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Establishment and Characterization of a new Human Extragonadal Germ Cell Line, SEM-1, and its Comparison With TCam-2 and JKT-1

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Abstract

OBJECTIVE—To describe the establishment and characterization of a human cell line, SEM-1, from a patient diagnosed with a mediastinal seminoma.

METHODS—A small percentage of germ cell tumors develop as primary lesions in extragonadal sites, and the etiology of these tumors is poorly understood. Currently, only 2 cell lines from seminoma patients have been reported, JKT-1 and TCam-2, both derived from the testis. The cell line was characterized by heterotransplantation in Nude mice, cytogenetic studies, immunohistochemical and flow cytometry staining for germ cell tumor biomarkers, quantitative reverse-transcription polymerase chain reaction for cancer testis antigen expression, and *BRAF* mutation screening with quantitative polymerase chain reaction.

RESULTS—Characterization studies confirmed the human extragonadal seminoma origin of SEM-1 and demonstrated that it had more features in common with TCam-2 than JKT-1. Specifically, SEM-1 was positive for Sal-like protein 4 (SALL-4), activator protein-2γ (AP-2γ), and cytokeratin CAM5.2, and demonstrated heterogeneous expression of stem cell markers octamer-binding transcription factor 3/4, NANOG, c-KIT, SOX17, and SOX2. Cytogenetic analysis revealed a hypotriploid chromosome number, with multiple copies of 12p, but isochromosome 12p and the *BRAF* mutation V600E were not identified. The cell lines also did not contain the *BRD4/NUT* gene rearrangement [t(15,19)] seen in midline carcinomas nor did they contain overexpressed nuclear protein in testis (*NUT*) genes.

CONCLUSION—SEM-1 is the first cell line derived from an extragonadal germ cell tumor showing intermediate characteristics between seminoma and nonseminoma, and as such, is an important model to study the molecular pathogenesis of this malignancy.

Germ cell tumors (GCT) are the most frequently diagnosed malignancy in men aged between 20 and 40 years and have been increasing in incidence worldwide.^{1,2} Type II GCT, which are classified as seminomatous or nonseminomatous,^{1,2} most commonly arise in the testis; however, 5% are extragonadal in origin.^{1,3} Pure classical seminoma accounts for approximately 50% of all GCT, and a seminomatous component is present in nearly 20% of all mixed or nonseminomatous tumors.¹ By comparison, the more aggressive nonseminomas demonstrate a heterogeneous phenotype and include embryonal carcinoma (EC), yolk sac tumor, choriocarcinoma, teratoma, and tumors of mixed histology.¹ All type II GCT arise from intratubular germ cell neoplasia of unspecified type (ITGCNU; carcinoma in situ, testicular intraepithelial neoplasia), the result of an arrest in the differentiation of primordial germ cells or gonocytes.^{1,4} Seminomas most closely resemble ITGCNU, whereas nonseminomas are the result of a dedifferentiation of ITGCNU to pluripotent EC cells.¹⁻⁴

With the exception of primary tumor samples, few tools are available to study the molecular pathogenesis of seminoma.⁵ Only 2 cell lines, TCam-2, a human seminoma cell line derived from a 35-year-old man established in 1993 by Mizuno et al,⁶ and JKT-1, established in 1998 from a 40-year-old man by Kinugawa et al,⁷ have been described in the literature. Unlike TCam-2, however, JKT-1 has been shown to be dissimilar from its original tumor in that it does not express pure classical seminoma markers.⁸⁻¹¹

