Male and female germline specific expression of an EGFP reporter gene in a unique strain of transgenic rats

Jennifer T. Cronkhite, Carola Norlander, Jenny K. Furth, Göran Levan, David L. Garbers, Robert E. Hammer*

A rat line was generated in which genomic integration of a ROSA-EGFP transgene resulted in exclusive expression of EGFP in the germ cells of both sexes. EGFP expression was uniform and robust in cleavage stage embryos beginning at the late 2-cell stage and continuing through blastocyst development where expression became restricted to cells of the inner cell mass. Subsequent analysis showed high EGFP expression exclusively in primordial, embryonic, and adult germ cells. This unique expression pattern makes this EGFP marked locus the first molecular marker of the germline lineage in both sexes in mammals. FISH was used to localize the transgene insertion to chromosome 11q11–q12, proximal to Grik1 and near Ncam2. Analysis of the region did not identify known germ cell-specific genes but did identify 19 ESTs or transcribed loci present in testes, ovary, or pre-implantation libraries from mice or rats. To assess the utility of the transgenic line for germ cell transplantation studies, non-selected, freshly isolated seminiferous tubule cells were transferred to the testis of recipient males. The donor cell population colonized the testis at a surprisingly high efficiency within 30 days following transfer. Since EGFP is a vital marker, the colonization process can be followed in vivo and the extent of colonization quantified. The unique germ cell specific expression of EGFP makes this line of transgenic rats an excellent novel tool to study germ cell origin, development, and differentiation, and to assess the plasticity of adult somatic stem cells to become male germ cells.

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Introduction

Germ cells have the ability to transmit genetic information from one generation to the next. The precursors to these cells, primordial germ cells (PGCs), were first identified in the mouse and shown to contain high alkaline phosphatase activity (AP). The PGCs form a small group of cells located in the extraembryonic region of the proximal epiblast of E7.25 mouse embryos (Chiquoine, 1954; Ginsburg et al., 1990; Lawson and Hage, 1994), which then migrate from the base of the allantois through the dorsal mesentery to the genital ridges; in the mouse, almost all PGCs reach the genital ridge by E11.5 (Bendel-Stenzel et al., 1998). Within the genital ridge, the PGCs interact with somatic cells to form sex cords that serve as precursors to the seminiferous tubules of the testis and the ovarian follicles. The sex cords can be identified by E13.5 (McLaren, 1992; Wylie, 2002) and develop into the embryonic gonads and finally into the adult testis and ovary.
Although PGCs contain high alkaline phosphatase activity (Chiquoine, 1954; Ginsburg et al., 1990), other molecules such as Oct3/4 (Okazawa et al., 1991; Yoshimiz et al., 1999), SSEA1 (stage specific embryonic antigen 1) (Fox et al., 1981), fragillis (Lange et al., 2003), nanog (Chambers et al., 2003), nanos (Tsuda et al., 2003), vasa (Tanaka et al., 2000), and GCNA (germ cell nuclear antigen) (Enders and May, 1994) also have been suggested as reliable molecular markers for identifying developing germ cells. However, AP, oct3/4, SSEA1, nanog, and fragillis are also expressed in undifferentiated cells of the early mammalian embryo (blastocyst to epiblast in the mouse), and therefore do no distinguish emerging germ cells from other undifferentiated cells of the embryo. Markers such as vasa and GCNA are germ cell specific, but are not expressed during all stages of germ cell development and GCNA is sex-specific. Thus, a single molecule that marks the germ cell lineage throughout development has not been reported in mammals. Here, we describe the generation and characterization of a novel transgenic rat line that expresses EGFP specifically in germ cells during virtually all stages of male and female germ cell development. We refer to this transgenic line as Germ Cell Specific-EGFP (GCS-EGFP).

