

1 **Genetic selection of mouse male germline stem cells in vitro: Offspring from single**  
2 **stem cells<sup>1</sup>**

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14

15 Short title: Offspring from single stem cells

16 One sentence summary: We demonstrate the feasibility of genetic selection of mouse  
17 male germline stem cells.

18

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22 Gametogenesis

23 **Abstract**

24

25 Spermatogenesis originates from a small population of spermatogonial stem cells. These  
26 cells are believed to divide infinitely and support spermatogenesis throughout life in the  
27 male. In this investigation, we examined the possibility of deriving transgenic offspring  
28 from single spermatogonial stem cells. Spermatogonial stem cells were transfected in  
29 vitro with a plasmid vector containing a drug resistance gene. Stably transfected stem cell  
30 clones were isolated by in vitro drug selection, and these were expanded and used to  
31 produce transgenic progeny following spermatogonial transplantation into infertile  
32 recipients. An average of 49% of the offspring carried the transgene, and the recipient  
33 mice continued to produce monoclonal transgenic progeny a year after transplantation.  
34 Thus, a somatic cell-based genetic approach can be used to modify and select clones of  
35 spermatogonial stem cells in a manner similar to embryonic stem (ES) cells. The  
36 feasibility of genetic selection using postnatal spermatogonial stem cells demonstrates  
37 their extensive proliferative potential and provides the opportunity to develop new  
38 methods for generating stable animal transgenics or for germline gene therapy.

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45 **Introduction**

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47 Spermatogenesis can be divided into three phases: mitotic expansion of  
48 spermatogonia, meiotic diversification in spermatocytes and maturation into  
49 spermatozoa to acquire mobility. At the foundation of this sophisticated process are the  
50 spermatogonial stem cells [1,2]. Because of their unique ability to self-renew, these cells  
51 are considered to proliferate infinitely and support spermatogenesis throughout life [3].  
52 Thus, spermatogonial stem cells are the only stem cells in the postnatal animal that can  
53 transmit genetic information to the offspring, and transduction of spermatogonial stem  
54 cells would produce numerous transgenic sperm, providing a new approach for animal  
55 transgenesis [4,5]. However, there is little evidence that supports the infinite proliferative  
56 potential of spermatogonial stem cells, and it has not been possible to modify the genome  
57 of spermatogonial stem cells in a manner similar to ES cells [6-9].

58 Recently, we described a method to expand mouse spermatogonial stem cells in  
59 vitro [10]. In this culture system, gonocytes isolated from neonatal mouse testis  
60 proliferated logarithmically over a five-month period ( $>10^{14}$ -fold expansion in stem cell  
61 number) in the presence of glial cell line-derived neurotrophic factor (GDNF), leukemia  
62 inhibitory factor (LIF), epidermal growth factor (EGF), and basic fibroblast growth factor  
63 (bFGF) on mitomycin C-inactivated mouse embryonic feeder cells. These cells were  
64 proved to retain stem cell activity by transplantation into seminiferous tubules of the  
65 testes, and donor cell haplotype was transmitted to the progeny by mating the recipient  
66 animals that underwent transplantation of the cultured cells. Based on these facts, we

67 designated the cultured cells as germline stem cells or GS cells. In addition to ES and  
68 embryonic germ (EG) cells that are derived from embryonic cells [11-14], GS cells  
69 represent a new target for the expansion and manipulation of germline cells.

70           Since stem cells are considered to be able to proliferate infinitely, in vitro  
71 transduction and expansion/selection of transfected stem cell clones comprise the central  
72 techniques in manipulating stem cells. Although reconstitution of an entire self-renewing  
73 tissue from single stem cells was previously reported in hematopoietic system [15], in  
74 vitro clonal selection of stem cells remains to be a major challenge in stem cell  
75 technology. In this investigation, we examined the feasibility of in vitro selection of  
76 spermatogonial stem cells using GS cell culture system. A plasmid vector that contains  
77 both enhanced green fluorescent protein (EGFP) marker and drug selection genes were  
78 transfected, and GS cell clones were selected in vitro for clonal offspring production.

