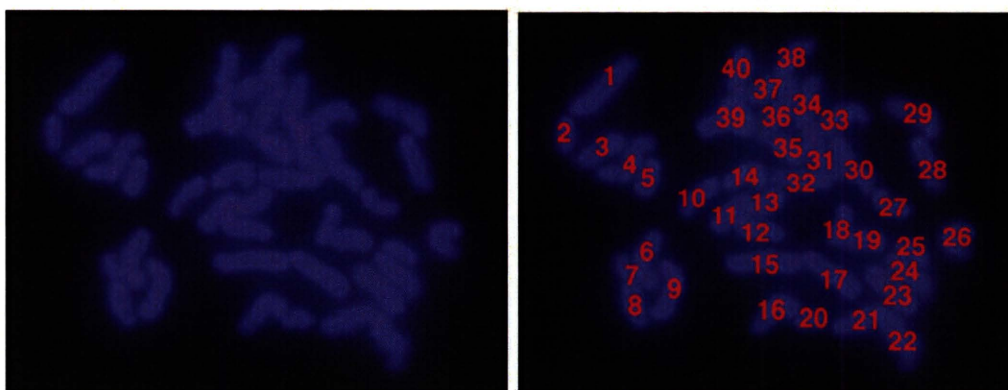図１７：RT-PCRによるSerum&Feeder-free条件における精子幹細胞培養のマーカーの発現。

図１８： Serum&Feeder-free条件にて長期培養した精子幹細胞の核型解析。
４０本の染色体があることを示す。

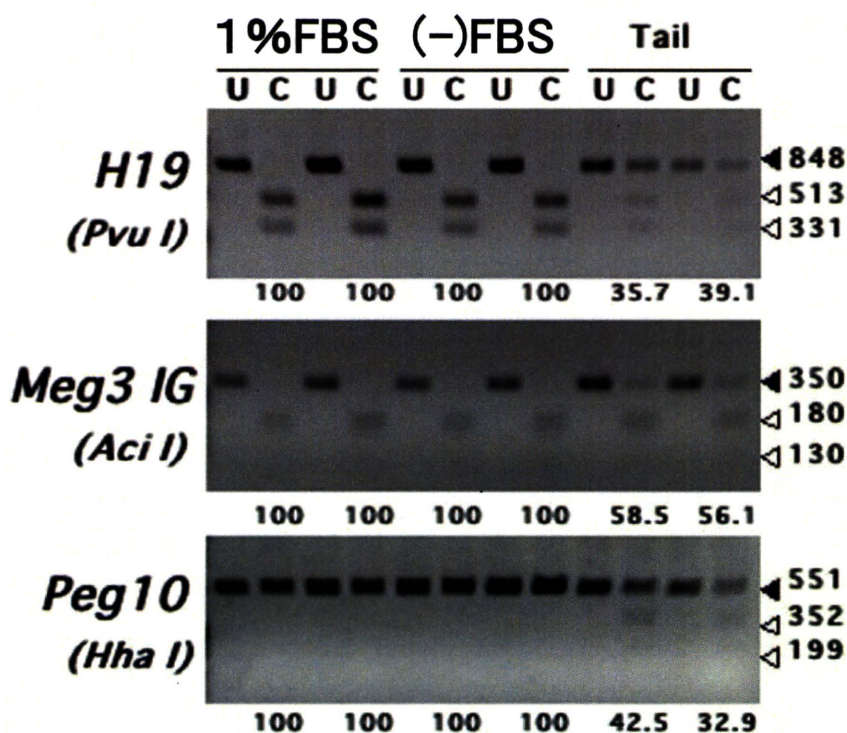図１９： Serum&Feeder-free条件にて長期培養した精子幹細胞のゲノムインプリンティング。
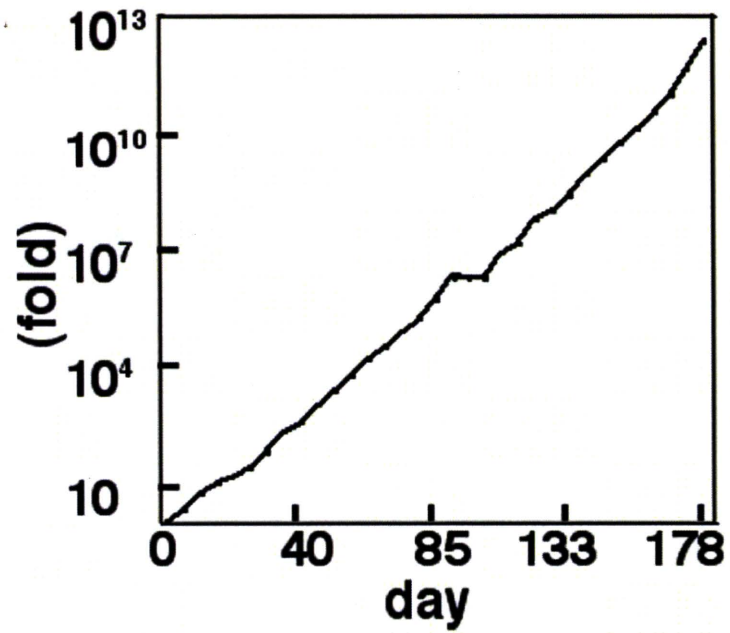COBRA法による。インプリンティング遺伝子DMRのメチル化。数値はメチル化のレベル（％）を示す。U: uncut (切断なし), C: cut (切断あり)。コントロール：マウス尻尾DNA。