### Materials and methods

**Construction of ROSA-EGFP transgene and production of transgenic rats**

A 0.8 Kb SalI–BamHI ROSA26 fragment (Zambrowicz et al., 1997) was inserted between the SalI and BamHI restriction sites of the EGFP-N1 plasmid from Clontech to generate the ROSA-EGFP transgene (Kisseberth et al., 1999; Zambrowicz et al., 1997). The 1.8 Kb SalI and AluII ROSA-EGFP fragment was separated from vector DNA by gel electrophoresis and the fragment was isolated from the gel by perchlorate elution. Transgenic rats were produced by microinjection of the 1.8 Kb ROSA-EGFP fragment into the pronucleus of Sprague–Dawley rat eggs as described (Young et al., 1999); 6 transgenic rats were produced. Founders were mated with Sprague–Dawley wild-type (WT) rats and 4 independent lines were established. Genotyping of founders was by dot blot analysis and progeny by either dot blot or PCR analysis of genomic DNA isolated from tail biopsies. PCR was performed using the forward primer EGFP5-1 (5’ AACCTAGGGTGCTAGCTTCGTTG) and the reverse primer EGFP3-1 (5’ TGTGGTTCTGCCTTAGTGTC) corresponding to nucleotides 971 to 1492 of the ROSA-EGFP DNA fragment that amplified a DNA product of 521 bp. Unless otherwise specified, homozygous transgenic rats and WT Sprague–Dawley rats were used for all described experiments. Animals were housed in SPF condition cages with a 12-h light and 12-h dark cycle and fed Teklad Mouse/Rat Diet (Harlan Teklad, Madison, WI) ad libitum.

**Imaging of EGFP fluorescence in embryos**

Prepubertal female rats were superovulated by a standard regimen (Young et al., 1999) and placed overnight with stud males. The presence of sperm in the vaginal lavage or a copulatory plug the following morning indicated mating had occurred and was scored as day E0.5. Pre-implantation embryos were collected on the specified day of development in R1ECM medium (Specialty Media, Phillipsburg, NJ), washed in R1ECM and held in R1ECM drop cultures overlaid with oil until use. Blastocyst implantation was delayed as previously described (McLean-Hunter and Evans, 1999). Briefly, WT females were mated with GCS-EGFP homozygous males and the morning of finding a copulatory plug was designated as E0.5. On E4.5, 50 μg tamoxifen (Sigma-Aldrich, St. Louis, MO) in an aqueous solution of corn oil was injected intraperitoneally and 5 mg Depo-Provera (Upjohn Co., Kalamazoo, MI) was injected subcutaneously. Delayed blastocysts were collected on E8.5 in R1ECM, washed in R1ECM and held in drop cultures in R1ECM until use. Epiblasts were dissected on day E8.5 and held in PBS until use. The genital ridge was dissected from E12.5 and E15.5 embryos in PBS, and gonads were dissected from E19.5 embryos in PBS. Embryos and epiblasts were visualized with a Nikon Eclipse TE2000-U inverted microscope using an EGFP filter. The genital ridges and embryonic gonads were visualized with a Nikon SMZ1500 stereoscope using an EGFP filter.

**Imaging of EGFP fluorescence in adult tissues**

Tissues were either directly visualized for EGFP expression using a Nikon SMZ1500 stereoscope or fixed in 4% paraformaldehyde overnight at 4°C, washed in PBS, placed in 30% sucrose overnight at 4°C to equilibrate, embedded in freezing medium Tissue Tek OCT (optimal cutting temperature) (Sakura Finetek U.S.A., Inc. Torrance, CA) and frozen in a biocooler Histobath 2 (Shandon Lipshaw, NY). Imaging of EGFP fluorescence in mature sperm

Mature spermatozoa were collected from the cauda epididymis of WT and homozygous GCS-EGFP male rats that had mated with a female within the previous 7–10 days. The cauda epididymis was placed into 1 ml of a fertilization medium (Brinster medium for oocyte culture with 30 mg/ml of BSA) under oil and the body of the cauda epididymis was cut and gently squeezed to express
the sperm cells. The spermatozoa were allowed to disperse by swimming out for 1 h during incubation at 37°C in 5% CO₂ atmosphere. An aliquot of the sperm preparation was then placed directly on to a glass slide and viewed using Leica TCS SP2 AOBS: confocal microscope (Leica Microsystems, Wetzlar, Germany).

**Alkaline phosphatase staining**

Epiblasts from E8.5 and genital ridges from E12.5 embryos were isolated by microdissection and fixed in 4% paraformaldehyde for 2 h at 4°C. The tissues were washed three times in PBS, incubated for 1 h in 70% ethanol, and washed three times in distilled water. Tissues were stained with α-naphthyl phosphate/fast red TR (Sigma-Aldrich. St. Louis, MO) for 15 min at room temperature (Ginsburg et al., 1990), mounted on slides, overlaid with 70% glycerol and viewed on a Nikon Eclipse TE2000-U inverted microscope.

