Production of knockout mice by random or targeted mutagenesis in spermatogonial stem cells

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Stem cells represent a unique population of cells with self-renewal capacity. Although they are important therapeutic targets, the genetic manipulation of tissue-specific stem cells has been limited, which complicates the study and practical application of these cells. Here, we demonstrate successful gene trapping and homologous recombination in spermatogonial stem cells. Cultured spermatogonial stem cells were transfected with gene trap or gene targeting vectors. Mutagenized stem cells were expanded clonally by drug selection. These cells underwent spermatogenesis and produced heterozygous offspring after transplantation into the seminiferous tubules of infertile mouse testes. Heterozygous mutant mice were intercrossed to produce homozygous gene knockouts. Using this strategy, the efficiency of homologous recombination for the *occludin* gene locus was 1.7% using a nonisogenic DNA construct. These results demonstrate the feasibility of altering genes in tissue-specific stem cells in a manner similar to embryonic stem cells and have important implications for gene therapy and animal transgenesis.

spermatogenesis | germ cell | testis | transplantation

Stem cells represent a unique cell population with self-renewal potential (1). Although stem cells are low in number, these cells proliferate extensively to sustain the various self-renewing tissues, such as bone marrow and intestine. Although these tissue-specific stem cells normally divide very slowly, stem cells are the last cell type to be destroyed after cytotoxic damage, and they regenerate the entire tissue in a relatively short time. In addition, stem cells often have migratory activities, and they can be transplanted between animals; transplanted stem cells migrate to a specific niche and regenerate the self-renewing tissue. Because of their unique properties, stem cells have become the attractive target of cell and gene therapies.

Among the many types of tissue-specific stem cells, spermatogonial stem cells are unique in that they have germ-line potential (2, 3). Genetic modification of spermatogonial stem cells creates permanent changes in the germ line, which are transmitted to the offspring by means of fertilization. In contrast to female germ-line cells, which cease to divide after birth, male germ-line cells proliferate continuously and produce sperm throughout the life of the animal. If these stem cells could be cultured and manipulated in a manner similar to embryonic stem (ES) cells (4, 5), they could be used to create knockout animals. As a first step toward this goal, a germ cell transplantation technique was developed in 1994 (6, 7), in which dissociated donor testis cells colonized the seminiferous tubules of infertile recipient testis and produced donor-derived spermatogenesis and offspring. Although this technique was an opportunity to produce offspring from manipulated spermatogonial stem cells, it has been difficult to produce transgenic animals using spermatogonial stem cells, because their number is very low in the testis, and the lack of methods to expand spermatogonial stem cells has restricted genetic manipulation (8, 9).

Recently, we described a culture system for expanding spermatogonial stem cells (10). Germ cells formed colonies of a unique shape in the presence of glial cell line-derived neurotrophic factor (GDNF) (11, 12), a critical factor for the self-renewing division of spermatogonial stem cells (13). The addition of GDNF maintains logarithmic proliferation of spermatogonial stem cells and achieved $>10^{85}$-fold expansion of the initial cell population (14). The cells retained germ-line potential after 2 years of culture and underwent spermatogenesis and produced offspring after germ cell transplantation. These cells can now be cultured under serum-free or feeder-free conditions (11, 15) and can be transfected to produce transgenic offspring (16). Based on these properties, we designated the cultured spermatogonia as germ-line stem (GS) cells (10). In this work, we describe the derivation of knockout mice using GS cells. GS cells were mutated by gene trap or targeting vectors, both of which are commonly used to create mutant animals using ES cells. Our results have important implications for future gene therapies and for animal mutagenesis.

Results

Mutagenesis of GS Cells Using the Gene Trap Vector. To induce mutations in the GS cells (Fig. 1*A*), the cells were infected with ROSAβgeo virus, which has been used to mutagenize ES cells (17). For selection using G418, the transfected GS cells were transferred onto neomycin (neo)-resistant mouse embryonic fibroblasts. Single clones were allowed to proliferate in 96-well plates, and the individual colonies were expanded. From the total of 4.5×10^6 GS cells that were infected, we selected 99 clones for *in vitro* expansion. Approximately 80% of the resulting neo-resistant colonies stained positively for LacZ expression.

The cDNA fragments that contained the junction between the endogenous exons and the site of retroviral insertion were amplified from the RNA using the 5′-rapid amplification of cDNA ends (RACE) method (Table 1). The RACE products were recovered from 89 of 99 (89%) clones. Multiple insertions of the retroviral tag sequence were found in 8% of the samples. Sequence analysis of the RACE cDNAs showed that the splice acceptor sequence had functioned in ≈71% of the clones, resulting in the generation of a single ORF that was in-frame with the ROSAβgeo sequence. The retrovirus insertions were randomly distributed on the chromosomes. The mutations occurred predominantly in the upstream regions of the mouse genes (95%), particularly in the first intron (63%). Sequence analysis identified several classes of mutated

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Abbreviations: EGFP, enhanced GFP; ES, embryonic stem; GS, germ-line stem; neo, neomycin.

See Commentary on page 7939.

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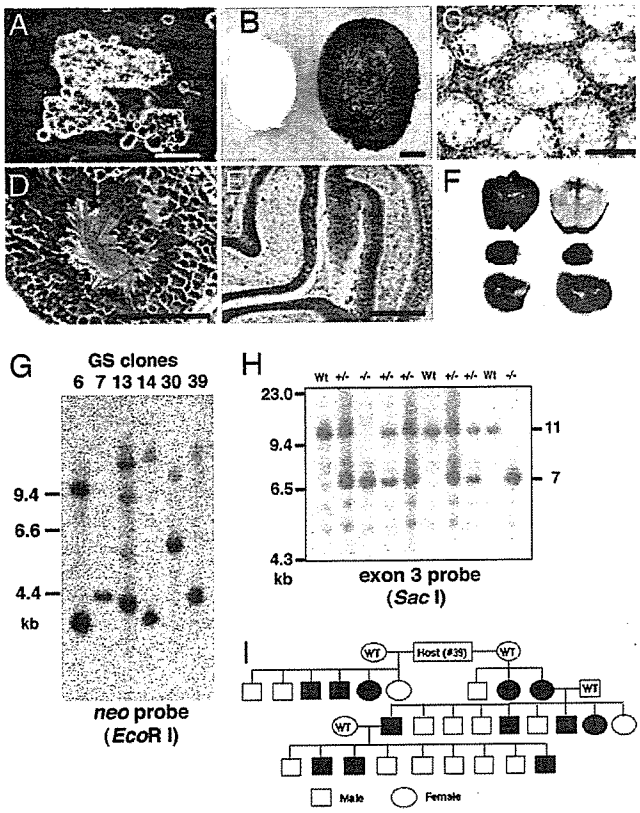

Fig. 1. Gene trapping in GS cells using the ROSAβgeo vector. (*A*) Appearance of the GS cells. (*B*) Appearance of recipient testes after transplantation with GS cells (clone 30) that express the *lacZ* reporter gene (*Right*). The testis of a control W mouse (*Left*) is smaller and does not stain positively for LacZ. (*C*) The nontransplanted recipient testis shows no spermatogenesis. (*D*) The recipient testis after transplantation of GS cells (clone 30) shows normal spermatogenesis. (*E*) *LacZ*-expressing spermatozoa in the epididymis (clone 30). (*F*) Macroscopic appearance of X-Gal-stained brain (*Top*), kidney (*Middle*), and liver (*Bottom*) of transgenic (*Left*) and WT (*Right*) F_1 offspring from clone 30 GS cells. *LacZ* is expressed in the brain and kidney but not in the liver. (*G*) Southern blot analysis of F_1 animals derived from different clones, showing variable integration patterns. (*H*) Southern blot analysis of offspring identified as WT animals and heterozygous or homozygous clone 39 (occludin) mutants. (*I*) Descendents of a recipient W male that was transplanted with clone 39 GS cells. The filled symbols indicate the presence of the transgene. (Scale bars: *A*, 25 μm; *B*, 1 mm; *C–E*, 100 μm.)

genes, including those that encode membrane proteins (ATP synthetase and occludin), metabolic enzymes (pyruvate kinase), DNA repair enzymes (methylguanine DNA methyltransferase and RAD18 homologue), transcription factors (erg and zinc finger protein 111), and proteins associated with the cytoskeleton (Rho GTPase activating protein gene 8 and kinesin-related protein KIFC1). Eleven of the cDNAs obtained with 5′-RACE could not be assigned functions based on database searches (see Table 3, which is published as supporting information on the PNAS web site).

Gene Targeting in GS and mGS Cells. To generate targeted mutations (18, 19), we chose the *occludin* gene (20), which is one of the genes that was successfully trapped in the previous section (Fig. 2*A*). Occludin is one of the tight junction proteins and is expressed in various organs (20). This gene was chosen for two reasons. First, we assumed that gene targeting in GS cells would occur at a higher rate in a transcriptionally active locus (Fig. 2*B*). Second, occludin knockout mice have been produced by ES cell technology (20), which allows comparisons of the efficiency of homologous recombination. Homologous recombination between the vector and the *occludin* gene would result in the deletion of exon 3. We electroporated the knockout vector into enhanced green fluorescent protein (EGFP)-expressing GS cells. The transfected cells were plated on neo-resistant mouse embryonic fibroblasts and exposed to G418 to select for neo-resistant clones. Of the total of 2.4×10^8 GS cells, selection with G418 yielded 120 neo-resistant colonies. Approximately 12–14 weeks of culture elapsed between the transfection and collection of $\approx 2 \times 10^6$ cells for cryopreservation and PCR analysis. In this gene-targeting experiment, we also used ES-like cells (mGS cells) (Fig. 3*A*), which could be generated from the neonatal testis cell culture (21). The same *occludin* gene targeting vector that was used for the GS cells was transfected into 1.9×10^8 cells, and neo selection was conducted on neo-resistant mouse embryonic fibroblasts. The frequency of neo-resistant colonies was $\approx 10^{-5}$ to 10^{-6}. Of the neo-resistant colonies, a total of 139 clones were chosen, and $1–2 \times 10^6$ cells were recovered within 1 month of transfection.

To check for homologous recombination, DNA samples were screened by PCR, which was designed to amplify the 2.4-kb fragment spanning the 3′ junction of the targeted locus. By using this strategy, we demonstrated that 1.7% (2/120) or 2.2% (3/139) of the neo-resistant GS or mGS cell colonies contained correctly targeted cells (Figs. 2*C* and 3*B*). These rates were somewhat lower than that (4.5%) reported for experiments with ES cells (20). The probe for the neo-resistance gene as well as those for exon 2 (internal) and intron 4 (external) hybridized to fragments of the correct sizes. Further confirmation of successful gene targeting was obtained by sequencing the PCR products (data not shown). Cytogenetic analysis using quinacrine plus Hoechst 33258 staining showed that 85–90% of the GS cells retained the normal karyotype, whereas the frequency of euploidy in the mGS cells ranged from 15–75% after genetic selection (Fig. 4*A*). Furthermore, combined bisulfite restriction analysis (COBRA) revealed that the androgenetic imprint patterns were stable in the GS cells, whereas those in the mGS cells changed dramatically (Fig. 4*B*).

Germ-Line Potential of Mutated GS Cells. To confirm the germ-line potential of the mutated GS cells, eight clones, which included seven randomly chosen trapped clones (clone 6, 7, 13, 14, 27, 30, and 39) and one of targeted clones (clone 101), were microinjected into the seminiferous tubules of three to nine infertile WBB6F1-W/W^v (W) mice. Because the germ cells of W mice are unable to differentiate beyond the spermatogonium stage owing to mutations in the *c-kit* gene, spermatogenesis in the recipient testis must originate from transplanted cells (22). After transplantation, the testes grew significantly (Fig. 1*B*), and normal spermatogenesis was confirmed (Fig. 1 *C–E*). The recipients began to produce offspring by natural mating with wild-type (WT) females as early as 77 days after GS cell transplantation; 18 of the 48 (37.5%) recipient mice became fertile within 4 months of transplantation (Table 2). When *in vitro* microinsemination was used, heterozygous offspring were obtained as early as 69 days after transplantation.

To date, we have generated F_1 offspring from all of the injected clones, and these offspring have produced F_2 progeny. The F_1 offspring from the fertile males were examined for the presence of transgenes in their genomes by PCR or Southern blot analysis using a *neo*-specific probe. In total, 65% (115/178) and 71% (10/14) of the F_1 mice were heterozygous for the transgenes for trapped and targeted clones, respectively. The patterns of retrovirus integration and LacZ staining in the offspring differed among the clones (Fig. 1 *F* and *G*). The transgene was transmitted in a Mendelian fashion (Fig. 1 *H* and *I*). The F_1 offspring from targeted GS cells showed the normal imprint pattern (Fig. 4*B*).