図２０： Serum&Feeder-free培養における増殖曲線。
１７８日間の培養にて1.9 x 10 12 倍に増幅した。Bar=50 μm

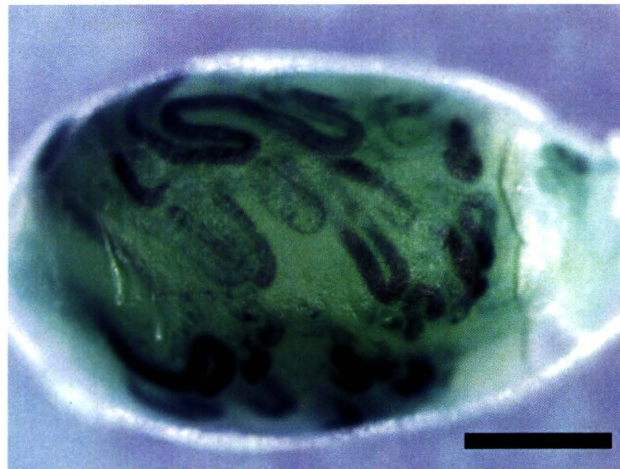図２１： Serum&Feeder-free培養後の細胞の精巣内移植によるコロニー形成像。
LacZ染色によりドナー細胞が染色されたもの。
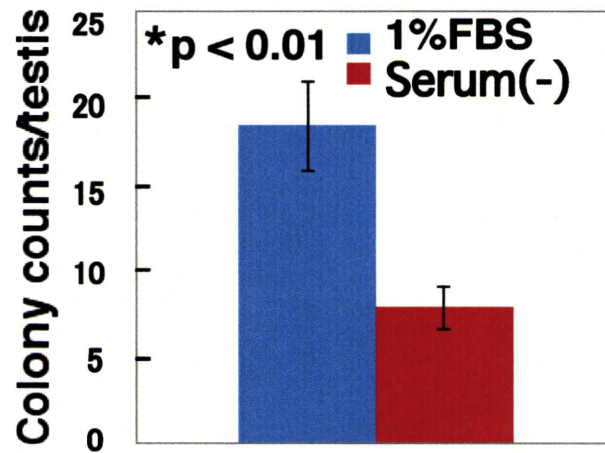
図２２： Serum&Feeder-free条件にて長期培養した精子幹細胞の幹細胞活性の低下。
8 x 103 個細胞を精巣内移植し、形成されたコロニー数を示した。
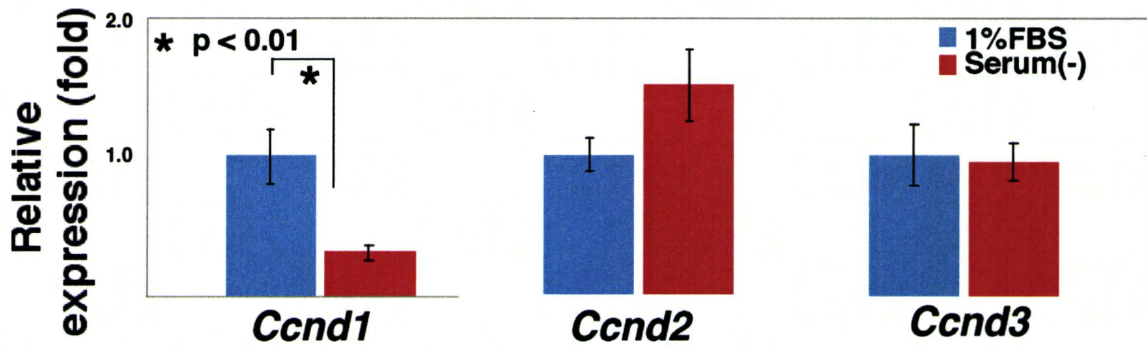
図２３： Serum&Feeder-free条件にて長期培養した精子幹細胞のG1サイクリンの発現。
Real-time PCR法による。縦軸はHprt遺伝子の発現との相対比を表す。
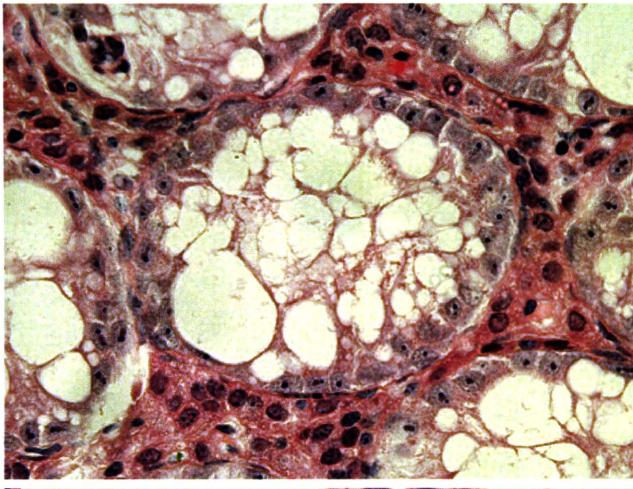
W   **Serum(-) GS**
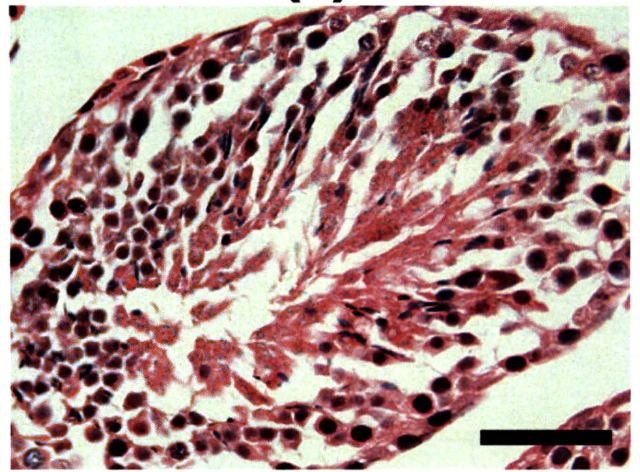
図２４： Serum&Feeder-free培養細胞の精巣内移植による精子形成と産仔の作成。
内因性の精子形成の欠損したＷマウス精巣に移植した。矢印：精子。
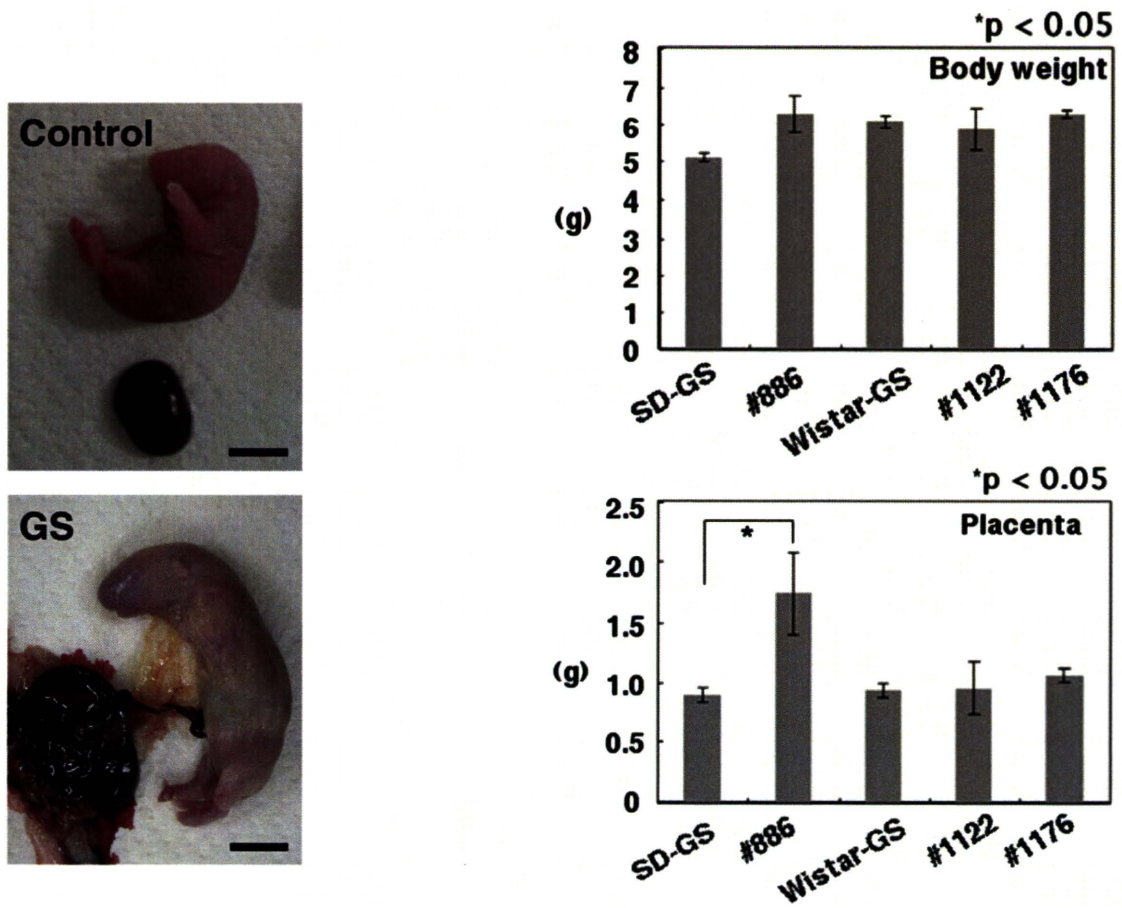
図２５： GS細胞クローン由来の産仔における巨大胎盤
（上）SDラット由来round spermatidにて顕微授精を行った産仔とその胎盤。（下）
トラップクローン由来round spermatidにて顕微授精を行った産仔とその胎盤。コン
トロールに比し胎盤が大きい。Bar=1cm

図２６： GS細胞クローン由来の産仔における巨大胎盤
（上）GS細胞クローンをヌードマウス精巣に移植して形成されたround spermatid
を用いて顕微授精を行い、産まれた仔の体重。（下）胎盤重量。クローン＃886由
来の胎盤はコントロールに比し、有意に重い（p<0.05）。コントロール：SDおよ
びWistarラット由来round spermatidにて顕微授精を行ったもの。
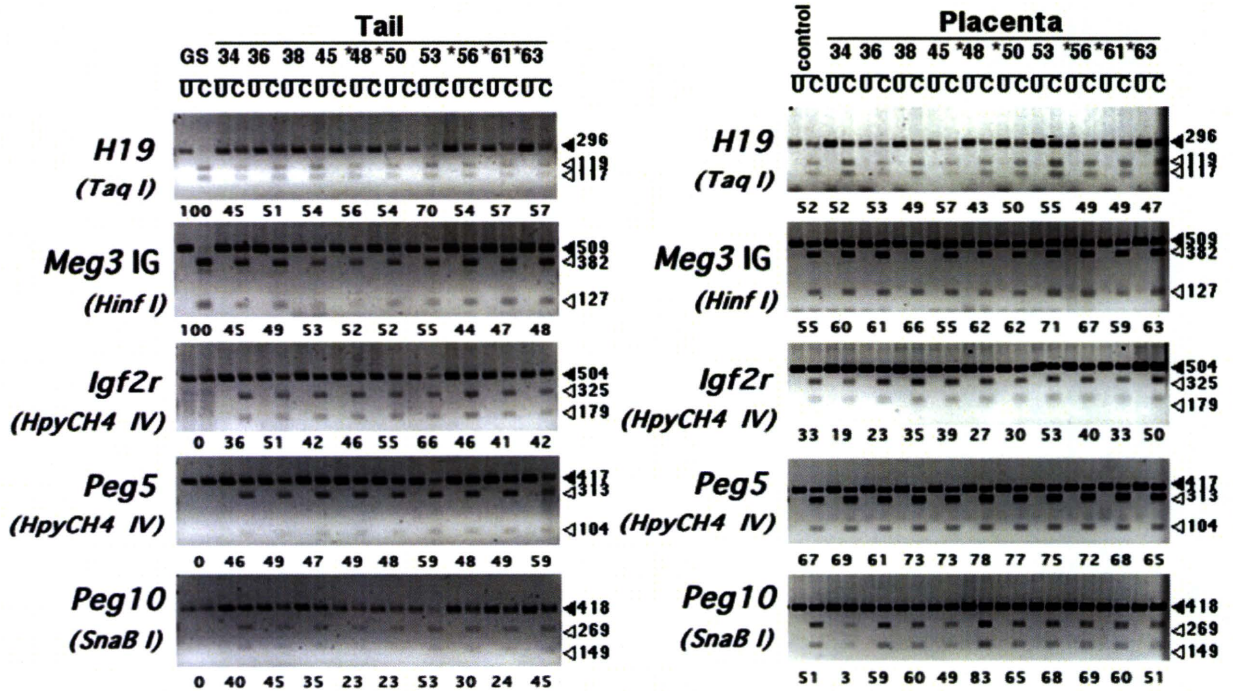
図２７：巨大胎盤を伴う産仔とその胎盤におけるゲノムインプリンティング
（左）GS細胞クローン#886をヌードマウス精巣に移植して形成されたround
spermatidを用いて顕微授精を行い、産まれた仔の尻尾DNAにてCombined bisulfite
restiction analysis によりインプリンティング遺伝子のDMRにおけるメチル化を調
べたもの。（右）胎盤におけるインプリンティング遺伝子DMRのメチル化。数値
はメチル化のレベル（％）を示す。U: uncut (切断なし), C: cut (切断あり)
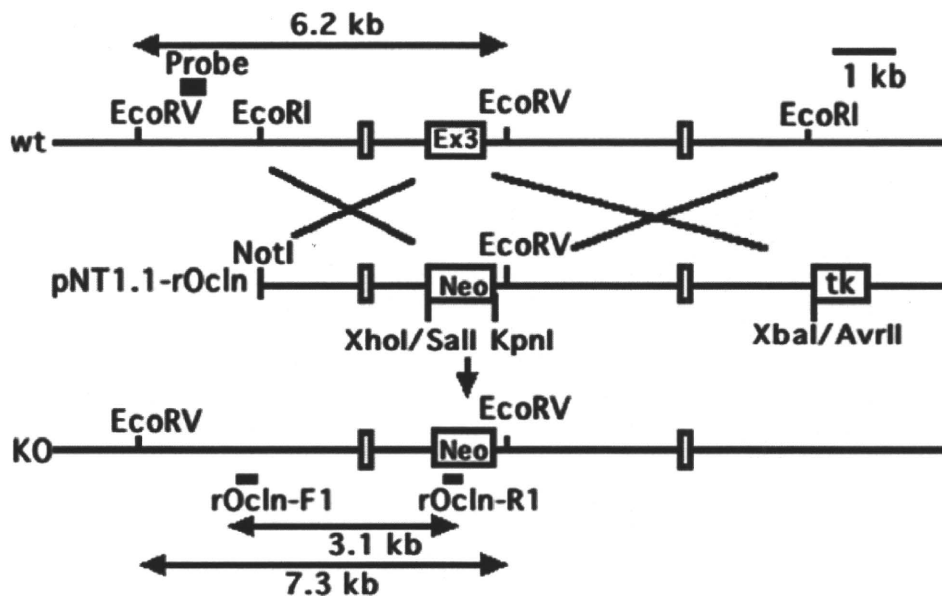
図２８：ターゲッティングベクターの設計

