Journal of Reproduction and Development, Vol. 51, No. 5, 2005

—Original—

Tetraploid Cells of Enhanced Green Fluorescent Protein Transgenic Mice in Tetraploid/Diploid-Chimeric Embryos

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Abstract. We succeeded in noninvasively analyzing the distribution of tetraploid (4n) cells in tetraploid↔diploid (4n↔2n) chimeric embryos by using enhanced green fluorescent protein (EGFP) transgenic (Tg) mouse embryos. We also evaluated whether this technique of analyzing 4n-cells in EGFP Tg 4n↔2n chimeric embryos could be used to determine which characteristics of 4n-cells cause the death of 4n-embryos and restricted distribution of 4n-cells in 4n↔2n-chimeric embryos after implantation. In our experiments, the distribution of 4n-cells in 4n↔2n-embryos was normal until an embryonic age of 3.5 days (E3.5). With respect to morphological development, there were no differences between 4n-, diploid (2n), 4n↔2n-, and diploid/diploid (2n↔2n) chimeric embryos, but the number of cells in the tetraploid (4n) blastocyst was smaller than expected. This decrease in the number of cells may have caused cell death or reduced the rate of cell division in 4n-cells, and may have restricted the distribution of 4n-cells in 4n↔2n-chimeric embryos. This study demonstrated the utility of EGFP transgenic mouse embryos for relatively easy and noninvasive study of the sequential distribution of cells in chimeric embryos.
Key words: 4n↔2n-chimeric embryo, EGFP transgenic mouse, Implantation

(**J. Reprod. Dev. 51:** 567–572, 2005)

Enhanced green fluorescent protein (EGFP) is a nontoxic marker that emits green fluorescence without exogenous substrates or cofactors, and it has been proven useful in numerous applications, such as in the selection of transgenic embryos [1]. Because the expression of EGFP can be easily observed by fluorescent microscopy, the use of EGFP transgenic (Tg) mouse embryos may be useful for analyzing the distribution of cells in chimeric embryos.

Accepted for publication: May 9, 2005
Published online: July 21, 2005
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Tetraploid (4n) mouse embryos with 80 chromosomes have been produced by either inhibition of cleavage or blastomere fusion [2-5]. These embryos develop into blastocysts *in vitro*, but with the exception of a report by Snow [6], they are generally not considered to be capable of completing their full-term development [4, 7]. On the other hand, *in vivo* tetraploid↔diploid (4n↔2n)-chimeric embryos are capable of continuing to develop after implantation, and tetraploid (4n) cells in those embryos tend to distribute in derivatives of the trophectoderm and primitive endoderm lineages, but not in those of the primitive ectoderm [8]. Because of this

characteristic of 4n-cells, tetraploid (4n) blastocysts are utilized as host embryos to produce embryonic stem cells that contribute effectively to the fetus [9-11]. Moreover, it has been reported that 4n↔2n-chimeric embryos display a restricted distribution of 4n-cells among the mural trophectoderm even at an embryonic age of 3.5 days (E3.5) [12, 13], and the contribution of 4n-cells to 4n↔2n-embryos decreases from E3.5 to E4.5 [14].

A noninvasive method for measuring the distribution of cells in chimeric embryos from EGFP Tg mice might provide additional information about living cells without the need for complicated techniques. We therefore examined whether the use of EGFP Tg mice would lead to new insights about the characteristics of 4n-cells in the development of 4n- and 4n↔2n-chimeric embryos.

Materials and Methods

Collection and culture of embryos

C3H/He female mice were superovulated by i.p. injection of 7.5 IU of equine chorionic gonadtropin (Sankyo, Tokyo, Japan) and, after an interval of 48 h, 7.5 IU of human chorionic gonadotrophin (hCG; Sankyo). After hCG injection, these females were mated with male EGFP Tg C57BL/6 [15] or non-Tg C57BL/6 mice. Noon on the day when a vaginal plug was detected was counted as E0.5. Two-cell-stage embryos were collected at E1.5 by flushing of the oviducts with 1 mg/ml of hyaluronidase (Sigma, St. Louis, MO) in M2 medium (Sigma). The embryos were cultured in M16 (Sigma) medium in a humidified atmosphere of 5% CO_2 in air at 37 C.