Table 1. Examples of fusion transcripts identified by 5′ RACE from the gene trap GS cell lines

No.	Sequences of the trapped gene	Homology (%)	GenBank	Symbol	Chromosome	Insertion	Definition
39	ggtgagcacc ttgggattcc ggccgccaag ctcgcgg	100/100 (100)	NM_008756	Ocln	13	First intron	Occludin
67	gctgctcagt gtggtggatc atttcaaccg ctcgcgg	100/100 (100)	NM_010817	Psmd7	8	First intron	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 7
78	nngaaagact tggtaatggc gacgggtttg ctcgcgg	28/28 (100)	NM_177993	Hbp1	12	First intron	High-mobility group box transcription factor 1
97	attcccacct gctgacttaa gtgcgcccag ctcgcgg	100/100 (100)	NM_009686	Apbb2	5	Second intron	Amyloid β (A4) precursor protein-binding, family B, member 2
101	ccgccgcgcc cggccccgcg cgcgacccgg ctcgcgg	100/100 (100)	NM_010757	Mafk	5	First intron	V-maf musculoaponeurotic fibrosarcoma oncogene family, protein K
104	ccggtgcggc ccggatccgt ggcacgggag ctcgcgg	98/100 (98)	NM_030730	Srisnf21	9	First intron	Steroid receptor-interacting SNF2 domain protein
112	tctgtacctg acccagcagn tnatattaan ctcgcgg	88/100 (88)	NM_133659	Erg	16	First intron	Avian erythroblastosis virus E-26 (v-ets) oncogene related
114	tcttcaagta catcatcatc ggcgacacag ctcgcgg	99/100 (99)	NM_021518	Rab2	4	First intron	RAB2, member RAS oncogene family

Failure to Produce Germ-Line Chimeras Using Mutated mGS Cells. To generate offspring from mGS cells, we microinjected one of the mGS cell clones (clone 118) into blastocysts. This clone was able to produce teratomas with three germ layers when injected s.c. (Fig. 3*C*). After microinjection into the blastocyst, 192 chimeric embryos were derived. Of these, 58 offspring were born, 43 of which developed into mature normal adults, 14 males and 29 females. To determine the level of chimerism, we killed 7 males and 15 females and examined the contribution of the EGFP-positive mGS cells to the mature adult animals. In all, 18% (4/22) of the animals were chimeric. The EGFP-positive cells were distributed throughout the body, i.e., in the gut, stomach, liver, lung, brain, heart, pancreas, muscle, and kidney (Fig. 3 *D–G*). Significantly, EGFP-positive cells also were found in the ovaries of two individual females (Fig. 3*H*), which suggests their contribution to the germ-line. However, no EGFP-positive offspring were produced from the remainder of the chimeras, and fluorescence was not found in the testes.

Phenotype of *occludin*-Deficient Mice Derived from Mutated GS Cells. To examine whether the retroviral insertion and gene targeting in GS cells disrupted the *occludin* gene, tissue samples were prepared from the lungs and kidneys of the WT and homozygous mutant F_2 offspring. RT-PCR and Western blot analysis showed that the both

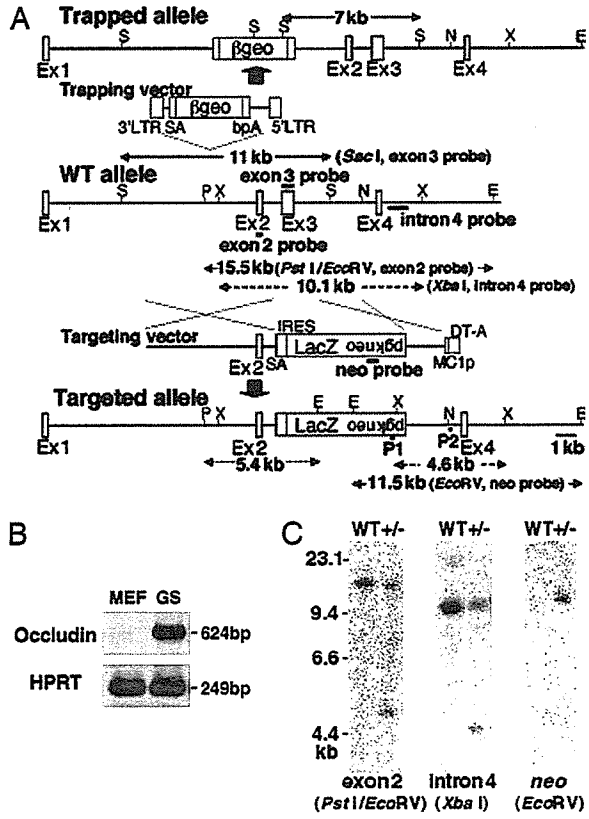

Fig. 2. Disruption of the *occludin* gene. (*A*) Insertion of the gene trap and gene-targeting vectors in the mouse *occludin* locus. SA, splice acceptor; bpA, bovine growth hormone gene polyadenylation signal; E, EcoRV; N, NheI; P, PstI; S, SacI; X, XbaI. P1 and P2 represent the primers used for PCR. (*B*) Expression of occludin in GS cells by RT-PCR. (*C*) Southern blot analysis of a GS cell clone (clone 101). Genomic DNA was digested with the indicated restriction enzymes and hybridized with three different probes.

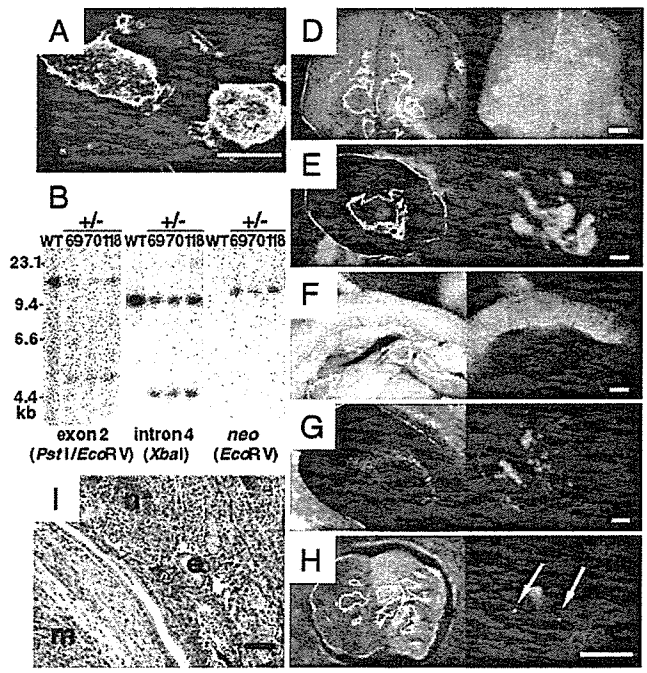

Fig. 3. Production of chimeric animals by heterozygous occludin knockout mGS cells. (*A*) Appearance of the mGS cells. (*B*) Southern blot analysis of the mGS cell lines. (*C*) Section of a teratoma from the mGS cells after gene targeting. Note the presence of tissues from three germ layers, which include muscle (m), neural (n), and epithelial (e) tissues. (*D–H*) Macroscopic appearance of the brain (*D*), heart (*E*), gut (*F*), kidney (*G*), and ovary (*H*), showing fluorescence under UV light. Arrows indicate oocyte-like cells in the ovary. (Scale bars: *A* and *C*, 100 μm; *D–H*, 1 mm.)

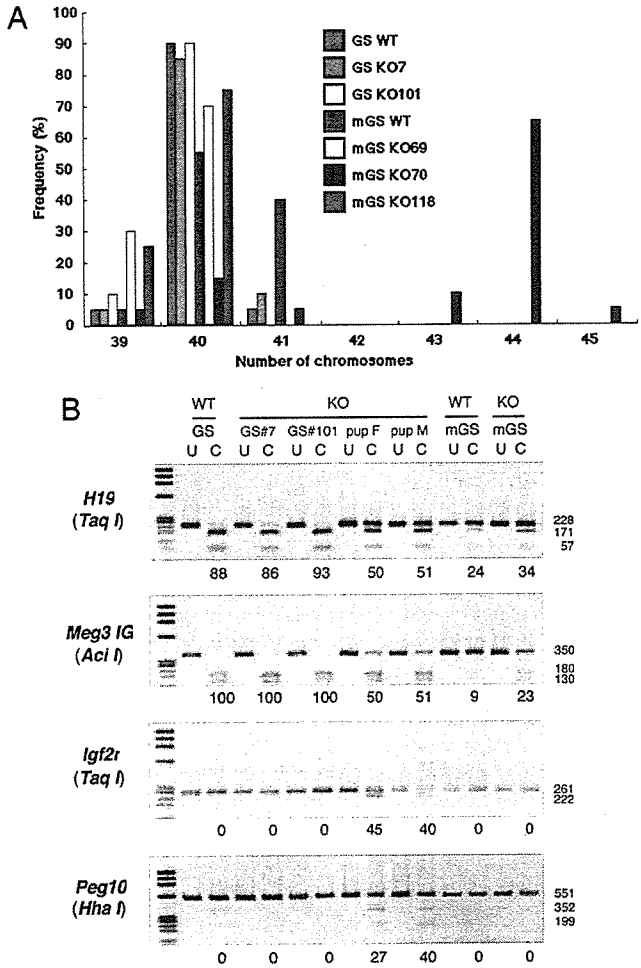

Fig. 4. Effects of genetic manipulation on karyotype and imprint patterns in GS and mGS cells. (*A*) Karyotype analysis of the heterozygous knockout GS and mGS cells before and after genetic selection. At least 20 cells were counted. (*B*) Combined bisulfite restriction analysis (COBRA) of imprinted genes. DNA was isolated from heterozygous knockout GS (clones 7 and 101) and mGS (clone 118) cells. Heterozygous F_1 offspring were produced from GS clone 101. PCR products from differentially methylated regions were digested with enzymes with a recognition sequence containing CpG in the original unconverted DNA. The percentage methylation is indicated below the gel. Enzymes used to cleave each locus are indicated in parentheses. U, uncleaved; C, cleaved. F, female; M, male.

types of homozygous mutant mouse lack the occludin mRNA and protein (Fig. 5 *A* and *B*).

We prepared several histological sections from the salivary gland, stomach, and femoral bone of the WT and homozygous mutant mice. In the salivary gland, histological analysis showed that striated ductal cells of the homozygous mutant mice lacked the characteristic cytoplasmic granules (Fig. 5 *C* and *D*). Defects in the stomach were more dramatic: most of the gastric mucosa of the homozygous mice consisted of regenerating foveolar cells with infiltrating neutrophils and lymphocytes (Fig. 5 *E–G*); there was a marked decrease in the number of parietal as well as chief cells. Degeneration of the proper muscle of the stomach also was observed. The femoral bones showed signs of osteoporosis, revealing decreased thickness of bone trabeculae in the mutant animals (Fig. 5 *H* and *I*). We could not observe any other abnormalities including brain and testis by morphological analyses during the observation period (≈6 weeks), probably because these abnormalities become apparent at later stages. These findings confirm the previous findings that occludin-deficient mice derived by gene targeting in ES cells are viable and have chronic gastritis (20).

Discussion

Ideally, a pure population of stem cells could be transfected to manipulate specific genes and produce mature differentiated cells. However, although site-specific homologous recombination has been achieved by using hematopoietic progenitor cells in one study (23), it has not been possible to achieve this goal using tissue-specific stem cells. The present study demonstrates that it is possible to apply the concept and techniques of ES cell manipulation to spermatogonial stem cells (24). Currently, knockout animals are generally produced by microinjection of mutated ES cells into blastocysts or by nuclear transfer of mutated somatic cells into oocytes (24, 25). Our results now demonstrate a third method to create mutant animals and suggest that the genomes of other stem cells can be similarly manipulated.

There has been uncertainty that homologous recombination can occur at a usable frequency in tissue-specific stem cells (23). Several studies have indicated a low mutation frequency in spermatogonial stem cells. For example, sister chromatid exchange, a measure of homologous recombination (26), occurs less frequently in spermatogonia than in other somatic cell types (27). In addition, there are indications that the level of mutations induced by ethylnitrosourea in spermatogonia is similar to or lower than that induced in ES cells (28). Nevertheless, the frequency of homologous recombinants was within the range reported for ES and other somatic cells (25). In contrast, the frequency of neo-resistant stable clones was low (1 of $\approx 2 \times 10^6$ transfected cells), and it influenced the efficiency

Table 2. Germ-line transmission of mutagenized GS cell lines by germ cell transplantation

Type of experiments	Clone	LacZ stain	Days from transfection to transplantation*	Days to first transgenic†	No. (%) of fertile out of total recipients	No. of litters analyzed	No. (%) of transgenic out of total offspring‡
Gene trap	6	+	64	109	2/7 (28.6)	2	9/12 (75.0)
	7	+	64	82	1/7 (14.3)	5	10/21 (47.6)
	13	+	52	77	3/5 (60.0)	6	38/46 (82.6)
	14	−	78	85	3/8 (37.5)	3	6/15 (40.0)
	27	+	52	80	2/3 (66.7)	5	26/35 (74.3)
	30	−	57	89	2/9 (22.2)	5	15/24 (62.5)
	39	−	57	89	2/4 (50.0)	4	12/27 (44.4)
Gene targeting	101	NA	109	109 (69)§	3/5 (60.0)	2	10/14 (71.4)

Results at 4 months after transplantation. NA, not applicable.
*Time in days from transfection of GS cells to transplantation into infertile recipients.
†Time in days from transplantation of donor cells to birth of first transgenic progeny.
‡Numerator is the number of transgenic progeny; denominator is the total number of progeny from all recipient mice.
§In parentheses is the number of days for first offspring by *in vitro* microinsemination.

