

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

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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 (SCID) 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. In order 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 non-clonal 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 are involved in the acquisition of metastatic behavior in the cancer stem cell population of tumors derived from pluripotent cells.

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, pre-pubertal teratomas are 46,XY and benign

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whereas post-pubertal testicular teratoma is aneuploid and often metastatic [1]. The cells in post-pubertal 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 re-transplantation assays to generate secondary tumors in 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 experimentally 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, post-pubertal 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 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-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 post-pubertal 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 (E) gestational day E7.5-E12.5. *In vivo* PGCs are unipotent, and in males 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 genes (Dppa)* such as *Dppa2*, *Dppa3* and *Dppa4* which are concomitantly down regulated as PGCs differentiate from E13.5-E15.5 [19] [20-23] [16]. Culture of human and mouse PGCs expressing *Nanog* and *Oct4 in vitro* results in conversion from unipotent PGCs to pluripotent embryonic germ cells (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 post-pubertal human disease that closely resembles EGCs and ESCs. Furthermore, once a murine testicular teratoma is generated, it can be serially transplanted by re-injecting 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 and generation of teratomas in 4-8 weeks 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, as well as 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].

METHODS

Cells

C57BL/6 EGCs were derived from PGCs at E12.5 and maintained on irradiated STO cells as previously described [30]. The H9 (WA09) and HSF-6 (UC06) hESC lines were cultured according to previous reports [39]. Karyotype 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 ATCC (Manassas, VA). All work with hESCs was conducted with prior approval from the UCLA Human Embryonic Stem Cell Research Oversight (ESCRO) 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 μ M retinoic acid (Sigma) for four 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 x 10⁶ cells in a volume of 50 μ l PBS were transplanted into the testis of

adult SCID mice. 4-6 weeks after surgery, mice were euthanized.

Generation of secondary testicular tumors by serial transplantation

SSEA1 positive cells were isolated by either Magnetic Activated Cell Sorting (MACS) or by Fluorescent activated cell sorting (FACS) following labeling with primary antibody against SSEA1. Secondary antibodies involved labeling with either a magnetic bead or APC-labeled secondary antibody for MACS or FACS respectively.

Live animal imaging

Mice were anesthetized with 2% isoflurane and injected with 100 μ l 15mg/ml D-luciferin. Following the injection, mice were placed in the chamber of an IVIS optical imaging system (Xenogen) and imaged for up to 30 minutes. Photons were collected for a period of 10s per reading and images were analyzed using the Living Image software v. 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/s/cm²/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 imaging system.

Flow cytometry

A single cell suspension of the tumors and testis was generated by dissection of the tumor into 1mm² pieces followed by incubation in 1mg/ml collagenase in high glucose DMEM for 2 hours at 37°C in 5% CO₂. Staining was performed in PBS containing 0.5% BSA. SSEA1 antibody from the Developmental Studies Hybridoma Bank was used at 1:100 with 1-2 million cells per ml of staining buffer. All samples were analyzed using a BD Biosciences LSR II. Apoptosis was evaluated using 7-AAD (Calbiochem, San Diego, CA) according to 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 2mM EDTA. Before adding the fluorescently labeled secondary antibody, MACS bead conjugated Rat anti mouse IgM antibody (Miltenyi Biotech) was added to the cells at a ratio of 20 μ l antibody to 80 μ l staining buffer per 1×10^7 cells and incubated at 4°C for 20 minutes. For MACS separation was performed on MS columns in a MiniMACS separation unit (Miltenyi Biotech) following the manufacturer's instructions. Purity of the separated populations was determined by flow cytometry. For FACS cells were sorted on a FACS Vantage (Becton Dickson).

Immunofluorescence

Tumors were fixed in 4% paraformaldehyde for at least 24 hours at room temperature. Tissue was embedded in paraffin and sectioned at 5 μ m. Antigen retrieval in 10 mM Tris Base, 1mM EDTA 0.05% Tween 20 at 95°C for 40 minutes was used for all antibodies. Primary antibodies included SSEA1 (1:50 – DSHB), Oct3/4 (1:100 – Santa Cruz Biotech), Dazl (1:100 – Abcam Inc) and Mvh/Ddx4 (1:100 – Abcam Inc). Secondary antibodies (1/200) were from Jackson Immuno Research. Sections were mounted in Prolong Gold Anti-fade Reagent with DAPI (Invitrogen) and photographed on an LSM 510 confocal microscope.

Real time polymerase chain reaction

RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse-transcribed with Superscript II Reverse Transcriptase (Invitrogen). Following reverse transcription, real-time polymerase chain reaction (RT-PCR) was performed on the cDNA according to the manufacturer's protocol using a BioRad iQ iCycler with SYBR green as the fluorophore. Primer sequences are included in Supplemental Table 1.

