

## A Self-Renewal Program Controls the Expansion of Genetically Unstable Cancer Stem Cells in Pluripotent Stem Cell-Derived Tumors

ANNE E. CONWAY,<sup>a,b</sup> ANNE LINDGREN,<sup>a,b</sup> ZORAN GALIC,<sup>c,d,e</sup> APRIL D. PYLE,<sup>d,e,f,g,h</sup> HONG WU,<sup>d,e,g,h,i</sup>  
JEROME A. ZACK,<sup>d,e,f,g,h</sup> MATTEO PELLIGRINI,<sup>a,b</sup> MICHAEL A. TEITELL,<sup>d,e,g,h,j</sup> AMANDER T. CLARK<sup>a,b,e,g,h</sup>

Departments of <sup>a</sup>Molecular Cell and Developmental Biology, <sup>c</sup>Medicine, <sup>f</sup>Microbiology, Immunology and Molecular Genetics, <sup>i</sup>Biological Chemistry, and <sup>j</sup>Pathology and Laboratory Medicine, <sup>b</sup>College of Letters and Science, <sup>d</sup>David Geffen School of Medicine, <sup>e</sup>Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, <sup>g</sup>Jonsson Comprehensive Cancer Center, and <sup>h</sup>Molecular Biology Institute, University of California, Los Angeles, California, USA

**Key Words.** Embryonal carcinoma • Embryonic stem cells • Germ line • Retinoic acid

### ABSTRACT

Human germ cell tumors are often metastatic, presumably due to distal site tumor growth by cancer stem cells. To determine whether cancer stem cells can be identified in a transplantation model of testicular germ cell tumor, we transplanted murine embryonic germ cells (EGCs) into the testis of adult severe combined immunodeficient mice. Transplantation resulted in a locally invasive solid tumor, with a cellular component that generated secondary tumors upon serial transplantation. The secondary tumors were invariably metastatic, a feature not observed in the primary tumors derived from EGCs. To characterize the differences between EGCs and the tumor-derived stem cells, we performed karyotype and microarray analysis. Our results show that generation of cancer stem cells is associated with the acquisition of nonclonal genomic rearrangements not

found in the originating population. Furthermore, pretreatment of EGCs with a potent inhibitor of self-renewal, retinoic acid, prevented tumor formation and the emergence of these genetically unstable cancer stem cells. Microarray analysis revealed that EGCs and first- and second-generation cancer stem cells were highly similar; however, approximately 1,000 differentially expressed transcripts could be identified corresponding to alterations in oncogenes and genes associated with motility and development. Combined, the data suggest that the activation of oncogenic pathways in a cellular background of genetic instability, coupled with an inherent ability to self-renew, is involved in the acquisition of metastatic behavior in the cancer stem cell population of tumors derived from pluripotent cells. *STEM CELLS* 2009; 27:18–28

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

Teratomas contain disorganized endoderm, mesoderm, and ectoderm tissues and can be derived experimentally by transplanting pluripotent cells into the testis of immunocompromised mice. In human males, prepubertal teratomas are 46,XY and benign, whereas postpubertal testicular teratoma is aneuploid and often metastatic [1]. The cells in postpubertal testicular teratoma responsible for metastatic activity are not known. However, in testicular teratocarcinoma, which is a malignant relative of teratoma, a subpopulation of cancer stem cells termed embryonic carcinoma (EC) cells can be isolated and used in retransplantation assays to generate secondary tumors in severe combined immunodeficient (SCID) mice [2–4]. The mechanism by which these stem cells emerge is not known. A recent report determined that stem cell-like cells can be identified in experi-

mentally induced teratomas derived from pluripotent human embryonic stem cells (hESCs) [5]. However, their potential to generate secondary tumors in serial transplantation assays has not been evaluated.

In the clinical setting, postpubertal testicular teratomas arise from transformed germ cells called carcinoma in situ (CIS) cells or intratubular germ cell neoplasia. These cells are localized within the seminiferous tubule epithelia of the testis. CIS cells are strongly positive for classic markers of embryonic stem cells (ESCs), including the pluripotent transcription factors NANOG and OCT4, which are normally at or below the level of detection in the adult testis [6–8]. Expression of these pluripotency-associated transcription factors suggests a close relationship between cancer-originating cells and ESCs [9, 10]. Evidence for this relationship is further supported by gene expression profiling, which reveals that ESC-expressed genes are enriched in human testicular samples containing CIS [11]. Furthermore, in

Author contributions: A.E.C., A.L., and A.C.: concept and design, collection and assembly of data, data analysis and interpretation, manuscript writing; Z.G.: collection and assembly of data, data analysis and interpretation; A.D.P.: collection of data, data analysis and interpretation; H.W. and J.A.Z.: data analysis and interpretation; M.P.: assembly of data, data analysis; M.A.T.: data analysis and interpretation, manuscript writing. A.E.C. and A.L. contributed equally to this work.

Correspondence: Amander T. Clark, Ph.D., 621 Charles E. Young Drive South, Los Angeles, California 90019, USA. Telephone: 310-794-4201; Fax: 310-206-0484; e-mail: clarka@ucla.edu Received May 28, 2008; accepted for publication September 18, 2008; first published online in *STEM CELLS EXPRESS* October 2, 2008. ©AlphaMed Press 1066-5099/2008/\$30.00/0 doi: 10.1634/stemcells.2008-0529

mouse models of testicular teratoma the initial lesion prior to the locally invasive stage of the disease involves the accumulation of pluripotent embryonic germ cell (EGC)-like cells in the fetal testis [12]. These findings have led to an important hypothesis that testicular tumor pathogenesis is driven in part by deregulation of transcription factors that normally regulate pluripotency and self-renewal [6, 11, 13–16]. We hypothesize that these same pathways may act not only in the initiating events in postpubertal testicular teratoma but also in the generation and maintenance of cancer stem cells derived from these tumors.

The progenitor germ cells from which testicular germ cell tumor CIS cells originate are called primordial germ cells (PGCs) [17]. In the mouse, PGCs exist from embryonic gestational day (E) 7.5–E12.5. In vivo PGCs are unipotent, and in males they are fated to generate spermatogonial stem cells. In the adult testis, these cells respond to retinoic acid by transcribing *Stimulated by Retinoic Acid 8 (Stra8)* and enter into meiosis [18]. During normal embryonic development, PGCs express ESC-specific transcription factors, including *Nanog* and *Oct4*, as well as *developmental pluripotency associated (Dppa)*, such as *Dppa2*, *Dppa3*, and *Dppa4*, which are concomitantly down-regulated as PGCs differentiate from E13.5 to E15.5 [16, 19–23]. Culture of human and mouse PGCs expressing *Nanog* and *Oct4* in vitro results in conversion from unipotent PGCs to pluripotent EGCs [19, 24–33]. In murine models of testicular teratoma, such as deletion of the tumor suppressor *Pten*, the initial transformation event is described as the conversion of PGCs to pluripotent EGCs [12]. Therefore the generation of testicular teratomas in mice begins with a pluripotent cell type similar to CIS in the postpubertal human disease that closely resembles EGCs and ESCs. Furthermore, once a murine testicular teratoma is generated, it can be serially transplanted by reinjecting the entire tumor into new mice, suggesting the presence of cancer-initiating cells in experimental teratomas [34]. However, the identity of these cancer-initiating cells has never been reported.

Given that the formation of EGCs from PGCs is the first step in the generation of locally invasive teratoma in vivo, we addressed whether transplantation of EGCs into the testis of SCID mice results in the formation of a testicular teratoma that contains a self-renewing cancer stem cell population capable of serial transplantation. Transplantation of pluripotent cells, such as ESCs, into the testis results in the formation of teratomas in 4–8 weeks. This is one of the standard methods for evaluating ESC pluripotency [5, 35, 36]. However, characterization of cancer-initiating cells in these experimentally induced teratomas has not been performed in detail. Our hypothesis is that transplantation of EGCs or ESCs into the adult testis will serve as an in vivo model for adult-type teratoma formation and the generation of cancer stem cells capable of recapitulating tumor growth upon transplantation. Results from this work will aid in the identification of molecular pathways that are deregulated in the formation of cancer stem cells in both malignant germ cell tumors and tumors derived from other pluripotent cells and may provide insight into pathways that are deregulated in the generation of somatic cancers with an embryonic stem cell signature [37, 38].

## MATERIALS AND METHODS

### Cells

C57BL/6 EGCs were derived from PGCs at E12.5 and maintained on irradiated mouse embryonic fibroblast cell lines (STOs) as previously described [30]. The H9 (WA09) and HSF-6 (UC06) hESC lines were cultured according to previous reports [39]. Karyo-

www.StemCells.com

type of the HSF-6 line was of normal female karyotype, 46XX, whereas the karyotype of the H9 line was 46XX,add(7). The NTERA-2 cl.D1 cell line was obtained from American Type Culture Collection (Manassas, VA, <http://www.atcc.org>). All work with hESCs was conducted with prior approval from the UCLA Human Embryonic Stem Cell Research Oversight Committee. To generate EGC reporter lines, EGCs were genetically modified by lentiviral transduction of a trifusion reporter construct [40]. EGC differentiation involved culture with 10  $\mu$ M retinoic acid (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) for 4 days.

### Generation of Testicular Tumors

Surgery was performed following institutional approval for appropriate care and use of laboratory animals. Briefly, a single incision was made in the peritoneal cavity and the testis was pulled through the incision site. Using a 27-gauge needle, 0.5–1  $\times$  10<sup>6</sup> cells in a volume of 50  $\mu$ l of phosphate-buffered saline (PBS) were transplanted into the testis of adult SCID mice. Four to 6 weeks after surgery, mice were euthanized.