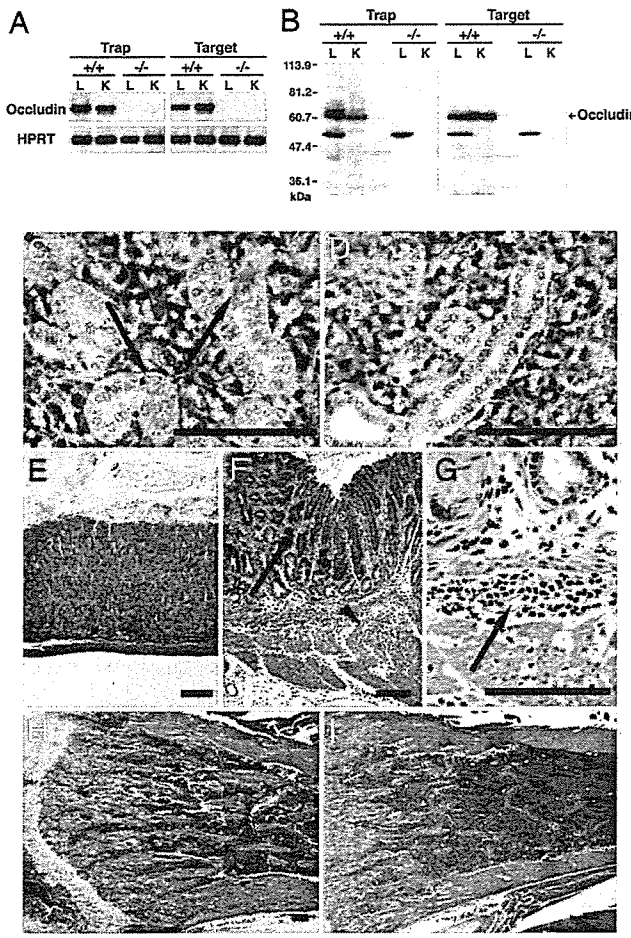

Fig. 5. Phenotype of the homozygous knockout mice. (*A*) RT-PCR analysis of RNA samples from the lungs (L) and kidneys (K) of the WT and homozygous knockout mice. Homozygous knockout offspring lack expression of occludin mRNA (624-bp fragment). (*B*) Western blot analysis with anti-occludin polyclonal antibody of samples from the lungs and kidneys of the WT and homozygous knockout mice. The occludin protein (≈66 kDa) is absent in the homozygous knockout mouse. (*C* and *D*) Histological analysis of the salivary glands of 6-week-old WT (*C*) and homozygous (*D*) mice produced by gene targeting, showing a loss of cytoplasmic granules in the mutant, which can be found in the WT (*C*, arrow). (*E–G*) Histological analysis of the gastric mucosa of 4-week-old WT (*E*) and homozygous (*F* and *G*) mice produced by gene trapping. The predominant hyperplasia of the foveolar cells (*F*, arrow) is accompanied by a significant loss of chief and parietal cells and the degeneration of the proper muscle of the stomach with chronic inflammation (*F*, arrowhead). Infiltration of neutrophils and lymphocytes is widely apparent (*G*, arrow). Similar histology was found in the homozygous mutant made by gene targeting. (*H* and *I*) Histological analysis of the femoral bones of 6-week-old WT (*H*) and homozygous (*I*) mice produced by gene targeting. Trabecular development is decreased in the mutant. Hematoxylin and eosin staining was used. (Scale bars: *C–I*, 100 μm.)

of our experiments. This result was probably caused by the relatively low concentrations of stem cells in GS cell cultures; only up to 1–2% of the cells have repopulation potential (15). Although the current degree of success is modest, our protocol is open to several improvements. For example, the enrichment of stem cells before transfection or changes in the culture condition would probably improve transfection efficiency (29). Although we used a nonisogenic construct in the current study (129 vs. DBA/2), the use of an isogenic construct would further enhance (10- to 50-fold) the gene targeting frequency (30).

Although mutagenesis in GS cells led to the production of knockout animals, we could not generate knockout mice using mGS cells. Although the gene-targeting efficiency in mGS cells was comparable with that in GS cells (2.2% vs. 1.7%), the current study revealed unstable germ-line potential in culture. This situation is somewhat similar to ES cells (31–33), which suggests that the multipotentiality of germ-line cells is maintained at the expense of stability. However, although ES cells acquire aberrant karyotypes during repeated passages (31, 32), they are commonly used for gene targeting, which indicates that they have an acceptable level of instability. In contrast, no targeted mutations have been reported using embryonic germ (EG) cells (34, 35), which suggests that EG cells are less stable than ES cells in germ-line potential. Nevertheless, it will be necessary to test independent mGS cell lines before their utility for germ-line modification can be assessed.

Although the strategies for generating knockout mice from GS and ES cells differ significantly, they require comparable amounts of time. In the present case, transfected cells were expanded for 3–4 months before transplantation. The recipients produced heterozygous progeny 2–3 months after germ cell transplantation, and these animals could be mated to produce homozygous offspring in 3–4 months. Thus, the knockout mice were obtained ≈8–11 months after DNA transfection. In contrast, because ES cells depend on chimera formation with cells of embryos, the donor cells distribute in a mosaic pattern in the F_1 offspring. Because it is necessary to produce an F_2 generation to confirm germ-line transmission of the transgene, it generally takes 6–12 months after transfection to produce knockout mice from ES cells (36). Although the speed of gene targeting is comparable, the GS cell-based approach may confer an advantage in gene trapping, because gene trapping in GS cells permits the direct expression analysis of whole embryos in the F_1 generation.

The most exciting application of our results will be the mutagenesis of other animal species. Given that >60% of genes have been disrupted by using ES cells (37), this technology may have little impact on mouse functional genomics. However, ES cells with germ-line potential are available only in mice. Even in mice, mutations are commonly made in the strain 129 background, and, owing to several genetic weaknesses in this strain, mutant animals are often backcrossed to another background (usually C57BL/6, B6), which takes 18–24 months even with speed congenics (38). Although targeted mutations have been produced recently in several animal species by using nuclear cloning, primary somatic cells have limited proliferative potential, which makes gene targeting more difficult in somatic cells than in ES cells (25). Furthermore, problems associated with nuclear cloning, such as low birth rate and abnormalities in the offspring, need to be resolved (39). In this sense, GS cells may provide the solutions to these problems. The ability to derive GS cells is now extended to different murine genetic backgrounds and to other species (29, 40). Extension of the methodology described here may lead to useful applications in diverse mammalian species.

Materials and Methods

Cell Culture and Genetic Modification. GS cells were established from newborn DBA/2 (Japan SLC, Hamamatsu, Japan) or Green mice (gift from M. Okabe, Osaka University, Osaka, Japan) using a two-step enzymatic digestion, and the culture was initiated as described in ref. 10.

For the gene-trapping experiments, GS cells were infected with the ROSAβgeo retrovirus plasmid (gift from P. Soriano, Fred Hutchinson Cancer Research Center, Seattle) (17). Virus particles were produced by transfection of Plat-E cells and were concentrated as described in ref. 41. The virus titer was $\approx 10^8$ colony forming units/ml. The infection of GS cells with the retrovirus was carried out as described in ref. 42. In brief, a single-cell suspension of 1.5×10^6 dissociated GS cells on mouse embryonic fibroblasts

was mixed with the virus suspension, and the mixture was centrifuged at 1,750 × *g* for 1 h at 32°C.

For the gene-targeting experiments, we used a gene-targeting vector for the mouse *occludin* gene (gift from S. Tsukita, Kyoto University, Kyoto, Japan) (20). Five micrograms of the targeting vector was electroporated into GS cells by using Nucleofector (Amaxa Biosystems, Cologne, Germany). In brief, GS cells were suspended in 100 μl of Nucleofector Solution T, mixed with 5 μg of DNA, and subjected to electroporation. For gene targeting in mGS cells, the cells were suspended in 500 μl of PBS with 20 μg of DNA and subjected to a pulse of 250 V and 500 μF by using the Bio-Rad Gene Pulser II. The mutated cells were selected with 40–200 μg/ml G418 (Invitrogen). The colonies were screened individually by PCR using the following specific primers, P1, 5′-TTGTCACGTCCTGCACGACG-3′, and P2, 5′-CTTAGCTAGGAACCATCAGA-3′.

Transplantation. For s.c. injection, 4×10^6 cells were injected into KSN mice (Japan SLC). For germ cell transplantation, $\approx 10^5$ cells were microinjected into the seminiferous tubules of the testes of 5- to 10-day-old immunosuppressed W recipients (Japan SLC) (15).

Chimera Formation and Microinsemination. Recipient blastocysts for chimera production were obtained by *in vitro* fertilization of $(C57BL/6 \times DBA/2)F_1$ (BDF1) oocytes with B6 sperm. Cells were microinjected into the blastocoels by using a Piezo-driven micromanipulator (43). Microinsemination was carried out by using BDF1 oocytes, as described in ref. 43. The embryos were transferred into the oviducts of ICR pseudopregnant females on the day after culture.

Histological Analysis. The sample tissues or GS cells were fixed with 4% paraformaldehyde for 2 h and stained for LacZ activity (40). The tissues were embedded in paraffin, cut into sections, and stained with hematoxylin and eosin.

5′-RACE. The 5′-RACE technique was performed by using a 5′-RACE system (Invitrogen) according to the manufacturer's protocol, with a primer for cDNA synthesis (5′-TGGGATAGGTTACGTTGGTG-3′) and primers for the nested PCR (5′-CATTCGCCATTCAGGCTGCG-3′ and 5′-TGTTGGGAAGGGCGATCGGT-3′). The PCR products were sequenced directly by using the primer 5′-CTCTTCGCTATTACGCCAGC-3′.

Southern Blotting. DNA was extracted from tail or brain tissue samples. For the gene trapping, a fragment that contained exon 3 of the *occludin* gene (≈630 bp) was amplified by PCR using the 5′-ATGTATGGCGGAGAGATGCAT-3′ and 5′-GGATCAACCACACAGTAGTGA-3′ primers and was used as a hybridization probe. For gene targeting, DNA samples were examined by both the exon 2 and intron 4 probes (20). The clones were further examined for single-site integration by hybridization with the *neo* gene (nucleotides 187–804).

RT-PCR and Western Blotting. First-strand cDNA synthesis and subsequent PCR were carried out as described in refs. 15 and 20. For Western blotting, samples were separated by SDS/PAGE, transferred to poly(vinylidene difluoride) membranes (Hybond-P; Amersham Pharmacia Biosciences), and probed with rabbit polyclonal anti-mouse occludin antibody (F4) (gift from S. Tsukita).

Combined Bisulfite Restriction Analysis (COBRA). The methylation statuses of the imprinted genes were assessed by COBRA using specific primers (14).

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Clonal Origin of Germ Cell Colonies after Spermatogonial Transplantation in Mice[1]

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ABSTRACT

Spermatogenesis originates from a small number of spermatogonial stem cells that can reinitiate spermatogenesis and produce germ cell colonies following transplantation into infertile recipient testes. Although several previous studies have suggested a single-cell origin of germ cell colonies, only indirect evidence has been presented. In this investigation, we tested the clonal origin hypothesis using a retrovirus, which could specifically mark an individual spermatogonial stem cell. Spermatogonial stem cells were infected in vitro with an enhanced green fluorescence protein-expressing retrovirus and subsequently transplanted into infertile recipient mice. Live haploid germ cells were recovered from individual colonies and were microinjected into eggs to create offspring. In total, 45 offspring were produced from five colonies, and 23 (51%) of the offspring were transgenic. Southern blot analysis indicated that the transgenic offspring from the single colony carried a common integration site, and the integration site was different among the transgenic offspring from different colonies. These results provide evidence that germ cell colonies develop from single spermatogonial stem cells.

developmental biology, gametogenesis, Sertoli cells, spermatogenesis, testis

INTRODUCTION

Spermatogenesis originates from a self-renewing population of spermatogonia called spermatogonial stem cells [1, 2]. These cells are the only stem cells in the body that can transmit genetic information to offspring, and they have enormous proliferative potential to sustain spermatogenesis throughout the life span of animals [3]. Spermatogonial stem cells are potentially useful in infertility treatments, animal transgenesis, or regenerative medicine because of their unique biological properties [4–7]. Despite their importance, the study of these cells has been hampered by the lack of a functional assay system to identify stem cells. Because stem cells can be identified only by their ability to self-renew, it is difficult, on the basis of morphological analysis, to distinguish these cells from spermatogonia that are committed to differentiate.

To overcome this problem, Brinster et al. reported a functional assay for spermatogonial stem cells in 1994 [8]. Dissociated donor testis cells produce colonies of spermatogenesis following transplantation into the seminiferous tubules of an infertile recipient testis. The transplanted stem cells migrate through the tight junction between Sertoli cells and proliferate on the basement membrane [9]. The colonies first expand horizontally on the basement membrane, but they start to differentiate vertically in the center of the colony and undergo meiotic division and spermiogenesis [9]. Stem cells are thought to increase their number at the margin of the colony, whereas they start to differentiate at the center. The growth of colonies continues for several months, and mature spermatozoa are observed 3 mo after transplantation [9]. The recipient animals eventually produce offspring from the donor cells [10]. By definition, only stem cells can initiate and maintain long-term spermatogenesis and produce these results. This transplantation technique provided a new opportunity for studying and manipulating spermatogonial stem cells.

Although the transplantation technique was pioneered more than a decade ago, one of the questions that remains to be answered is the clonal origin of germ cell colonies. The technique has been used for quantifying the number of stem cells [11, 12], but this has been based on the assumption that colonies are derived from single stem cells, which remains to be proven. The single-cell origin of germ cell colonies was supported by the linearity of the curve relating the number of germ cells transplanted to the number of colonies that developed in the recipient testis [13]. In another study, Zhang et al. transplanted the mixed testis cell populations from two different transgenic mice, excised the individual colony, and examined its origin by PCR analysis [14]. Their study revealed that only one genotype was present in the colony when there were less than 20 colonies in the testis. Although these studies lend support to the clonal origin of stem cell colonies, they do not eliminate the possibility that the colonies originated from two or more cells that had remained aggregated during the preparation of the testis cell suspension. In addition, PCR-based assays are often accompanied by problems in specificity and reproducibility, and different approaches are required to resolve this problem.

