Correction

For the article “Production of transgenic rats by lentiviral transduction of male germ-line stem cells,” by F. Kent Hamra, Joel Gatlin, Karen M. Chapman, Dana M. Grellhesl, J. Victor Garcia, Robert E. Hammer, and David L. Garbers, which appeared in number 23, November 12, 2002, of Proc. Natl. Acad. Sci. USA (99, 14931–14936; First Published October 21, 2002; 10.1073/pnas.222561399), on page 14936, the last sentence of the Acknowledgments should read “This work was supported in part by the Cecil H. and Ida Green Center for Reproductive Biology Sciences, the Cecil H. and Ida Green Distinguished Chair in Reproductive Biology Sciences, the Howard Hughes Medical Institute, and National Institutes of Health Grant AI39416.” In addition, on the same page, left column, line 5, “zygates” should read “zygotes,” and in lines 6 and 7, “(6, 28)” should read “(22, 28).”

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Production of transgenic rats by lentiviral transduction of male germ-line stem cells

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Contributed by David L. Garbers, September 16, 2002

Primary cultures of rat spermatogenic cells that did not bind to collagen matrices were able to colonize and form mature spermatocytes when transferred to testes of recipient males. Up to 73% of the progeny from matings with recipient males were derived from the transferred spermatogenic cells. Subsequently, two populations of germ cells were obtained by selection on laminin matrices. Both populations expressed the spermatogenic cell marker, DAZL, but not the somatic cell marker, vimentin. The cells that bound to laminin represented ~5% of the total population and were greatly enriched in ability to colonize a recipient testis, suggesting an enrichment in germ-line stem cells. The colonization potential was maintained for at least 7 days in culture. These cells were subsequently transduced with a lentiviral enhanced GFP reporter vector and then transferred to WT recipient males. After mating, 26 of 44 pups were derived from the cultured donor germ cells, and 13 pups carried the lentiviral transgene. Based on Southern analysis, the transgene was integrated at a different genetic locus in each animal and was transmitted to ~50% of pups in the F1 generation. Thus, by using these procedures, ~30% of pups in the F1 generation inherited and stably transmitted a lentiviral transgene that integrated at various genomic loci.

The laboratory rat represents one of the most comprehensively studied mammals (1, 2). More than a million publications in a wide range of areas, including development, nutrition, physiology, pharmacology, genetics, microbiology, toxicology, parasitology, and pathology, have focused on this mammal. Various qualities of the rat, such as size, fecundity, ease of surgical techniques, tissue sampling, and general laboratory management make it a particularly attractive animal model (1, 2). However, gene-targeting protocols have not been developed in the rat despite the establishment >12 years ago of the first transgenic rats by pronuclear injection (3, 4). This failure results from an inability to derive and culture rat embryonic stem cells that maintain pluripotency (5). In contrast, gene targeting is well established in the mouse (6). Expansion of the technology to produce genetically modified rats through the use of stem cells is the genetic modification of spermatozoa through the rat germ line is the genetic modification of spermatozoa that maintain pluripotency (5). In contrast, gene targeting is well established in the mouse (6). Expansion of the technology to produce genetically modified rats through the use of stem cells is the genetic modification of spermatozoa through the rat germ line is the genetic modification of spermatozoa.

Methods

Materials and Chemicals. Porcine pepsin A and 2-mercaptoethanol (ME) were obtained from Sigma. Dispase, rat-tail collagen I-coated culture dishes, mouse laminin, and spectra-mesh were obtained from Fisher. Culture medium, calcium- and magnesium-free heat-inactivated horse serum (HS), and antibiotic-anitmycotic solution were obtained from Gibco/BRl. FBS was obtained from Atlanta Biologicals (Norcross, GA); PBS was from JRH Biosciences (Lenexa, KS); protease inhibitors (EDTA-free tablets) were from Roche Molecular Biochemicals. Hoechst 33342 and ethidium homodimer-2 were obtained from Molecular Probes.