Western blot

Protein was harvested using Pierce M-PER cell lysis reagent (Thermo Scientific, Rockford, IL). Protein concentration measured using the Pierce BCA Protein assay (Thermo Scientific, Rockford, IL). 25 μ g of total protein was electrophoresed through 12% NuPAGE Novex Bis-Tris gels and transferred according to standard procedures. For immunoblotting, primary antibodies were mouse anti-human Rb 1/250 (BD Pharmingen), mouse anti-human p53 1/500 (Santa Cruz), rabbit anti mouse nanog 1/500 (Abcam), and rabbit anti human beta-actin (1/5000) (loading control).

Statistics

In order to determine statistical significance between retinoic acid treated and control EGCs, Student's T-tests were performed and significance was accepted with $p < 0.05$

Microarray

Microarrays were performed using the Affymetrix Mouse Genome 430 2.0 Array. 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) and 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.

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 $0.5 - 1 \times 10^6$ EGCs into the testes of SCID mice resulted in tumor formation in 25/26 transplants at 4 to 6 weeks following surgery (Fig 1A). EGCs transplanted directly into the testes result in locally invasive testicular tumors with tri-lineage embryonic differentiation that included large amounts of primitive neuroepithelium (Figure 1B) and endodermally derived gut epithelium (Figure 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 stage-specific embryonic antigen 1 (SSEA1), which is present on the cancer initiating EGCs used to make the primary tumor. SSEA1 is not expressed on adult testicular cells (Figure 1D and Supplemental Figure 1A and B). 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 to the tumor initiating EGCs cultured on MEFs which are >90% positive for SSEA1 prior to transplantation (Figure 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 (Supplemental Table 2). Our results show that 4-6 weeks after transplantation into the testis, teratomas derived from mouse ESCs have a detectable SSEA1 positive population at 12.17%, which is very similar to the proportion of SSEA1

positive cells in EGCs 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 2/3 tumors suggesting that pluripotent ESCs are more tumorigenic than EGCs *in vivo*. With regard to human pluripotent cells, the pluripotent embryonal carcinoma line (called NTERA2), which was derived from a metastasized teratocarcinoma exhibits a SSEA4 positive population at 6.89% following transplantation and tumor formation, whereas tumors from karyotypically normal hESCs results 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 (Figure 2A). Co-staining with anti-Oct4 demonstrated that all SSEA1 positive tumor cell clusters expressed this pluripotent transcription factor in the nucleus (Figure 2A). Given the abundance of immature neural structures in the primary tumors, Nestin and Oct4 co-staining was performed and showed that Nestin and Oct4 did not co-localize, suggesting that the SSEA1 positive cells in tumors were not related to neural precursor or neural stem cells (Figure 2B). Two additional germ cell markers, deleted in azoospermia-like (Dazl) and mouse vasa homolog (Mvh), were also evaluated because these markers label the spermatogonia and spermatocytes in adult testis (Supplemental Figure 2A and B). These germ line markers did not co-localize with SSEA1 or Oct4 staining in

the testicular tumors (Figure 2C and D). In sum, the lack of germ line or neural identity, as indicated by the absence of Dazl, Mvh and Nestin co-staining, 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 2.5×10^5 positive cells resulted in not only a testicular tumor, but also metastasis throughout the peritoneum and also to the kidney (Figure 3A). This was observed in 100% of secondary tumors at this cell number (Table 1). In contrast metastatic activity was only observed 1/26 transplants of primary EGCs at numbers of 5×10^5 cells and greater. As controls, transplantation of mouse embryonic fibroblasts (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 LIF. Together, this strongly suggests 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 (Figure 3B) bone (Figure 3C) and muscle (not shown). In order to determine whether stem cell populations can be isolated from the secondary tumors, we performed MACS sorting before culturing on STOs (Figure 3D). This resulted in the generation of cell lines capable of robust self-renewal (Figure 3D). Re-transplantation of tumor derived SSEA1 positive

cells that had been cultured for >10 passages resulted in highly metastatic tumors six 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 either genetic, epigenetic and/or transcriptional differences between primary EGCs and the subsequent cancer 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 non-clonal sublines, and a minor clone with two non-clonal 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 (Supplemental Figure 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 (Supplemental Figure 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 four days disrupted self-renewal as monitored by changes in colony morphology, and significantly reduced mRNA expression of pluripotent transcription factors (Supplemental Figure 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 (Supplemental Figure 5). Western blot was also performed to confirm loss of NANOG protein, and also that RA treatments result in reduced Rb phosphorylation. We did not identify appreciable differences in P53 expression between control and RA treated cells (Supplemental Figure 5). Next we transplanted 5×10^5 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 (Figure 4A). Tumors derived from control cells were localized to the testis (no metastasis) and were detected in 2/8 cases beginning at 5 days following transplantation. By day 10, 6/8 transplants had a detectable signal above background, and by day 14, 8/8 transplants had a detectable signal. In contrast, tumors derived from RA treated EGCs were first detectable in 2/4 transplants at day 14. By day 21, 2/4 transplants had a signal above background and one transplant lost the signal (Figure 4A). By day 41 only 1/4 transplants had retained a bioluminescent signal above background. In the cohort treated with RA, 1/4 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.