79

## 80 **Methods and methods**

### 81 *Culture of GS cells*

82 Neonatal testis cells were collected from newborn DBA/2 mice (purchased from Japan  
83 SLC, Shizuoka, Japan) by two-step enzymatic digestion [16]. A total of three mice were  
84 used to establish GS cells and used in the present study. The dissociated cells were  
85 cultured overnight on 0.2% gelatin-coated plate to remove somatic cells. GS cells were  
86 established according to the previously published protocol [10], and early passage (8 to  
87 13 passages) GS cells were used for experimental studies. All GS cell clones were  
88 established from a GS cell culture derived from a total of three mice through three

89 independent transfection experiments. Transfection was performed when more than 70%  
90 of GS cell colonies attached to the feeder cells.

91

#### 92 *Transfection of GS cells*

93 Single cell suspension of GS cells was obtained by treatment with 0.25% trypsin  
94 (Invitrogen, Carlsbad, CA), and used for all transfection experiments. The plasmid vector  
95 used for the transfection was based on the pCX vector [17], which carries a neo cassette  
96 consisting of a neomycin resistant gene driven by a synthetic mutant polyoma enhanced  
97 *HSVtk1* promoter (MC1) [18](gift from Dr. T. Honjo). The cDNA encoding the EGFP  
98 gene (Clontech, Palo Alto, CA) was inserted in the Xho I site of the vector under the  
99 control of the CAG promoter (pCAG-EGFP2). For electroporation,  $10^7$  GS cells were  
100 suspended in 0.5 ml PBS and mixed with 20  $\mu$ g DNA. Cells were then exposed to a single  
101 320 V, 200  $\mu$ F pulse using the BioRad Gene Pulser II (0.4 cm gap cuvette; BioRad,  
102 Hercules, CA). Cells were incubated for 10 min on ice and then plated at a density of  $10^5$   
103 cells/cm<sup>2</sup> on embryonic fibroblast feeder. For calcium phosphate-mediated and  
104 DEAE-Dextran-mediated transfections, GS cells were transduced with the CellPfect  
105 transfection kit (Amersham Biosciences, Piscataway, NJ), according to the manufacturer's  
106 instructions. For lipofection, FuGENE 6 transfection reagent (Roche Diagnostics, Tokyo,  
107 Japan) was used according to the manufacturer's instructions. GS cells that had been  
108 transfected with the plasmid vector were cultured on a G418-resistant mouse embryonic  
109 fibroblast feeder layer at a density of  $10^5$  cells/cm<sup>2</sup>.

110 On the third day after transfection, the expression of the EGFP gene was

111 examined using either a fluorescence microscope or a FACS-Calibur system (BD  
112 Biosciences, Franklin Lakes, NJ) as described [19]. In brief, cultured cells were  
113 incubated with 0.25% trypsin for 5 min followed by strong pipetting. The collected cells  
114 were suspended in PBS supplemented with 1% fetal calf serum. Cells were kept in the  
115 dark on ice until analysis. At least 10,000 events were acquired for each sample. Survival  
116 rate after transfection was determined by trypan blue exclusion. To minimize the effect of  
117 cell proliferation, we plated the same number of GS cells without treatment, and took the  
118 ratio to normalize the survival rate.

119

#### 120 *Expansion of GS cells*

121 For clonal expansion, GS cells were transduced with FuGENE 6 transfection reagent.  
122 Dissociated GS cells were plated at a density of  $2 \times 10^6$  cells/55 cm<sup>2</sup> with 7 ml medium  
123 and cultured with 9 µg DNA and 27 µl FuGENE 6. Cells were maintained for 3 weeks  
124 after transfection, and then G418 selection (0.2-0.4 mg/ml Geneticin; Invitrogen) was  
125 performed 2 days after the passage on G418 resistant embryonic fibroblast feeder. Ten  
126 days after selection with G418, single colonies were picked and transferred to a 96-well  
127 plate coated with feeder cells. As the growth of GS cells is influenced by cell density,  
128 individual colonies were mixed with 1,000 non-transfected GS cells. Transduced cells  
129 populations were expanded by repeating G418 selection and mixing procedures until all  
130 colonies consisted of EGFP-expressing cells.