**FISH analysis**

Rat embryonic fibroblasts were isolated from E15.5 homozygous embryos by standard procedures (Hogan et al., 1994). Slides for cytogenetic analysis were prepared essentially as previously described (Islam and Levan, 1987). Briefly, cell cultures were treated with 0.2 mg/ml of 5'-bromo-2'-deoxyuridine (BrdU) for 17 h. Subsequently, the cells were washed three times and cultured for 6 h in medium supplemented with 0.05 μg/ml thymidine. Mitotic figures were accumulated by adding 0.05 μg/ml Colcemid (Sigma Aldrich. St. Louis, MO) during the final 30 min, and metaphase cells were harvested by mitotic shake-off; a procedure which selects cells which have entered mitosis and have become morphologically rounded which allows them to become easily detached from the culture plate by gentle shaking. The cells were resuspended in 0.07 M KCl at room temperature for 10 min, washed, and fixed in three dilutions of methanol:acetic acid (9:1, 5:1, and 3:1). Dual-

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**Fig. 1. EGFP is expressed in spermatozoa and eggs in GCS-EGFP rats.** (A–H) Germ cells from GCS-EGFP rats; (A–D) DIC photomicrographs and (E–H) fluorescent photomicrographs of the same specimens as in panels (A–D). (A, E) Isolated seminiferous tubules dissected from the testis, (B, F) cauda epididymal sperm cells, (C, G) unfertilized eggs in the associated cumulus masses, and (D, H) a denuded unfertilized, metaphase-II egg. (I–P) Germ cells from WT Sprague–Dawley rats; (I–L) DIC photomicrographs and (M–P) fluorescent photomicrograph of the same specimens in panel (I–L). (H and P) Eggs were counterstained with Hoechst 33442 to visualize DNA (blue).
color FISH analysis was performed using a biotinylated (Nick Translation Systems, GibcoBRL, Carlsbad, CA) BAC DNA probe for the \textit{Grik1/Ncam2/Kcjn6/Mx1} genes and a digoxigenin-11-dUTP labeled (DIG-NICK Translation Mix, Roche Diagnostics GmbH, Mannheim, Germany) DNA probe for \textit{EGFP} (Behboudi et al., 2002). Approximately 500 ng of the co-precipitated probes along with about 15-fold excess of sonicated total rat genomic DNA were co-hybridized to each slide. Detection of the dual-color labeling was performed using a mixture of Rhodamine-conjugated antidigoxigenin and FITC-conjugated avidin (Invitrogen/ GibcoBRL Carlsbad, CA). The chromosome preparations

<table>
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<th>Tissue Survey</th>
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<tr>
<td><strong>Line GCS-EGFP</strong></td>
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<tr>
<td>Testis</td>
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<tr>
<td>Ovary</td>
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<tr>
<td>Muscle</td>
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<td>Jejunum</td>
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<td>Heart</td>
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<td>Liver</td>
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<td>Kidney</td>
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Fig. 2. EGFP expression is specific to germ cells in the GCS-EGFP line. Tissues (testis, ovary, muscle, jejunum, heart, brain, liver, and kidney) from adult rats were fixed, snap frozen, sectioned, and imaged using an EGFP fluorescent filter and photographed. Adjacent sections were stained with H&E. In GCS-EGFP rats, only germ cells in the seminiferous tubules in the testis and eggs in the ovary express EGFP. There was very little auto fluorescence in the tissues from the WT Sprague–Dawley rats. EGFP was expressed in a subset of cells in all of the tissues examined in the 7-10 ROSA-EGFP transgenic line.
were washed, counter-stained, and the fluorescence signals were visualized as described previously (Helou et al., 1999).