In this study, we attempted to test the hypothesis that colonies are derived from single stem cells using retroviral transduction of spermatogonial stem cells and microinsemination [7, 15, 16]. Retroviruses integrate into the genome randomly; they have been used extensively to mark stem cells clonally to trace their differentiation patterns in various stem cell systems [17–19]. Donor germ cells were transduced with enhanced green fluorescence protein (EGFP)-expressing retrovirus, and offspring were produced using germ cells that developed from EGFP-expressing colonies by microinsemina-

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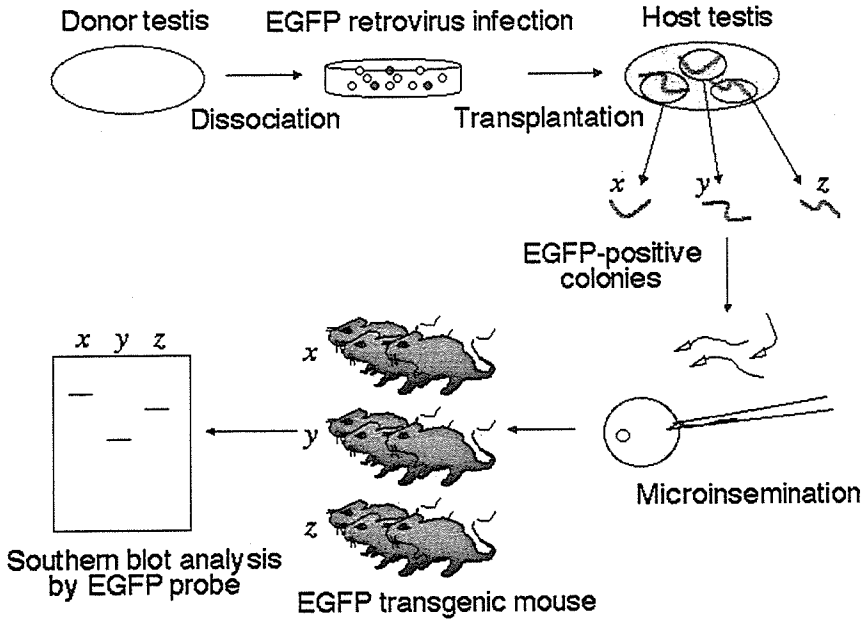

FIG. 1. Diagram of the experimental protocol. Donor testis cells were dissociated by enzymatic digestion and infected in vitro with retroviruses. The infected cells were transplanted into syngeneic infertile recipient testes. At 20 wk following transplantation, EGFP-positive colonies were harvested, and elongated spermatids were isolated by mechanical dissociation. The elongated spermatids were then microinjected into oocytes to produce transgenic offspring. DNA from the transgenic offspring was subjected to Southern blot analysis using an *EGFP* probe. See *Materials and Methods* for details.

tion. The genotype of the resulting animals was determined to see whether each colony arose from a single stem cell.

MATERIALS AND METHODS

Animals and Transplantation

The Institutional Animal Care and Use Committee of Kyoto University approved all animal experimentation protocols. Donor testis cells were collected from 5- to 10-day-old C57BL6 (B6) male mice (Japan SLC, Shizuoka, Japan). Testis cells were dissociated using a previously described two-step enzymatic digestion method [20]. For transplantation, busulfan-treated C57BL/6 × DBA/2 F1 (BDF1) mice or congenitally infertile WBB6F1-Kit^{W}/Kit^{W-v} mice (designated W; both from Japan SLC) were used as recipients. BDF1 mice were used for germ cell transplantation at least 4 wk after the intraperitoneal injection of busulfan (44 mg/kg) at 4 wk of age [20]. W mice lack all stages of differentiating germ cells because of mutations in the gene that encodes KIT receptor tyrosine kinase [21]. For the microinjections, approximately 10 μl of the donor cell suspension were introduced into the seminiferous tubules of a BDF1 testis, whereas 2 μl were microinjected into a W testis [22]. Cells were microinjected through the efferent duct [20], and 75%–85% of the tubules in each recipient testis were filled.

Retrovirus Preparation

An overview of the experimental design is outlined in Figure 1. To introduce retroviral genes into spermatogonial stem cells, we used a recently reported in vitro retrovirus-mediated gene transfer technique [7], which led to the production of transgenic offspring after the transplantation of transduced stem cells into recipient testes. We used two types of vectors that express *EGFP* genes under different promoters. The pMy-internal ribosomal entry site (IRES)-EGFP vector (designated pMy) was a generous gift from Dr. T. Kitamura (Tokyo University, Japan). The pGpig vector was constructed from a replication-defective ecotropic retroviral vector pGen- [23]. An *EGFP* (Clontech, Palo Alto, CA) attached downstream of an encephalomyocarditis virus IRES sequence was inserted under phosphoglycerate kinase 1 (*Pgk1*) promoter (gift from Dr. T. Nomura, Kyoto University). Whereas the pMy vector depends on the modified long-terminal repeat (LTR) for EGFP expression, the LTR is inactivated in pGpig, so *EGFP* gene expression depends on the internal *Pgk1* promoter. These viral vectors were previously used to transduce different kinds of stem cells [7, 24].

Viral particles were produced by the transient transfection of packaging cells. The Plat-E packaging cell line was transiently transfected with retrovirus cDNA by Fugene6 (Roche Diagnostics, Tokyo, Japan) [25]. The supernatant, which was concentrated as previously described [26], was collected 2 days later. The final titer of the virus was ~10^9 or 10^8 colony-forming units/ml for pMy or pGpig, respectively.

Retroviral Infection of Spermatogonial Stem Cells

For the donor cells, we collected testes from 5- to 10-day-old B6 pups. The testes of this stage contain an enriched population of spermatogonial stem cells and have a higher frequency of retrovirus infection than those from mature testes [7, 22]. After dissociation, 1.2–1.8 × 10^7 testis cells were placed on mitotically inactivated mouse embryonic fibroblasts, and the mixture was centrifuged at 1750 × *g* for 1 h at 32°C in the presence of 4 μg/ml polybrene [27]. After a 1- to 2-day culture at 37°C in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), the infected cells were washed twice with PBS, and 55%–95% of the input cells were harvested by trypsin digestion. The cells were suspended in DMEM supplemented with 10% FCS and transplanted into recipient testes. This technique allows competent donor cells to colonize into empty niches to permit the regeneration of donor stem cells [8]. Busulfan-treated adult BDF1 mice were used for the transplantation of pMy-infected cells, and congenitally infertile W pups were used for the transplantation of pGpig-infected cells. In a total of six experiments, approximately 1.4–2.1 × 10^6 pMy-infected cells were transplanted into BDF1 recipients, whereas 1.1–1.5 × 10^5 pGpig-infected cells were transplanted into W recipients. The viability of the cells was greater than 95% as determined by trypan blue exclusion.

Microinsemination

The seminiferous tubules of recipient testes were dissected under UV illumination, which allowed for the specific identification of donor cells because the recipient testis does not exhibit endogenous fluorescence. Fragments of EGFP-expressing seminiferous tubules were recovered, and each fragment was dissociated mechanically to collect the germ cells. We chose colonies that were longer than 1 mm to ensure sufficient recovery of germ cells. In these colonies, germ cells occupied 100% of the basal surface of the tubule. The number of colonies was quantified by counting the total number of colonies observed under a stereomicroscope. Microinsemination was performed as previously described by the intracytoplasmic injection into BDF1 oocytes [15]. After in vitro culture, two- or four-cell-stage embryos were transferred to the oviducts of Day 1 pseudopregnant Institute for Cancer Research (ICR) female mice (Japan SLC). Offspring were born by cesarean section on Day 19.5.

Southern Blot

Genomic DNA was isolated from the offspring by phenol/chloroform extraction, followed by ethanol precipitation [5]. Twenty micrograms of DNA

TABLE 1. Transduction of spermatogonial stem cells by EGFP-expressing vectors.

Vector	Host[a]	No. of experiments	No. of recipient animals	No. of testes injected	No. of testes colonized	Total no. of colonies
pMy	B6 × DBA/2 F1	4	19	35	0	0
pGpig	Kit^{W}/Kit^{W-v}	2[b]	5	10	10	47
Total		6	24	45	10	47

[a] Only immature pups (5- to 10-day old) were used as recipients for transplantation of pGpig-infected cells.
[b] In one experiment involving three recipients, cells were incubated with retrovirus for 1 day.

were digested with indicated restriction enzymes and separated on a 1.0% agarose gel. DNA was transferred and blotted onto a nylon membrane (Hybond-N+; Amersham Biosciences, Buckinghamshire, UK). Hybridization was performed according to a conventional protocol. The full-length *EGFP* cDNA was used as a probe for hybridization. The membrane was hybridized for 16 h at 65°C with a ^{32}P-labeled probe.

Statistical Analysis

Statistical analysis was performed using ANOVA.

RESULTS

Retroviral Infection of Spermatogonial Stem Cells In Vitro

To assess the single-cell origin of germ cell colonies, we used two retroviral vectors to mark spermatogonial stem cells. Although we failed to observe EGFP-positive colonies after transplantation of pMy-infected cells, we found that following the transplantation of pGpig-infected cells into W pup testes, the transplantation of cultured cells resulted in the colonization of all recipient testes (Table 1) and that EGFP-expressing colonies were observed (Fig. 2, A and B). The number of colonies ranged from 2 to 18 per testis.

After mechanical dissociation of the fluorescent colonies (Fig. 2B), we observed EGFP-expressing germ cells and found that 60.9% ± 5.0% (mean ± SEM, n = 3) of the cells showed fluorescence under UV light. Round spermatids and spermatocytes are round cells without tail and could be identified by their morphology. They commonly show low nuclear/cytoplasmic ratio and characteristic nuclear appearance. Because of weak activity of the *Pgkl* promoter in the later stage of spermatogenesis [28], EGFP expression in germ cells became weaker as the cells differentiated. Although round spermatids showed relatively strong fluorescence, only a portion of elongated spermatids showed weak fluorescence (Fig. 2, C and D). EGFP expression in spermatozoa was barely detectable. Nevertheless, these results indicated that pGpig-infected donor stem cells colonized the recipient testes and completed spermatogenesis.

Production of Transgenic Offspring by In Vitro Microinsemination

We chose two different W recipients that received a transplantation of pGpig-transduced testis cells to examine whether each colony was derived from a single stem cell. In total, six EGFP-positive colonies were removed from the recipient testes under UV light 20 wk after the transplantation of donor cells. The length and intensity of EGFP fluorescence varied among the six colonies, suggesting the different integration sites of the retrovirus. Of these six colonies, elongated spermatids with tails were recovered from five colonies after mechanical dissociation (designated colonies 1–5). These cells were generally nonmotile and were picked by micromanipulation for injection into BDF1 oocytes. In total, 212 embryos were constructed, and 162 embryos that reached the two- or four-cell stage were transferred into 11 pseudopregnant ICR mothers. Although 1 of the 11 recipients died after embryo transfer, the remainder gave birth to normal offspring by cesarean section at term. The number of offspring ranged from 4 to 19 for each group; 45 offspring were obtained in total (Fig. 2E).

To determine the presence of the retroviral vector in the genome, we recovered DNA from all the offspring and performed Southern blot analysis using an *EGFP*-specific probe. Genomic DNA was digested with *Sca* I or *Dra* I, both of which do not cleave within the probe. Thus, the *EGFP* probe hybridized with a single unique-size fragment from each retroviral integration. Of the 45 offspring, 23 (51.1%) contained the retroviral transgene. Transgenic offspring were produced from four of five colonies, and 33.3%–73.7% of the offspring from each colony contained the transgene (Table 2).

Analysis of the offspring from colonies 2–4 showed that all the transgenic animals that were derived from the same colony showed bands of identical size by *Sca* I digestion (Fig. 3A). This relationship was conserved with *Dra* I digestion, indicating that they represent a common integration event. Furthermore, transgenic animals from different colonies showed bands of different sizes, indicating that they represent different integration events. The differences among these groups were statistically significant ($P < 0.0001$ by ANOVA). In contrast, offspring from colony 1 had three different patterns when they were analyzed by the same approach. Of the 19 total offspring, 8 of the 14 transgenic offspring had two bands (16.5 kb and 7.3 kb), and the remaining six offspring had only one band (two at 16.5 kb and four at 7.3 kb). A similar relationship was confirmed with *Dra* I digestion (Fig. 3B). These results show that two retroviral integrations occurred at different positions in this stem cell and that the two transgenes were transmitted independently by segregation during meiosis, eventually producing three patterns in the offspring. Taken together, our results indicate that the transgenic offspring from individual colonies were identical in stem cell origin.

DISCUSSION

The colonization of spermatogonial stem cells into recipient testes has provided the first functional assay for spermatogonial stem cells [8]. It involves a complicated process of cell migration into the niche and subsequent expansion of stem cells and differentiation into progenitor cells. The process of stem cell colonization is apparently nonphysiological because stem cells migrate to the basal side and colonize a niche when they are injected into the adluminal side of seminiferous tubules. While germ cells normally differentiate from the basal part to the adluminal lumen [1, 2], the colonized stem cells could still perform spermatogenesis, producing gametes that are normal in appearance and function. Although several

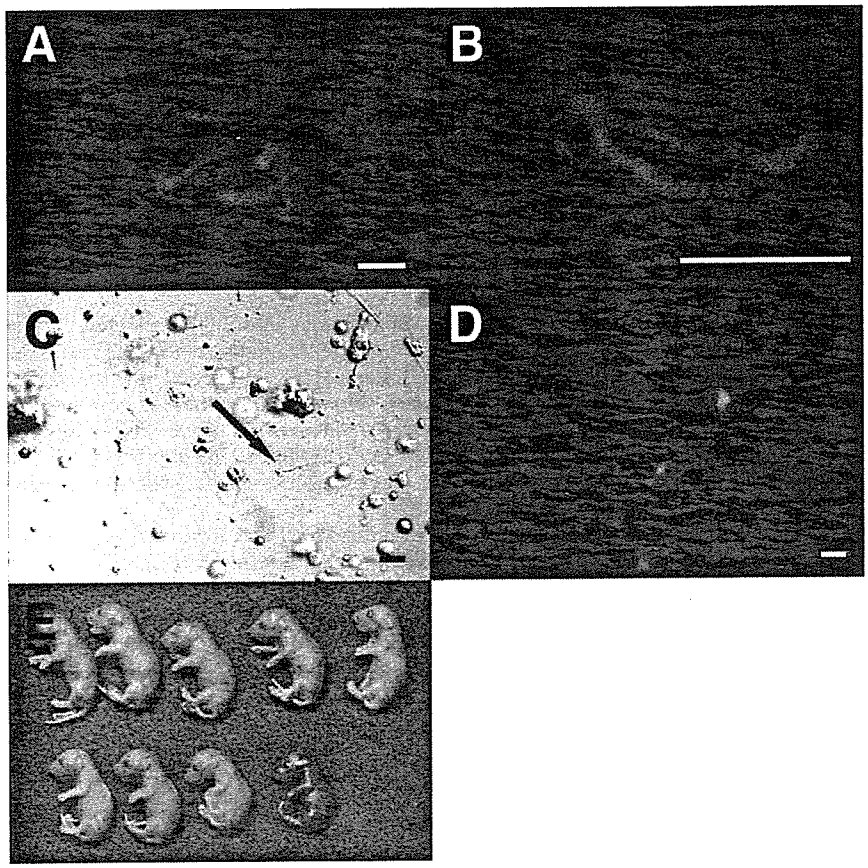

FIG. 2. Offspring produced from a single colony. **A**) Macroscopic appearance of a recipient testis that received pCpig-infected donor testis cells. Testes were placed under UV light to visualize the EGFP-positive colonies originating from transduced spermatogonial stem cells. **B**) Single colony dissected out from the testis in **A**. **C**) Single-cell suspension made from the colony in **B** by mechanical dissociation, shown in bright field. A round spermatid or elongated spermatid (arrow) was found in the cell suspension. **D**) The same cell suspension fluorescing under UV light. Note the faint fluorescence in transduced germ cells. **E**) Nine offspring derived from colony 4. One of the offspring died immediately after birth. Bar = 1 mm (**A**, **B**) and 20 μm (**C**, **D**).

morphological studies were conducted on how stem cell colonies develop after transplantation [9, 29, 30], it remains unclear whether the colonies developed from clonal proliferation of a single stem cell.

