GFP Expression in Mouse Fetuses Derived from Aggregation of Embryonic Stem Cells with Diploid or Tetraploid Embryos

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Abstract: *It is necessary to use a suitable marker gene to select embryos expressing transgene prior to transfer into recipient females especially in an efficient production of transgenic farm animals. Recently a green fluorescent protein (GFP) gene has been used as a marker gene for this purpose, because it is a self-fluorescent substance and does not require a substrate such as X-Gal in a lacZ gene. Using these characteristics of GFP, we transfected the GFP gene with the neomycin-resistance gene under the control of the phosphoglycerase kinase promoter into mouse embryonic stem (ES) cells, and attempted to produce a transgenic mouse derived from aggregates of GFP-positive ES cells after the selection with G418 for 8 days with diploid or tetraploid embryos. There were some ES colonies expressing GFP at different levels after the selection with G418. These GFP-positive ES cells were used as a complete tight adhered clump for aggregation with diploid or tetraploid embryos and resulted in GFP expression in the inner cell mass (ICM) cells of aggregates, but no embryos expressing GFP in trophoblasts. At 9.5 days p.c., 6 out of 10 live fetuses (60%) for diploid⇔ES chimera, and all of 14 live fetuses (100%) for tetraploid⇔ ES chimera, in spite of head deformity, were transgenic fetuses. At 14.5 days p.c., among 7 normal embryos obtained from 21 embryos transferred to recipients, one fetus showed GFP expression in five organs (heart, kidney, liver, gonad and intestine), and 4 fetuses strongly expressed GFP in more than one organ as seen under the fluorescent dissecting microscope. These results indicate that enough transfected ES cells selected in a short time with G418 were able to be used for the selection of transgenic embryos prior to transfer to the recipient. Moreover, by using a complete tight adhered clump of ES cells for aggregation, the clump is able to completely contribute to a part of the ICM. The GFP gene may be useful and efficient as a marker especially for farm animals which have a long term pregnancy and a small litter size.*

Key words: *GFP, Mouse embryo, Aggregation chimera.*

Some transgenic cloned farm animals were produced from somatic cells transfected with foreign genes [1, 2], so that we have great expectations for application to the animal industry or in biotechnology, for example to produce medicines in milk from transgenic farm animals. We have also produced chimeric calves derived from aggregates of bovine embryonic stem (ES)-like cells derived from inner cell mass (ICM) and tetraploid embryos [3]. In order to increase the production efficiency of transgenic cloned or chimeric farm animals by using biotechnological skills, it is important to select genes for transfection as a marker. Chalfie et al. [4] transfected the green fluorescent protein (GFP) gene into *Caenorhabditis elegans* and they observed GFP expression under the fluorescent microscope in real time. And Takada et al. [5] microinjected the GFP gene into the pronucleus of mouse and bovine fertilized oocytes and they reported the possibility of producing transgenic embryos selectively in the preimplantation period by using GFP as a marker. It is therefore possible to select transgenic embryos prior to transfer to recipient by simply using a GFP gene as a marker for farm animals, especially cattle which have long term pregnancy and a small litter size. With this technique, it is possible to produce transgenic cloned or transgenic chimeric farm animals much more efficiently. In the present paper, first by using a completely tightly adhered clumps of ES

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cells, we produced blastocysts from aggregates of GFP-positive ES cells with diploid or tetraploid embryos and observed a localization of GFP-positive cells in them. Secondly, blastocysts showing GFP expression in ICM cells were transferred into the recipient mouse and there occurred a localization of GFP expression in fetuses at 9.5 days p.c. and in various tissues of fetuses at 14.5 days p.c.

Materials and Methods

Mouse ES cells

We purchased ES (TT2) cells (XY) from Oriental Yeast Co. Ltd. These ES cells were cultured on feeder layers obtained from mouse fibroblasts treated with mitomycin C (Sigma) in ES culture medium (Dulbecco's Modified Eagle Medium (DMEM: Gibco BRL), 20% FBS, 10^{-4} M β-mercaptoethanol, nonessential amino acid (Gibco BRL), nucleosides (0.03 mM each of guanosine, cytidine, uridine and 0.01 mM thymidine), 10^3 IU/ml recombinant human leukaemia inhibitory factor (ESGRO, Gibco BRL). After passaging regularly, the cells were stocked in liquid nitrogen. For electroporation, the thawed cells were cultured on gelatinized culture flasks and passaged for three times to remove the feeder cells.