Quantitative real-time PCR

Total cellular RNA was isolated from multiple organs of WT rats, homozygous GCS-EGFP rats, and line 7–10 rats using RNA Stat-60 (Tel-Test, Friendswood TX). One microgram of total RNA was reverse transcribed using random primers and Superscript III reagents (Invitrogen, Carlsbad, CA). Samples were diluted 1:10 and 3 μl was used for the PCR reaction. The PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and EGFP primers: forward EGFP2-5′ GGGCACAAGCTGGAGTACAAC and reverse EGFP2-3′ TCTGCTTGTCGGCCATGATA, which were designed in Primer Express Ver. 2.0. Real-time PCR analysis was performed with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), and the PCR reaction was analyzed using the Sequence Detection System Ver. 2.1. For expression analysis, all samples were normalized to the ribosomal RNA 18S signal and the expression of EGFP in transgenic tissues was compared to the background signal in WT testis.

Seminiferous tubule cell isolation from testis

Seminiferous tubules were isolated from the testes of 23 day-old homozygous GCS-EGFP rats. The tubules were mechanically disaggregated and enzymatically digested with dispase (Invitrogen/GibcoBRL Carlsbad, CA), disso- ciated into a cellular suspension, and filtered through a 20 μm nylon mesh. Cells were then counted and resuspended at a concentration of 1 × 10⁷ cells/ml (Hamra et al., 2004).

Germ cell transplantation

Twelve day-old WT Sprague–Dawley male rats were injected intraperitoneally with 12.5 mg/kg of busulfan (4 mg/ml in 50%DMSO) and used as recipient males at 24 days of age. Donor cells were loaded into an injection needle fashioned from a 100 μl glass capillary microcaps (Cole-Parmer Instruments Co., Vernon Hills, IL) and cells were transplanted into the seminiferous tubules of an anesthetized recipient rat by retrograde injection through the rete testis (Hamra et al., 2002; Ogawa et al., 1997). Trypan blue was added to the cell suspension to visualize transfer into the tubules. Recipient rats were analyzed for donor cell colonization on day 30 or 60 after transfer by direct visualization of EGFP expression using a fluorescent Nikon SMZ1500 stereomicroscope. The seminiferous tubules were dissected from the testis and processed for the quantitation of soluble GFP as described (Hamra et al., 2004). The testis lysates were assayed for fluorescent intensity using recombinant EGFP with a carboxyl-terminal histidine tag as a standard.

Results

Generation and characterization of ROSA-EGFP transgenic rat lines

We generated four lines of ROSA-EGFP transgenic rats, designated as lines Hsd:SDTgN (ROSA-EGFP) 2–4, 4–2, 7–9 and 7–10 Reh; three of which contained the transgene on autosomes and one (7–9) that harbored the transgene on the Y chromosome. The murine ROSA promoter sequences used in the EGFP reporter transgene to target expression had been used in both transgenic mice and rats (Kisseberth et al., 1999; Zambrowicz et al., 1997) to drive nearly ubiquitous expression of reporter genes. We expected a similar expression pattern for our ROSA-EGFP transgenic rats. In two (lines 4–2 and 7–9) of the four lines, one with an autosomal integration and the other Y- linked, there was no apparent EGFP expression in any of the 15 tissues examined, either macroscopically or micro- scopically. In the 7–10 line, EGFP was expressed in every organ examined, but the abundance of EGFP fluorescence varied between organs and often appeared cell-specific within an organ (Fig. 2). In the fourth line (line 2–4, designated GCS-EGFP), expression of EGFP appeared limited to the germ cells (Fig. 1). The two lines without obvious transgene expression were discarded and the other two were bred to transgene homozygosity. Progeny from both lines develop normally and do not display obvious abnormalities associated with transgene insertion or expression.

We characterized the EGFP expression pattern in lines 7–10 and GCS-EGFP more extensively. In the GCS-EGFP

