Establishment of a Versatile Seminoma Model Indicates Cellular Plasticity of Germ Cell Tumor Cells

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In western countries, 60% of all malignancies diagnosed in men between 17–45 years of age are germ cell tumors (GCT). GCT arise from the common precursor lesion carcinoma in situ, which transforms within an average of 9 years into invasive Type-II GCTs. Seminomas are considered to be the default developmental pathway of carcinoma in situ cells and the seminoma-like cell line TCam-2 has been used to study seminoma biology in vitro. However, the generation of an animal model, which would allow for the in vivo analysis of seminoma formation, remained elusive. We applied transplantation approaches using TCam-2 cell transfer into ectopic (skin, brain) and orthopic (testis) sites of immunodeficient mice. We demonstrate that a transplantation into the seminiferous tubules results in formation of a carcinoma in situ/seminoma. In contrast, TCam-2 cells adopt an embryonal carcinoma-like fate when grafted to the flank or corpus striatum and display downregulation of the seminoma marker SOX17 and upregulation of the embryonal carcinoma markers SOX2 and CD30. Grafted TCam-2 cells reduce AKT-, ERK-, EphA3-, and Tie2/TEK-signaling to levels comparable to embryonal carcinoma cells. Hence, TCam-2 cell transplantation into the testis generated a carcinoma in situ/seminoma mouse model, which enables addressing the biology of these tumors in vivo. The fact that TCam-2 cells give rise to a carcinoma in situ/seminoma or embryonal carcinoma in a transplantation site specific manner implies that conversion of carcinoma in situ/seminoma to an embryonal carcinoma does not require additional genetic aberrations but relies on signals from the tumor-micro-environment. © 2012 Wiley Periodicals, Inc.

INTRODUCTION

Germ cell tumors (GCTs) are the most common cancer type in young men with increasing incidence in western countries (Adami et al., 1994). GCTs arise from carcinoma in situ (CIS) cells, which are thought to originate from a gonocyte blocked in maturation (Sonne et al., 2009). This precursor lesion transforms within an average of 9 years into an invasive GCT (Jorgensen et al., 1990). These tumor entities were classified into different groups (Oosterhuis and Looijenga, 2005). Teratomas and yolk-sac tumors of infants and young adults belong to Type I, while seminomas and nonseminomas are Type II GCTs. Seminomas and embryonal carcinomas (EC) represent the most frequent subtypes of Type II GCTs. While seminomas are considered the default developmental pathway of CIS cells (de Jong et al., 2008b) and resemble undifferentiated germ cells, ECs are pluripotent and eventually differentiate into teratomas, choriocarcinomas and yolk-sac tumors. The tumor biology of ECs can be recapitulated and analyzed in vitro by taking advantage of several human EC cell lines and in vivo by EC-xenografts in mice (Bronson et al.,

1980; Damjanov et al., 1993; Damjanov, 2004; Josephson et al., 2007). However, the molecular pathways involved in seminoma formation remain elusive. While the well characterized human seminoma-like cell line TCam-2 (de Jong et al., 2008a; Eckert et al., 2008a,b) allows for studying seminoma biology in vitro, to date, no animal model is available to analyze seminoma formation and progression in vivo.

Here, we demonstrate that transfer of TCam-2 cells into the seminiferous tubules of mice resulted in formation of a CIS/seminoma, while transplantation into the trunk (flank) or central nervous system

Additional Supporting Information may be found in the online version of this article.

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Supported by: DFG, Grant number: Scho-503/9; Deutsche Krebshilfe, Grant number: 108249; Kompetenznetzwerk Stammzellforschung NRW.

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Received 23 January 2012; Accepted 2 March 2012

DOI 10.1002/gcc.21958

Published online in Wiley Online Library (wileyonlinelibrary.com).