### Generation of Secondary Testicular Tumors by Serial Transplantation

Stage-specific embryonic antigen 1 (SSEA1)-positive cells were isolated by either magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) following labeling with primary antibody against SSEA1. Secondary antibodies involved labeling with either a magnetic bead or allophycocyanin (APC)-labeled secondary antibody for MACS or FACS, respectively.

### Live-Animal Imaging

Mice were anesthetized with 2% isoflurane and injected with 100  $\mu$ l of 15 mg/ml D-luciferin. Following the injection, mice were placed in the chamber of the IVIS<sup>®</sup> Imaging System (Caliper Life Sciences, Hopkinton, MA, <http://www.caliperls.com>) and imaged for up to 30 minutes. Photons were collected for a period of 10 seconds per reading, and images were analyzed using the Living Image software, version 2.50. Signal intensity was quantified in defined regions of interest as photon count rate per body unit body area per unit solid angle subtended by the detector (units of photons/second/cm<sup>2</sup>/steradian). The maximum signal from each region of interest was collected for every time point. Images were constructed using the three-dimensional photograph capability of the IVIS<sup>®</sup> imaging system.

### Flow Cytometry

A single-cell suspension of the tumors and testis was generated by dissection of the tumor into 1-mm<sup>2</sup> pieces, followed by incubation in 1 mg/ml collagenase in high-glucose Dulbecco's modified Eagle's medium for 2 hours at 37°C in 5% CO<sub>2</sub>. Staining was performed in PBS containing 0.5% bovine serum albumin (BSA). SSEA1 antibody from the Developmental Studies Hybridoma Bank (Iowa City, IA, <http://www.uiowa.edu/~dshbwww>) was used at 1:100 with 1–2 million cells per milliliter of staining buffer. All samples were analyzed using a BD Biosciences LSR II (BD Biosciences, San Diego, <http://www.bdbiosciences.com>). Apoptosis was evaluated using 7-aminoactinomycin D (Calbiochem, San Diego, <http://www.emdbiosciences.com>) according to the manufacturer's instructions followed by flow cytometry.

### Sorting

Cells were prepared for MACS and FACS as for flow cytometry with the following exceptions. The staining buffer used contained PBS, 0.5% BSA, and 2 mM EDTA. Before adding the fluorescently labeled secondary antibody, MACS bead-conjugated rat anti-mouse IgM antibody (Miltenyi Biotec, Bergisch Gladbach, Germany, <http://www.miltenyibiotec.com>) was added to the cells at a ratio of 20  $\mu$ l of antibody to 80  $\mu$ l of staining buffer per 1  $\times$  10<sup>7</sup> cells and incubated at 4°C for 20 minutes. For MACS separation was performed on MS Columns in a MiniMACS separation unit (Miltenyi Biotec) following the manufacturer's instructions. Purity of the separated populations was determined by flow cytometry. For

FACS cells were sorted on a FACSVantage (Becton, Dickinson and Company, Franklin Lakes, NJ, <http://www.bd.com>).

### Immunofluorescence

Tumors were fixed in 4% paraformaldehyde for at least 24 hours at room temperature. Tissue was embedded in paraffin and sectioned at 5  $\mu$ m. Antigen retrieval in 10 mM Tris base, 1 mM EDTA 0.05% Tween 20 at 95°C for 40 minutes was used for all antibodies. Primary antibodies included SSEA1 (1:50; Developmental Studies Hybridoma Bank), Oct3/4 (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, <http://www.scbt.com>), deleted in azoospermia-like (Dazl; 1:100; Abcam, Cambridge, MA, <http://www.abcam.com>), and mouse vasa homolog (Mvh)/Ddx4 (1:100; Abcam). Secondary antibodies (1:200) were from Jackson ImmunoResearch Laboratories (West Grove, PA, <http://www.jacksonimmuno.com>). Sections were mounted in Prolong Gold Antifade Reagent with 4,6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) and photographed on a laser scanning microscope 510 confocal microscope (Carl Zeiss, Oberkochen, Germany, <http://www.zeiss.com>).

### Real-Time Polymerase Chain Reaction

RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany, <http://www1.qiagen.com>) and reverse-transcribed with Superscript II Reverse Transcriptase (Invitrogen). Following reverse transcription, real-time polymerase chain reaction was performed on the cDNA according to the manufacturer's protocol using a Bio-Rad iQ iCycler (Bio-Rad, Hercules, CA, <http://www.bio-rad.com>) with SYBR Green as the fluorophore. Primer sequences are included in supporting information Table 1.

### Western Blot

Protein was harvested using M-PER cell lysis reagent (Thermo Scientific, Rockford, IL, <http://www.thermo.com>). Protein concentration measured using BCA Protein assay (Thermo Scientific). Twenty-five micrograms of total protein was electrophoresed through 12% NuPAGE Novex Bis-Tris gels (Invitrogen) and transferred according to standard procedures. For immunoblotting, primary antibodies were mouse anti-human retinoblastoma (Rb) protein (1:250; BD Pharmingen, San Diego, [http://www.bdbiosciences.com/index\\_us.shtml](http://www.bdbiosciences.com/index_us.shtml)), mouse anti-human p53 (1:500; Santa Cruz Biotechnology), rabbit anti-mouse nanog (1:500; Abcam), and rabbit anti-human  $\beta$ -actin (1:5,000; loading control).

### Statistics

To determine statistical significance between retinoic acid-treated and control EGCs, Student's *t* tests were performed, and significance was accepted with  $p < .05$ .

### Microarray

Microarrays were performed using the Affymetrix Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, <http://www.affymetrix.com>). Three biological replicates were analyzed for each. The arrays were processed using the Affymetrix Mas5.0 software, and downstream analyses were performed using Matlab to perform *t* tests, hierarchical and k-means clustering. Gene Ontology enrichment calculations were performed using DAVID (<http://david.abcc.ncifcrf.gov/home.jsp>). Comparisons were made between primary EGCs used at the same passage as transplantation (passage 29), cancer stem cell (CSC) line 66 (first-generation tumor) (passage 6), and CSC line 84 (second-generation tumor) (passage 11).

### Karyotype

Karyotyping and G-banding of HSF-6 (passage 52), H9 (passage 23), EGC (passage 29), and CSC66 (passage 6) were performed by Cell Line Genetics (Madison, WI, <http://www.clgenetics.com>).

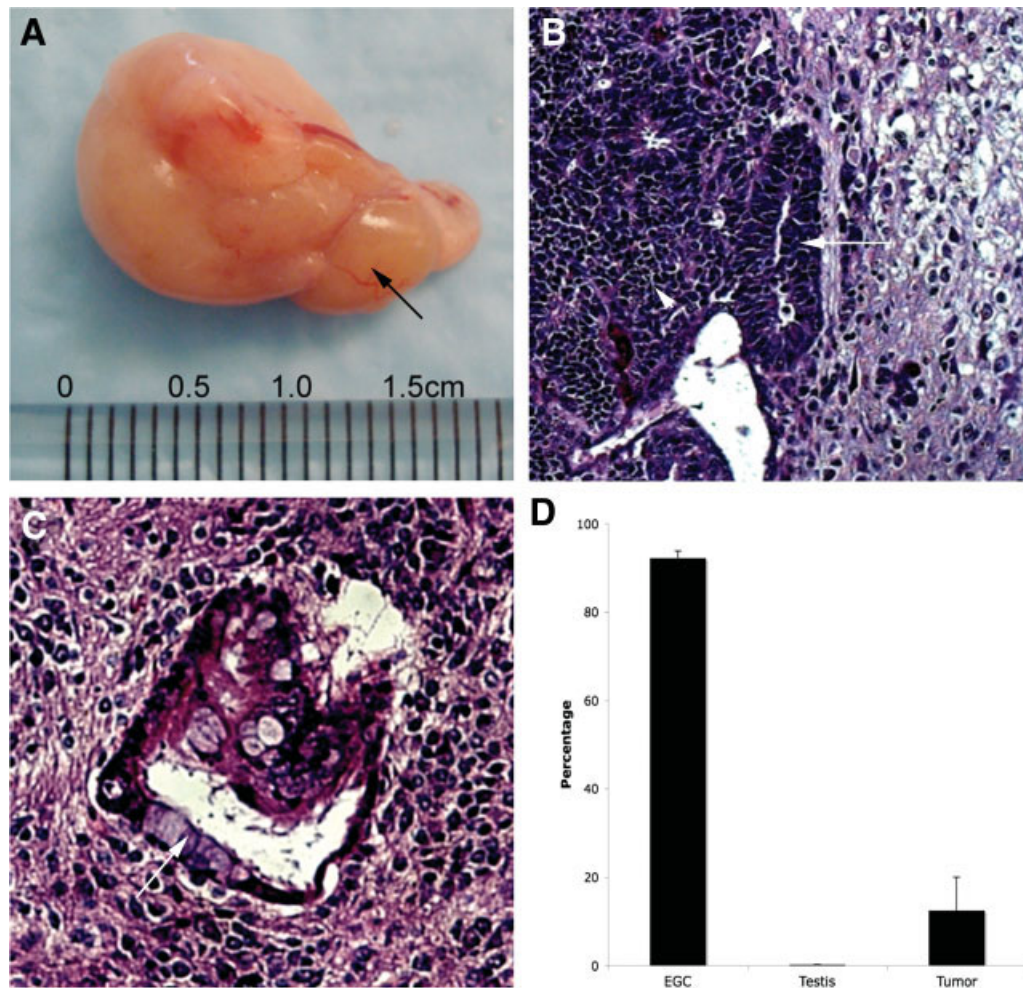
## RESULTS

Cultured PGCs (EGCs) were derived from E12.5 C57BL/6 mice. Karyotype analysis of the primary EGC line determined that this line was 40,XY. Transplantation of  $0.5\text{--}1 \times 10^6$  EGCs into the testes of SCID mice resulted in tumor formation in 25 of 26 transplants at 4–6 weeks following surgery (Fig. 1A). EGCs transplanted directly into the testes result in locally invasive testicular tumors with trilineage embryonic differentiation that included large amounts of primitive neuroepithelium (Fig. 1B) and endodermally derived gut epithelium (Fig. 1C). In the majority of cases, the testis architecture was completely destroyed by tumor cells.