Occludin遺伝子のexon 3をneoにて置換することにより遺伝子破壊を行う。neo と thymidine kinase 遺伝子を含有するpNT1.1 ベクターにOccludin 遺伝子のexon3上流のNotI-XhoI 断片（2.8-kb）と 下流 KpnI-XbaI 断片（6.5-kb）を挿入した。プライマー（rOcln-F1, rOcln-R1）によるPCRにて相同組み換え体では3.1kbのバンドが検出される。相同組み換え体の検出にはサザンブロッティングにはゲノムDNAをEcoRVにて切断し、Intron1領域のプローブによりハイブリダイゼーションを行った。野生型にて6.2 kbのバンドが、相同組み換え体では7.3 kbのバンドが検出される。
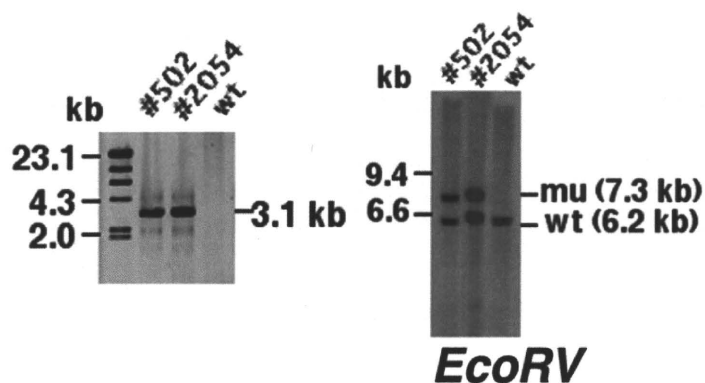
図２９：PCR法による相同組み換え体の検出

図３０：サザンブロッティング法による相同組み換え体の検出

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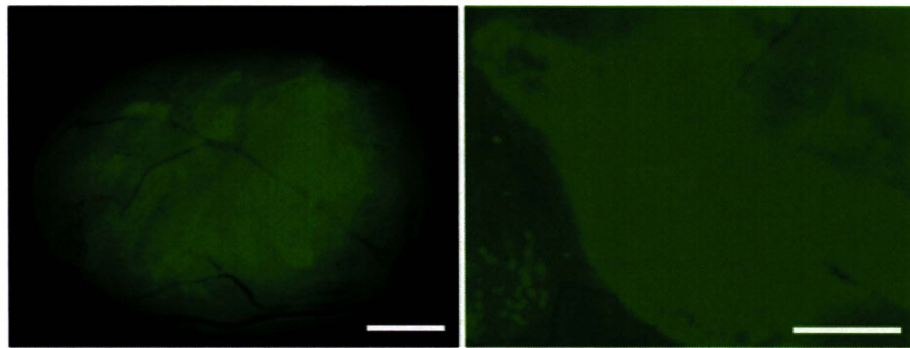
図３１：相同組み換えクローンの精巣内移植によるコロニー形成
クローン#2054-GS細胞をヌードマウス精巣に移植後３ヶ月におけるコロニー形成
を示した。ドナーマーカーのEGFP を発現している。Bar=1 mm （左）、200 μm（右）
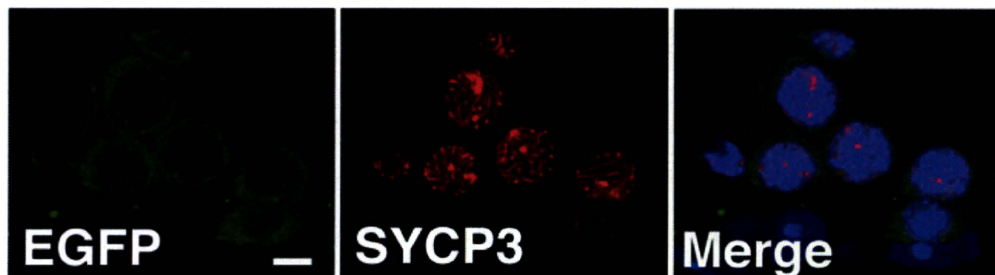
図３２：相同組み換えクローンの精巣内移植による減数分裂像
クローン#2054-GS細胞をヌードマウス精巣に移植後３ヶ月におけるコロニーの
SYCP発現。抗SYCP抗体にて免疫組織染色を行った。Hoechst 33258により核染色
（青）、ドナーマーカーのEGFP（緑）、SYCP（赤）。Bar=1 mm （左）、200 μm（右）

表 1 　　ラット精子幹細胞長期培養における増殖

培養	培養日数[a] (継代数)	コロニー/10^5 GS 細胞 (レシピエント数)	細胞の増殖率 (倍)[b]	幹細胞の数の増加 (倍)[c]
#1	100 (8)	251.4 ± 13.1 (4)	-	-
	172 (16)	70.6 ± 15.7 (4)	1.7×10^3	466.2
	351 (37)	493.3 ± 149.6 (6)	3.4×10^{13}	6.7×10^{13}
	443 (48)[d]	310.0 ± 63.4 (6)	5.2×10^{18}	6.4×10^{18}
	922 (114)	210.0 ± 58.6 (3)	2.6×10^{55}	2.2×10^{55}
#2	116 (10)[d]	137.3 ± 20.3 (4)	-	-
	338 (35)	332.5 ± 33.8 (8)	2.5×10^{11}	6.1×10^{11}

数値は平均 ± 標準誤差で示した。　4.0-8.0 $\times 10^4$ 個の GS 細胞を精細管内に移植した。

[a] 培養開始からの日数を示した。

[b,c] 培養開始からの総細胞数または幹細胞数の増加率を示した。

[d] 顕微授精により産仔を作成した。

表2 顕微授精による産仔の作成

クローン	ホスト卵の系統	卵子の数(%)				No.(%)	
		Injected	Survived	Cleaved	Transferred	Implantations	Pups born
886	SD	1482	1184 (80)	176 (15)	1089	75 (7)	18 (2)
1122	SD	154	115 (75)	4 (3)	100	61 (61)	33 (33)
1176	SD	587	401 (68)	103 (26)	334	154 (46)	64 (19)
Total		2223	1700 (76)	283 (17)	1523	290 (19)	115 (8)

表3 遺伝子改変したラット GS 細胞由来の産仔における体重と胎盤重量

クローン	系統	ID	性別	Dead/Alive	体重 (g)	胎盤重量 (g)	クローン	系統	ID	性別	Dead/Alive	体重 (g)	胎盤重量 (g)
886	SD	34	♂	Alive	5.51	1.85	1176	Wistar	306	♂	Alive	7.4	0.81
		36[a]	♂	Alive	6.84	0.71			308	♂	Alive	7.2	1.15
		38	♂	Alive	7.64	0.79			310	♂	Alive	6.9	0.72
		40	♀	Dead	6.75	0.74			312	♂	Alive	4.4	1.57
		42	♀	Dead	ND	ND			314	♂	Alive	7.0	0.60
		43	♀	Dead	5.80	0.69			316	♀	Dead	6.3	1.00
		45	♀	Alive	6.84	1.32			319	♀	Alive	7.0	0.88
		48[a]	♂	Alive	8.40	2.31			321	♂	Alive	5.4	0.83
		50	♀	Dead	4.84	4.66			323	♂	Alive	7.8	1.3
		52	♀	Dead	ND	ND			325	♀	Alive	7.21	1.92
		53	♀	Dead	7.24	0.94			327	♀	Alive	6.47	0.95
		55	♀	Dead	ND	ND			327	♀	Alive	6.71	1.12
		56	♂	Alive	8.51	2.35			331	♂	Alive	7.43	0.88
		61	ND	Dead	4.42	2.07			333	♂	Alive	7.39	0.70
		63	♀	Alive	7.91	2.4			335	♂	Alive	7.33	0.81
									337	♂	Alive	7.04	0.53
		Total		n=12	6.73±0.39[b]	1.74±0.33[b]			339	♂	Alive	6.94	0.68
									341	♀	Alive	7.6	1.10
1122	Wistar	282	♂	Alive	6.81	0.89			343	♀	Alive	7.2	1.88
		284	♀	Alive	6.60	0.88			345	♂	Alive	7.0	0.74
		286	♂	Alive	6.62	0.50			347	♂	Alive	7.3	0.78
		288	♂	Alive	6.47	0.55			349	♂	Alive	6.8	0.68
		290	♂	-	ND	3.29			351	♀	Alive	7.4	0.81
		291	♀	Dead	5.48	0.63			364	♀	Alive	6.6	1.35
		293	♀	Alive	ND	1.01			366	♂	Alive	7.2	0.85
		295	♂	Alive	ND	0.58			368	♂	Alive	7.3	1.52
		297	♀	Alive	ND	0.83			370	♂	Alive	7.4	0.76
		299	♀	Alive	ND	0.76			372	♂	Alive	7.1	1.40
		301	♂	Alive	ND	0.44			374	♀	Alive	6.7	1.46
		303	♂	Dead	3.07	1.06			376	♀	Alive	6.3	1.28
									378	ND	Alive	ND	1.40
		Total		n=12	5.84±0.59[b]	0.95±0.22[b]			379	♂	Alive	8.0	1.27
									381	♀	Alive	6.3	1.30
									383	♀	Alive	6.5	1.07
									Total		n=34	6.90±0.13[b]	1.02±0.06[b]