In concept, our approach mirrors those previously taken to demonstrate the clonal origin of hematopoietic stem cells. It was shown in the early 1960s that transplanted hematopoietic cells produce spleen colonies after intravenous injection into lethally irradiated recipient mice [31]. To demonstrate their single-cell origin, the transplantation of mixed donor cells was attempted, and the results of these studies supported this hypothesis [32–34]. These studies, however, could not exclude the possibility that colonies are derived from aggregated donor cells. The most rigorous evidence of the clonal nature of colonies was provided by transplanting irradiated bone marrow cells [35]. Irradiation induced unique chromosomal damage to the donor cells, which served as a specific marker to trace the progenitor cells that developed from single cells. However, we did not apply this technique for two reasons. First, we speculated that radiation-induced chromosomal damages might influence the normal differentiation of germ cells. It has been known that chromosomal abnormalities are often found in infertile males [36]. Second, accurate cytological analysis of a small number of cells is technically difficult. In our previous study with single-colony analysis, only $\sim 10^4$–10^5 cells could be recovered from single colonies [37].

We used a retroviral marking technique to overcome these problems. Retroviral marking of stem cells has been widely

TABLE 2. Embryo development after microinsemination.[a]

Colony[b]	No. of cultured eggs	Two-cell (%)	Four-cell (%)[e]	No. of eggs transferred	Implantation sites (%)	No. of offspring (%)	Transgenic (%)
1[c]	65	59 (90.8)	NA	59	34 (57.6)	19 (32.2)	14/19 (73.7)
2[d]	53	49 (92.5)	48 (90.6)	30[f]	13 (43.3)	7 (23.3)	3/7 (42.9)
3[d]	44	36 (81.8)	34 (77.3)	34	13 (38.2)	6 (17.6)	2/6 (33.3)
4[d]	34	31 (91.2)	30 (88.2)	30	23 (76.7)	9 (30.0)	4/9 (44.4)
5[d]	16	9 (56.3)	9 (56.3)	9	5 (55.6)	4 (44.4)	0/4 (0)
Total	212	184 (86.8)	NA	162	88 (54.3)	45 (27.8)	23/45 (51.1)

[a] Elongated spermatids were used for microinsemination.
[b] Colonies 2–5 were collected from a single testis, but colony 1 was collected from a different recipient.
[c] Embryos were cultured for 24 h and transferred into the pseudopregnant mother.
[d] Embryos were cultured for 48 h and transferred into the pseudopregnant mother.
[e] NA, not applicable because embryos were transferred at the two-cell stage.
[f] One of three recipients died during pregnancy and the results were omitted from the data.

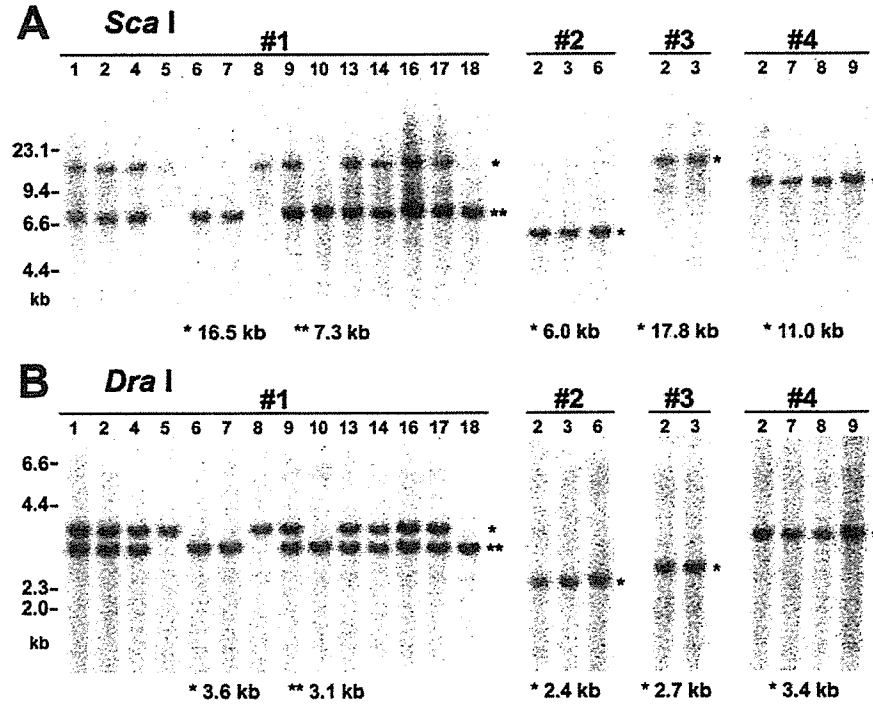

FIG. 3. Southern blot analysis of DNA samples from transgenic offspring. Genomic DNA from all transgenic progeny was digested with *Sca* I (A) or *Dra* I (B) and hybridized with an *EGFP* gene-specific probe. Note the variance in the hybridization pattern, indicating the different integration site of the transgene.

used to study the kinetics or fate of stem cells from several self-renewing systems, including the hematopoietic system [17–19]. Retroviruses can randomly integrate into the genome of donor cells, which allows for the clonal identification of stem cells. In the spermatogenic system, it was recently shown that the in vitro transduction of donor cells and subsequent transplantation into infertile recipients led to the labeling of spermatogonial stem cells [7, 38]. These studies showed that the unique marking of stem cells by a retrovirus is an effective method for tracing the dynamics of stem cells, which may be applicable to demonstrate the clonal origin of stem cell colonies. Another important advantage of the current approach is in vitro microinsemination [39, 40], which allows the development of offspring from a small number of germ cells that could be recovered from pieces of seminiferous tubules. Not only mature spermatozoa but also more immature germ cells, such as round spermatids or primary spermatocytes, can be used to fertilize eggs for offspring production [39, 40]. Because of these advantages, the technique has revolutionized conventional assisted reproduction techniques and is widely used to treat infertile animals and men [15, 16]. In this study, these techniques were combined and used as tools for the clonal analysis of germ cell colonies. This is in contrast to traditional approaches for lineage analysis of somatic cells, which depend on DNA analysis of small tissues or the retransplantation of colonies into secondary recipients. Microinsemination provided a unique and reliable method of analyzing the donor origin of germ cell colonies.

The results in the present study provide new evidence in support of the clonal origin of germ cell colonies. In the present experiments, the injected testes of the recipient mice were analyzed 4–5 mo after transplantation, which corresponds to approximately four spermatogenic cycles in mice [1, 2]. Therefore, differentiated progenitor cells, even if they were infected, would disappear, and only stem cell-derived colonies would be generated [9]. Although periodic infection during several days was necessary for infection of mouse and rat spermatogonia [7, 41], infection could be achieved even after overnight culture, suggesting that a combination of concentration and centrifugation is a more efficient method for spermatogonia transduction. However, the infection of stem cells was clearly demonstrated only with pGpig vector, and we did not observe EGFP signal after pMy infection. A possible explanation is that expression silencing occurred with pMy vector. Alternatively, the lower seeding efficiency of stem cells in adult recipients may not have allowed the colonization of the small number of transduced stem cells [22].

Overall, the efficiency of transgenic offspring production reached ~50% and surpassed the results of previous studies (5%–30%) in which transgenic offspring were produced by natural mating [7, 38]. The current efficiency (~50%) is likely the upper limit expected with this vector because the *EGFP* transgenic marker was expressed predominantly in the diploid stage and we could not distinguish elongated spermatids that carried the transgene for microinsemination. The efficiency could be improved by the use of a haploid promoter for EGFP expression. Partly because of this problem, we failed to produce transgenic offspring from colony 5 because of the low amounts of spermatozoa that were present in the colonies. Nonetheless, transgenic offspring were produced from four other colonies, which validated our approach for the genetic analysis of colony formation. Of the four colonies that produced transgenic offspring, three colonies (colonies 2–4) were shown to produce offspring with a consistently identical viral integration site. The pattern was specific to each colony and differed from one to another. The viral integration must have occurred at the stem cell level because differentiated progenitor cells, even if they were infected, would have differentiated and disappeared by the time of analysis. Therefore, these results clearly indicate that the transgenic progeny originated from different stem cells and that each

infected stem cell underwent self-renewal division to produce committed progenitors bearing the same viral integration site.

In contrast, the Southern blot analysis of colony 1 showed that their transgenic offspring had three patterns of viral integration. However, further analysis using different restriction enzymes showed that the integration event in the offspring having a single band was identical to one of the two events that occurred in different offspring. It suggests that these patterns resulted from the segregation of two transgenes during meiosis; some of the progeny inherited only one of the transgenes, and others inherited two transgenes. Therefore, we can conclude that this result was produced by the two viral integrations that occurred at the diploid stem cell level, supporting the clonality for colony 1. Although multiple integrations of the transgenes do not generally pose this type of problem in the lineage analysis of somatic stem cell systems, the present case suggests that care should be taken for the interpretation of results when it is applied to germ-line cells; meiotic recombination may alter the distribution of viral integration sites.

Although our results suggest the clonal origin of spermatogonial stem cell colonies, they do not exclude the possibility that multiple stem cells exist in a niche [42]. Indeed, our retransplantation experiment previously showed that a single colony contains multiple stem cells after spermatogonial transplantation [37]. However, we still do not know how many stem cells maintain spermatogenesis, the normal life span of the spermatogonial stem cell, or their possible regulatory mechanisms. Although these questions are difficult to study directly in normal testes, approaches taken in this study, namely, retroviral marking and single colony analysis after transplantation, may provide a simple model system to dissect the complex processes of spermatogenesis. The use of EGFP will be particularly useful because it allows the vital analysis of stem cells and their progenitors. These types of studies will ultimately lead to a better understanding of the spermatogenesis mechanism.

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Complementation of placental defects and embryonic lethality by trophoblast-specific lentiviral gene transfer

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Placental dysfunction underlies many complications during pregnancy, and better understanding of gene function during placentation could have considerable clinical relevance. However, the lack of a facile method for placenta-specific gene manipulation has hampered investigation of placental organogenesis and the treatment of placental dysfunction. We showed previously that transduction of fertilized mouse eggs with lentiviral vectors leads to transgene expression in both the fetus and the placenta[1,2]. Here we report placenta-specific gene incorporation by lentiviral transduction of mouse blastocysts after removal of the zona pellucida. All of the placentas analyzed, but none of the fetuses, were transgenic. Application of this method substantially rescued mice deficient in *Ets2*, *Mapk14* (also known as *p38α*) and *Mapk1* (also known as *Erk2*) from embryonic lethality caused by placental defects. Ectopic expression of *Mapk11* also complemented *Mapk14* deficiency during placentation.

The placenta, the first organ to be formed during gestation, is responsible for gas and nutrient exchange as well as hormone production to support fetal development. Over the past decade, gene manipulation in the mouse has substantially advanced our understanding of the genetic control of placental development[3,4]. However, in most cases, it is not clear whether phenotypes originate in the placenta, the fetus, or both. Direct injection of gene-therapy vectors into the placenta has resulted in complications such as injury and patchy expression[5,6], underscoring the need for an efficient, noninvasive approach for gene manipulation exclusively in the placenta.

In the preimplantation embryo, trophoblast cells are the first differentiated cells that can be distinguished from the pluripotent inner cell mass (ICM) and they form the outermost layer of the blastocyst. The trophoblast cell lineage provides the most important cell types for the main structural and functional components of the placenta. To introduce a foreign gene into trophoblast cells, we chose a self-inactivating lentiviral vector based on HIV-1[1,2,7] and transduced preimplantation blastocysts, expecting placenta-specific gene expression (**Fig. 1a,b**). The strong and ubiquitously expressed CAG promoter[2] was used in all of the experiments. As the zona pellucida functions as a physical barrier to viral infection, we removed it by treatment with acidic Tyrode's solution[8] before exposing blastocysts for 4 h to a lentiviral vector LV-EGFP at 1×10^3 ng p24/ml (p24 is a capsid protein in the HIV virus particle). When blastocysts were transduced with an *EGFP*-containing construct, fluorescence was observed only in trophoblasts after 6–8 h of incubation, whereas when two cell– to four cell–stage embryos were transduced, uniform expression of *EGFP* in both ICM and trophoblast cells was observed (**Fig. 1c**). The capacity for transgene expression before implantation suggests the potential of this approach for investigating trophoblast cell function during implantation.

