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# Germ cell nuclei of male fetal mice can support development of chimeras to midgestation following serial transplantation

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## SUMMARY

**Chimeric embryos between fertilized eggs from $F_1$ (C57BL×CBA) and 15.5-16.5 days post coitum (dpc) male fetal germ cells (FGCs) from CD-1 strain (glucose phosphate isomerase, Gpi-1a/a) mice were produced by nuclear transfer. Briefly, a single FGC was fused with enucleated oocytes and activated, and the reconstituted oocytes were cultured to the 2-cell stage. The nucleus from the reconstituted 2-cell embryos was then transferred into an enucleated blastomere of the same stage embryos derived from $F_1$ mice to produce chimeric embryos. The reconstituted 2-cell embryos, which synchronously divided to the 4-cell stage after treatment with nocodazole, were further cultured in vitro. Compacted morula and blastocysts were transferred to the uteri of pseudopregnant female mice. Some recipients were allowed to develop to term and the others were killed at mid gestation to analyze the contribution of donor FGC-derived cells. Survival to term was low with no chimeric animals. Glucose phosphate isomerase (GPI) analysis at midgestation revealed that some conceptuses had chimerism in the fetuses, trophoblast and yolk sac at day 10.5 of pregnancy. The contribution of donor cells was 37-47%, 19-65% and 12-63%, respectively. It was concluded that the nucleus from 15.5-16.5 dpc male fetal germ cells had the potency to develop into fetus, trophoblast and yolk sac after serial nuclear transfer with oocytes and fertilized embryos. The reason for the low viability of chimeric embryos is discussed.**

Key words: fetal germ cells, chimerism, nuclear transfer, mouse, serial transplantation

## INTRODUCTION

In mice, chimeric animals have been produced mainly by aggregation of embryonic cells with precompacted embryos (Tarkowski, 1961; Tokunaga and Tsunoda, 1992), injecting them into blastocysts (Gardner, 1968) or by nuclear transfer of their nuclei into 2-cell-stage embryos (Kono et al., 1989). ICM cells isolated from 3.5 and 4.5 day blastocysts can form adult chimeric animals following injection into blastocysts or aggregation with 8-cell-stage embryos (Gardner, 1970). The 5.5- to 7.5-day embryonic or extraembryonic endoderm or ectoderm cells formed chimeric conceptuses after injection into blastocysts (Rossant et al., 1978; Cockroft and Gardner, 1987). Recently, stem cells derived from primordial germ cells (embryonic germ cells, EG cells) at day 8.5 were established in vitro and the EG cells could produce chimeric animals following injection into blastocysts (Matsui et al., 1992), as in the case of embryonic stem cells (ES cells). However, it is not clear whether primordial germ cells (PGCs) or fetal germ cells (FGCs) isolated from fetuses without exposure to in vitro culture have a potency to produce chimeras. So far, we aggregated FGCs isolated from 12.5-day to 16.5-day fetuses with 8-cell embryos (Kato and Tsunoda, 1991) or transferred them to enucleated blastomeres of 2-cell-stage embryos (Kato and Tsunoda, 1992a), but no chimeric mice were obtained after transfer to recipients despite the age and sex of FGCs used in the experiments. It is known that embryonic nuclei transplanted into enucleated oocytes could be reprogrammed to some extent. Male fetal germ cells fused with enucleated oocytes lost their nuclear membrane immediately after fusion, and showed premature chromosome condensation by maturation promoting factor (MPF) in the cytoplasm, reformed nuclear membrane after activation, duplicated DNA and showed normal diploid chromosome constitution (Tsunoda et al., 1989, 1992). So far no fetuses were obtained at 10.5 to 13.5 days of pregnancy after transfer of reconstituted eggs receiving male FGCs, but they developed to blastocyst stage in vitro with a time course similar to that of fertilized eggs (Tsunoda et al., 1989, 1992 and unpublished observation). The present study examined whether nuclei of FGCs isolated from gonads of male fetuses at 15.5 days of pregnancy formed chimeras when they were reprogrammed in the enucleated oocytes.

