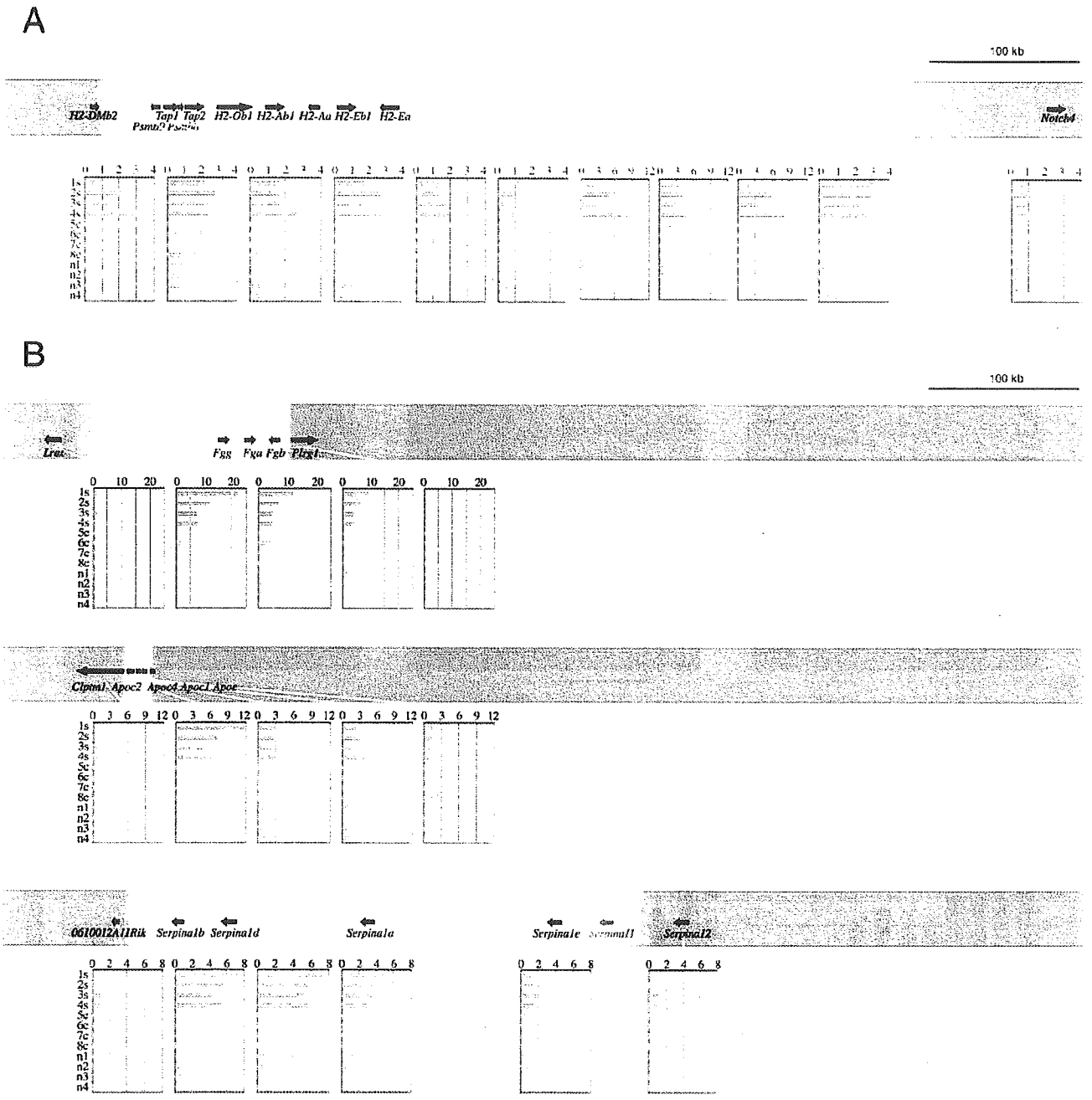

FIG. 3. Chromosome regions containing multiple upregulated genes in cloned mice. The physical maps of the genes were drawn using the mouse genome sequence assembly coordinates from NCBI Build 33, version 1. Black and gray arrows represent genes with and without expression profiles, respectively. The lack of expression profiles for some genes was the result of the absence of a probe in the DNA microarray or expression levels below the detection threshold. The gray background in the genome maps indicates regions outside the hypothesized area of coregulation. Scale bars = 100 kb. The gene expression profiles are charted as follows. The fold changes in gene expression were calculated from the signal intensities on the microarrays, normalized to the mean value of the controls. Panels with a white background represent the profiles of coregulated genes, while panels with a gray background represent the profiles of genes adjacent to the coregulated gene clusters. A) Chromosome region containing the class II MHC gene cluster. B) Chromosome regions containing the fibrinogen, apolipoprotein, and serine protease inhibitor gene clusters.

markers of the acute phase of inflammation, such as *Trf*, *Alb1*, *Ahsg*, *Hp*, *Serpina1a*, *Serpina1d*, and *Serpina1e*. Semiquantitative RT-PCR confirmed that the expression of *Trf*, *Alb1*, and *Ahsg* was increased 5- to 25-fold in the kidneys of Sertoli cell-derived clones compared with the normal controls (Fig. 2B). The microarray signal intensities for these genes were more than 50 times greater in the liver samples compared with brain and kidney samples (data not shown), indicating that the expression of these genes is highly tissue specific and may be under the control of a common gene regulatory mechanism. We found that the genes showing upregulation in a Sertoli-clone-specific manner also formed clusters in three chromosome regions, including apolipoproteins (*Apoc2*, *Apoc4*, *Apoc1*), fibrinogens (*Fgg*, *Fga*, *Fgb*), and serine protease

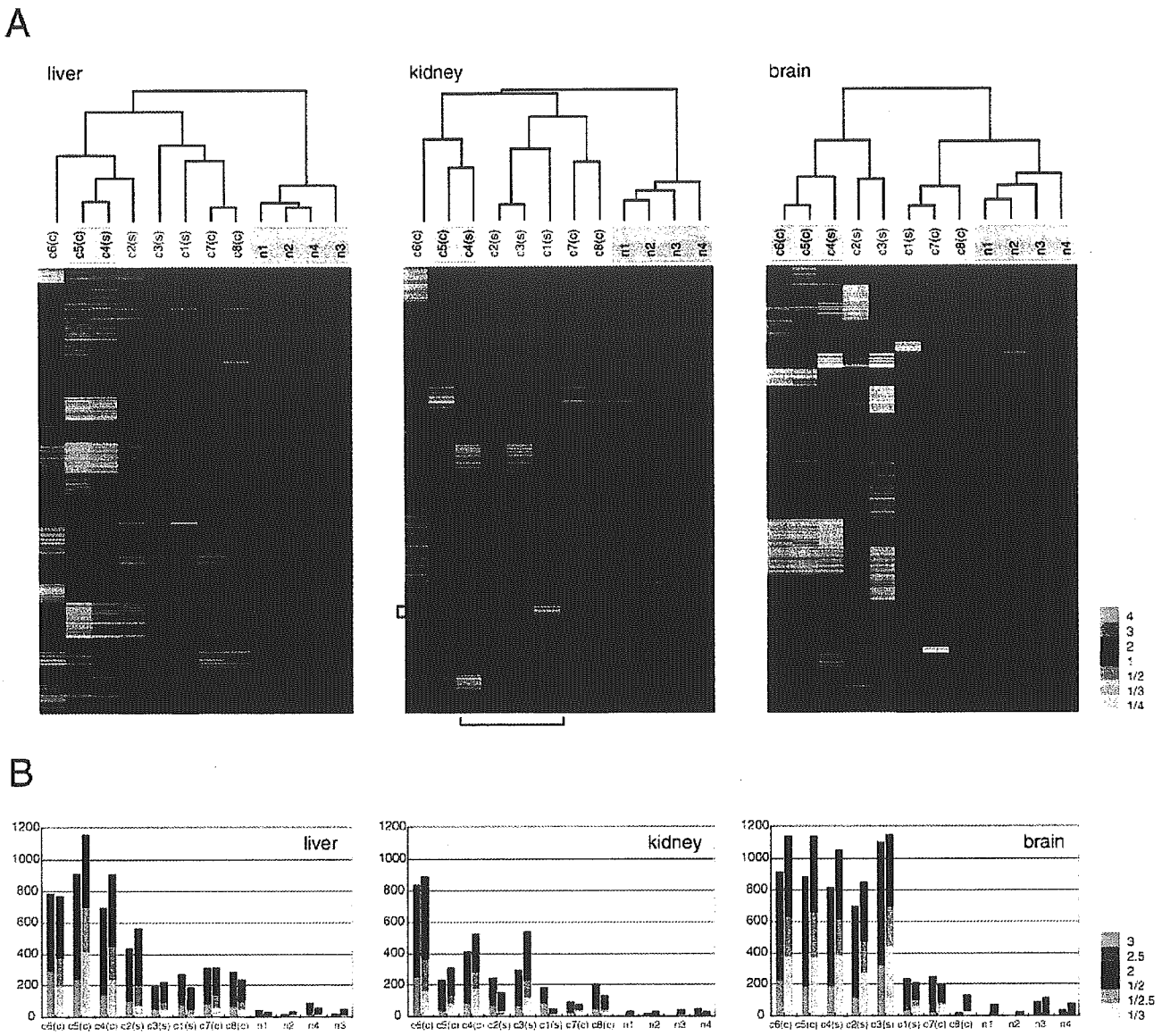

FIG. 4. Clustered gene expression profiles and the numbers of genes with altered levels of expression. A) Dendrogram (upper part): cloned and control individuals were clustered according to their gene expression profiles using a hierarchical clustering algorithm and GeneCluster version 3.0. The dendrogram was drawn using Java TreeView. Heat map representation (lower part): genes with similar expression patterns in the clones were clustered using GeneCluster version 3.0 and illustrated using Java TreeView. The mean expression level in the normal controls is indicated in black. The expression profiles are presented as a heat map in which red indicates increased expression levels above the mean and increasing green intensity indicates reduced expression. The intensity of the red or green corresponds to the extent of the change from normal. Sertoli cell clone-specific abnormally expressed genes (red bands in c2, c3, c1, and c4 in kidney) are clearly seen in the middle column as indicated by the parentheses. B) The numbers of genes affected in each individual are indicated on a cumulative bar graph. Red and green bars indicate values for genes with increased and decreased expression, respectively.

inhibitors (*Serpina1b*, *Serpina1d*, *Serpina1a*, *Serpina1e*; Fig. 3B).

The large difference between the total number of aberrantly expressed genes and the number of genes exhibiting aberrant expression in all individual clones indicated that mean expression levels did not represent the real expression profiles of individual somatic cell clones. Therefore, another analytical method is needed to define the genetic character of cloned individuals more precisely. Closer examination of the data revealed that the number of genes showing increased or decreased expression differed among the cloned individuals (Table 1 and Fig. 1), suggesting that the extent of the initial perturbation on the affected chromosome regions and the extent of the abnormalities caused by nuclear transfer differed among cloned individuals.