<table>
<thead>
<tr>
<th>Tissue Transgenic lines</th>
<th>GCS-EGFP</th>
<th>Lines 7–10</th>
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<tr>
<td>Skeletal muscle (soleus)</td>
<td>Bkg</td>
<td>2.7</td>
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<tr>
<td>White fat</td>
<td>Bkg</td>
<td>5.6</td>
</tr>
<tr>
<td>Liver</td>
<td>Bkg</td>
<td>5.8</td>
</tr>
<tr>
<td>Jejunum</td>
<td>Bkg</td>
<td>5.7</td>
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<tr>
<td>Colon (proximal)</td>
<td>Bkg</td>
<td>5.6</td>
</tr>
<tr>
<td>Spleen</td>
<td>Bkg</td>
<td>8.6</td>
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<tr>
<td>Kidney</td>
<td>Bkg</td>
<td>7.1</td>
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<tr>
<td>Heart (right ventricle)</td>
<td>Bkg</td>
<td>6.9</td>
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<tr>
<td>Submandibular</td>
<td>Bkg</td>
<td>1.5</td>
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<tr>
<td>Brain (frontal lobe)</td>
<td>Bkg</td>
<td>5.2</td>
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<tr>
<td>Stomach</td>
<td>Bkg</td>
<td>6.9</td>
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<tr>
<td>Ovary</td>
<td>5.1*</td>
<td>8.6</td>
</tr>
<tr>
<td>Testis</td>
<td>11.7</td>
<td>9.6</td>
</tr>
<tr>
<td>Epididymis (cauda)</td>
<td>6.1</td>
<td>9.7</td>
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Total RNA was subjected to real-time quantitative PCR as described in Materials and methods. Each value represents the ratio of EGFP mRNA relative to the background (Bkg) value in WT. * 6 week-old GCS-EGFP females were injected intraperitoneally with 20 units of Gestyl (Organon Pharmaceuticals, West Orange, NJ) sacrificed and ovaries collected 72 h after treatment.
rats, EGFP fluorescence was not detectable in the brain, heart, jejunum, kidney, liver, or skeletal muscle (Fig. 2). EGFP expression was robust in ovulated unfertilized eggs (Figs. 1G and H) and in adult male germ cells including mature sperm cells (Figs. 1E and F). Expression of EGFP mRNA, analyzed by quantitative PCR, demonstrated that expression of EGFP was confined to the testis and ovary (not present in muscle, fat, liver, small intestine, large intestine, spleen, kidney, heart, submandibular, brain, and stomach) (Table 1). In line 7–10, EGFP expression was observed in all tissues examined (Fig. 2); there was robust EGFP fluorescence in the testis, ovary, and kidney. Expression in the testis was evident in germ, Sertoli and Leydig cells. In the ovary, EGFP fluorescence was substantial in both germ and cumulus cells. Quantitative PCR of RNA isolated from this subset of organs confirmed the expression of EGFP and identified the ovary, testis, epididymis, and spleen as the sites of highest EGFP expression (Table 1). Given the unique pattern of germ cell specific expression in the GCS-EGFP line, we performed a more thorough examination of the EGFP expression pattern and characterized the site of transgene insertion.

### Characterization of the transgene insertion and assignment of the transgene chromosomal position

Based on the nearly ubiquitous EGFP expression pattern in line 7–10 and on previously described ubiquitous expression patterns of ROSA-EGFP and ROSA-alkaline phosphatase in mice and rats (Kisseberth et al., 1999), we surmised that the unique germ cell-specific expression in line 2–4 was due to the chromosomal position of the transgene. As a first approach to identify the DNA regulatory regions that target the germ cell lineage, and to ultimately identify the structural gene that marks this lineage, we used two PCR based methods, the Universal Genome Walker Kit (BD Biosciences, San Jose, CA) and inverse PCR on the 3′ transgene sequence. Using both methods, we were able to obtain at most, 50 bp of flanking sequence; this was insufficient to identify a unique sequence in the rat genomic database. Most transgenes generated by microinjection are present in multiple copies and may contain small stretches of endogenous DNA between copies of the transgene (Palmiter and Brinster, 1986). A genomic dot blot analysis
of the transgene insertion using an EGFP probe indicated that 5 copies of the transgene were incorporated in the genome. Southern analysis using probes to the EGFP and SV40 regions of the transgene indicated that there were two copies oriented in a tail–tail pattern (data not shown). With multiple copies of the transgene present, PCR based methods to identify flanking DNA are often less effective due to multiple priming sites.

To determine the chromosomal location of the transgene, fluorescence in situ hybridization (FISH) analysis was performed on GCS-EGFP homozygous colcemid-treated embryonic fibroblasts. The chromosome preparation was spread onto slides and an EGFP probe was used for hybridization (Fig. 3A). The transgene was assigned to chromosome 11 in the q11–q12 region. To narrow the region, dual hybridization was performed using an EGFP probe and a BAC clone probe that contained a known gene on chromosome 11; (Mx1) (Fig. 3B). The EGFP signal was proximal to Mx1. Another BAC clone containing Kcnj6, a known gene on rat chromosome 11 was also used as a probe and the EGFP was proximal to Kcnj6 (data not shown). We then chose two other genes that were proximal to Mx1 and Kcnj6, namely Ncam2 (Fig. 3C) and Grik1 (data not shown). The EGFP insertion site was proximal to Grik1 and in very close proximity to Ncam2 (Fig. 3D).