The recent controversy over cell line origin highlights the need for sensitive and specific biomarkers for different types of GCTs. The most important and difficult distinction is often between seminoma and EC (Table 1). Classical GCT markers include placental alkaline phosphatase (PLAP), positive in both seminomas and EC, surface marker cluster of differentiation (CD) 30, positive only in EC, β -human chorionic gonadotropin, and alpha-fetoprotein, both typically negative in seminomas and EC.^{1,12} Recent advances have led to the identification of several novel markers that may allow better characterization of seminomas and EC.^{1,12} Both express pluripotency-associated transcription factors octamer-binding transcription factor (OCT) 3/4, NANOG, Sal-like protein 4 (SALL4),^{13,14} and LIN28.⁹ Seminomas also express transcription factors c-KIT, activator protein-2 γ (AP-2 γ), and SOX17, whereas EC express SOX2.^{12,15-17} Testicular-specific protein on the Y chromosome (TSPY) and general germ cell marker VASA are also specific to seminoma. Transmembrane glycoprotein M2A is another highly sensitive marker for seminoma.^{18,19} Finally, recent reports suggest that extragonadal seminomas may represent a more mature phenotype characterized by the expression of the same markers and increased expression of PLAP, cytokeratin CAM5.2 (cytokeratin 8/18), vimentin, AP-2 γ , and M2A when compared with testicular seminomas.²⁰⁻²²

This report describes the establishment and characterization of a unique cell line, designated SEM-1, derived from an extragonadal seminoma. This is the first seminoma cell line of extragonadal origin that has been shown to be distinct from testicular seminoma in its pathogenesis and biomarker expression.²² In addition, although most seminomas are easily treated with chemotherapy, SEM-1 is derived from a tumor that later recurred and thus provides an excellent model in which to study this more aggressive clinical entity. This report compares the newly derived SEM-1 cell line with the established testicular seminoma cell lines TCam-2 and JKT-1 to provide a more comprehensive analysis of their phenotype

and cellular characteristics. SEM-1 represents an important preclinical model for extragonadal seminoma and has been made available to the scientific community by its submission to the American Tissue-type Cell Collection (<http://www.atcc.org>).

MATERIALS AND METHODS

Cell Lines

Tumor cell lines TCam-2 and JKT-1 were gifted to the Epstein Laboratory from Dr. Chris Lau (University of San Francisco, VA Medical Center, San Francisco, CA). Tumor cell line authenticity was performed by DNA (short tandem repeats) profiling by the American Tissue-type Cell Collection. All cell lines were maintained in complete medium (Roswell Park Memorial Institute Medium-1640 with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin) in a humidified 5% CO₂, 37°C incubator.

Establishment of Cell Line SEM-1

A tumor biopsy specimen obtained from surgical resection was used to develop the SEM-1 cell, as described previously.²³

Heterotransplantation in Nude Mice

Early passaged SEM-1 cells were injected subcutaneously (5×10^6 cells) in the flank of 8-week-old female Nude mice (Harlan Sprague Dawley, Indianapolis, IN) that were pretreated 2 days before implantation with 4 Gy total body irradiation. Tumors were removed 3 weeks after implantation and fixed in 10% neutral buffered formalin overnight at room temperature for paraffin-embedded procedures. Institutional Animal Care and Use Committee-approved protocols and institutional guidelines for the proper and humane use of animals in research were followed.

Cytogenetics

Karyotype analysis was performed by the Division of Anatomic Pathology, City of Hope (Duarte, CA) using cultured SEM-1 cells from an early passage. Analysis included Giemsa banding of metaphase spreads and fluorescence in situ hybridization (FISH) procedures performed routinely by this laboratory.

Electron Microscopy

A cell suspension of cultured SEM-1 cells was pelleted and fixed in Karnovsky's fixative for 1 hour, followed by osmium tetroxide, graded dehydration, and transfer to 7 BEEM capsules for plastic embedment. Thin sections were stained with toluidine blue and examined with an electron microscope.

