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# Development of rat tetraploid and chimeric embryos aggregated with diploid cells

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## Summary

In the present study, we examined the preimplantation and postimplantation development of rat tetraploid embryos produced by electrofusion of 2-cell-stage embryos. Developmental rate of tetraploid embryos to morula or blastocyst stage was 93% (56/60) and similar to that found in diploid embryos (95%, 55/58). After embryo transfer, rat tetraploid embryos showed implantation and survived until day 8 of pregnancy, however the conceptuses were aberrant on day 9. In mouse, tetraploid embryos have the ability to support the development of blastomeres that cannot develop independently. As shown in the present study, a pair of diploid blastomeres from the rat 8-cell-stage embryo degenerated immediately after implantation. Therefore, we examined whether rat tetraploid embryos have the ability to support the development of 2/8 blastomeres. We produced chimeric rat embryos in which a pair of diploid blastomeres from an 8-cell-stage green fluorescent protein negative (GFP−) embryo was aggregated with three tetraploid blastomeres from 4-cell GFP-positive (GFP+) embryos. The developmental rate of rat $2n$(GFP−) ↔ $4n$(GFP+) embryos to the morula or blastocyst stages was 93% (109/117) and was similar to that found for $2n$(GFP−) ↔ $2n$(GFP+) embryos (100%, 51/51). After embryo transfer, $2n$(GFP−) ↔ $4n$(GFP+) conceptuses were examined on day 14 of pregnancy, the developmental rate to fetus was quite low (4%, 4/109) and they were all aberrant and smaller than $2n$(GFP−) ↔ $2n$(GFP+) conceptuses, whereas immunohistochemical analysis showed no staining for GFP in fetuses. Our results suggest that rat tetraploid embryos are able to prolong the development of diploid blastomeres that cannot develop independently, although postimplantation development was incomplete.

Keywords: Embryo, Preimplantation development, Postimplantation development, Rat, Tetraploid

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## Introduction

Tetraploid mouse embryos are produced either by the inhibition of cleavage at an early stage or by fusion of blastomeres. Inhibition of cleavage, in order to cause duplication of the genome without cell division, has been accomplished using inhibitors of cytokinesis, such as colchicines (Edwards, 1958) and cytochalasin B (Snow, 1973; Tarkowski *et al.*, 1977). In addition, two diploid blastomeres can be fused using polyethylene glycol (Eglitis, 1980; Spindle, 1981), inactivated Sendai virus (O'Neill *et al.*, 1990) or electrofusion (Berg,

1982; Kubiak & Tarkowski, 1985). Stimulation of 2-cell embryo fusion by electrical pulses appears to be the most efficient method and has been used to produce tetraploid embryos in a number of species including mouse (Kubiak & Tarkowski, 1985), rabbit (Ozil & Modlinski, 1986), cow (Iwasaki *et al.*, 1989; Curnow *et al.*, 2000), pig (Prather *et al.*, 1996; Prochazka *et al.*, 2004) and rat (Krivokharchenko *et al.*, 2002).

Although preimplantation development of electrofusion-generated tetraploid embryos has been examined in a number of species, the results of post-implantation development have been limited. In the mouse, the most advanced viable homogenously tetraploid embryo has been recovered on the 15th day of gestation and its morphological features suggest that it was developmentally equivalent to a normal embryo of around 13.5 to 14 days *post coitus*. (Kaufman & Webb, 1990). In contrast, fused tetraploid rabbit embryos generated by an electronic pulse can develop normally at least two-thirds of the way through pregnancy, whereas rabbit embryos electrofused at the 2-cell stage display occasional mosaic preimplantation development (Ozil & Modlinski, 1986).

It has been reported that most mouse tetraploid cells do not contribute to the inner cell mass (ICM) in chimeric embryos with diploid cells at the blastocyst stage, although tetraploid cells tended to colonize in the trophectoderm (Everett & West, 1996, 1998; Everett *et al.*, 2000). Furthermore, during postimplantation, although diploid cells contribute to both embryonic and extraembryonic lineages, most tetraploid cells contributed to the extraembryonic lineage (Tarkowski *et al.*, 1977; Nagy *et al.*, 1990; James *et al.*, 1995; Wang *et al.*, 1997; Tang & West, 2000; Goto *et al.*, 2002). Indeed, the aggregation of tetraploid embryos with cells involved in embryonic stem (ES) cells that could not develop independently and injection of these into tetraploid blastocysts allowed the production of mice (Nagy *et al.*, 1993; Ueda *et al.*, 1995; Wang *et al.*, 1997). Furthermore, in mouse, aggregation of 1/4 and/or 2/8 blastomeres with 2-, 3- or 4-cell carrier tetraploid embryos allowed development to normal and fertile adults, although mouse single 'quarter' blastomeres were no longer totipotent, because they were not able to develop independently into mice (Tarkowski *et al.*, 2001).

In rat, recently, electrofused blastomeres of rat 2-cell embryos were reported to develop to the blastocyst stage *in vitro* as homogenously as tetraploid embryos (Krivokharchenko *et al.*, 2002). However, postimplantation development of rat tetraploid embryos has not been determined. Moreover, it has not been assessed whether rat tetraploid embryos have the ability to support the development of diploid cells that cannot develop independently.

In the present study, we examined the pre-implantation and postimplantation development of rat tetraploid embryos produced by electrofusion of 2-cell-stage embryos. We also examined whether rat tetraploid embryos have the ability to support the development of blastomeres that cannot develop independently.