**Transgene is expressed in the epiblast, genital ridge, embryonic gonads, and adult germ cells**

We examined the EGFP expression pattern in post-implantation embryos (E8.5–E19.5) by fluorescence microscopy. EGFP fluorescence was first observed in the proximal epiblast with a small population of cells displaying strong fluorescence on E8.5 (Figs. 4A and I); these are conceivably primordial germ cells (PGC). In E13.5 embryos, the genital ridge (Figs. 4B and J) showed strong fluorescence while the adjacent mesonephros was devoid of an EGFP signal. In E15.5 embryos, the male and female genital ridges can be distinguished from each other in that the male genital ridge (Figs. 4D and L) exhibits a distinct
According pattern while the female ridge is mottled (Figs. 4C and K). The genital ridges from both sexes of the GCS-EGFP rats were strongly fluorescent. The male and female embryonic gonads (Figs. 4M and N) also showed strong EGFP fluorescence while the surrounding somatic support tissues were negative. EGFP fluorescence in the adult ovary of GCS-EGFP rats was confined to eggs in all stages of maturation (Figs. 4G and O) while in the adult testis EGFP fluorescence was robust only within the seminiferous tubules (Figs. 4H and P).

As PGCs are identified principally by location, morphology and high alkaline phosphatase activity, we designed experiments to determine if there was concordance between cells showing GFP expression and cells expressing high alkaline phosphatase activity. Cells in the proximal region of the epiblast and cells in the genital ridge had very high alkaline phosphatase activity (Figs. 5B and D), and this same region also displayed abundant number of cells with robust expression of EGFP (Figs. 5A and C). Thus, early and late primordial germ cells in the GCS-EGFP rat appear to express EGFP.

Transgene expression in pre-implantation embryos

We characterized EGFP expression in pre-implantation embryos to determine the temporal and spatial patterns of expression during early germ line delineation. To accomplish this, homozygous females and males were mated to WT rats and embryos were collected at 1-cell, 2-cell, 4-cell, morula, and blastocyst stages of development and examined for EGFP fluorescence. In fertilized eggs collected from transgenic females, there was uniform robust EGFP expression from the 1-cell to the morula stages of development (Fig. 6A, parts a–f and h–m). There was a significant, reproducible increase in the expression of EGFP at the late 2-cell stage onward, the known time of transition from maternal to zygotic based transcription (Zernicka-Goetz, 1994). The initiation of EGFP expression at the late 2-cell stage was confirmed in embryos collected from WT females mated with transgenic males. In eggs from this cross, there is no maternal EGFP message or protein and thus we could definitely establish the onset of transgene expression. There was no EGFP fluorescence in either the one or early 2-cell eggs (Fig. 6B, parts a–b and h–i). In contrast, there was weak but discernable expression in both blastomeres of the late 2-cell stage eggs (Fig. 6B, parts c and j) indicating that transgene expression is initiated during the earliest period of zygotic transcriptional activation.

Somewhat surprisingly, expression in the early to mid blastocyst stage of development was localized to both the inner cell mass and trophoblast (Figs. 6A and B, parts g and n). This non-ICM restricted pattern of expression may be due to active transcription of the transgene in these two compartments or more likely, may be a reflection of the extended half-life of EGFP (~20 h). To discriminate between these two possibilities, we induced delayed implantation in WT females mated with transgenic males, collected blastocysts at E8.5 and examined them for the localization of EGFP expression. In all of the blastocysts...
examined ($n = 50$), EGFP fluorescence was localized exclusively to the ICM with no discernable expression in trophoectoderm (Fig. 6D, parts a–c). This result strongly suggests that EGFP expression is limited to the ICM, the sole derivative of the germ cell lineage.