To identify whether the testicular tumors had a stem cell subpopulation capable of self-renewal, we evaluated the proportion of tumor cells that expressed a unique cell surface marker called SSEA1, which is present on the cancer-initiating EGCs used to make the primary tumor. SSEA1 is not expressed on adult testicular cells (Fig. 1D; supporting information Fig. 1A, 1B). We reasoned that tumor stem cells would maintain SSEA1 expression, whereas the majority of tumor cells would lose expression of SSEA1 in the process of differentiation. Indeed, we determined that SSEA1-positive cells derived from the testicular tumors averaged 12.5% of the total cell population compared with the tumor-initiating EGCs cultured on mouse embryonic fibroblasts, which are >90% positive for SSEA1 prior to transplantation (Fig. 1D). Therefore, the majority of cells within the testicular tumor are SSEA1-negative.

To determine whether stem cell-like cells could also be identified in tumors derived from ESCs, we performed transplantations with mouse ESCs; diploid human ESCs (HSF-6); karyotypically abnormal hESCs (H9) with a karyotype of 46, XX, add(7); and NTERA-2 cl.D1 EC cells with a hypotriploid karyotype (supporting information Table 2). Our results show that 4–6 weeks after transplantation into the testis, teratomas derived from mouse ESCs had a detectable SSEA1-positive population at 12.17%, which is very similar to the proportion of SSEA1-positive cells in EGC-derived tumors (12.5%). However, in contrast to the murine EGC line, transplantations of murine ESCs into the testis of SCID mice resulted in metastasis in two-thirds of tumors, suggesting that pluripotent ESCs are more tumorigenic than EGCs *in vivo*. With regard to human pluripotent cells, the pluripotent embryonic carcinoma line (called NTERA2), which was derived from a metastatic teratocarcinoma contains a 6.89% SSEA4 positive population following transplantation and tumor formation, whereas tumors from karyotypically normal hESCs result in a 1% positive SSEA4-positive population. Interestingly, analysis of a karyotypically abnormal hESC line results in a population of SSEA4-positive cells equivalent to NTERA2 at 5.52%. These results suggest that similar to the EGCs in the current study, murine ESCs transplanted into the testis generate teratocarcinomas with a clearly reproducible stem cell population, whereas human pluripotent cells with aneuploidies are generated teratocarcinomas with a clearly distinguishable stem cell population.

Next we used immunofluorescence and confocal microscopy to determine the location of SSEA1-positive cells within the EGC-derived tumors. We found that SSEA1-positive cells within testicular tumors were arranged in small clusters (Fig. 2A). Costaining with anti-Oct4 demonstrated that all SSEA1-positive tumor cell clusters expressed this pluripotent transcription factor in the nucleus (Fig. 2A). Given the abundance of immature neural structures in the primary tumors, Nestin and Oct4 costaining was performed and showed that Nestin and Oct4 did not colocalize, suggesting that the SSEA1-positive cells in tumors were not related to neural precursor or neural



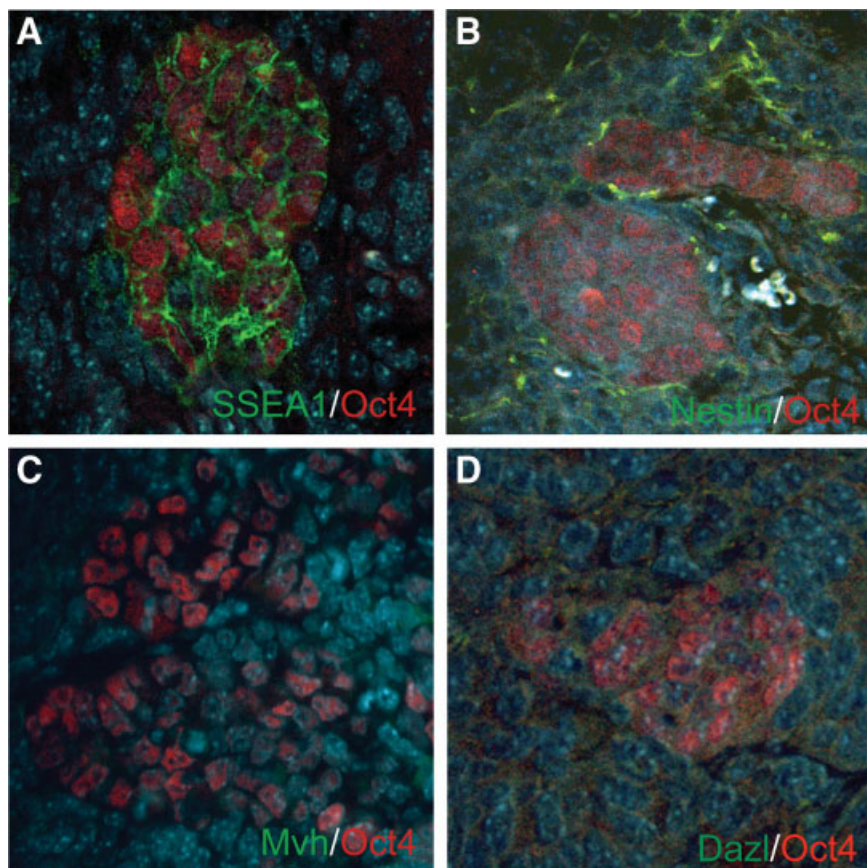
**Figure 1.** Testicular transplantation of EGCs. (A): Solid tumor generated by transplantation of  $5 \times 10^5$  EGCs directly into the testis (arrow shows testis). (B): Primitive neural tissue (arrowhead) and neural epithelium (arrow) (magnification,  $\times 100$ ). (C): Endodermal derivative, arrow showing a goblet cell (magnification,  $\times 200$ ). (D): The average percentage of stage-specific embryonic antigen 1-positive cells in testis and testicular tumors ( $n = 5$ ) induced from EGCs, compared with EGCs cultured on mouse embryonic fibroblast cell lines in the presence of leukemia inhibitory factor. Abbreviation: EGC, embryonic germ cell.

stem cells (Fig. 2B). Two additional germ cell markers, Dazl and Mvh, were also evaluated because these markers label the spermatogonia and spermatocytes in adult testis (supporting information Fig. 2A, 2B). These germ line markers did not colocalize with SSEA1 or Oct4 staining in the testicular tumors (Fig. 2C, 2D). In sum, the lack of germ line or neural identity, as indicated by the absence of Dazl, Mvh, and Nestin containing, coupled with the expression of SSEA1 and Oct4, strongly suggests an immature stem-like tumor cell population rather than a primitive germ or neural cell population within these small tumor clusters.

To determine whether the SSEA1-positive population was capable of self-renewal and differentiation, SSEA1-positive cells at various numbers were serially transplanted into recipient testes following MACS or FACS sorting without intervening culture (Table 1). We found that transplantation of just  $2.5 \times 10^5$  positive cells resulted in not only a testicular tumor but also metastasis throughout the peritoneum and also to the kidney (Fig. 3A). This was observed in 100% of secondary tumors at this cell number (Table 1). In contrast, metastatic activity was only observed 1 of 26 transplants of primary EGCs at numbers of  $5 \times 10^5$  cells and greater. As controls, transplantation of STOs did not result in tumors, and SSEA1-negative tumor cells isolated by FACS did not self-renew when plated on STOs in

the presence of leukemia inhibitory factor. Together, these observations strongly suggest that the SSEA1-negative population does not contain a stem cell component capable of self-renewal.

Given that SSEA1-positive cells had an undifferentiated identity in the primary tumors, we performed histology on both the testicular tumors and metastases to evaluate the degree of differentiation. Similar to the results shown in Figure 1, the secondary tumors were highly differentiated and contained cells from all three embryonic layers, including primitive neuronal tissue (Fig. 3B), bone (Fig. 3C), and muscle (not shown). To determine whether stem cell populations can be isolated from the secondary tumors, we performed MACS sorting before culturing on STOs (Fig. 3D). This resulted in the generation of cell lines capable of robust self-renewal (Fig. 3D). Retransplantation of tumor-derived SSEA1-positive cells that had been cultured for  $>10$  passages resulted in highly metastatic tumors 6 weeks following surgery. Together, these results demonstrate that the SSEA1-positive cells within a primary tumor can differentiate and regenerate a new teratoma with a new stem cell population upon transplantation into a new host testis. Furthermore, these tumors are more aggressive than the primary tumors. This suggests that genetic, epigenetic, and/or transcriptional differences between primary EGCs and the subsequent cancer



**Figure 2.** Immunofluorescent staining of testicular tumors at 6 weeks (A–C). (A): Double immunofluorescence for SSEA1 (green) and Oct4 (red). (B): Double immunofluorescence for Nestin (green) and Oct4 (red). (C): Double immunofluorescence for Mvh (green) and Oct4 (red). (D): Dazl (green) and Oct4 (red). Magnifications,  $\times 400$ . Abbreviations: Dazl, deleted in azoospermia-like; Mvh, mouse vasa homolog; SSEA1, stage-specific embryonic antigen 1.

