Analysis of Gene Expression Profiles of Microdissected Cell Populations Indicates that Testicular Carcinoma In situ Is an Arrested Gonocyte

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Abstract

Testicular germ cell cancers in young adult men derive from a precursor lesion called carcinoma in situ (CIS) of the testis. CIS cells were suggested to arise from primordial germ cells or gonocytes. However, direct studies on purified samples of CIS cells are lacking. To overcome this problem, we performed laser microdissection of CIS cells. Highly enriched cell populations were obtained and subjected to gene expression analysis. The expression profile of CIS cells was compared with microdissected gonocytes, oogonia, and cultured embryonic stem cells with and without genomic aberrations. Three samples of each tissue type were used for the analyses. Unique expression patterns for these developmentally very related cell types revealed that CIS cells were very similar to gonocytes because only five genes distinguished these two cell types. We did not find indications that CIS was derived from a meiotic cell, and the similarity to embryonic stem cells was modest compared with gonocytes. Thus, we provide new evidence that the molecular phenotype of CIS cells is similar to that of gonocytes. Our data are in line with the idea that CIS cells may be gonocytes that survived in the postnatal testis. We speculate that disturbed development of somatic cells in the fetal testis may play a role in allowing undifferentiated cells to survive in the postnatal testes. The further development of CIS into invasive germ cell tumors may depend on signals from their postpubertal niche of somatic cells, including hormones and growth factors from Leydig and Sertoli cells. [Cancer Res 2009;69(12):5241–50]

Introduction

Testicular germ cell cancer is the most common malignant disease among young adult men in Europe, affecting up to 1% of all men (1). All testicular germ cell tumors of young adult men derive from carcinoma in situ (CIS). The CIS cells are believed to arise from fetal germ cells and reside dormant in the testis until they start proliferating after puberty and eventually develop into an overt tumor (2). Overt testicular germ cell tumors can be divided into two major classes: the seminomas, which retain a CIS-like phenotype and germ cell features; and the more pluripotent embryonic stem cell (ESC)–like nonseminomas, which consist of tumors resembling embryonic tissues (e.g., embryonal carcinoma and teratoma) as well as extraembryonic tissues (e.g., choriocarcinoma and yolk sac tumor).

Testicular germ cell tumors are part of the testicular dysgenesis syndrome (3), a group of disorders believed to arise as a result of disturbed development of the somatic cells in the gonad, probably due to an imbalanced hormonal environment of the fetus (reviewed in ref. 4). The exact trigger for the neoplastic transformation is unknown, but it is probably initiated at the stage of primordial germ cells or gonocytes. This assumption is based on the morphology of CIS (5) and overlap in expression of markers in CIS, primordial germ cells, and gonocytes, but not in infantile spermatogonia and adult germ cells, including several embryonic pluripotency genes (6). In accordance, our recent study showed a striking resemblance between the gene expression profiles of CIS and ESCs because up to 34% of the identified CIS genes were previously reported in ESCs (7). Further, when ESCs are cultured for a prolonged time, gain of chromosome arms 17q and 12p is repeatedly observed (8). Interestingly, the same chromosomal regions are implicated in the progression of CIS to invasiveness, emphasizing the resemblance between CIS and ESCs (9, 10).

When the primordial germ cells migrate through the hindgut toward the gonadal ridge, they remain sexually bipotent. After an initial proliferation in the gonadal ridge, the female germ cells, oogonia, enter meiosis, while male germ cells, gonocytes, continue to proliferate until their differentiation to the quiescent prespermatogonia. One possible explanation for the development of CIS could be that an insufficient virilization of somatic cells surrounding the germ cells could lead to a more female-like differentiation and perhaps a premature initiation of meiosis (11).

Due to the cellularity of the testis, where CIS cells maximally constitute about 5% of the cells, it is difficult to make a satisfactory expression profile of CIS. Previous studies of global gene expression in CIS cells have analyzed testis tissues containing increasing proportions of CIS cells (7) or simply compared testis tissue with CIS to normal testis tissue (12, 13). Although giving useful results, these approaches are limited by a considerable background noise from other cell types in the testis.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/). Requests for reprints: Si Brask Sonne, Righospitalet, Blegdamsvej 9, Copenhagen DK-2100, Denmark. Phone: 45-3545-5127; Fax: 45-3545-6054; E-mail: sbsonne.dk. ©2009 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-08-4554

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We have addressed this issue by developing a fast and specific staining procedure for CIS and fetal germ cells (14), allowing laser microdissection and RNA isolation from relatively pure cell populations. This resulted in RNA of a quality sufficient to perform two rounds of amplification, producing microgram amounts of RNA, which allowed microarray analysis.