Immunohistochemical Staining

Formalin-fixed paraffin-embedded (FFPE) cell pellets prepared from SEM-1, TCam-2, and JKT-1 cells, along with FFPE tissue sections of heterotransplant tumors, were used for immunohistochemical studies. Wright-Giemsa staining (Protocol Hema 3, Fisher, Kalamazoo, MI) of cytospin preparations and hematoxylin and eosin staining of tissue

sections was performed to assess morphology. Specific markers for seminoma cells (OCT3/4 [O3839], NANOG [ab80892], c-KIT [YR145], D2-40 [D2-40], and SOX17 [09-03840]) and general germ cell markers (SALL4 [6E3], VASA [ab13840], CAM5.2 [53D], AP-2 γ [EP2692Y], and PLAP [A89]) were applied. Samples were stained for CD45 (X16/99) to identify hematopoietic cells, and SOX2 (ab97959) was used as a marker for EC. Relevant positive and negative controls were used for each stain. Observation, evaluation, and image acquisition were made as described previously.²³

Flow Cytometry

Single-cell suspensions (10^6 cells in 100 μ L) of SEM-1, TCam-2, and JKT-1 were prepared in 2% fetal calf serum in phosphate-buffered saline and stained with fluorescence-conjugated antibodies. For intracellular staining, cells were fixed with 2% paraformaldehyde and permeabilized with 1% triton 100 $\times$ in phosphate-buffered saline before staining. The following antibodies were used: OCT3/4 (40/oct-3), NANOG (N31-355), c-KIT (104D2; Santa Cruz Biotechnology, Santa Cruz, CA), CD30 (Ber-H2), SOX2 (245610), SOX17 (P7-969; BD Biosciences, San Diego, CA), and isotype controls (eBioscience, San Diego, CA). All samples were done in duplicate. Samples were run on a FACSCalibur flow cytometer (BD), and analyses were performed using Cell Quest Pro (BD).

Immunoblotting

Reduced whole-cell lysates in radioimmunoprecipitation assay buffer were separated by Tris-glycine polyacrylamide gel electrophoresis, transferred onto nitrocellulose, and probed with antihuman and rat nuclear protein in testis (*NUT*; C52B1; Cell Signaling Technology, Danvers, MA) or antihuman glyceraldehyde-3-phosphate dehydrogenase (FL-335; Santa Cruz Biotechnology). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cell Signaling Technology) was used as secondary. Normal whole rat testis lysate was purchased from Cell Signaling Technology, and normal whole human testis lysate was purchased from Novus Biological (Littleton, CO).

Quantitative Reverse-Transcription Polymerase Chain Reaction With and Without 5-Azacytidine Treatment

The effect of dedifferentiation, using 5-azacytidine treatment, on messenger RNA expression levels was assessed using quantitative reverse transcription polymerase chain reaction (qRT-PCR). SEM-1, TCam-2, and JKT-1 cells were plated in 24-well plates at a density of 5×10^5 cells/mL. After 24 hours, duplicate samples of cells were exposed to medium alone or with 10 μ M 5-azacytidine. After another 6 hours, total RNA was extracted from each sample with RNAeasy Mini Kit (Qiagen, Valencia, CA), and DNase was treated using Turbo DNase (Applied Biosciences, Foster City, CA). RNA (100 ng) was amplified using Power SYBR Green RNA-to-CT 1-step Kit (Applied Biosciences). Gene-specific primer sequences from the National Institutes of Health qRT-PCR database (<http://primerdepot.nci.nih.gov>) were synthesized by the University of Southern California Core Facility. Specific markers analyzed included embryonic stem-cell markers *OCT3/4*, *SOX2*, *NANOG*, *PLAP*, *c-KIT*, *AP2 γ* , and cancer testis antigens *TPTE*, *MAGE-A*, *MAGE-C*, *SYCP1*, *SSX2*, *SPANX*, *CTCF*, *ACRBP*, *TRO*, *CTAG*, and *TSP50*.²⁴ Gene-specific amplification

was normalized to *GAPDH*, and fold-change in gene expression was calculated relative to Universal Human Reference RNA (Stratagene, La Jolla, CA). qRT-PCR was performed with a Stratagene Mx3000P cyclor with MxP QPCR software (Stratagene).