[a] #36 と #48 の産仔はそれぞれ 10 匹と 9 匹の産仔を作成した。
[b] 数値は平均 ± 標準誤差を表す。

Serum- and Feeder-Free Culture of Mouse Germline Stem Cells[1]

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Japan Science and Technology Agency (CREST),[5] Kyoto University, Kyoto, Japan

ABSTRACT

Spermatogonial stem cells (SSCs) undergo self-renewal divisions to support spermatogenesis. Although several in vitro SSC culture systems have been developed, these systems include serum or fibroblast feeders, which complicate SSC self-renewal analyses. Here, we developed a serum- and feeder-free culture system for long-term propagation of SSCs. In addition to the SSC self-renewal factors, including glial cell line-derived neurotrophic factor, supplementation with fetuin and lipid-associated molecules was required to drive SSC proliferation in vitro. Cultured cells proliferated for at least 6 mo at a rate comparable to that of serum-supplemented cultured cells. However, germline potential was reduced under serum- and feeder-free conditions, as indicated by a lower SSC frequency after germ cell transplantation. Nevertheless, the cultured cells completed spermatogenesis and produced offspring following spermatogonial transplantation into seminiferous tubules of infertile mice. This culture system provides a basic platform for understanding the regulation of SSC fate commitment in vitro and for improving SSC culture medium.

culture, developmental biology, gametogenesis, self-renewal, Sertoli cells, serum, spermatogenesis, testis, transplantation

INTRODUCTION

Spermatogonial stem cells (SSCs) undergo self-renewal division and support spermatogenesis. A single SSC can produce two SSCs or two differentiating cells [1]. The two types of division are thought to occur with equal frequency, thereby maintaining a constant SSC population size while producing many differentiating cells. SSC identification has been challenging, both because of the difficulty in distinguishing SSCs from other committed, undifferentiated spermatogonia and because of the lack of SSC-specific markers. However, this problem was resolved by development of a germ cell transplantation technique [2] in which transplanted SSCs

reinitiate long-term spermatogenesis after microinjection into the seminiferous tubules of infertile mice, which can then produce offspring by mating with wild-type females.

The estimated number of SSCs in the testis is very small, because SSCs comprise 0.02–0.03% of the total testis germ cell population [3, 4]. However, the SSC frequency changes significantly according to the levels of the SSC self-renewal factor glial cell line-derived neurotrophic factor (GDNF) [5]. GDNF overexpression induced the accumulation of undifferentiated spermatogonia in *Gdnf* transgenic mice, whereas decreased GDNF levels in heterozygous *Gdnf* knockout mice resulted in hypospermatogenesis and male infertility. Based on this in vivo finding, a long-term SSC culture system was developed in which SSCs proliferated in the presence of GDNF supplemented with epidermal growth factor (EGF) or basic fibroblast growth factor (FGF2) on mitomycin C-treated mouse embryonic fibroblasts (MEFs) in the presence of fetal bovine serum (FBS) [6]. The cultured cells, deemed germline stem (GS) cells, proliferated as spermatogonia in vitro but reinitiated spermatogenesis following transplantation into seminiferous tubules. GS cells maintained fertility after 2 yr of consecutive culture [7], whereas embryonic stem (ES) cells lost germline potential after several months [8, 9]. GS cells have been used to produce knockout mice after homologous recombination [10]. Moreover, they have pluripotency potential and convert into ES-like multipotent GS (mGS) cells [11].

The initial GS cell culture protocol was subsequently simplified and improved. Although GS cells proliferated more rapidly than SSCs in vivo (doubling every 2–3 days) [6, 7], the serum and fibroblast feeder cells used for GS cell culture prevented SSC self-renewal analyses. When propagated without MEFs, GS cells proliferated successfully over 6 mo on laminin-coated plates and retained fertility [12]. Moreover, GS cells could be maintained in suspension without feeder or laminin support [13]. Although GS cells proliferated more slowly in suspension than on MEFs (doubling every 4–5 days), the cells expressed spermatogonial markers and produced offspring following spermatogonial transplantation. This result was unexpected, because ES cells readily differentiate and lose germline potential in suspension. Thus, GS cells have a relatively flexible requirement for structural support in maintaining their stem cell potential.

Conversely, the effect of serum in GS cell culture is not well understood. Generally, serum varies from batch to batch and often induces stem cell differentiation. Although bone morphogenic protein (BMP) is important for derivation, maintenance, and proliferation of ES cells, only a minute amount of BMP in serum induced differentiation when leukemia-inhibitory factor (LIF) was removed [14]. Moreover, neural stem cells initiated differentiation following the addition of serum to the medium [15]. One study demonstrated an inhibitory role of serum for SSCs and showed the feasibility of serum-free culture on LIF-secreting STO (S, Sandoz inbred mouse; T, 6-thioguanine-

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resistant; O, ouabain-resistant) embryonic fibroblasts [16]. However, throughout that study, serum was continuously used to neutralize trypsin [17], which complicated the interpretation of results. We also developed another serum-free culture system for GS cells using MEFs [12], but we could not exclude the possibility that despite extensive washing, residual serum in MEFs might have caused GS cell proliferation. In fact, when GS cells were transferred onto laminin-coated plates in the same culture medium, they failed to propagate, and proliferation ceased. Serum was also necessary when we derived cultured SSCs from embryos or hamster testes [18, 19]. However, the component in the serum that caused GS cell self-renewal remained unknown.

In the present study, we report, to our knowledge, the first successful culture of SSCs under serum- and feeder-free conditions. Using these conditions, the cells proliferated for 6 mo and reinitiated spermatogenesis after germ cell transplantation. However, the cells demonstrated a lower SSC frequency, suggesting that serum enhanced the self-renewal division. Nevertheless, the development of a new GS cell culture system may be useful for understanding the self-renewal machinery and the biological potential of these cells.

MATERIALS AND METHODS

Cell Culture

The GS cells were established from the transgenic mouse line B6-*TgR(ROSA26)26Sor* (The Jackson Laboratory) bred into a DBA/2 background [6]. For primary culture of gonocytes, we used 0-day-old pups in ICR background (Japan SLC). Gonocytes were enriched by gelatin selection and cultures initiated as described previously [6]. For primary culture of spermatogonia, we used 8-day-old pups in DBA/2 background (Japan SLC). Spermatogonia were collected by using a magnetic bead selection technique with biotinylated rat anti-mouse CD9 antibody (KMC8; BD Bioscience) and streptavidin-conjugated Dynabeads (Invitrogen) as previously described [20]. After selection, 2–3 × 10^5 cells were plated in six-well plates coated with laminin (20 µg/ml; BD Biosciences).

Medium A (original GS cell medium) was made by supplementing StemPro-34 serum-free medium (Invitrogen) with 20 ng/ml of mouse EGF, 10 ng/ml of human FGF2, and 15 ng/ml of rat GDNF (all from PeproTech EC) as described previously [6]. This medium contained 1% FBS (Thermo Fisher Scientific). Medium B was modified from medium A by replacing serum and bovine serum albumin (BSA; ImmunO, fraction V; MP Biomedicals) with 3 mg/ml of lipid-rich BSA (Albumax II; Invitrogen), 1 mg/ml of fetuin (Sigma), 1:100 Lipid Mixture 1 (Sigma), and 1:1000 Lipoprotein-Cholesterol Concentrate (MP Biomedicals). Cells were cultured on laminin-coated dishes at 37°C under of 5% CO_2 in air. The cells were passaged by incubation with 0.25% trypsin for 5 min. Trypsin reaction was stopped by adding two volumes of medium B. In some experiments, we also used sphingosine kinase inhibitor 2 (SKI II; 10 µM; Cayman Chemical) at the time of cell plating. When indicated, heat-inactivated FBS or charcoal-treated FBS (Thermo Fisher Scientific) was added to the medium as a control. Sera from sheep, horse, goat, rabbit, and pig (all from Invitrogen) were used after heat inactivation for 30 min at 55°C. The cells were cryopreserved after adding 10% dimethyl sulfoxide (DMSO; Sigma).

For laminin adhesion assays, 2 × 10^5 cells/well were plated in six-well plates coated with laminin and incubated overnight at 37°C. Adherent cells were recovered by trypsin digestion after washing the plates twice with PBS as previously described [21].