In order to determine whether SSEA1 positive cells could be identified in tumors derived from

RA treated cells, flow cytometry was performed (Figure 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 derived 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 (Figure 4B, arrow) without differentiated structures. Furthermore, SSEA1 and Oct4 did not stain the tumor cells (Figure 4C). Although we did detect isolated Vasa-positive cells intermingling with the negatively staining stroma (Figure 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 (Figure 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%) 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, 1 of the 2 mice died at 5 weeks, and in septenary tumors 1 of 2 mice died at 4 weeks due to tumor burden and increased metastasis now including the liver. This suggests that the cancer stem cells are 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 (Supplemental Figure 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 Supplemental table 3. The most up-regulated 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 1000 differentially expressed genes based on a ttest p-value of 0.00001 to account for multiple testing corrections (Figure 5B). K-means clustering (Figure 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 CSC84 compared to CSC66 and EGCs (cluster 1). The second-generation tumor CSC84 exhibited reduced expression of tumor suppressors *Pten* and *APC*, and interesting, some genes associated with pluripotency and germ cell development including *Deleted in azoospermia like*, *Developmental pluripotency associated (Dppa)2*, *Dppa3*, *Dppa4*, *Sox2*, *Utf1* (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*, *Stat3* (cluster 4). CSC84 was also enriched

in genes associated with motility and migration including *SDF-1*, *SCF*, *lasp1*, *lpp*, *Insig2*, which may account for the more aggressive behavior of these cells with serial transplantation. Cluster 2 contained enriched transcripts in both 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 *Igfbp4*. For the list of differentially expressed genes and the GO analysis of clusters derived from K-mean clustering refer to Supplemental Chart 1. Therefore, our results show 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 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 (for review see [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 post-pubertal 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 have 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 pre-treatment 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 towards 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 were 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 (for review see [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 (CNV) 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) (for review see [50, 55]). Progression from CIS to the invasive disease is associated with additional chromosomal rearrangements and duplications at 12p, dup(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 (5-10Mb), however more sensitive methods such as CGH, spectral karyotyping or aCGH could be used to test this in future studies (for review see [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 retinoic acid (RA), and RA is essential for growth and embryo development (for review see [58]) Due to its anti-proliferative, pro-apoptotic and pro-differentiation properties, all-trans RA is currently approved to treat promyelocytic leukemia [59]. However, treatment of testicular germ cell tumor with RA has been met with mixed success. In one case [60], use of the NCI-sponsored Institutional protocol resulted in complete remission, however in other cases, RA has had little benefit (for review see [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 to CSC66) and in the next generation there is an even greater shift in active and repressed transcriptional programs (comparing EGC and CSC66 to 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 by-product 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* over expression 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 down regulated 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 together with the significant increase in oncogenes, we hypothesize are together 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 re-initiate cancer. We have shown that isolation of SSEA1 cells from primary tumors by FACS re-initiates 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 to 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/ SUMMARY

Together, our model (Figure 6) provides supporting *in vivo* evidence 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] or 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 have 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 tumorigenic potential and the risk of tumor formation from pluripotent cell-derivatives *in vivo*.

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Figure 1. Testicular transplantation of EGCs. (A) Solid tumor generated by transplantation of 5×10^5 EGCs directly into the testis (arrow shows testis). (B) Primitive neural tissue (arrow head) and neural epithelium (arrow) (100X) (C) Endodermal derivative, arrow showing a goblet cell (200X). (D) The average percentage SSEA1 positive cells in testis and testicular tumors (n=5) induced from EGCs, compared to EGCs cultured on STOs in the presence of LIF.