131

#### 132 *Transplantation*

133 Cultured cells were transplanted into 5- to 10-day-old WBB6F1-W/W<sup>v</sup> (W) pups (Japan  
134 SLC). The colonies were dissociated by trypsin digestion, and approximately 2 µl of each  
135 donor cell suspension (~10<sup>5</sup> cells/testis ) was injected into the seminiferous tubules of a  
136 W pup's recipient testis through the efferent duct [16]. At least six males were  
137 transplanted per GS cell clone. Recipients were placed on ice to cause  
138 hypothermia-induced anesthesia [20]. The different MHC haplotype of the cultured cells  
139 necessitated giving the recipient mice 50 µg anti-CD4 antibody (GK1.5) intraperitoneally  
140 on days 0, 2, and 4 after transplantation to induce tolerance to the allogeneic donor cells  
141 [21]. At 4 to 5 weeks after transplantation, the males were introduced to two or three  
142 wild-type female mice in order to generate progeny. The Institutional Animal Care and  
143 Use Committee of Kyoto University approved all of the animal experimentation  
144 protocols.

145

#### 146 *DNA analysis*

147 Recipient males were mated with wild-type females, and genomic DNA was isolated  
148 from tail samples of each offspring by phenol/chloroform extraction, followed by ethanol  
149 precipitation. Genomic DNA (10 µg) isolated from tail tissue samples was digested  
150 overnight with Sph I, separated by electrophoresis, and blotted onto a nylon membrane  
151 (Hybond-N+, Amersham Biosciences) according to conventional protocols. The  
152 full-length EGFP cDNA was used as a probe for hybridization. The membrane was  
153 hybridized for 16 h at 65°C with a <sup>32</sup>P-labeled probe.

154

155 *Analysis of testis*

156 Donor cell colonization was evaluated by observation of fluorescence under UV light  
157 [22]. This method allows the specific identification of donor germ cells, as the  
158 endogenous cells of the host testis do not fluoresce. The testes were fixed in 10%  
159 neutral-buffered formalin (Wako Pure Chemical Industries, Osaka, Japan), and processed  
160 for paraffin sectioning. All histological sections were stained with hematoxylin and  
161 eosin.

162

163 **Results**

164

165 To set up a system for transfecting GS cells, GS cells were established from  
166 neonatal DBA/2 mice using a cocktail of cytokines [10]. Cells that had been cultured for  
167 8 to 10 weeks (8 to 13 passages) were used for transduction studies. At this stage, GS cell  
168 populations expand logarithmically; thus,  $5 \times 10^8$  GS cells could be collected from a  
169 newborn testis (composed of  $\sim 5 \times 10^5$  cells). Only cultures in which GS cell appeared  
170 undifferentiated were selected for transfection (Fig. 1A, left top). In order to gauge their  
171 efficacies for transfection, four conventional methodologies were assayed:  
172 electroporation, lipofection, calcium phosphate-mediated transfection, and  
173 DEAE-dextran-mediated transfection. A neo-containing plasmid vector expressing the  
174 EGFP gene (pCAG-EGFP2) was used to facilitate the assessment of the transfection  
175 efficiency. Assuming that GS cells proliferated in the same speed after transfection, we  
176 determined the transfection efficiency using flow cytometry on the third day after

177 transfection. Although electroporation proved the most efficient method of transduction  
178 ( $20.3 \pm 6.0\%$ ,  $n=3$ ; mean  $\pm$  SEM.), only small numbers of cells ( $8.7 \pm 0.8\%$ ,  $n=3$ )  
179 subsequently survived the procedure (Fig. 1B). In contrast, lipofection demonstrated both  
180 a relatively high transfection efficiency ( $3.9 \pm 2.5\%$ ,  $n=3$ ) and a higher number of  
181 subsequent survivors of the transfected cells ( $95.8 \pm 7.0\%$ ,  $n=3$ )(Fig. 1A, right top). We  
182 did not obtain sufficient numbers of EGFP-positive cells by either calcium  
183 phosphate-mediated ( $0.6 \pm 0.3\%$ ,  $n=3$ ) or DEAE-dextran-mediated methods ( $0.6 \pm 0.3\%$ ,  
184  $n=3$ ) to warrant further consideration. Owing to concerns that adverse culture condition  
185 after electroporation might negatively affect GS cell culture, lipofection was selected as  
186 the optimal methodology for clonal expansion.