GFP construction

Phosphoglycerate kinase (PGK) promoter was inserted between the *Eco*R1 and *Pst*1 sites of pEGFP 1 vector (Clontech) with a neomycin-resistance cassette (*neo*^{r}) and it was linealized by digestion with *Hind*III.

Electroporation

Seven-hundred μl of HBS (25 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4 \cdot 2H_2O$, 6 mM glucose, pH 7.05) including 1×10^7 ES cells and 40 μg of the GFP vector was put into a cuvette with a 0.4 cm electrode gap. After the cooling on ice for 10 min, electroporation was carried out by means of an electroporation system (Gene pulsar II, Bio Rad, USA) under a condition of 960 μF-250 V. Forty-eight h after transfection, ES cells were split into 1:10 and G418 was added to a final concentration of 600 μg/ml for 8 days. By means of a mouth controlled pipette, colonies showing signs of morphology of ES cells and expressing GFP in a whole colony were picked up under the fluorescent microscope, and the colonies, washed with PBS without magnesium or calcium (PBS(–)), were tripsinized, and the single cells were cultured with ES culture medium on feeder layers. At the fifth passage, the ES cells were kept in liquid nitrogen.

Preparation of diploid embryos

Crj:CD1 (ICR) females (6–7 wks of age) and males (8 wks of age) were purchased from Charles River Japan Inc. Crj:CD1 female mice were superovulated with 5 units of pregnant mare serum gonadotropin (PMSG) followed by 5 units of human chorionic gonadotropin (hCG) 48 h later, then they were mated with ICR male mice. Two-cell stage embryos collected at 44 to 45 h after treatment with hCG were cultured with M16 and embryos at the the compacted or compacting 8-cell stage were used for aggregation.

Production of tetraploid embryos

Late two-cell stage embryos collected at 45 h post-hCG treatment from Crj:CD1 female mice likewise superovulated were put between two platinum electrodes including 0.3 M mannitol solution (0.3 M D(-)-mannitol, 0.1 mM magnesium sulfate, 0.05 mM calcium chloride and 0.05% bovine serum albumin (BSA, Wako, Japan)). Electrofusion was carried out in a condition of 1 kV/cm for the strength of the field and two pulses of 50 µsec duration. The treated embryos were washed with M16 and an embryo fused within 30 min was recognized as a tetraploid embryo. These tetraploid embryos were cultured in M16, and compacted or compacting 4-cell stage embryos were used for aggregation.

Aggregation

Droplets (15 μl) of M16 were dropped onto a plastic dish (Falcon #1008) and covered with mineral oil (Squibb, USA). A small depression was made in each culture droplet with a darning needle (Clover 55-042, Japan). Thawed ES cells were cultured on feeder layers with ES culture medium, a colony consisting of approximately 20 ES cells were picked up with a mouth controlled pipette. We selected ES colonies expressing GFP under the fluorescent microscope. After washing with M16, each of the colonies was put into a depression. Zona pellucida of diploid or tetraploid embryos were removed with 0.5% pronase E (Kaken Kagaku, Japan) at the compacting or compacted 8-cell or 4-cell stage, respectively. Two zona-free embryos were put into each depression to form a triangle with the ES colony. These aggregates were cultured at 37°C in a humidified gaseous atmosphere of 5% CO_2 in air up to the blastocyst stage.

Embryo transfer and observation of GFP expression

The aggregates developed to blastocysts were transferred into the uteri of pseudopregnant Crj:CD1 females. The procedure for pseudopregnancy is as follows; Crj:CD1 females (6–7 wks of age) in estrus were mated to vasec-

tomized Crj:CD1 males in the evening, and the females were approximately 2.5 day p.c. pregnant at the time of the embryo transfer. At 9.5 days p.c. and 14.5 days p.c., fetuses were removed from the uteri. After washing them in PBS(–), GFP expression in the fetuses was observed under a fluorescent dissecting microscope (Leica, Germany). Localization of GFP expression in the fetuses at 9.5 days p.c. and in various tissues and organs of the fetuses at 14.5 days p.c. were also investigated.