Flow Cytometry

The primary antibodies used were mouse anti-FUT4 (MC-480; Chemicon), rat anti-mouse EPCAM (G8.8), rat anti-human ITGA6 (CD49f) (GoH3), biotinylated hamster anti-rat ITGB1 (CD29) (Ha2/5), biotinylated rat anti-mouse CD9 (KMC8), and rat anti-mouse KIT (CD117) (2B8) (all from BD Biosciences). Allophycocyanin (APC)-conjugated goat anti-rat immunoglobulin G (Cedarlane Laboratories) and APC-conjugated streptavidin (BD Biosciences) were used to detect the primary antibodies. Antibodies were used at 5 µg/ml.

Transplantation Procedure

For germ cell transplantation, cultured cells were microinjected into the seminiferous tubules of WBB6F1-W/W^v (W) mice via an efferent duct (Japan

SLC). Approximately 80–90% of the tubules were filled in each recipient testis. To avoid rejection of donor cells, the recipient mice were treated with anti-CD4 antibody (GK1.5) to induce tolerance to the donor cells [22]. The Institutional Animal Care and Use Committee of Kyoto University approved all of the animal experiments.

Analysis of Recipient Testes

The donor cell colonization levels were determined by staining the recipient testes for the LacZ gene product, β-galactosidase with X-gal (Wako Pure Chemical Industries), as previously described [23]. Tubules that stained blue were counted under a stereomicroscope. Colonies were defined as germ cell clusters longer than 0.1 mm that occupied the entire circumference of the tubule. For histological analyses, the samples were embedded in paraffin blocks and processed for sectioning. All sections were 12 µm in thickness and were stained with hematoxylin and eosin.

Analysis of Gene Expression

Total RNA was isolated using TRIzol reagent (Invitrogen), and first-strand cDNA was produced by Superscript II (RNase H$^-$ reverse transcriptase; Invitrogen). RT-PCR was performed using the specific primers listed in Supplemental Table S1 (available online at www.biolreprod.org). To quantify mRNA expression by real-time PCR, we used a StepOnePlus real-time PCR system and *Power* SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's recommendations. The transcript levels were normalized to those of *Hprt*. The PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The experiments were performed on six independent samples, and each PCR reaction was run in triplicate.

Combined Bisulfite Restriction Analysis

Genomic DNA was treated with sodium bisulfite, which deaminates unmethylated cytosines to uracils but does not affect 5-methylated cytosines. Using this template, we amplified the indicated differentially methylated regions (DMRs) by PCR with the specific primers listed in Table S1. The PCR products were digested with the indicated restriction enzymes, which had recognition sequences containing CpG in the original unconverted DNA. The intensity of the digested bands was assessed using Image Gauge software (Fuji Photo Film).

Karyotype Analysis

Cultured cells were harvested, treated with 75 mM KCl for 15 min, and fixed with methanol/acetic acid (3:1). Metaphase spreads were prepared using standard procedures, and the slides were stained with Hoechst 33258 (Sigma).

Microinsemination

Spermatozoa were collected by mechanically dissociating the seminiferous tubule segments of fresh or frozen recipient testes [24]. These cells were microinjected into C57BL/6 × DBA/2 (BDF1) oocytes using a piezo-driven micropipetter (PrimeTech) [25]. Embryos at the 2-cell stage after 24 h in culture were transferred to the uteri of ICR recipient females.

Statistical Analyses

Results are presented as the mean ± SEM. Data were analyzed by Student *t*-test.

RESULTS

Requirement of Fetuin in Feeder-Free Culture of GS Cells

The GS cells changed their morphology when they were transferred onto laminin-coated plates (Fig. 1A). Original GS cell culture medium (medium A) contained serum. However, removal of serum prevented GS cells from attaching to the laminin-coated plates in the next passage, and proliferation ceased. The failure to attach to laminin was not ameliorated by reducing the trypsin concentration or by using other proteases, such as type I collagenase or dispase, suggesting that destruction of cell surface molecules during passage is not
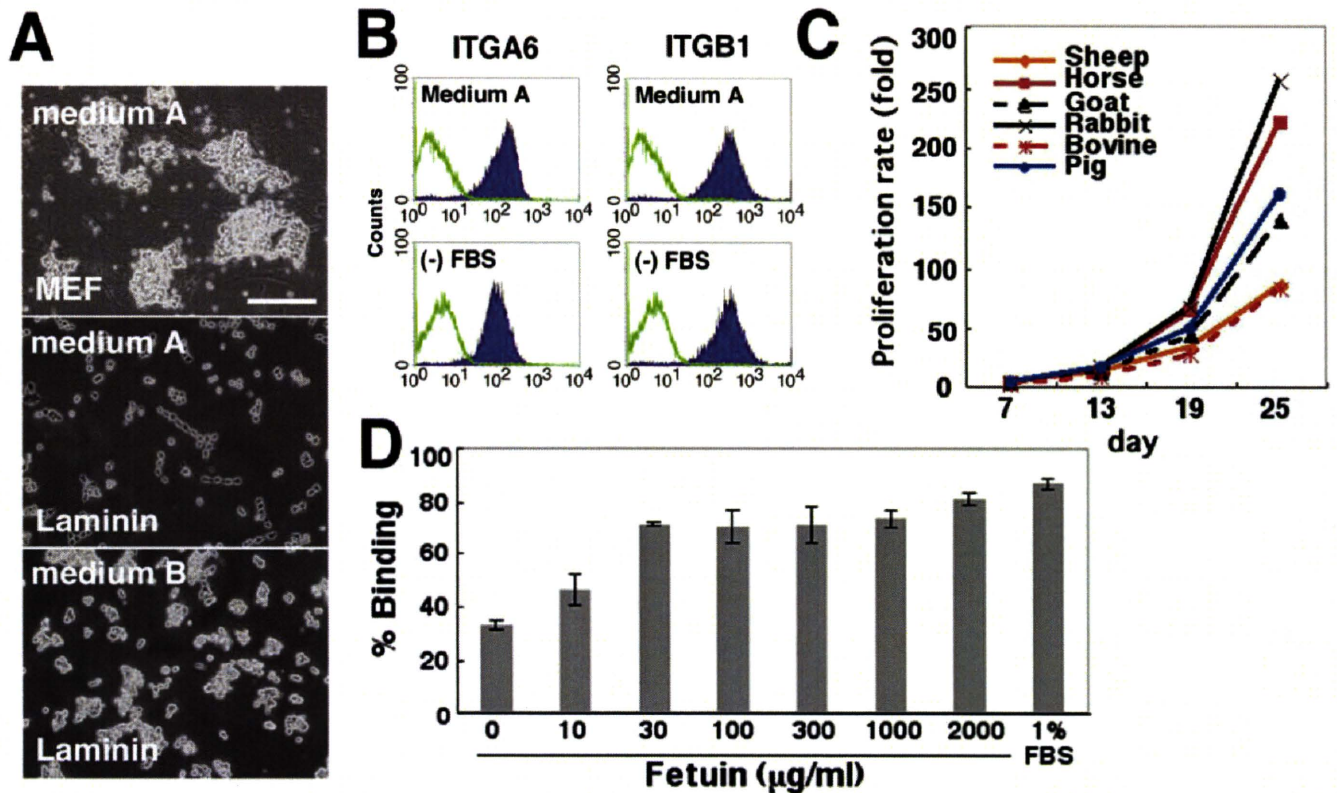
FIG. 1. GS cells cultured under serum- and feeder-free conditions. **A**) Morphology of GS cells under different culture conditions. **Top**) GS cells on MEFs with 1% FBS. Note the morula-like morphology. **Middle**) GS cells on laminin with 1% FBS. Note the chain-type colonies. **Bottom**) GS cells on laminin without serum. Note the clump-like colonies. **B**) Flow cytometric analysis of ITGB1 and ITGB6 expression after removing serum from medium A. Although cells were detaching from the laminin, no significant changes in ITGB1 and ITGA6 were noted. Green lines indicate controls. **C**) Effects of serum from various animal species on GS cell proliferation. Each serum type was added at a concentration of 1%. **D**) Promotion of GS cell attachment to laminin by fetuin supplementation. GS cells were plated overnight on laminin (20 μg/ml) in the presence of different concentrations of fetuin (0–2 mg/ml). After washing twice with PBS, the plates were treated with trypsin to recover adherent cells. Results are from three independent experiments (n = 3). Bar = 100 μm (**A**).

responsible for growth inhibition. Flow cytometric analyses did not reveal significant changes in the expression levels of ITGB1 and ITGA6, which comprise laminin receptor (Fig. 1B). Conversely, no species-specific serum requirement was observed for GS cell culture: Sera (at 1%) from sheep, horse, goat, rabbit, and pig maintained GS cells for at least 2 wk on laminin-coated plates without noticeable changes (Fig. 1C). Furthermore, charcoal/dextran-treated FBS, which is reduced in hormonal or growth factor content, maintained GS cells at a comparable efficiency (data not shown).

Because only a small amount of serum was necessary for proliferation, we hypothesized that the effective components must be present as a significant proportion of the serum. Supplementation with fetuin, a predominant glycoprotein comprising approximately 45% of the total FBS protein [26], allowed GS cell attachment to laminin under serum-free conditions. In the present study, GS cells from ROSA26 mice were incubated overnight on laminin-coated dishes with different concentrations of fetuin (0–2 mg/ml) in serum-free medium, and the cells were analyzed for attachment efficiency (Fig. 1D). GS cells attached to laminin efficiently when fetuin was added at concentrations of greater than 30 μg/ml: 70–80% of the cells bound to the laminin after overnight culture, which was comparable to the effect of 1% serum. Increasing the fetuin concentration to 2 mg/ml did not improve attachment.