We transplanted the LV-EGFP–transduced embryos into pseudopregnant females and determined the efficiency of transgene incorporation by PCR-mediated amplification of genomic DNA prepared from the fetus and placenta at embryonic day (E) 13.5 (**Supplementary Table 1** online). Consistent with previously reported efficiencies of the transduction of material spanning the range from fertilized eggs to morula-stage embryos[1,2,9], 61% (51/83) of the embryos transduced at the two-cell stage showed transgene incorporation in both the fetus and placenta. By contrast, transduction of blastocysts resulted in transgene incorporation exclusively in the placentas of all embryos (81/81). More efficient transduction of trophoblast cells than two-cell stage blastomeres was also supported by an increased range (3–11 versus 1–9) and average number (6.6 versus 4.4) of proviral insertions per cell, as determined by fluorescent *in situ* hybridization analysis (**Supplementary Fig. 1** online). Lentiviral vector transduction of preimplantation embryos was not deleterious. The percentages of newborn pups per implanted blastocysts were comparable in the following three groups: nontransduced embryos (38.4%; 56/146), embryos transduced at the two-cell stage as a control (34.0%; 53/156) and embryos transduced at the blastocyst stage (43.3%; 78/180). More importantly, the 78 newborn pups obtained from blastocyst transduction developed to term normally (data not shown).

Whereas genes introduced with certain other retroviruses tend to be silenced, transgenes introduced lentivirally are stably expressed *in vivo*[2,9,10]. As expected, consistent *EGFP* transgene expression was

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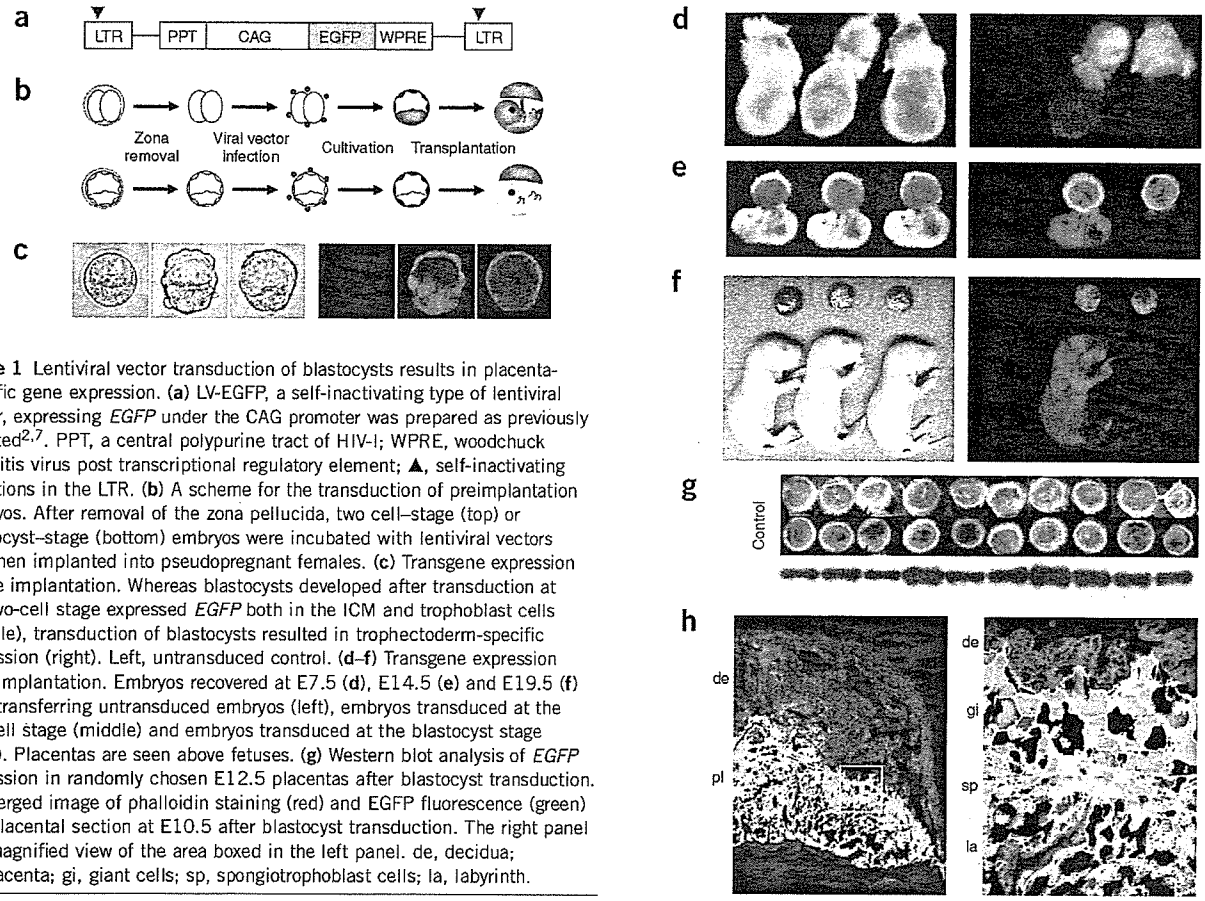

Figure 1 Lentiviral vector transduction of blastocysts results in placenta-specific gene expression. (**a**) LV-EGFP, a self-inactivating type of lentiviral vector, expressing *EGFP* under the CAG promoter was prepared as previously reported[2,7]. PPT, a central polypurine tract of HIV-I; WPRE, woodchuck hepatitis virus post transcriptional regulatory element; ▲, self-inactivating mutations in the LTR. (**b**) A scheme for the transduction of preimplantation embryos. After removal of the zona pellucida, two cell–stage (top) or blastocyst–stage (bottom) embryos were incubated with lentiviral vectors and then implanted into pseudopregnant females. (**c**) Transgene expression before implantation. Whereas blastocysts developed after transduction at the two-cell stage expressed *EGFP* both in the ICM and trophoblast cells (middle), transduction of blastocysts resulted in trophectoderm-specific expression (right). Left, untransduced control. (**d–f**) Transgene expression after implantation. Embryos recovered at E7.5 (**d**), E14.5 (**e**) and E19.5 (**f**) after transferring untransduced embryos (left), embryos transduced at the two-cell stage (middle) and embryos transduced at the blastocyst stage (right). Placentas are seen above fetuses. (**g**) Western blot analysis of *EGFP* expression in randomly chosen E12.5 placentas after blastocyst transduction. (**h**) Merged image of phalloidin staining (red) and EGFP fluorescence (green) of a placental section at E10.5 after blastocyst transduction. The right panel is a magnified view of the area boxed in the left panel. de, decidua; pl, placenta; gi, giant cells; sp, spongiotrophoblast cells; la, labyrinth.

confirmed throughout pregnancy by fluorescence and western blot analysis (**Fig. 1d–h**). In the transgenic embryos developed by lentiviral vector transduction at the two-cell stage, *EGFP* expression and fluorescence in both placenta and fetus were ubiquitous. However, when blastocysts were transduced, green fluorescence was restricted to the placenta. Histology revealed uniform and ubiquitous expression of *EGFP* in all three major layers of the placenta: the labyrinth, spongiotrophoblast and giant cells (**Fig. 1h**).

It has been reported that the direct injection of lentiviral vectors into the blastocoel of rhesus monkeys resulted in chimeric transgene expression in all three placentas analyzed[11]. Our replication of this approach in mice confirmed the uneven transduction of placentas (**Supplementary Fig. 2a** online), possibly owing to limited transduction of trophoblast cells as polar trophoblast cells underlying the ICM are not accessible from the blastocoel. By the onset of implantation, mural trophoblast cells cease to proliferate, whereas polar trophoblast cells remain mitotic. Moreover, the transduction was not limited in placentas after blastocoel injection, at least in mice. We observed transgene expression in 5 of 58 fetuses examined (**Supplementary Fig. 2b** online), indicating that this method did not enable placenta-specific transduction. This is consistent with a report that 65% of the murine pups were transgenic after blastocoel injection of retroviral vector–producing cells[12]. On the contrary, incubation of blastocysts in the presence of lentivirus after zona pellucida removal transduced both mural and polar trophoblast cells and resulted in even transgene expression throughout the placenta without generating transgenic pups. Our method is also less damaging to embryos, more cost effective and technically less demanding.

To determine whether trophoblast cell–specific gene manipulation might complement genetic aberrations of the placenta, we chose mice deficient in *Ets2* (ref. 13), *Mapk1* (refs. 14,15) and *Mapk14* (refs. 16–18) from over 100 mutant animals with placental defects that cause embryonic lethality[3,4]. *Ets2* is a member of the Ets family of transcription factors that mediate transcriptional responses of many signaling pathways and is highly expressed in a subset of cells in the trophectoderm and extra-embryonic ectoderm during early embryogenesis. Targeted disruption of *Ets2* results in abnormal, cone-shaped, yolk-sac envelopes, much less trophoblastic tissue and abnormal placentation, leading to embryonic lethality by E8.5 (ref. 13). Placental defects in $Ets2^{-/-}$ can be rescued by aggregating a mutant eight-cell embryo with a wild-type tetraploid embryo (tetraploid complementation).

We prepared a lentiviral vector expressing *Ets2*, LV-Ets2 and transduced blastocysts from $Ets2^{+/-}$ intercrosses (**Fig. 2a**). Transducing blastocysts with LV-Ets2 restored placentation and embryonic development in $Ets2^{-/-}$ embryos (**Fig. 2b**), yielding 24 $Ets2^{-/-}$ pups from 154 transplantation attempts (**Fig. 2c** and **Table 1**). At birth, the average body weights of rescued $Ets2^{-/-}$ newborn pups were comparable to those of wild-type pups. Average placental weight was slightly lower than in the wild type, but no overt abnormalities were observed in either newborn pups or placentas (**Supplementary Fig. 3** online). PCR analysis confirmed placenta-specific gene incorporation in the

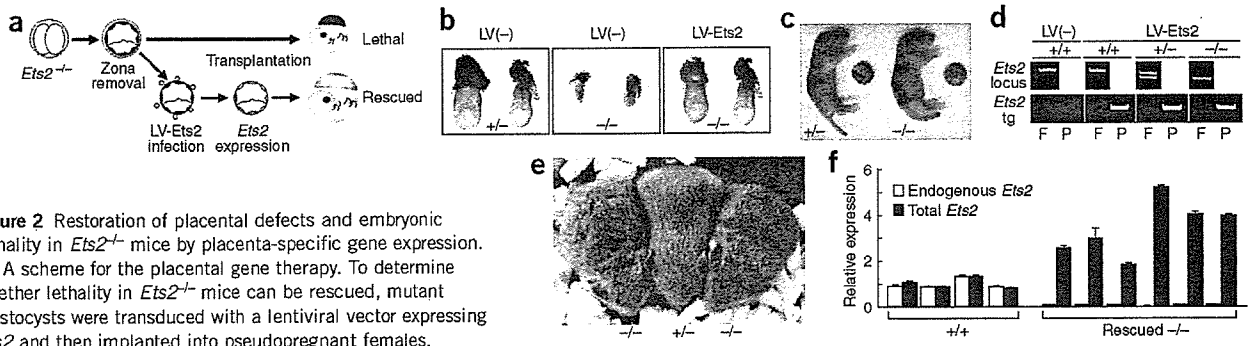

Figure 2 Restoration of placental defects and embryonic lethality in *Ets2*$^{-/-}$ mice by placenta-specific gene expression. (**a**) A scheme for the placental gene therapy. To determine whether lethality in *Ets2*$^{-/-}$ mice can be rescued, mutant blastocysts were transduced with a lentiviral vector expressing *Ets2* and then implanted into pseudopregnant females. (**b**) E7.5 embryos after LV-Ets2 treatment. (**c**) Live *Ets2*$^{-/-}$ pups obtained by LV-Ets2 treatment. (**d**) Fetal (F) and placental (P) tissue subjected to PCR analysis for transgene detection. The lentiviral vector transgene was detected only in the placenta. tg, transgene. (**e**) Rescued *Ets2*$^{-/-}$ mice with wavy hair. (**f**) Relative amounts of *Ets2* mRNA determined by quantitative real-time RT-PCR. Amounts of endogenous and total *Ets2* mRNA were normalized to the *β-actin* control and relative amounts to the mean amounts of endogenous *Ets2* mRNA in +/+ mice were plotted.

rescued *Ets2*$^{-/-}$ pups (**Fig. 2d**). The rescued *Ets2*$^{-/-}$ pups developed wavy hair but were otherwise normal (**Fig. 2e**) and fertile, as originally reported[13]. Thus, restoration of placental function by lentiviral vector–mediated transgene expression was as efficient as tetraploid embryo complementation, an approach that has proven difficult and inefficient for many nonrodents[19,20]. Therefore, prospects for applying lentiviral vector–mediated gene transfer to species currently recalcitrant to tetraploid embryo complementation seem promising. Fewer −/− fetuses were rescued than anticipated from Mendelian segregation. When we compared the amounts of endogenous and transgenic *Ets2* mRNA by quantitative real-time RT-PCR, all of the rescued −/− placentas analyzed expressed more *Ets2* mRNA than endogenous *Ets2* mRNA in +/+ placentas (**Fig. 2f**). This may imply that a critical level of *Ets2* expression is required to rescue the lethal phenotype and that some of the transduced −/− placentas do not attain this threshold. As the timing and cell specificity of transgene expression may also reduce the frequency of mutant complementation, the use of endogenous promoters might improve the efficiency. As far as we know, this is the first case of gene therapy–mediated correction of placental dysfunction that normally leads to pregnancy loss. The rescue of *Mapk1*$^{-/-}$ mice from embryonic death by LV-Mapk1 transduction confirmed the effectiveness of this method (**Table 1**).