Results

To avoid differentiation of ES cells during long-term selection for GFP expression, we cultured cells for only 8 days with ES medium including G418. Then some colonies expressing GFP in a whole colony showing signs of an ES-specific morphology were picked up under the fluorescence microscope. There were some clonies expressing GFP at different levels in a colony.

GFP expression in blastocysts from aggregates of GFP-positive ES colonies with diploid (Fig. 1a-1d) and tetraploid embryos (Fig. 1e-1h) are shown in Fig. 1. Blastocysts from aggregations with diploid or tetraploid embryos both showed that GFP expression was located in the ICM (Fig. 1d or 1h).

Efficiencies of GFP expression in blastocysts and fetuses at 9.5 days p.c. from aggregates of ES cells with diploid or tetraploid embryos are shown in Table 1. The percentages of GFP expression in ICMs of the aggregates which developed to blastocysts were 75.9% (60/79) and 86.3% (120/139) for ES⇔diploid chimera (ES⇔2N), and ES⇔tetraploid chimera (ES⇔4N), respectively, but we observed no GFP expression in trophoblast of ES⇔2N or ES⇔4N blastocysts. The ratios of implanted embryos to transferred embryos were 16/56 (28.6%) for ES⇔2N and 33/104 (31.7%) for ES⇔4N. Furthermore, the ratios of heart beating fetuses at 7 days after embryo transfer to transferred embryos were 10/56 (17.9%) for ES⇔2N and 14/104 (13.5%) for ES⇔4N. All ES⇔4N fetuses at 7 days after embryo transfer showed signs of heartbeat, but they had head deformity (Fig. 2). Six out of 10 heart beating fetuses (60%) for ES⇔2N emitted green fluorescence in their bodies and all heart beating fetuses for ES⇔4N emitted green fluorescence (Fig. 2).

In Table 2, development of ES⇔2N and ES⇔4N

Table 1. Development of aggregates from ES cells with diploid or tetraploid embryos and GFP expression in blastocysts and fetuses at 9.5 days p.c.[a]

Type of embryos	No. of		No. of blastocyst GFP expressing in		No. of embryos		No. of fetuses	
	aggregates	blastocysts	ICMs (%)[b]	trophoblast	transferred	implanted (%)[c]	viable (%)[d]	GFP expressing (%)[e]
Diploid	80	79	60 (75.9)	0	56	16 (28.6)	10 (17.9)	6 (10.7)
Tetraploid	141	139	120 (86.3)	0	104	33 (31.7)	14[f] (13.5)	14 (13.5)

[a]: These results are from four individual trials. [b]: The rate of GFP expressing embryos to blastocysts. [c~e]: The numbers of placentae, viable embryos which have a heartbeat and fetuses expressing GFP to numbers of transferred embryos, respectively. [f]: All fetuses had head deformity.

Table 2. Development of aggregates from ES cells with diploid or tetraploid embryos and GFP expression in embryos and fetuses at 14.5 days p.c.

Type of embryos	No. of		No. of embryos expressing GFP in		No. of embryos		No. of viable fetuses at 14.5 days p.c. (%)[c]
	aggregates	blastocysts	ICMs (%)[a]	trophoblast	transferred	implanted (%)[b]	
Diploid	25	21	21 (100)	0	21	12 (57.1%)	7 (33.3)
Tetraploid	13	12	12 (100)	0	12	0 (0)	0 (0)

[a]: The rate of GFP expressing embryos to blastocysts. [b, c]: The numbers of implanted embryos and viable fetuses which show heart beating to numbers of transferred embryos.