## Materials and methods

### Animals

Wistar Imamichi rats were purchased from Japan SLC and bred in our laboratory. GFP-transgenic rats with a CAG (cytomegalovirus enhancer, chicken actin enhancer–promoter and rabbit globin polyA signal) promoter were developed from Wistar rats (Hakamata *et al.*, 2001; Takeuchi *et al.*, 2003). Lights were switched on between 9:00 and 21:00 daily. The estrus cycle of females was monitored by daily examination of vaginal smear. Rats with at least three consecutive regular cycles were used in this study. At proestrus, females were caged with males for mating and presence of vaginal plugs and/or sperm in the vaginal smear the next morning was taken to indicate the first day of pregnancy. All procedures adhered to the *Guide for the Care and Use of Laboratory Animals* published by Tohoku University.

### Embryo collection and culture

Embryos were collected as previously described (Matsumoto *et al.*, 2002*a*). Early rat 2-cell embryos were collected from mated females at 12:00 on day 2 of pregnancy and were then cultured in 100 µl droplets of mR1ECM medium (Miyoshi *et al.*, 1997) overlaid with liquid paraffin (Nacalai Tesque) in a humidified atmosphere of 5% $CO_2$ in air at 37 °C.

### Production of tetraploid embryos

For production, 2-cell-stage embryos were placed between the electrodes of a fusion chamber filled with 0.3 M mannitol containing 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$ and 0.1 mg/ml polyvinyl alcohol at 37 °C. The fusion of 2-cell embryos was induced by an alternating current field (12 V, 500 kHz, 15 s) and two direct current pulses of 1.20 V/cm for 70 µs with a 1 s interval between pulses. The embryos were then placed in a culture droplet and checked every 10 min. Embryos usually fused within 20 min.

### Examination of the karyotype of the diploid or tetraploid blastocysts

The karyotype of the diploid or tetraploid blastocysts was examined as described previously with slight modifications (Tarkowski, 1966). Briefly, blastocysts

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were incubated in 0.4 μg/ml demecolcine (Sigma) for 6 h to synchronize the blastocyst cells at metaphase. Next, the blastocysts were cultured in hypotonic solution consisting of 25% culture medium and 75% deionized water for 1 h. Cells were then spread and fixed with glacial acetic acid. Chromosome slides were prepared by air drying and stained with Giemsa. We only examined embryos showing more than five metaphase spread cells to define as either diploid or tetraploid embryo.

### Production of two blastomeres in a zona pellucida derived from 8-cell-stage embryos

All micromanipulation procedures were carried out in mR1ECM medium buffered with 10 mM HEPES and 15 mM $NaHCO_3$ and supplemented with 2.4 μg/ml cytochalasin B at room temperature. A pair of blastomeres at the 8-cell stage were removed from the zona pellucida with a micropipette and separated from each other by gentle pipetting. Each pair of separated blastomeres was then inserted into an evacuated zona pellucida at the 8-cell stage. Manipulated embryos, two blastomeres in a zona pellucida, were cultured as described above.

### Production of $2n \leftrightarrow 4n$ chimeric embryos

The production of $2n \leftrightarrow 4n$ chimeric embryos was performed as described previously with slight modifications (Tsunoda *et al.*, 1987). One blastomere was removed from a 4-cell-stage 4*n*(GFP+) embryo using a pipette (20 to 25 μm external diameter) driven by a piezo-actuated unit (Prime Tech). Next, two blastomeres isolated from the 8-cell-stage 2*n*(GFP−) embryos were inserted into the 4*n* embryos. As a control, two blastomeres were removed from the 8-cell-stage 2*n*(GFP+) embryos, followed by insertion of two blastomeres isolated from the 8-cell-stage 2*n*(GFP−) embryos into the 2*n*(GFP+) embryos.

### Embryo transfer to recipient rats and examination of implantation sites

Embryos at the morula to blastocyst stage were transferred to the uteri of pseudopregnant recipients on day 5. On days 7 to 9, the implantation sites were collected after intravenous injections of Chicago Blue B dye in saline solution (Matsumoto *et al.*, 2002*b*). Small pieces of tissue were fixed with 3.7% formaldehyde and then embedded in paraffin wax. Sections (10 μm) were stained with hematoxylin–eosin. To examine the localization of GFP, immunohistochemical analysis was performed as described previously with slight modifications (Takeuchi *et al.*, 2003). Deparaffinized sections were incubated sequentially with rabbit anti-GFP antibody (Molecular Probes), biotinylated anti-rabbit IgG antibody (Vector Laboratories) and horseradish peroxidase-conjugated streptavidin (Vector Laboratories). Reactions were visualized using 3-amino-9-ethyl carbazole (Zymed Laboratories) as a chromogen.

### Statistical analysis

$\chi^2$ analysis was used to evaluate differences. A *p*-value less than 0.05 was considered significant. Comparisons with expected values of less than 5 were analysed using Fischer's exact probability test.