In this study, we aimed at elucidating the origin of CIS cells based on comparative gene expression profiling. For this purpose, we compared the gene expression profiles of microdissected CIS cells, gonocytes, and oogonia and cultured ESCs with and without genomic aberrations. To correct for contamination with RNA from Sertoli cells, in which gonocytes and CIS cells are embedded, we also microdissected Sertoli cells from tubules with CIS and included these data in the analysis.

Materials and Methods

Tissue samples and ESC lines. The Regional Committee for Medical Research Ethics in Denmark approved the use of adult testicular samples, and collection of human fetal gonads in the United Kingdom was done in agreement with the Polkinghorne guidelines, following ethical approval and informed consent of women who underwent elective abortions at 10 to 12 wk of pregnancy. Adult testicular tissues containing CIS were residual tissues from orchidectomies collected at the Department of Pathology at Rigshospitalet after diagnosis of testicular cancer. One of the normal testes RNA samples was from apparently normal tissue adjacent to a tumor, and the other two were commercial samples (Applied BioSystems/Ambion and Clontech). Fetal gonads were collected in Sheffield from abortion material; the gestational age was estimated by ultrasound scanning and measurements of hand size. Cultures of the human ESC lines (H7 and SHE5) were maintained in Sheffield under the direction of two of the authors (P.W.A. and H.D.M.). H7 cells with or without genomic aberrations were sorted using a fluorescence-activated cell sorter and only cells expressing the pluripotency marker SSEA3 (undifferentiated cells) were analyzed (see Table 1 for a more thorough description of the samples).

Preparation of cryosections for microdissection. Adult testicular tissue and fetal gonads were embedded in optimum cutting temperature compound (Sakura Fintek Europe) and snap frozen at −80°C in isopentan. Sections of 10 μm (fetal tissue) and 20 μm (adult tissue) were cut on a Shandon SME Cryotome (Life Sciences International Europe Ltd.), collected on nuclease- and acidic–free membrane slides (Molecular Machines & Industries), immediately fixed in 75% RNase-free ethanol for 10 min, and stored in absolute ethanol at −80°C. Serial sections of fetal gonads and testicular tissues containing CIS were analyzed by immunohistochemistry (Fig. 1A) for AP-2γ to identify gonocytes, oogonia, and CIS (15); fetal antigen-1 (FA-1) to identify Leydig cells (16); and AMH (17) and MIC-2 (18) to identify fetal and adult Sertoli cells, respectively. An additional serial section was stained for alkaline phosphatase activity (14), which is only to identify fetal and adult Sertoli cells, respectively. An additional serial section was stained for alkaline phosphatase activity (14), which is only to identify fetal and adult Sertoli cells, respectively. An additional serial section was stained for alkaline phosphatase activity (14), which is only to identify fetal and adult Sertoli cells, respectively.

Microdissection and RNA amplification. Before microdissection, slides were transferred to room temperature and stained with nitroblue tetrazolium (NBT)-5-bromo-4-chloro-3-indolyl phosphate (BCIP) by direct histochemistry as previously described (14). The cells were microdissected within 2 h at room temperature using the MMI CellCut or SmartCut system (Olympus/Molecular Machines & Industries; Fig. 1B). Only CIS tubules with a classic appearance with CIS cells along the edge of tubules and stained areas that resembled fetal germ cells were excised to avoid CIS cells at a more advanced stage or unspecifically stained areas.

RNA was purified using the Ambion RNAqueous Micro Kit (Applied Biosystems/Ambion). The RNA quality was tested with the Bioanalyzer Picokit (Agilent Technologies), and samples were amplified in two rounds using the MessageAmp II aRNA Amplification Kit (Applied Biosystems/Ambion). Microarray analysis. The following samples were analyzed: three ESC samples, three microdissected oogonia samples, three microdissected gonocyte samples, three microdissected CIS samples (CIS), and three microdissected Sertoli cell samples from tubules containing CIS. In addition, three samples of testis tissue containing CIS (CIST) from the same patients as the microdissected CIS and three normal testis samples were included (Table 1). All samples underwent two rounds of amplification as described above.

For microarray analysis, we used Agilent Whole Human Genome Microarray 4×44K chips (design no. 014850, Agilent Technologies). Hybridization and scanning of one-color arrays were done as described by the manufacturer (Agilent Technologies) and analyzed using the Agilent Feature extraction software (version 9.1.3.1).