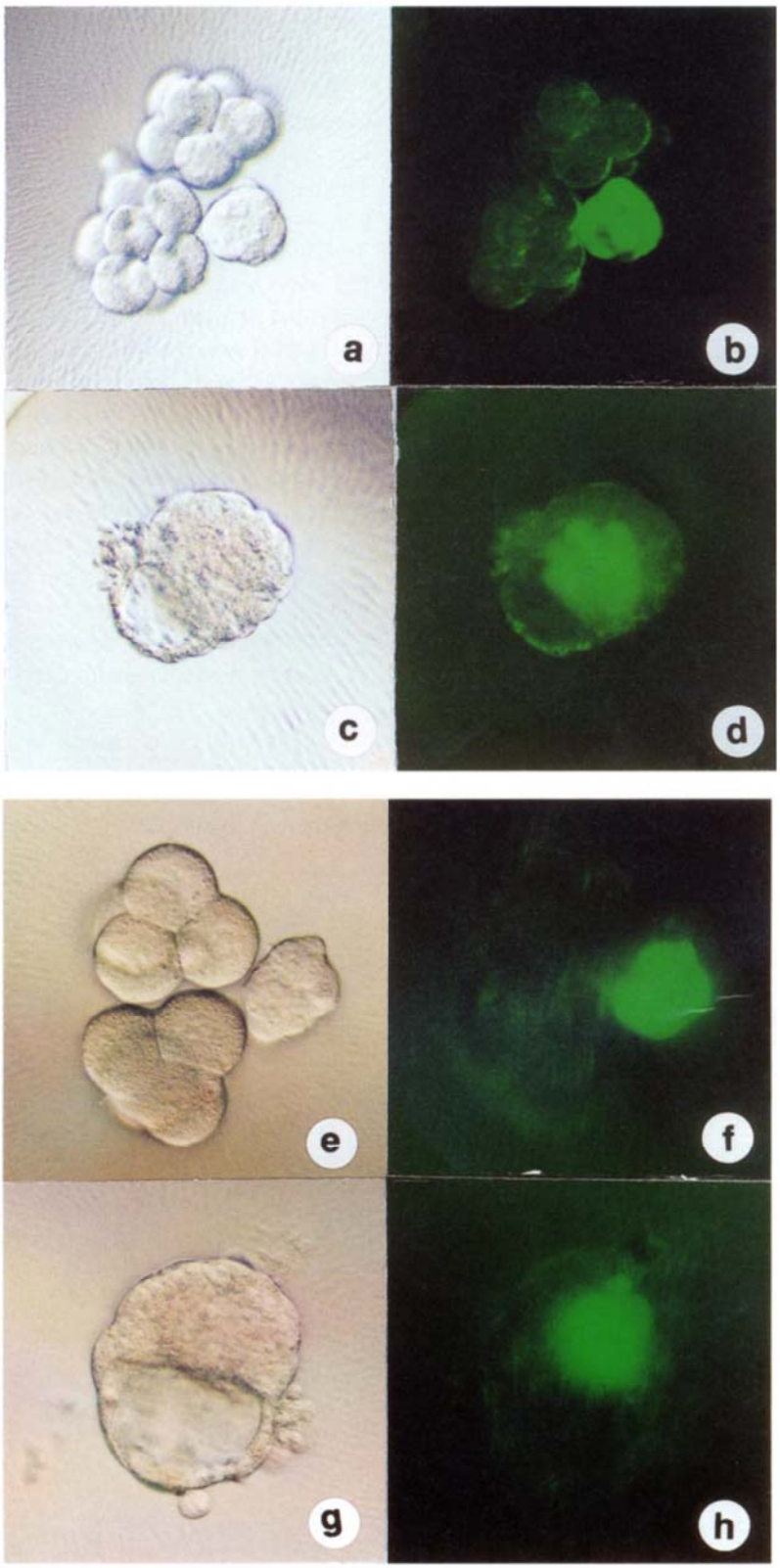

Fig. 1. Aggregation of GFP expressing ES cells with diploid (a~d) or tetraploid embryos (e~h). d, f: Completely tightly adhered ES clumps were located in the ICMs of aggregates.