## Results

### Development of tetraploid embryos

Developmental rate to the morula/blastocyst stage of tetraploid embryos was 93% (56/60) at 72 h after the start of culture (Table 1). This was not significantly different from that of diploid embryos (95%, 55/58). Compaction in both diploid and tetraploid embryos occurred at 54 h (Fig. 1*A*, *B*, *D*, *E*). Specifically, diploid embryos compacted at the 8-cell stage (Fig. 1*B*), whereas compaction of tetraploid embryos occurred at the 4-cell stage (Fig. 1*D*). Chromosomes examined in all blastocysts that were developed from fused and untreated embryos showed diploid (100%, 30/30) and tetraploid (100%, 21/21) karyotypes, respectively (Fig. 1*C*, *F*). After embryo transfer, both diploid and tetraploid embryos showed normal implantation (Fig. 2*A*, *B*, *F*, *G*) until day 8 of pregnancy, although tetraploid conceptuses were aberrant on days 9 (Fig. 2*H*) and 10 (Fig. 2*I*, *J*).

### Development of embryos from two diploid blastomeres isolated at the 8-cell stage

To address whether rat tetraploid embryos have the ability to support the development of diploid cells that cannot develop independently, we examined the developmental potential in pairs of diploid blastomeres isolated at the 8-cell stage. Embryos developed from two diploid blastomeres isolated at the 8-cell stage (Fig. 3*A*) reached the morula/blastocyst stage (Fig. 3*B*, *C*;

**Table 1** Preimplantation development of electrofused tetraploid rat embryos *in vitro*

| | Examined 2-cell-stage embryos | Fused embryos | Developed to morula/blastocyst[a] |
|---|---|---|---|
| Diploid | 58 | – | 55 (95) |
| Tetraploid | 63 | 60 (95) | 56 (93) |

Values represent no. (and %).
[a]Based on the number of 2-cell stage (diploid) or fused embryos (tetraploid).

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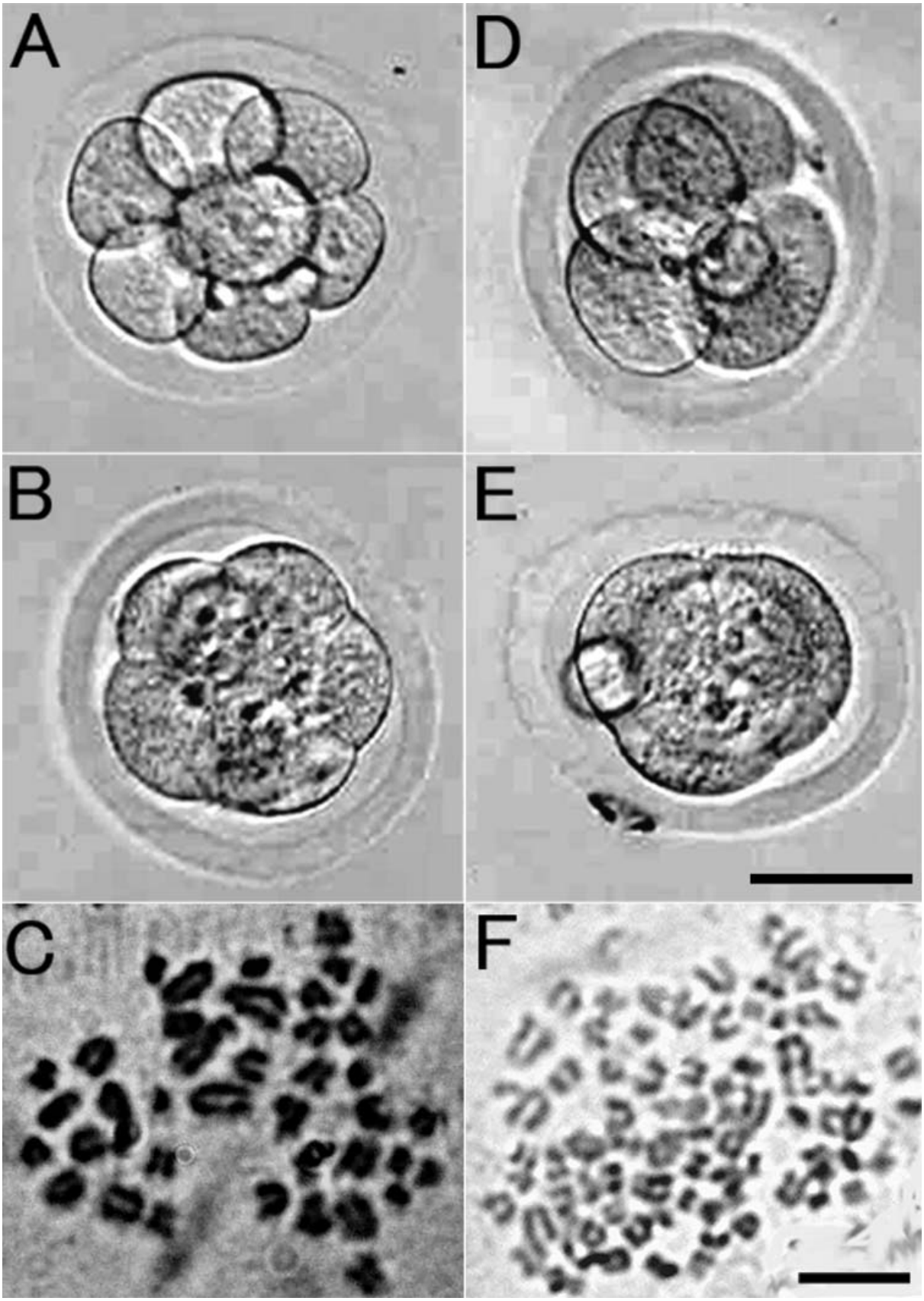


**Figure 1** Preimplantation development of diploid and tetraploid rat embryo *in vitro.* Untreated (*A*, *B*) and fused (*D*, *E*) embryos at 48 h (*A*, *D*) and 54 h (*B*, *E*) after the start of culture. Untreated and fused embryos showed diploid (*C*) and tetraploid (*F*) karyotypes, respectively. Note the compaction at 4-cell stage in tetraploid embryos. Bars in (*E*) and (*F*) indicate 30 μm and 2 μm, respectively.