However, some clones had similar gene expression profiles, including clones 1, 7, and 8 and clones 4, 5, and 6, which exhibited similar general expression patterns (Figs. 1 and 4A). Interestingly, this similarity in the expression profiles did not depend on the donor cell type; both groups mentioned above included Sertoli cell- and cumulus cell-derived clones. When the genes that exhibited Sertoli-specific expression abnormal-

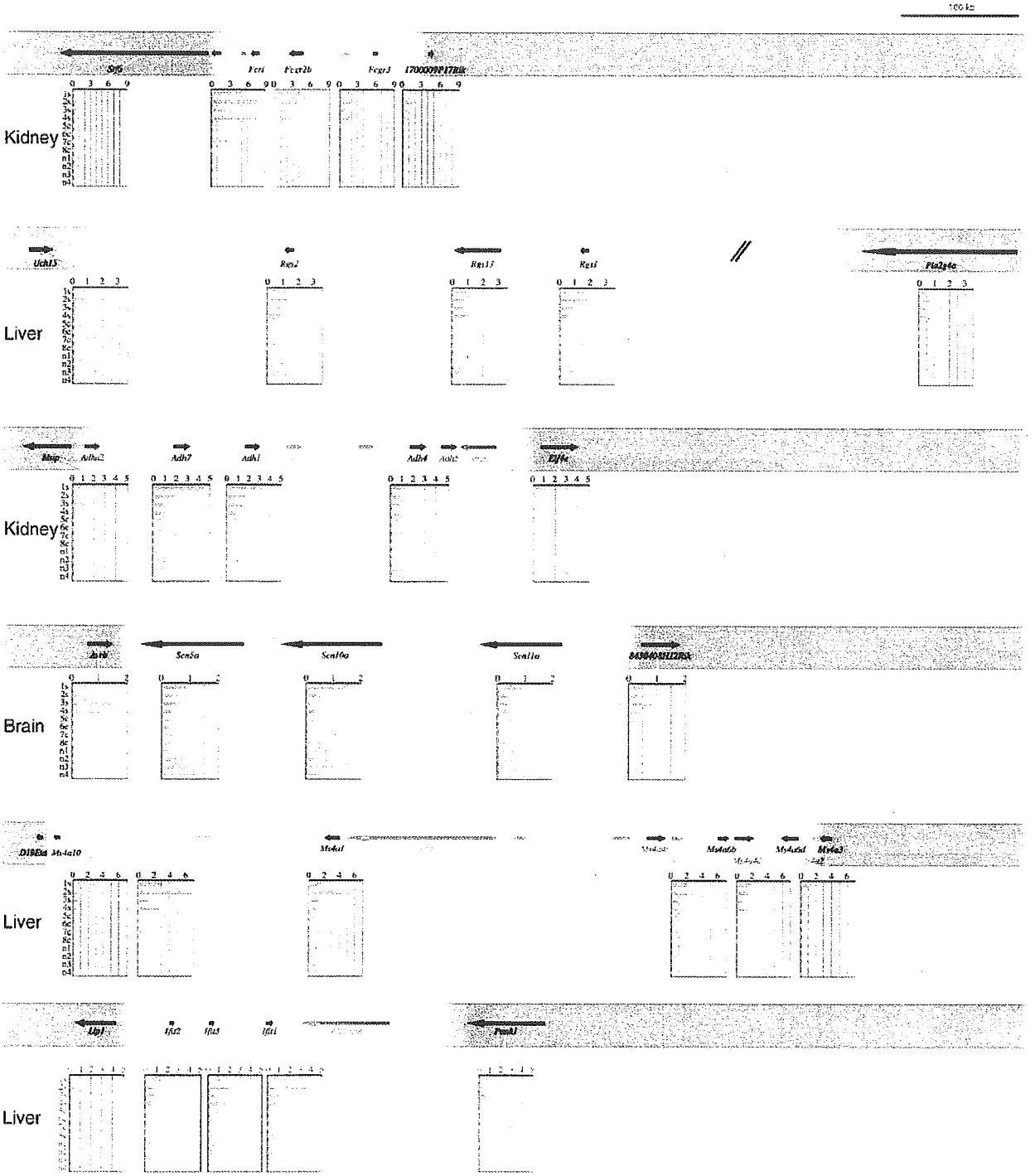

FIG. 5. Chromosome regions containing genes with related functions or structures that were coregulated in the clones. The physical maps of the genes and the gene expression profiles are as shown in Figure 3. Scale bars = 100 kb. As shown in Figure 4B, each clone individual showed increased or decreased expression by more than a factor of two in 2–10% of genes. Assuming that the gene expression of all clones was affected independently, the probability of a nearest neighbor gene showing the same pattern of aberrant gene expression in at least three clone individuals is less than 10^{-3} to 10^{-6}. Therefore, the gene clusters observed here are significant.

ities in the kidney were excluded, comparable pattern similarities were also observed for the expression profiles in the brain and kidney. The two groups corresponded to the individuals exhibiting fewer gene expression abnormalities (9–12%) and to those exhibiting many abnormalities (34–41%; Table 1 and Fig. 4). These results suggest that the susceptibility of genes to dysregulation caused by somatic cloning varies in a nonrandom manner.

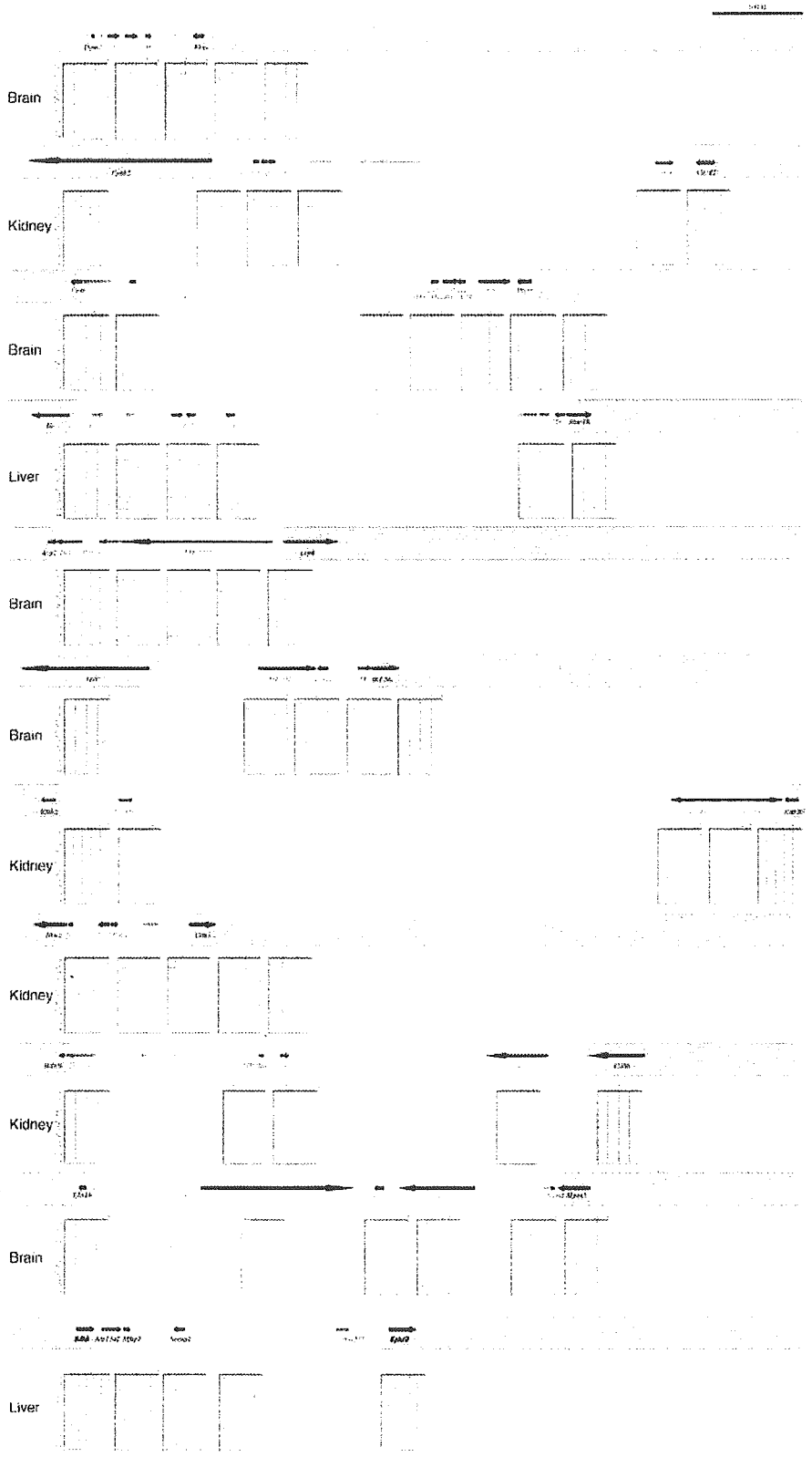

FIG. 6. Chromosome regions containing genes that were coregulated in the clones, including gene(s) with nonrelated function(s). The physical maps ofthe genes and the gene expression profiles are as shown in Figure 3 and Supplementary Figure 5 (available online at http://www.biolreprod.org).

Next, we clustered the genes into 20 groups and assigned group numbers using *k*-means clustering with nonparametric analysis of similarities according to the expression pattern among cloned and control individuals in each tissue, as presented in Figure 4. Then, the assigned group numbers were aligned along the mouse genome according to their gene order using assembled mouse genome sequences (NCBI mouse build 33) so that regions with the same assigned numbers could be abstracted automatically. Finally, we checked the gene expression pattern of each gene in these regions manually, in detail, and confirmed that genes with similar patterns of varied expression were clustered. Twenty-one such gene clusters consisting of three or more neighboring genes (Figs. 5 and 6) and more than 70 gene pairs (Supplementary Table, available online at http://www.biolreprod.org) were identified in the genome, in addition to the genes that showed aberrant expression in all clones (described above; Figs. 2 and 3). This analysis revealed that gene expression is regulated abnormally in clusters within many chromosome regions of somatic clones and that the dysregulating effect of somatic cloning sometimes influences genes within one to several hundred kilobases in the genome.

Approximately half of these clusters consisted of genes with similar functions (Fig. 5), while the other half contained genes with no functional or structural similarities (Fig. 6). Coregulation in the former clusters may arise from the abnormal expression of common upstream regulatory genes. In contrast, coregulation of the nonrelated gene clusters implies the presence of functional chromosome domains for regional gene regulation and perturbation of these regulatory systems by somatic cell cloning.

Note that cluster analysis based on variation in gene expression was useful for identifying abnormalities in several metabolic pathways of the clones. We found that genes in some important pathways, including glycolysis, heme synthesis, and steroid synthesis, were aberrantly coregulated in individual cloned mice (Supplementary Figs. 1–3, available online at http://www.biolreprod.org).

DISCUSSION

Our data demonstrated a general disturbance of gene expression in cloned mice from somatic cells that included dysregulation associated with the donor cell type, as well as dysregulation inherent to cloning; whereas a few genes were consistently affected in all clones, many genes showed variation in gene expression among the clones. Unexpectedly, normal-appearing mice cloned from somatic cells had a large number of affected genes compared with normal controls, and the expression profiles differed among the cloned individuals despite their identical genotypes and normal appearance. This was in marked contrast with the observations in the offspring of the control produced by in vitro fertilization, which exhibited homogeneous, strictly regulated gene-expression profiles. Most cloned individuals die before developing to term: the birth rate of mice cloned using somatic cells is only 2–5%. Therefore, the clones we analyzed represented selected individuals, and the number of genes affected was likely the minimum number that met all essential conditions for survival and development, i.e., the changes in gene expression observed here fell within the tolerance levels of mouse development and subsequent growth.

As shown in Figures 2 and 3, the abnormally expressed genes in the neonatal cloned mice included many genes associated with the immune system, including cytokines, specific markers of immune system cells, such as histocompatibility antigens, and markers of inflammation. Although we did not assess the impact of this variation in gene expression on the physiology or life span of the clones [14], these abnormalities may represent a disturbance of the immune system, as reported for other clones [18].

Our data clearly demonstrated major disturbances of gene expression in cloned animals, including effects that were inherent to cloning (donor cell-independent) and donor cell-dependent effects. Note that these common effects accounted for only a small portion of the affected genes; the remaining abnormally expressed genes showed wide variation across the individual somatic clones. One important consequence of this variation is that the calculation and comparison of mean expression levels tend to mask many of the gene expression abnormalities in each individual. Genes that were expressed abnormally in all of the individual Sertoli cell- or cumulus cell-derived clones accounted for only 0.4% or 4% of the total examined genes, respectively (red dots in the lower panels of Fig. 1, A–C, and Table 2). These values were consistent with those in a previous report, which found that less than 0.1% of the genes in the liver of neonatal clones derived from cumulus or embryonic stem cells showed expression levels that differed by more than two-fold [8].