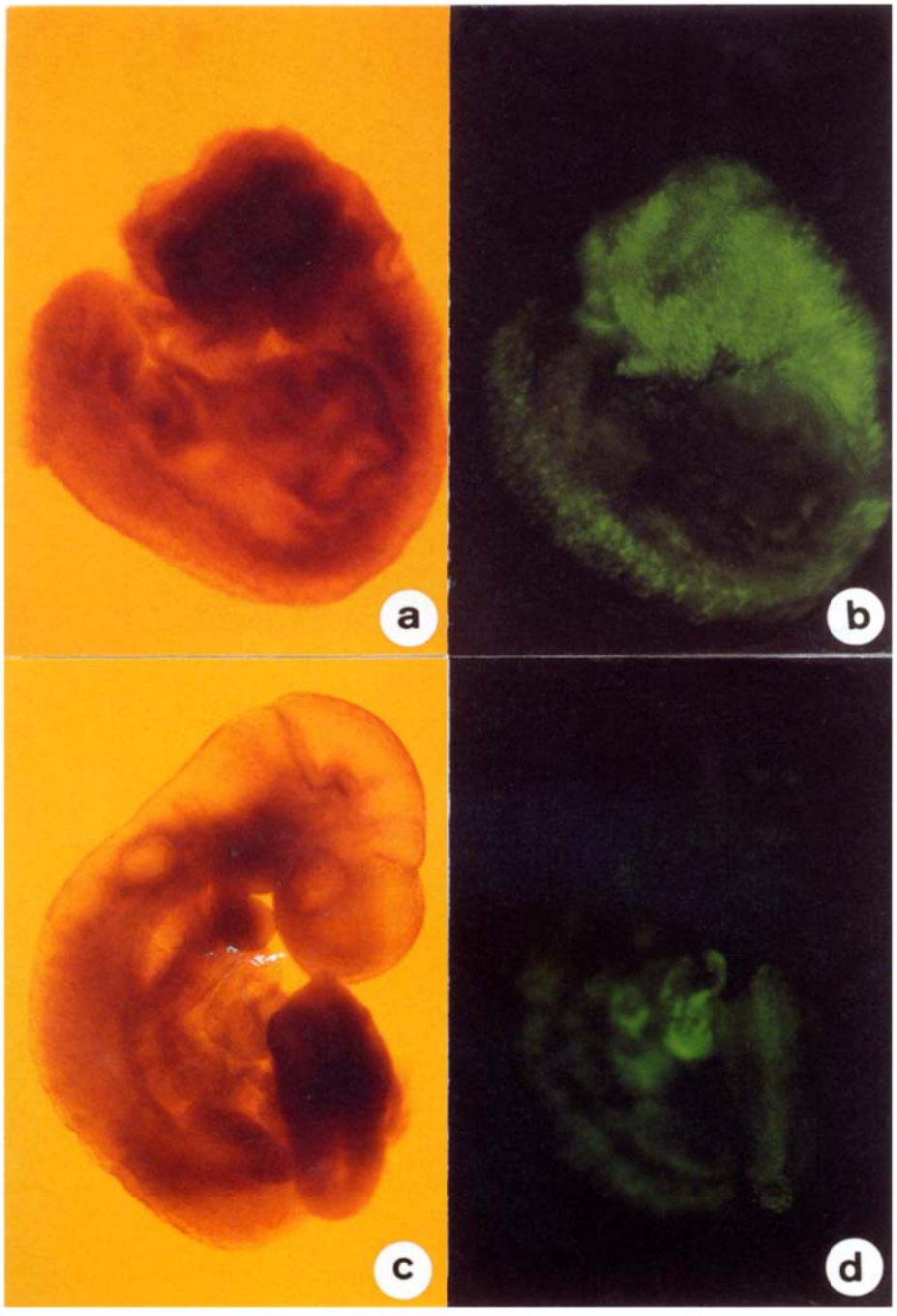

Fig. 2. Aggregation chimera at 9.5 days p.c. derived from GFP expressing ES cells with tetraploid embryos (a, b) or diploid embryos (c, d). a, b: All aggregation chimeras of ES cells with tetraploid embryos showed had a heartbeat, but all of them had head deformity. c, d: This aggregation chimera of ES cells with diploid embryos was normal and expressed GFP strongly in its heart.