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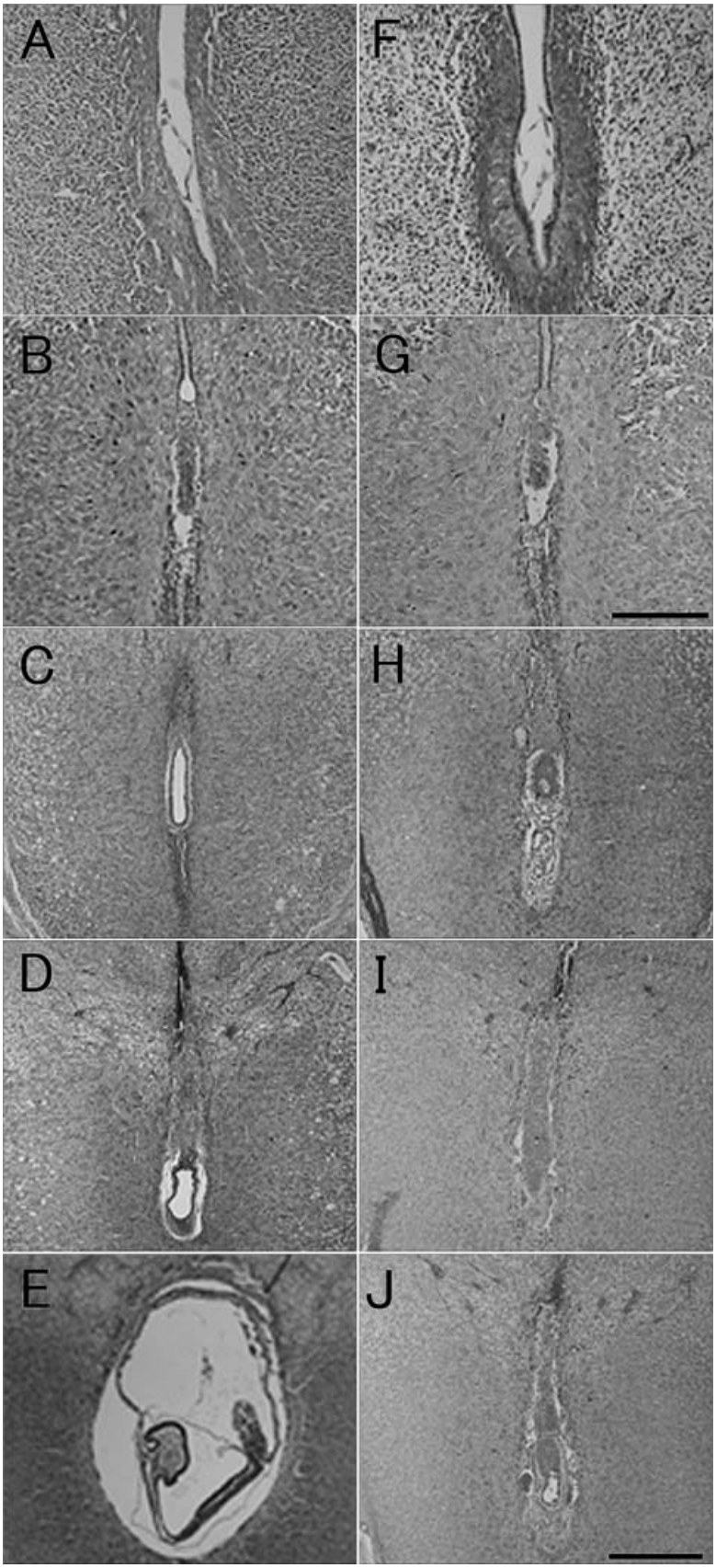


**Figure 2** Postimplantation development of diploid and tetraploid rat embryos after embryo transfer. Diploid (*A–E*) and tetraploid (*F–J*) conceptuses on days 7 (*A*, *F*), 8 (*B*, *G*), 9 (*C*, *H*), 10 (*D*, *I*, *J*) and 11 (*e*) of pregnancy. Note the aberration in tetraploid embryos on days 9 and 10. Bars in (*G*) and (*J*) indicate 150 μm and 1 mm, respectively.