Cell Cultures. Testes from 20- to 22-day-old homozygous MT-lacZ (11) or Rosa-EGFP [a germ-line-specific, enhanced GFP (EGFP)-expressing strain developed by J. T. Cronkhite and R.E.H.] Sprague–Dawley rat strains were transferred to plastic culture dishes containing DMEM: Ham’s F12 medium 1:1 supplemented with 100 units/ml penicillin G sodium/100 μg/ml streptomycin sulfate/250 mg/ml amphotericin B (DHF12) under ambient conditions (22–24°C). The tunica albuginea was dissected free of seminiferous tubules and discarded. The tubules were then washed by sequentially transferring them with forceps to fresh culture dishes (35 mm²) containing 6 ml of DHF12 (two dishes), then to one dish containing 6 ml of PBS before being minced with scissors in a fourth culture dish containing 6 ml of a 1 M glycine buffer, pH 7.2 for 5 min (15). After an additional 5 min of incubation, the minced tubules were suspended in 35 ml of glycine buffer and pelleted by centrifugation at 500 × g. Tubules were washed by suspension in 35 ml of DHF12 containing 8.5% FBS (DHF12-FBS) and then pelleted at 500 × g (procedure for wash steps). After a second wash, tubules were suspended in dispase solution (50 casinolytic units per testis) and digested for 30 min with gentle rocking at 32.5°C. The digest was adjusted to 35 ml with DHF12-FBS, positioned vertically on a G24 environmental shaker (New Brunswick Scientific) set to 220 rpm and agitated for 10 s (22–24°C). The digested tubules were pelleted at 500 × g, washed twice in DHF12-FBS, filtered through 20-μm mesh, and washed twice with DHF12 supplemented with 5.5% HS and 2.4% FBS (DHF12-TC). Cells were plated into 10-cm² plastic culture dishes at ~2 × 10⁶ cells per cm² containing 10 ml of DHF12-TC and incubated at 32.5°C, 5.5% CO₂ for ~65 h.

Matrix Selection of Cells. Medium was removed after ~65 h and testis cell cultures were gently washed once with 4 ml of DHF12 and once with 4 ml of PBS. Bound germ cells were harvested from monolayers of adherent somatic cells by repeated pipetting with 4 ml of DHF12. The pooled suspension from five 10-cm² dishes was pelleted at 500 × g, suspended in 10 ml of DHF12-TC.
filtered through 20-µm mesh, transferred to a 10-cm² plastic dish coated with collagen I, and incubated at 32.5°C for 4 h. Cells that did not bind to collagen I dishes (ColNb cells) were harvested and pelleted at 500 × g. The ColNb cells were suspended in DHI2-FBS and plated at 0.5–1 × 10⁶ cells per ml per well in 12-well plates precoated with mouse laminin (4.4 μg/cm²). The plated ColNb cells were incubated for 45 min at 32.5°C and unbound cells (LamNB cells) were removed from bound cells (Lam cells) by pipetting. The Lam cells were rinsed twice with 1 ml of DHI2-FBS and then incubated with 1 ml of PBS containing 0.5% BSA at 32.5°C for 5 min. The Lam cells then were harvested by gentle pipetting, washed in DHI2 containing 10% (vol/vol) PBS and 30 μm ME (DHI2-10% PBS) and counted for use in transplantation experiments. For viral transduction, Lam cells were replated on laminin-coated culture dishes (1–1.5 × 10⁵ cells per cm²) containing DHI2-10% PBS, exposed once to VSV-G-pseudotyped, lentiviral particles (multiplicity of infection = 4.5) produced as described (16) by cotransfection of the plasmids pHR'CMVENS1-18, pBII10' E−, and pLVSV-G. Cells were maintained overnight at 32.5°C, 5.5% CO₂ before harvesting and used as donor cells. The mean viability of testicular cells after isolation was 94.5 ± 1.3%, 93.6 ± 2.3%, and 97.1 ± 1.3% for ColNb, LamNB, and Lam cells (± SEM, n = 4, 6, and 6), respectively, based on labeling with membrane-permeable Hoechst 33342 and impermeable ethidium homodimer-2.

