# Efficient reproduction of cynomolgus monkey using pronuclear embryo transfer technique

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**One of the technical bottlenecks in producing nonhuman primate models is that current assisted reproductive techniques, such as *in vitro* culture and frozen conservation of multicell-stage embryos, often result in poor embryo quality and subsequently lead to low birth rates. We investigated whether pronuclear embryo transfer can be used as an effective means for improving pregnancy and live birth rates of nonhuman primates. We collected 174 metaphase II oocytes by laparoscopy from 22 superovulated mature females and then fertilized these eggs using either *in vitro* fertilization or intracytoplasmic sperm injection, resulting in a 33.3% and a 50% fertilization rate, respectively. These 66 fertilized pronuclear-stage embryos were then tubally transferred to 30 recipients and led to 7 births and 1 abortion. Importantly, we observed that the highest live birth rate of ≈64% was obtained when the transfer of pronuclear embryos was performed in the presence of new corpus luteum in the ovary of recipients between 24 h and 36 h after estradiol peak. Therefore, our experiments demonstrate that by matching the critical time window in the recipient's reproductive cycle for achieving optimal embryo-uterine synchrony, pronuclear embryo transfer technology can significantly improve the pregnancy rate and live birth of healthy baby monkeys. This efficient method should be valuable to the systematic efforts in construction of various transgenic primate disease models.**

*in vitro* fertilization | intracytoplasmic sperm injection | test-tube monkey | transgenic monkey | embryo-uterine synchrony

Considering the great genetic and physiologic similarities with humans, nonhuman primates such as monkeys represent the most ideal experimental models for detailed analysis of biologic processes under physiologic or pathologic conditions. As a result, new drug candidates are typically required to go through systematic assessment of drug efficacy, pharmacokinetics, and toxicity in monkeys before their evaluation in human clinical trials. Furthermore, monkeys are the most favored organisms for in-depth analysis of neural mechanisms underlying high cognition and complex behavior. It is conceivable that the potential of the nonhuman primate model systems can be further expanded when researchers introduce or remove genes of interest by transgenic methods that have been proven so powerful in lower organisms such as mice, zebra fish, and fruit flies.

One of the major bottlenecks in producing transgenic monkeys is the low birth rate of "test-tube" baby monkeys with assisted reproductive technologies. Although the first birth of a monkey (a rhesus macaque) after *in vitro* fertilization (IVF) was reported in 1984 (1), constraints on materials and resources for systematic experimentation with various *in vitro* culture conditions and procedures have contributed to the slow progress of the field. There are also many factors that can greatly affect the quality of embryo development and the rates of pregnancy and live birth. For example, the success of IVF methods is critically dependent on the oocyte quality after extensive *in vitro* culture from the single-cell stage to the 8-cell or even blastocyst stage, embryo transfer methods, and embryo-uterine synchrony. It has been speculated that extensive *in vitro* culture of zygotes into expanded blastocysts may reduce the viability of the embryos (2, 3). Furthermore, freezing and thawing of cultured cleavage-stage embryos, a typical practice owing to lack of proper foster mothers in the colony at the time of experiments, may further reduce embryo quality, thereby leading to low birth rates of test-tube baby monkeys.

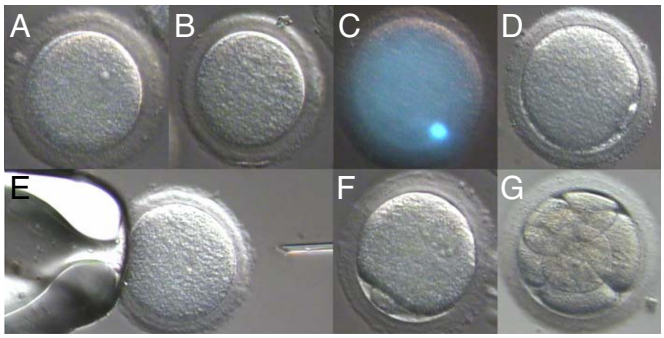


**Fig. 1.** Early stages of cynomolgus monkey embryos. (*A*) Oocyte at germinal vesicle stage. (*B*) Oocyte at metaphase I stage (M-I). The germinal vesicle breaks down before the appearance of the first polar body. (*C*) Oocyte at M-I stained with the Hoechst method. The nucleus can be clearly visualized. (*D*) M-II oocyte with first polar body. (*E*) ICSI. (*F*) Embryo with 2 pronuclear and the polar body after ICSI. (*G*) ICSI-fertilized embryos entered the 8-cell stage.