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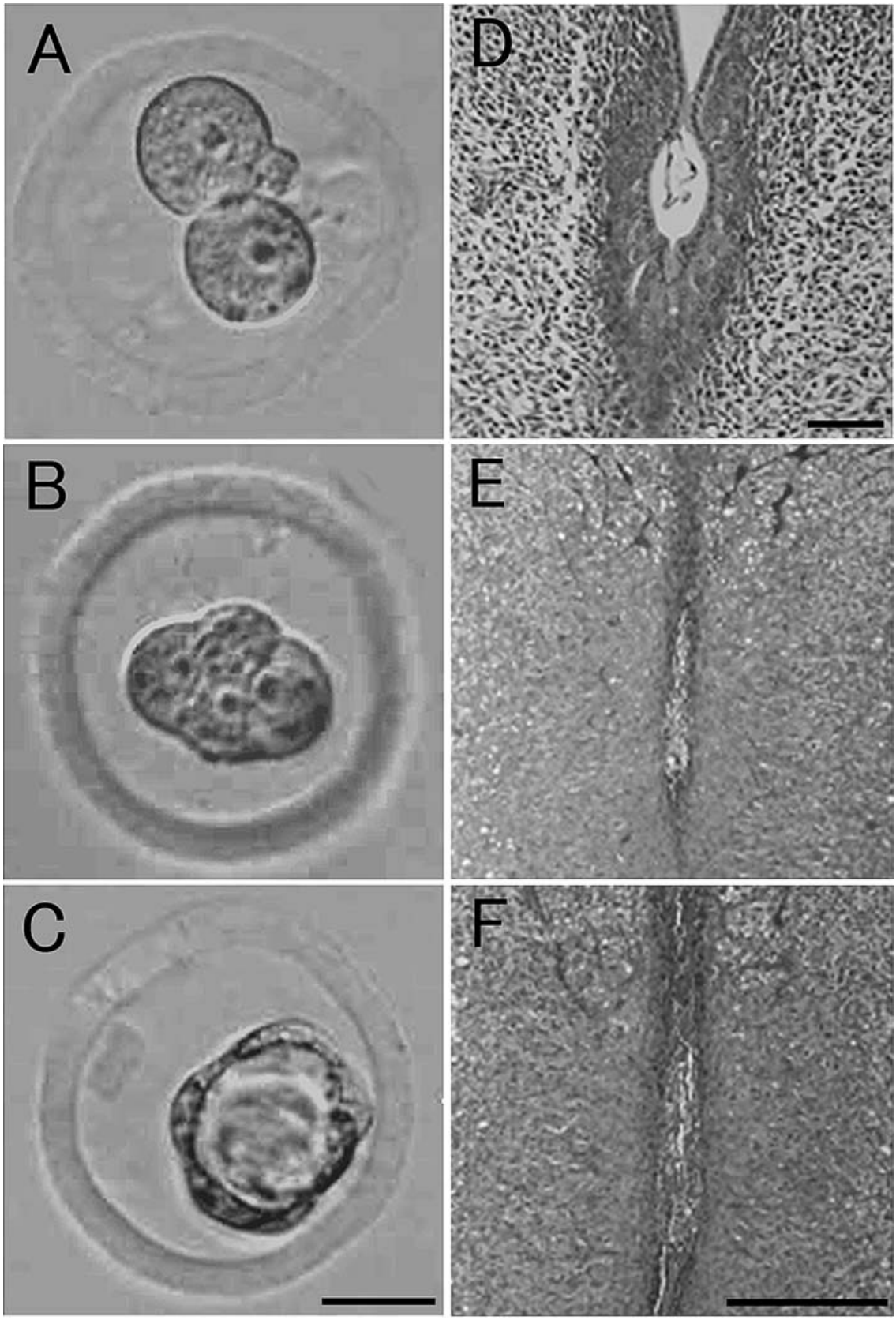


**Figure 3** Development of embryos from two blastomeres isolated at the 8-cell stage. An isolated pair of blastomeres (*A*), a morula (*B*) and a blastocyst (*C*). Postimplantation development of embryos from two blastomeres isolated at the 8-cell stage after embryo transfer on days 7 (*D*), 8 (*E*) and 9 (*F*) of pregnancy. Bars in (*C*), (*D*) and (*F*) indicate 30 μm, 100 μm and 500 μm, respectively.

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**Table 2** Preimplantation and postimplantation development of chimeric rat embryos[a]

| Aggregation patterns | Manipulated embryos | Developed to morula/blastocyst | Recipients | Rats with IS | Transferred embryos | IS | Fetuses |
|---|---|---|---|---|---|---|---|
| $2n \leftrightarrow 2n^b$ | 51 | 51 (100) | 4 | 4 (100) | 51 | 19 (37) | 11 (22) |
| $2n \leftrightarrow 4n^b$ | 117 | 109 (93) | 14 | 14 (100) | 109 | 42 (39) | 4 (4) |

Implantation and fetus were examined on day 14 of pregnancy. Values represent no. (and %).
[a]Generated by aggregating a pair of diploid blastomeres from an 8-cell-stage embryo with three tetraploid blastomeres from a 4-cell-stage embryo.
[b]Carrier of the GFP transgene; IS, implantation sites.

89%, 41/46) at 24 h after isolation (at 72 h after the start of culture). Blastocysts appeared as trophectoderm vesicles. The timing of compaction and blastocyst formation in these embryos resembled that of intact 8-cell embryos (72 h after the start of culture). After embryo transfer, implantation sites were identified as blue bands along the uterine horns after injection of a Chicago blue B (Matsumoto *et al.*, 2002*b*). Although the rate of Chicago blue-positive sites was 50% (10/20) on day 7 of pregnancy, only one embryo attached (5%, 1/20; Fig. 3*D*). On days 8 and 9, the rates of Chicago blue positive sites were 31% (5/16) and 32% (7/22), whereas one degenerated conceptus was obtained on each day (6%, 1/16 and 5%, 1/22; Fig. 3*E*, *F*).