## MATERIALS AND METHODS

### Preparation of donor and recipient cells

Donor male fetal germ cells (FGCs) were obtained from albino CD-1 strain female mice (Gpi-$1^{a/a}$) mated with the same strain males at 15.5-16.5 days post coitum (dpc), according to the procedures of Brinster and Harstad (1977), Felici and McLaren (1983), Hogan et al. (1986) and Pesce et al. (1993). The cell cycle of FGCs at this age was $G_0$ phase (Monk and McLaren, 1981). Isolated FGCs were stored in M2 (Fulton and Whittingham, 1978) medium supplemented with 10%

fetal bovine serum (FBS) at room temperature until use. Recipient oocytes were collected from superovulated $F_1$ (C57BL/6×CBA, Gpi-$1^{b/b}$) females 14-19 hours after injection with human chorionic gonadotrophin (hCG) and the 2-cell embryos were recovered from $F_1$ females mated with the same strain males 43 hours after injection of hCG. In some cases, 2-cell embryos recovered from CD-1 females mated with same strain males were used for the donor nuclei of the control experiments. The oocytes were denuded by treatment with hyaluronidase (300 i.u./ml) in M2 medium and washed three times with M2 medium.

### Nuclear transfer into enucleated oocytes

Nuclear transfer technique was based on the report of McGrath and Solter (1983) with some modifications. Before nuclear transfer, the zonae pellucidae of oocytes and embryos were partially cut (Tsunoda et al., 1986) and pretreated with or without cytochalasin B (CB) and nocodazole according to the previous reports (Tsunoda et al., 1992). Removal of oocyte nuclei at the MII chromosome stage was performed under a fluorescence microscope after staining with Hoechst 33342 (Tsunoda et al., 1988). Successfully enucleated oocytes were fused with a single FGC by inactivated Sendai virus (HVJ, 2500 haemagglutinating activity unit). Nuclear transferred oocytes were cultured with M16 medium (Whittingham, 1971) with or without CB (5 μg/ml) for 20 minutes before electrical stimulus to induce parthenogenetic activation (DC pulse 150V for 50 microseconds). Stimulation was repeated two more times using a 50 V DC pulse for 50 microseconds at 20 minute intervals (Tsunoda and Kato, 1993). Reconstituted oocytes were examined for nuclear formation 5-6 hours after incubation.

### Nuclear transfer into enucleated 2-cell embryos for producing chimeric embryos

The reconstituted oocytes forming one nucleus were cultured to the 2-cell stage with M16 medium overnight. Fig. 1 shows the experimental design. One nucleus of reconstituted 2-cell embryos was fused with the enucleated blastomere of fertilized 2-cell-stage embryos (from $F_1$ females mated with $F_1$ males) by HVJ. The remaining blastomere of the 2-cell embryos was left intact (A). Controls were (1) 2-cell embryos (from $F_1$ females mated with $F_1$ males) with one blastomere was enucleated (B) and (2) 2-cell embryos in which one blastomere was enucleated and fused with one nucleus of fertilized 2-cell embryos from CD-1 females (C).

### Treatment with nocodazole and in vitro culture

The chimeric 2-cell embryos that successfully fused and control embryos were stored at 4°C for several hours for experimental convenience and then cultured with M16 medium supplemented with nocodazole (3 μg/ml) for 14 hours to synchronize the cell division to the 4-cell stage according to previous reports (Kato and Tsunoda, 1992b, 1993). There was no effect of nocodazole treatment on the developmental ability of 2-cell mouse embryos in vitro and after transfer to recipients. Briefly, embryos that ceased development at the 2-cell stage during culture were washed with M2 medium three times and cultured with M16 medium for 1-1.5 hours. Embryos that synchronously divided to the 4-cell stage were cultured further in vitro for 2 days, whereas 3-cell and 2-cell embryos were discarded. However, 3-cell embryos were selected in control 1 for further culture since the nucleus of one blastomere had been removed.

### Transfer to pseudopregnant females

Compacted morulae or blastocysts were transferred to the day 3 uteri of pseudopregnant females. CD-1 recipient females were allowed to complete to term or Caesarean operation at 19 days p.c. and $F_1$ females (Gpi-$1^{b/b}$) were killed at mid gestation for analysis of the contribution of the donor cells demonstrated below. The contribution of FGCs to the offspring was assessed visually by eye and by coat color. The young were mated with CD-1 strain mice several times to assess further the contribution to the germ line.