**Table 1.** Comparison of tumor potential between EGC transplants to generate primary tumors and SSEA1-positive serial transplants without intervening culture to generate secondary tumors

| Number of transplants   | Cell number transplanted | Generation of a testis tumor | Metastasis |
|---|--------------------------|------------------------------|------------|
| Primary tumor generated with EGCs                                       |                          |                              |            |
| 12  | 1,000,000                | 12/12                        | 1          |
| 14  | 500,000                  | 13/14                        | 0          |
| 2   | 100,000                  | 1/2                          | 0          |
| 1   | 10,000                   | 0                            | 0          |
| Total: 29   |                          |                              |            |
| Secondary tumor generated with SSEA1-positive cells from primary tumors |                          |                              |            |
| 3   | 250,000                  | 3/3                          | 3/3        |
| 2   | 10,000                   | 2/2                          | 2/2        |
| 1   | 2,500                    | 1/1                          | 0          |
| 2   | 1,000                    | 2/2                          | 0          |
| Total: 8  |                          |                              |            |

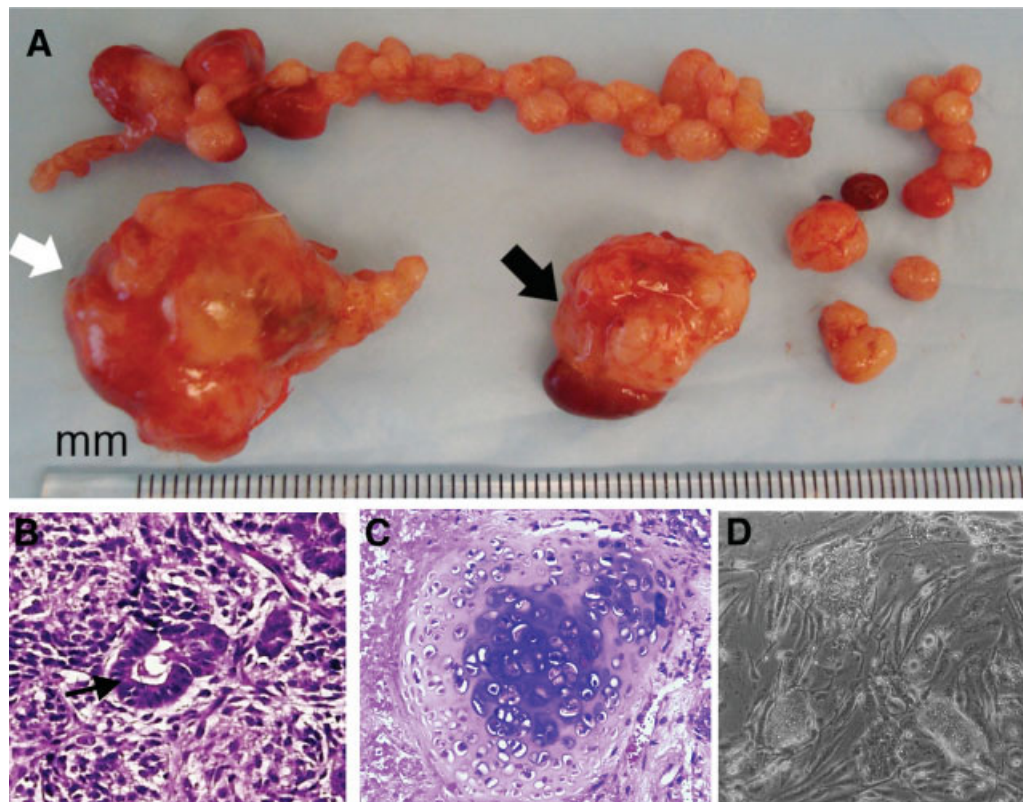
Abbreviations: EGC, embryonic germ cell; SSEA1, stage-specific embryonic antigen 1.

stem cell population are responsible for this malignant potential.

We first addressed genetic stability between the EGCs and a first-generation cancer stem cell line by performing G-banding karyotype analysis. The EGC line was 40,XY; however, G-banding identified two stable structural changes in 100% of cells involving a duplication of B2-C7 on chromosome 4 and a

deletion of C3-F1 on chromosome 6. In contrast, the SSEA1-positive cells derived from primary tumors displayed a number of genomic rearrangements, including a major clone with three nonclonal sublines, and a minor clone with two nonclonal sublines. The gross genomic changes included isochromosomes iso(8) and iso(15), translocations (t) t(6;8) and t(11;14), as well as loss of the Y chromosome del(Y) and in one case a hypotriploid karyotype (supporting information Fig. 3). This result indicates that generation of cancer stem cells in vivo are not the result of clonal selection from a single karyotypically abnormal cell in the original EGC population and instead occurs via more generalized genetic instability during tumorigenesis.

Given that teratomas are generated through a process of differentiation from self-renewing cells, our next aim was to determine whether disruption of self-renewal prior to transplantation was sufficient to block the formation of tumors and the generation of SSEA1-positive cancer stem cells. To achieve this we pretreated the EGCs with all-trans retinoic acid (RA) prior to transplantation [41]. Tumor growth was quantified in vivo using live-animal imaging of EGC lines that stably expressed a trifusion-imaging vector (supporting information Fig. 4A). Imaging was performed to quantify tumor growth and also to determine whether metastases arose from RA-treated primary EGCs. Treatment of EGCs with RA for 4 days disrupted self-renewal, as monitored by changes in colony morphology, and significantly reduced mRNA expression of pluripotent transcription factors (supporting information Fig. 5). Furthermore, RA-treated EGCs displayed increased expression of genes associated with differentiation, as well as increased numbers of apoptotic cells and a decreased proliferation rate (supporting information Fig. 5). Western blot was also performed to confirm loss of NANOG protein and to confirm that RA treatments resulted in reduced Rb phosphorylation. We did not identify



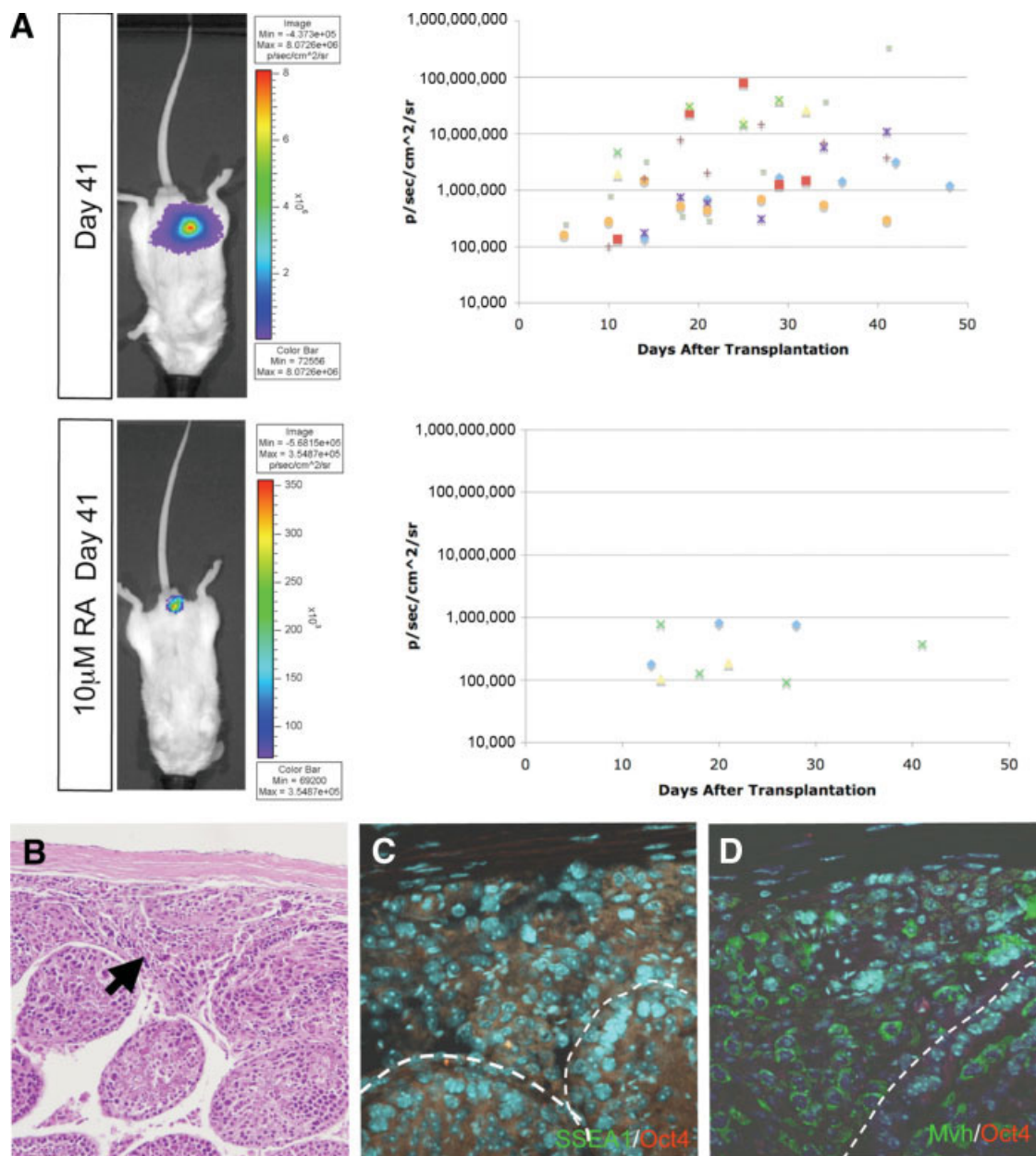
**Figure 3.** Secondary tumors derived following re-transplantation of stage-specific embryonic antigen 1 (SSEA1)-positive cells without intervening culture. **(A):** Testicular tumor (white arrow) and metastasis from the primary tumor into the kidney (black arrow) and dissected tumors that had seeded the peritoneum (scale bar in millimeters). **(B):** Histology of secondary tumor shows differentiated cell types, including neural epithelium (arrow). **(C):** Bone (magnification,  $\times 100$ ). **(D):** SSEA1-positive cells isolated by magnetic-activated cell sorting and recultured on inactivated mouse embryonic fibroblast cell lines in the presence of leukemia inhibitory factor.