### Tetraploid embryos support the postimplantation development of two diploid blastomeres isolated at the 8-cell stage

To assess the ability of tetraploid embryos to act as carrier blastomeres, as has been previously demonstrated in the mouse, we examined the development of diploid (GFP−) ↔ tetraploid (GFP+) chimeric embryos. Chimeric rat embryos, in which two diploid blastomeres from an 8-cell embryo were aggregated with three tetraploid blastomeres from a 4-cell embryo, developed to the morula/blastocyst stage (93%, 109/117) at 24 h after the manipulation (72 h after the start of culture; Table 2 and Fig. 4). This did not differ from the development of diploid embryos (100%, 51/51). Analysis of GFP expression showed that the contribution of 4*n*(GFP+) cells to chimeric blastocysts was mosaic and similar to 2*n*(GFP−) ↔ 2*n*(GFP+) embryos (Fig. 4). Our goal was to assess whether the rat tetraploid embryos could support the development of embryos that lacked full developmental potential to term by themselves. We usually estimate the fetal development on days 13–14 after manipulated embryo transfer (Shinozawa *et al.*, 2004). Therefore, we examined the development of chimeric conceptuses and the contribution of tetraploid cells on development on day 14 of pregnancy. On day 14, four conceptuses (4%, 4/109) were obtained (Table 2), although all of them were aberrant and smaller than those of the 2*n*(GFP−) ↔ 2*n*(GFP+) chimeric fetuses (Fig. 5*A*, *D*).

To examine the localization of 4*n*(GFP+), immunohistochemical analysis was performed for three conceptuses out of four and showed neither staining for GFP in fetus nor placenta (Fig. 5*E*, *F*). These results indicate that rat tetraploid embryos are able to prolong the development of diploid blastomeres that cannot develop independently, although postimplantation development was incomplete.

## Discussion

In a previous study in mouse, morphological features of day 15 tetraploid embryos suggested that they were developmentally equivalent to a normal embryo of around 13.5 to 14 days *post coitus* (Kaufman & Webb, 1990). In contrast, rat tetraploid embryos implant normally and survive until day 8 of pregnancy. However, these embryos showed signs of aberrant development on day 9. These results suggest that rat tetraploid embryos are less able to undergo normal development beyond day 8 than those from the mouse. Because compaction of rat tetraploid embryos occurred at the 4-cell stage while diploid embryos compacted at the 8-cell stage, the number of cells in the tetraploid embryos transferred into uteri may be half that in diploid embryos. Therefore, reduced cell number in the tetraploid rat embryos may the cause of aberrant fetal development, although these embryos have the ability to implant.

It has been reported previously that the aggregation of ES cells with tetraploid embryos or injection of ES cells into tetraploid blastocysts has allowed the production of mice that were entirely derived from ES cells (Nagy *et al.*, 1993; Ueda *et al.*, 1995; Wang *et al.*, 1997). These results suggest that rat tetraploid embryos may also allow the production of rats entirely derived from ES cells. However, rat ES cells that are able to access to germ line have not been established (Iannaccone *et al.*, 1994; Brenin *et al.*, 1997; Buehr *et al.*, 2003). In contrast, in mouse, aggregation of 1/4 and/or 2/8 blastomeres with 2-, 3- or 4-cell

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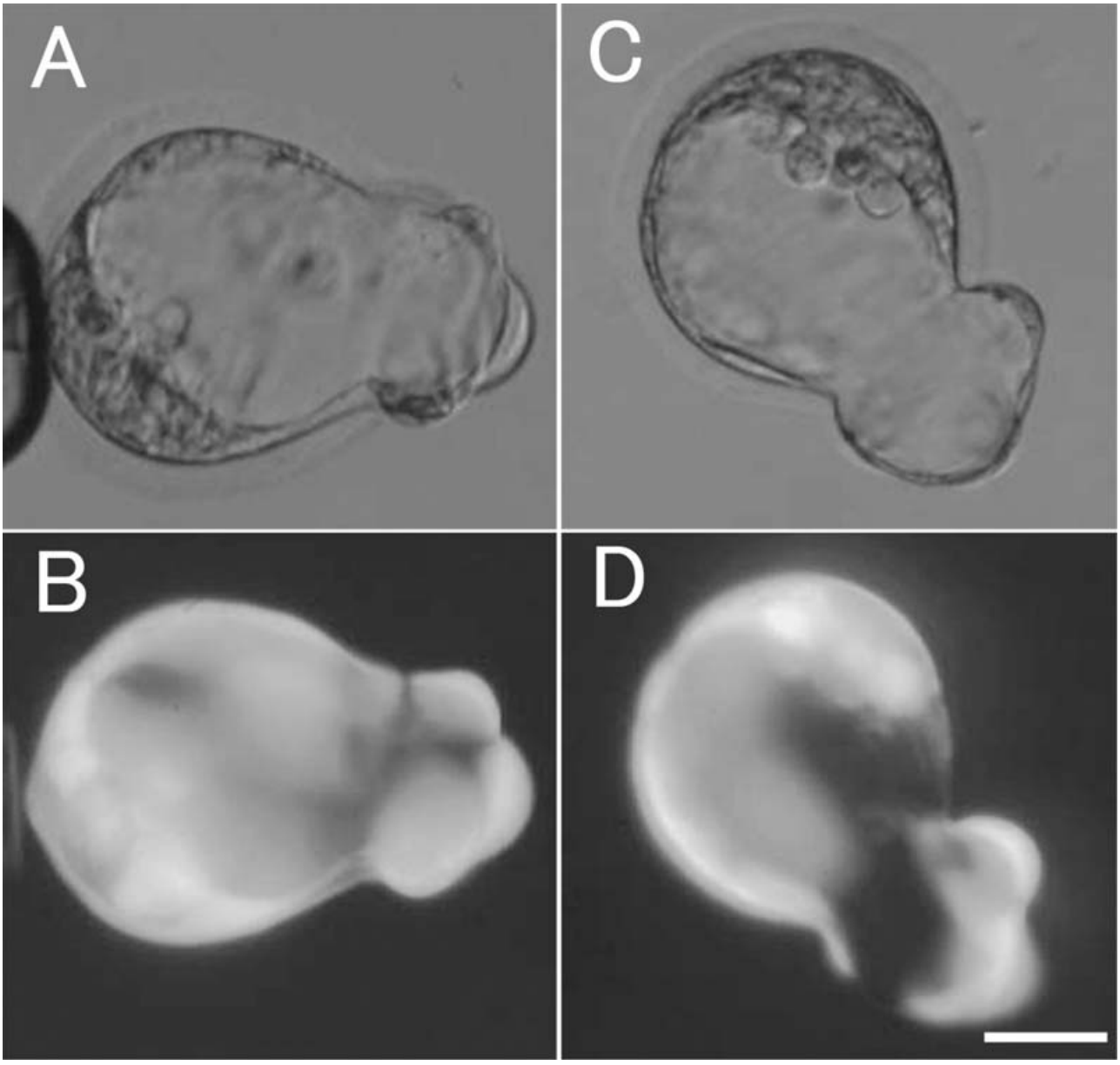