BRAF Mutation Analysis

Automatic sequencing was used to investigate the presence of the *BRAF* V600E point mutation. Total DNA was extracted from SEM-1 cells using TRIreagent (Sigma). PCR to amplify exon 15 of the *BRAF* gene was performed as described previously²⁵ Purified PCR products were sequenced in duplicate by Genewiz (South Plainfield, NJ) using an ABI 3730xI DNA Analyzer.

FISH for *NUT* Rearrangement

Dual-color FISH assays evaluating chromosome 15q13 *NUT* break points were carried out on FFPE, 4- μ m-thick sections of cell pellets as described.²⁶ Probes used were those flanking a 181-kb region that contained the 15q13 *NUT* break point, and included telomeric BAC clones 1H8 and 64o3B (digoxigenin-labeled, FITC antidigoxigenin-detected, green) and centromeric clones 1084a12 and 3d4 (biotin-labeled, rhodamine-streptavidin-detected, red). Slides with >80% hybridization efficiency in 4 areas (200 cells/area) were regarded as interpretable.

Statistical Analysis

To identify statistically significant differences in gene expression, one-way analysis of variance, followed by the Dunnett post-test were applied. Statistical tests were performed using GraphPad Prism software (La Jolla, CA) at a significance level of $\alpha = 0.05$.

RESULTS

Case Report of a Patient With Extragonadal Seminoma

The patient was a 58-year-old Hispanic man who presented in November 1986 for assessment of an asymptomatic anterior mediastinal mass identified on routine chest x-ray imaging. The patient underwent thoracotomy with resection of a 7- $\times$ 10- $\times$ 6-cm tumor on November 26, 1986. After surgical resection, the patient received 6 cycles of chemotherapy with BOP (bleomycin, vincristine, predinsone) and VP-16.

Microscopic examination of the tumor revealed large cells with abundant pale cytoplasm, prominent nucleoli, and euchromatic nuclei with finely dispersed chromatin (Fig. 1A). Giant cells were numerous, with abnormal mitoses. Immunohistochemistry showed negative staining for cutaneous lymphocyte-associated antigen, standard lymphoid markers, keratin, alpha-fetoprotein, β -human chorionic gonadotropin, and vimentin but was positive for ferritin. On the basis of these data, the tumor was diagnosed as an extragonadal seminoma. In April 1988, the patient was diagnosed with tumor recurrence and received additional chemotherapy before being lost to follow-up.

Establishment and Growth Parameters of SEM-1

The SEM-1 cell line was derived from the first biopsy specimen of the mediastinal seminoma resected in 1986. In culture, small tissue fragments were seen to adhere to the plastic surface, from which distinctive, flat tumor cells were seen to grow peripherally from the central area of each fragment. After 3 weeks, the tumor cells were removed by differential trypsinization and subcloned in large petri dishes using metal rings to establish the cell line. The doubling time for SEM-1 was 50 hours, which was similar to that of the TCam-2 cell line but longer than that of JKT-1 (37.5 hours).^{6,7,10} SEM-1, like TCam-2, required a higher initial cell density in culture to prevent delay in the exponential growth phase compared with JKT-1.

Heterotransplantation of SEM-1 in Nude Mice

Three weeks after subcutaneous implantation in irradiated Nude mice, the tumors demonstrated morphology consistent with the original tumor, including sheets of relatively uniform, large tumor cells with pale eosinophilic, vacuolated cytoplasm (Fig. 1B). Giant cell forms were identifiable but were less evident than in the primary tumor. Staining for OCT3/4, NANOG, SOX17, and PLAP was positive, whereas staining for c-KIT was negative. SOX2 showed heterogeneous staining, with only some highly positive cells. These findings indicated that SEM-1 is transplantable into xenograft models and that heterotransplanted tumors closely resembled the original tumor biopsy specimen described in the original pathology report.

