transcriptional expression of cold-inducible transcription factors *Cirp* and *Rbm3*, implicated in mild hypothermia-induced cellular protection against various types of stress. In the normothermic control (38.5°C), viability of the embryos decreased rapidly after day 10, and most samples were degenerated on day 13. However, mild hypothermia, particularly at 33°C, resulted in maintenance of high embryonic survival rates until day 13 (77.1% on day 13) and significant increases in transcriptional expression of *Rbm3* in day 11 embryos compared with those at 38.5°C. Thus, our results suggested that upregulation of *Rbm3* may occur in response to mild hypothermia in many bovine embryos, providing insights into the effects of mild hypothermia on embryo quality.

Key words: Bovine blastocyst, *Cirp*, Embryo holding, Mild-hypothermia, *Rbm3*

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In recent years, the number of *in vitro*-produced (IVP) bovine embryos transferred into surrogates for cattle production has increased significantly, similar to the number of *in vivo*-produced embryos worldwide [1]. However, pregnancy rates following transfer of IVP bovine embryos into cows are lower than those of *in vivo*-produced bovine embryos [2] owing to poor IVP embryo quality. In cryopreservation of bovine embryos, IVP embryos are more susceptible to freezing-induced damage, which can reduce embryo survival, than *in vivo*-produced embryos [3]. To avoid cryodamage caused during the freeze-thaw process, the development of methods that enable bovine embryos to be held for up to 7 days at suprazero temperatures has recently been achieved; *in vivo*-produced blastocysts can survive at 4°C for 7 days in 25 mM HEPES medium 199 with 50% fetal bovine serum [4], and IVP blastocysts can survive at 4°C for 4 days or at 20°C for 3 days in a serum-free medium with BSA and the small molecules CHIR99021 and Y-27632, which can maintain the self-renewal and pluripotency of embryonic stem cells by inhibiting differentiation [5].

When cells are placed at low physiological temperatures, global protein synthesis is generally repressed, thereby altering the cellular program from cell growth to cell senescence. Interestingly, cold-inducible RNA-binding protein (*Cirp*) and RNA-binding motif protein 3 (*Rbm3*) are transcriptionally upregulated in response to low temperature in hibernating animals [6, 7]. Recent studies have shown that *Cirp* and *Rbm3* are involved in protection against cold stress and freezing damage during the processes of slow freezing and vitrification in mouse oocytes [8] and embryos [9, 10]. The expression levels of both proteins are higher in mild hypothermia (32–35°C) than in deep hypothermia (< 25°C) [11–13], normothermia, and hyperthermia (> 39°C) [13, 14]. Notably, mild hypothermia has some beneficial biological effects, including cellular protection against pathological and physiological stressful conditions [15, 16] and inhibition of the differentiation of embryonic and induced pluripotent stem cells [17]. Accordingly, we hypothesized that IVP bovine blastocysts could be held under *in vitro* culture conditions with mild hypothermia owing to their high transcriptional expression of *Cirp* and/or *Rbm3* genes. However, few studies have reported the effects of mild hypothermia on mammalian embryos.

Accordingly, in this study, we examined the effects of mild hypothermia (33°C and 35°C) on the viability of IVP bovine blastocysts during *in vitro* culture from 7 days after IVF (day 7) to day 13 and evaluated their transcriptional expression of *Cirp* and *Rbm3* genes.

We first examined the effects of mild hypothermia on the viability and hatching rates of day 7 blastocysts cultured for 6 days. As shown in Fig. 2, when cultured under normothermia (38.5°C) as a control, most embryos survived, and the hatching rate was increased by day 10, with a maximum rate of 81.8%. Subsequently, the rate of degenerated embryos was increased, and most embryos degenerated on day 13. In contrast, under mild hypothermia, the timing of embryo