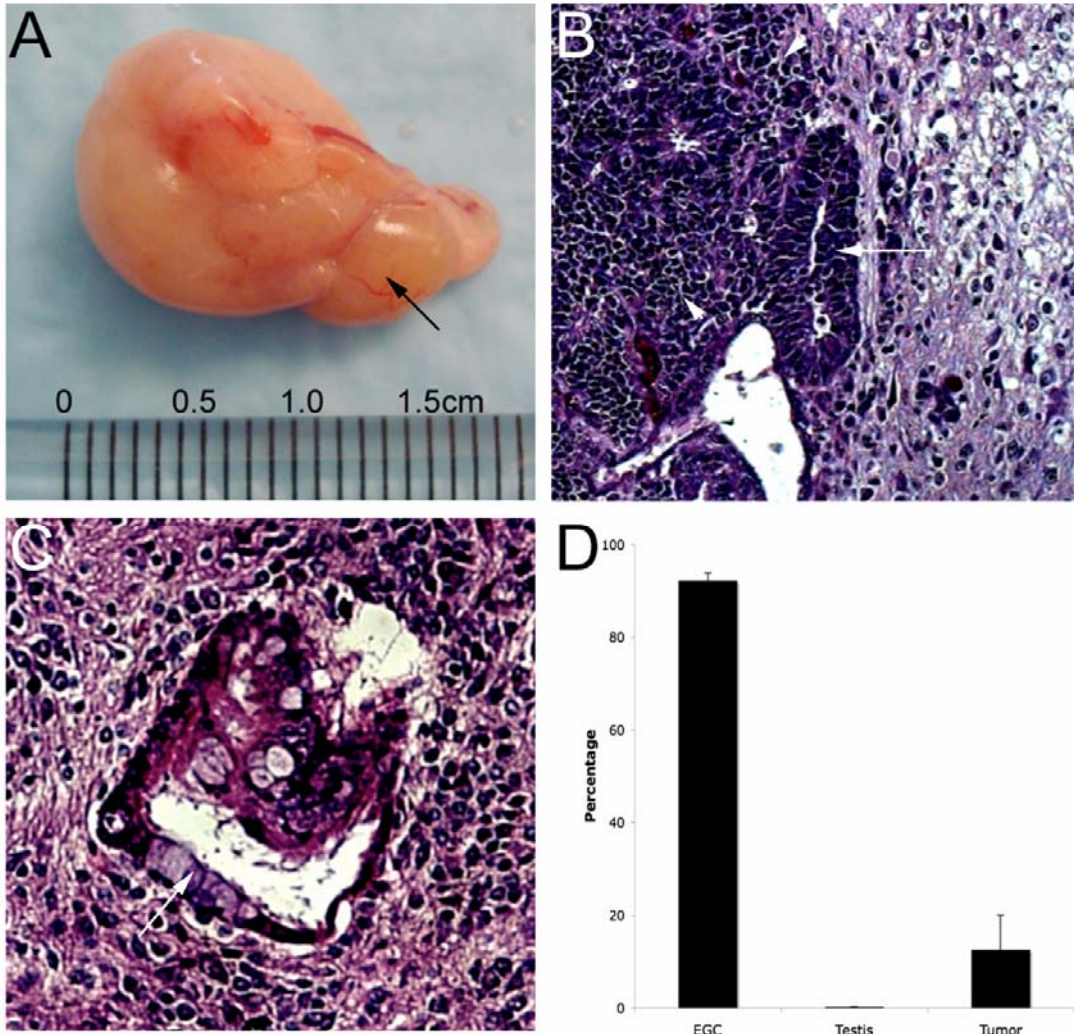


Figure 2. Immunofluorescent staining of testicular tumors at 6 weeks (A-C). (A) Double immunofluorescence for SSEA1 (green) and Oct4 (red). (B) Double immunofluorescence for Nestin (green) and Oct4 (red). (C) Double immunofluorescence for Mvh (green) and Oct4 (red). (D) Dazl (green) and Oct4 (red). Magnifications: 400x