Morphology of SEM-1

Phase-contrast photomicrographs of cultured cells and Wright-Giemsa stained cytopins were used to assess the morphology of SEM-1 compared with established cell lines TCam-2 and JKT-1 (Fig. 1C). Cytology of SEM-1 shows malignant cells with abundant cytoplasm, occasionally multiple nuclei, and one to several nucleoli. Mitoses were readily seen among these cells. There was no evidence of glandular or epithelial differentiation. Electron microphotographs of the SEM-1 cells showed tumor cells to have abundant cytoplasm, large oval and indented nuclei, and fairly abundant euchromatin, with one to several moderately sized nucleoli (Fig. 1D). A simplified cytoplasm with polysomes and one focus of cytoplasmic glycogen were also seen. The lack of more abundant glycogen, which is a hallmark of this tumor, could be due to cell culture conditions.

Biomarkers Analysis by qRT-PCR, Flow Cytometry, Immunohistochemistry, and Immunoblotting

The expression of pertinent GCT markers was examined for the 3 cell lines using qRT-PCR techniques. SEM-1 showed a statistically significant increase in mean expression of *AP-2γ*, and *SOX2* (Fig. 2A, $P < .05$). Expression of *NANOG*, *OCT3/4*, *PLAP*, and *c-KIT* was not increased. JKT-1 showed a similar expression profile to SEM-1, with the exception of elevated *PLAP* expression levels ($P < .05$). TCam-2 showed increased expression of *NANOG*, *OCT3/4*, and *AP-2γ*, but showed no increase in the expression of *SOX2*, *PLAP*, and *c-KIT* ($P < .05$), which concurs with prior literature describing low levels of *SOX2* expression in TCam-2.⁸ Similarly, flow cytometry studies of SEM-1 and JKT-1 displayed

negative staining for NANOG, c-KIT, OCT3/4, and CD30, whereas staining for SOX2 was positive in all cell lines (Fig. 2B).

In contrast, immunohistochemical staining for the seminoma markers OCT3/4, NANOG, c-Kit, AP-2 γ , PLAP, SOX17, and SAL4 were positive for SEM-1 (Fig. 3A). For c-Kit, TCam-2 and JKT-1 were negative. The EC marker SOX2 showed Golgi staining for SEM-1 and nuclear staining for TCam-2, but was negative in JKT-1 (Fig. 3A). A very weak positive stain was found for the germ cell—specific marker VASA. The heterogeneity in expression of OCT3/4, NANOG, c-Kit, and SOX2 may be explained by the extragonadal origin of SEM-1¹⁵ or by its passage in cell culture, or both.¹¹ In contrast to TCam-2, which showed a more classical testicular seminoma phenotype, the overall expression profile of SEM-1 demonstrated a phenotype that is intermediate between seminomatous and nonseminomatous GCT.

Another potential biomarker for GCT is *NUT*, a protein normally confined to germ cells of the testis. Rearrangement of *NUT* on chromosome 15 defines *NUT* midline carcinoma, a rare, aggressive cancer arising from the body midline.²⁷ FISH was performed to rule out the possibility that the GCT cell lines may harbor the *NUT* rearrangement. FISH studies revealed no rearrangement of the *NUT* locus in TCam-2, JKT-1, or SEM-1. Looking at wild-type *NUT*, JKT-1 had weak protein expression compared with normal testis, whereas SEM-1, and TCam-2 were both negative for *NUT* (Fig. 3B). *NUT* expression in JKT-1, SEM-1, and TCam-2 is consistent with clinical data, where only 6% of seminoma cases were reported as staining positive for wild-type *NUT* by immunohistochemistry.²⁸