Enhanced Proliferation of GS Cells by Lipid Supplementation

Although GS cells adhered to laminin-coated plates following fetuin supplementation, the cells failed to grow after two to three passages, despite increasing the concentrations of cytokines or adding soluble GDNF receptor α-1. This suggests that enhanced cytokine signaling alone does not support GS cell proliferation. We then studied the effect of the lipid components by replacing the lipid-poor albumin (ImmunO, fraction V) in the original GS cell culture medium (medium A) with lipid-rich BSA (Albumax II) and adding other lipid-associated components in the medium, such as high-density lipoprotein (HDL), cholesterol, and lipid mixtures. In the improved medium, the colony morphology changed from flat-type to morula-like clumps, and cell proliferation was stimulated (Fig. 2, A and B). The enhanced amplification ratio following lipid supplementation suggests that lipids are an important component of the serum. Because sphingosine kinase has been involved in regulation of cell proliferation, differentiation, senescence, and apoptosis in stem cells and their progenitors by producing bioactive sphingosine 1-phosphate [27], we tested the effect of SKI II, a specific inhibitor of sphingosine kinase, on GS cell proliferation. Addition of SKI II significantly suppressed GS cell proliferation within 5 days in 1% serum-containing medium (medium A) (Fig. 2C).

FIG. 2. Improvement of GS cell proliferation by lipid supplementation under serum- and feeder-free conditions. **A**) Effect of Albumax II and lipid supplementation on GS cell proliferation (n = 8). Cells were cultured on laminin-coated plates for 6 days. Note the improved growth of GS cells with fetuin and lipid supplementation. **B**) Effect of lipid supplementation on GS cell morphology on laminin-coated plates. **Top**) GS cells on laminin without lipid supplementation. Note the epithelial morphology. **Bottom**) GS cells on laminin with lipid supplementation. Note the clump-like morphology. **C** and **D**) Decreased cell recovery (**C**) and SSC expansion (**D**) after 5 days of culture with SKI II (n = 16 for cell recovery, n = 8–10 for SSC number). SSC numbers were determined by germ cell transplantation. The same numbers of cells were cultured on laminin in 1% serum-supplemented medium (medium A) with or without SKI II for 5 days. The number of SSCs was quantified by multiplying the total cell recovery by the SSC concentration, as estimated by colony counting after germ cell transplantation. Results are from three independent experiments. **E**) Growth curve for GS cells. Cells were maintained for 178 days, and the total number of cells was counted at each passage. **F**) Morphology of GS cells established by culturing newborn testis cells in medium B. **G**) Growth curve for GS cells from neonatal testes during culture initiation. Bar = 50 μm (**B** and **F**).
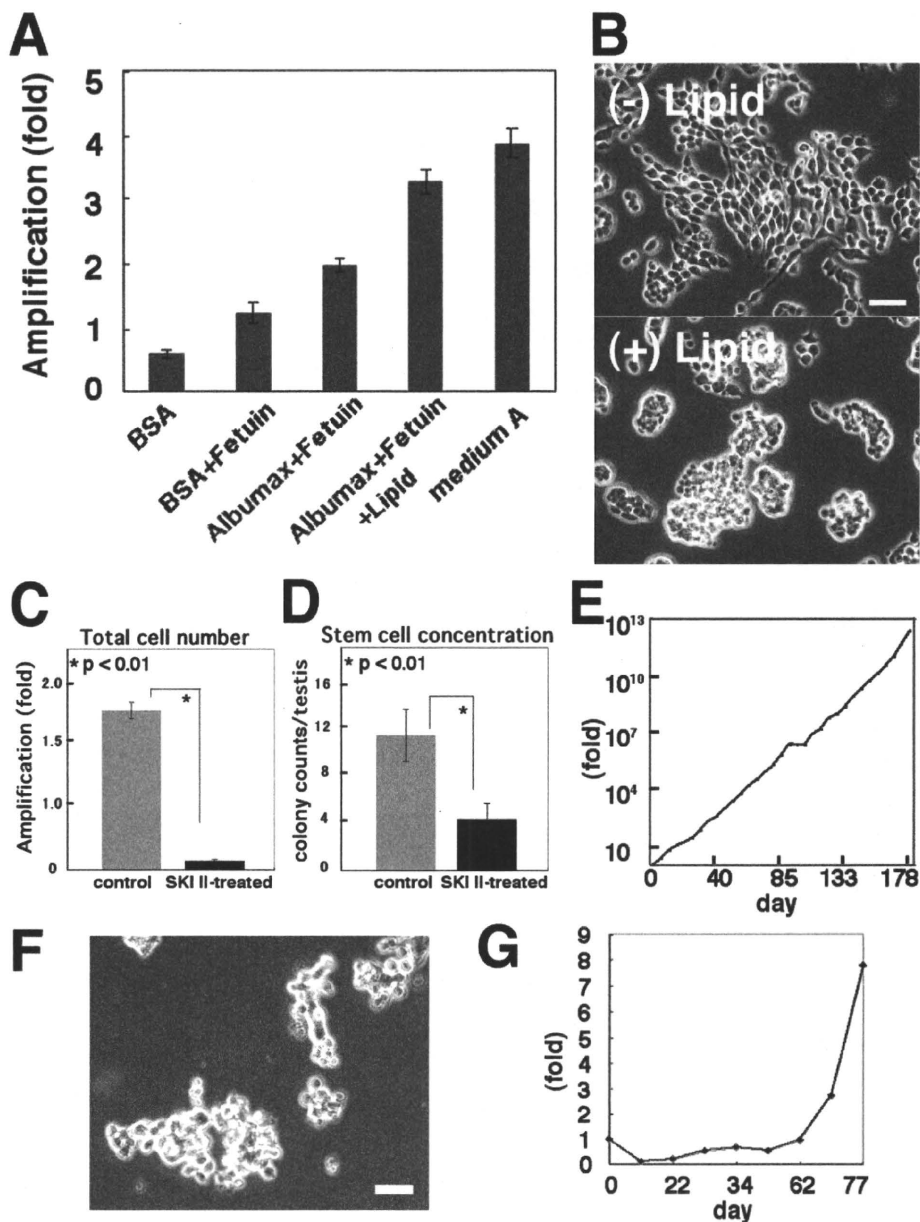
We confirmed the inhibition of SSC proliferation by transplanting cells that had been cultured for 5 days in the presence of SKI II into the seminiferous tubules of W mice (Fig. 2D). The inhibitor treatment suppressed the SSC number; the SKI II-treated group exhibited only 40% of the SSCs observed in the DMSO-treated controls. These results indicate that sphingosine-mediated signaling is necessary for SSC proliferation.

The cytokine requirements of the GS cells remained unaltered in the improved medium (medium B), and the cells were trypsinized and passaged at a ratio of 4:1 to 6:1 every 5–6 days. Overall, the cells expanded by a factor of 1.9×10^{12}-fold during 178 days in vitro, with 32 passages (Fig. 2E). The colony morphology of cells was somewhat different from those cultured in the serum (Figs. 1A and 2B), which resembled chains of spermatogonia during early phases of germ cell colonies in vivo (Fig. 1A) [23]. However, the colonies in the serum- and feeder-free culture conditions (medium B) did not form chains but, instead, occurred in clumps. The morphological change was not irreversible, and the cells exhibited a typical morula-like appearance when plated on MEFs in medium A. The improved medium was also useful for culturing SSCs from other mouse strains, such as BDF1 or ICR. In addition, we were able to establish serum- and feeder-free GS cultures using primary germ cells of 0- or 8-day-old pup testes, indicating that the culture medium was useful for initiating and maintaining GS cell cultures (Fig. 2, F and G). The cells were cryopreserved after adding 10% DMSO to the serum-free culture medium. We did not observe mGS cells at any point during the entire culture period [11].

Phenotypic Characterization of GS Cells under Serum- and Feeder-Free Conditions

We next examined the phenotype of the cultured cells. Despite morphological differences, flow cytometric analyses demonstrated that the cultured cells expressed normal SSC markers, including ITGB1 and ITGA6, EPCAM, and CD9 (Fig. 3A). The cells also weakly expressed KIT, suggesting that most cells were undifferentiated spermatogonia. However, the