The lowess normalized, gProcessedSignal from each array was loaded into the marray and limma R/Bioconductor package, normalized between arrays using a quantile normalization procedure, and log transformed. Normalized data were then imported into TIGR's MeV v4.0 (19) for subsequent statistical analysis using the significance analysis of microarrays (SAM; ref. 20) with standard settings. Partition clustering of selected gene lists was made by the Partitioning Around Medoids clustering algorithm (R library ‘‘cluster’’), and simple Correspondence Analysis (R library ‘‘MASS’’; ref. 21) was done to elucidate the correspondence between profiles of selected genes across a set of cell types.

Reverse transcription-PCR, immunohistochemistry, and in situ hybridization. cDNA synthesis was made with 50 ng/μl random hexamer primers. Reverse transcription-PCR (RT-PCR) was done using gene-specific primers placed just upstream of the polyA site. Primer sequences, cycle numbers, and annealing temperatures are summarized in Supplementary Table S1. RPS20 was used as a positive control for cDNA synthesis and PCR efficiency. Representative bands for each primer set were excised from the gels and sequenced.

Serial sections of frozen tissues (10 μm) used for microdissection were fixed in phosphate buffered formalin (4% w/v, pH 7; VW, Bier & Berntsen) for 10 min. Immunohistochemistry was done as previously described (17) with the following antibodies: AP-2γ (Santa Cruz Biotechnology), GA-1 (provided by Charlotte Harken Jensen, Odense University, Odense, Denmark), AMH (provided by Richard L. Cate, Biogen, Cambridge, MA), MIC-2 (clone 12E7, Dako), and MYCL1 [1–MyC (C-20), Santa Cruz Biotechnology]. The antibodies were diluted 1:50, 1:200, 1:150, 1:50, and 1:100, respectively.

In situ hybridization was done as previously described (22). The DNA template for the THCH240734 RNA probe was amplified using nested primers: 1st PCR, GCCAAACAAGAGGAAGCTCATGGA and GTATGGAAGATGTAAGCTGT; 2nd PCR, AATTAACCCCTCACTAAAGGACATCATC-TAGACCT and TAATAGACCTACATAGGGTGTGTTCCCTGT (boldface indicates T3 and T7 promoters).

Results

Purity of microdissected CIS and fetal germ cells. Although it was impossible to completely avoid contamination with surrounding somatic cells, we obtained enriched cell populations that, based on visual inspection, contained up to 60% oogonia, 80% gonocytes, and 80% CIS cells (Fig. 1; Table 1). Before we proceeded to microarray analysis, we tested the enrichment of RNA from CIS and fetal germ cells by RT-PCR analysis of genes with cell type–specific expression (Fig. 1C). The selected genes were POU5F1 (OCT3/4), expressed in CIS, gonocytes, and oogonia (23); VIM, expressed in Sertoli cells, endothelial cells, Leydig cells (24), and granulosa cells (25); INS3, specific for Leydig cells (26); FOXL2, expressed in granulosa cells and undifferentiated Sertoli cells in fetal testes and adult testes with signs of testicular dysgenesis syndrome (27); and PRM1, expressed in round and elongating spermatids (28). RPS20 was used as a cDNA synthesis and PCR control.

POU5F1 was as expected, present in microdissected samples of oogonia, gonocytes, and CIS, and when compared with RPS20, the bands were generally stronger in the microdissected samples than in
the whole tissue preparations. VIM was present in the fetal ovary, fetal testis, and whole CIS testis preparations, but weak bands were also seen in some of the microdissected oogonia, gonocyte, and CIS samples, indicating contamination with neighboring Sertoli and granulosa cells. The Leydig cell gene INSL3 was only detected in samples containing total testis RNA and, at a very low level, in one of the CIS and a few of the gonocyte samples. FOXL2 was detected in all oogonia samples, reflecting the granulosa cell contamination, and was also weakly detectable in gonocyte and CIS samples, probably due to contamination with undifferentiated and partially undervirilized Sertoli cells (27). PRM1 was, as expected, expressed in all CIST samples because of the presence of normal tubules with spermatogenesis, but not in any of the microdissected or fetal samples.

Based on these results, we concluded that the microdissected cell populations were adequately enriched to proceed to microarray analysis.

**Evaluation of microarray gene expression profiles.** Gene expression was analyzed using Agilent microarrays covering more than 41,000 unique human genes and transcripts; the analyzed samples are summarized in Table 1. The raw data have been submitted to Array Express at the European Bioinformatics Institute (accession no. E-TABM-488).