appreciable differences in P53 expression between control and RA treated cells (supporting information Fig. 5). Next we transplanted  $5 \times 10^5$  control (untreated) and RA-treated EGCs into the testis of SCID mice, and tumor growth was quantified by bioluminescent imaging from 5 to 41 days post-transplantation. Bioluminescent images of mice are shown at day 41 following transplantation (Fig. 4A). Tumors derived from control cells were localized to the testis (no metastasis) and were detected in two of eight cases beginning at 5 days following transplantation. By day 10, six of eight transplants had a detectable signal above background, and by day 14, eight of eight transplants had a detectable signal. In contrast, tumors derived from RA-treated EGCs were first detectable in two of four transplants at day 14. By day 21, two of four transplants had a signal above background, and one transplant had lost the signal (Fig. 4A). By day 41 only one of four transplants had retained a bioluminescent signal above background. In the cohort treated with RA, one of four transplants never developed a detectable signal, and the testis containing the transferred cells was of normal size at necropsy except for a small tuft of tubules at the site of injection, suggesting that tumor formation never progressed.

To determine whether SSEA1-positive cells could be identified in tumors derived from RA-treated cells, flow cytometry was performed (Fig. 4). SSEA1-positive cells derived from control testicular tumors averaged 9.2% (+ SD) of the total population. In contrast, testes from transplants of RA-treated EGCs contained, on average, 1.8% SSEA1-positive cells (+ SD). Cell lines were attempted from the SSEA1-positive cells derived from RA-treated tumors; however, no cells survived after the first split, whereas SSEA1-positive cell lines were derived from the control tumors. Transplants were performed using the SSEA1-positive cells de-

rived from RA-treated tumors, and no tumors formed. This result indicates that the small population of SSEA1-positive cells in teratomas derived from RA-treated EGCs are not capable of self-renewal and are therefore not cancer stem cells. Histology of the primary tumors derived from RA-treated EGCs revealed small foci of stromal cells (Fig. 4B, arrow) without differentiated structures. Furthermore, SSEA1 and Oct4 did not stain the tumor cells (Fig. 4C). Although we did detect isolated Vasa-positive cells intermingling with the negatively staining stroma (Fig. 4D), we hypothesize that these correspond to isolated germ cells migrating from the tubules, which have degenerated as a consequence of the initial transplant. Taken together, these results suggest the generation of genetically unstable SSEA1-positive cells in teratomas is dependent upon an ability to self-renew via RA-sensitive pathways.

Finally, we assessed transcriptional differences between SSEA1-positive cells derived from primary and secondary tumors and EGCs using microarray analysis (Fig. 5). For this experiment, EGCs were used to generate a primary tumor, and 6 weeks later the SSEA1-positive cells (11.83%) were isolated by MACS and used to generate secondary tumors ( $n = 2$  mice) without intervening culture, as well as an SSEA1-positive cancer stem cell line (CSC66). The secondary tumors were harvested at 6 weeks, and the SSEA1-positive cells (21.5%) were collected by MACS and used to generate tertiary tumors ( $n = 2$  mice) without intervening culture, as well as an SSEA1-positive cancer stem cell line (CSC84). The history of this tumor series is shown in Figure 5A. Serial transplants are still continuing; however, at the quaternary tumor stage, one of the two mice died at 5 weeks, and in septenary tumors one of two mice died at 4 weeks due to tumor burden and increased metastasis now including the liver. This suggests that the cancer stem cells are

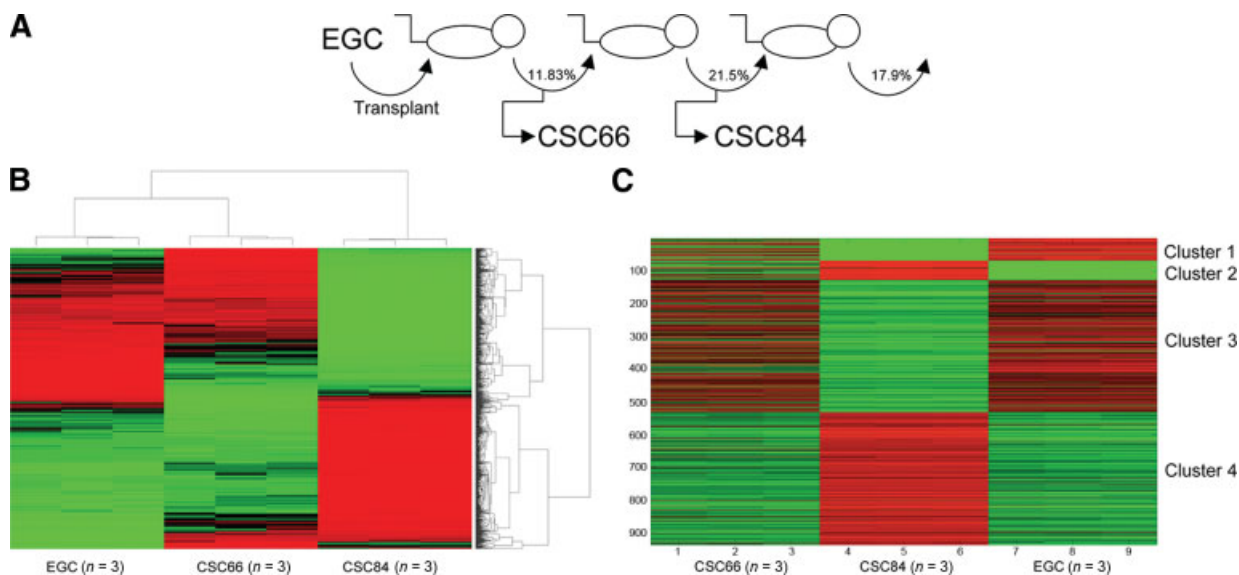


**Figure 4.** Analysis of tumor growth following treatment of embryonic germ cells (EGCs) with RA. Shown is live-animal imaging of tumor growth. **(A):** Dynamics of tumor growth following transplantation of EGCs into the testis as monitored by live-animal imaging. Each spot refers to one mouse imaged over multiple time points. Shown are time points taken at day 41 with the corresponding bioluminescence color scale. Tumor growth increased from  $1 \times 10^5$  p/sec/cm<sup>2</sup>/sr by day 5 to  $1 \times 10^8$  p/sec/cm<sup>2</sup>/sr by day 30 in mice transplanted with untreated EGCs. In mice transplanted with EGCs treated with RA, signal was detected in three of five mice, and the signal ranged from  $1 \times 10^5$  to  $1 \times 10^6$  up to day 30, with only one mouse showing a signal at the conclusion of the experiment (day 41). Each colored symbol represents the signal of one mouse over time. **(B):** Histology of testis tumor transplanted with RA-treated EGCs shows a stromal-like morphology (arrow) with no evidence of differentiation (magnification,  $\times 200$ ). **(C):** Stromal-like cells were negative for OCT4 and SSEA1. Dotted lines outline an intact seminiferous tubule, which was also negative for OCT4 and SSEA1 (magnification,  $\times 400$ ). **(D):** Stromal-like cells were negative for Oct4, with some isolated VASA-positive cells intermingled with the stroma. The dotted lines outline an intact seminiferous tubule containing VASA-positive spermatogonial and spermatocytes (magnification, 400). Abbreviations: Max, maximum; Min, minimum; Mvh, mouse vasa homolog; p, photons; RA, retinoic acid; sec, seconds; sr, steradian; SSEA1, stage-specific embryonic antigen 1.

becoming more functionally aggressive with serial transplantation, which we hypothesize will be reflected by significant changes in the transcriptional profile.

Grouping of the transcriptional profiles was first performed using cluster analysis (supporting information Fig. 6). This analysis revealed that the transcriptome of the SSEA1-positive cancer stem cell lines clustered independently from the primary EGCs. The top 10 differentially expressed genes are shown in supporting information Table 3. The most upregulated gene in second-generation

cancer stem cells was *Igf2*, which has previously been associated with human testicular cancers [42]. Although the EGCs CSC66 and CSC84 were highly similar, we could identify 1,000 differentially expressed genes on the basis of a *t* test *p* value of .00001 to account for multiple testing corrections (Fig. 5B). *K*-Means clustering (Fig. 5C) of these 1,000 most differentially expressed genes revealed that the transcriptional change from EGC to CSC84 was associated with repression of fibroblast growth factor signaling pathways (*FGF-4*, *FGF-17*) and repression of *inhibin*, *nodal*, and *lefty1* in



**Figure 5.** Microarray comparing primary EGCs with the stage-specific embryonic antigen 1 (SSEA1)-positive cells derived from primary tumors and secondary tumors. **(A):** First- and second-generation tumors were generated by serial transplantation without intervening culture. An aliquot of SSEA1-positive cells from primary and secondary tumors were expanded for 6 passages (CSC66) or 11 passages (CSC84) before microarray was performed. **(B):** Heat map showing the 1,000 most differentially expressed genes. Differential expression was determined by *t* test of mean intensities. Abbreviations: CSC, cancer stem cell; EGC, embryonic germ cell.