Antibody Production and Purification. Synthetic peptides, DAZL-3 (CSTVQDDYFKKRHHFRRRSRAVLKSDH) and DAZL-4 (CKD-KRVHHHFRRSSRAVLKSDH), were produced to amino acid residues 269–296 of the mouse DAZL protein (GenBank accession no. U46694). Amino terminal cysteine residues were added to allow conjugation with maleimide-activated keyhole limpet hemocyanin (Pierce). Purified conjugates were used to raise polyclonal antisera in rabbits. Preimmune and immune sera were processed over protein A affinity columns (Hi-Trap, Amersham Pharmacia). Purified IgG fractions from immune sera were further purified by affinity chromatography by using the antigenic DAZL-3 and DAZL-4 peptides (Sulfolink kit, Pierce). All preimmune (PI-3 and PI-4) and immune (anti-DAZL-3 and anti-DAZL-4) IgG preparations were adjusted to ~2 μM by protein assay (Bradford, Bio-Rad) and Coomassie blue staining (GelCode Blue, Pierce) on reducing SDS gels. Commercial sources of purified, rabbit IgG (Zymed and Research Diagnostics, Flanders, NJ) served as standards to determine IgG concentrations.

Immunohistochemistry. Histological sections (5 μm) were prepared from Bouins-fixed, paraffin-embedded testes of 22-day-old rats. Testis sections were deparaffinized, washed in PBS, treated with pepsin (23 units per ml) for 15 min at 37°C, washed in PBS, and incubated at 80°C in 0.01 M sodium citrate, pH 6 for 30 min. Sections were washed with PBS once and incubated in blocking buffer [CAS blocking reagent (Zymed) plus 10% goat serum] for 30 min at 22–24°C. Sections then were incubated overnight at 4°C with 10 nM of each IgG preparation (PI-3, PI-4, anti-DAZL-3, anti-DAZL-4, and commercial rabbit IgGs) in blocking buffer. Sections were washed three times with PBS and incubated at 22–24°C for 45 min with AlexaFluor 488, goat anti-rabbit IgG (Molecular Probes) at a dilution of 1:150 in PBS alone or in combination with Cy3-conjugated, anti-vimentin monoclonal IgG (clone V9, Sigma) diluted 1:200 in PBS. Sections were washed three times in PBS and mounted using Gel/Mount (Electron Microscopy Sciences, Fort Washington, PA).

Immunocytochemistry. Medium was removed from culture dishes (Costar, Fisher) or chambered slides (Perminox, Nunc) and replaced with Hoechst 33342 (10 μg/ml) solution in Hepes-buffered saline solution (HBSS: 135 mM NaCl/5 mM KCl/1 mM MgSO4/1.8 mM CaCl2/10 mM Hepes). Cultures were incubated for 20 min at 32.5°C in Hoechst 33342 solution, washed once with HBSS, and fixed for 90 s on ice by using cold methanol. Isolated testis cell populations (GC, ColNb, and LamNB, see Fig. 3) required washing and plating in serum-free DHI2 to permit binding (30 min at 32.5°C) to culture dishes before Hoechst 33342 labeling and fixation. Fixed cultures were washed twice with HBSS, incubated in blocking buffer (see immunohistochemistry above) for 1 h, and then incubated overnight with primary IgGs diluted in fresh blocking buffer at ~10 nM. Unbound IgGs were removed by three washes with PBS, then treated with secondary reagents and mounted as described for immunohistochemistry. Incubations and wash steps after fixation were at 22–24°C. The percentage of cells labeled with Hoechst 33342 plus anti-DAZL-3 or plus anti-vimentin IgGs were scored by using an IX70 inverted fluorescent microscope (Olympus, New Hyde Park, NY) with SIMPLEPCI imaging software (C-Imaging Systems, Compix, Cranberry Township, PA).

The average number of cells labeled with each reagent per experiment was determined from eight fields (20–200 cells per field). The sum of DAZL/Vimentin−/imm− cells plus vimentin−/DAZL−/imm− cells collected per cellular population represented 99.3 ± 4.2%, 101.3 ± 4.4%, and 99.5 ± 3.2% of the Hoechst 33342-labeled nuclei collected per well when using ColNb, LamNB, and Lam cells, respectively (± SEM, n = 4, 6, and 6, respectively).