187           After G418 selection, isolated single colonies of undifferentiated GS cells were  
188 picked by micromanipulation and transferred to individual wells of a 96-well plate for  
189 clonal expansion. To facilitate clonal proliferation, individual colonies were mixed with  
190 1,000 non-transfected GS cells. This was because we did not obtain any clones when 100  
191 GS cell colonies were cultured without non-transfected GS cells. A total of 180 clones  
192 were picked, and 35 (19.4%) G418-resistant clones were successfully established. Five of  
193 these clones were randomly chosen for production of offspring by spermatogonial  
194 transplantation (Fig. 1A, left bottom, right bottom) [23]. No gross abnormalities in  
195 colony morphology or growth speed were noted after selection; cells were typically  
196 passaged every 5 days at dilutions of 1:3 to 1:5, while still in their undifferentiated state.  
197 The transfected clones could then be stored using a technique commonly employed to  
198 freeze somatic cells [24,25]. The time scale required from initial transfection to

199 transplantation was typically 2 to 3 months.

200 To generate offspring from the transfected GS cell clones, single cell  
201 suspensions of the colonies were microinjected into the seminiferous tubules of W mice.  
202 W mice are congenitally infertile but can produce offspring from transplanted donor cells  
203 [20]. By mating with wild-type females, recipient mice started to produce offspring as  
204 early as 77 days after transplantation, and 14 of 51 (27%) recipients became fertile within  
205 9 months of transplantation. All progeny that resulted from the mating were examined for  
206 the presence of the transgene by Southern blotting of tail DNA samples using an  
207 EGFP-specific probe. In total, 517 offspring from the 14 recipient mice were analyzed; of  
208 these, 251 of the offspring (48.5% of the F1 progeny) contained the transgene (Table 1).  
209 The offspring showed different degrees of fluorescence under UV light, and not all the  
210 offspring showed fluorescence despite the presence of the transgene. The donor transgene  
211 had inserted at the same site in DNA samples from progeny derived from a single  
212 recipient clone (Fig. 2A). However, each individual clone represented a unique  
213 integration event of the transgene (Fig. 2B). Furthermore, transgenic offspring from  
214 different recipient males that had received the same clone all had identical patterns on  
215 Southern blots, confirming the common integration event (Fig. 2C). At least four of the  
216 recipients continued to successfully produce offspring with the transgene at the same site  
217 of integration a year after transplantation, indicating the continued proliferation of donor  
218 stem cells. DNA from the transgenic offspring, digested with an enzyme that cuts once  
219 within the plasmid, produced a 7.4 Kb band representative of the complete plasmid.  
220 Significantly, this band was present in all samples and was at elevated levels compared

221 with all other bands, indicating that the transgene had integrated as multiple copies.  
222 Densitometric analysis showed that up to 6 copies of the transgenes were present in the  
223 genome (data not shown). EGFP-positive donor GS cells extensively colonized host  
224 recipient testis, as was evident under UV light (Fig. 3A). The testes of the fertile males  
225 were significantly larger than those of the untreated, infertile males (Fig. 3B), reflecting  
226 the extensive donor cell colonization. Histological analysis confirmed that complete  
227 spermatogenesis was occurring in the testis (Fig. 3C), and that spermatozoa were present  
228 in the epididymides (Fig. 3D). These results show that the donor GS cells proliferate  
229 monoclally and colonized the recipient testes through spermatogonial transplantation.

230           The inheritance of the transgene occurred in a Mendelian fashion, and both  
231 male and female progeny could successfully transmit the transgene to the next generation  
232 (Fig. 3E). Tail DNA samples from F2 offspring demonstrated that the transgene was  
233 integrated into the same site as in the cultured cells and F1 offspring (Fig. 2D),  
234 demonstrating that the transgene was stably transmitted to the following generation. To  
235 confirm whether the transgene is fully expressed in the F2 offspring, cells were collected  
236 from kidney and testis, and cultured in vitro in the presence of G418. Only cultures from  
237 EGFP-positive offspring showed resistance to G418. In contrast, cells from  
238 EGFP-negative mice died within 10 days of selection. The transgene was similarly  
239 expressed in germline cells, as GS cells established from the EGFP-positive males  
240 continued to proliferate in G418 containing medium (Fig. 3F). The transgene continued  
241 to be expressed in the F3 generation in a manner similar to that in the F1 generation (data  
242 not shown). Thus, transfected GS cells can stably transmit the transgene through the

243 germline to subsequent generations.