The large variation in gene expression among individuals may be attributable to initial errors in the reprogramming process after nuclear transfer or may simply represent an abnormal epigenetic state that arises in the donor cells despite avoiding in vitro culture steps in their preparation. In either case, the first event affects target genes at random and may induce many secondary effects and ultimately lead to genome-wide, multilayered, mosaic abnormalities in somatically derived clones. In this respect, applications of nuclear transfer to human or veterinary medicine, such as therapeutic cloning, have serious limitations at present.

Recently, extensive evidence has accumulated for the clustering of coexpressed genes in mammalian genomes. The importance of the hierarchical architecture of epigenetic regulation that partitions the mammalian genome functionally, including very large domains such as isochors, as well as relatively small chromosomal domains of several hundred kilobase pairs, has now been recognized [19]. However, detailed, genome-wide surveys of gene regulation in these chromosome regions have not yet been reported for mammalian systems. The abnormal expression patterns observed in some gene clusters in this study may represent disturbances of a regionally regulated architecture of the chromosome. The gene expression variation-based cluster analysis used in this study may be generally applicable to the classification of genes that are coordinately regulated in the mammalian genome by permitting systematic analysis of apparently random biological phenomena that have previously been difficult to study.

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nature genetics

Deletion of *Peg10*, an imprinted gene acquired from a retrotransposon, causes early embryonic lethality

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By comparing mammalian genomes, we and others have identified actively transcribed Ty3/gypsy retrotransposon-derived genes with highly conserved DNA sequences and insertion sites[1–6]. To elucidate the functions of evolutionarily conserved retrotransposon-derived genes in mammalian development, we produced mice that lack one of these genes, *Peg10* (paternally expressed 10)[1–3,7], which is a paternally expressed imprinted gene on mouse proximal chromosome 6. The *Peg10* knockout mice showed early embryonic lethality owing to defects in the placenta. This indicates that *Peg10* is critical for mouse parthenogenetic development and provides the first direct evidence of an essential role of an evolutionarily conserved retrotransposon-derived gene in mammalian development.

Recently, we and others discovered the evolutionarily conserved retrotransposon-derived gene *Peg10* (refs. 1–3,8). Mouse *Peg10* is a single-copy gene located in an imprinted gene cluster on proximal chromosome 6, and its human homologue *PEG10* is in the syntenic imprinted region with identical gene order on human

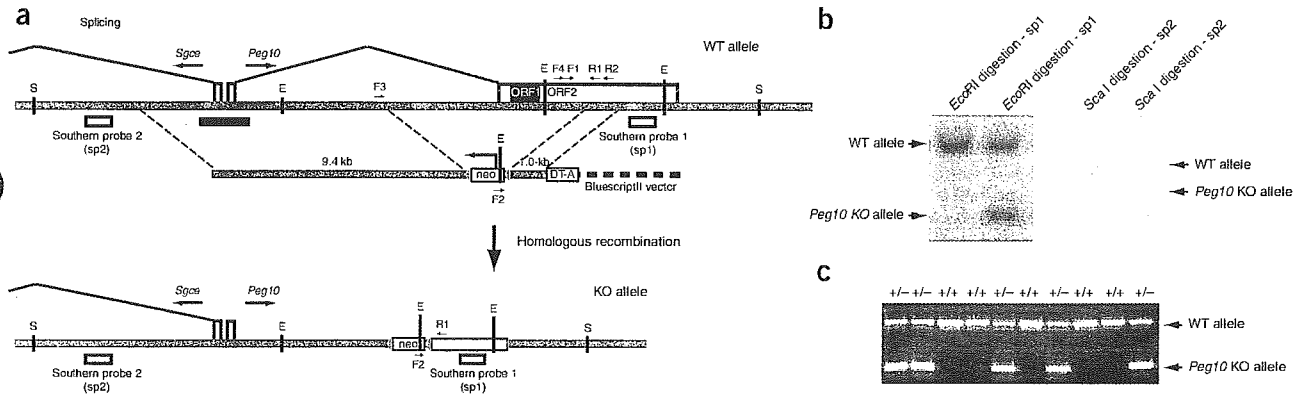

Figure 1 Targeted disruption of the *Peg10* locus. Schematic representation of the complete *Peg10* locus, the targeting vector and the targeted *Peg10* allele. Relevant restriction sites are indicated: E: *Eco*RI; S: *Sca*I. Primers are indicated by small arrows. (**a**) In the targeted allele, ORF1 and ORF2 were completely replaced by a neomycin resistance gene cassette. The *lox*P sites are shown as green triangles. WT, wild-type; KO: knockout. Black bar represents position of differentially methylated region (DMR). (**b**) DNA blot analysis of targeted ES cell clones. Genomic DNA was digested with *Eco*RI or *Sca*I and hybridized with Southern Probe 1 (SP1) or SP2. Left: wild-type ES cells; right: ES cells heterologous for the *Peg10* knockout allele. (**c**) Genotype analysis of 9.5-d.p.c. embryos by PCR using the *Peg10* F1, F2 and R1 primers. The results for each wild-type and Pat-KO embryo derived from mating a *Peg10* knockout chimera male with a wild-type female are shown.

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embryos that contain two maternally derived genomes lack expression of all paternally expressed imprinted genes. Parthenogenetic embryos die before 9.5 d.p.c. and show early embryonic lethality with poorly developed extraembryonic tissues[13,14]. Morphological defects of the most developed parthenotes are very similar to those of *Peg10*-Pat KO embryos; they lack the diploid trophoblast cells of the labyrinth layer and the spongiotrophoblast but have some giant cells and some chorion at days 9 and 10 (ref. 15). However, the majority of parthenotes show more severe phenotypes; the diploid trophoblast cells of the ectoplacental cone almost completely fail to develop, leading to lack of extraembryonic ectoderm, and therefore chorion, so that at 6.5 d.p.c. the conceptus is abnormal, despite the vigorous embryonic ectoderm (S. Barton and M.A. Surani, personal communication). Therefore, it is clear that some other genes could also contribute to the parthenogenetic phenotypes[13,14].

The genetic conflict hypothesis predicts that genes that promote embryonic and placental growth have become paternally expressed,

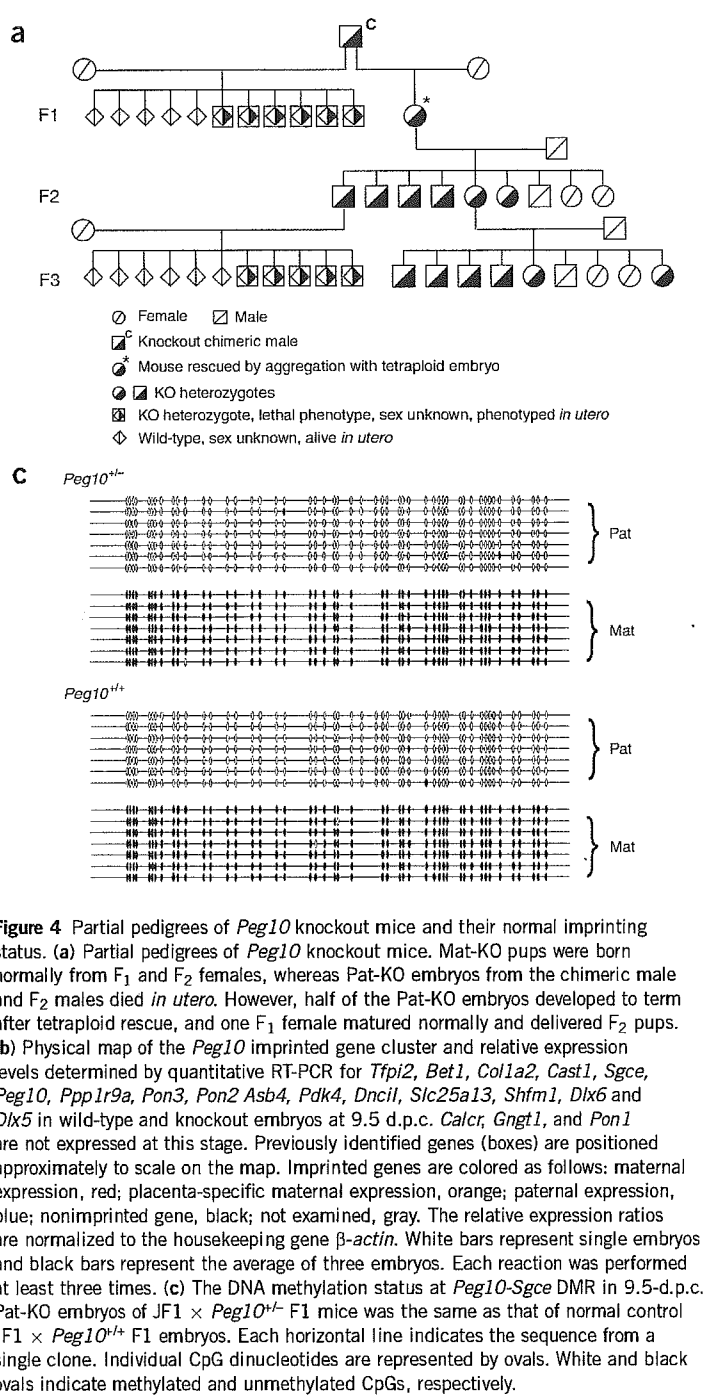

Figure 4 Partial pedigrees of *Peg10* knockout mice and their normal imprinting status. (**a**) Partial pedigrees of *Peg10* knockout mice. Mat-KO pups were born normally from F_1 and F_2 females, whereas Pat-KO embryos from the chimeric male and F_2 males died *in utero*. However, half of the Pat-KO embryos developed to term after tetraploid rescue, and one F_1 female matured normally and delivered F_2 pups. (**b**) Physical map of the *Peg10* imprinted gene cluster and relative expression levels determined by quantitative RT-PCR for *Tfpi2, Bet1, Col1a2, Cast1, Sgce, Peg10, Ppp1r9a, Pon3, Pon2 Asb4, Pdk4, Dncil, Slc25a13, Shfm1, Dlx6* and *Dlx5* in wild-type and knockout embryos at 9.5 d.p.c. *Calcr, Gngt1,* and *Pon1* are not expressed at this stage. Previously identified genes (boxes) are positioned approximately to scale on the map. Imprinted genes are colored as follows: maternal expression, red; placenta-specific maternal expression, orange; paternal expression, blue; nonimprinted gene, black; not examined, gray. The relative expression ratios are normalized to the housekeeping gene β-*actin*. White bars represent single embryos and black bars represent the average of three embryos. Each reaction was performed at least three times. (**c**) The DNA methylation status at *Peg10-Sgce* DMR in 9.5-d.p.c. Pat-KO embryos of JF1 × *Peg10*$^{+/-}$ F1 mice was the same as that of normal control JF1 × *Peg10*$^{+/+}$ F1 embryos. Each horizontal line indicates the sequence from a single clone. Individual CpG dinucleotides are represented by ovals. White and black ovals indicate methylated and unmethylated CpGs, respectively.