### Analysis of the chimerism at mid gestation

Glucose phosphate isomerase (GPI) was used as a marker for donor cells (Gpi-$1^{a/a}$) and analyzed according to the procedures of Eicher and Washburn (1978) and Mikami and Onishi (1985). Briefly,

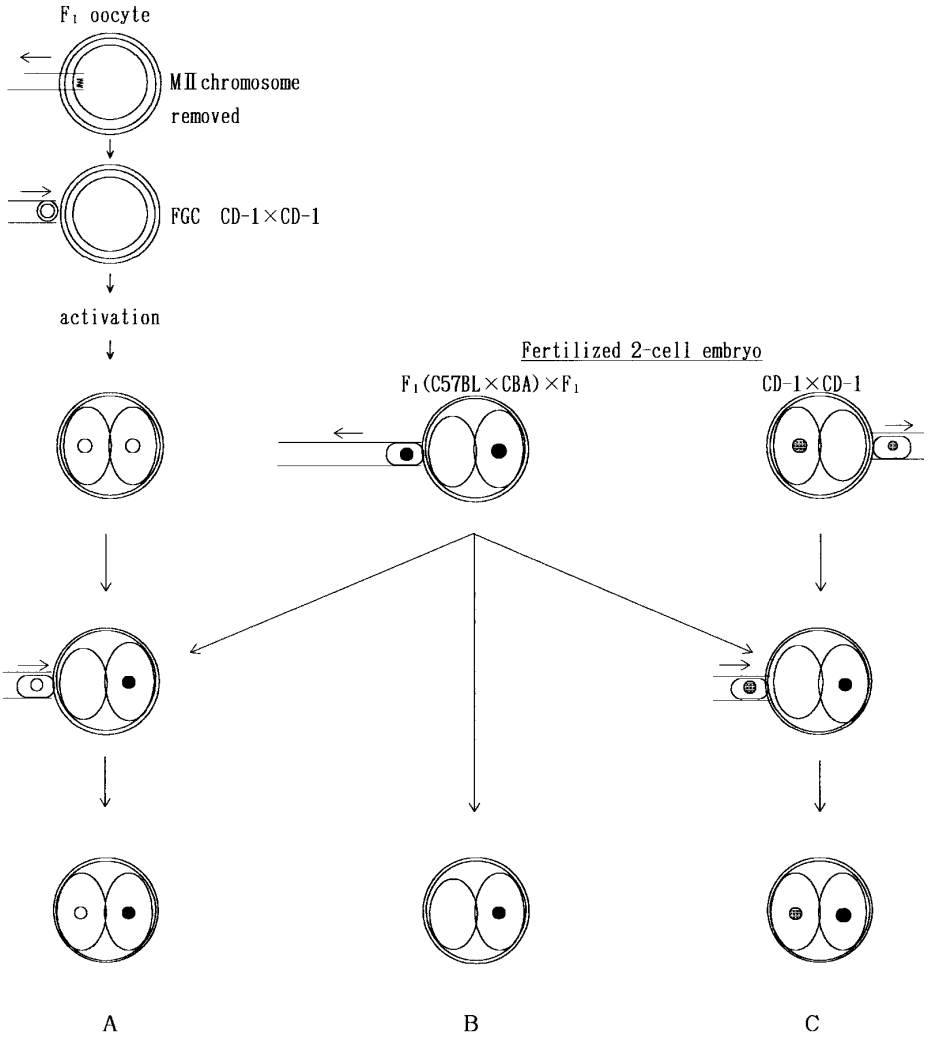


**Fig. 1.** Scheme of experimental design. (A) Chimeric embryos with nucleus from FGC; (B) 2-cell embryos whose nucleus was removed (control 1); (C) Chimeric embryos with nucleus from 2-cell embryo (control 2). Nucleus of $F_1$ fertilized embryos (●), CD-1 fertilized embryos (◍) and CD-1 fetal germ cells (○).

**Table 1. In vitro development of reconstituted chimeric embryos**

| | No. of embryos cultured | Developed to (%) | |
|---|---|---|---|
| | | 4-cell | Morula-blastocyst |
| Reconstituted | 333 | 249 (75)[a] | 192 (58)[a] |
| Control 1 | 101 | 101 (100)[b]* | 98 (97)[b] |
| Control 2 | 37 | 37 (100)[b] | 33 (89)[b] |

[a-b]$P<0.05$.
*Development to 3-cell stage.