Western Blot Analysis. Proteins were extracted from tissues or cultured cells with lysis buffer: 1% Triton X-100, protease inhibitor tablet, 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 50 mM Hepes. Supernatant fluids obtained after centrifugation of lysates at 14,000 × g at 4°C were used for Western blotting. Protein (~50 μg) was separated on SDS gels (10–20% acrylamide gradient, Invitrogen) and transferred to nitrocellulose membranes. Nonspecific, protein-binding sites were blocked by incubating membranes overnight at 4°C in blotting buffer: Tris-buffered saline with Tween-20 (TBST: 10 mM Tris-HCl, pH 7.5/150 mM NaCl/0.1% Tween-20) containing 5% nonfat dry milk. Membranes were washed three times in TBST and incubated for 1 h at 22–24°C with a 1:1,000 dilution of primary antibody in blotting buffer. Membranes were washed three times in TBST (0.3% Tween-20) and incubated for 45 min at 22–24°C with peroxidase-conjugated, anti-rabbit IgG diluted 1:50,000 in blotting buffer. Membranes were washed three times in TBST; protein bands were detected by using the enhanced chemiluminescence detection method (ECL, Amersham Pharmacia).

Germ Cell Transplantation. WT Sprague–Dawley rats (Harlan Breeders, Indianapolis) at 10–12 days of age were injected (i.p.) with 11.5 or 12.5 mg/kg busulphan (4 mg/ml in 50% DMSO) to generate germ cell-depleted males that were used as recipients 12–14 days after treatment. Donor cell suspensions isolated from cultures of testis cells were established from homozygous MTlacZ, or Rosa-EGFP transgenic rats were loaded into injection needles fashioned from 100-μl glass capillary tubes and then transplanted into seminiferous tubules of anesthetized rats by retrograde injection through the rete testes (12). Recipients were analyzed for donor cell colonization by 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) staining of testes (9) and/or direct visualization of EGFP expression in anesthetized animals after surgical exposure of testes by using a fluorescent Nikon SMZ1500 stereomicroscope. Acquired images were analyzed by color thresholding on blue regions of seminiferous tubules observed after X-Gal staining (17) by using SIMPLEPCI imaging software to determine the area of donor cell colonization per testis.
Derivation of Transgenic Rat Strains. Male recipients from select transplantation studies were paired at 12 weeks of age (60 days after transplantation) with WT Sprague–Dawley rats of similar age. Genotypes of all pups born were verified by Southern analysis and RT-PCR by using rat-tail genomic DNA to determine the presence of the MT-lacZ and/or lentiviral EGFP reporter genes. DNA was digested with the restriction endonucleases NcoI and BamHI, which cut within each transgene at single sites on the 5' side of sequences used for probes (see legend to Fig. 5). The relative MT-lacZ transgene copy number in the F2 progeny was determined by dot blot hybridization of probe (lacZ ORF) to DNA spotted on Hybond N+ membranes (Amersham Pharmacia). Concentrations for DNA samples were determined by using PicoGreen reagent (Molecular Probes).

Results

Isolation of Germ Cells from Testis Cell Cultures. Spermatogenic cells were purified from primary cultures by using established methods. Antisera generated to the germ-line-specific gene product, DAZL, was used to distinguish spermatogenic cells from somatic cells (19, 20). The antisera selectively labeled all germ cell populations in testis sections from young rats (Fig. 1A).

Fig. 1. Detection of spermatogenic cells in testis sections and cultures. (A Left) Hematoxylin- and eosin-stained section from testis of 22-day-old rat. (Center) Adjacent section labeled with anti-DAZL3 (green) plus anti-vimentin IgGs (red). (Right) Adjacent section labeled with preimmune (green) plus anti-vimentin IgGs (red). (Bar = 150 μm.) (B Upper Left) Testis cell culture from 22-day-old rat showing Dazl immunoreactivity (green) in germ cells on top of anti-vimentin-labeled somatic cells (red). (Upper Right) Preimmune (green) plus anti-vimentin IgGs (red). (Lower Left and Right) Bright-field microscopy of cultures at Upper Left and Right, respectively. (Bar = 40 μm.)

Fig. 2. A germ cell-enriched fraction isolated from testis cell cultures. (A Left) Testis cell cultures on plastic (21-day-old animals); germ cells (green arrows) overlie the somatic cells (red arrows). (Center) Somatic cell monolayer after removal of germ cells from testis cell cultures. (Right) Germ cells (green arrows) harvested from testis cell cultures shown 4 h after plating on collagen I. Somatic cells (red arrows) bind to the collagen I matrix. (Bar = 40 μm.) (B) Immunoblot detection of DAZL in rat tissues: L = liver, Lg = lung, K = kidney, In = small intestine, H = heart, Ep = epididymis, T = testis, GC = germ cell culture, SC = somatic cell culture. The blot was probed with DAZL3 IgG (Upper) and then stripped and probed again with anti-α-tubulin IgG (Lower).