Transfer of GCS-EGFP seminiferous tubule cells to recipient testes

Unsorted seminiferous tubule cells from GCS-EGFP rats (Figs. 7A and B) were transferred to the testis of recipient

Fig. 7. Disaggregated seminiferous tubule cell preparations from GCS-EGFP rats colonize the seminiferous tubules of wild-type rats depleted of endogenous germ cells. (A) Unsorted seminiferous tubule preparation from a GCS-EGFP homozygous male. (B) Only a subset of unsorted seminiferous tubule cells shown in panel (A) express EGFP and are germ cells. (C) Recipient testis 60 days post-transfer. (D) Fluorescent photomicrograph of specimen shown in panel (C) showing extensive colonization by GCS-EGFP germ cells. (E) Quantitation of the abundance of GFP in recipient testis 30 days ($10^{-1}$, $10^{-2}$, $10^{-4}$, $10^{-5}$, $10^{-7}$, $10^{-9}$) and 60 days ($8^{-2}$, $8^{-3}$, $8^{-4}$) following transfer. Values represent the quantity of extracted GFP/testis. NT (uninjected testis).
males to establish the efficiency of this heterogenous population of cells to take residence in a recipient testis. Colonization efficiency is the measure of the inherent stem cell activity of a given population of cells and presumably the germline stem cells constitute a very small proportion of the total seminiferous tubule cell preparation of an adult testis (Brinster and Zimmermann, 1994; Dobrinski et al., 2000; Hamra et al., 2002; Nagano and Brinster, 1998). Recipient males were sacrificed at either 30 or 60 days following cell transfer, the testis examined by fluorescent microscopy and the entire tubular mass processed for quantification of the abundance of GFP. The testis from all recipient males that were sacrificed 30 days after receiving seminiferous tubule cells from GCS-EGFP rats had abundant GFP expressing cells distributed throughout the tubules (Figs. 7C and D), and had quantities of GFP/testis that ranged between 200 and 300 ng. Extending the time of colonization from 30 to 60 days approximately doubled the abundance of GFP per testis (Fig. 7E).

Discussion

In this study, we describe and characterize a line of transgenic rats in which a reporter transgene is fortuitously expressed exclusively in the germ cell lineage in both males and females. We have established that expression is initiated at the late 2-cell stage of embryogenesis and is localized to blastomeres of cleavage stage eggs, cells in the ICM of the blastocyst, proximal epiblast, and in primordial, embryonic, and adult germ cells. We have localized the transgene insertion site to rat chromosome 11q11–q12, a region that interestingly harbors a number of testes-specific ESTs and transcribed loci (Online Supplementary Table 1 in Appendix A). We have shown that the EGFP marked locus makes this strain of rats a powerful tool for the study of germ cell origin, development and migration, and potentially for the derivation of rat embryonic stem (ES) cells, germline stem (GS) cells (Zwaka and Thomson, 2005), and embryonic germ (EG) cells.

The generation of transgenic animals expressing a reporter gene cassette such as EGFP, LacZ, or growth hormone is a very common strategy for defining and characterizing the regulatory regions of a gene that direct tissue or cell specific expression (Hadjantonakis and Nagy, 2001; Sasaki and Hogan, 1996). In most instances, the random integration of a transgene into the host genome does not alter expression of the neighboring genes, and therefore does not perturb the normal physiology of the transgenic animal (Tidhar et al., 2001). However, in approximately 10% of transgene insertions, the integration event seems to disrupt the expression of endogenous genes, leading to unexpected phenotypes most often discernable when the line is bred to homozygosity (Palmiter and Brinster, 1986). A second phenomenon common to transgene insertions generated by microinjection is improper expression of a transgene due to the influence of neighboring strong regulatory elements. This generally occurs when one uses “ubiquitous” or minimal promoters to drive transgene expression. The ROSA promoter sequences used in our transgene are known to drive the reporter genes, EGFP and alkaline phosphatase in a ubiquitous fashion (Kisseberth et al., 1999), and thus we assume that the novel pattern of transgene expression in the GCS-EGFP line is due to positional effects.