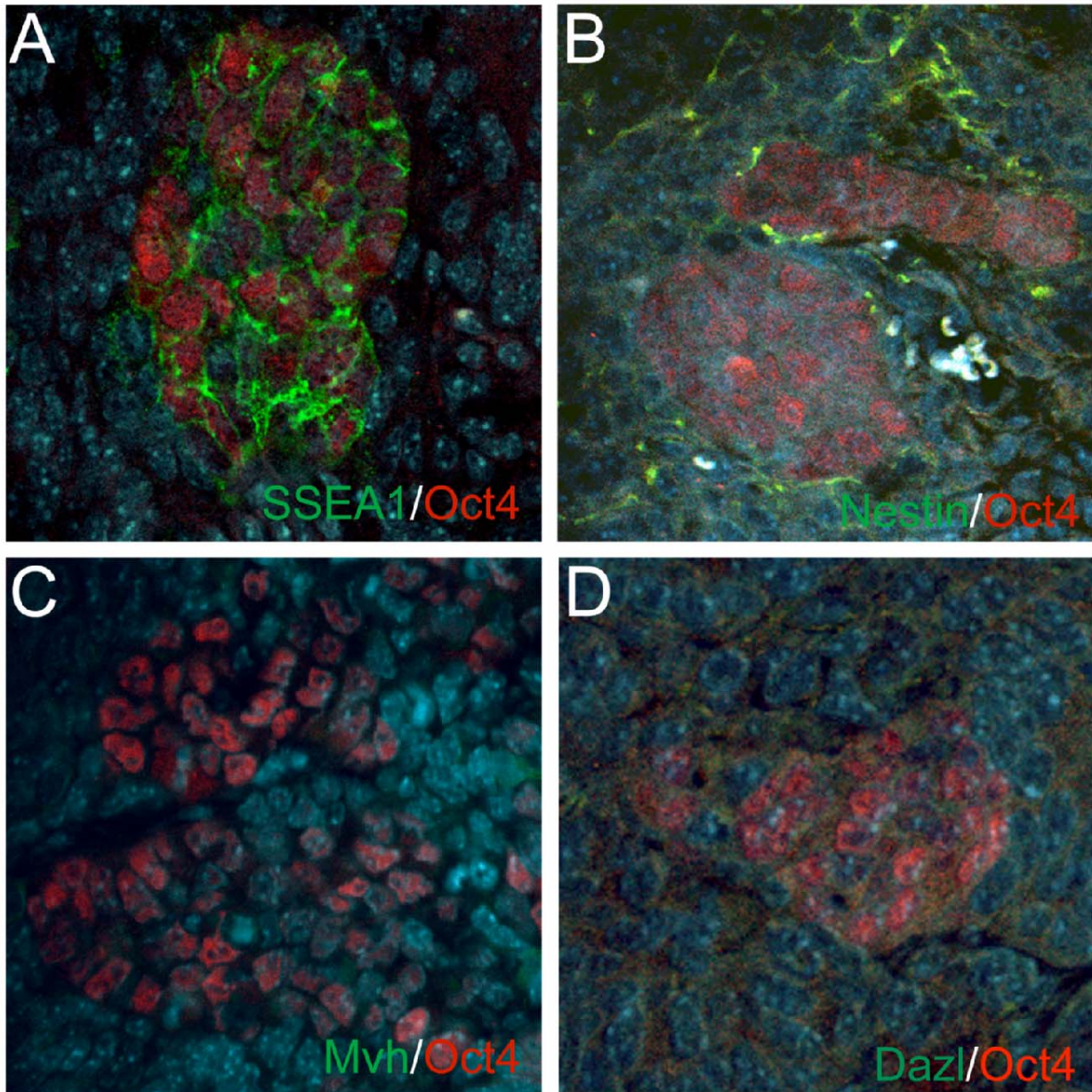


Figure 3. (A) Testicular tumor (white arrow) and metastasis from the primary tumor into the kidney (black arrow) and dissected tumors that had seeded the peritoneum (scale bar in mm). (B) Histology of secondary tumor shows differentiated cell types including neural epithelium (arrow), and (C) Bone (100X). (D) SSEA1 positive cells isolated by MACS and re-cultured on inactivated STOs in the presence of LIF.