Fig. 3. Purification of male germ cells by selection on extracellular matrices. (A) Percent anti-DAZL and anti-vimentin immunopositive testicular cells obtained following selection on plastic, collagen I (Col), and laminin (Lam). Testis Cells, initial testis cell population from ~65-h cultures on plastic; Somatic Cells, on plastic after removal of germ cells; Germ Cells, germ cell-enriched population isolated from testis cells; ColB, cells from germ cell-enriched population that did bind to Col; ColNB, germ cell-enriched population that did not bind to Col; LamB, cells from ColNB that did not bind to Lam; LamNB, cells from ColNB that did bind to Lam. Bars represent mean % ± SD, n = 4–7. (B) DAZL (green) and vimentin (red) expression in LamB cells. (Upper Left) DAZL3 plus vimentin IgGs; (Bottom Left) Preimmune plus vimentin IgGs. Asterisk denotes vim+ somatic cells. (Right) Respective bright-field images for panels at Left. (C) DAZL (green) and vimentin (red) expression in LamNB cells. (Upper Left) DAZL3 plus vimentin IgGs; (Bottom Left) Preimmune plus vimentin IgGs. (Right) Respective bright-field images for panels at Left.
culture, the antisera specifically detected germ cells (DAZL⁺) lying over a monolayer of vimentin-positive (vim⁻) somatic cells (Fig. 1B; ref. 21). The DAZL⁺ population was harvested from the monolayer independent of most somatic cells; the vim⁻ cells remained bound to the culture dish (Figs. 2A and B). By immunocytochemistry, the germ cell-enriched fraction contained 83 ± 8% DAZL⁺/vim⁻ cells (Fig. 3A). Somatic cells in the germ cell-enriched population bound avidly to collagen-I (ColB cells), whereas germ cells did not (ColNB cells; Figs. 2 and 3A). This step provided a ColNB population consisting of 91 ± 4% DAZL⁺/vim⁻ cells (Fig. 3A) that was subsequently tested in vivo for spermatogenic stem cell activity.

**Recipient Males Generate a High Proportion of Pups from Donor Cells.** The ColNB population contained cells that displayed spermatogenic developmental capacity after transplantation to seminiferous tubules of WT rats (Table 1). Crosses between males transplanted with the ColNB cells and WT females indicated

### Table 1. Cultured spermatogenic cells colonize and produce mature spermatozoa capable of fertilization

<table>
<thead>
<tr>
<th>Recipient*</th>
<th>Donor cells</th>
<th>Days to analysis</th>
<th>Gram wt per R, L testis</th>
<th>Area β-gal⁺/ R, L testis</th>
<th>Testis EGFP expression†</th>
<th>Days to first litter</th>
<th>Litters</th>
<th>Total born</th>
<th>Pups from donor sperm, % total</th>
<th>Pups with LvTg, % total</th>
</tr>
</thead>
<tbody>
<tr>
<td>R41</td>
<td>ColNB</td>
<td>190</td>
<td>0.6, 0.5</td>
<td>22.1, 9.9</td>
<td>n.a.</td>
<td>126</td>
<td>4</td>
<td>37</td>
<td>27 (73)</td>
<td>n.a.</td>
</tr>
<tr>
<td>R43</td>
<td>ColNB</td>
<td>215</td>
<td>0.9, 0.6</td>
<td>14.0, n.i.</td>
<td>n.a.</td>
<td>135</td>
<td>3</td>
<td>18</td>
<td>5 (28)</td>
<td>n.a.</td>
</tr>
<tr>
<td>R45</td>
<td>ColNB</td>
<td>258</td>
<td>0.8, 0.3</td>
<td>12.5, 0.7</td>
<td>n.a.</td>
<td>136</td>
<td>3</td>
<td>22</td>
<td>9 (41)</td>
<td>n.a.</td>
</tr>
<tr>
<td>R51</td>
<td>LamB</td>
<td>190</td>
<td>0.3, 0.3</td>
<td>0.2, n.i.</td>
<td>+</td>
<td>n.p.b.</td>
<td>0</td>
<td>0</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>R52</td>
<td>LamB</td>
<td>215</td>
<td>0.5, 0.2</td>
<td>17.2, n.i.</td>
<td>+ ++</td>
<td>127</td>
<td>4</td>
<td>44</td>
<td>26 (59)</td>
<td>13 (30)</td>
</tr>
<tr>
<td>R53</td>
<td>LamB</td>
<td>190</td>
<td>0.2, 0.3</td>
<td>0.4, n.i.</td>
<td>+</td>
<td>n.p.b.</td>
<td>0</td>
<td>0</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