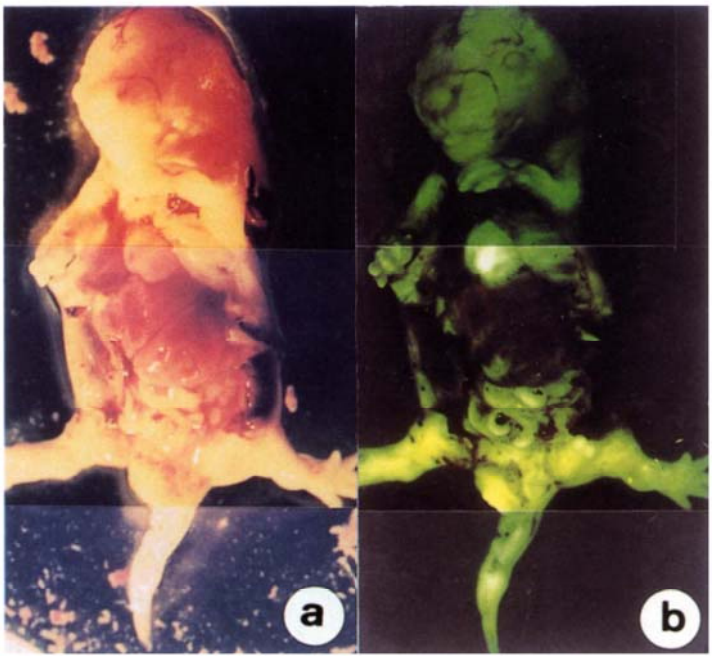

Fig. 3. Aggregation chimera at 14.5 days p.c. derived from GFP expressing ES cells with diploid embryos (Fetus No. 7). This chimera was normal and expressed GFP strongly throughout the body except for the liver.

Table 3. GFP expression in organs of viable ES⇔ diploid chimeric fetuses at 14.5 days p.c.

Fetus No.	Phenotypic sex	Heart	Kidney	Liver	Gonad	Intestine
1	male	N	W	N	W	P
2	male	N	N	N	N	N
3	female	N	N	N	N	P
4	male	N	N	N	W	P
5	male	W	W	N	N	W
6	female	N	N	N	N	N
7	male	W	W	P	W	W

N, W and P indicate no, whole and partial GFP expression in each organ observed under the fluorescent dissecting microscope, respectively.

aggregates and GFP expression in embryos and fetuses at 14.5 days p.c. are shown. For ES⇔2N, the rate of embryos expressing GFP in ICM to aggregates developed to blastocysts was 100% and twelve out of 21 transferred embryos were implanted. And seven fetuses were alive at 14.5 days p.c. For ES⇔4N, 12 out of 13 aggregates developed to blastocysts, and the rate of embryos expressing GFP in ICM to aggregates developed to blastocysts was 100%, but no embryo was implanted. Table 3 shows sex and GFP expression in the five main organs (heart, kidney, liver, gonad and intestine) of fetuses from ES⇔2N. No GFP expression was observed in the five main organs from two out of seven fetuses, but strong GFP expression was observed in at least one organ in the remaining five fetuses (Fig. 3).

Discussion

A nuclear transfer technique using transgenic somatic cells as a donor has recently been established and this technique can be applied to many fields, especially to pharmaceutical production in milk. That is, some cloned sheep derived from somatic cells tansfected with a human factor IX gene connected with an ovine β-

lactoglobin gene promoter have been produced to secrete recombinant proteins in milk [1]. Similarly, some cloned goats derived from fetal fibroblasts generated from a male transgenic founder transfected with a human antithrombin III gene connected with a goat-β-casein promoter have already been produced [6]. On the other hand, we recently produced chimeric calves from aggregates of bovine ES-like cells with tetraploid embryos [3]. The aggregation technique used in this experiment does not require expensive equipment or a high level of skill for the manipulation of embryos. For highly efficient production of transgenic farm animals in both methods, nuclear transfer and aggregation chimera, selection of embryos prior to transfer to the recipient is very important. In the present study, we attempted aggregating mouse tetraploid or diploid embryos with ES cells transfected with a GFP gene, selected with G418 for a short time to avoid differentiation of ES cells, and the production efficiency of transgenic embryos was investigated. Furthermore, GFP expression in fetuses at 9.5 days p.c. for ES⇔2N or ES⇔4N chimeras and in the five main organs for ES⇔2N or ES⇔4N chimera at 14.5 days p.c. was observed.

So far the β-galactosidase gene has been used as a reporter gene, but additional substrates are required to detect the gene expression. *Vargula* luciferase [7] is known as another reporter gene as well, because the gene expression can be observed in living cells, but because it requires an imaging system to determine the activity of luciferase secreted by transgenic embryos, it is a little complicated. Chalfie *et al.* [4] reported that GFP derived from *Aequoea victoria* is not an enzyme and it does not require the addition of substrates to detect the gene expression because it is a self-fluorescent substance. Using these characteristics, we tried a short term selection of ES cells transfected with the GFP gene construction connected to the PGK promoter to prevent differentiation during long term selection with G418 followed by passaging for these undifferentiated and GFP expressing cells. At 8 days after the start of the selection with G418, ES colonies expressing GFP in their entirety were picked up by means of a mouth controlled pipette under the fluorescent microscope, and then these ES cells were cultured on feeder layers to proliferate and were stocked in liquid nitrogen. In this study, thawed ES cells were cultured on feeder layers and some colonies including about 20 cells adhering tightly and expressing GFP in their entirety were used for aggregation. The degree of GFP expression was different in each colony. It is thought that the expression level differs depending on the location in the chromosome when the GFP gene was inserted into the chromosome [8].