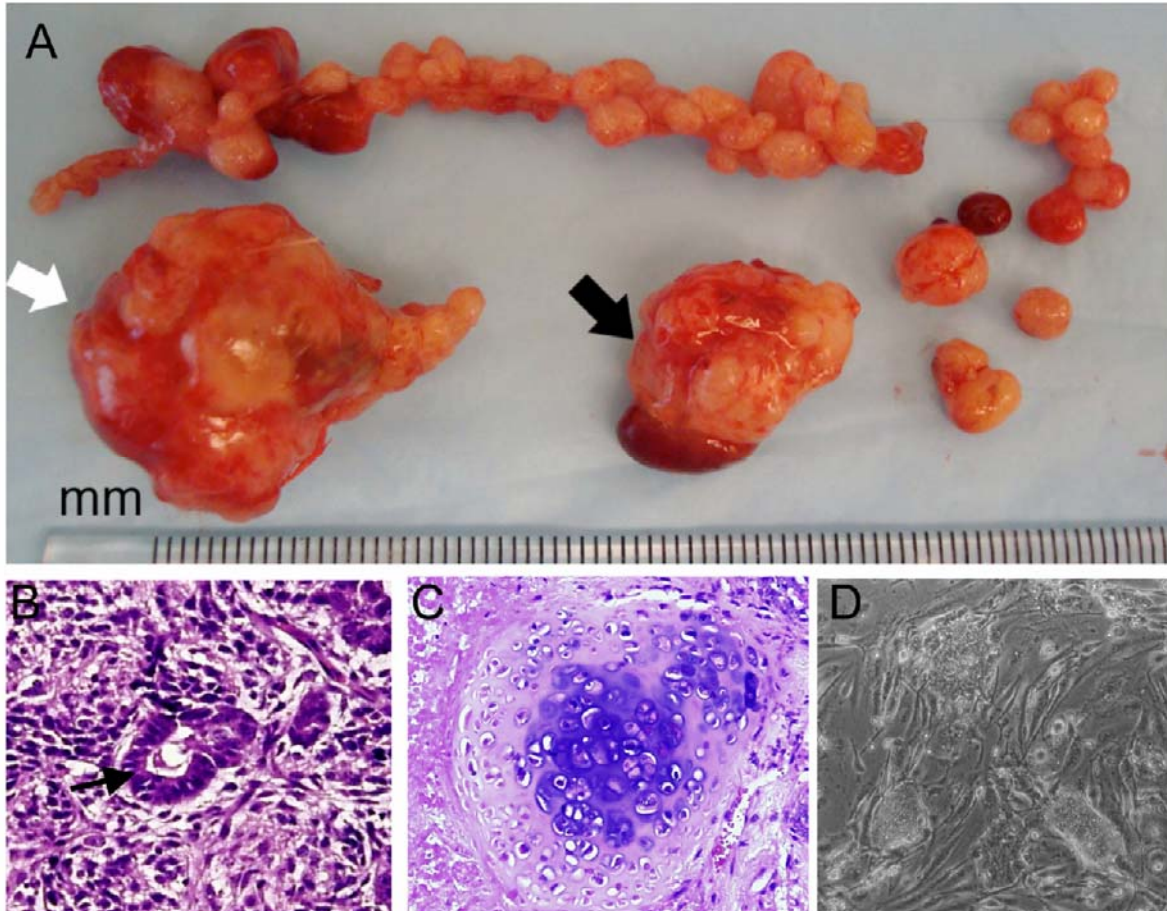


Figure 4. Analysis of tumor growth following treatment of EGCs with RA. Live animal imaging of tumor growth. (A) Dynamics of tumor growth following transplantation of EGCs into the testis as monitored by live animal imaging. Each spot refers to one mouse imaged over multiple time points. Shown are time points taken at day 41 with the corresponding bioluminescence color scale. Tumor growth increased from 1×10^5 p/sec/cm²/sr by day 5 to 1×10^8 p/sec/cm²/sr by day 30 in mice transplanted with untreated EGCs. In mice transplanted with EGCs treated with RA, signal was detected in 3/5 mice and the signal ranged from 1×10^5 to 1×10^6 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 (200X). (C) Stromal-like cells were negative for OCT4 and SSEA1. Dotted lines outline an intact seminiferous tubule, which is also negative for OCT4 and SSEA1 (400X). (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 (400x).

A

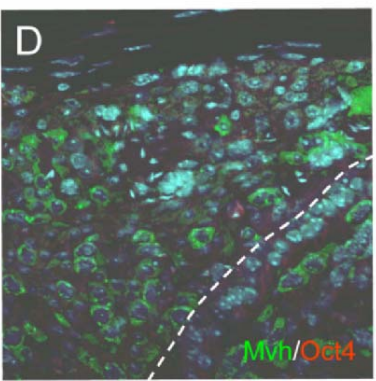
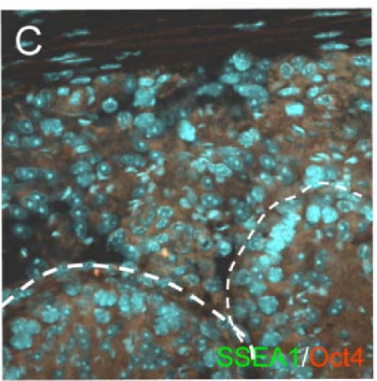
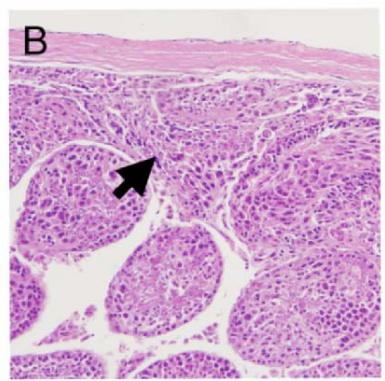
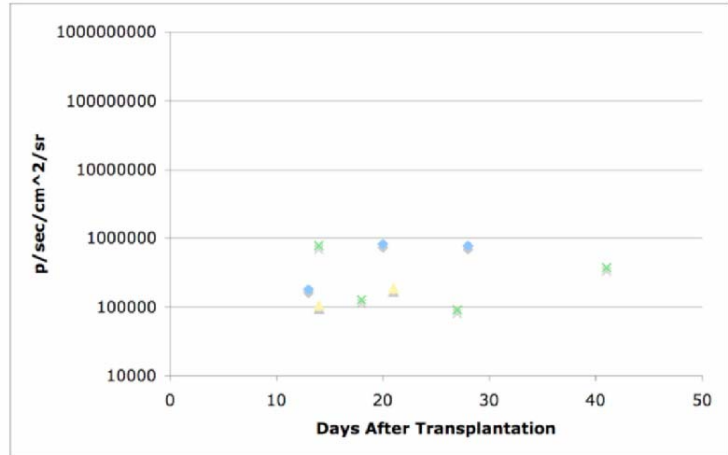
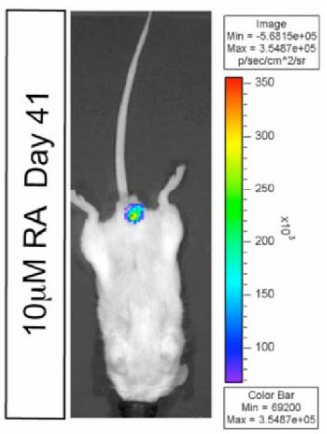
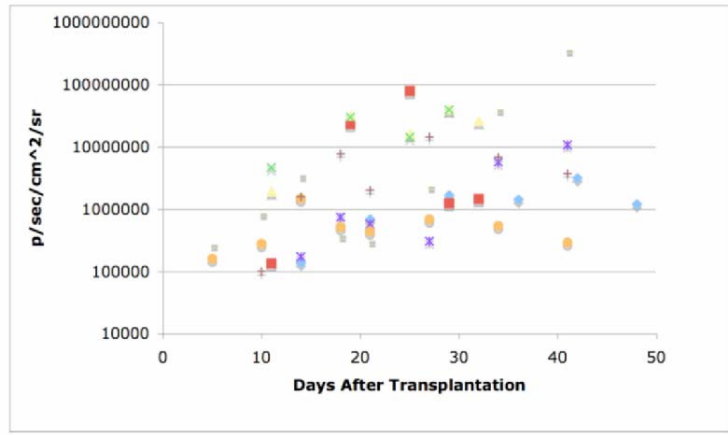
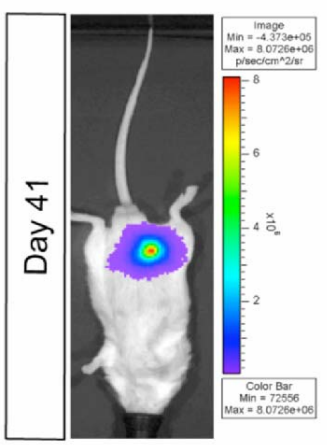