To examine those important issues, we explored the feasibility of using a pronuclear embryo transfer technique for the reproduction of IVF-derived monkeys. We used cynomolgus monkeys (*Macaca fascicularis*) because they have been used extensively in biomedical research experiments, in particular those connected with neuroscience. Moreover, similar to human reproductive physiology, cynomolgus monkeys are known to exhibit monthly menstrual cycles all year. The cynomolgus monkey is also one of the first to have been flown into space for assessing physiologic changes and safety issues for manned space flight.

In addition, the reported rate for cynomolgus monkey zygotes to develop into expanded blastocysts is only approximately 15% (even with the support cell coculture method) and 20% when using sequential culture medium (4, 5). To date there has been no report of the successful development of *in vitro*-fertilized cynomolgus monkey embryos to blastocyst stage without coculture with supporting cells (6, 7). In the present study we demonstrate that pronuclear embryo transfer technology results in a high birth rate of test-tube monkey babies. Furthermore, we

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**Table 1. Number and developmental stages of oocytes at retrieval after hCG injection**

| No. of monkeys | No. of oocytes | Oocytes per cycle | M-II (%) | GVBD (%) | GV (%) |
|---|---|---|---|---|---|
| 22 | 579 | 26.3 ± 10.4 | 174 (30.1) | 210 (36.3) | 195 (33.7) |

GV, germinal vesicle; GVBD, germinal vesicle breakdown.

have identified the critical physiologic parameters by which transfer of pronuclear embryos can work best for achieving optimal embryo-uterine synchrony and a subsequent high pregnancy rate and live birth rate of healthy baby monkeys.

## Results

Mature cynomolgus males and females were housed in single-unit cages with perches and other enrichment devices. From the colony, we selected sexually mature female cynomolgus monkeys, whose menstrual cycles were confirmed to be normal by examination of vaginal bleeding for at least 5 cycles (26–34 days per cycle). For those females exhibiting normal menstrual cycles, we used a regimen of exogenous gonadotrophic hormone injections to achieve superovulation. Recombinant human follicle-stimulating hormone was administered as 25 international units (IU) i.v. twice daily for 7 to 8 days, beginning on day 3 or 4 of the menstrual cycle. Human chorionic gonadotrophin (hCG; 1000 IU) was administered after the last injection of recombinant human follicle-stimulating hormone, to achieve final follicular maturation.

We then used laparoscopy to collect the oocytes from anesthetized females. The collected oocytes were assessed for nuclear maturity, and those showing extruded first polar body were selected and matured in Tyrode's medium for approximately 4–6 h until IVF or intracytoplasmic sperm injection (ICSI). A total of 579 oocytes were retrieved from 22 females, with an average of 26.3 ± 10.4 oocytes per cycle. Of these, 174 (30.1%) were classified as metaphase II (M-II) (with first polar body; Fig. 1*A*). Of the remaining oocytes, 210 (36.3%) were classified as germinal vesicle breakdown and 195 (33.7%) as germinal vesicle (Table 1).

A total of 174 M-II oocytes were used for insemination (IVF or ICSI). We collected semen from male monkeys by penile electro-ejaculation. For IVF, we first treated sperm with 1 mmol/liter dibutyryl cyclic adenosine monophosphate (db-cAMP) and 1 mmol/liter caffeine for 30 min to induce hyperactivation before addition to M-II oocytes. We allocated 63 M-II oocytes for IVF and coincubated them with hyperactivated sperm for 12–16 h at 37°C. Twenty-one oocytes were fertilized, for a fertilization rate of 33.3%. Of these 21 embryos, 18 exhibited normal fertilization (85.7%), evident from the formation of 2 distinct pronuclei and extrusion of the second polar body (Fig. 1*C*).

We also allocated 105 M-II oocytes for insemination by ICSI (Fig. 1*B*). This method resulted in 52 fertilized oocytes, for a success rate of 49.5%. Of these 52 embryos, 48 (92.3%) showed normal fertilization. Therefore, these results suggest that the rate of pronuclear formation after ICSI is significantly higher than that after IVF ($P < 0.01$) (Table 2).