TABLE 1. Oligonucleotides Used in RT-PCR Experiments

Marker gene	Forward primer 5'-3'	Reverse primer 5'-3'	Tan (°C)	Cycles
AFP	AGCTTGGTGGTGGATGAAAC	CCCTCTTCAGCAAAGCAGAC	60	30
TFAP2C	CCCACTGAGGTCTTCTGCTC	AGAGTCAC ATGAGCGGCTTT	60	25
BLIMP1	GGGTGCAGCCTTTATGAGTC	CCTTGTTTCATGCCCTGAGAT	60	30
BRACHYURY	GGGTGGCTTCTTCCTGGAAC	TTGGAGAATTGTTCCGATGAG	60	35
CD30	AGCTAGAGCTTGTGGATTCCAG	GTCTTCTTCCCTTCCTCTTCC	58	30
cKIT	TCATGGTCGGATCACAAAGA	AGGGGCTGCTTCCTAAAGAG	60	25
GAPDH	TGGTATCGTGGAAGGACTCATG AC	ATGCCAGTGAGCTTCCCGTTCAGC	60	22
GDF3	AGACTTATGCTACGTAAAGGAGCT	CTTTGATGGCAGACAGGTTAAAGTA	60	30
HAND1	ACATCGCCTACCTGATGGAC	CGGCTCACTGGTTTAACTCC	60	30
MCFD2	CCCTGTGGCTTTGTAGGGTA	GCAGAAGGGAAATGTGGTGT	60	30
NANOG	GATTTGTGGGCCTGAAGAAA	AAGTGGGTTGTTTGCCTTTG	58	25
OCT3/4	CGAAAGAGAAAGCGAACCAG	GCCGGT TACAGAACCACACT	60	22
PAX6	ACCCATTATCCAGATGTGTTTCCCCGAG	CTGCCGCCTATGCCAGCTTCACCAT	60	30
SOX17	GAGGGCCAGAAGCAGTGTTA	AGTGATTGTGGGGAGCAAGT	60	25
SOX2	ATGCACCGCTACGACGTGA	CTTTTGCACCCCTCCATT	60	25
VASA	AGAAA GTAGTGATACTCAAGGACCAA	TGA CAGAGATTAGCTTCTTC AAAAGT	60	35

(CNS) (corpus striatum) resulted in growth of EC-like tumors—a process that is accompanied by up-regulation of EC-specific genes and adaptation to EC-characteristic pathway signaling.

MATERIAL AND METHODS

Cell Culture

TCam-2 and 2102EP cells (obtained from Dr. Janet Shipley, Institute of Cancer Research, Sutton, England and Dr. Stefanie Gläsener, UKE Hamburg, Germany) were cultivated in RPMI (TCam-2) or DMEM (2102EP) medium supplemented with 10% FCS, 1% penicillin/streptomycin, 200 mM L-glutamine at 37°C and 5% CO₂.

RT-PCR

RNA was extracted by TRIzol (Sigma, Taufkirchen, Germany) according to manufacturer's instructions. RNA quality and concentration was measured by NanoDrop spectrophotometer (Pep-Lab, Erlangen, Germany). First-strand synthesis was performed according to "RevertAid First Strand cDNA Synthesis Kit" manual (Fermentas, St. Leon-Rot, Germany). About 500 ng of total RNA were used for each first-strand synthesis. About 1.5 µl (37.5 µg) cDNA was used for each PCR reaction. PCR was carried out at 95°C for 1 min, followed by 25–35 cycles of 94°C for 30 sec, 58–60°C for 30 sec and 72°C for 45 sec. For primer details, see Table 1.