Tetraploidy induction and chimera production

The production of 4n-embryos was carried out by treatment of 2-cell-stage embryos with cytochalasin B (CB) [Sigma; 1 mg/ml in dimethyl sulfoxide (DMSO)]. CB was diluted to give a final concentration of 10 μg/ml in M16 medium (CB-M16). Two-cell-stage embryos were cultured in CB-M16 medium for 12 h, and washed thoroughly in M16 medium. As a control, 2-cell-stage embryos were cultured in M16 medium containing 1% DMSO for 12 h. Chimeric embryos were produced by the aggregation method as follows. Eight-cell-stage embryos were collected from the oviduct at E2.5, as described for 2-cell-stage embryos. To remove the zona pellucida, the embryos were incubated in pronase (Sigma) solution (0.5% in M2 medium) for 5-10 min at 37 C. Eight-cell-stage embryos were aggregated with 8-cell-stage EGFP Tg diploid (2n) embryos of or 4-cell-stage EGFP Tg tetraploid (4n) embryos. Then, 8-cell-stage non-Tg 2n-embryos were gently pressed onto 8-cell-stage EGFP Tg 2n-embryos or 4-cell-stage EGFP Tg 4n-embryos with a glass capillary.

Cytogenetic and cell number analysis

The number of chromosomes and the number of cells in embryos were counted [15]. To increase metaphase chromosomes, embryos were precultured in 2% KaryoMAX colcemid (Gibco/Invitrogen, Carlsbad, CA; 10 μg/ml)/M16 medium at 37 C for 2-3 h. The embryos were incubated in 0.5% pronase (Sigma)/M2 medium solution for 5-20 min at room temperature (RT) until swelling of the zona pellucida occurred, and then they were transferred to 1% trisodium citrate (hypotonic treatment) for 20-40 min at RT. After fixation in 50% methanol and 10% acetic acid solution for 4 min at RT, these embryos were pushed out onto grease-free dry slides and vibrated immediately. The slides were then dried at 37 C, stained with 5% Giemsa solution (Merck, Frankfurt, Germany), and examined with a light microscope.

BrdU assay for cell division

To analyze cell division in 4n- and 2n-embryos, an immunofluorescence assay for the detection of BrdU incorporated into cellular DNA was performed with a BrdU Labeling and Detection Kit I (Roche, Indianapolis, IN). Namely, 4n- and 2n-embryos were cultured in BrdU labeling M16 medium (final BrdU concentration: 10 μM) for 1 h or 12 h at 37 C, and then they were washed 3 times with 0.1% polyvinylpyrolidone (PVP) (Sigma)/phosphate buffered sodium (PBS). To remove the zona pellucida, the embryos were incubated with acidic Tyrode's solution. The embryos were fixed in methanol for 30 min and washed 6 times with 0.1% PVP/PBS. Then, the embryos were incubated in 2% Triton X-100 for 1 h, washed 6 times with 0.1% PVP/PBS, and blocked for non-specific antigen-antibody reaction in 5% bovine serum albumin/PBS for 1 h. These embryos were incubated in anti-BrdU mouse monoclonal antibody (diluted 1:10 with incubation buffer) overnight at RT, washed 3 times with blocking

solution, and incubated in anti-mouse-Ig-fluorescein from sheep (diluted 1:10 with PBS) for 2 min at RT. Embryos were photographed under a fluorescence microscope, and embryos with BrdU positive nuclei were counted.

Measurement of maximal projected areas

Maximal projected areas of diploid (2n)-, 4n-, diploid/diploid (2n↔2n)-, and tetraploid/diploid (4n↔2n)-blastocysts were measured with the NIH Image 1.62 software.