244

## 245 **Discussion**

246           Although ES cells are now widely used for germline modification, ES cells  
247 with germline potential have only been obtained in mice [26], which limits their  
248 application. Our results show that germline cells from testis could provide an alternative  
249 approach. There are several advantages associated with GS cell-based germline  
250 modification compared with conventional approaches. First, because GS cells are derived  
251 from postnatal testis, it is not necessary to sacrifice the conceptus or embryos. Second,  
252 because male founder animals can produce enormous numbers of transgenic spermatozoa  
253 [3], the number of possible transgenic progeny far exceeds the comparative potential  
254 number achievable by female founders. Third, it is possible to obtain transgenic offspring  
255 at a higher efficiency. Typically, with conventional technologies, only 5-10% of the  
256 offspring will contain a transgene [27,28]; whereas, GS cell-based germline modification  
257 demonstrated significantly higher transfection efficiency, approaching ~50% in the  
258 present experiments. Finally, GS cells are stably committed to the germline and do not  
259 lose germ cell potential even after long-term culturing [10]. In contrast, ES cells often  
260 lose germ cell potential owing to differentiation or abnormal karyotype [29,30], which is  
261 difficult to predict before chimera formation [26]. The stable germline transmission of  
262 GS cells makes the procedure simple and more reliable. Thus, GS cells may overcome  
263 difficulties associated with conventional transgenic technologies.

264           The next obvious goal is to generate knockout animals using site-directed

265 homologous recombination. Our experiments are encouraging because they demonstrate  
266 that GS cells will allow the in vitro selection that is necessary to obtain clones of desirable  
267 mutations. This “gene targeting” technology was initially developed in somatic cells  
268 [31,32], and is widely used in ES cells [18,33]. However, very little is known about the  
269 efficiency of homologous recombination in tissue-specific stem cells. Because stem cells  
270 are significantly more resistant to cytotoxic damages than are differentiating progenitors,  
271 they might possess different DNA repair machinery [34], which may have an impact on  
272 the efficiency of homologous recombination. Current GS cell culture condition may also  
273 be problematic, because the clonal growth of GS cells required the presence of other GS  
274 cells. Nonetheless, the induction of specific changes in DNA is an important goal in stem  
275 cell-based technology, and the GS cell system will provide a useful model to determine  
276 the feasibility of such a procedure. Possibly it will lead to new developments in animal  
277 transgenics and gene therapy.

278

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284

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374

375

376 **Figure Legends**

377 **Fig. 1.** (A) In vitro drug selection of GS cells. (left top) A typical colony of GS cells.  
378 (right top) GS cells were transfected, using lipofection, with an EGFP-expressing  
379 plasmid possessing a neo-resistant gene. Cells were observed under UV light at 3 days  
380 after transfection. (left bottom) Colonies of a stably transfected GS cell clone (Clone #49)  
381 originating from a single colony, shown in bright field. Cells were plated at the same  
382 density as in (right top). (right bottom) The same colonies fluorescing under UV light. (B)  
383 Flow cytometric characterization of transfected cells. Only live cells were gated to  
384 exclude dead cells. Bar = 50  $\mu\text{m}$  (left top) and 250  $\mu\text{m}$  (right top, left bottom, right  
385 bottom).

386

387 **Fig. 2.** Southern blot analysis of DNA samples from transgenic progeny. Genomic DNA  
388 was digested with Sph I and hybridized with an EGFP gene-specific probe. (A) DNA  
389 from transplanted cultured cells or from transgenic progeny derived from recipient #3734  
390 (Left) or #3719 (Right). Representative offspring, born between 78 and 130 days (#3734),  
391 or between 89 and 126 days (#3719), after donor cell transplantation are shown. Similar  
392 results were obtained from all transgenic offspring. The band at 7.4 Kb represents the size  
393 of the entire plasmid vector. (B) DNA from transgenic progeny of recipients #3737,  
394 #3765, and #3770. Note the variance in patterns, representative of differing integration  
395 events of the transgene, in offspring derived from different clones (See Table 1). (C) DNA  
396 from transgenic progeny of recipients #3736, #3737, and #3738 that received the same

397 donor cells (Clone #23). Note the similarity of the patterns, representative of a single  
398 integration event of the transgene. (D) Transmission of the transgene to the F2 generation.  
399 DNA from cultured cells (Clone #23) and from F1 and F2 progeny of recipient #3924 is  
400 shown.