*Recipient* males were treated with 11.5 mg/kg of busulphan at 10 days of age and used as recipients at 23 days of age.
†Recipient males were treated with 11.5 mg/kg of busulphan at 10 days of age and used as recipients at 23 days of age. Recipient Males Generate a High Proportion of Pups from Donor Cells. The ColNB population contained cells that displayed spermatogenic developmental capacity after transplantation to seminiferous tubules of WT rats (Table 1). Crosses between males transplanted with the ColNB cells and WT females indicated

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**Fig. 4.** Enrichment of germ-line stem cells. (A) Expression of donor-cell transgene in seminiferous tubules of testes from a WT recipient rat R41, 190 days after transplantation with ~2 × 10⁵ ColNB cells per testis (Left). Blue areas in tubules represent colonization by donor cells visualized after X-Gal staining. Expression of β-galactosidase in testes of representative F1 progeny from R41 and a WT female rat that did not inherit (Center) or that did inherit (Right) the donor transgene. (Bar = 1 cm.) (B) Number of ColNB, LamNB, and LamB cells obtained from rat testis cell cultures after selection on collagen-I and laminin. Bars represent ± SD, n = 6 cultures. (Upper Right) Relative SSC activity in ColNB, LamNB, and LamB testis cell populations. Recipients were injected with 2 × 10⁵ ColNB, 1 × 10⁵ LamNB, or 1 × 10⁵ LamB cells per testis. Blue areas in tubules represent colonization by donor cells visualized after staining with X-Gal 85 days after transplantation (ColNB 10.4 ± 5.1, n = 3; LamNB 0.24 ± 0.52, n = 6; LamB 8.9 ± 5.2, n = 4; ± SEM mm² per testis). Bars represent normalized values for area of donor cell colonization per testis by 10⁵ cells transplanted. (Lower) Illustration of donor cell colonization in testes of recipient rats injected with −10⁵ LamNB (Left) or −10⁵ LamB (Right) cells per testis. (Bar = 1 cm.) (C) Spermatogonial stem cell activity of LamB cells is maintained after time in culture with STO fibroblasts. (Left) Fluorescent (Upper) and bright-field (Lower) images of WT rat testis 40 days after transplantation with −10⁵ LamB cells freshly isolated from Rosa-EGFP rat testis cell cultures. (Right) Seminiferous tubules from WT rat testis 31 days after transplantation with −10⁵ LamB cells that had been cultured with STO fibroblasts for 4 days (Upper). Contralateral, noninjected testis (Lower). (Bar = 1 mm.) (D) Cross-section through seminiferous tubule (stage V) of recipient rat displaying donor cell-derived spermatogenesis (~10⁵ LamB cells used as donor cell population). Analysis was at 85 days after transplantation. (Left) Culture of LamB cells isolated from MT-lacZ rats (~1.5 × 10⁵ cells per cm²) before treatment with lentivirus and transplantation into recipient rat testes. (Bar = 40 μm.)
that donor haplotypes were transmitted to 41 of 77 pups (n = 10 litters; Table 1). Hemizygous F1 progeny from donor-derived spermatooza appeared normal, expressed β-galactosidase in testes (Fig. 4A), and transmitted the MT-lacZ gene at Mendelian ratios to the F2 progeny (48 pups, 4 litters; 21% −/−, 52% −/+ , 27% +/+ ). Thus, SSCs isolated after 3 days in culture as the ColNB population developed into mature, functional spermatooza.