It is widely believed that the pregnancy success rate is critically dependent on the synchrony between the ages of the transferred embryos relative to the predicted ovulation day of recipients. To obtain synchronous transfers in the cynomolgus monkey we systematically assessed the receptivity of the recipients by 2 methods: hormone assay and laparoscopic observation. The 66 fertilized pronuclear-stage embryos were tubally transferred to 30 synchronized monkeys at various time points within 1–3 days after recipients exhibited an estradiol (E2) peak. The status of the ovaries was simultaneously observed by laparoscopy (Fig. 2). The total pregnancy rate was 27.6% (8/29), resulting in 7 infants (1 set of twins) and 1 abortion. From the analysis (Table 3), we found that the highest pregnancy rate of 54.5% (6/11) and the live birth rate of ≈64% (7/11) were obtained from the recipient females showing new corpus luteum in their ovaries (Fig. 2*B*). Importantly, there was no pregnancy in the group without stigma or new corpus luteum in their ovaries (Table 3). This suggests that the receptivity of the recipients is optimal at the time of exhibiting new corpus luteum. With the exception of a male baby monkey (from the only twin birth) dying within the first week (natural abandonment), all other baby monkeys grew normally (Fig. 3).

## Discussion

Since the first birth of a rhesus macaque after IVF in the mid-1980s (1), several laboratories have reported successfully obtaining infants of rhesus, cynomolgus monkey, and pigtailed macaque by IVF or ICSI (8–14). However, the success rate has been low in comparison with that for rodents and humans. Interestingly, the birth rate of the cynomolgus monkey by assisted reproduction techniques is even lower than that of other types of monkey. Moreover, according to the literature, all of the embryos transferred were cultured to the 4–8-cell stage or beyond. Unlike rodent and human embryo culture (15, 16), there is still no appropriate culture condition for achieving long-term development of monkey embryos *in vitro*. In rhesus monkey, the developmental percentage for early morula, morula, and blastocyst was 61%, 44%, and 33%, respectively (with supporting culture) (17). Although Ng (12) reported a 100% cleavage rate (40/40) for ICSI embryos *in vitro*, the viability of development to the 4-cell stage was only 65% (26/40). However, in cynomolgus monkey, the rate for *in vitro*-fertilized embryos cultured *in vitro* in the absence of cell support was zero (6, 7). It is likely that embryonic impairment occurs during *in vitro* culture and may contribute to the low birth rate in monkeys. Data from the literature indicate a birth rate of zero for *in vitro*-fertilized cynomolgus monkey blastocysts cultured in the absence of cell support (6, 7), whereas oocytes cultured with cell support and sequential culture medium achieved an approximately 15–20% success rate in producing *in vitro*-fertilized cynomolgus monkey (4, 5).

**Table 2. Percentage of fertilized oocytes after ICSI vs. IVF**

| Fertilization method | No. of M-II oocytes | No. (%) of surviving oocytes | No. (%) of fertilized oocytes | | | |
|---|---|---|---|---|---|---|
| | | | Total fertilized | With 2 PN + 2 PB | With 1 PN + 2 PB | With >3 PN |
| IVF | 63 | 63 (100) | 21 (33.3)* | 18 (28.6) | 2 | 1 |
| ICSI | 111 | 105 (90.3) | 52 (49.5)* | 48 (45.7) | 4 | 0 |
| Total | 174 | 168 | 73 (42) | 66 (37.9) | 6 (3.5) | 1 |

Values are expressed as mean. PN, pronucleus; PB, polar body.
*Statistically significant difference in terms of number between IVF and ICSI methods ($P < 0.01$).