**Table 2. In vivo development of reconstituted chimeric embryos**

| | No of embryos transferred | No. of recipients | | No. of young | |
|---|---|---|---|---|---|
| | | Total | Pregnant | Total | Chimera |
| Reconstituted | 63 | 8 | 1 | 2 (3)[a] | (0) |
| Control 1 | 98 | 7 | 7 | 24 (24)[b] | – |
| Control 2 | 33 | 3 | 3 | 15 (45)[b] | 7 (47) |

[a-b]$P<0.05$.

recipient females (Gpi-$1^{b/b}$) were killed at 10.5 or 12.5-13.5 days p.c. and the embryos, yolk sacs and trophoblasts were dissected and homogenated in 20 volumes of distilled water at 4°C, respectively. Electrophoresis was performed on Titan III Iso-vis cellulose acetate plates (Helena Laboratories, USA) with phsophate buffer (pH 6.8) at 160 V for 1 hour. After electrophoresis, the plates were affixed to plates impregnated with staining mixture (Eicher and Washburn, 1978). The proportion of chimerism was estimated with a densitometer (Auto Scanner FIUR-VIS, Helena Laboratories, USA).

### Statistical analysis

The data were analysed by $\chi^2$ analysis.

## RESULTS

### Developmental ability of reconstituted chimeric embryos in vitro

After transfer of fetal germ cells into enucleated oocytes, 47% of nuclear transferred oocytes (367/774) formed nuclei and 93% of those developed to the 2-cell stage. A few oocytes emitted a polar body (17/367, 5%) and cleaved (13/17, 76%), but they were not used for further study due to suspected chromosomal abnormality. Table 1 shows the results of in vitro development of the reconstituted chimeric embryos. 333 chimeric embryos were successfully produced from 316 2-cell embryos that did not emit a polar body. After treatment with nocodazole, 75% of them synchronously cleaved to the 4-cell stage and 58% developed to the morula and blastocyst stage. All the control embryos cleaved once to the 3-cell stage in control 1 or to the 4-cell stage in control 2 after elimination from nocodazole treatment. 97% or 89% of embryos in control 1 or 2 developed to the morula and blastocyst stage, respectively. There were significant differences in the developmental ability of embryos in vitro between chimeric and control groups ($P<0.05$).

### Developmental ability of reconstituted chimeric embryos in vivo

Table 2 shows the results of in vivo development of reconstituted chimeric embryos after transfer to the pseudopregnant recipient female mice. When 63 blastocysts with normal morphological appearance were transferred to 8 recipients, one became pregnant and two non-chimeric young (3%, 2/63) were obtained. This proportion was significantly lower than those of both control 1 (24/98, 24%) and control 2 (15/33, 45%). The 2 young males were each mated with CD-1 female mice twice, but no contribution of donor cells was observed in the germ line.

### Contribution of FGCs derivation in conceptus at mid gestation

It was assumed from the above result that derivation from FGCs would hinder normal development in the chimeric embryos. Therefore, the contribution of donor cells at mid gestation was examined. Development of chimeric embryos at mid gestation is shown in Table 3. When recipients were dissected at 10.5 days of pregnancy, conceptuses (66%) were obtained, and 53% of them had normal size fetuses and 17 (45%) exhibited retarded development, including 1 dead fetus. When recipients were analysed at 12.5-13.5 days p.c., 60% conceptuses (15/25) were obtained and absorptions were frequently observed (47%). The results of analysis of GPI type of these conceptuses are shown in Table 4. The GPI type of all conceptuses at 12.5 day p.c. was BB, which indicates no contribution of FGCs. However, GPI analysis showed that 2 of 20 normal fetuses (10%), 4 of 16 trophoblasts (25%) and 6 of 19 yolk sacs (32%) of conceptuses at 10.5 days p.c. had an A band in addition to the B band. Moreover, 4 of 16 retarded conceptuses showed chimerism. The proportion of the contribution of FGCs in chimeric conceptuses by GPI analysis is shown in Fig. 2. The code number corresponds to that in Table 4. The development of conceptuses 1-7 were normal and the proportions of FGCs contribution in fetuses, trophoblasts and in yolk sacs were 37 and 47%, 19-65% and 8-63%, respectively. The development of conceptuses 8-11 were retarded at the egg cylinder stage. Donor contribution was 34-100%.