Immunohistochemistry

Tumor tissues were dissected, fixed in 4% phosphate-buffered formalin for 1–2 days at room

temperature and processed in paraffin wax. Dewaxed, 4-µm thick tissue sections were microwave pretreated in Citrate-buffer (10 mM citric acid, pH 6). Endogenous peroxidase was blocked for 5 min in 3% hydrogen peroxide (diluted in distilled water). Sections were washed with 1× PBS and unspecific binding sites were blocked with 1.5% BSA/PBS + 10% horse serum for 1 hr at room temperature. Primary antibodies, diluted in 1.5% BSA/PBS, were applied for 2 hr at room temperature. Thereafter, a biotin-conjugated secondary antibody was applied for 1 hr at room temperature. The staining was visualized with 3-amino-9-ethyl-carbazole (AEC Substrate-Chromogen, DAKO, Hamburg, Germany) and counterstained with haematoxylin. Negative controls were performed using blocking solution instead of the primary antibody. For antibody details see Table 2.

Western Blot

Proteins were isolated by RIPA-buffer (50 mM TRIS-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS + 1 tablet of protease inhibitor cocktail (Roche, Mannheim, Germany)). The lysates were incubated for 30 min at 4°C, followed by a 20-min centrifugation step at 12,000 g at 4°C. The supernatant was used for western blot analysis. The protein concentrations were determined by BCA Protein Assay Kit (Pierce, Rockford, IL). For protein analysis, we used the Mini-PROTEAN Electrophoresis Cell and Mini Trans-Blot system (Bio-Rad, Munich, Germany). SDS-PAGE was carried out at 90 V for approximately 90 min. Gels were blotted onto a polyvinylidene fluoride membrane

TABLE 2. Primary and Secondary Antibodies Used for Immunohistochemistry and Western Blotting

	Clone/order code	Company	Source	IHC dilution	WB dilution
Primary antibody					
AFP	A0008	Dako	Rabbit	1:200	
Beta-actin	AC-74	Sigma Aldrich	Mouse	–	1:20,000
BLIMP1	–	–	Rabbit	1:200	
CD30	Ber-H2/M0761	Dako	Mouse	1:200	
cKIT	A4502	Dako	Rabbit	1:200	
hCG (β)	AHP536	Serotec	Rabbit	1:300	
MAGEA4	57B	–	Mouse	1:200	
OCT3/4	C-10	Santa Cruz	Mouse	1:200	
pERK1/2	9101	Cell Signaling	Rabbit	–	1:1,000
PGP9.5	Z5116	Dako	Rabbit	1:300	
PRMT5	AB3349	Chemicon	Rabbit	1:200	
SOX17	GT15094	Neuromics	Goat	1:50	
SOX2	AF2018	R&D systems	Goat	1:100	
VASA	ab13840	Abcam	Rabbit	1:200	
Secondary antibody					
Anti-goat HRP	61–1620	Invitrogen	Rabbit	1:400	
Anti-mouse HRP	61–0120	Invitrogen	Rabbit	1:400	
Anti-rabbit HRP	65–6120	Invitrogen	Goat	1:400	
Anti-rabbit HRP	sc2955	Santa cruz	Chicken	1:100	
Anti-goat HRP	sc2961	Santa cruz	Chicken	1:100	

in a BioRad blotting chamber overnight at 30 V at 4°C. After the membrane had been blocked in 5% low fat dry milk powder (LFDMP)/PBS for at least 1 hr at room temperature, primary antibodies, diluted in 5% LFDMP/PBS, were applied for 3 hr at room temperature, followed by HRP-conjugated secondary antibodies for 1 hr. Finally, the membrane was incubated in 1 ml PierceSuper Signal West Pico chemiluminescent substrate (Perbio, Bonn, Germany) for 5 min and the signal was detected by using Kodak X-Ray film (Sigma-Aldrich, Taufkirchen, Germany). For antibody details, see Table 2.

Gene Expression Profiling by Affymetrix Array

TCam-2 cells were analyzed using the Affymetrix GeneChip Human Genome U133A plus 2.0 array. The sample preparation, Affymetrix array, data normalization, data analysis and visualisation were performed as described in depth by Looijenga et al. (2006). Ten micrograms of total RNA were used to prepare anti-sense biotinylated RNA, according to the Affymetrix GeneChip eukaryotic one-cycle target preparation protocol (Affymetrix, Santa Clara, CA).