Observation of EGFP-expressing cells in chimeric embryos

The distribution and behavior of EGFP-expressing cells were compared between 2n↔2n- and 4n↔2n-chimeric embryos from E3.75 to E4.5 with a fluorescence microscope.

Statistical analysis

Statistical differences between groups were analyzed by Student's *t*-test or the Tukey-Kramer method when appropriate.

Results

Behaviour of EGFP-expressing cells in chimeric embryos

EGFP-expressing 4n- and 2n-cells were distributed in a mass in 4n↔2n- and 2n↔2n-chimeric morulae and blastocysts, respectively. These cells, in each chimeric blastocyst, were distributed in both the areas of the trophectoderm (TE) and the inner cell mass (ICM). The observation of EGFP-expressing cells revealed that the distribution of 4n-cells in 4n↔2n-chimeric embryos did not differ from that of 2n-cells in 2n↔2n-chimeric embryos (Fig. 1), and 4n-cells were not eliminated from 4n↔2n-chimeric embryos from E3.75 to E4. 5 (Fig. 2).

Development and cell division of 4n embryos

CB-treated embryos possessed about 80 chromosomes in cytogenetic analysis. Tetraploid embryos developed to the 4-cell-stage at 71.4 h post-hCG injection, and 2n-embryos developed to the 8-cell-stage at 69.6 h. No significant difference