qRT-PCR With and Without 5-azacytidine Treatment

The expression of specific cancer testis antigens, a group of genes with expression restricted to normal male germ cells in the testis and to various malignancies,²⁴ was assessed in the 3 cell lines before and after treatment with the dedifferentiating agent 5-azacytidine. The typical expression pattern of these genes during normal gametogenesis is shown in Figure 2C. The relative changes in gene expression levels after 5-azacytidine treatment compared with the untreated cell lines are shown in Figure 2D. SEM-1 demonstrated a statistically significant decrease in the expression of *OCT3/4*, *SCYPI1*, *TRO*, *SPANXA1*, and *ACRBP*, and an increase in the expression of *TSP50* ($P < .05$). These changes in gene expression show that SEM-1 has cancer testis antigens expression consistent with later stages of gametogenesis, before and after dedifferentiation treatment. Conversely, TCam-2 had a notable increase in expression of *NANOG* and *OCT3/4* ($P < .05$), showing that this cell line more closely resembles early stages of gametogenesis.

Cytogenetic Analysis

Analysis of G bands produced by trypsin and Giemsa indicated the cell line was hypotriploid, with a range from 52–64 chromosomes, and had a modal number of 64, consistent with prior reports for extragonadal seminomas.¹ Owing to tumor cell heterogeneity, a composite karyotype was created containing all clonally occurring abnormalities (Fig. 3C). The aberrations noted are mostly nonspecific, but like previously reported seminomas, showed gains of chromosomes 17, 12, and X.¹ There was no evidence

of isochromosome 12p (i(12p)), an aberration that is frequently present in all subtypes of GCT,¹ but multiple copies of 12p, 12q, and whole-number 12 chromosomes were noted. FISH analysis on 183 SEM-1 cells was completed to determine the 12p copy number using a TEL/AML t(12;21) probe set. From these studies, 20.2% showed a normal disomy signal pattern for both probes, 72.7% showed a pattern of 12p trisomy/12q disomy, 3.3% showed 12p and 21q trisomy, and 3.8% showed a pattern of 12p tetrasomy and 21q disomy.

BRAF Mutation Analyses

Although most solid tumors demonstrate frequent mutations in protooncogenes and tumor-suppressor genes, type II GCT are unique in having a relatively low mutation rate.¹ Previous studies on TCam-2, however, have identified a point mutation in the protooncogene *BRAF* that may provide the growth advantage needed for in vitro culture.⁸ Mutation analysis by automatic sequencing of SEM-1 did not demonstrate the presence of the *BRAF* V600E point mutation, indicating that this cell line may use a different mechanism of oncogenesis.

COMMENT

GCT tumorigenesis is of particular interest not only because of their high incidence but also because of the close relationship they have with normal germ cell development.¹ Seminomas most closely resemble ITGCNU, the first step in the development of all GCT,^{1,4} and although patient tumor samples provide some information, in vitro or animal models are needed for more complex studies. Seminoma cell lines are particularly important because they can be used to study the tumorigenesis of seminoma as well as early transformations that may take part in the development of nonseminomatous GCTs.¹ In this study, we describe the establishment and characterization of SEM-1, which was derived from the initial tumor biopsy specimen of a 58-year-old patient with recurrent mediastinal seminoma. Furthermore, in light of the recent debate surrounding previously established seminoma cell lines, this model is described in relationship to the widely accepted seminoma cell lines TCam-2 and JKT-1.^{7–11}

Morphologic, phenotypic, cytogenetic, and gene expression studies performed on this new cell line demonstrate that in vitro cultures and xenografts had features characteristic of a mediastinal GCT. As reported in Table 1, SEM-1 was positive, staining for several seminoma markers, and was negative for the EC surface marker CD30. SEM-1 also demonstrated positive staining for CAM5.2, consistent with reports documenting positive expression of CAM5.2 in 80% of mediastinal vs 20% of testicular seminoma.^{20,22} Although SEM-1 did not show increased expression of *PLAP* by qRT-PCR or M2A by immunohistochemistry, there was increased expression of AP-2 γ , which has been shown to be of value in the detection of extragonadal seminoma specifically.^{15,17,21} Stem cell markers OCT3/4, NANOG, and c-KIT showed heterogeneous expression in SEM-1, indicating that there may be multiple subclones present with features characteristic of both seminomatous and nonseminomatous tumors. The partial negative staining of SEM-1 for these markers is consistent with reports that extragonadal seminoma may represent a more mature/differentiated phenotype compared with their testicular counterparts.²² This finding is

further supported by the novel 5-azacytidine dedifferentiation studies completed on SEM-1, as described above.