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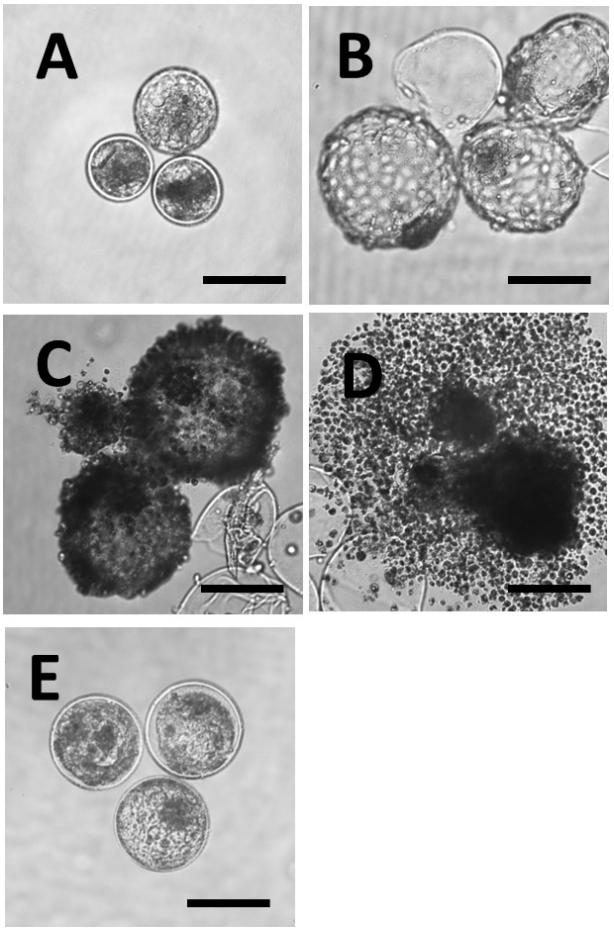

Fig. 1. Photomicrographs of bovine embryos cultured *in vitro* for 6 days in blastocyst culture medium. Day 7 blastocysts (A), hatching blastocysts (B), degenerated embryos that appeared to have a number of darkened cells (C) or to be dark and shrunken with no cellular integrity (D), and nonhatching blastocysts held for 6 days at 33°C (E). Bars = 200 μm.

hatching was delayed compared with that at 38.5°C, with maximum hatching rates on day 11 or 12 at 35 or 33°C, respectively, and on day 10 at 38.5°C. After day 11, the survival rates were significantly higher than that at 38.5°C ($P < 0.05$). Interestingly, after day 10, the rate of nonhatching blastocysts was significantly higher at 33°C than at 35°C ($P < 0.05$), and the rate of degenerated embryos on day 13 was lower at 33°C than at 35°C ($P < 0.05$).

We then measured the diameters of embryos cultured for 6 days at 38.5°C or under mild hypothermia. As shown in Fig. 3, when cultured at 38.5°C, the embryos greatly expanded during the period from day 7 to day 12 from 172.2 ± 2.8 μm to 570.4 ± 56.0 μm in diameter. Most of the embryos then quickly shrunk or degenerated. In contrast, in mild hypothermia, the embryos modestly expanded for 6 days at 35°C or 33°C from 162.3 ± 2.7 and 167 ± 2.8 μm to 345.1 ± 24.9 and 249.8 ± 13.8 μm in diameter, respectively.

Taken together, these results showed that mild hypothermia, particularly at 33°C, could retain the high viability of blastocysts, but lowered their abilities to expand and hatch from the zona pellucida.

We then verified whether the transcriptional expression of the cold-inducible transcription factors *Cirp* and *Rbm3* was upregulated in our *in vitro* cultured bovine blastocysts under mild-hypothermic temperatures (33°C and 35°C). As shown in Fig. 4, in the normothermia control, the expression level of *Rbm3* mRNA was slightly increased (3.3-fold) during *in vitro* culture from day 7 to day 11. In contrast, in response to 4 days of mild hypothermia, the expression levels of *Rbm3* mRNA were greatly increased (8.5- and 11.2-fold at 35°C and 33°C, respectively; $P < 0.05$). However, we found that there were no significant differences in the expression levels of *Cirp* mRNA between day 7 blastocysts and day 11 blastocysts cultured *in vitro* under normothermia or mild hypothermia. We further examined the effects of mild hypothermia on the expression of *Oct4* and *Cdx2* mRNAs. In bovine embryos developed *in vivo* (normothermia) from day 7 to day 11, constant levels of *Cdx2* mRNA have been reported; in contrast, *Oct4* mRNA levels have been shown to be downregulated on day 11 compared with that on day 7 [18]. Here we found that blastocysts cultured *in vitro* for 4 days under normothermia or mild hypothermia displayed *Cdx2* and *Oct4* mRNA expression profiles similar to those of *in vivo* developed blastocysts, and the expression levels of *Cdx2* and *Oct4* mRNAs in day 11 blastocysts did not differ significantly between normothermia and mild hypothermia (Fig. 4). Furthermore, on day 11, when the nonhatching blastocysts that had been held for 4 days at 33°C were warmed for 24 h at 38.5°C in 500-μl droplets of 199 medium with 10% NBCS covered with mineral oil in 4-well dishes, 61.5% (8/13) of the embryos hatched from the zona pellucida.