Figure 5. Micoarray comparing primary EGCs to the SSEA1 positive cells derived from primary tumors and secondary tumors. (A) First and second generation tumors were generated by serial transplantation without intervening culture. An aliquot of SSEA1 positive cells from primary and secondary tumors were expanded for 6 passages (CSC66) or 11 passages (CSC84) before performing microarray. (B) Heat map showing 1,000 most differentially expressed genes. Differential expression was determined by ttest of mean intensities.

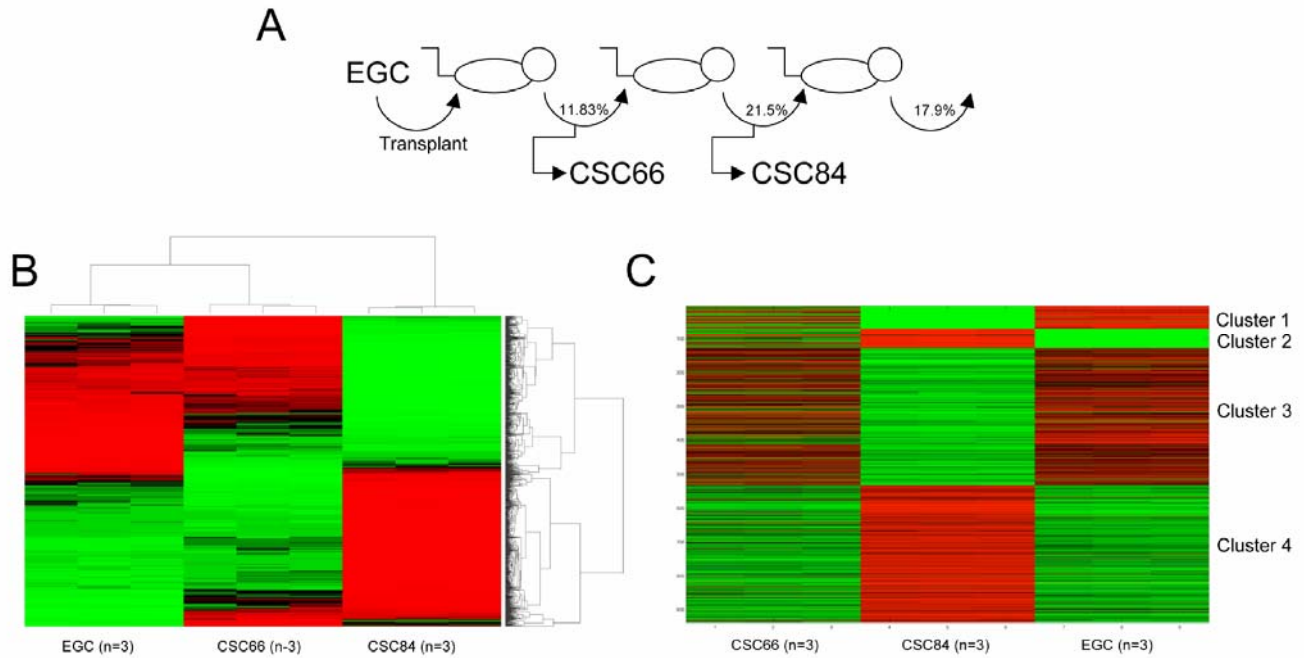


Figure 6. Model for the role of the stem cell self-renewal program in the generation of testicular cancer and formation of cancer initiating cells. Primordial germ cells (PGCs) normally enter the gonad at E10.0 and undergo mitotic arrest by E15.5, which is associated with a significant decrease in the expression of ESC expressed genes. Spermatogonia subsequently respond to retinoic acid signaling *in vivo* to initiate meiosis [18]. PGCs that fail to reduce the expression of the embryonic stem cell program develop CIS 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.