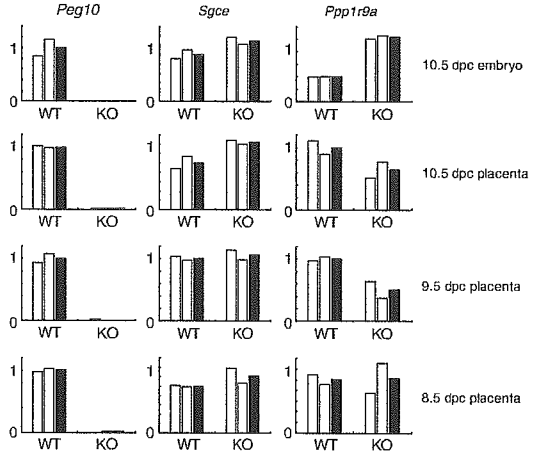

Figure 5 Relative expression levels of *Peg10, Sgce* and *Ppp1r9a* at different stages, as determined by quantitative RT-PCR The relative expression levels of 8.5- to 10.5-d.p.c. Pat-KO placentas and 10.5-d.p.c. Pat-KO embryos were determined by quantitative RT-PCR (as in **Fig. 4b**). Each reaction was performed at least three times. White bars represent single embryos and black bars represent the average of two embryos.

whereas genes that inhibit these growth have become maternally expressed during mammalian evolution[16]. Our results clearly show that *Peg10* conforms well to this hypothesis, as *Peg10* is paternally expressed and essential for the formation of the placenta, which functions to promote nutrient transfer from mother to fetus, as shown for the *Igf2* P0 transcript[17]. Therefore, we provide further evidence that the functional bias predicted by this hypothesis exists among imprinted genes.

Recently, RAG (the V(D)J recombinase)[18] and telomerase[19] have been shown or suggested to be derived from transposons and retrotransposons, respectively, indicating that some of these transposable elements have contributed to the acquisition of several new important genomic functions during evolution[20]. There are other reports that the rat IgE-binding protein contains a partial coding sequence of the retrotransposon intracisternal A particle (IAP)[21] and that the human *syncytin* gene[22], which functions in syncytiotrophoblast formation in placental tissues, is derived from a human endogenous retrovirus (HERV-W). Recently, mouse *syncytin-A* and *syncytin-B* genes have been isolated and, notably, have been found to be derived from similar but different retroviruses derived from the human HERV-W[23]. These findings also provide evidence that various species-specific retrotransposons play roles in different species. However, as far as we know, this is the first demonstration that an evolutionarily conserved mammal-specific, retrotransposon-derived gene has an essential function in development, at least in mice.

Recently, it has been reported that human PEG10 may have a carcinogenetic function in affecting cell cycle progression[24] or inhibiting apoptosis mediated by SIAH1 (ref. 25), and/or inhibiting TGF-β signaling by interacting with the TGF-β receptor ALK1 (ref. 26). Therefore, *PEG10* may have a wide variety of functions such as the regulation of cell growth and differentiation as well as placental function[1,8].

It is of great interest to learn the role of *Peg10* in the acquisition of the placenta during mammalian evolution, as it is highly conserved in eutherian mammals. Based on database searches, *Peg10* is not present in the *Fugu rubripes* (fish) or chicken genomes (**Supplementary Fig.** 7). Ancestral mammals might have developed this new organ, the placenta, from newly acquired, retrotransposon-derived genes, or endogenous genes present in oviparous animals might have been modified for placenta formation some time after the divergence of mammals and birds, more than 92 million years ago[27]. Further comparative genome analyses among eutherians marsupials, and monotremes may help to uncover the origin of *Peg10* in mammalian evolution. We found another ten homologues to the Sushi-ichi retrotransposon (called *Sirh* family genes) in the mouse and human genomes, including another paternally expressed gene, *Rtl1 (also* called *PEG11* in sheep)[3–6], which is located on the mouse distal chromosome 12, and *Ldoc1* (refs. 3,28) on the X chromosome. Similar conclusions have recently been reported by other researchers[29]. It will be fascinating to discover the functions of these evolutionarily conserved retrotransposon-derived genes, as well as those of *Peg10*.

METHODS

Deletion of the *Peg10* gene. We obtained 9.4-kb (nucleotides 6903–16254; AC084315) and 1.0-kb (nucleotides 20116-21135; AC084315) genomic fragments by screening the 129SvJ lambda genomic library (Stratagene). We used these fragments as the right- and left-arm sequences of a construct in which both *Peg10* ORFs were replaced with the neomycin resistance gene. After a 2-week incubation under G418 selection followed by electroporation of the linearized DNA into ES cells (CCE) of 129/SvEv mouse origin, we obtained 120 colonies. The genomic DNA of these colonies was checked by DNA blot analysis using DNA fragments of nucleotides 5300–6525 and nucleotides 21271–21766 as 5′-end and 3′-end probes, respectively.

The *Peg10*-targeted ES cells that resulted from homologous recombination of the construct were used to generate chimeric mice by blastocyst injection. From two male chimeras, germline transmission of the knockout allele was confirmed when pregnant female mice that had mated with a *Peg10* male chimera were dissected and their embryos analyzed.

PCR. Genomic DNA and total RNA samples were prepared from embryos and placentas at various stages using ISOGEN (Nippon Gene), as described previously[8]. The cDNA was synthesized from 1 μg of RNA using Superscript II reverse transcriptase (Life Technologies) with oligo-dT primer. For RT-PCR, 10 ng cDNA in a 100-μl reaction mixture containing 1× ExTaq buffer (TaKaRa), 2.5 mM dNTP mixture, primers and 2.5 units (U) ExTaq (TaKaRa) was subjected to 25–30 PCR cycles of 96 °C for 15 s, 65 °C for 30 s and 72 °C for 30 s in a Perkin Elmer GeneAmp PCR System 2400. Gene expression profiles were deduced from agarose gel electrophoresis of RT-PCR products with ethidium bromide staining. The primer sequences are available upon request.

Quantitative RT-PCR. The expression levels of 15 genes in the *Peg10* cluster and *Ascl2* were measured with the ABI PRISM 7700 using SYBR Green PCR Core Reagents (Applied Biosystems), which were designed to detect these cDNAs. The target cDNA fragments were cloned into plasmids to be used as standards in the quantitative analysis of gene expression. The relative expression ratios were normalized to the housekeeping gene β-*actin*. The primers for *Dncil* have been described previously[30]. The other primer sequences and conditions for their use are available upon request.

DNA methylation analyses of the *Peg10-Sgce* DMR. Genomic DNA samples were isolated from 9.5-d.p.c. embryos of JF1 × *Peg10*$^{+/-}$ F1 mice using ISOGEN, as described in the RT-PCR section. Purified genomic DNA was treated with a sodium bisulfite solution, and *Peg10-Sgce* DMR was amplified by PCR[8].

Generation of tetraploid aggregation chimeras. Electrofusion of two-cell–stage blastomeres collected from B6D2 F_1 females was used to produce wild-type tetraploid embryos. The fused embryos were cultured overnight in embryo culture medium in 5% CO_2 at 37 °C. Each eight-cell–stage diploid *Peg10* $^{+/-}$ embryo was aggregated overnight with a four-cell tetraploid embryo. Successfully aggregated embryo pairs at the morula or blastocyst stage were transferred to 2.5-d.p.c. pseudopregnant ICR recipients.

Histology. Pregnant mice that had mated with a *Peg10* knockout male were killed at 7.5, 8.5, 9.5, 10.5 and 11.5 d.p.c. Whole embryos were collected and immediately embedded in OCT. Sections from embedded embryos were cut in 7-μm sections. For hematoxylin and eosin staining or *in situ* hybridization, the sections were fixed in 4% paraformaldehyde before the standard staining procedures. The nuclei of the cell samples for *in situ* hybridization were stained with 2% methyl green.

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

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Anchorage-Independent Growth of Mouse Male Germline Stem Cells In Vitro[1]

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ABSTRACT

Spermatogenesis originates from a small number of spermatogonial stem cells that reside on the basement membrane and undergo self-renewal division to support spermatogenesis throughout the life of adult animals. Although the recent development of a technique to culture spermatogonial stem cells allowed reproduction of self-renewal division in vitro, much remains unknown about how spermatogonial stem cells are regulated. In this study, we found that spermatogonial stem cells could be cultured in an anchorage-independent manner, which is characteristic of stem cells from other types of self-renewing tissues. Although the cultured cells grew slowly (doubling time, ~4.7 days), they expressed markers of spermatogonia, and grew exponentially for at least 5 months to achieve 1.5×10^{10}-fold expansion. The cultured cells underwent spermatogenesis following transplantation into the seminiferous tubules of infertile animals and fertile offspring were obtained by microinsemination of germ cells that had developed within the testes of recipients of the cultured cells. These results indicate that spermatogonial stem cells can undergo anchorage-independent, self-renewal division, and suggest that stem cells have the common property to survive and proliferate in the absence of exogenous substrata.

developmental biology, gametogenesis, sertoli cells, spermatogenesis, testis

INTRODUCTION

Spermatogenesis depends on a small population of spermatogonial stem cells [1, 2]. These cells are the only cells in the spermatogenic system that can self-renew, and spermatogonial stem cells support male reproduction throughout life. Spermatogonial stem cells reside on the basement membrane and are distributed nonrandomly within seminiferous tubules [3]. Stem cells are thought to grow in a special microenvironment within which factors that regulate proliferation and differentiation are provided [4]. However, despite the importance of spermatogonial stem cells, very little is known about the factors that regulate these cells. Although spermatogonial stem cells can be identified by a functional transplantation assay [5], the small number (20 000 to 30 000 in mice) of stem cells within a testis has made direct in vivo analysis of spermatogonial stem cells very difficult [2, 6].

To overcome this problem, we recently developed an in vitro system to culture spermatogonial stem cells [7]. In the presence of glial cell line-derived neurotrophic factor (GDNF), which promotes self-renewing division of spermatogonial stem cells [8], germ cells from neonatal testes proliferated to form uniquely shaped colonies on mitomycin-C-treated mouse embryonic fibroblasts (MEFs) [7]. The cultured cells expressed spermatogonia markers and exhibited logarithmic proliferation for several months. When the cells were microinjected into the seminiferous tubules of infertile animals, the transplanted cells colonized the basement membrane and underwent spermatogenesis, and the recipient males sired normal fertile offspring. The cell cultures could be established from testes at various stages of development [7, 9–11], and these cells are an efficient vehicle for the production of transgenic animals [12]. Because of these unique properties, we called these cells germline stem (GS) cells to distinguish them from embryonic stem (ES) and embryonic germ (EG) cells that can be incorporated into the germline following injection into blastocysts [13–16]. Recapitulation of spermatogonial stem cell division in vitro will likely provide valuable information about how the self-renewal division of spermatogonial stem cells is regulated. In addition, the culture technique allows us to obtain large numbers of stem cells for biochemical or molecular analysis. Thus, the establishment of GS cell culture has provided an opportunity to study the factors that control spermatogonial stem cell division.

Generally, stem cells are associated closely with basement membrane [4]. This association probably provides signals via integrins that promote the survival and proliferation of the stem cells [4, 17]. However, several recent reports revealed that stem cells from various types of self-renewing tissue could be cultured in suspension [18–20]. For example, neuronal stem cells grow as neurospheres that do not depend on a particular substrate [18]. Similarly, mammary stem cells or stem cells from skin can also grow in suspension using similar culture conditions [19, 20]. These cells differentiate into various cell lineages after exposure to extracellular matrix molecule (ECM) or serum. These results demonstrate that stem cells can survive and proliferate in anchorage-independent culture conditions. Spermatogonial stem cells preferentially attach to laminin, and they express α6 and β1-integrins that in combination comprise the laminin receptor [21]. However, it is not known whether GS cells will remain in an undifferentiated state in suspension or whether suspension will induce differentiation or cell death.

In the present study, we investigated whether self-renewal division of spermatogonial stem cells could occur in the absence of substrate. The functional activity of GS cells

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cultured in suspension was assessed using a functional transplantation assay.