**Table 3. Development of chimeric embryos at the mid gestation**

| Days of pregnancy | No. of pregnant recipients (%) | No. of embryos transferred in pregnant recipient | No. of conceptus (%) | | | |
|---|---|---|---|---|---|---|
| | | | Total | Normal | Retarded | Absorptions |
| 10.5 | 8/9 (89) | 64 (58) | 38 (66) | 20 (53) | 17 (45) | 1 (2)[a] |
| 12.5-13.5 | 6/10 (60) | 48 (25) | 15 (60) | 5 (33) | 3 (20) | 7 (47)[b] |

[a-b]$P<0.05$.

**Table 4. Chimerism of embryos**

| Tissue | Days of pregnancy | No. of conceptuses (%) Analyzable | Chimeric | Chimera code number |
|---|---|---|---|---|
| Fetus | 10.5 | 20 | 2 (10) | 1, 2 |
| | 12.5 | 5 | 0 | |
| Trophoblast | 10.5 | 16 | 4 (25) | 1, 2, 3, 4 |
| | 12.5 | 5 | 0 (0) | |
| Yolk sac | 10.5 | 19 | 6 (32) | 1, 3, 4, 5, 6, 7 |
| | 12.5 | 5 | 0 (0) | |
| Retarded* | 10.5 | 16 | 4 (25) | 8, 9, 10, 11 |
| | 12.5 | 3 | 0 (0) | |
| Absorption | 10.5 | 1 | 0 (0) | |
| | 12.5 | 2 | 0 (0) | |

*Egg cylinder stage.

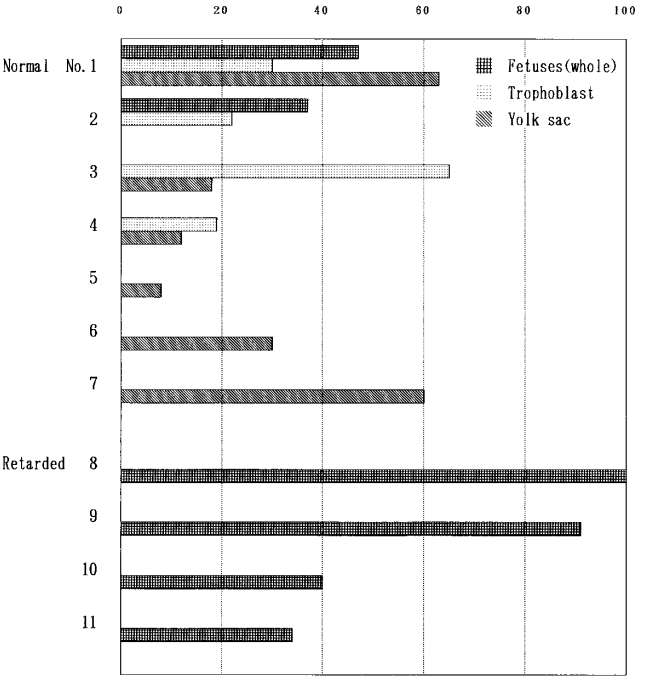


**Fig. 2.** Chimerism of conceptuses. The code number corresponds to that in Table 4. Conceptuses were examined at day 10.5. Nos. 1 to 7 were normal and Nos. 8 to 11 were retarded at the egg cylinder stage.