A

MEF medium A | Laminin medium A | Laminin medium B

ITGB1
ITGA6
EPCAM
KIT
CD9
FUT4

B

GS medium A
GS meidum B
mGS

Stra8
Neurog3
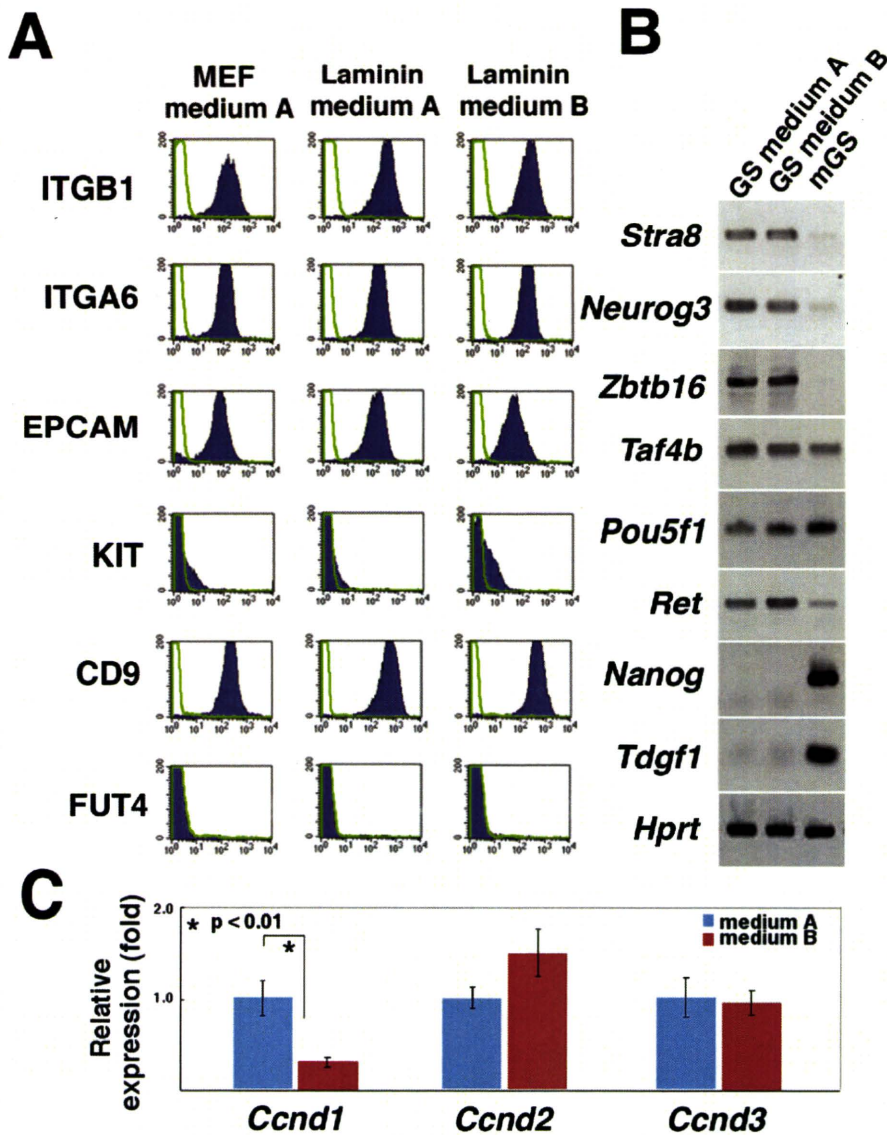Zbtb16
Taf4b
Pou5f1
Ret
Nanog
Tdgf1
Hprt

FIG. 3. Phenotypic analyses of GS cells cultured under serum- and feeder-free conditions. **A**) Flow cytometric analysis of cell surface marker expression. Green lines indicate controls. **B**) RT-PCR analyses. Gene-specific primers for each gene were used to amplify cDNA from GS cells cultured under different conditions. **C**) Real-time PCR analysis of cyclin D gene expression. Cells were cultured on laminin under the indicated conditions. The values are normalized to *Hprt* expression levels in GS cells cultured in medium A.

C

Relative expression (fold)

* p < 0.01

medium A
medium B

Ccnd1   Ccnd2   Ccnd3

cells did not express FUT4 (SSEA-1), a marker of primordial germ cells (PGCs) and ES cells. This overall pattern of marker expression was unaltered regardless of serum or MEFs. The RT-PCR analyses confirmed normal spermatogonia marker expression; the GS cells expressed *Stra8*, *Neurog3*, *Zbtb16*, *Taf4b*, *Pou5f1*, and *Ret*, but not *Nanog* or *Tdgf1*, both of which are strongly expressed in ES cells (Fig. 3B). Thus, the cultured cells maintained a normal spermatogonial phenotype.

We previously showed that GS cells had a normal chromosome number and a stable DNA methylation pattern even after long-term in vitro culture [7]. Consistent with those observations, cytogenetic analysis by Hoechst 33258 staining in the present study revealed that the cultured cells had a normal karyotype after 6 mo of continuous culture (Fig. 4A). In addition, combined bisulfite restriction analysis of DNA methylation patterns on imprinted genes showed that the DNA methylation at DMRs of two paternally imprinted genes (*H19* and *Meg3 IG*) and a maternally imprinted gene (*Peg10*) displayed typical androgenetic DNA methylation patterns, including hypermethylation of *H19* and *Meg3 IG* DMRs and hypomethylation of *Peg10* DMRs (Fig. 4B). Taken together, these results indicate that the cultured cells retained normal genetic and epigenetic properties of spermatogonia during in vitro culture.

Functional Analyses of GS Cells under Serum- and Feeder-Free Conditions

To confirm the increase in SSC number, we transplanted the cultured cells into seminiferous tubules at three different time points during 172 days of continuous culture (Table 1). In these experiments, at least three recipients received donor cell transplantation. Two months after transplantation, the recipients were killed, and their testes were examined for donor cell colonization by LacZ staining (Fig. 5A). Assuming that 10% of the SSCs were able to colonize seminiferous tubules [23], the SSC concentration of the transplanted cells was calculated as 0.4–1.0% (average, 0.6%); the SSC frequency in traditional GS cell cultures is usually 1–2% [28]. A direct comparison between the GS cell cultures in medium A and medium B showed that the GS cells cultured with serum produced 230.3 colonies per 10^5 cells whereas those cultured without serum produced 98.2 colonies per 10^5 cells, suggesting that certain serum components enhanced the SSC frequency (Fig. 5B). Given the significant impact of the cyclin D expression pattern on SSC frequency [28], we also analyzed the expression levels of cyclin D genes (Fig. 3C). Although we did not observe significant differences in *Ccnd2* and *Ccnd3* expression, *Ccnd1* was significantly downregulated under serum- and feeder-free
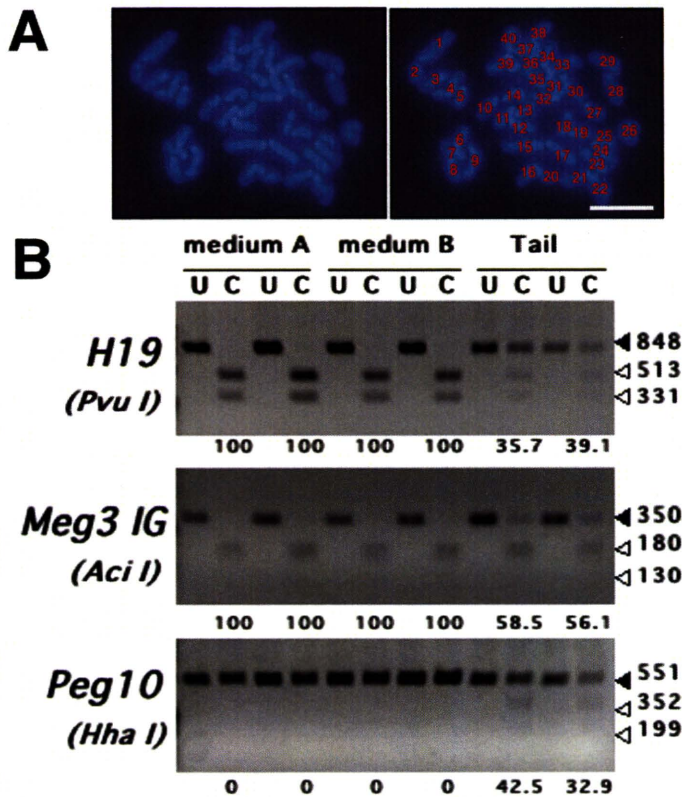
FIG. 4. Genetic and epigenetic characterization of GS cells under serum- and feeder-free conditions. **A)** Metaphase spread of GS cells. The two images are the same; the right image indicates that 40 chromosomes are in this cell. Original magnification ×100. **B)** Combined bisulfite restriction analysis of GS cells. Open arrowheads indicate the size of methylated DNA. Closed arrowheads indicate the size of unmethylated DNA. The enzymes used to cleave each locus are indicated in parentheses. C, cleaved; U, uncleaved. The numbers at the bottom of each gel indicate the percentage of digested DNA/undigested DNA (i.e., C/U).

days before ICSI. In the second set of experiments, GS cells were cultured for 124 days, and one of the recipient mice was killed at 175 days after transplantation for ICSI without cryopreservation. Spermatogenic cells were recovered mechanically by repeated pipetting of the seminiferous tubules. Spermatozoa, identified by their unique morphology, were microinjected into oocytes from BDF1 females using a piezo-driven microinjector (Fig. 5D). Of the 67 embryos formed, 31 (46.3%) developed to 2-cell stage (Table 2) and were transferred into the uteri of three pseudopregnant female mice. One offspring resulted from the sperm derived from fresh recipient testes, indicating the fertility of the donor GS cells (Fig. 5E). However, the efficiency of offspring production was apparently reduced in this experiment, because in our previous experiments using serum-supplemented culture medium [12, 13], 22–74% of the constructed embryos underwent implantation and 15–50% developed to term. We were unable to obtain offspring using sperm from the frozen-thawed testes.

DISCUSSION

Serum plays important roles in SSC culture. We used serum in our original GS cell culture medium, because spermatogenic cells cultured without serum readily reaggregated with somatic cells to form cell clumps, preventing germ cell proliferation. Conversely, high concentrations of serum stimulated proliferation of testicular somatic cells and interfered with the germ cell growth. We resolved this dilemma by decreasing the serum concentration to 1%, which limited the proliferation of testicular somatic cells while providing factors necessary for germ cell responses to self-renewal factors. A similar strategy was used to develop culture systems for PGCs/gonocytes and hamster SSCs [18, 19], although a lower serum concentration (0.04%) was sufficient for promoting germ cell proliferation. To our knowledge, the active components in serum have yet to be identified.