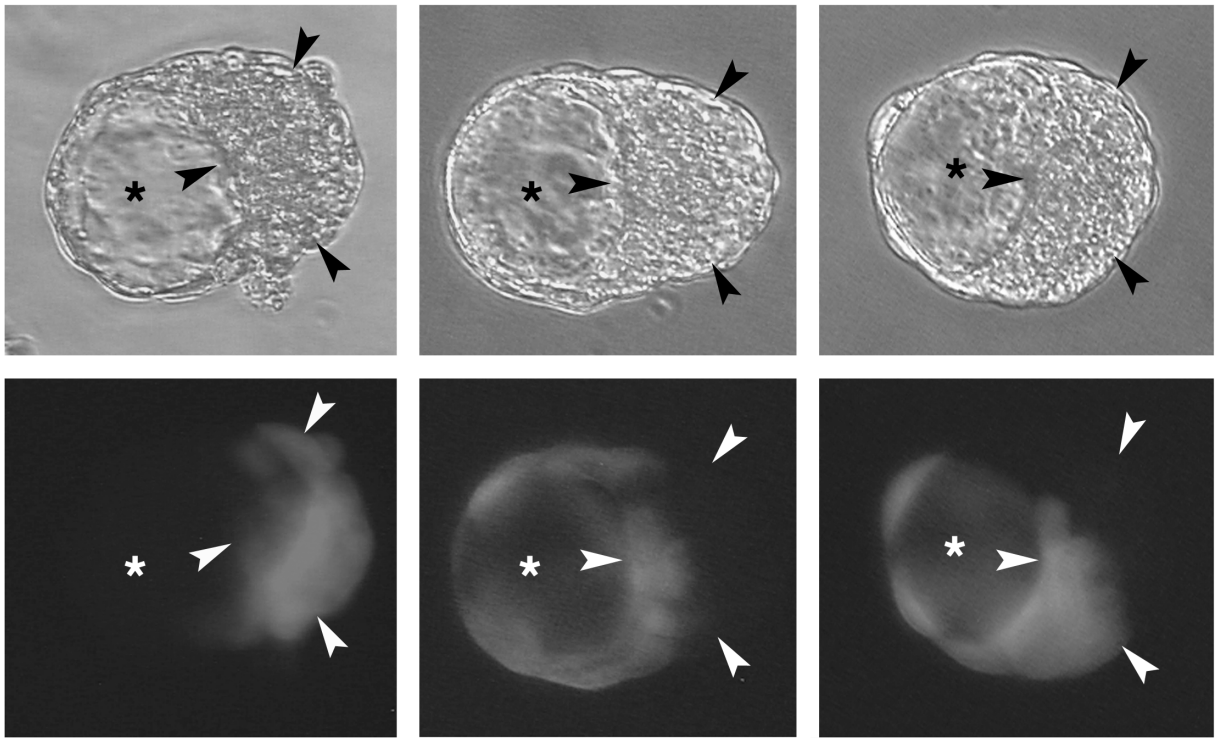

Fig. 1. Distribution of EGFP-expressing 4n- and 2n-cells among 4n↔2n- and 2n↔2n-chimeric embryos. Upper panels show blastocysts under a phase contrast microscope, and lower panels show the same field under a fluorescence microscope. EGFP-expressing 4n- and 2n-cells in each chimeric blastocyst were distributed in every area. The asterisk indicates the cavity of the blastocyst, and arrowheads indicate the inner cell mass.