**Figure 4** Distribution of GFP-positive cells in chimeric rat embryos. Blastocysts derived from $2n$(GFP−) ↔ $2n$(GFP+) embryos (*A*, *B*) and $2n$(GFP−) ↔ $4n$(GFP+) embryos (*C*, *D*). Bar = 30 μm.

carrier tetraploid embryos has allowed development to normal and fertile adult, although mouse single 'quarter' blastomeres are no longer totipotent, because they are not able to develop independently into a mouse (Tarkowski *et al.*, 2001). These results suggest that rat tetraploid embryos may have the ability to support the development of blastomeres that cannot develop independently. However, developmental potential of isolated rat blastomeres from early-stage embryos has not been well understood. In the present study, our results showed that a pair of rat diploid blastomeres isolated at the 8-cell stage developed to the morula/blastocyst stage, while the ability of implantation and postimplantation development was very poor. Developed blastocysts appeared to resemble trophectoderm vesicles without a clear inner cell mass. Therefore, developmental failure of these embryos may be due to lack of sufficient number of ICM cells.

To assess whether the rat tetraploid embryos possess the ability to support the poor developmental potential in a pair of rat diploid blastomeres isolated at the 8-cell stage, we produced chimeric $2n$(GFP−) ↔ $4n$(GFP+) rat embryos. After transfer $2n$(GFP−) ↔ $4n$(GFP+) chimeric embryos into uteri, all of the conceptuses obtained on day 14 of pregnancy (four conceptuses) were aberrant and smaller than

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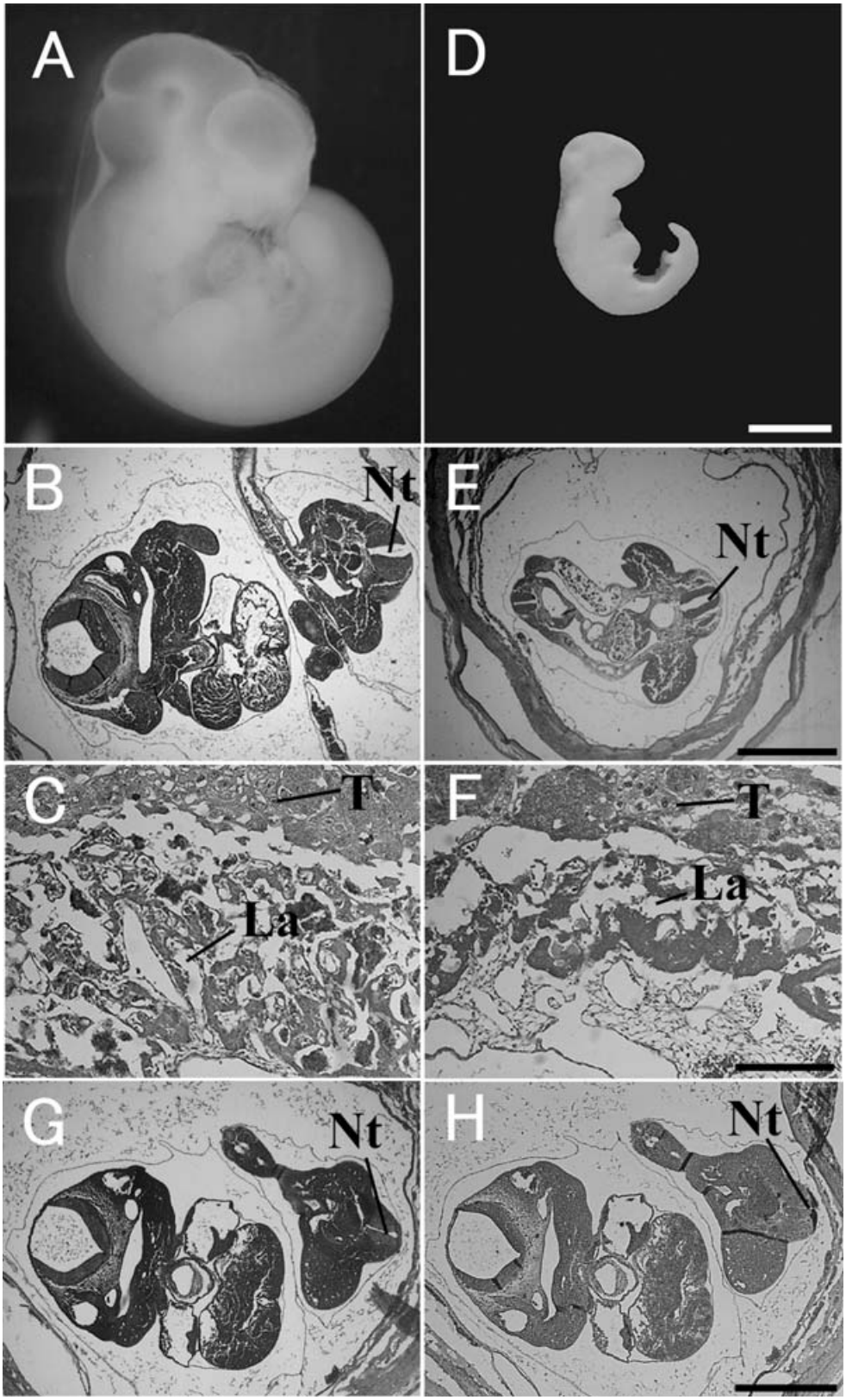