CSC84 compared with CSC66 and EGCs (cluster 1). The second-generation tumor CSC84 exhibited reduced expression of tumor suppressors *Pten* and *APC* and, interestingly, some genes associated with pluripotency and germ cell development, including *Deleted in azoospermia like*, *Developmental pluripotency associated (Dppa) 2*, *Dppa3*, *Dppa4*, *Sox2*, *Uf1* (cluster 3). However, there was no change in *Oct4* or *Nanog*. The CSC84 line was particularly enriched in oncogenes, including *JunD*, *Rad23a*, *Rb-like*, *R-Ras2*, and *Rab8b* as well as exhibiting increased expression of the Wnt receptor *Frizzled2* and *Jak2* and *Stat3* (cluster 4). CSC84 was also enriched in genes associated with motility and migration, including *SDF-1*, *SCF*, *lasp1*, *lpp*, and *Insig2*, which may account for the more aggressive behavior of these cells with serial transplantation. Cluster two contained enriched transcripts in both of the cancer stem cells lines, CSC84 and CSC66, relative to EGCs. This cluster contains genes associated with developmental processes and metabolism, as well imprinted genes such as *Insulin growth factor 2* and *H19*, as well as *Igf1bp4*. The list of differentially expressed genes and the Gene Ontology analysis from *K*-mean clustering are given in supporting information Chart 1. Therefore, our results show that the cancer stem cell populations derived from the pluripotent tumors are not identical at the transcriptional level to EGCs used to generate the primary tumors, and with sequential transplantation they become progressively more distinct and tumorigenic.

## DISCUSSION

Testicular germ cell neoplasms are solid or cystic, often disorganized tissue masses that can be either benign or malignant (reviewed in [43]). The experimental counterpart generated by the transplantation of pluripotent cells into the testis, under the kidney capsule, subcutaneously or by intramuscular injection is considered benign. However, in the current study we have determined that experimental teratomas in the testis generate tumors corresponding to a malignant, metastasizing postpubertal type of human teratoma with a self-renewing cancer stem cell population [44–47]. It is not known whether alternate transplantation sites of pluripotent cells will mediate the generation

of self-renewing tumor-initiating cells; however, analysis of stem cell markers in experimentally induced subcutaneous teratomas has revealed the presence of stem cell-like cells, suggesting that a testicular transplantation site is not essential [5]. Our results also show that emergence of cancer stem cells in teratomas is regulated by RA-sensitive self-renewal pathways, because pretreatment with RA suppresses tumorigenesis and the generation of a self-renewing population. Furthermore, the serial transplantation of cancer stem cells in this model is associated with genomic instability and transcriptional differences toward pathways associated with motility and oncogenesis indicating that the cancer stem cells are not identical to the original EGC population used to generate the primary tumor.

The original work on murine models of teratocarcinoma involved transplantation of genital ridges into host mice, which were serially transplanted into subsequent mice. It was later determined that the tumors were initiated from PGCs prior to their differentiation into spermatogonia; however, the cancer-initiating cells within each tumor were not characterized [17, 48, 49]. In the current study, our model represents an extension of this historical experiment in which a subpopulation of the teratoma cells corresponding to the most stem cell-like SSEA1-positive cells was identified as being responsible for the self-renewal capacity of teratomas derived from EGCs in serial transplantation assays. Furthermore, using this model, our results demonstrate that genetic instability is associated with cancer stem cell formation in vivo, and this is particularly evident with the transplantation of human pluripotent cells.

Genetic instability is a hallmark of testicular germ cell tumor (reviewed in [50]). Furthermore, genetic instability in pluripotent cells is an important consideration before using these cells in regenerative medicine [51–53]. Genetic stability of hESCs is determined routinely through G-banding karyotype analysis. Using this technique, characteristic changes associated with the pluripotent state, including duplications of human chromosomes 12 and 17, have been identified [53]. High-resolution techniques, such as array comparative genomic hybridization (aCGH) have shown that copy number variants differ between hESC lines [54]. Although these differences can act as a signature to distinguish the hESCs from each other, it is not known whether some of these variations

are culture-induced, or whether particular genetic changes will relate to tumor susceptibility and the generation of cancer stem cells. In testicular germ cell tumor, the generation of tumor-initiating CIS cells from PGCs is associated with profound chromosomal aneuploidies, including del(Y) (reviewed in [50, 55]). Progression from CIS to the invasive disease is associated with additional chromosomal rearrangements and duplications at 12p. Here, we observed genetic instability and del(Y), which is consistent with the current model. With regard to human chromosome 12p, this region is syntenic with mouse chromosomes 6 and 15. In the current model, karyotype analysis did not identify duplications at 6; however, iso(15) was identified. It is possible that small duplications of mouse chromosome 6 occurred below the level of detection by karyotype (5–10 megabases); however, more sensitive methods such as comparative genomic hybridization, spectral karyotyping, or aCGH could be used to test this in future studies (reviewed in [56]).

The importance of RA-mediated self-renewal in cancer stem cells was recently highlighted in malignant breast cancer in which aldehyde dehydrogenase (ALDH) activity was associated with the generation of malignant mammary stem cells [57]. ALDH converts retinal to RA, and RA is essential for growth and embryo development (reviewed in [58]). Due to its antiproliferative, proapoptotic, and prodifferentiation properties, all-trans RA is currently approved to treat promyelocytic leukemia [59]. However, treatment of testicular germ cell tumor with RA has met with mixed success. In one case [60], use of the National Cancer Institute-sponsored institutional protocol resulted in complete remission; however, in other cases, RA has had little benefit (reviewed in [61]). The reasons for these differences are not clear. However, recent studies suggest that alternate chromosomal rearrangements are associated with poor response to RA in promyelocytic leukemia [62]. Therefore, severe genetic instability in testicular cancer may abrogate the positive effects of RA resulting in treatment resistance. In future studies, the effect of RA on cancer stem cells derived from secondary tumors should help address the effect of genetic instability on efficacy of RA. In the current model, the broad effects of RA do not enable a distinction between loss of specific regulators of pluripotency and self-renewal or loss of additional retinoic acid sensitive pathways (such as hypophosphorylation of Rb) in mediating the suppressive effects on cancer stem cell generation. However, the fact that cancer stem cells are not generated in these tumors indicates that self-renewal pathways mediated by RA are required before genetically unstable cancer stem cells will emerge. In future studies, more targeted approaches will be undertaken to address specific pathways.

In the current study we used microarray analysis to determine whether the transcriptome of SSEA1-positive cells derived from primary and secondary tumors was identical to EGCs. We found that within one generation there is a change in transcription (comparing EGCs with CSC66), and in the next generation there is an even greater shift in active and repressed transcriptional programs (comparing EGC and CSC66 with CSC84). The most differentially expressed genes in second-generation SSEA1-positive cells included expected signaling pathways, such as Jak2/Stat3, Igf, Igfbp, and *Scf*, as well as various oncogenes, such as *Ras*, *Rab*, and *Rad* family members. Increasing the expression of the Jak/Stat activating ligand, *unpaired*, in *Drosophila* germ line stem cells results in self-renewal and testicular germ cell tumor [63]. Furthermore, *Igf2* consistently undergoes loss of imprinting in germ cell tumors [42]. This is usually considered a byproduct of PGC reprogramming and therefore an indicator of the stage at which PGCs transform to CIS. A tantalizing hypothesis is that increased expression of *Igf2* due to abnormal epigenetic reprogramming in the germ line may be a functional contributor to germ cell tumor formation. Indeed, *Igf2* overexpression is a consistent finding in various somatic malignancies [64, 65].

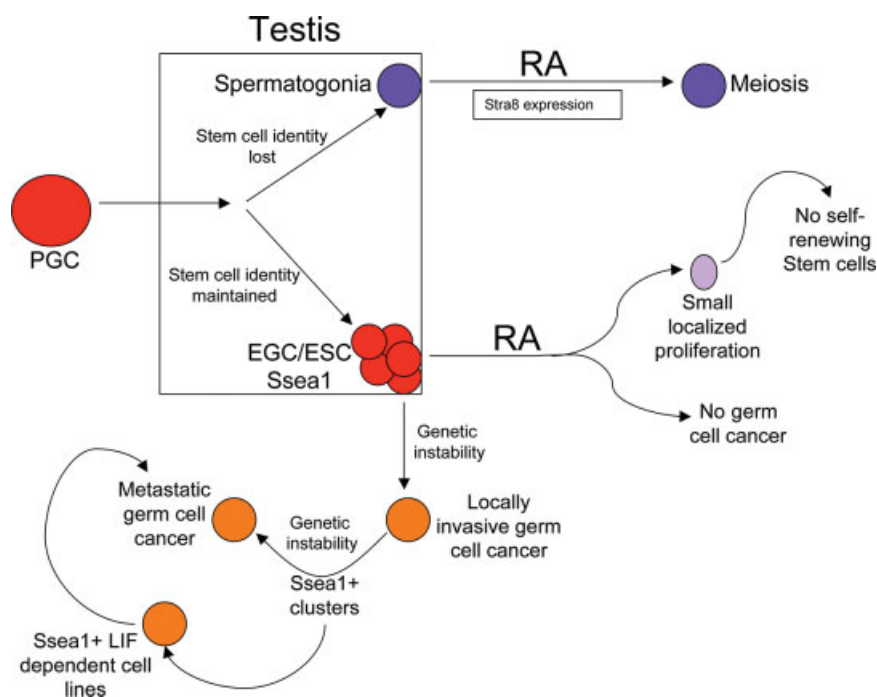
Unexpected findings included a decrease in some of the pluripotency genes, including *Dppa2*, *Dppa3*, and *Dppa4*, as well as *Sox2* and *Gdf3*, in second-generation SSEA1-positive tumor cells. However, expression levels of *Nanog*, *Oct4*, and the recently identified *Caspase-3* interacting protein *Thap11* (*Ronin*) [66], remained high and unchanged across cell types. Even though some pluripotent genes were decreased, continuing serial transplants indicate that these cells are still pluripotent in vivo and exhibited metastatic potential. These types of transcriptional imbalances should be important when studying alternate methods for generating pluripotent cells.