Injection of TCam-2/2102EP Cells into the Flank

TCam-2 and 2102EP cells were cultivated for 8 days in standard growth medium. Cells were trypsinized, counted and 1×10^7 cells were resuspended in 500 μ l of 4°C cold Matrigel (BD,

Heidelberg, Germany) and injected into the flank of CD1 nude mice using a cold 27G syringe (BD, Heidelberg, Germany).

Injection of TCam-2/2102EP Cells into the Seminiferous Tubules

Five weeks before TCam-2/2102EP injection, busulfan (40 mg/kg) was injected intraperitoneally into male NRMI-Foxn^{nu}/Foxn^{nu} mice. At the day of injection, TCam-2/2102EP cells were trypsinized and counted. About 1×10^5 cells were resuspended in 10 μ l standard growth medium plus 0.05% trypan-blue to monitor the injection process. About 10 μ l of the cell suspension were injected under pressure (100–400 hPa, FemtoJet, Eppendorf, Hamburg, Germany) into the seminiferous tubule via the efferent duct.

Injection of TCam-2/2102EP Cells into the Corpus Striatum

At the day of injection, TCam-2/2102EP cells were trypsinized and counted. Per injection 1×10^5 cells were resuspended in 2 μ l PBS. CD1 nude mice were anaesthetized by ketamine-xylazine into the peritoneum and fixed afterwards in the injection apparatus. After the cranial bone was laid open, a small hole was drilled into the cranial bone at the injection site. Cells were injected through this hole into the corpus striatum via a syringe.

PathScan Signaling Array

The PathScan Signaling Array (Cell Signaling Technology, Frankfurt, Germany) was performed according to the manufacturers recommendations. About 150 μ g total protein extract was used per assay and samples were run in duplicates. The chemiluminescent signals were detected using Kodak BioMax light films (Sigma Aldrich, Steinheim, Germany). Pixel intensities were quantified by ImageJ-software (<http://rsbweb.nih.gov/ij/>) and normalized to individual background intensities.

Statistical Analysis

Standard deviations were calculated after normalization of the PathScan Signaling Array data set to the background noise and after weighting the testes and seminal vesicles 1 and 3 months after transplantation. A Student's *t*-test was performed to determine the significance of the measured differences in testes and seminal vesicle weight. Significance level $\alpha = 5\%$.

RESULTS

The seminoma derived cell line TCam-2 cells display a seminoma-like character in vitro and can be induced to convert into a mixed nonseminoma by defined conditions (Eckert et al., 2008a,b; Nettersheim et al., 2011). These results suggested that seminomas might be instructed by signals from the tumor-microenvironment to convert to nonseminoma (i.e. embryonal carcinoma) without the requirement of an additional mutation. Unfortunately, there is no in vivo seminoma model available to test this hypothesis. In order to further extend these findings to the in vivo situation, we decided to transplant cell lines representing seminoma (TCam-2) and nonseminoma (2102EP) to different sites in mice.

In humans, CIS-cells reside as small nests within the seminiferous tubules of the testes (intratubular neoplasia). Also, seminomas and ECs initially grow within these tubules. Hence, we decided to perform a heterologous transfer of human GCT cells into the seminiferous tubules of busulfan-treated immunodeficient mice. Histological analysis of the testes 1 month after transplantation revealed that the TCam-2 cells had formed clusters of large, round cells with clear cytoplasm and big, round nuclei (Fig. 1A, red arrow). These are morphological hallmarks of CIS/seminoma formation (Hoei-Hansen et al., 2005). Three months after transplantation, the CIS-like nests persisted, but parts