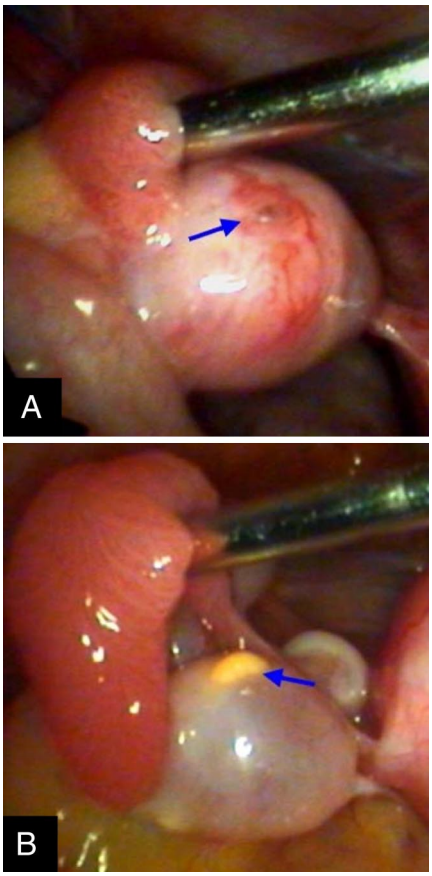


**Fig. 2.** Ovary state of recipient monkey. (*A*) Ovary with a new stigma (arrow). (*B*) Ovary with a new corpus luteum (arrow). The photos were obtained by laparoscopy.

To date there has been no report using a pronuclear embryo transfer technique for the production of *in vitro*-fertilized nonhuman primates, including cynomolgus monkey (1–14). Based on our and other researchers' extensive experiences in producing transgenic mice (15, 18–22), it is reasonable to postulate that transferring embryos at the pronuclear stage can come with the certain benefits, such as decreasing the risk of losing embryo quality during the course of long-term *in vitro* culture. Thus, we performed a set of experiments to investigate whether the pronuclear embryo transfer method can be used for efficient reproduction in primates. As such, we adapted the pronuclear embryo transfer method to the monkey experiments and transferred pronuclear embryos rather than 4-cell or 8-cell embryos into the recipients (23–25). The present study demonstrates the high efficiency of using a pronuclear embryo transfer technique for the production of IVF-derived baby monkeys. The shortening of *in vitro* culture time to the pronuclear, single-cell stage can minimize the potential damage that is typically associated with extensive *in vitro* culture. Therefore, this pronuclear transfer approach should be the preferred method if the timed synchrony in foster mothers can be achieved.

The number of oocytes obtained from superovulation in this study seems not as high as those previously reported in other monkey studies (26). On average, we obtained 26 oocytes per cycle from cynomolgus monkeys, and the percentage of M-II oocytes was approximately 30%. This might be in part because the time course after hCG injection was shorter than 36 h in our experiments. Moreover, we also noted that the fertilization rate of *in vivo* M-II oocytes subjected to ICSI was significantly higher

**Table 3. Summary of embryo transfer experiments with pronuclear embryos after IVF and ICSI in cynomolgus monkey**

| Ovary E2 peak | No stigma | | | | Stigma | | | | New corpus luteum | | | | Total | | | | Birth rate, % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Recipients | Oocytes | Pregnancies | Births | Recipients | Oocytes | Pregnancies | Births | Recipients | Oocytes | Pregnancies | Births | Recipients | Oocytes | Pregnancies | Births | Pregnancy rate, % |
| 24 h | 3 | 9 | 0 | 0 | 9 | 17 | 1 | 1 | 1 | 3 | 1 | 1 | 13 | 29 | 2 | 2 | 15.4 | 15.4 |
| 48 h | 1 | 2 | 0 | 0 | 2 | 3 | 1 | 1 abortion | 5 | 13 | 3 | 4 | 8 | 18 | 4 | 4 | 50 | 50 |
| 72 h | 1 | 1 | 0 | 0 | 2 | 5 | 0 | 0 | 5 | 11 | 2 | 2 | 8 | 17 | 2 | 2 | 25 | 25 |
| Total | 5 | 12 | 0 | 0 | 13 | 25 | 2 | 1 | 11 | 27 | 6 | 7 | 29 | 64 | 8 | 8 | 27.6 | 27.6 |
| % | | | | | | | 15.4 | 7.7 | | | 54.5 | 63.6 | | | | | | |