Profiling revealed a gene of particular interest called *Nuclear receptor binding SET domain protein 1* (NSD1). This gene is significantly downregulated in both CSC66 and CSC84 relative to EGCs. Mutations in NDS1 result in Sotos syndrome, which is associated with neurological disorders, overgrowth, and cancer [67]. Recently there have been four case reports of children with Sotos syndrome presenting with childhood germ cell tumors [68–70]. Nothing is known about expression and function of NDS1 in the germ line, and it will be important to address this in light of the current findings. Finally, the increase in genes associated with motility in second-generation SSEA1-positive cells (CSC84) was striking, and we hypothesize that this, together with the significant increase in oncogenes, is responsible for metastasis in this model.

Are these SSEA1-positive cells cancer stem cells? Our results support this conclusion. First, a cancer stem cell should reinitiate cancer. We have shown that isolation of SSEA1 cells from primary tumors by FACS reinitiates a robust metastatic secondary tumor. Second, secondary tumors contain a persistent stem cell population that can be isolated and serially passaged in culture. Third, tumors generated from the tumor stem cells were more aggressive with metastatic properties not observed in the original primary tumors, a feature of less well differentiated tumors in general. Fourth, RA treatment inhibited cancer stem cell formation in primary tumors, suggesting that the emergence of these stem cells occurs during tumorigenesis in vivo and not the expansion of a genetically unstable clone originating in the EGC population. Finally, the transcriptional profile of SSEA1-positive cells derived from primary and secondary tumors is different compared with the original EGC population, indicating that these cells are not just EGCs that have failed to differentiate, but a cancer stem cell type that has evolved through the tumorigenic process.

## CONCLUSION

In conclusion, our model (Fig. 6) provides supporting in vivo evidence that the ESC self-renewal networks regulate progenitor cell proliferation and tumorigenesis [71]. In addition, our data show that self-renewal is integral to the formation of genetically unstable cancer stem cells during tumorigenesis in vivo. Future experiments will determine the relationship between RA-responsive genes in ESCs and additional regulators of malignant teratoma development. For example, loss of PTEN [12, 72] and loss of the Dead end (DND1) protein [73–75] both result in the generation of testicular tumors in murine models in vivo; however, the generation of cancer stem cells in these models has not been evaluated. Finally, this study primarily addressed the use of EGCs as a model for testicular germ cell tumor metastasis; however, it also extends to studies on the use of pluripotent stem cells, such as ESCs or induced pluripotent stem cells. Currently the only method for predicting tumorigenic potential in human cells is abnormal karyotype [52]. In future studies, more detailed analysis of teratoma formation and emergence of cancer stem cells may provide an additional tool with which to predict



**Figure 6.** Model for the role of the stem cell self-renewal program in the generation of solid tumors and the formation of cancer initiating cells. PGCs normally enter the gonad at embryonic gestational day (E) 10.0 and undergo mitotic arrest by E15.5, which is associated with a significant decrease in the expression of ESC-expressed genes. Spermatogonia subsequently respond to RA signaling in vivo to initiate meiosis [18]. PGCs that fail to reduce the expression of the embryonic stem cell program develop carcinoma in situ, which resembles clusters of ESCs/EGCs [12]. We have shown that transplantation of EGCs into the adult testis is sufficient to model the invasive stage of the disease, with the generation of localized immature teratomas experimentally called teratocarcinoma because of their stem cell population that is capable of self-renewal in vitro, and also for generating new teratomas in vivo. We also demonstrated that reducing the stem cell identity and self-renewal of EGCs by addition of RA prior to transplantation is sufficient to block testicular cancer of the immature teratoma/teratocarcinoma class and prevent and the generation of cancer-initiating cells. Abbreviations: EGC, embryonic germ cell; LIF, leukemia inhibitory factor; PGC, primordial germ cell; RA, retinoic acid; Ssea1, stage-specific embryonic antigen 1; Stra8, *Stimulated by Retinoic Acid 8*.

tumorigenic potential and the risk of tumor formation from pluripotent cell derivatives in vivo.

## ACKNOWLEDGMENTS

We thank Dr. Martin Pera, Ph.D., for instruction in testicular transplantation and Dr. Gayane Ambartsumyan, M.D., for instruction in aseptic surgical technique. The SSEA1 antibody developed by D. Solter and B.B. Knowles was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA. Microarray was performed by the UCLA Clinical Microarray Core, directed by Xinmin Li. We also acknowledge John Vincent, Caroline Sham, and Ziwei Li for critical reading of the manuscript. This work

was supported by funding from the Department of Molecular, Cell and Developmental Biology, UCLA (to A.T.C.); the Lance Armstrong Foundation (to A.T.C.); STOP Cancer (to A.T.C.); a Developmental Project (to A.T.C.) with the UCLA In Vivo Cellular and Molecular Imaging Center (PI, Herschmann); the Fuller Foundation and the Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research at UCLA (to A.T.C.); California Institute for Regenerative Medicine RS1 (to M.A.T.); and NIH Grant P01-GM081621-01A1 (to J.A.Z., Z.G., M.A.T., and A.T.C.). A.L. is supported by an Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research Center Training Grant.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

## REFERENCES

- 1 Simmonds P, Lee A, Thacker J et al. Primary pure teratoma of the testis. *J Urol* 1996;155:939–942.
- 2 Pera M, Cooper S, Mills J et al. Isolation and characterization of a multipotent clone of human embryonal carcinoma cells. *Differentiation* 1989;42:10–23.
- 3 Pera MF, Blasco-Lafita MJ, Cooper S et al. Analysis of cell-differentiation lineage in human teratomas using new monoclonal antibodies to cytostructural antigens of embryonal carcinoma cells. *Differentiation* 1988;39:139–149.
- 4 Pera MF, Blasco-Lafita MJ, Mills J. Cultured stem-cells from human testicular teratomas: The nature of human embryonal carcinoma, and its comparison with two types of yolk-sac carcinoma. *Int J Cancer* 1987; 40:334–343.
- 5 Prokhorova TA, Harkness LM, Frandensen U. Teratoma formation by human embryonic stem cells is site-dependent and enhanced by the presence of Matrigel. *Stem Cells Dev* 2008 [Epub ahead of print].
- 6 Clark A. The stem cells of testicular cancer. *Stem Cell Reviews* 2007; 3:49–59.

- 7 Hoei-Hansen C, Alumstrup K, Nielsen J et al. Stem cell pluripotency factor NANOG is expressed in human fetal gonocytes, testicular carcinoma in situ and germ cell tumors. *Histopathology* 2005;47:48–56.
- 8 Cheng L. Establishing a germ cell origin for metastatic tumors using OCT4 immunohistochemistry. *Cancer* 2004;101:2006–2010.
- 9 Skotheim RI, Lind GE, Monni O et al. Differentiation of human embryonal carcinomas in vitro and in vivo reveals expression profiles relevant to normal development. *Cancer Res* 2005;65:5588–5598.
- 10 ISCI. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol* 2007;25:803–816.
- 11 Almstrup K, Hoei-Hansen CE, Wirkner U et al. Embryonic stem cell-like features of testicular carcinoma in situ revealed by genome-wide gene expression profiling. *Cancer Res* 2004;64:4736–4743.
- 12 Kimura T, Suzuki A, Fujita Y et al. Conditional loss of PTEN leads to testicular teratoma and enhances embryonic germ cell production. *Development* 2003;130:1691–1700.
- 13 Looijenga L, Gills A, Stoop H et al. Chromosomes and expression in human testicular germ cell tumors: Insight into their cell of origin and pathogenesis. *Ann NY Acad Sci* 2007;1120:187–214.
- 14 Gillis A, Stoop H, Hersmus R et al. High-throughput microRNAome analysis in human germ cell tumours. *J Pathol* 2007;213:319–328.
- 15 Giuliano C, Kerley-Hamilton J, Bee T et al. Retinoic acid represses a