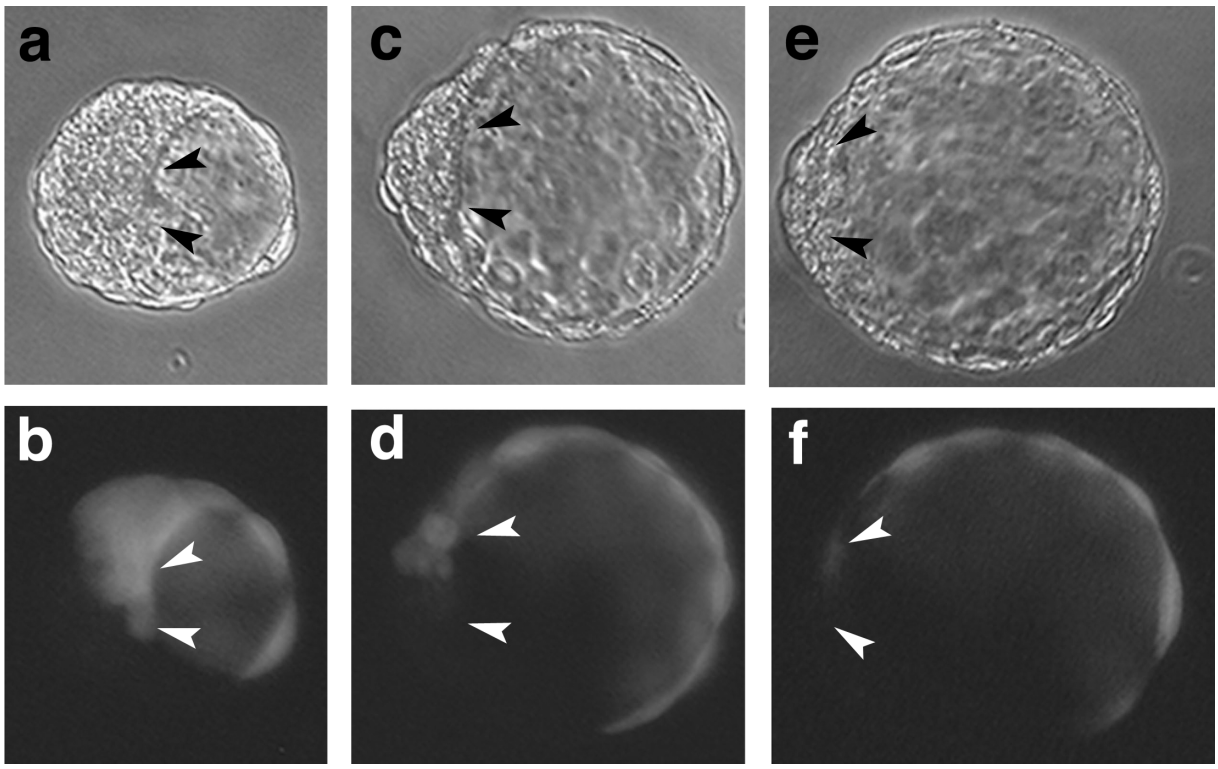

Fig. 2. Sequential distribution of EGFP-expressing 4n-cells in 4n↔2n-chimeric embryos. Upper panels show the identical blastocysts from E3.75 to E4.5 under a phase contrast microscope, and lower panels show the same field under a fluorescence microscope. Panels a) and b): E3.75; c) and d): E4.0; and e) and f): E4.5. Arrowheads indicate the inner cell mass.

in developmental rate was observed between 4n- and 2n-embryos. BrdU was incorporated in 87.5% of 4n- and 85.7% of 2n-embryos after 1 h incubation in BrdU-labeling medium, and was incorporated in 100% of 4n- and 2n-embryos after 12 h incubation (Fig. 3). BrdU was incorporated in all nuclei of BrdU-positive embryos. Blastocysts of 4n, 2n, 4n/2n, and 2n/2n had 22.6 cells, 47.9 cells, 83.9 cells, and 121.3 cells, respectively (Fig. 4). All of these values were significantly different (Tukey-Kramer: $p<0.01$).

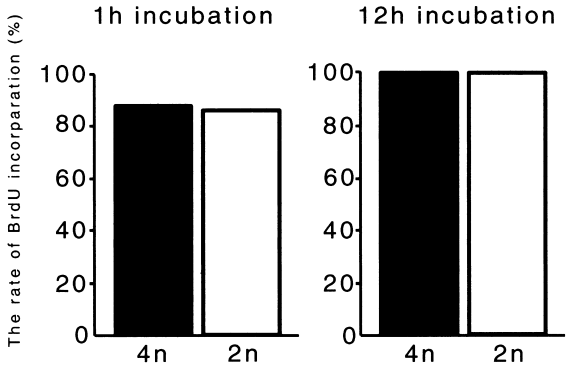

Fig. 3. Rate of incorporation of BrdU in 4n and 2n-embryos. BrdU was incorporated in all blastomeres after 1 h or 12 h incubation in BrdU labeling medium.

Morphological development

There was no significant difference in morphological appearance between 4n- and 2n-embryos, or between 4n↔2n- and 2n↔2n-chimeric embryos during development. The 4n and 2n embryos began to compact from 2 to 8 cells and from 6 to 10 cells, respectively. But there was no significant difference among the groups in either the timing of the compaction or the formation of the blastocyst cavity (Table 1). The mean maximal projected areas of the 4n-, 2n-, 4n↔2n-, and 2n↔2n-blastocysts were 8319.9 μm^2, 8154.4 μm^2, 14063.7 μm^2, and 15997.9 μm^2, respectively. There was no significant difference in these values between 4n- and 2n-blastocysts, or between 4n↔2n- and 2n↔2n-blastocysts.

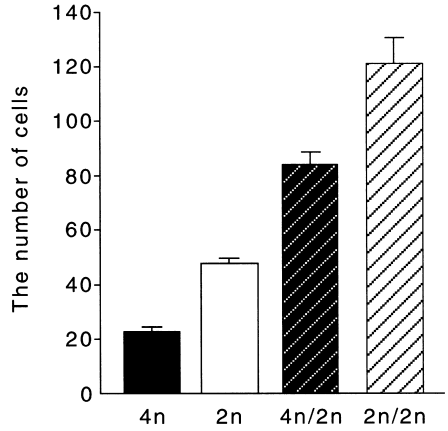

Fig. 4. Numbers of cells in 4n-, 2n-, 4n↔2n-, and 2n↔2n-blastocysts with expanded cavities. Error bars indicate the SEM. The numbers of cells in blastocysts were as follows: 4n (n=8), 2n (n=9), 4n/2n (n=8), and 2n/2n (n=6). The numbers of cells were significantly different among groups (Tukey-Kramer: $p<0.01$ for all comparisons).