401

402 **Fig. 3.** Macroscopic and histological appearance of recipient testes possessing transduced  
403 stem cells. (A) A testis of recipient #3765 under UV light. Note the extensive  
404 colonization of EGFP-expressing donor cells. (B) Histological section of W recipient  
405 testis (no transplantation). Note the complete absence of differentiating germ cells. (C)  
406 Spermatogenesis in the testis of recipient #3765. Complete spermatogenesis is apparent.  
407 (D) Mature spermatozoa in the epididymis of recipient #3765. (E) Expression of the  
408 EGFP transgene in F2 offspring (arrow) from recipient #3734 under UV light.  
409 Fluorescence was not observed in non-transgenic progeny (arrowhead). (F) Expression of  
410 the EGFP transgene in GS cells established from F2 offspring (#3734). Bar = 1 mm (A),  
411 250  $\mu\text{m}$  (B-D), and 50  $\mu\text{m}$  (F). Stain, Hematoxylin and eosin.

412

413

Table 1. Transgenic mice from spermatogonial stem cell clones

Recipient mouse	Days from transfection to transplant	Clone	testis surface area covered (%) <sup>a</sup>		Days to first transgenic <sup>b</sup>	No. of litters	No. (%) of transgenic /total offspring <sup>c</sup>
			right	left			
3718	82	23	90	N.I.	85	2	7/16 (43.8)
3719	82	23	80	20	89	12	35/67 (52.2)
3734	89	49	40	90	78	6	31/63 (49.2)
3736	89	23	70	90	78	14	44/108 (40.7)
3737	89	23	90	20	109	10	33/69 (47.8)
3738	89	23	90	20	157	5	14/23 (60.9)
3761	96	62	N.I.	90	151	1	1/1 (100)
3765	103	32	90	N.I.	104	4	17/36 (47.2)
3768	103	56	90	90	200	5	16/32 (50)
3769	103	56	90	N.I.	77	7	19/41 (46.3)
3770	103	56	80	80	108	5	19/34 (55.9)
3858	131	23	90	90	88	4	12/22 (54.5)
3903	138	32	90	40	222	1	2/4 (50)
3924	145	23	90	90	101	1	1/1 (100)

Results at 9 months after transplantation. # 3765 was sacrificed at 209 days for histological analysis (See Fig. 3). N. I., testis not injected with donor cells.

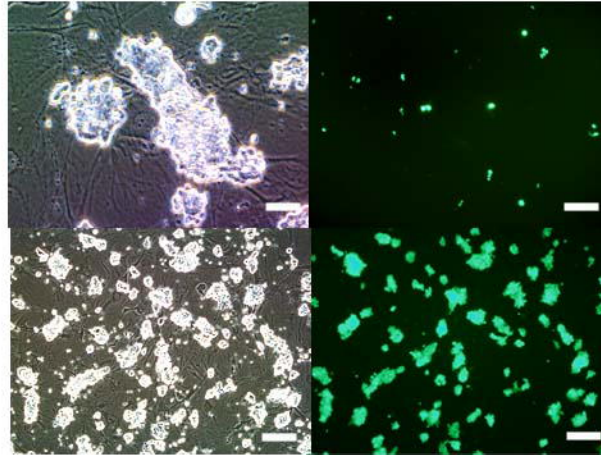
<sup>a</sup>Percentage of the surface seminiferous tubules in recipient testes filled by the injected cell suspension. Cells were transplanted 12 to 24 passages after transfection.

<sup>b</sup>Time in days from transplantation of donor cells to birth of first transgenic progeny, which generally appeared in the first litter, except in one case (#3768).

<sup>c</sup>Numerator is the number of transgenic progeny; denominator is the total number of progeny from each recipient mouse.