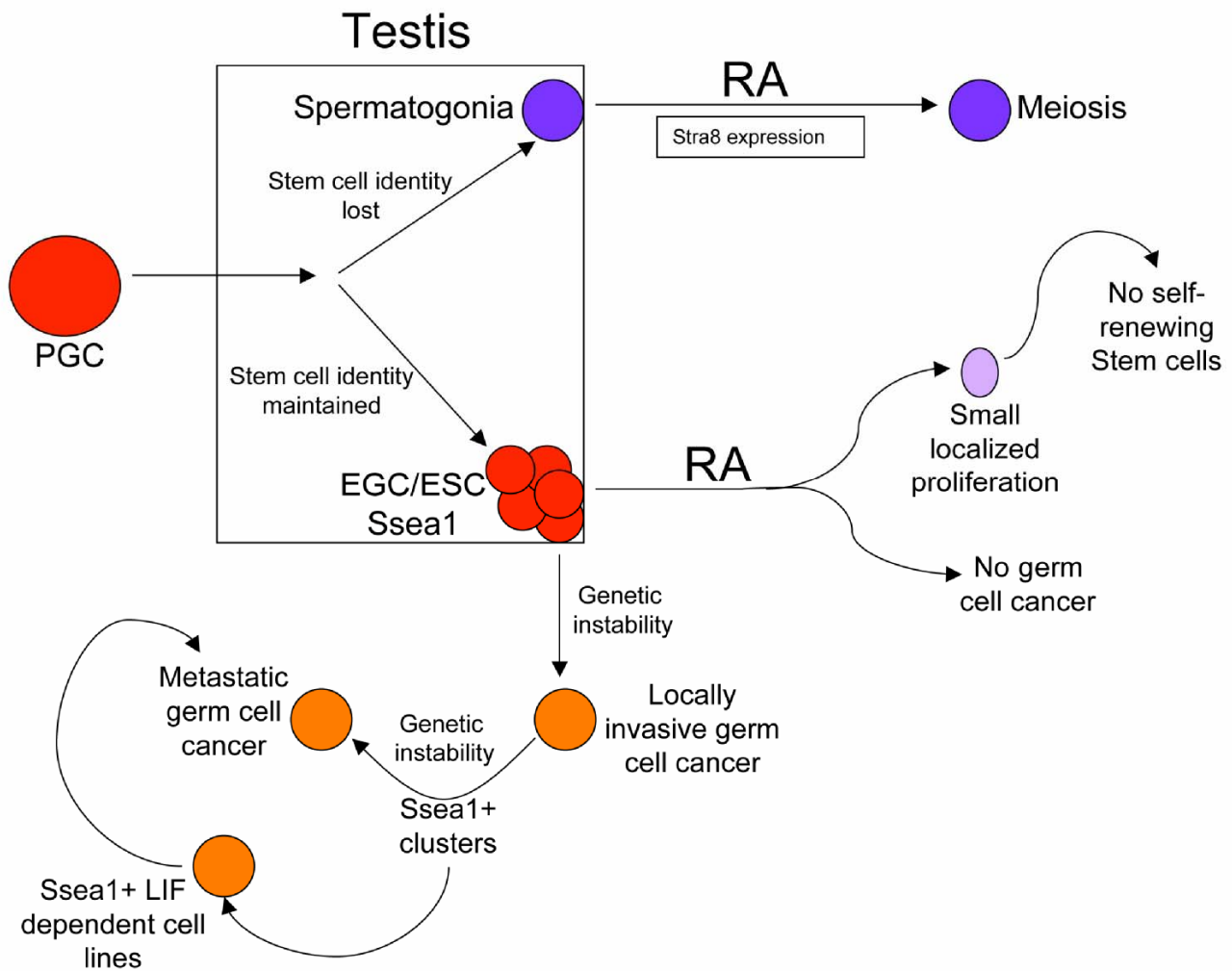


Table 1: Comparison of tumor potential between EGC transplants to generate primary tumors and SSEA1 positive serial transplants to generate secondary tumors.

Primary Tumor Generated with EGCs

Number of transplants	Cell number transplanted	Generation of a Testis Tumor	Metastasis
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

Number of transplants	Cell number transplanted	Generation of a Testis Tumor	Metastasis
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			

A Pluripotency and Self-Renewal Program Controls the Expansion of Genetically Unstable Cancer Stem Cells in Pluripotent Stem Cell-Derived Tumors
Anne E. Conway, Anne Lindgren, Zoran Galic, April D. Pyle, Hong Wu, Jerome A. Zack,
Matteo Pelligrini, Michael A. Teitell and Amander Clark
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