**Enrichment of SSC Activity from Testis Cell Cultures.** The SSC activity in the ColNB population was enriched by selection on culture dishes coated with laminin. Selection of ColNB cells on a laminin matrix provided two distinct germ cell populations: cells that bound to laminin (LamB) and cells that did not bind (LamNB) (Fig. 3A). The LamB population was 97 ± 1% DAZL+/vimentin− (Fig. 3A and B) and consisted of single or paired cells representing 4.7 ± 1% of the total ColNB suspension (Fig. 4B). The LamNB cells were 98.7 ± 0.2% DAZL+/vimentin− (Fig. 3A and C) and were 20 times more abundant than the LamB cells within the ColNB population (Fig. 4B). Based on the detectable area of donor cell colonization per rat testis (X-Gal staining), the LamB population was enriched ~30 fold for SSC activity when compared with the same number of LamNB cells transplanted per testis (Fig. 4B). Testes receiving ~106 LamB cells per testis showed a similar extent of X-Gal staining as rats receiving 20× the number of ColNB cells per testis (Fig. 4B), a level capable of transmitting the donor transgene to over 50% of progeny (Table 1). Further, LamB cells from Rosa-EGFP transgenic rats maintained apparent stem cell activity for 4 additional days after isolation when cultured with STO fibroblasts, equaling a total of 7 days in culture (Fig. 4C). Rosa-EGFP rats express high levels of EGFP specifically in the germ line (J. T. Cronkhite and R.E.H., unpublished data). Thus, based on differential expression of DAZL and vimentin, two relatively pure germ cell populations were obtained by selection of testis cells on plastic, collagen-1, and laminin. The spermatogonic potential and degree of purity of the LamB cells provided a population of spermatoozoal progenitors for testing in gene delivery studies (Fig. 4D).

**Germ-Line Transmission by Lentivirus-Transduced SSCs from Culture.** To determine whether cells within the LamB population could be genetically modified and used to produce transgenic rats, cells were transduced in culture on laminin matrix with VSVG-pseudotyped lentiviral vector (16), then transplanted to a single testis in each of three recipient rats. The proviral EGFP-reporter gene was expressed in the seminiferous tubules of all three recipient testis (Fig. 5A; Table 1). Two recipients, R51 and R53, did not produce litters after pairing with female rats, which correlated with low levels of donor cell colonization per testis (Table 1). One recipient, R52, did regain fertility ~100 days after transplantation and sired four litters with four different WT females (Table 1). In total, 26 of 44 pups were positive for the lacZ transgene by Southern analysis, confirming derivation from donor, LamB cells (Table 1). One (eight pups) or two copies (five pups) of the lentiviral transgene were transmitted per haploid genome to 13 of 26 F1 pups (Fig. 5B and C), which further transmitted viral integrants to ~50% of F2 progeny (Fig. 5D). Expression of EGFP could be visualized in the skin of only one transgenic rat (Fig. 5E), a finding consistent with weak expression of transgenes under the control of the CMV promoter in animals transduced by this vector type (22). Thus, by transplantation of ~106 lentivirus-treated, LamB cells into a single testis, 59% of progeny were from donor cells, of which 30% were newly derived strains of transgenic rats (Table 1).
Discussion

Here, male rat germ cells were efficiently transduced in culture by a VSV-G-pseudotyped, HIV-1-based self-inactivating vector. A recent report also demonstrated that mouse male germ cells can be transduced in culture by lentiviral vectors (23). Transgenic mice and rats have been produced by using zygotes and preimplantation embryos transduced with this vector type (6, 28). The direct modification of the genome in the rat germ line by lentiviral-based vectors enabled multiple transgenic rat strains to be produced from a single recipient/founder, with each strain defined by a unique site of viral integration. The potential to generate rats containing viral integrations in biologically important genes provides a prospective genetic approach for gene disruption or transfection with DNA-targeting constructs, a method similar to that used for gene disruption in mouse embryonic stem cells (6). Thus, spermatogenesis developing from the selected populations could directly transmit targeted genomic modifications.

The procedure reported here for producing transgenic rats is relatively easy, requiring only the germ-line culture selection method, the appropriate lentiviral vectors, and subsequent transfer of transduced cells to recipient males. About 30% of pups obtained from a recipient male/WT female cross carry a lentiviral-transduced gene, with integrations in each pup at a different genomic site. The integrations are stable and are transferred to the F2 generation at the expected frequency of 50%.

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