In this study, our findings supported the holding of bovine IVP blastocysts in *in vitro* culture under mild hypothermia, particularly at 33°C, for 6 days and demonstrated the potential involvement of the cold-inducible gene *Rbm3* in embryo holding. We first demonstrated that culturing under mild hypothermia, particularly at 33°C, resulted in holding of blastocysts at high survival rates for 6 days; however, these blastocysts showed low ability to expand and hatch from the zona pellucida. There were positive correlations between the cell number and diameter in bovine blastocysts [19]. Therefore, mild hypothermia is thought to reduce the cell numbers in surviving blastocysts. Although the mechanisms associated with these hypothermic effects still remain unclear, the effects of the embryos may be explained by previous studies, in which mild hypothermia-induced cellular protection was found to involve various cellular mechanisms, such as reduction of cell metabolisms [20] and cell cycle arrest [21]. Recent studies have shown that these protective effects are attributed to upregulation of the cold-inducible transcription factors *Cirp* and *Rbm3* [13, 14], both of which are highly expressed in mammalian testes, where hypothermia is critical for normal spermatogenesis [16]. Herein, we also demonstrated the significant upregulation of *Rbm3* mRNA in blastocysts cultured *in vitro* under mild hypothermia (33°C and 35°C) compared with that in the normothermia control. Therefore, our findings suggested that the beneficial effects of mild hypothermia on embryo holding at the blastocyst stage during *in vitro* culture may be associated with transcriptional upregulation of the *Rbm3* gene because RBM3 is involved in the regulation of cell growth [22] and viability [23]. Further studies are needed to elucidate the mechanisms through which mild hypothermia affects