Each tightly adhering ES colony was put into a droplet to form a triangle with two tetraploid or diploid embryos. After the aggregation, ES cells completely contributed to the ICMs of aggregates with two types of embryos. Hillman *et al.* [9] showed that each blastomere of a 4-cell mouse embryo placed on the outer surface of a mass of aggregated blastomeres formed a trophoblast. They indicated that cell position after compaction can determine whether the cell is able to form an ICM. Moreover, Wood *et al.* [10] reported that when ES cells transfected with *lacZ* were introduced into morula by a co-culture method, the majority of ES cells contributed to the ICM. They suggested that this may reflect the more committed nature of the ES cells to an ICM fate or simply their much smaller size. In our previous paper [11], when clumps of bovine mammary cells or bovine ICM stained with Hoechst were used for aggregation with bovine putative tetraploid embryos, all clumps contributed to the ICM. Recently Hadjantonakis *et al.* [12] produced green fluorescent mice by germline transmission of green fluorescent ES cells. They performed diploid aggregation of one diploid embryo with a slightly loosely adhered clump, and showed that some ES cells were present in the trophoblast. These results indicated that it was very important for a considerable contribution of ES cells to the ICM that cell-clumps consisted of completely tight adhered cells, not loosely adhered cells.

Nagy *et al.* [13] reported that ES cells tended to contribute fetuses and tetraploid cells tended to contribute extraembryonic lineages in ES⇔4N chimeras. Moreover they produced completely ES derived mice from early-passage ES⇔4N embryos [14]. We also produced heart-beating fetuses at 9.5 days p.c. from ES⇔4N chimeras and they expressed GFP in the whole body, but we did not detect GFP expression in extraembryonic membranes at all. This means that ES cells greatly contributed to chimeras. Unfortunately all of these fetuses had head deformities showing defective formation of the mesencephalon and telencephalon in spite of complete closure of the neurocoel. The anterior neuropore region is usually closing while the posterior neuropore remains open [15]. It was thought that these embryos formed brain anlagen, but complete head formation did not occur because of the failure of neural plate fusion in the head region, and we do not know whether it might be caused by differentiation of ES cells. On the other hand, becausse some fetuses expressing GFP at 9.5 days p.c. were obtained from only ES⇔2N

chimeras, it was thought that ES cells used in these experiments had at least pluripotency in support of diploid embryos. Furthermore, one fetuses at 12 days after embryo transfer obtained from ES⇔2N chimera emitted strong green fluorescence throughout the body. Saburi *et al.* [16] indicated that ES cells underwent one to two cycles of mitosis between the 8-cell and the blastocyst stage when they were introduced as single cells, whereas those introduced as groups of two to five cells did not proliferate during the same period of development. They suggested that the ES cells in the embryos are under the influence of certain growth inhibiting factors secreted in paracrine fashion by the neighboring ES cells or by the ICM cells. It was thought that ES cells which were introduced into the ICM as a clump developed to organs as groups, and under the fluorescent dissecting microscope, GFP expression was observed in the whole organ or in a part of an organ. And, although embryos which expressed GFP in the ICM at blastocyst stage were transferred into recipients, we obtained fetuses in which no GFP expression was observed because of inhibiting development of ES cells.

These results indicated that GFP transfected ES cells selected with G418 in a short term were able to be used sufficiently for the selection of transgenic blastocysts and that transgenic chimeric embryos were produced efficiently by confirming GFP expression in the ICMs under the fluorescent microscope. Moreover, by using a completely tightly adhered clump of ES cells for aggregation, the clump is able to completely form a part of the ICM. By using the GFP gene as a marker, it is possible to select transgenic cells and transgenic blastocysts easily and in a shorter time. Accordingly, GFP is useful for farm animals which have a long term pregnancy and a small litter size.

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