Figure 1. (A) HE or PAS staining of testis sections 1 and 3 months after injection of TCam-2 (HE) and 2102EP cells (PAS) into the seminiferous tubules. Red arrows point at CIS-like cells, while the green arrow points at more differentiated appearing cells. Scale bars: 100 μ m. (B) RT-PCR of indicated markers of in vitro cultivated TCam-2 and testis tissues 3 months after transplantation of TCam-2 (TCam-2, 3 months). GAPDH was used as endogenous control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of the transplant presented as small, round to polygonal and eosinophilic cells indicating progression of the CIS cells to the invasive GCT entities (Fig. 1A, green arrow). The testis-transferred 2102EP cells grew as an EC from the beginning (Fig. 1A, 2102EP).

To validate the CIS/seminoma-like nature of the lesions, we performed RT-PCR and demonstrated expression of the CIS/seminoma markers *NANOG*, *OCT3/4*, *KIT*, *BLIMP1*, *TFAP2C*, *MCFD2*, and *SOX17* (Fig. 1B). The lesions were negative for EC markers (*SOX2*, *CD30*) as well as mesoderm markers (*BRACHYURY* (*T*), *HAND1*) but expressed the markers for endoderm (*AFP*) and ectoderm (*PAX6*). IHC analyses further verified expression of *OCT3/4*, *KIT*, *SOX17*, and *AFP* (Figs. 2A–2H). Of note, expression of *AFP* was confined to cells having lost the CIS-like morphology. Like the parental TCam-2 cells in vitro, the grafted cells remained negative for *MAGEA4* and displayed only focal β -hCG staining. Furthermore, expression of the late germ cell marker *VASA* or the spermatogonia marker *PGP9.5* was not detected (Figs. 2I–2P), indicating that the grafted TCam-2 cells did not initiate germ cell differentiation. The results suggested that intratesticular transplantation of the

Figure 2. IHC staining of indicated markers 1 and 3 months after TCam-2 injection into the seminiferous tubules of nude mice. Secondary antibody controls are given in the lower, right corners. Scale bars: A–B, E–F, K–P, 200 μ m; C–D, I–J, 100 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

seminoma-like cell line TCam-2 results in initiation of a CIS/seminoma based on morphology, gene expression, and cellular localization of the proteins.

Of note, the left and right testis weights did not differ strongly 1 and 3 months after TCam-2 injection (Supporting Information Fig. 1A) and derived tumors remained within the testis. Transplantation of 2102EP cells resulted in a significant increase of the left testis weight (Supporting Information Fig. 1A) and huge tumor masses could be found that had already penetrated the confines of the testes (Supporting Information Fig. 1B). Furthermore, the seminal vesicles of the TCam-2-injected mice were significantly heavier compared

to those of 2102EP-injected mice (mean 529/521 vs. 320 mg; *P*-value TCam-2, 1 month vs. 2102EP: 0.013; *P*-value TCam-2, 3 months vs. 2102EP: 0.014 (Supporting Information Fig. 1C).

Mizuno et al. (1993) have reported that TCam-2 cells present as EC-like tumors when transplanted into the flank of mice. We hypothesized that, in order to convert from seminoma to EC, TCam-2 cells must downregulate the seminoma marker SOX17 and upregulate the EC markers SOX2 and CD30. Therefore, TCam-2 cells were transplanted into the flank of nude mice and tumors ($n = 3$ for each time point) were analyzed for expression of SOX2, SOX17, and CD30 by RT-PCR and/or IHC. Up to 2 weeks after