We next tried to rescue *Mapk14*-deficient animals that die at midgestation because of impaired placentation[16–18]. The p38 mitogen-activated protein kinase (MAPK) family comprises four members—*Mapk14* (refs. 21,22), *Mapk11* (ref. 23), *Mapk12* (ref. 24) and *Mapk13* (ref. 25) (also known as *p38α*, *p38β*, *p38γ* and *p38δ*, respectively)—and plays important roles in cell proliferation, differentiation and survival[26]. Approximately 75% amino acid identity, as well as overlaps in the upstream and downstream pathways suggest functional redundancy between *Mapk14* and *Mapk11*. On the other hand, differences in their signaling pathways as well as their different patterns of tissue expression may reflect specific functions *in vivo*[23,26,27]. Interestingly, *Mapk11* cDNA was first isolated from a placental cDNA library, but later shown to be poorly expressed in placenta[25]. To determine whether *Mapk14* has a unique function in the placenta, the effect of ectopically expressed *Mapk11* was examined in *Mapk14*-deficient mice.

Blastocysts from *Mapk14*$^{+/-}$ intercrosses were transduced with the lentiviral vectors LV-Mapk14 or LV-Mapk11, expressing either *Mapk14* or *Mapk11*, respectively. Histological analysis showed full recovery of the thickness of the labyrinthine layer, with normal vasculogenesis in *Mapk14*$^{-/-}$ placenta treated with LV-Mapk14 (**Fig. 3a**). Complementation of the placental defects was associated with full restoration of normal fetal development. Notably, ectopic expression of *Mapk11* could also complement *Mapk14* function *in vivo*: live *Mapk14*$^{-/-}$ pups were obtained with the same efficiency (16/106) as upon LV-Mapk14 transduction (29/180) (**Fig. 3a–c** and **Table 1**). The normal appearance and comparable average weights of pups and placentas also imply successful substitution of *Mapk14* with *Mapk11* (**Fig. 3b,d**). Overexpression of *Mapk14* or *Mapk11* in placenta did not seem to be deleterious because +/+ and +/− littermates that developed from transduced blastocysts seemed normal. Elucidation of the common cascade between *Mapk14* and *Mapk11* may facilitate identification of an essential signaling pathway(s) for normal placentation.

Our demonstration that lentiviral vector–transduction of blastocysts after zona pellucida removal ensures trophoblast cell–specific gene expression addresses the need for a placenta-specific gene expression system for elucidating the molecular basis of placental development and function. Combination of this approach with technologies such as RNAi, Cre/loxP and Tet On/Off systems will provide versatile systems to elucidate mechanisms of implantation and placentation. Certain human genetic defects known to cause placental dysfunction and/or

Table 1 Genotypes of newborn pups after lentiviral vector transduction

Deficient gene	Treatment	Genotypes of newborn pups +/+	+/−	−/−	Total
Ets2	(−)	19	37	0	56
	LV-EGFP	14	30	0	44
	LV-Ets2	45	84	24	154
Mapk1	(−)	17	33	0	50
	4 N	4	8	2	14
	LV-Mapk1	14	30	7	51
Mapk14	(−)	16	33	0	49
	4 N	19	37	3	59
	LV-Mapk14	51	100	29	180
	LV-Mapk11	31	59	16	106

Preimplantation embryos collected from heterozygous intercrosses were transduced with lentiviral vectors at blastocyst stage and implanted into pseudopregnant females. Tetraploid complementation (4N) was performed as described previously[8].

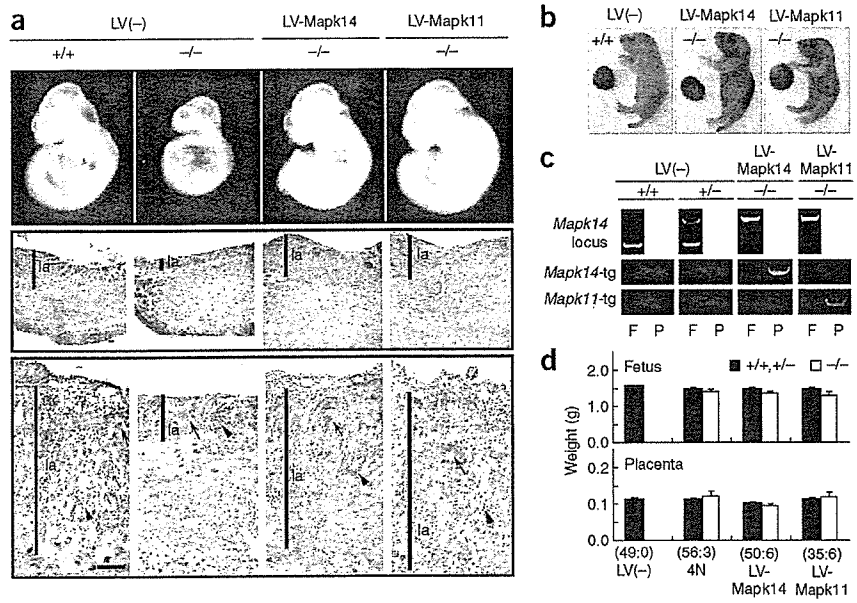

Figure 3 Functional complementation of *Mapk14* with *Mapk11*during placentation. **(a)** Embryos collected at E10.5 after LV-Mapk14 or LV-Mapk11 transduction at the blastocyst stage. (Top) Fetuses. (Middle and bottom) Placental sections stained with hematoxylin and eosin observed at 4 × magnification (middle) or at 20 × magnification (bottom). Vertical bars labeled la mark the labyrinthine layer. Fetal vessels filled with nucleated erythrocytes are indicated by arrowheads and maternal blood vessels containing enucleated erythrocytes are indicated by arrows. **(b)** Live *Mapk14–/–* newborn pups obtained by LV-Mapk14 or LV-Mapk11 treatment. **(c)** Fetal (F) and placental (P) tissue collected at birth subjected to PCR analysis for transgene detection. The lentiviral vector transgene was detected only in the placenta. tg, transgene. **(d)** Average body and placenta weight with standard error at birth after LV-vector treatment or tetraploid complementation (4N). There were no significant differences between wild-type (+/+ and +/–, black bars) and rescued –/– embryos (white bars). Numbers of mice examined are indicated in parentheses.

complications during pregnancy[28,29] can be diagnosed by isolating and examining a blastomere from preimplantation embryos (preimplantation genetic diagnosis)[30]. Our gene manipulation method may be used to explore novel assisted reproduction technologies to overcome defects in implantation and placental function.

METHODS

Animals. *Ets2*- or *Mapk1*-deficient mice used were prepared as previously reported[13,14]. Mapk14-deficient mice were generated by inserting a gene trap cassette between exon 10 and 11 (ref. 13 and unpublished data). Mice were genotyped by 40 cycles of PCR amplification from genomic DNA with the following primers; *EGFP*-F (5′-accatggtgagcaagggcgag-3′) and *EGFP*-R (5′-tcaccttgatgccgttcttct-3′) for the *EGFP* transgene, *Ets2*-1 (5′-cgtccctactggatgtacagcgg-3′), *Ets2*-2 (5′-tgctttggtcaaataggagccactg-3′), and *Ets2*-3 (5′-aatgacaagacgctgggcgg-3′) for the *Ets2* locus, *Ets2*-tg-F (5′-gcggatccgggaccgccgcgatctcctctc-3′) and *Ets2*-tg-R (5′-gcctcgaggtctttggagcaagtgggtcag-3′) for the *Ets2* transgene, *Mapk14*-1 (5′-tagatacagagccatcagaccacca-3′), *Mapk14*-2 (5′-tgaatggtgtagcataggctgga-3′), and *Mapk14*-3 (5′-atctggacgaagagcatcag-3′) for the *Mapk14* locus, *Mapk14*-tg-F (5′-gatgtcgcaggagaggcccacgttctac-3′) and *Mapk14*-tg-R (5′-gatatccatgagaaggtcttccc-3′) for the *Mapk14* transgene, *Mapk11*-tg-F (5′-gcgtctagacttctgtggatatgtcgggtc-3′) and *Mapk11*-tg-R (5′-gcctcgagtgcctcactgctcaatttcatggggtgc-3′) for the *Mapk11* transgene, and *Mapk1*-1 (5′-taactgggtcgagcacagtgatgc-3′), *Mapk1*-2 (5′-tcagaattgatctggtcttcaagaccttg-3′), and *Mapk1*-3 (5′-atgtatgctatacgaagttattagggc-3′) for the *Mapk1* locus. All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University.

Lentiviral vector production. HIV-I based, self-inactivating, lentiviral vector plasmid pLV-EGFP was constructed by replacing the PGK promoter with the CAG promoter fragment in pRRLsin-hPGK-*EGFP*[2,7]. Other constructs such as pLV-Ets2, pLV-Mapk14, pLV-Mapk11 and pLV-Mapk1 were generated by replacing the *EGFP* fragment of pLV-EGFP with PCR-amplified murine cDNAs. VSV-G pseudotyped lentiviral vectors were generated as described[7]. Briefly, 293T cells were transfected with the pLV plasmid, packaging plasmid and VSV-G– expressing plasmid by the calcium phosphate method. Lentiviral vectors were harvested at 2 and 3 days after transfection, and then concentrated 1,000 times by ultracentrifugation (50,000*g*, 2 h × 2 times). After resuspension with HBSS buffer, lentiviral vector concentration was determined by measuring p24 gag antigen by ELISA (Retrotek, Zeptometrix).

Transduction of preimplantation embryo. Wild-type or heterozygous mutant female mice with B6D2F1 background were superovulated by intraperitoneal injection of pregnant mare's serum gonadotropin (5 units) followed by human chorionic gonadotropin (5 units) 48 h later and then mated with wild-type or heterozygous mutant males respectively. Two cell–stage embryos were collected from the females at 1.5 days after copulation, and then incubated for 2 days to obtain blastocysts. Prior to lentiviral vector transduction, the zona pellucida was removed by treatment with acidic Tyrode's solution[8]. Zona pellucida–free embryos were incubated individually in 5 μl of medium containing lentiviral vector (1×10^3 ng p24/ml) for 2 days for two-cells or 4 h for blastocysts. For blastocoel injection, lentiviral vector (1×10^4 ng p24/ml) was injected into blastocoel using an Eppendorf Transjector 5246 micromanipulator[11]. Transduced blastocysts were observed under a fluorescence microscope (KEYENCE, BZ-8000) or implanted into pseudopregnant females. Blastocyst images were obtained by computer-assisted Z-stack and haze reduction.

Fluorescence *in situ* hybridization analysis (FISH). pLV-EGFP plasmid DNA was labeled with digoxigenin-11-dUTP by nick translation for use as a probe. Slides were denatured for 2 min in 70% formamide/2 × SSC at 70 °C and dehydrated. The probe was denatured for 10 min in at 75 °C and applied to the slides. After hybridization, the slides were washed in 50% formamide/2 × SSC at 37 °C and 1 × SSC at 20 °C for 20 min each. Detection of the probe signals was performed with Cy3-labeled anti-digoxigenin. The FISH images were captured under a Leica DMRA2 microscope with the CW4000 FISH application program of Leica Microsystems Imaging Solution Ltd.

Tetraploid complementation. Tetraploid embryo and aggregation chimera were prepared as described previously[8]. In brief, B6D2F1 two cell–stage embryos were placed in fusion buffer and electrofusion was performed by applying 140 V for 50 μs after aligning embryos between the electrodes. A wild-type tetraploid four-cell embryo and a mutant diploid eight-cell embryo were aggregated and then the developed blastocysts were implanted into pseudopregnant females.

Histological examination. Placentas were fixed in 4% paraformaldehyde (PFA) for 12 h, then rinsed with PBS for 1 h, dehydrated in acetone for 1 h and embedded in glycol methacrylate (Technovit 8100; Heraeus Kulzer). Sections were prepared at a thickness of 5 μm and stained with hematoxylin and eosin. For the fluorescence staining, 10 μm frozen sections were prepared after PFA fixation. The sections were stained with TRITC-phalloidin (Sigma) to visualize actin distribution and observed with a confocal imaging system (FV1000MP, Olympus).