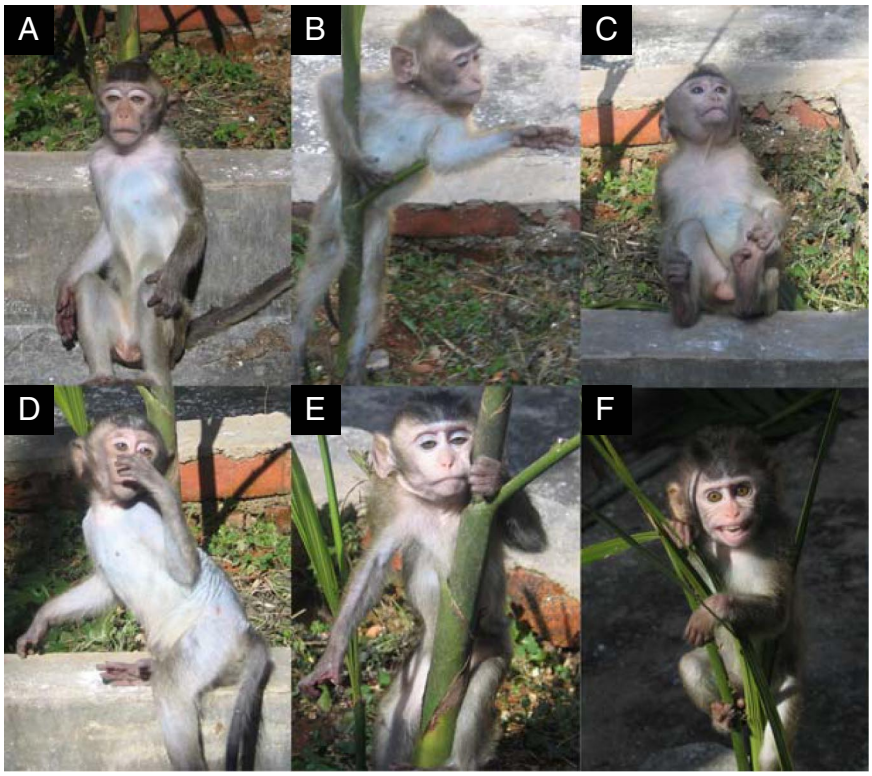


**Fig. 3.** Test-tube baby monkeys derived from pronuclear embryo transfer. (*A*) The first test-tube baby monkey was born on February 4, 2007 and was 1 year old at the time of being photographed. (*B*) The second test-tube monkey at age 37 weeks. (*C*) The third test-tube monkey at age 35 weeks. (*D*) The fourth test-tube monkey at age 32 weeks. (*E*) The fifth test-tube monkey at age 26 weeks. (*F*) The seventh test-tube monkey at age 24.5 weeks.

than that with IVF (49.5% vs. 33.3%, $P < 0.01$). This is consistent with observations made in humans (27, 28).

It is crucial to identify the optimal time window in which one can increase the chance for transferred embryos to develop *in vivo*. We find that synchronization of the development of embryos with proper timing of the ovulation of the recipient is a crucial procedure. Follicular ovulation in primate is known to be related to change of hormones. The M-II-arrested oocytes ovulate ≈12 h after the surge of leuteinizing hormone (29), which typically occurs 24 h before the E2 peak. Thus, we estimated ovulation by assaying the E2 peak in blood serum. Our observations suggest that the surge of E2 combined with the presence of corpus luteum is a good index for timing the transfer of pronuclear embryos. Transfer of pronuclear embryos at 24–36 h after E2 peak in the presence of new corpus luteum in the ovary resulted in a live birth rate of 64% in this study, significantly higher than in previous reports on cynomolgus monkey (12) and rhesus monkey (24).

In conclusion, we have shown that by identifying the critical time window in the recipient's reproductive cycle for achieving optimal embryo-uterine synchrony, pronuclear embryo transfer is an efficient method for increasing the birth rate of IVF-derived monkeys. This method should be valuable for systematic construction and establishment of various transgenic primate disease models.

## Materials and Methods

**Animals.** Sexually mature cynomolgus males and females were purchased from Yunnan Primate Experimental Animals and housed at the Yunnan Banna Primate Disease Model Research Center. They were individually caged in a temperature-controlled (24°C), light-regulated (12 h light/12 h dark) room and fed commercial nonhuman primate diets twice daily, supplemented with fresh fruits and water ad libitum.

**Superovulation.** Hyperstimulation of female cynomolgus monkeys exhibiting normal menstrual cycles (26–34 days for at least 5 sequential cycles) was induced by a regimen of exogenous gonadotrophic hormones. Recombinant human follicle-stimulating hormone (rhFH) (Gonal-F; Serono) was administered as 25 IU twice daily i.v. for 7 or 8 days, beginning on day 3 or 4 of menses. Human chorionic gonadotrophin (1000 IU) (Sigma) was administered after the last rhFSH injection to achieve final follicular maturation.