Figure 3. (A) HE and IHC staining of SOX2 or SOX17 3 and 8 weeks after TCam-2 injection into the murine flank as well as RT-PCR of indicated markers 3 (1–3) and 8 weeks (4–6) after transplantation. The white arrow points at a CIS/seminoma-like cell. Secondary antibody controls are given in the lower, right corners. Scale bars: 100 μ m. (B) HE and IHC staining of indicated markers in tumor tissues 8 weeks after TCam-2 flank injection. 2102EP cells served as

control. Secondary antibody controls are given in the right, lower corners. Scale bars: 100 μ m. (C) RT-PCR of indicated markers of in vitro cultivated and 8 weeks in vivo grown TCam-2/2102EP cells. GAPDH was used as endogenous control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 4. (A) HE and IHC staining of indicated markers in TCam-2 and 2102EP cells 1 month after injection into the corpus striatum of nude mice. Secondary antibody controls are given in the lower, right corners. Scale bars: 100 μm. (B) RT-PCR of indicated markers of

in vitro cultivated and in vivo grown TCam-2/2102EP cells 4 weeks after injection into the corpus striatum of nude mice. GAPDH was used as endogenous control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

transplantation, the tumors displayed the typical seminoma-like morphology (Supporting Information Figs. 2A and 2B). After 3 weeks, cells with a seminoma-like morphology were still detectable; however, many of cells presented with an EC-like morphology (Fig. 3A, white arrow) and by 8 weeks, all cells of the tumor had adopted the EC-like morphology. On the molecular level, during the first 2 weeks the majority of cells remained positive for SOX17 (Supporting Information Figs. 2C and 2D). After 3 weeks, the number of SOX17 positive cells was markedly reduced and by 8 weeks none of the cells within the tumors expressed SOX17 (Fig. 3A). On the other hand, few SOX2 positive cells could be detected after 1 week, but the number increased steadily over time and after 8 weeks virtually all cells were positive for SOX2 (Supporting Infor-

mation Figs. 2E and 2F; Fig. 2A). RT-PCR confirmed these findings and demonstrated that after 3 weeks the tumors already expressed robust amounts of *SOX2* and *CD30* while displaying variable amounts of *SOX17* (Fig. 3A, 1–3, 4–6). These findings indicate that the transition to an EC-like tumor involves an initial and rapid induction of SOX2 within the first 3 weeks, while downregulation of SOX17 as well as a change in morphology takes 3–8 weeks.

To validate the EC-like nature of the tumors derived after injection of TCam-2 cells into the flank, we next compared them to tumors originating from the EC-line 2102EP after injection into the flank. Here, IHC analyses revealed that both tumors expressed comparable levels of SOX2 and CD30 and lacked expression of SOX17 (Fig. 3B). Furthermore, cytoplasmic location of BLIMP1 and

Figure 5. PathScan Signaling Array data of indicated signaling molecules of in vitro cultivated TCam-2 or 2102EP cells and TCam-2/2102EP cells injected into the flank (Flank, after 3 and 8 weeks/4 weeks) or corpus striatum (Striatum, after 4 and 8 weeks/4 weeks). Standard deviation is indicated as error bars. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

PRMT5 further underscored the EC-like nature of the tumors (Eckert et al., 2008a,b). Next, RT-PCR was performed to compare the expression of marker genes from the TCam-2 derived tumors in the flank to the parental TCam-2 line and 2102EP cells (Fig. 3C). TCam-2 derived tumors displayed upregulation of *SOX2* and *CD30* and downregulation of *SOX17* to levels comparable to 2102EP controls (Fig. 3C). In addition, upregulation of *AFP*, *PAX6*, and *BRACHYURY (T)* might indicate a beginning nonseminoma-like differentiation. The results suggest that the seminoma-like cell line TCam-2 is instructed by signals from the microenvironment to convert into a tumor resembling an EC after transplantation to the flank (Oosterhuis and Looijenga, 2005; Lind et al., 2007).

Since GCTs are also found in the CNS, we additionally transplanted TCam-2 and 2102EP cells into the corpus striatum of mice. One month after injection, tumors from TCam-2 cells displayed a typical EC-phenotype (Fig. 4A; HE) comparable to tumors generated by transplanting 2102EP cells. IHC staining and RT-PCR verified the EC-like character of the tumor cells (Fig. 4B; *OCT3/4*, *SOX2*, *CD30* positive, and *SOX17* negative). Similar to tumors derived from TCam-2 in the flank, a moderate upregulation of *PAX6* and *BRACHYURY (T)* could be detected. Taken together these data indicate that TCam-2 cells, when injected into the corpus striatum, also adopt an EC-like character.