Currently, there are no known markers for germ cells that are either expressed throughout germ cell development or that are exclusive to the germ cells. Markers such as vasa, oct4, and alkaline phosphatase identify germ cells, but are somewhat of limited value in that they either are not germ cell specific or they are not present throughout germ cell development (Chiquoine, 1954; Gertz, 1999; Tanaka et al., 2000). Identifying the regulatory sequences and ultimately the gene that drives germ cell-specific expression would provide a valuable tool to study germ cell delineation throughout development. The GCS-EGFP line is fully fertile in both males and females suggesting that the coding sequence of the putative germ cell-specific gene was likely not functionally disrupted by the insertion event. This type of positional effect on transgene expression is common and has been shown to occur in at least the following examples: the hsp68-LacZ transgene was expressed in developing neural tissue due to the influence of the dystonia enhancer (Kothary et al., 1988), the IE-LacZ transgene was expressed only in the apical ectodermal ridge (Gardner and Kappen, 2000) and HSVtk-LacZ transgene was expressed only in developing neurons (Allen et al., 1990). Alternatively, the transgene insertion may have disrupted the coding sequence of a gene whose function is not essential for the development of germ cells. The identification of the hypothesized gene that targets germ cells may lead to an understanding of the mechanisms that determine and maintain germ cell fate.

Since we were unable to obtain sufficient flanking DNA sequence for further analysis, we used FISH to identify the chromosomal position and localize the transgene to rat chromosome 11q11–q12. This chromosomal region is syntenic to mouse chromosome 16 and human chromosome 21. Interestingly, this region encompasses the Down syndrome critical region genes 1–6. Based on dual FISH analysis, we were able to narrow down this region (q11–q12) even further. There are 35 rat and 62 mouse locuses mapped to this region and of these 19 rat and 21 mouse genes have been identified (NCBI mouse and rat genome database and Celera mouse database). We looked at the expression pattern of the assigned genes in this region in an attempt to identify candidate genes that had a similar pattern of expression, or that had germ cell expression at any stage of germ cell development. We did not find a gene that was known to be exclusively expressed in both the male and female germ cells; however, there were 10 rat or mouse transcribed loci expressed in the testis, ovary or pre-implantation embryo. Recently, 9 Riken clones from a
tests-specific library were mapped to mouse chromosome 16 in the region that is syntenic to rat chromosome 11 in our region of interest (Online Supplementary Table 1 in Appendix A). We intend to determine whether any of these genes or ESTs also are exclusively expressed in female germ cells.

We have previously shown that matrix-selected male germ line stem cells isolated from the GCS-EGFP rats were capable of forming functional spermatozoa when transferred to WT recipient testes (Hamra et al., 2002, 2004). Here, we demonstrate that a non-selected, crude preparation of adult seminiferous tubule cells contains a sufficient number of germ line stem cells to efficiently colonize a recipient testis within 30 days following transfer and the extent of colonization doubles with an additional 30 days. The surprisingly efficient colonization of a recipient testis using only a crude preparation of cells suggests that adult male germ line stem cells are remarkably effective at reconstituting the germ cell compartment. Given that all of the previous estimates of the colonization potential of a population of non-selected tubule cells have relied on the use of β-galactosidase marked donor cells, we suggest that these studies have likely underestimated the colonization potential due to the necessity of performing histochemical staining on fixed tissue. The use of donor stem populations with robust expression of a vital marker, such as EGFP, will facilitate the accurate assessment of colonization potential and allow ease of manipulation of such a population of cells while maintaining viability.

In conclusion, the unique germ cell specific expression pattern of this transgene makes this rat line a very powerful tool to be used to address important questions in the germ cell field. Novel genes could be identified and then along with known genes could be studied to understand their involvement in the delineation of the germ line during development. Since EGFP is a vital marker, germ cells at all stages of embryonic development can be isolated by FACS and the RNA can be analyzed using microarrays to develop markers for distinct stages of germ cell development. Additionally, these genes could be studied to determine their function during germ cell development and may eventually lead to an understanding of the signaling pathways involved in this complex process. Currently, PGCs in the mouse are first identified at E7.5 by high alkaline phosphatase activity; earlier identification is not possible because AP activity levels are high in many cells in the embryo at earlier time points (McLaren, 2000). The GCS-EGFP rat may allow the identification of markers of the germ lineage at the earliest embryonic stages. Finally, the GCS-EGFP rat can be used to determine when PGCs first acquire stem cell activity. Purified populations of PGCs can be collected at different developmental stages and then assayed for stem cell activity by transplanting them to a recipient testis. These are just a few examples demonstrating the value of GCS-EGFP rat as a tool to study germ cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.05.015.

References