Compared with previously established cell lines, SEM-1 was found to have morphologic and growth parameters similar to TCam-2 and different from the more rapidly proliferating JKT-1 cell line. SEM-1 and TCam-2 demonstrated the classical over-representation of chromosome 12p found in all invasive type II GCT, whereas JKT-1 has been shown to lack this classic molecular marker.^{8–11} The 3 cell lines demonstrated heterogeneous protein expression of SOX2, a transcription factor expressed by embryonic stem cells that is typically positive in EC and negative in seminoma.¹² These data are inconsistent with previous reports by de Jong et al,⁸ who found TCam-2 was negative for SOX2 expression. The positive expression of SOX2 in SEM-1 and TCam-2 may represent a partial dedifferentiation of the cell lines in culture.^{1,4}

CONCLUSIONS

Therapy remains difficult for a subset of aggressive, treatment-resistant seminomas. Recent reports indicate that among this group, extragonadal tumors have a worse prognosis than testicular tumors.^{2,22} Although TCam-2 represents a model for testicular cisplatin-resistant seminoma,²⁹ an in vitro model for extragonadal treatment-resistant seminoma was not available. Our conclusions from the analysis of the 3 cell lines demonstrate that SEM-1 is intermediate between seminomatous and nonseminomatous GCT and provides investigators with the first extragonadal seminoma tumor model.

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Figure 1.

Original tumor, heterotransplant, and SEM-1 cell line morphology. (A) Original archival thin sections prepared for electron microscopy and stained by toluidine blue showed large, undifferentiated tumor cells with pale cytoplasm and numerous vacuoles. Giant cell forms are frequent. Nuclei are large with finely dispersed chromatin, prominent nucleoli, and distinct nuclear membranes. Abnormal mitoses are also present. Immunohistochemistry performed at diagnosis on formalin-fixed paraffin-embedded tissue, was reported to show no staining for cluster of differentiation (CD) 45, broad-spectrum keratin, alpha-fetoprotein,

human chorionic gonadotropin, or vimentin. Ferritin was reported to be positive. **(B)** Formalin-fixed paraffin sections of heterotransplanted SEM-1, stained by hematoxylin and eosin, showed relatively more uniform, large tumor cells with pale, eosinophilic cytoplasm and vacuolation. Nuclei are large with distinct nuclear membranes and conspicuous nucleoli. Giant cell forms are present but are less frequent than in the primary tumor, whereas mitotic figures are more numerous in the cell line (original magnification $\times 400$). **(C)** Phase-contrast photomicrographs of TCam-2, JKT-1, and SEM-1 cells growing in culture (*top*). Cytopsin preparations (*bottom*) of the 3 cell lines show large undifferentiated cells with a primitive chromatin pattern, prominent nucleoli, and cytoplasmic vacuoles. SEM-1 cells demonstrate more pleomorphism, with giant cell forms and mitoses (cytopsin, Wright-Giemsa stain, original magnification $\times 400$). **(D)** Electron microscopy images of cultured SEM-1 cells show a primitive nuclear morphology, with prominent nucleoli and finely dispersed chromatin.

Figure 2.

Analysis of germ cell tumors (GCT) biomarkers and cancer testis antigen (CTA) expression.