The tumor-microenvironment clearly instructed the fate of TCam-2 cells in vivo. We next aimed to elucidate signaling cascades altered during conversion of TCam-2 into an EC-like tumor. PathScan Signaling Arrays were performed and

revealed that phosphorylation of AKT (Thr308/Ser743), ERK1/2 (Thr202/Tyr204), angiopoietin receptor Tie2/TEK (pan-Tyr), and ephrin Type-A receptor 3 (EphA3, pan-Tyr) was decreased in the EC-like tumors compared to the parental TCam-2 cells (Fig. 5, Supporting Information Fig. 3A). Interestingly, the phosphorylation levels of these signaling molecules in TCam-2 derived EC-like tumors were highly similar to the levels seen in 2102EP in vitro and in vivo control cells (Fig. 5, Supporting Information Fig. 3A). The reduced ERK1/2-phosphorylation (Thr202/Tyr204) in vivo was verified by western blotting (Supporting Information Fig. 3B). In summary, these data indicate that the TCam-2 derived tumors not only express markers comparable to ECs, but also adopt an EC-like signaling signature in addition.

DISCUSSION

Here, we demonstrate that transplantation of the seminoma-like cell line TCam-2 into the seminiferous tubules results in CIS/seminomatous growth, while injection into the murine flank or brain leads to transition into and growth as an EC. Hence, we have established a versatile murine model system that allows analyzing human Type II GCTs and their progression from CIS in vivo.

Transplantation of TCam-2 cells into the testes resulted in growth of cells with a typical CIS-like morphology. Expression of markers indicative for CIS/seminoma and germ cells (*NANOG*, *OCT3/4*, *SOX17*, *BLIMP1*, *TFAP2C*, *KIT*) further demonstrated their CIS-like character (Jones et al., 2004; Oosterhuis and Looijenga, 2005). Additionally,

cells with a different morphology could be detected after 3 months. Here, expression of PAX6 and AFP indicate differentiation into nonseminomatous GCT. These data demonstrate that TCam-2 cells grow as a CIS/seminoma in the seminiferous tubules for at least 3 months. Hence, our model recapitulates GCT development in vivo from intratubular CIS/seminoma to invasive growth and differentiation into nonseminoma. Further, the procedure of efferent duct transplantation is tolerated well by the mice (survival rate 28/30) and can be performed efficiently (up to 12 transplantations per day). In addition, the experiments can be monitored over 3 months, allowing for drug-response studies. In contrast to murine transgenic model systems, whose generations are time consuming, TCam-2 cells can be genetically modified quickly and easily in vitro before transplantation, allowing the fast in vivo analysis of a gene's role in GCT formation. After busulfan treatment (and TCam-2 transfer), regular spermatogenesis recovers and a functional testis microenvironment is provided until the tumor cells start invasion. Therefore, our animal model is highly comparable to human GCT formation in vivo.

We demonstrated further that TCam-2 cells resemble an EC after injection into the murine flank or corpus striatum, indicated by the cellular morphology and downregulation of SOX17 as well as upregulation of SOX2 and CD30 (de Jong et al., 2008a,b). The rapid upregulation of SOX2 1 week after transplantation into the flank, which is neither accompanied by morphological changes nor downregulation of SOX17, might indicate that induction of SOX2 represents an early step in the transition of a CIS/seminoma into an EC. Furthermore, our data demonstrate that the transition process is accompanied by a decrease in AKT-, ERK1/2-, EphA3-, and Tie2/TEK-phosphorylation.