- cassette of candidate pluripotency chromosome 12p genes during induced loss of human embryonal carcinoma tumorigenicity. *Biochem Biophys Acta* 2005;1731:48–56.
- 16 Maldonado-Saldivia J, van den Bergen J, Krouskos M et al. Dppa2 and Dppa4 are closely linked SAP motif genes restricted to pluripotent cells and the germ line. *STEM CELLS* 2007;25:19–28.
  - 17 Stevens L. Origin of testicular teratomas from primordial germ cells in mice. *J Natl Cancer Inst* 1967;549–552.
  - 18 Koubova J, Menke D, Shou Q et al. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proc Natl Acad Sci U S A* 2006;103:2474–2479.
  - 19 Kerr CL, Hill CM, Blumenthal PD et al. Expression of pluripotent stem cell markers in the human fetal testis. *STEM CELLS* 2008;26:412–421.
  - 20 Yamaguchi S, Kimura H, Tada M et al. Nanog expression in mouse germ cell development. *Gene Expr Patterns* 2005:639–646.
  - 21 Schöler H, Ruppert S, Suzuki N et al. New type of POU domain in germ line-specific protein Oct-4. *Nature* 1990;344:435–439.
  - 22 Schöler H, Dressler G, Balling R et al. Oct-4: A germ line specific transcription factor mapping to the mouse t-complex. *EMBO J* 1990;9:2185–2195.
  - 23 Sato M, Kimura T, Kurokawa K et al. Identification of PGC7, a new gene expressed specifically in preimplantation embryos and germ cells. *Mech Dev* 2002;113:91–94.
  - 24 Resnick JL, Bixler LS, Cheng L et al. Long-term proliferation of mouse primordial germ cells in culture. *Nature* 1992;359:550–551.
  - 25 Resnick JL, Ortiz M, Keller JR et al. Role of fibroblast growth factors and their receptors in mouse primordial germ cell growth. *Biol Reprod* 1998;59:1224–1229.
  - 26 Matsui Y, Zsebo K, Hogan B. Derivation of pluripotent embryonic stem cells from murine primordial germ cells. *Cell* 1992;70:841–847.
  - 27 Labosky PA, Hogan BL. Mouse primordial germ cells. Isolation and in vitro culture. *Methods Mol Biol* 1999;97:201–212.
  - 28 McLaren A, Durcova-Hills G. Germ cells and pluripotent stem cells in the mouse. *Reprod Fertil Dev* 2001;13:661–664.
  - 29 Durcova-Hills G, Adams IR, Barton S et al. The role of exogenous fibroblast growth factor-2 on the reprogramming of primordial germ cells into pluripotent stem cells. *STEM CELLS* 2006;24:1441–1449.
  - 30 Labosky PA, Barlow DP, Hogan BL. Mouse embryonic germ (EG) cell lines: Transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. *Development* 1994;120:3197–3204.
  - 31 Labosky PA, Barlow DP, Hogan BL. Embryonic germ cell lines and their derivation from mouse primordial germ cells. *Ciba Found Symp* 1994;182:157–168, discussion 168–178.
  - 32 Shambloot MJ, Axelman J, Wang S et al. Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci U S A* 1998;95:13726–13731.
  - 33 Kerr CL, Hill CM, Blumenthal PD et al. Expression of pluripotent stem cell markers in the human fetal ovary. *Hum Reprod* 2008;23:589–599.
  - 34 Stevens L. The Origin and Development of Testicular, Ovarian and Embryo-Derived Teratomas. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1983.
  - 35 Reubinoff B, Pera M, Fong C-Y et al. Embryonic stem cell lines from human blastocysts: Somatic differentiation in vitro. *Nature* 2000;18:399–404.
  - 36 Blum B, Benvenisty N. Clonal analysis of human embryonic stem cell differentiation in teratomas. *STEM CELLS* 2007;25:1924–1930.
  - 37 Wong D, Liu H, Ridky T et al. Module map of stem cell genes guides creation of epithelial cancer stem cells. *Cell Stem Cell* 2008;10:333–344.
  - 38 Ben-Porath I, Thomson M, Carey V et al. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* 2008;40:499–507.
  - 39 Clark AT, Bodnar MS, Fox M et al. Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. *Hum Mol Genet* 2004;13:727–739.
  - 40 Cao F, Lin S, Xie X et al. In vivo visualization of embryonic stem cell survival, proliferation, and migration after cardiac delivery. *Circulation* 2006;113:1005–1014.
  - 41 Ivanova N, Dobrin R, Lu R et al. Dissecting self-renewal in stem cells with RNA interference. *Nature* 2006;442:533–538.
  - 42 van Gurp RJ, Oosterhuis JW, Kalscheuer V et al. Biallelic expression of the H19 and IGF2 genes in human testicular germ cell tumors. *J Natl Cancer Inst* 1994;86:1070–1075.
  - 43 Ulbright T. Germ cell tumors of the gonads: A selective review emphasizing problems in differential diagnosis, newly appreciated, and controversial issues. *Mod Pathol* 2005;18(suppl):S61–S79.
  - 44 Damjanov I, Andrews PW. The terminology of teratocarcinomas and teratomas [Comment]. *Nat Biotechnol* 2007;25:1212.
  - 45 Lensch M, Ince TA. The terminology of teratocarcinomas and teratomas. *Nat Biotechnol* 2007;25:1211.
  - 46 Damjanov I, Andrews P. The terminology of teratocarcinoma and teratomas. *Nat Biotechnol* 2007;25:1212.
  - 47 Atala A, Furth M. Response to The terminology of teratocarcinomas and teratomas. *Nat Biotechnol* 2007;25:1211–1212.
  - 48 Stevens L. The biology of teratomas including evidence indicating their origin from primordial germ cells. *Annee Biol* 1962;1:585–610.
  - 49 Stevens L, Hummel K. A description of spontaneous congenital testicular teratomas in strain 129 mice. *J Natl Cancer Inst* 1957;8:719–747.
  - 50 Rajpert-De Meyts E, Bartkova J, Samson M et al. The emerging phenotype of the testicular carcinoma in situ germ cell. *APMIS* 2003;111:267–278, discussion 278–279.
  - 51 Herszfeld D, Wolvetang E, Langton-Bunker E et al. CD30 is a survival factor and a biomarker for transformed human pluripotent stem cells. *Nat Biotechnol* 2006;24:351–357.
  - 52 Baker D, Harrison N, Maltby E et al. Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. *Nat Biotechnol* 2007;25:207–215.
  - 53 Draper J, Smith K, Gokhale P et al. Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nat Biotechnol* 2003;22:53–54.
  - 54 Wu H, Kim K, Mehta K et al. Copy number variant analysis of human embryonic stem cells. *STEM CELLS* 2008;26:1484–1489.
  - 55 Bianchi N, Richard S, Pavicic W. Y chromosome instability in testicular cancer. *Mutat Res* 2006;612:172–188.
  - 56 Teitell M. How to assess a stem cell genome. *Nature Reports Stem Cells* 2008 [Epub ahead of print].
  - 57 Ginstier C, Hur M, Charafe-Jauffret C et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 2007;1:555–567.
  - 58 Duester G. Genetic dissection of retinoid dehydrogenases. *Chem Biol Interact* 2001;130–132:469–480.
  - 59 Sanz M, Montesinos P, Vellenga E et al. Risk-adapted treatment of acute promyelocytic leukemia with all-trans-retinoic acid and anthracycline monotherapy: Long-term outcome of the LPA 99 multicenter study by the PETHEMA Group. *Blood* 2008;112:3130–3134.
  - 60 Gordon M, Battiato L, Finch D et al. Dramatic response of teratoma-associated non-germ-cell cancer with all-trans retinoic acid in a patient with nonseminomatous germ cell tumor. *Am J Clin Oncol* 2001;24:269–271.
  - 61 Kollmannsberger C, Mayer F, Kuczyk M et al. Treatment of patients with metastatic germ cell tumors relapsing after high-dose chemotherapy. *World J Urol* 2001;19:120–125.
  - 62 Luatti S, Marzocchi G, Ottaviani E et al. Acute promyelocytic leukemia with amplification of PML-RARalpha rearrangement: Clinical implications. *Leuk Res* 2008;32:1941–1943.
  - 63 Kiger AA, Jones DL, Schulz C et al. Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. *Science* 2001;294:2542–2545.
  - 64 Wise T, Pravtcheva D. Delayed onset of Igf2-induced mammary tumors in Igf2r transgenic mice. *Cancer Res* 2006;66:1327–1336.
  - 65 Kaneda A, Feinberg A. Loss of imprinting of IGF2: A common epigenetic modifier of intestinal tumor risk. *Cancer Res* 2005;65:11236–11240.
  - 66 Dejosez M, Krumenacker J, Zitur L et al. Ronin is essential for embryogenesis and the pluripotency of mouse embryonic stem cells. *Cell* 2008;133:1162–1174.
  - 67 Faravelli F. NSD1 mutations in Sotos syndrome. *Am J Med Genet C Semin Med Genet* 2005;137C:24–31.
  - 68 Jin Y, Chen R, Wang P et al. Sacrococcygeal germ cell tumor and spinal deformity in association with Sotos syndrome. *Med Pediatr Oncol* 2002;38:133–134.
  - 69 Leonard N, Cole T, Bhargava R et al. Sacrococcygeal teratoma in two cases of Sotos syndrome. *Am J Med Genet* 2000;95:182–184.
  - 70 Muraishi O, Kumamaru T, Nozaki Y et al. Testicular yolk sac tumour in a patient with Sotos syndrome. *BJU Int* 1999;83:357–358.
  - 71 Hochedlinger K, Yamada Y, Beard C et al. Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. *Cell* 2005;121:465–477.
  - 72 Di Cristofano A, Pesce B, Cordon-Cardo C et al. Pten is essential for embryonic development and tumour suppression. *Nat Genet* 1998;19:348–355.
  - 73 Noguchi T, Noguchi M. A recessive mutation (ter) causing germ cell deficiency and a high incidence of congenital testicular teratomas in 129/Sv-ter mice. *J Natl Cancer Inst* 1985:385–392.
  - 74 Youngren K, Coveny D, Peng X et al. The Ter mutation in the dead end gene causes germ cell loss and testicular germ cell tumors. *Nature* 2005;435:360–364.
  - 75 Kedde M, Strasser MJ, Boldajipour B et al. RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. *Cell* 2007;131:1273–1286.



See [www.StemCells.com](http://www.StemCells.com) for supporting information available online.