MATERIALS AND METHODS

Animals and Transplantation

The Institutional Animal Care and Use Committee of Kyoto University approved all animal experimentation protocols. Testis cells were collected from the testes of a transgenic mouse line C57BL6/Tg14(act-EGFP-OsbY01) that was bred on a DBA/2 background (designated Green) (originally provided by Dr. M. Okabe, Osaka University). GS cells were established from newborn animals (0–2 days old), using a two-step enzymatic digestion method described previously [22]. The transgenic mice ubiquitously express the gene for enhanced green fluorescent protein (EGFP) under the control of the β-actin promoter [23].

The cultured cells were transplanted into the testes of WBB6F1-W/W^v mice (designated W; purchased from Japan SLC). W mice are congenitally infertile, and lack all stages of differentiating germ cells owing to mutations in the gene that encodes *Kit* receptor tyrosine kinase [24]. Cultured cells were transplanted into 6- to 10-week-old W mice. As these mice are not histocompatible with the donor cells, the recipient mice were treated with anti-CD4 antibody (clone GK1.5) to induce tolerance to the allogeneic donor cells [25]. For microinjections, approximately 3 μl (1.5×10^4 cells per testis) of the donor cell suspension were introduced into the seminiferous tubules of a W testis by efferent duct injection [22]; this filled 75–85% of the tubules in each recipient testis.

GS Cell Culture and Adhesion Assay

The standard culture medium was StemPro-34 SFM (Invitrogen) supplemented with StemPro Supplement (Invitrogen), 25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 30 nM sodium selenite, 6 mg/ml D-(+)-glucose, 30 μg/ml pyruvic acid, 1 μl/ml DL-lactic Acid (Sigma), 5 mg/ml BSA (ICN Biomedicals), 2 mM l-glutamine, 5×10^{-5} M 2-mercaptoethanol, MEM Vitamin Solution (Invitrogen), MEM nonessential amino acids solution (Invitrogen), 10^{-4} M ascorbic Acid, 10 μg/ml d-biotin, 30 ng/ml β-estradiol, and 60 ng/ml progesterone (Sigma). The growth factors that were used were 20 ng/ml mouse epidermal growth factor (EGF; BD Biosciences), 10 ng/ml human basic fibroblast growth factor (FGF2; BD Biosciences), 10^3 U/ml murine leukemia inhibitory factor (LIF; Invitrogen), and 10 ng/ml recombinant rat GDNF (R&D Systems). In some cases (where indicated), cells were cultured in the presence of 300 ng/ml rat GDNF sRα1/Fc chimera or 10 ng/ml human neurturin (NRTN; both from R&D Systems). The cells were cultured in the presence of 1% or 15% fetal bovine serum (FBS; JRH Biosciences).

To initiate suspension cultures, GS cells that had been maintained on MEFs for 120 days were trypsinized at 37°C for 5 min before being transferred to a 15-ml centrifuge tube. After centrifugation ($440 \times g$ for 5 min), the pellet was resuspended in GS cell culture medium, and 3×10^5 cells were plated onto a 35-mm Petri dish (BD Biosciences, cat. no. #351008). To passage the cells, the plated cells were flushed gently by repeated pipetting using a blue tip pipette. The cells were then centrifuged at $440 \times g$ for 5 min. After the removal of the supernatant, 1 ml of 0.25% trypsin/EDTA was added to the pellet, and the cells were incubated for 5 min at 37°C. To stop the trypsin activity, 3 ml of Iscove's modified Dulbecco medium supplemented with 5 mg/ml BSA was added. Dissociated cells were triturated and were then centrifuged at $440 \times g$ for 5 min. Live cells were counted in a hemocytometer, and 3×10^5 cells were resuspended in a Petri dish that contained 1.5 ml of standard GS cell culture medium. Three days after each passage, the cells were fed with 0.5 ml fresh medium (a total of 2 ml), and thereafter were passaged every 4–5 days. Feeder-free GS cell culture was performed as described previously [26]. All cultures were maintained at 37°C in an atmosphere of 5% carbon dioxide in air. Cryopreservation was performed as described previously [27].

For the adhesion assay, GS cells were trypsinized before being plated in GS cell culture medium on MEFs or laminin-coated (20 μg/ml) dishes at a density of 2.5×10^5 cells per 9.5 cm^2. After overnight culture, the dishes were washed twice with PBS to collect nonadherent (floating) cells. Adherent cells were collected after 5 min of trypsin digestion. To remove serum in the MEF culture, the plates were washed four times with PBS before the cells were plated. Data were analyzed using the Student *t*-test.

Flow Cytometry and Immunohistochemistry

Flow cytometric analyses were performed as described previously [28]. Briefly, 10^6 cells were suspended in 0.1 ml of PBS that contained 1% FBS (PBS/FBS). The suspended cells were then incubated with primary antibodies. The cells were washed twice with PBS/FBS before being stained by exposure to secondary antibodies The stained cells were analyzed using the FACS-Calibur system (BD Biosciences). We collected 10000 events for flow cytometric analysis. For immunohistochemical staining, whole spheres were gently centrifuged onto 3-aminopropyltriethoxy silane-coated slides (Matsunami Glass) at $600 \times g$ for 6 min. After fixation in 3.7% formaldehyde for 10 min, the slides were washed twice, and stained by primary antibodies.

The primary antibodies used were: rat anti-EpCAM (G8.8); mouse anti-SSEA-1 (MC-480) (Developmental Studies Hybridoma Bank, University of Iowa); rat anti-human α6-integrin (CD49f) (GoH3); biotinylated hamster anti-rat β1-integrin (CD29) (Ha2/5); biotinylated rat anti-CD9 antigen (KMC8); rabbit anti-human fibronectin (DAKO), rabbit anti-mouse laminin (Sigma); rabbit anti-human POU5F1; rabbit anti-human GFRA1 (Santa Cruz Biotechnology), or allophycocyanin (APC)-conjugated rat anti-mouse KIT (CD117) (2B8) (BD Biosciences). APC-conjugated goat anti-rat IgG (Cedarlane Laboratories); APC-conjugated streptavidin (BD Biosciences); Alexa Fluor 568 goat anti-rabbit IgG; Alexa Fluor 680-allophycocyanin goat anti-rabbit IgG; or Alexa Fluor 633-conjugated goat anti-mouse IgM (Molecular Probes) were used as secondary antibodies.

Analysis of Testes

To quantify the number of donor-derived cell colonies, recipient mice were killed 2 mo after the transplantation of donor cells. Because the donor cells express EGFP, donor-derived colonies were detected by excitation with UV illumination. This method allowed for specific identification of donor cells because the recipient testis does not exhibit endogenous fluorescence. A cluster of germ cells was defined as a colony when it occupied more than 50% of the basal surface of the tubule and was at least 0.1 mm long [29]. The number of colonies was quantified by counting the total number of colonies observed under a stereomicroscope. To prepare histological sections, recipient testes were fixed in 10% neutral-buffered formalin before being embedded in paraffin wax. Sections were stained with hematoxylin-eosin. Data were analyzed using the Student *t*-test.

Analysis of Marker Gene Expression

Total RNA was isolated from whole spheres using Trizol reagent (Invitrogen). For the reverse transcriptase-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized using Superscript II (RNase H$^-$ reverse transcriptase; Invitrogen). The PCR was carried out using appropriate primer sets, as described previously [11, 26, 30]. PCR amplifications of fibronectin1 and laminin α1 was carried out using specific primers (5′-TGTGACAACTGCCGTAGACC-3′ and 5′- TGCTGAAGCTGAGAA-CATGG-3′ for fibronectin1, and 5′- TGTGGACAGGTGCTATGTCG-3′ and 5′-AGGTGGCTGTTATCCTTCCG-3′ for laminin α1).

Microinsemination

The seminiferous tubules of recipient testes were dissected under UV illumination when the recipient mice were 7 mo old. EGFP-expressing seminiferous tubules were recovered, and the germ cells were collected mechanically from the tubules by fine forceps. Microinsemination was performed as described previously using elongated spermatids or spermatozoa [31]. Embryos that reached the two-cell stage after 24 h in culture were transferred to the oviducts of Day 1 pseudopregnant Imperial Cancer Research (ICR) female mice. Fetuses that were retrieved on Day 19.5 were raised by ICR foster mothers.

RESULTS

Anchorage-Independent Growth of GS Cells

We first examined whether GS cells adhered to MEFs and/or laminin, both of which can be used for GS cell culture [7, 26]. GS cells were established from a newborn Green mouse using a standard protocol [7]. After dissociation into single-cell suspensions by enzymatic digestion, germ cells from neonatal testis formed uniquely-shaped colonies, and proliferated in the presence of GDNF and 1% FBS. Within 1 mo, germ cells were cultured on mitomycin C-treated MEF. After 120 days in culture, GS cells on MEFs were plated on MEFs or laminin in GS cell culture medium (Fig. 1A, left, top). After overnight culture, although some cells had attached to the substrate, a

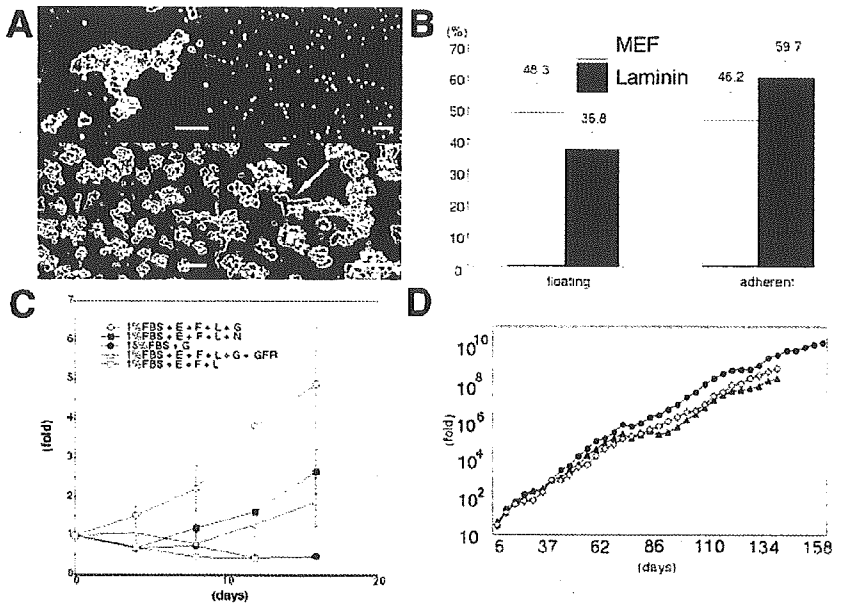

FIG. 1. Growth of GS cells in suspension. A) GS cells. Left, top: GS cells on MEFs. Note the morula-like structure. Right, top: GS cells in suspension culture, immediately after plating. Left, bottom: GS cells in suspension culture, 4 days after passage. Right, bottom: Higher magnification. Note the chain-type colony (arrow). B) Adhesiveness of GS cells to MEFs and laminin. GS cells were plated overnight on MEFs or laminin (20 μg/ml). Nonadherent (floating) cells were recovered by repeated washing of the culture plates. The values on the vertical axis represent percentages of cells that could be recovered after treatment. Results are from three independent experiments (mean ± SEM, n = 6). C) Expansion of GS cells cultured with various cytokines. The same number of cells (3×10^5 cells) was cultured under the conditions indicated. Cells were passaged three times over 16 days. Abbreviations: E, EGF; F, FGF2; G, GDNF; L, LIF; N, NRTN; GFR, GFRα1. D) Cumulative growth curves for three separate cultures of nonadherent GS cells. Cells were maintained for 134 or 158 days. The total number of cells was counted at at each passage. Note the steady and consistent exponential increase in total cell number over time. Bar = 100 μm (A).

substantial number of nonadherent cells were observed. The average recoveries of adherent cells from three experiments were 46.2 ± 8.9% and 59.7 ± 5.4% of cells plated on MEFs and laminin, respectively (mean ± SEM, n = 6 for each). By contrast, 48.3 ± 9.5% and 36.8 ± 5.6% of cells failed to adhere to MEFs and laminin, respectively (n = 6 for each) (Fig. 1B). The proportions of cells that adhered to MEFs and laminin were not significantly different ($P > 0.3$ by t-test). Similar results were obtained under serum-free culture conditions (data not shown).