## DISCUSSION

In the present study, we obtained chimeric fetuses on day 10.5 p.c. after transfer of chimeric embryos produced with 15.5 to 16.5 dpc male fetal germ cells by serial nuclear transplantation. Furthermore, a fetal germ cell derived contribution was also found in trophoblasts and yolk sacs of conceptuses on day 10.5 p.c. If pluripotency means the ability to produce chimeras, this is the first report that demonstrates the nuclear pluripotency of mouse fetal germ cells, although in a very restricted sense. Since 2-cell embryos, from which the nucleus from one blastomere had been removed and which received a fetal germ cell at 11.5-16.5 days, divided at a very low frequency (Kato and Tsunoda, 1992a), cytoplasm or nucleus of 2-cell embryo seemed to have no beneficial effect on the developmental potential of FGCs. Enucleated unfertilized oocytes fused with male fetal germ cells, especially small ones, developed to blastocysts with the same time course as zygotes (Tsunoda et al., 1989, 1992). In that system, the nuclei of fetal germ cells at the $G_0$ stage showed premature chromosome condensation immediately after fusion with enucleated oocytes. After parthenogenetic activation, they formed nuclear membranes and duplicated chromosomal DNA before division to the 2-cell stage. So far, no fetuses have been obtained after transfer of reconstituted eggs with FGCs although implantation sites were observed at 10.5-13.5 dpc (Tsunoda et al., 1989, 1992 and unpublished observation). From this information, it is possible that nuclear pluripotency of FGCs is induced by the reprogramming in the oocytes cytoplasm and intensified by the adjacent cells originating from fertilized embryos. In a preliminary study, the division of enucleated blastomeres of 2-cell fertilized embryos, fused with a nucleus from 2-cell embryos originating from oocytes receiving a FGC, was delayed compared with that of an intact blastomere originating from fertilized embryos. Such chimeric embryos showed abnormal development in vitro. So, chimeric 2-cell embryos were treated with nocodazole to synchronize cell divisions to the 4-cell stage without decreasing the viability of embryos (Kato and Tsunoda, 1992b).

Analysis at mid gestation suggested that some lethality must occur prior to day 10.5 since retarded conceptuses were obtained even at that age. The reason why the chimeric blastocysts died at mid gestation was not clear. One explanation might be inappropriate imprinting of male fetal germ cells. We did not examine the developmental ability of chimeric embryos with female germ cells as a control in this study, since enucleated oocytes receiving female germ cells isolated from fetuses 12.5-17.5 dpc did not develop beyond 4-cell stage (1 of 141 oocytes developed to 4-cell and none to blastocyst, Tsunoda unpublished observation). Although the exact reason for the low developmental ability of reconstituted oocytes with female germ cells was not clear, abnormal DNA duplication after fusion with enucleated oocytes might be one reason since most of them were at the prophase of the first meiotic division. Nagy et al. (1990, 1993) demonstrated that completely ES-cell-derived mice were obtained after aggregation with tetraploid embryos, but the aggregated embryos exhibited high mortality in utero and at birth, which might be due to a loss of appropriate genetic imprints in subpopulations the ES cells used (Nagy et al., 1993). Surani et al. (1988) examined the development of aggregated chimeric embryos with parthenogenetic and androgenetic embryos. Although no androgenetic cells were detected in young, they were observed in one dead embryo at day 10 p.c. and with greater frequency in yolk sacs and trophoblasts. However, there was no evidence that androgenetic cells were deleterious to the development of chimeric embryos since the proportion of young after transfer of aggregated 4-cell androgenones with fertilized 2-cell embryos was no different from control embryos. Mann and Stewart (1991) reported that 129/sv androgenetic aggregation chimeras could occasionally develop to term. The rate of development to term of chimeric embryos was not particularly low (24%), but the presence of androgenetic cells in the extraembryonic tissue had a deleterious effect on development of embryos. Skeletal abnormalities in chimeric fetuses were also reported in blasto-

cyst injection chimeras produced with androgenetic embryonic stem cells of the 129/sv strain (Mann et al., 1990). Since such abnormalities were not observed in adult chimeras produced by aggregation with parthenogenetic eggs (Paldi et al., 1989), the differences observed between androgenetic and parthenogenetic chimeras might be due to paternal imprinting in androgenetic eggs and embryonic stem cells. Although we did not examine the details of the morphology of fetuses obtained after transfer of chimeric embryos, obvious abnormalities were not observed.

The other explanation is that chromosomal abnormalities of 2-cell embryos developed from oocytes fused with FGC decreased the viability of chimeric embryos. Although they had 40 normal chromosomes (Tsunoda et al., 1992 and in the present study), it was not clear whether all the chromosomal DNA originating from fetal germ cells at the $G_0$ stage could be normally duplicated in the cytoplasm of oocytes. The other possibility is the incompatibility between the nucleus of 2-cell embryo originating from FGC and 2-cell embryo cytoplasm originating from fertilized embryo.

Since Mann and Stewart (1991) reported that the developmental potential of androgenetic aggregation chimeras was dependent on mouse strain, further studies are clearly necessary to examine the developmental ability of chimeric embryos, not only with male but also female germ cells from other strains of mice.

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