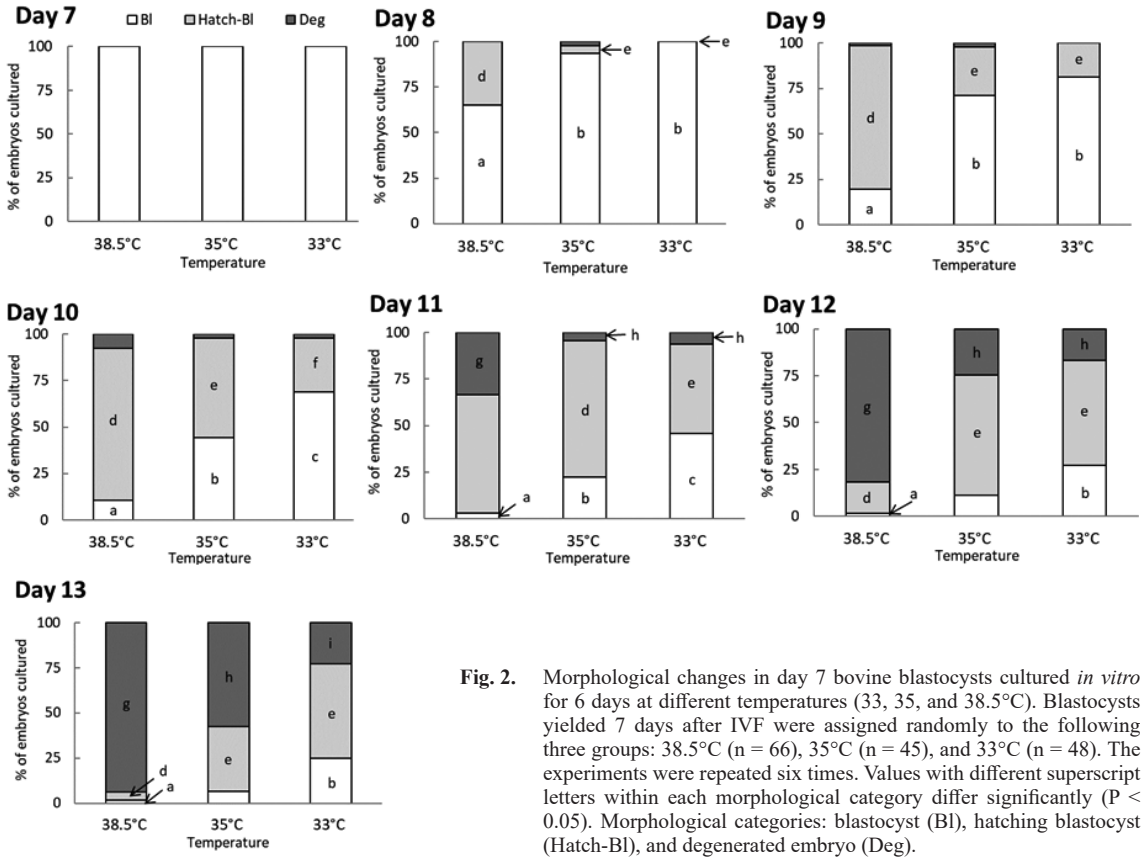

Fig. 2. Morphological changes in day 7 bovine blastocysts cultured *in vitro* for 6 days at different temperatures (33, 35, and 38.5°C). Blastocysts yielded 7 days after IVF were assigned randomly to the following three groups: 38.5°C (n = 66), 35°C (n = 45), and 33°C (n = 48). The experiments were repeated six times. Values with different superscript letters within each morphological category differ significantly ($P < 0.05$). Morphological categories: blastocyst (Bl), hatching blastocyst (Hatch-Bl), and degenerated embryo (Deg).

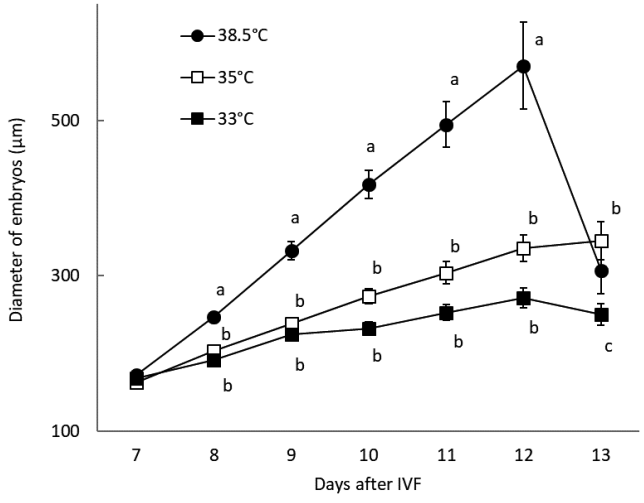

Fig. 3. Diameters of bovine embryos cultured *in vitro* during the period from day 7 to day 13 at different temperatures (33, 35, and 38.5°C). Blastocysts yielded 7 days after IVF were assigned randomly into the following three groups: 38.5°C (n = 66), 35°C (n = 45), or 33°C (n = 48). The experiments were repeated six times. Data are presented as the means ± SEM. Different superscript letters (a–c) within the same column indicate significant differences ($P < 0.05$).

bovine IVP blastocysts. Our findings are expected to contribute to the development of practical methods to enable the transport and *in vitro* holding of bovine IVP blastocysts under mild hypothermia.

Methods

All reagents used in this study were purchased from Merck KGaA (Darmstads, Germany), unless otherwise indicated.