To examine whether the nonadherent cells would continue to proliferate in vitro, cells were dissociated by trypsin digestion, and 3×10^5 cells were plated as single cells onto uncoated bacteriological Petri dishes that do not bind GS cells (Fig. 1A, right, top). Although GS cells did not grow in serum-free medium, the addition of 1% serum triggered cell growth. Some of the plated cells died, and we did not observe dividing cells 1 day after plating. Within 3 days, the plated cells grew and formed colonies with various morphologies (Fig. 1A, bottom). Although some colonies grew as chains of interconnecting cells, most colonies exhibited a morula-like appearance that resembled GS cells plated on MEFs. These colonies merged to form aggregate when maintained in a small volume of medium. We did not observe sphere-shaped colonies, which is the predominant morphology of cell aggregates in suspension cultures of other types of stem cell [18–20]. As with other types of GS cell cultures, growth of nonadherent cells depended on the presence of GDNF (Fig. 1C). However, cells did not proliferate in the absence of EGF and FGF2, even when a higher concentration of serum (15%) was added to the medium. The action of GDNF was mimicked by NRTN, albeit at a lower degree, which suggested that other members of the GDNF family of ligands can maintain the proliferation of GS cells. The addition of soluble GDNF receptor alpha-1 (GFRA1) did not improve the growth of nonadherent GS cells [9]. Floating colonies could not be derived directly from newborn testes, which suggested that in vitro maturation of gonocytes into spermatogonia is necessary for stating suspension culture.

Although these colonies could be partially dissociated and passaged by trituration into small clumps, it was not possible to obtain a suspension of single cells using trituration alone. We therefore used trypsin digestion to obtain a suspension of single cells to measure the increase in cell number during culture. Cultures were passaged serially every 4–5 days and were replated in fresh medium. The proliferation of viable cells was concentration-dependent, but the cells proliferated efficiently at a constant rate when 2×10^4 to 3×10^5 cells were plated per dish (20–300 cells/mm^2). The cells stopped proliferating when 1×10^6 cells were plated per dish. Three independent cultures grew in a logarithmic manner through repeated passages for at least 158 days with a doubling time of ~112 h (Fig. 1D). The GS cells plated on MEFs grew faster and had a doubling time of ~65 h when growing logarithmically after being transferred to MEFs. Nonadherent cells could be frozen successfully at any stage of suspension culture. After thawing, the mean viability index of the freeze-thawed testis cells, as assessed by trypan blue exclusion, was 60.6 ± 3.6% (mean ± SEM; n = 3). The cells could expand 8.8-fold in 24 days after thawing. We did not observe any ES-like cell development during the entire culture period by morphological criteria [11].

To examine the phenotypes of the cultured cells, we performed flow cytometric analysis of surface antigen

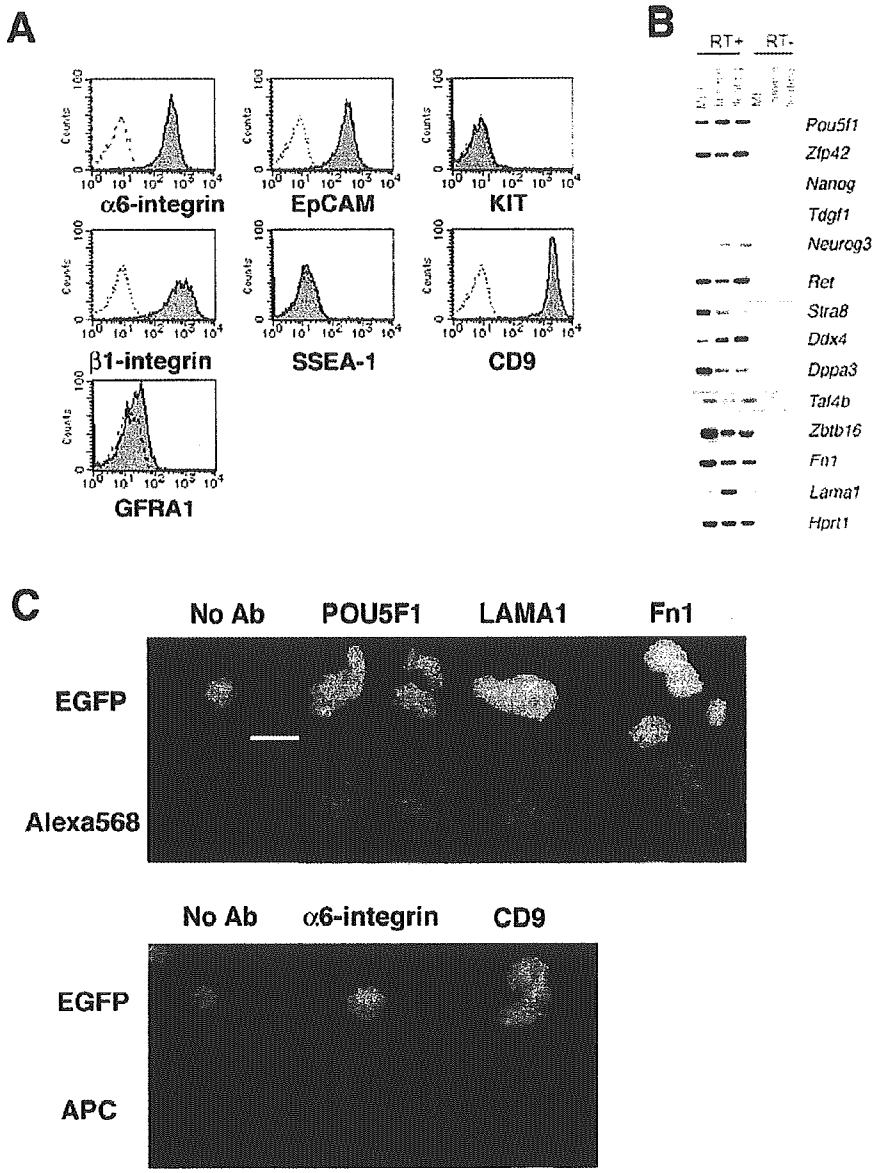

FIG. 2. Phenotypic analysis of nonadherent GS cells. **A**) Characterization of nonadherent GS cells using flow cytometry. The horizontal axis represents fluorescence (log scale) measured in arbitrary units. Floating GS cells were exposed to trypsin and were incubated with antibodies against α6-integrin, β1-integrin, EpCAM, SSEA-1, CD9, KIT, or GFRA1. Unfilled peaks represent staining by control immunoglobulin, whereas filled peaks represent staining by specific antibody against each antigen. **B**) RT-PCR analysis of nonadherent GS cells. Specific primers were used to amplify cDNA from different types of GS cell cultures. **C**) Expression of spermatogonia markers and ECM molecules on spheres. EGFP-expressing spheres were stained with specific antibodies. Secondary antibodies with APC or Alexa Fluor 568 were used to detect primary antibodies. Bar = 500 μm (**C**).

expression (Fig. 2A). Cultured cells expressed α6- and β1-integrins, EpCAM, CD9, and GFRA1 (spermatogonial stem cell markers) [9, 21, 32, 33], but did not express KIT (differentiated spermatogonia marker) or SSEA-1 (ES or primordial germ cell markers) [34, 35], These results indicated that the cultures were relatively enriched with undifferentiated spermatogonia. We also examined other ES and germ cell markers using RT-PCR (Fig. 2B). Floating GS cells expressed the ES cell markers *Pou5f1* and *Zfp42*, but did not express ES cell markers such as *Nanog* and *Tdgf1* [36–40]. In agreement with the flow cytometric analysis, PCR showed that the cells expressed all of the expected germ cell and spermatogonia markers, including *Neurog3*, *Ret*, *Stra8* (spermatogonia markers), *Ddx4*, and *Dppa3* (germ cell markers) [8, 41–44]. Floating GS cells also expressed *Zbtb16* and *Taf4b*, both of which are essential for spermatogonia self-renewal division [30, 45, 46]. These markers were similarly expressed in other types of GS cell cultures. The cells in the sphere appeared to be relatively uniform by immunohistochemistry (Fig. 2C). On the basis of a recent report that neurospheres expressed ECM [47], we examined whether GS cells expressed ECM. RT-PCR and immunohistological analyses revealed that laminin or fibronectin was expressed under all culture conditions: MEF-based, feeder-free, and nonadherent cultures. These results indicate that nonadherent cells have the spermatogonia phenotype and suggest that these cells also express ECM molecules, which has been reported for neural stem cells [47].

Spermatogonial Stem Cell Activity of the Cultured Cells

To determine whether the GS cells exhibited spermatogonial stem cell activity, we performed germ cell transplantation. In this technique, transplanted spermatogonial stem cells colonize the vacant germline niche in the seminiferous tubules of infertile animals [5]. EGFP-expressing, nonadherent GS cells were collected at four different times after initiation of the suspension culture. The collected cells were trypsinized, and a suspension of single cells was microinjected into the

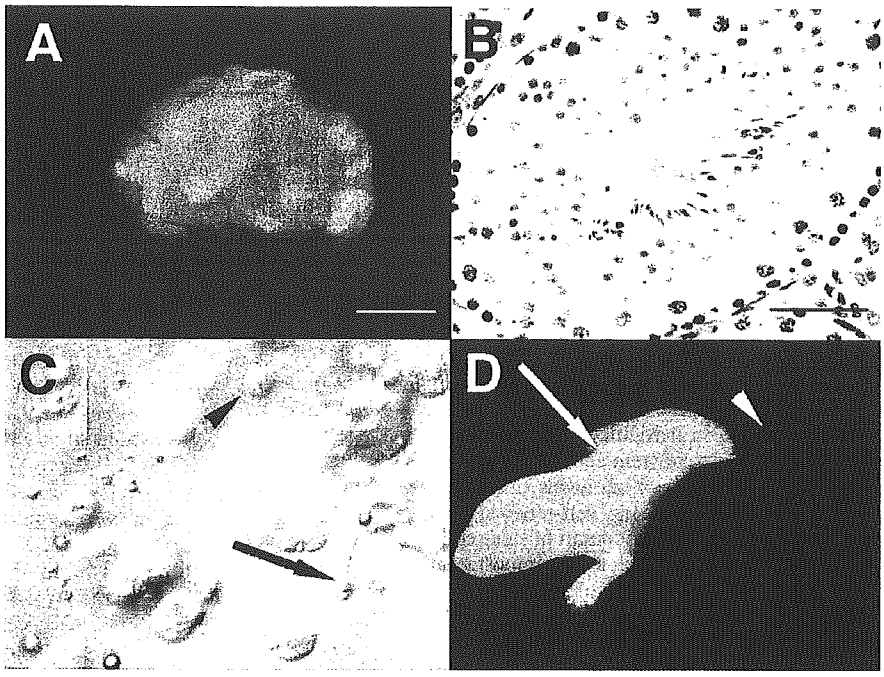

FIG. 3. Production of fertile offspring from suspension-cultured GS cells. **A**) Macroscopic appearance of a recipient testis after the transplantation of floating GS cells. The green stretches in the seminiferous tubules indicates the presence colonies of spermatogenic donor cells, which express EGFP. **B**) Histological appearance of a recipient testis. Note the normal appearance of spermatogenesis. Elongated spermatids are observed. **C**) Spermatogenic cells released from a recipient testis. A spermatozoon (arrow) or a round spermatid (arrowhead) are observed within the cell suspension. **D**) Expression of the EGFP transgene in F2 offspring (arrow) produced by mating EGFP-expressing F1 males with wild-type females. EGFP fluorescence is not observed in nontransgenic progeny (arrowhead). Bar = 1 mm (**A**), 50 μm (**B**). Hematoxylin and eosin staining (**B**).

seminiferous tubules of immunosuppressed congenitally infertile W recipient mice. These mice lack endogenous spermatogenesis [24]; therefore, any spermatogenesis in the recipient mice is derived from transplanted donor cells. The recipient mice were killed 2 mo after transplantation, and donor cell colonization was analyzed under UV illumination to detect EGFP expression in donor cells. Donor cell colonization was observed in all recipient testes (Fig. 3A). Assuming that stem cell colonization after transplantation occurred with an efficiency of 10% [29], the concentration of stem cells ranged from 0.2 to 2.0% of the original number of cultured cells (Table 1). Although the total number of cells increased 4×10^6-fold, the number of stem cells increased $\sim 3.2 \times 10^7$-fold through 101 days. Histological analysis revealed that normal spermatogenesis occurred within the recipient testis (Fig. 3B). These results indicate that nonadherent GS cells exhibit spermatogonial stem cell activity.