**Oocyte Collection.** The oocytes were collected by laparoscopy. In brief, 30–36 h after the hCG injection, the female was anesthetized. Recovery of the oocytes was performed with a laparoscope (Olympus) attached to a video system. The oocytes were aspirated from follicles 2–8 mm in diameter into a container with warmed Hepes-buffered Tyrode's lactate medium supplemented with 100 IU/ml penicillin and 100 $\mu$g/ml streptomycin (TH3 medium) with 10 IU/ml heparin using a 19-gauge needle attached to the Cook aspiration system. All oocytes treated with 1 mg/ml hyaluronidase (Sigma) were retrieved from aspirates using a 70-$\mu$m mesh size EM Con filter (ImmunoSystems). The collected oocytes were immediately assessed for nuclear maturity under an inverted microscope. Those in which the first polar body was extruded were selected and matured in Tyrode's lactate medium with 100 IU/ml penicillin and 100 $\mu$g/ml streptomycin (T3 medium) in an incubator at 37°C in a humidified 5% $CO_2$ for 4–6 h until IVF or ICSI.

**Semen Collection and Processing.** Semen was collected from males by penile electro-ejaculation as described in ref. 30. Briefly, ejaculated semen was liquefied at 37°C for 15–20 min. Liquid sperm was washed twice with TH3 medium and diluted to $1 \times 10^7$ with T3 medium, then incubated in an incubator at 37°C in humidified 5% $CO_2$ for 3 to 4 h. For IVF, sperm were treated with 1 mmol/liter dbcAMP (Sigma) and 1 mmol/liter caffeine to induce hyperactivation for 30 min before coincubation with oocytes.

***In Vitro* Fertilization.** Sperm ($1 \times 10^6$) were coincubated with oocytes in T3 medium for 12–16 h at 37°C and 5% $CO_2$. Sperm were then removed manually by pipetting through a finely pulled glass pipette. Oocytes were cultured in T3 medium with 10% FBS and examined at 400× magnification to conform the formation of pronuclei.

**Intracytoplasmic Sperm Injection.** Denuded M-II oocytes were transferred to a 30-$\mu$l T3 droplet and sperm were transferred to a 30-$\mu$l 10% polyvinylpyrrolidone (Sigma) droplet on the injection dish. ICSI was conducted using a DMIRE-2 inverted microscope (Leica Microsystems) with attached micromanipulators. Briefly, a single sperm was immobilized by crushing the mid-piece with the tip of the injection pipette (Cook Ireland). The immobilized sperm was aspirated with the tail first. Thereafter, the injection pipette was moved into the T3 droplet. A single oocyte was fixed by the holding pipette, fixing the polar body at the 6 o'clock or 12 o'clock position. The injection pipette was pushed through the zona pellucida and subsequently through the oolemma into the cytoplasm at the 3 o'clock position. A small amount of ooplasm was aspirated into the injection pipette to ensure oocyte membrane penetration. Subsequently the immobilized spermatozoon was released into the cytoplasm. The temperature was maintained at 37°C throughout the procedure. After ICSI, the oocytes were placed in T3 medium with 10% FBS. At 6 to 7 h after injection, the oocytes were examined at 400× magnification to confirm the formation of pronuclei. All of the culture medium was overlaid with light mineral oil that had been equilibrated overnight.

**Hormone Assay and Embryo Transfer.** Ovulation in the menstrual cycle of the females was estimated by measuring the serum concentrations of E2 using a commercially available enzyme immunoassay kit (Cayman Chemical). Blood samples were taken in the morning, and serum separated by centrifuge was frozen until the measurement was taken. The day of ovulation was estimated as the day that the E2 concentration significantly decreased. To synchronize the developmental stage of embryos with the menstrual cycle of the recipient, tubal embryo transfer was performed 0–3 days after ovulation according to E2 peak. At the same time, laparoscopic observations were made to investigate a stigma or a new corpus luteum on the ovary (Fig. 2*A* and *B*).

Females were anesthetized and received abdominal incision. Embryos at the pronuclear stage were selected for tubal transfer. Embryos were picked from the culture dishes into a microglass capillary containing 1 to 2 $\mu$l of culture medium and transferred surgically into one oviduct through the infundibulum. The microglass capillary containing the embryos was introduced through the fimbriated end of the fallopian tube; 2 to 3 embryos were transferred into the mid-ampullary portion of the oviduct. The pregnancy was carefully monitored all the way through to natural delivery.

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