In vitro embryo production

In vitro-embryo production was performed as described previously [24]. Briefly, ovaries were obtained from Japanese Black beef cows at a local slaughterhouse and were transported to the laboratory within 2 h, stored in physiological saline containing 500 ng/ml kanamycin sulfate at 25°C. Cumulus-oocyte complexes (COCs) were collected from 2–6 mm-wide follicles with 18-gauge needles containing HEPES-buffered TCM199 (M199; Thermo Fisher Scientific, Waltham, MA, USA) with 10 ng/ml gentamicin sulfate. The *in vitro* maturation (IVM) medium consisted of M199 supplemented with 10% newborn calf serum (NBCS; heat-inactivated; Thermo Fisher Scientific), 0.02 AU/ml follicle-stimulating hormone (Antorin-R10; Kyoritsu Seiyaku, Tokyo, Japan), 50 ng/ml epidermal growth factor, 5.0 µg/ml dbcAMP, and 10 ng/ml gentamicin sulfate. COCs with two or more layers were washed three times with IVM medium. Groups of 30–50 COCs were cultured in 4-well dishes in 500-µl droplets

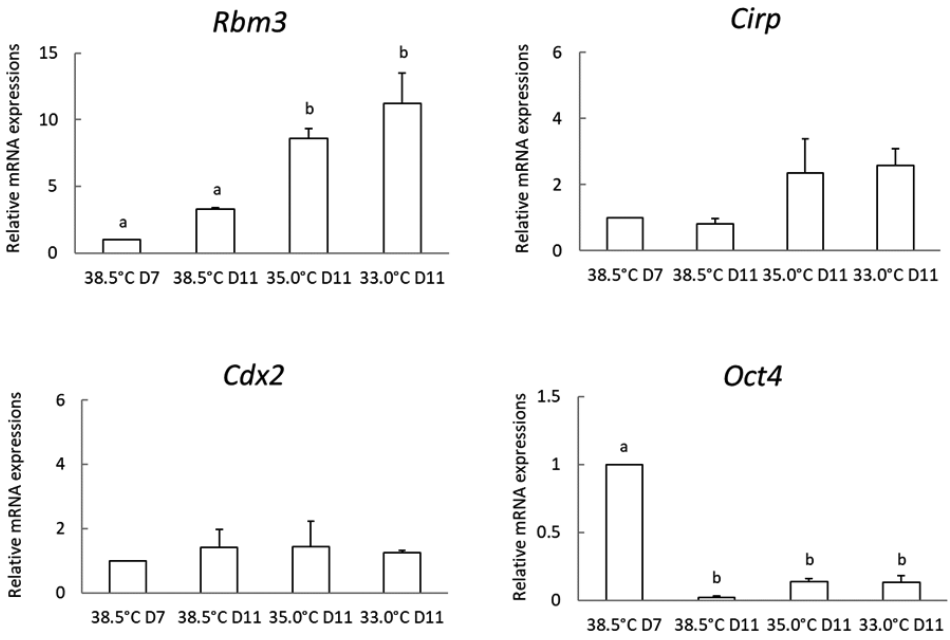

Fig. 4. Quantitative real-time polymerase chain reaction analysis of relative mRNA levels of *Rbm3*, *Cirp*, *Oct4*, and *Cdx2*, normalized to β-actin levels in bovine blastocysts cultured *in vitro* from day 7 to day 11 at different temperatures (33, 35, and 38.5°C). The experiments were repeated three times. Data are presented as the means ± SEM. The different superscript letters (a, b) indicate significant differences ($P < 0.05$).

of IVM medium, covered with mineral oil, and incubated for 21 h at 38.5°C in 5% CO_2 and saturated humidity. Frozen semen from Japanese Black bulls stored in straws (Livestock Improvement Association of Japan, Tokyo, Japan) was thawed in water at 37°C for 30 sec and washed twice with IVF100 (Research Institute for the Functional Peptides, Yamagata, Japan) by centrifugation (600 × *g*, 5 min). Spermatozoa were removed from the pellets and added to IVF100 to obtain a suspension with a final sperm concentration of 5.0×10^6/ml. This suspension served as the IVF medium. After IVM, the COCs were removed from the maturation medium and washed three times with IVF100. Up to 30 COCs were incubated in 35-mm dishes containing 100-µl droplets of IVF medium covered with mineral oil for 6 h at 38.5°C in 5% CO_2 and saturated humidity. The IVC medium used was Charles Rosenkrans 1 medium with amino acids [25] containing 10% NBCS and 10 ng/ml gentamicin sulfate. After IVF, cumulus cells and spermatozoa were removed from the surface of the zona pellucida by high-speed vortexing. The putative zygotes were washed three times with IVC medium. Up to 50 zygotes were cultured in 4-well dishes in 500-µl droplets of IVC medium covered with mineral oil for 7 days at 38.5°C in 5% CO_2/5% O_2/90% N_2 with saturated humidity.