(A) Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis of GCT biomarker messenger RNA levels in TCam-2, JKT-1, and SEM-1. (B) Flow cytometry studies of TCam-2, JKT-1, and SEM-1 show the percentage of positive-staining cells for each antibody relative to its isotype control. Flow cytometry data are consistent with qRT-PCR data, with the exception of SOX2 expression in TCam-2 and JKT-1. (C) Schematic diagram demonstrates the typical expression pattern of CTA during normal gametogenesis in the testis. Genes in **bold** were assessed for expression levels before and after treatment with 5-azacytidine. (D) Analysis of relative changes in gene expression levels is shown after 5-azacytidine treatment compared with the untreated cell lines using qRT-PCR. For panels A, B, and D, mean ($n = 2$) data $\pm$ standard deviation are shown. $*P < .05$.

Figure 3.

Immunohistochemical, immunoblotting, and karyotype analysis are shown for the SEM-1 cell line. **(A)** Photomicrographs of immunoperoxidase staining of paraffin-embedded SEM-1, TCam-2, and JKT-1 cell pellets for c-KIT, NANOG, OCT3/4, and SOX2 (original magnification $\times 400$). Immunohistochemistry showed c-KIT membrane positivity in SEM-1 only, NANOG nuclear staining in all 3 cell lines, OCT3/4 cytoplasmic staining in all 3 cell lines, and SOX2 Golgi staining in SEM-1, nuclear staining in TCam-2, and no staining in JKT-1. **(B)** Immunoblot of nuclear protein in testis (*NUT*) in TCam-2, SEM-1, and JKT-1 lysates. *NUT* bands for positive controls, rat, and human testis are shown at a lighter exposure (5 seconds) compared with bands in TCam-2, SEM-1, and JKT-1 (1 minute). hGAPDH, human glyceraldehyde-3-phosphate dehydrogenase. **(C)** Karyotype of SEM-1 containing all clonally occurring abnormalities demonstrates vast aneuploidy with features suggestive of seminoma including gains of chromosome 7, 12p, and X.

Table 1

Comparison of biomarkers for cell lines SEM-1, JKT-1, and TCam-2 as reported from this and other laboratories

Cell Lines (First Author)	PLAP	OCT3/4	NANOG	c-KIT	AP-2 γ	M2A (D2-40)	CD30	SOX2	SOX17	CAM5.2	VASA	SALL4
Seminoma *	+	+	+	+	+	+	-	-	+	Var	+	+
Embryonal carcinoma *	+	+	+	-	-	Var	+	+	-	+	-	+
TCam-2	-	+	+	+	+	+	-	-	+	+	-	+
TCam-2 (Eckert, 2008)	-	+	+	+	+	+	-	-	+	+	+	+
TCam-2 (de Jong, 2007)	+	+	+	+	+	+	-	-	+	+	+	+
TCam-2 (Mizuno, 1993)	+	+	+	+	+	+	-	-	+	+	+	+
JKT-1	+	+	+	+	+	+	-	-	+	+	+	+
JKT-1 e (Bouskine, 2009)	+	+	+	+	+	+	-	-	+	+	+	+
JKT-1 i (Bouskine, 2009)	+	+	+	+	+	+	-	-	+	+	+	+
JKT-1 (Eckert, 2008)	-	-	-	-	-	-	-	-	-	-	-	-
JKT-1 (de Jong, 2007)	-	-	-	-	-	-	-	-	-	-	-	-
JKT-1 (Kinugawa, 1998)	+	+	+	+	+	+	-	-	+	+	+	+
SEM-1	+	+	+	+	+	+	-	-	+	+	+	+

AP-2 γ , activator protein-2 γ ; CD30, cluster of differentiation 30; f, flow cytometry; i, immunohistochemistry; OCT, octamer-binding transcription factor; PLAP, placental alkaline phosphatase; r, quantitative reverse-transcription polymerase chain reaction; SALL4, Sal-like protein 4.

* Emerson et al, 2010.