Quantitative real-time RT-PCR. Total RNA prepared from placenta with ISOGEN (Nippon gene) was treated with DNase (TURBO DNA-free, Ambion), then 40 ng of each RNA sample was reverse transcribed with Superscript III RT (Invitrogen). One twentieth of each cDNA sample was subjected to quantitative RT-PCR analysis with an ABI PRISM 7900HT (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). To prepare a standard curve, serial dilutions (1:1, 1:5, 1:25, 1:125, 1:625) of wild-type cDNA was included in each set of reactions. The relative expression level of each target gene mRNA was then normalized to the amount of *β-actin* control. Each reaction was performed in triplicate and data are presented as the mean plus s.d. Primer sets used were as follows: 5′-aagtgtgacgttgacatccg-3′ and 5′-gatccacat ctgctggaagg-3′ for *β-actin*, 5′-ggcgtccagcctgatacaga-3′ and 5′-gtggaagaggcgtggat gaaag-3′ for internal *Ets2*, 5′-gcagtgatgagccaagcctt-3′ and 5′-ggtgcctgcattccatat gg-3′ for total *Ets2*.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

Y.O. performed research, analyzed data and wrote the manuscript; Y.U. performed research; A.I. performed tetraploid complementation; T.S.F. performed quantitative real-time RT-PCR; H.N. performed histological analysis; K.K. performed histological analysis; A.M. performed histological analysis and provided useful discussions; M.O. provided p38α mutant mice; Y.M. provided useful reagents; M.O. provided p38α and *Mapk1* mutant mice and useful discussions; R.G.O. provided *Ets2* mutant mice and useful discussions; M.O. provided useful discussions and wrote manuscript; M.I. designed, coordinated and performed research, analyzed data and wrote manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Aberrant Distribution of ADAM3 in Sperm from Both Angiotensin-Converting Enzyme (*Ace*)- and Calmegin (*Clgn*)-Deficient Mice[1]

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ABSTRACT

Male mice deficient for the calmegin (*Clgn*) or the angiotensin-converting enzyme (*Ace*) gene show impaired sperm migration into the oviduct and loss of sperm-zona pellucida binding ability in vitro. Since CLGN is a molecular chaperone for membrane transport of target proteins and ACE is a membrane protein, we looked for ACE on the sperm membranes from $Clgn^{-/-}$ mice. ACE was present and showed normal activity, indicating that CLGN is not involved in transporting ACE to the sperm membranes. The ablation of the *Adam2* and *Adam3* genes generated animals whose sperm did not bind the zona pellucida, which led us to examine the presence of ADAM2 and ADAM3 in $Clgn^{-/-}$ and $Ace^{-/-}$ sperm. ADAM3 was absent from $Clgn^{-/-}$ sperm. In the $Ace^{-/-}$ mice, while ADAM2 was found normally in the sperm, ADAM3 disappeared from the Triton X-114 detergent-enriched phase after phase separation, which suggests that ACE is involved in distributing ADAM3 to a location where it can participate in sperm-zona pellucida binding. This diminished amount of ADAM3 in the Triton X-114 detergent-enriched phase may explain the inability of $Clgn^{-/-}$ and $Ace^{-/-}$ sperm to bind to the zona pellucida.

gamete biology, in vitro fertilization, male sexual function, sperm capacitation

INTRODUCTION

Mammalian fertilization is accomplished through complex processes that involve cell–matrix and cell–cell interactions. The zona pellucida (ZP) is an extracellular matrix that serves as a barrier to protect the egg from physiological damage [1] and to block polyspermic fertilization. Based on biochemical analyses, different sperm proteins have been postulated to play roles in ZP binding, penetration and fusion. However, when genes, such as *Acr* (acrosin) [2], *B4galt1* [3], *Spam1* (previously known as PH-20) [4], *Mfge8* (previously known as SED1) [5], and *Cd46* [6] are ablated, the predicted phenotypes do not appear, which suggests that the various models used to explain fertilization need to be redefined. Among the various proteins that have been reported to have critical roles in fertilization, only the A Disintegrin And Metalloprotease 2 (*Adam2*) gene (previously known as fertilin-β) has been shown to be essential [7]. Even in this case, the observed phenotype, i.e., loss of ZP-binding ability, is different from that of the predicted phenotype, i.e., a role in sperm-egg fusion. Additional proteins that may play roles in sperm-ZP binding have emerged over the years. For example, we have shown that the calmegin (*Clgn*) gene is essential for sperm binding to the ZP [8]. Other genes, such as *Adam1a and Adam3*, as well as the angiotensin-converting enzyme *(Ace)*, have also emerged as essential factors for sperm-ZP binding [9–11].

It is intriguing that the disruption of certain genes, e.g., *Clgn*, *Ace*, *Adam1a*, *Adam2*, and *Adam3*, results in a similar sperm phenotype, i.e., failure of sperm to bind to the ZP. Since CLGN functions as a testis-specific molecular chaperone, we previously examined the possibility of ADAM2 misfolding in sperm from $Clgn^{-/-}$ mice [12]. In wild-type sperm, both ADAM1 and ADAM2 bind CLGN and this interaction probably leads to the correct tertiary folding of these proteins. However, in the absence of CLGN, the ADAM1/ADAM2 heterodimer disappears from testicular extracts. Subsequently, it has been shown that the *Adam1* gene comprises two similar genes, *Adam1a* and *Adam1b*, and that only ADAM1b is present in sperm, where it forms a heterodimer with ADAM2 [13, 14]. It is clear that when the ADAM1b/ADAM2 heterodimer fails to assemble in the testis, ADAM2 disappears from the sperm of the $Clgn^{-/-}$ male mouse. These results helped to clarify why $Clgn^{-/-}$ and $Adam2^{-/-}$ mice share a similar phenotype, i.e., the sperm cannot bind to the ZP because they lack ADAM2.

To continue our studies towards deciphering the role of candidate genes in sperm-ZP binding, we turned our attention to the angiotensin-converting enzyme. Somatic ACE (sACE) is a zinc metalloprotease that is involved in blood pressure regulation by cleaving bioactive peptides, such as angiotensin I and bradykinin. Testicular ACE (tACE) has also been described, and is generated by alternative promoter usage [15]. Both isoforms have similar enzymatic activities. Ablation of both sACE and tACE results in males with reduced fertility [16]. Males that lack only sACE are fertile, which indicates that tACE is responsible for the infertility [17, 18]. We now report a role of tACE in the localization of ADAM3 so that it can participate in sperm-zona pellucida binding.

MATERIALS AND METHODS

Animals

$Clgn^{+/-}$ and $Clgn^{-/-}$ male mice were obtained by mating $Clgn^{-/-}$ females with $Clgn^{+/-}$ males. The $Ace^{-/-}$ mice were originally produced by Krege et al. [9]. Mice heterozygous for alterations in exon 14 of the *Ace* gene, which contains critical amino acids for sACE and tACE functions [15], were purchased from the Jackson Laboratory (Bar Harbor, ME). $Ace^{+/-}$ and $Ace^{-/-}$

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male mice were obtained by mating *Ace*$^{+/-}$ females with *Ace*$^{+/-}$ males. All of the experiments were performed with the approval of the Animal Care and Use Committee of Osaka University.

Antibodies

Affinity-purified rabbit anti-ADAM1a (1aCysE), anti-ADAM1b (1bCysE), and anti-SPAM1 (previously known as PH-20) [4, 13] antibodies, which were produced by immunizing with recombinant proteins that correspond to a unique region within each protein, were kind gifts from Drs. Hitoshi Nishimura and Tadashi Baba (University of Tsukuba, Ibaraki, Japan). Monoclonal antibodies (Mabs) against mouse ADAM2 (fertilin-β; 9D2.2) and ADAM3 (cyritestin; 7C1.2) were purchased from Chemicon International Inc. (Temecula, CA). Rabbit anti-SPAM1 antiserum [19] was a kind gift from Dr. Paul Primakoff (University of California, Davis, CA). Affinity-purified antibodies against ADAM2, IZUMO1, CD46, and CLGN were obtained as described previously [6, 8, 12, 20]. The rat anti-mouse sperm tail antigen Mab (#124) was generated in our laboratory and was used to examine the separation of sperm heads and tails.

To produce monoclonal antibodies against ACE, we prepared an amino-terminal recombinant tACE protein (AA #31–140). A DNA fragment that contained 110 amino acids starting from the N-terminus of the mature form of tACE (without the signal peptide) was obtained by PCR-amplification using a forward primer that corresponds to nucleotides 89–113 (5′-TTCCATGGC-CACTGACCACGTGACAGCCAA-3′) and a reverse primer that corresponds to nucleotides 399–420 (5′-ACTCGAGAGAGTTTTGAAAGTTGCTCAC-3′). The DNA was ligated into the *Nco*I and *Xho*I restriction sites of pET28b (Novagen, Madison, WI) and transformed. The recombinant tACE protein was expressed as a fusion protein with the 6×His tag at the C-terminus, and purified by Ni-NTA resin affinity column chromatography (Qiagen, Hilden, Germany). Monoclonal antibodies against ACE were generated by MBL (Nagoya, Japan). Two 7-week-old, female *Ace*$^{-/-}$ mice were immunized i.p. with 50 μg of recombinant protein emulsified in complete Freunds adjuvant (Day 0) or incomplete Freunds adjuvant (Days 7, 14, 21, and 28). Spleen cells were harvested three days after the fifth immunization and fused with mouse myeloma cells using polyethylene glycol. Wells that contained hybridomas were screened by an enzyme-linked immunosorbent assay (ELISA) for recombinant tACE protein. Positive clones were grown, and their supernatants were used for further screening. The supernatant from one clone, 1D5, was used for immunoblot analysis.

Fractionation of Sperm Heads and Tails

Sperm from the cauda epididymis and vas deferens were collected in TBS. Sperm heads and tails were separated by mild sonication on ice (five times with flash set at output level 1) using the Ultrasonic Disruptor UD-201 (Tomy Digital Biology, Tokyo, Japan), followed by the addition of an equal amount of 1.8 M sucrose. The 2-ml sample was layered on a discontinuous sucrose gradient composed of 2 ml of 0.9 M sucrose, 1 ml of 2.05 M sucrose, and 1 ml of 2.2 M sucrose; all of the sucrose solutions contained 10 mM Tris-HCl (pH 7.5) and 0.15 M NaCl. The gradients were centrifuged at 100 000 × *g* for 12 h in a SW50.1 rotor (Beckman Coulter, Tokyo, Japan). Sperm tails banded at the 0.9 M/2.05 M interface, and the sperm heads were found in the pellet. Both fractions were resuspended in TBS and pelleted by centrifugation at 1000 × *g* for 10 min. The samples were subjected to immunoblot analysis, as described below.

Phase Separation of Sperm Triton X-114 Extracts

Cauda epididymal and vas deferens sperm were collected into PBS and centrifuged at 1000 × *g* for 10 min at 4°C. The sperm pellets were suspended in PBS that contained 1% Triton X-114 [21], 10 mM benzamidine, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. The sperm suspensions were placed on ice for 20 min with occasional vortexing. After centrifuging at 15 700 × *g* for 20 min at 4°C, the supernatants were collected, and layered onto 6% sucrose/PBS in microtubes. After incubation at 37°C for 15 min, the tubes were centrifuged at 1000 × *g* for 15 min at room temperature, to separate the Triton X-114 extract into the detergent-depleted phase, sucrose phase, and detergent-enriched phase. The separated detergent-depleted and detergent-enriched phases were mixed with SDS-sample buffer, boiled, and subjected to SDS-PAGE and immunoblot analysis.

Immunoblot Analysis

Sperm from the cauda epididymis and vas deferens of sexually mature male mice were collected and incubated in lysis buffer (10 mM Tris-HCl [pH 7.5], 50 mM KCl, 1% Triton X-100, 10 mM benzamidine, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) for 20 min on ice with occasional vortexing. The testis and kidney were excised, minced, and homogenized in lysis buffer, and then placed on ice for 1 h. The sperm and tissue extracts were pelleted at 15 700 × *g* for 5 min at 4°C, and the supernatants were collected. The protein concentration in each homogenate was determined using the Coomassie protein assay reagent (Pierce, Rockford, IL). Proteins were separated by SDS-PAGE under reducing conditions (CLGN, ADAM2, ADAM3, ACE, SPAM1, and #124) or non-reducing conditions (ADAM1b; IZUMO1, and CD46), and were transferred electrophoretically to PVDF membranes (Immobilon-P; Millipore Corp., Bedford, MA). After blocking in TBS-T buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween-20] that contained 5% skim milk, the blots were incubated with primary antibody overnight at 4°C, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The detection of immunoreactive bands was performed using the enhanced chemiluminescence (ECL) Western blotting detection kit (GE Healthcare Bio-Sciences). Signal intensities were determined using the Scion Image software (Scion Corp., Frederick, MD).

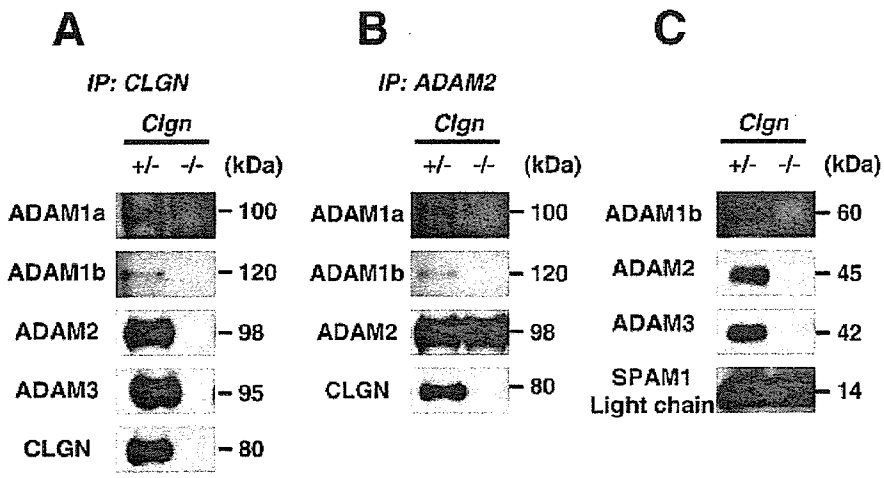

FIG. 1. The presence of ADAMs in the testes and sperm of *Clgn*$^{-/-}$ mice. Proteins from *Clgn*$^{+/-}$ and *Clgn*$^{-/-}$ testis and sperm were subjected to immunoprecipitation and immunoblot analysis. A) Association of ADAMs with CLGN. Testicular proteins from animals of each genotype were immunoprecipitated with the anti-CLGN antibody. The precipitates were subjected to immunoblot analysis using antibodies against ADAM1a, ADAM1b, ADAM2, ADAM3, and CLGN. B) Heterodimerization of ADAM2/1a and ADAM2/1b. Testicular proteins from animals of each genotype were immunoprecipitated using the anti-ADAM2 antibody, followed by immunoblot analysis using antibodies to ADAM1a, ADAM1b, and ADAM2. C) Detection of ADAMs in sperm. Sperm proteins from animals of each genotype were separated by electrophoresis and subjected to immunoblot analysis using antibodies to ADAM1b, ADAM2, and ADAM3. As a control, the light chain of SPAM1 is detected with anti-SPAM1 antiserum in both the *Clgn*$^{+/-}$ and *Clgn*$^{-/-}$ sperm.