Finally, to test whether the germ cells that developed from nonadherent GS cells were functionally normal, we performed in vitro microinsemination, a technique used commonly to derive offspring from animals and humans [31, 48]. At 167 days after transplantation, we killed one of the recipient mice that had received 122-day-old cultured GS cells. The recipient testis was examined under UV illumination, and fragments of the seminiferous tubules that exhibited EGFP fluorescence were dissected before being dissociated mechanically with fine forceps. Spermatozoa and elongated spermatids could be identified in the cell suspension that contained various stages of germ cells (Fig. 3C). These cells were microinjected into oocytes from C57BL/6 × DBA/2 F1 mice. Spermatozoa and elongated spermatids from four different segments were used, and 152 embryos were constructed. Of these, 122 embryos developed to the two-cell stage. These embryos were transferred into the oviducts of pseudopregnant mice after 24 h of culture. A total of 51 offspring were born by cesarean section; 48 of these developed into adults (21 males and 27 females; Table 2). Of these animals, 33 (69%) exhibited EGFP fluorescence that was consistent with the incorporation of the donor cells into the germline. These offspring grew up to be fertile (Fig. 3D).

DISCUSSION

Most macromolecular metabolic processes in normal cells require that the cells be adhered to an appropriate substrate [49]. The suspension of fibroblasts in methylcellulose inhibits the synthesis of RNA and proteins. Stem or progenitor cells

TABLE 1. Spermatogonial stem cell expansion in suspension conditions.[a]

Days to transplant (passage)[b]	Cells injected/testis ($\times 10^4$)	Colonies/testis	Colonies/10^5 cells	Stem cells/10^5 GS cells	Increase in GS cell number (fold)[c]	Increase in stem cell number (fold)[c,d]
21 (3)	1.5	3.8 ± 2.2	25.3 ± 14.3	253.3		
47 (8)	1.5	3.0 ± 1.0	20.0 ± 6.5	200.0	49.8	39.3
78 (16)	1.5	6.8 ± 1.9	45.0 ± 12.6	450.0	6.2×10^3	1.1×10^4
122 (27)	1.5	30.2 ± 3.1	201.1 ± 20.7	2011.3	4.0×10^6	3.2×10^7

[a] Values are mean ± SEM; results from at least four recipient testes for each transplantation.
[b] Number of days from initiation of culture to transplantation.
[c] Increase in the total cell or stem cell number from the first transplantation (Day 21).
[d] (Increase in GS cell number at indicated time point) × (Stem cells/10^5 GS cells at indicated time point)/(Stem cells/10^5 GS cells at first transplantation).

TABLE 2. Development of oocytes fertilized with germ cells derived from floating GS cells.

Type of cells	No. of embryos cultured	2-cell	No. of embryos transferred (%)[b]	No. of embryos implanted (%)	No. of pups (%)
Sperm[a]					
1	34	32 (94)	32 (94)	25 (74)	17 (50)
2	27	16 (59)	15 (56)	6 (22)	4 (15)
3	24	16 (67)	16 (67)	11 (46)	6 (25)
Elongated spermatid	67	58 (87)	58 (87)	40 (60)	24 (36)
Total	152	122 (80)	121 (80)	82 (54)	51 (34)

[a] Spermatozoa from three different seminiferous tubule fragments.
[b] Embryos were cultured for 24 h and transferred at the 2-cell stage.

from various tissues reside on basement membranes [4], and the behavior of these cells is regulated by modulatory signals from the ECM. In particular, signaling via β1-integrin acts as a negative regulator of stem cell differentiation in various tissues, and cells that express high levels of β1-integrin have a higher probability of undergoing self-renewal division [17, 47, 50]. Therefore, the close association of stem cells with the basement membrane is considered to be important for the biology of these cells. Nevertheless, stem cells exhibit differential affinities for, and responses to, different ECM molecules. Indeed, the ability to create suspension cultures of stem cells derived from several self-renewing tissues, such as brain, mammary glands, and skin, suggests that close association with the basement membrane may not be a prerequisite for self-renewal division of stem cells.

In testis, spermatogonial stem cells express β1- and α6-integrins and are located at the basal side of seminiferous tubules [1, 2, 21]. Based on the assumption that a cellular component is required for stem cells to proliferate, most previous attempts to culture spermatogonial stem cells included various types of substrates [51–60], such as Sertoli cells or bone marrow stroma cells, and it is possible to culture GS cells on embryonic fibroblasts [7]. However, by taking advantage of the fact that spermatogonial stem cells have an affinity to laminin [21], more recent studies revealed that cellular support is dispensable in GS cell cultures, because feeder cells could be replaced with laminin to support the in vitro expansion of GS cells [26]. One question that was not addressed in the aforementioned studies was whether spermatogonial stem cells can proliferate in suspension, which has been demonstrated for stem cells from other types of self-renewing tissue.

The success of suspension culture in the present study demonstrates that anchorage to the basement membrane is not required for the survival or self-renewal of spermatogonial stem cells. The transplantation experiments showed that GS cells in suspension were functionally normal. These cells formed colonies of spermatogenic cells and could be used to create fertile offspring, thereby satisfying the criteria for classification as spermatogonial stem cells [5]. The proportion of the stem cells that failed to adhere varied among different experiments but was within the range (0.1 to 4.9% of the total cell population) reported for similar studies of adhesion to MEFs and laminin [7, 9–11, 26]. Therefore, it would appear that the type of substrate does not have a significant effect on the germline potential of cultured cells. Interestingly, in the present study, GS cells could be grown on MEFs even after extensive passages under nonadherent culture conditions. These facts indicate that nonadherent GS cells and GS cells on MEFs are convertible to each other and that GS cells retain germline potential regardless of matrix support.

Although functional spermatogonial stem cells were obtained under three different culture conditions (suspended cells and cells grown on MEFs or laminin), several differences in the cells among the different conditions were noted. First, GS cells grew more slowly in suspension than on MEFs (doubling time, 4.7 vs. 2.7 days) [7]. This difference cannot be attributed only to a decrease in cell-matrix interaction, because cell growth on laminin was even slower (doubling time 5.6 days) [26]. One explanation is that MEFs secrete unknown growth factors that are beneficial to GS cells. Second, although the expression of most marker genes was consistent among the three culture conditions, GS cells on laminin aberrantly expressed the embryonic marker, SSEA-1 [26]. SSEA-1 is expressed by ES cells and early germ cells in the fetus, and is not normally expressed in the postnatal testis. GS cells on MEFs and suspension cultures of GS cells did not express SSEA-1. Interestingly, the suspension culture was more similar to the MEF culture than to the laminin culture. In addition, although GS cells cultured on MEFs or laminin express variable levels of *Kit* [7, 26], *Kit* expression was suppressed markedly in GS cells from suspension cultures in the present study. This suggests that association with the basement membrane may influence the differentiation of spermatogonia.

The success of suspension culture in the present study was critically dependent on GDNF. GDNF is normally secreted from Sertoli cells in vivo, and a decrease in GDNF expression results in defective spermatogenesis and infertility in mice [8]. Recent studies also showed that GDNF influences spematogonial stem cell activity in species other than rodents [61]. The results of our study confirmed previous reports that GDNF is a crucial regulator of stem cell self-renewal [7–12, 26]. In this study, we also demonstrated that NRTN, another member of the GDNF family of ligands, could also stimulate the growth of GS cells. Although Sertoli cells express NRTN, NRTN is believed to regulate the proliferation and differentiation of spermatogonia, spermatocytes and spermatids [62, 63]. The positive effects of NRTN on GS cells likely reflect cross-talk between NRTN and the GFRA1 receptor [60]. On the other hand, the negative effect of high serum concentration indicates that serum contains unknown factors that may stimulate apoptosis or differentiation of stem cells. However, the requirement of low concentration of serum in suspension culture suggests that it also provides some trace ingredients (e.g., hormones) that are necessary for self-renewal division of spermatogonial stem cells. Although these results concur with those of previous studies, the negative effect of soluble GFRA1 was unexpected. This is in contrast to a previous report that soluble GFRA1 enhanced the growth of GS cells [9]. A possible explanation is that soluble GFRA1 may have interfered with the binding of GDNF to GS cells. Alternatively, this difference could be attributable to the different genetic backgrounds of the mice that were used in the studies (C57 vs. DBA/2) [9]. Further studies are required to confirm the effect

of soluble GFRA1 on the self-renewal of spermatogonial stem cells.

Although we succeeded in culturing GS cells in suspension, there are at least three differences between our cultures and other suspension stem cell cultures of stem cells. First, GS cells grew as chains or morula-like clumps in suspension, whereas stem cells from other tissues form spheres in suspension [18–20]. Second, serum generally induces the differentiation of spheres in cultures of other stem cells, but GS cells required serum for propagation [18–20]. Third, GS cells could be passaged by trypsin, whereas trypsin has detrimental effects on sphere-forming stem cells, which are usually passaged using mechanical dissociation [18–20]. These differences may result in part from the fact that stem cells undergo limited proliferation in spheres [19, 64]. Spheres are not composed of a homogeneous population of cells. Indeed, differentiation markers and growth factor receptors are distributed heterogenously within neurospheres, which suggests that stem cells are located nonrandomly in spheres [47]. Generally, the expansion of adult stem cell populations does not occur readily ex vivo, presumably owing to asymmetric cell division kinetics that result in the production of a large number of progenitors and differentiated cells and a small, fixed number of stem cells. By contrast, GDNF induced the symmetric division of spermatogonial stem cells and caused an increase in the total number of these cells [7, 9–12, 26]. The morphology of colonies probably reflects the division patterns of stem cells [65], and this difference in stem cell division kinetics may reflect the unusual colony structure that we observed in the current study.

At present, why spermatogonial stem cells are capable of anchorage-independent growth is not known. Anchorage-independent growth is not a general characteristic of GS cells, because germline cells are associated closely with ECM throughout development and ES, EG, and multipotent GS cells form embryoid bodies and differentiate in suspension [11, 13–16, 66]. However, it is worth noting that very similar clusters of spermatogonia were found in the lumen of seminiferous tubules when GDNF was overexpressed in vivo. This occurred in the testes of GDNF transgenic mice and after in vivo elecctroporation of Sertoli cells with a GDNF transgene [8, 67]. Instead of proliferating as chains or networks on the basement membrane [29], many spermatogonia lost contact with the basement membrane and accumulated within the lumen of seminiferous tubules. In agreement with our findings, these clusters do not express KIT [8], which indicates that the clusters comprise undifferentiated spermatogonia. Furthermore, these cells grew more slowly than did wild-type cells; the peak proliferation index was higher in wild-type mice than in transgenic mice [8]. These results suggest that high concentrations of GDNF can influence cell-substrate adhesion and can create an environment in which stem cells can slowly undergo anchorage-independent growth. Further studies are required to clarify the physiological significance of this phenomenon.