In the present study, we focused on cell attachment and proliferation to improve the culture medium. At serum concentrations of less than 0.04%, the cells did not adhere to laminin-coated plates and did not proliferate. Because GS cells can proliferate in suspension [13], attachment is not an absolute requirement for GS cell proliferation, but GS cells proliferate more actively when attached to a matrix or to feeder cell layer. Serum contains many factors, such as fibronectin or vitronectin, that facilitate cell attachment. In particular, SSCs have a strong preference for laminin over other extracellular matrix substrates [28]. Although GS cells can attach to artificial substrate, such as poly-L-lysine, cell proliferation is not stimulated [21]. In the present study, fetuin, a major component of bovine serum, allowed GS cell attachment to laminin under serum-free conditions. In general, total serum protein concentration gradually increases during development, but the fetuin concentration is significantly higher in fetuses than in adults

conditions, suggesting that abnormal *Ccnd1* levels played a role in the reduced SSC frequency. However, transplantation analyses confirmed a consistent increase in SSC number during in vitro culture. Cultured SSCs expanded by 3.4×10^9-fold during 132 days, whereas the total cells increased by 1.3×10^9-fold. The doubling time of SSCs was 4.1 days. Histological analyses of the recipient testes confirmed normal spermatogenesis in the seminiferous tubules (Fig. 5C).

Finally, to test the fertility of these germ cells, we used intracytoplasmic sperm injection (ICSI), a technique commonly used to treat infertility in animals and humans [25, 29]. In the first set of experiments, GS cells were cultured for 78 days, and one of the mice was killed at 108 days after transplantation for cryopreservation of testes. The testes were frozen for 113

TABLE 1. SSC expansion in serum- and feeder-free culture.

Experiment[a]	Days to transplant (passage)[b]	Colonies/ 10^5 GS cells[c]	Increase in cell no. (fold)[d]	Increase in stem cell no. (fold)[d]
1	40 (7)	37.5 ± 21.7		
2	74 (14)	37.5 ± 12.5	260	260
3	172 (30)	102.1 ± 31.4	1.3×10^9	3.4×10^9

[a] In each experiment, 4.0–8.0 × 10³ cells were microinjected into the seminiferous tubules of infertile recipient testis.
[b] The number of days from initiation of culture to transplantation.
[c] Values are mean ± SEM; results from four to six recipient testes for each transplantation.
[d] The increase in the total cell or stem cell number from the initial transplantation (experiment 1).
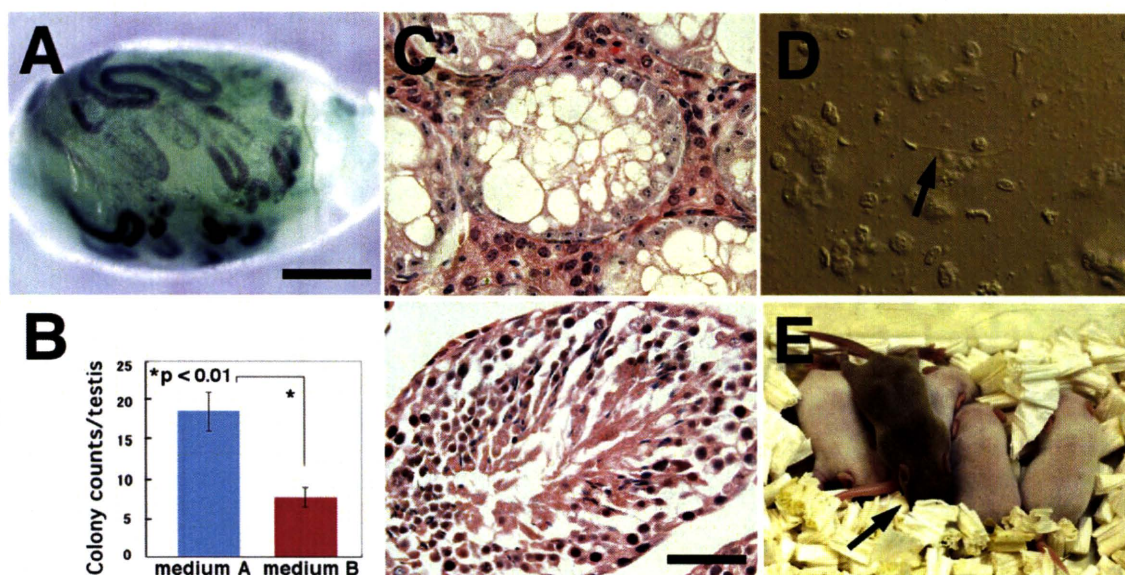
FIG. 5. Production of offspring from cultured cells by germ cell transplantation. **A**) LacZ staining of a recipient testis, indicating the presence of the donor cell maker. Blue stretches of tubules indicate the presence of donor cell-derived germ cell colonies. **B**) Decreased SSC activity of GS cells cultured under serum- and feeder-free conditions. Results are from three separate experiments. Approximately 8×10^3 cells were injected into each testis (n = 19–21). **C**) Histological appearance of recipient testis showing normal spermatogenesis. **Top**) Nontransplanted W recipient testis. **Bottom**) Normal-appearing spermatogenesis in testis transplanted with GS cells cultured under serum- and feeder-free conditions. **D**) Spermatozoon (arrow) recovered from recipient testis. **E**) Offspring (arrow) derived from spermatozoa produced from transplanted GS cells. Bars = 1 mm (**A**) and 50 μm (**C**).

[30]. Fetuin has positive effects on the growth and differentiation of various cells in culture [30], and it promotes cell attachment, by an unknown mechanism, depending on the cell type. We previously demonstrated that GS cells attached to laminin via integrins; our current results suggest that this interaction may involve additional factors.

Fetuin supplementation was effective for GS cell attachment to laminin under serum- and feeder-free conditions, but it was not sufficient for maintaining cell proliferation. Lipid signaling is another important process mediated by serum, and the inhibition of GS cell proliferation by SKI II indicates that sphingosine-mediated signaling was necessary for GS cell proliferation. Therefore, we added lipid components or HDL mixtures and lipid-rich BSA (Albumax II) to the GS cell culture medium. Albumin, a major serum component, functions as a carrier protein for thyroid and other steroid hormones and binds to fatty acids and other unknown metabolites. A critical role has been demonstrated for BSA in cultures of ES cells and other stem cells, including SSCs [16, 31, 32]. Albumax II contains free fatty acids (~54%), lysophosphatidylcholine (~17%), triacylglycerides (~15%), phosphatidylcholine (~8%), phosphatidic acid (~3%), cholesterol (~1%), and sphingomyelin (~1%) [32]. These additions to the medium, combined with fetuin, allowed GS cell proliferation in the absence of serum. Although our results indicated that sphingosine was necessary for proliferation, we

currently do not know how sphingosine influenced GS cells; it is important to study whether it directly stimulated proliferation of GS cells or influenced apoptosis. Because addition of sphingosine to the culture medium was not sufficient to drive GS cell proliferation (unpublished observation), sphingosine likely interacts with other lipid components in the serum or Albumax II. The identity of other lipid components must be further investigated.

An unexpected finding from the present study was the low SSC frequency under serum- and feeder-free culture conditions. Generally, 1–2% of GS cells exhibit SSC activity, as calculated from the colony number [28], and the presence of feeder cells did not significantly change this frequency. However, the SSC frequency in the present study was significantly lower (0.4–1.0%). Given that the cultured cells expanded at comparable levels in serum-free and serum-containing media, these results suggest reduced efficiency of self-renewing divisions in our serum-free medium. However, we did not detect significant changes in spermatogonial phenotype based on marker analyses, and we were able to obtain one normal offspring. Changes in SSC frequency were evident only in functional transplantation assays.

Presently, little is known about the mechanism of SSC fate commitment. Although GDNF levels apparently influence fate commitment in vivo, factors that influence SSC frequency in vitro have remained unknown for a long time. However, we recently found that cyclin D expression had a significant impact on SSCs activity [28], such that *Ccnd1* overexpression impairs the SSC activity of GS cells whereas GS cells overexpressing *Ccnd2* can proliferate without self-renewal factors and retain SSC activity. Our current results indicate that serum also contains one or more factors that may influence SSC fate commitment and cyclin D expression. Importantly, *Ccnd1* expression under serum- and feeder-free conditions was significantly downregulated compared with that under serum-supplemented conditions. Because *Ccnd1* overexpression also impaired SSC homing [28], it seems that *Ccnd1* levels must be

TABLE 2. ICSI using spermatozoa recovered from recipient mice.

Type of testis	No. of cultured embryos	No. of cleaved (%)	Embryo transfer[a]	Implantation (%)	Offspring (%)
Fresh	15	9 (60.0)	9	1 (6.7)	1 (6.7)
Freeze-thawed	52	22 (42.3)	22	1 (1.9)	0 (0.0)
Total	67	31 (46.3)	31	2 (3.0)	1 (1.5)

[a] Embryos were transferred after culturing 24 h in vitro.

maintained within an appropriate range for successful SSC homing. Although the link between cyclin D expression and SSC self-renewal awaits confirmation by further experiments, our finding suggests that SSC fate commitment is not entirely stochastic [33, 34] but, instead, may be influenced by environmental factors, such as serum. The culture system reported here may be useful for studying the effect of various test molecules on SSC self-renewal and differentiation.

Although we developed serum- and feeder-free culture conditions for GS cells, additional improvements in the medium are still necessary. Culture conditions are being developed for SSCs from other animal species [19, 35–38], but their growth in vitro is still reduced compared with that of mouse GS cells. This exposes a critical concern regarding the use of this technology for genetic modification of the germline: Slow growth will limit transfection efficiency and in vitro drug selection. We also must further define the culture medium. Although GS cells can proliferate faster in StemPro medium than in other media, StemPro may contain numerous factors that are not well optimized for GS cells. Establishing defined culture conditions for GS cells is apparently important for understanding the molecular requirements of the SSC self-renewal machinery and providing important information on stem cell niche/microenvironments in vivo. Hopefully, our culture system will stimulate future developments toward completely defined culture conditions for GS cells.

ACKNOWLEDGMENT

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