**Figure 5** Postimplantation development on day 14 of pregnancy. Fetuses derived from 2*n*(GFP−) ↔ 2*n*(GFP+) embryos (*A*) and 2*n*(GFP−) ↔ 4*n*(GFP+) embryos (*D*). Contribution of GFP(+) cells was assessed by immunohistochemical analysis in 2*n*(GFP−) ↔ 2*n*(GFP+) conceptus (*B*, fetus; *C*, placenta) and 2*n*(GFP−) ↔ 4*n*(GFP+) conceptus (*E*, fetus; *F*, placenta). Positive (*G*) or negative control (*H*) in 2*n*(GFP+) conceptus was performed using immunohistochemical analysis with or without primary antibody, respectively. Note that neither fetus nor placenta was GFP-positive in the 2*n*(GFP−) ↔ 4*n*(GFP+) conceptus. Bars in (*D*), (*E*, *H*) and (*F*) indicate 2 mm, 1 mm and 500 μm, respectively. La, labyrinth; Nt, neural tube; T, trophoblastic giant cell.

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the $2n$(GFP−) ↔ $2n$(GFP+) chimeric fetuses. Immunohistochemical analysis showed neither fetus nor placenta contained GFP-positive cells, although GFP expression showed that the contribution of $4n$(GFP+) cells to chimeric blastocysts was mosaic and similar to $2n$(GFP−) ↔ $2n$(GFP+) embryos. These results suggest that rat tetraploid embryos are able to prolong the development of a pair of diploid blastomeres from the 8-cell blastomeres, although it is incomplete.

In mouse, during preimplantation development, most tetraploid cells do not contribute to the inner cell mass in chimeric embryos with diploid cells at the blastocyst stage, although tetraploid cells tended to colonize in the trophectoderm (Everett & West, 1996, 1998; Everett *et al.*, 2000). During postimplantation development, tetraploid cells are abundant in extraembryonic tissues in $2n \leftrightarrow 4n$ aggregation chimeras, whereas they do not contribute to embryonic tissue (Tarkowski *et al.*, 1977; Nagy *et al.*, 1990; James *et al.*, 1995; Everett & West, 1996). Although the mechanism by which the developmental potential of tetraploid cells supports the development of diploid cells is not clearly understood, it has been reported that the tendency of tetraploid cells to colonize the trophectoderm occurs at the blastocyst stage and this colonization may be accompanied by selection against tetraploid cells in ICM in mouse (Everett & West, 1996, 1998; Everett *et al.*, 2000). In the present study in rat, both ICM and trophectoderm in chimeric blastocysts contained tetraploid cells with mosaic contribution during preimplantation development. Furthermore, our results showed rat tetraploid embryos are less able to undergo postimplantation development compared with mouse. Therefore, poor postimplantation development with different localization of rat tetraploid blastomeres could be associated with quite low developmental rate to chimeric fetus with aberrant and smaller size.

In conclusion, our results suggest that rat tetraploid embryos are able to prolong the development of diploid blastomeres that cannot develop independently, although postimplantation development was incomplete. In chimeric embryos, a different contribution of tetraploid cells compared with that of the mouse may be a cause of incomplete developmental support during postimplantation. Furthermore, the contribution of tetraploid and diploid cells to blastocysts may involve the postimplantation development stage. These results suggest that rat electrofused tetraploid embryos may not allow the development of rats that have been entirely derived from ES cells, if rat ES cells were established. It could be possible that improvement of the fusion procedure to create rat tetraploid embryos and/or culture system for rat embryos increases the ability of tetraploid embryos to act as carrier blastomeres during postimplantation development. Whereas an aberrant and smaller size could account for the lack of tetraploid cells that could develop into either the fetus or placenta on day 14 of pregnancy, it may be possible that they have just ceased to express GFP. Further investigation using other genetic markers might clarify this point.

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