Blastocyst culture at 33°C, 35°C, and 38.5°C

Blastocysts obtained 7 days after IVF were collected and classified into codes 1–2 [26]. After washing, groups of three blastocysts were transferred to wells of ultra-low attachment U-bottomed 96-well plates (PrimeSurface; Sumitomo Bakelite, Tokyo, Japan), which contained 100-µl blastocyst culture medium, and then cultured for 6 days under 5% CO_2 in air at 33°C, 35°C, or 38.5°C. The blastocyst culture medium used was glutamine and ethylenediaminetetraacetic acid-depleted KSOM medium [27] with 10% NBCS, 10 ng/ml gentamicin sulfate, 25 mM HEPES and the following supplements: 10 mM methionine, 100 µM β-mercaptoethanol, and 1 µg/ml resveratrol. β-mercaptoethanol and resveratrol, which suppress oxidative stress, are known to improve blastocyst quality [28, 29], and methionine has important roles in maintenance of the pluripotency and self-renewal of inner cell mass-derived embryonic stem cells [30].

Evaluation of normal viability and hatching rates after culturing at 33°C, 35°C, and 38.5°C

The viability and hatching rates of the embryos were estimated every 24 h for 6 days. Embryos that appeared to have a number of darkened cells or darkened and shrunken cells with no cellular integrity were judged to have degenerated, and embryos other than degenerated ones were assessed as survived (see Fig. 1C and D). Embryos that exhibited discarded zona pellucida were classified as hatching blastocysts.

Measurement of embryonic size

The diameters of embryos at the blastocyst and hatching blastocyst stages were measured along the long axis with an ocular micrometer.

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction

Total RNA was extracted using TRIzol (Invitrogen, ThermoFisher) according to the manufacturer's instructions. RNA was extracted from the pooled blastocysts in three replications; each pool consisted of 20 blastocysts. Isolated RNA was reverse-transcribed to cDNA

Table 1. Sequences of primers for quantitative reverse transcription polymerase chain reaction

Gene name	Primer sequences (5′-3′)	Product size (bp)	GenBank accession number
Rbm3	F: GTCCAGAGACTATGGTGGCAG R: TCATTTGGAGAGGTGGTCCTG	137	NM_001303463.1
Cirp	F: GCTCTGGTCGTTTTGCTCTC R: CTCAGCCCTCCGACGAAAAG	107	NM_001034278.1
Cdx2	F: GCTAGAACCCTCAGGAAAGACC R: CCTTTGTTTCTGCTCGGAGGG	83	NM_001206299.1
Oct4	F: GCAAATTAGCCACATCGCCC R: CACTGCTTGATCGTTTGCCC	98	NM_174580.3
β-actin	F: AACTTGCGCAGAAAACGAGA R: CTGTCACCTTCACCGTTCCA	126	NM_173979.3

using a ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. The resulting cDNA was stored at −20°C until use. Real-time quantitative polymerase chain reaction was performed in duplicate using an Applied Biosystems StepOnePlus Real Time PCR System (Life Technologies) on 96-well plates, with a 20-µl reaction mixture containing 0.3 µM of each primer, diluted cDNA as a template, and THUNDERBIRD SYBR qPCR Mix (TOYOBO). Quantification of *Rbm3*, *Cirp*, *Oct4*, and *Cdx2* transcripts was performed using the ΔΔCt method with β-actin as an internal control. Data analysis was performed with StepOne Software V 2.3. The primers used for the analysis are listed in Table 1.

Statistical analysis

The results were expressed as mean percentages ± standard errors of the means (SEM). Data on the diameters of embryos and gene expression levels were analyzed using one-way or two-way analysis of variance with correction for multiple comparisons. Other data were analyzed using Fisher's exact test. Differences with P values less than 0.05 were considered significant.

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