Table 1. Timing of morphological development in 4n-, 2n-, 4n↔2n-, and 2n↔2n-embryos

Embryo	Compaction	Blastocyst
4n	83.9 ± 1.3	99.1 ± 0.8
2n	82.4 ± 2.0	96.0 ± 0.8
4n/2n	NE	97.7 ± 1.0
2n/2n	NE	96.7 ± 1.0

NE, not examined. The values are the means ± SEM of the post hCG time in hours. The numbers of compactioned embryos were as follows: 2n (n=10) and 4n (n=10). The numbers of blastocysts were as follows: 2n (n=11), 4n (n=11), 4n↔2n (n=15), and 2n↔2n (n=15). There were no significant differences in the timing of compaction or blastocyst formation among the types of embryos.

Discussion

We succeeded in producing EGFP Tg 4n↔2n-chimeric embryos and in observing the distribution of EGFP-expressing 4n-cells in these embryos under a fluorescence microscope. Everett and West [12] and Everett *et al.* [13] reported that 4n-cells made a greater contribution to the TE than to the ICM of E3.5 chimeras; in the present experiments, however, we did not find a high population of 4n-cells in the TE of E3.5 chimeras (data not shown), and chimeric embryos did not eliminate 4n-cells until E4.5. The results of the BrdU incorporation assay revealed that the capabilities of cell division and DNA synthesis of 4n cells were almost the same as those of 2n-cells until the 4-cell-compaction stage. In addition, the timing of compaction and blastulation seemed to be similar among the 2n-, 4n-, and chimeric embryos (2n↔2n and 4n↔2n). Although the mechanisms of compaction and blastulation are still unclear, both 2n- and 4n-cells in embryos have the capacity to synchronize with each other and complete the developmental events in the preimplantation period. This suggests that 4n-embryos and 4n↔2n-chimeric embryos might have the capacity to cleave and undergo morphogenesis in the early preimplantation period in the same way as 2n-embryos and 2n↔2n-chimeric embryos. However, the number of cells in 4n-blastocysts with an expanded cavity (22.6) was slightly less than half of that in 2n blastocysts (47.9) at the same stage. Also, the number of cells in 4n↔2n-chimeric blastocysts (83.9) was less than that predicted from 2n↔2n-chimeric blastocysts. These findings might suggest that cell death or prolongation of the cell cycle in 4n-cells occurs at the blastocyst stage. In mouse embryos, cell cycle regulation begins to work after fertilization; however, judging from the fact that mouse embryos with a null mutation for the cyclin A2 gene can develop to the blastocyst stage [16, 17], there is a possibility that regulation of the embryonic cell cycle at the preimplantation period does not work as well as regulation of the somatic cell cycle. Therefore, 4n-cells may be permitted to cleave at the preimplantation period. After the blastocyst stage, however, the cell cycle period may begin to be prolonged in 4n-cells because the checkpoint control is completed. After implantation, 4n-cells may be able to survive only in extra embryonic tissues permitted to proliferate slowly because of the prolongation of the cell cycle period. These characteristics of 4n-cells may make some small contribution to the fetus during the postimplantation period.

In conclusion, analysis of chimeric embryos using EGFP Tg mice enabled us to examine the distribution of cells in chimeric embryos more clearly, noninvasively, sequentially, and easily than the ordinary method using immunostained sections. Moreover, this method of using EGFP-expressing cells provided the new insight that 4n-

cells contribute to the formation of both the TE and ICM. In addition, there is a possibility that cell death or prolongation of the cell cycle might affect the development of 4n- and 4n↔2n-chimeric embryos. This method using EGFP Tg mouse embryos will be very useful for the future analysis of chimeric embryos.

Acknowledgements

We would like to thank Dr. Hideaki Tojo from the University of Tokyo for his generous donation of EGFP transgenic mice.

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