The identification of GDNF as a spermatogonial self-renewal factor was a key to establishing the technique used to culture GS cells [7]. The successful elucidation of the conditions required for the suspension culture of GS cells has provided a simplified method that should facilitate large-scale spermatogonial stem cell culture. However, the GS cell culture technique is not yet perfect, and many improvements are needed to increase the usefulness of this technology. We believe that future refinements in culture technology will lead to the identification of additional factors that are essential for the self-renewal process of spermatogonial stem cells, which will provide greater insight into this unique biological process.

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Long-Term Culture of Mouse Male Germline Stem Cells Under Serum- or Feeder-Free Conditions[1]

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ABSTRACT

Spermatogonial stem cells are the only stem cells in the body that transmit genetic information to the next generation. These cells can be cultured for extended periods in the presence of serum and feeder cells. However, little is known about factors that regulate self-renewal division of spermatogonial stem cells. In this investigation we examined the possibility of establishing culture systems for spermatogonial stem cells that lack serum or a feeder cell layer. Spermatogonial stem cells could expand in serum-free conditions on mouse embryonic fibroblasts (MEFs), or were successfully cultivated without feeder cells on a laminin-coated plate. However, they could not expand when both serum and feeder cells were absent. Although the cells cultured on laminin differed phenotypically from those on feeder cells, they grew exponentially for at least 6 mo, and produced normal, fertile progeny following transplantation into infertile mouse testis. This culture system will provide a new opportunity for understanding the regulatory mechanism that governs spermatogonial stem cells.

developmental biology, gametogenesis, Sertoli cells, spermatogenesis, testis

INTRODUCTION

Spermatogonial stem cells are a unique population of cells with self-renewing potential. Unlike other spermatogonia that are committed to differentiate, spermatogonial stem cells can continuously self-renew and produce differentiated progenitor cells [1, 2]. Spermatogonial stem cells are believed to interact closely with Sertoli cells and to distribute nonrandomly in tissue [3]. Generally, stem cells reside within a special microenvironment, or niche [4], that provides factors that regulate proliferation or differentiation of the stem cell population. Although this niche was originally a hypothetical entity, recent studies with *Drosophila* have revealed the molecular machinery of the stem cell-niche interaction within the testis [5, 6]. For example, stem cells adhere within the niche by DE-cadherin [7], and the undifferentiated state of stem cells is maintained by Jak-Stat signaling [8, 9]. Similar studies in other self-renewing tissues uncovered the close interaction of stem cells and stroma cells that constitute the niche [10]. While these studies demonstrate the important roles of the microenvironment in stem cell biology, very little is known about the factors in the process of self-renewal of spermatogonial stem cells in mammals and how they influence stem cell behavior.

A valuable approach for studying this problem would be to reproduce stem cell self-renewal division in vitro. This would elucidate the minimal external requirement of the self-renewal process and provide important information on the stem cell-niche interaction in vivo. Recently, we described a method of culturing mouse spermatogonial stem cells [11]. In this method, spermatogonial stem cells from neonatal testis were able to proliferate for more than 5 mo on mouse embryonic fibroblasts (MEFs) in the presence of glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), leukemia inhibitory factor (LIF), and fetal calf serum (FCS). After transplantation into infertile recipient testes, the cultured cells retained stem cell activity, underwent spermatogenesis, and produced offspring. In addition to the systems for embryonic stem (ES) and embryonic germ (EG) cells [12–15], this method is the third method for expanding germline cells. Based on these results, we named these cells germline stem (GS) cells to distinguish them from the other two cell types. This GS cell culture system supports the self-renewal of spermatogonial stem cells and opens up a possible way to analyze stem cell-niche interactions in vitro.

However, the use of serum and feeder cells in this culture system is limited by our lack of knowledge of the factors that regulated spermatogonial stem cells. Serum contains complex undefined materials that occasionally affect cell differentiation [16]. For example, neural stem cells will differentiate into progenitor cells when they are cultured in medium containing serum [17]. In addition to providing physical support for stem cell attachment, feeder cells also affect stem cells by producing various undefined factors through their interactions with stem cells. Therefore, the presence of serum or feeder cells complicates the culture conditions, making them uncontrollable.

This study examined the possibility of establishing serum- or feeder-free cultures of germline stem cells. We show that GS cells continued to proliferate in the absence of serum or feeder cells, although they could not proliferate when both of them are absent. The cultured cells were as-

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sessed for stem cell activity by using spermatogonial transplantation and by the ability to produce offspring.

MATERIALS AND METHODS

Culture Conditions

GS cells were established from the transgenic mouse line C57BL6/Tg14(act-EGFP-OsbY01) bred into the DBA/2 background (designated Green; provided by Dr. M. Okabe, Osaka University) [11]. The spermatogonia and spermatocytes of these mice express the *EGFP* gene, although the level of EGFP expression decreases gradually after meiosis [18]. Therefore, donor cells can be identified readily following transplantation. All the GS cells used in our studies were derived from 0- to 2-day-old neonatal mice.

GS cells were established according to an existing protocol [11]. In brief, dissociated neonatal testis cells were cultured overnight on a gelatin-coated plate, and floating cells were passaged to secondary plates. These cells were passaged two to three times before they were transferred onto MEFs. For feeder-free culture, fully established GS cells were cultured on dishes that had been coated with laminin (BD Biosciences, Franklin Lakes, NJ) at a concentration of 20 μg/ml. The basal culture medium was StemPro-34 SFM (Invitrogen, Carlsbad, CA) supplemented with StemPro Supplement (Invitrogen), 25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 30 nM sodium selenite, 6 mg/ml D-(+)-glucose, 30 μg/ml pyruvic acid, 1 μl/ml DL-lactic acid (Sigma, St. Lois, MO), 5 mg/ml BSA (ICN Biomedicals, Irvine, CA), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, MEM Vitamin Solution (Invitrogen), MEM nonessential amino acids solution (Invitrogen), 10^{-4} M ascorbic acid, 10 μg/ml D-biotin, 30 ng/ml β-estradiol, and 60 ng/ml progesterone (Sigma). The growth factors used were 20 ng/ml mouse EGF (BD Biosciences), 10 ng/ml human bFGF (BD Biosciences), 10^3 U/ml ESGRO (a murine leukemia inhibitory factor; Invitrogen), and 10 ng/ml recombinant rat GDNF (R&D Systems, Minneapolis, MN). Growth factors were added as indicated. MEF-conditioned medium was prepared as described [19]. Serum-supplemented medium was prepared by adding fetal calf serum (JRH Biosciences, Lenexa, KS) to the basal culture medium. In the serum-free culture experiments on MEFs, the basal medium was supplemented with B27 (Invitrogen) with the indicated cytokines. The cells were maintained at 37°C in an atmosphere of 5% carbon dioxide in air. Cell number was determined at each passage. The number of cells seeded was 0.2 to 1.0×10^6 cells/9.6 cm^2 in a 6-well culture plate. The rest of the cells were discarded.

Antibodies and Staining

The primary antibodies used were rat anti-EpCAM (G8.8) and mouse anti-SSEA-1 (MC-480) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City), rat anti-human α6-integrin (CD49f) (GoH3), biotinylated hamster anti-rat β1-integrin (CD29) (Ha2/5), biotinylated rat anti-CD9 antigen (KMC8), and allophycocyanin (APC)-conjugated rat anti-mouse c-kit (CD117) (2B8) (BD Biosciences). APC-conjugated goat anti-rat-immunoglobulin G (IgG; Cedarlane Laboratories, ON, Canada), APC-conjugated streptavidin (BD Biosciences), and Alexa Fluor 633-conjugated goat anti-mouse IgM (Molecular Probes, Eugene, OR) were used as secondary antibodies. The cell-staining technique followed the process described previously [20]. Cells were analyzed using the FACS-Calibur system, and 10 000 events were collected (BD Biosciences).

Analysis of Marker Gene Expression

Total RNA was isolated using Trizol (Invitrogen). For reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized with Superscript II (Rnase H^- Reverse Transcriptase; Invitrogen), and PCR was carried out with rTaq (Takara, Shiga, Japan). RT-PCR for HPRT, Oct-4, Rex-1, neurogenin3 (ngn3), c-ret, Mvh, Fragilis, and Stella were carried out using specific primers, as described [21–25].

Transplantation and Analysis

Approximately 3 μl of the donor cell suspension was injected into the seminiferous tubules of a WBB6F1-W/W^v (designated W; purchased from Japan SLC, Shizuoka, Japan) recipient through the efferent duct [26, 27]. The injection filled 75% to 85% of the tubules in each recipient testis. To induce tolerance to the allogeneic donor cells, the recipient mice received 50 μg if anti-CD4 antibody (GK1.5) i.p. on Days 0, 2, and 4 after transplantation [28].

To count colonies, recipient mouse testes were recovered 2 mo after donor cell transplantation and analyzed by observing the fluorescence under UV light [18]. Donor germ cells were identified specifically because the host testis cells had no endogenous fluorescence. A cluster of germ cells was defined as a colony when it occupied the entire circumference of the tubule and was at least 0.1 mm long [29]. Statistical analysis was performed by *t*-test. The Institutional Animal Care and Use Committee of Kyoto University approved all the animal experimentation protocols.

Microinsemination

The seminiferous tubules of recipient testes were dissected carefully, and the germ cells were collected mechanically. Microinsemination was performed as described previously [30]. Embryos that reached the four-cell stage after 24 h in culture were transferred to the oviducts of Day 1 pseudopregnant ICR females. Live fetuses retrieved on Day 19.5 were raised by lactating foster ICR mothers.

RESULTS

Serum-Free Culture of GS Cells

To establish a serum-free culture system of spermatogonial stem cells, we initially examined whether serum was required to establish GS cells. Testis cells were prepared from a newborn Green mouse following an established protocol [11], and cultured on a gelatin-coated plate. The medium was supplemented with EGF, bFGF, LIF, and GDNF. In the absence of serum, few cells attached to the plate, and most floated in the medium. Cells occasionally formed aggregates, but there was no apparent growth of germ cells. In the presence of serum, however, some of the cells had attached to the gelatin-coated plate by the next day, and floating cells could be recovered by vigorous pipetting. These cells were relatively enriched for gonocytes and were transferred to a new culture plate for further culture. When the cells were cultured in a low concentration (0.3% to 2%) of serum, fibroblastic somatic cells began to grow on the culture plate by 2–3 days after culture initiation. The majority of germ cells attached to the somatic cells, and began to form germ cell colonies by 5 to 7 days. By contrast, when a high concentration (5% to 15%) of serum was added to the medium, the germ cells still proliferated on somatic cells and formed colonies, but they eventually disappeared owing to the extensive growth of fibroblastic cells. These results indicate that serum is required for establishing GS cells, although the presence of a high concentration of serum abrogates the propagation of germ cell colonies.

Next, we determined whether the established GS cells required serum for in vitro expansion. EGFP-expressing GS cells were established from a newborn Green mouse in the presence of 1% serum. The cells were passaged to a new plate (½× dilution) when the culture became confluent. Within 3 to 4 wk after culture initiation, there were few fibroblastic somatic cells with repeated passages, and the GS cells were transferred onto mitomycin C-treated MEFs for further expansion (Fig. 1A). Sixty-three days after culture initiation, we transferred the EGFP-expressing GS cells to a serum-free culture. In this culture, we removed serum from the medium and supplemented the medium with B27, a proprietary serum-free supplement designed for long-term viability of neuronal cultures [31, 32]. Upon transfer to MEFs, GS cells attached to MEFs, and the cells retained the characteristic morphology of GS cells (Fig. 1B). Similar to GS cells in serum-containing culture, the cells were passaged every 4 to 6 days under serum-free conditions. However, the cell proliferation was more dramatic in the serum-free condition; while the GS cells multiplied approximately