Figure 1

**A**



**B**

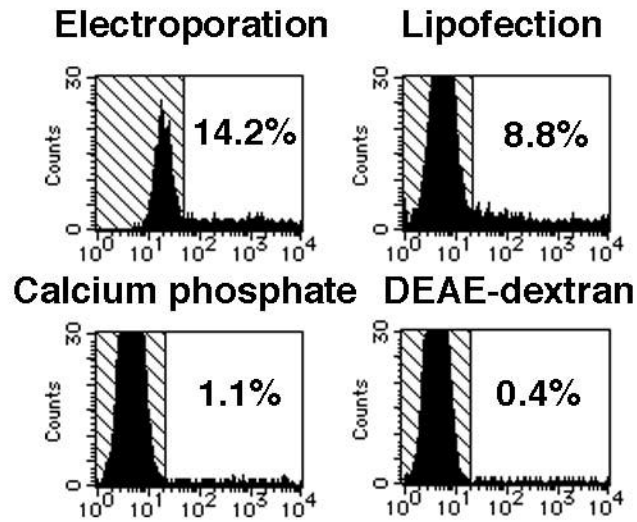


Figure 2

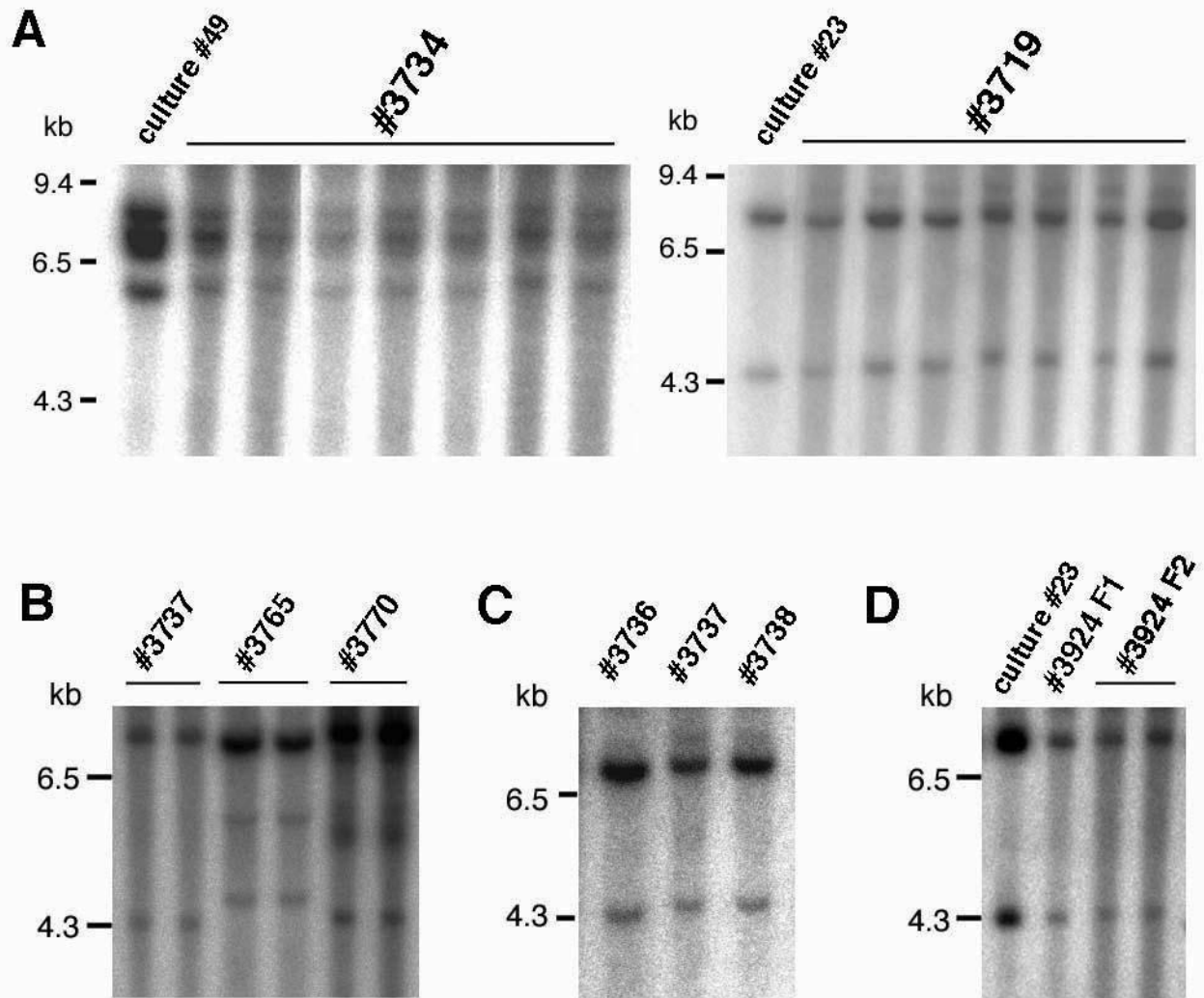


Figure 3