FGFs and EGFs transduce their signals via the AKT- and ERK-signaling cascade (Kunath et al., 2007; Mayshar et al., 2008). In TCam-2 cells, *FGF2* seems to be the predominant inducer of AKT- and ERK-signaling, since *FGF4* and *EGF* are expressed at low levels, as demonstrated by cDNA microarray analysis (Supporting Information Fig. 3C). Additionally, *AKT1* and *ERK2* seem to be the main downstream effectors of their corresponding pathways (Supporting Information Fig. 3C). In a previous study, we could show that FGF4- and EGF-supplementation induces TCam-2 cells to differentiate into a mixed nonseminoma. There, an EC-like interme-

diate was not detected (Nettersheim et al., 2011). In this study, we see a reduction of AKT1- and ERK2-activation, which accompanies the transition from seminoma to an EC-like tumor. Therefore, reduction of both signaling pathways is an important event in this transition.

What are the mechanisms leading to reduction of FGFR- and EGFR-signaling? Angiopoietins, the ligands of Tie2/TEK, drive angiogenesis and tumor growth in a context-dependent manner (Ward and Dumont, 2002). Thereby, different angiopoietins act agonistic (Ang1, Ang4) or antagonistic (Ang2) in Tie2/TEK-activation (Ward and Dumont, 2002). Parental TCam-2 cells show high levels of *Ang1* and low levels of the antagonist *Ang2* expression (Supporting Information Fig. 3C), indicating active Tie2/TEK-signaling. In contrast, ECs and EC-like cells show decreased Tie2/TEK-phosphorylation, which normally induces AKT- and ERK1/2-signaling. Therefore, reduced Ang1/Tie2/TEK-signaling might be responsible for diminished AKT- and ERK1/2-phosphorylation (Lee et al., 2004; Yuan et al., 2007). Of note, while our data demonstrate that EC-like tumor growth is accompanied by reduced Tie2/TEK-signaling, the biological effects of Ang/Tie2/TEK-signaling are strongly context dependent (Ward and Dumont, 2002). Hence, further studies are on the way to decipher the role of Ang/Tie2/TEK-signaling in CIS/seminomas and nonseminomas.

Ephrin (Eph)-receptors and their Ephrin-ligands are thought to be important regulators of aberrant cell–cell communication in tumor cells (Pasquale, 2010). Ephrin-As are the ligands to EphA-receptors (Pasquale, 2010) and Ephrins A1 and A5 are highly expressed in TCam-2 cells (Supporting Information Fig. 3C); hence they appear to be the main inducers of EphA3-signaling in these cells. The transition of TCam-2 cells to an EC-like state is accompanied by reduction of EphA3-signaling to levels detectable in the EC control cells. The reduction in EphA3-phosphorylation may be caused by downregulation of Ephrin-A1/A5. This observation may reflect a different dependence of seminomas and ECs to cell–cell communication and cell–stroma communication. This hypothesis is in line with the fact that seminomas, but not ECs, are strongly affected by anoikis in vivo (Olie et al., 1996).

In this study, we effectively established a versatile CIS/seminoma mouse model that allows recapitulating human GCT formation and progression from CIS in vivo. Additionally, we demonstrated a dependency of seminomatous

differentiation processes to the microenvironment and highlighted SOX2-induction as well as inactivation of AKT-, ERK, Tie2/TEK-, and EphA3-signaling as important regulators of these processes. Our study helps to obtain new insights into GCT biology and the developmental connection between CIS, seminoma, and EC.

ACKNOWLEDGMENTS

We gratefully thank Ad Gillis, Leendert H. J. Looijenga and the Erasmus Center for Bioinformatics (University Medical Center, Rotterdam) for performing the Affymetrix-microarray experiment and helping us to analyze the dataset. We also thank Angela Egert, Susi Steiner, and Mathilde Hau-Liersch for technical assistance; Hans-Martin Jaeck and Giulio C. Spagnoli for providing the BLIMP1 and MAGE-A4 antibody, respectively.

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