### Table 1. Samples used for RT-PCR and microarray analysis

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Age</th>
<th>Description</th>
<th>Estimated purity (%)</th>
<th>Ampli</th>
<th>RT-PCR</th>
<th>Array</th>
</tr>
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<tr>
<td>ESC1</td>
<td>—</td>
<td>H7 abnormal subline, SSEA3-positive</td>
<td>100</td>
<td>Yes</td>
<td>—</td>
<td>Yes</td>
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<td>ESC2</td>
<td>—</td>
<td>H7 normal subline, SSEA3-positive</td>
<td>100</td>
<td>Yes</td>
<td>—</td>
<td>Yes</td>
</tr>
<tr>
<td>ESC3</td>
<td>—</td>
<td>She5, not fluorescence-activated cell sorted</td>
<td>100</td>
<td>Yes</td>
<td>—</td>
<td>Yes</td>
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<tr>
<td>Oo1</td>
<td>12–13 wg</td>
<td>Microdissected fetal oogonia</td>
<td>60</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Oo2</td>
<td>11–12 wg</td>
<td>Microdissected fetal oogonia</td>
<td>60</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Oo3</td>
<td>10–11 wg</td>
<td>Microdissected fetal oogonia</td>
<td>60</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Oo4</td>
<td>11–12 wg</td>
<td>Microdissected fetal oogonia</td>
<td>60</td>
<td>Yes</td>
<td>Yes</td>
<td>—</td>
</tr>
<tr>
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<td>Total fetal ovary, same tissue as Oo4</td>
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<td>—</td>
<td>—</td>
</tr>
<tr>
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<td>11–12 wg</td>
<td>Microdissected fetal oogonia</td>
<td>60</td>
<td>Yes</td>
<td>Yes</td>
<td>—</td>
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<tr>
<td>FetO5</td>
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<td>Total fetal ovary, same tissue as Oo5</td>
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<td>Yes</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Gon1</td>
<td>10–11 wg</td>
<td>Microdissected fetal gonocytes</td>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Gon2</td>
<td>10–11 wg</td>
<td>Microdissected fetal gonocytes</td>
<td>80</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Gon3</td>
<td>11–12 wg</td>
<td>Microdissected fetal gonocytes</td>
<td>80</td>
<td>Yes</td>
<td>Yes</td>
<td>—</td>
</tr>
<tr>
<td>Gon4</td>
<td>10–11 wg</td>
<td>Microdissected fetal gonocytes</td>
<td>80</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
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<tr>
<td>FetT4</td>
<td>10–11 wg</td>
<td>Total fetal testis, same tissue as Gon4</td>
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<td>Yes</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Gon5</td>
<td>12–13 wg</td>
<td>Microdissected fetal gonocytes</td>
<td>80</td>
<td>Yes</td>
<td>Yes</td>
<td>—</td>
</tr>
<tr>
<td>FetT5</td>
<td>12–13 wg</td>
<td>Total fetal testis, same tissue as Gon5</td>
<td>—</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CIS1</td>
<td>37 y</td>
<td>Microdissected CIS from tissue with CIS invasion (seminoma-like)</td>
<td>80</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CIST1</td>
<td>37 y</td>
<td>Same tissue as CIS1, 95% CIS tubules</td>
<td>—</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CIST2</td>
<td>30 y</td>
<td>Microdissected CIS from tissue with CIS next to classic seminoma</td>
<td>80</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CIS3</td>
<td>26 y</td>
<td>Microdissected CIS from tissue with CIS next to nonseminoma, predominantly EC, immature Sertoli cells</td>
<td>80</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CIS4</td>
<td>27 y</td>
<td>Microdissected CIS from tissue with CIS next to nonseminoma, predominantly EC progressing to YST, focal immature TER</td>
<td>80</td>
<td>Yes</td>
<td>Yes</td>
<td>—</td>
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<tr>
<td>CIST4</td>
<td>27 y</td>
<td>Same tissue as CIS4, 30% CIS tubules</td>
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<td>Yes</td>
<td>Yes</td>
<td>—</td>
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<td>27 y</td>
<td>Same tissue as CIST4, not amplified</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>CIST5</td>
<td>55 y</td>
<td>Microdissected CIS from tissue with CIS next to nonseminoma, predominantly EC and TER</td>
<td>80</td>
<td>Yes</td>
<td>Yes</td>
<td>—</td>
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<tr>
<td>CIST5-Ampli</td>
<td>55 y</td>
<td>Same tissue as CIST5, not amplified</td>
<td>—</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
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<tr>
<td>NT1</td>
<td>—</td>
<td>Ambion</td>
<td>—</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
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<tr>
<td>NT2</td>
<td>54 y</td>
<td>Areas of impaired spermatogenesis, hyalinized tubules and lymphocyte infiltration</td>
<td>—</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
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<td>NT3</td>
<td>—</td>
<td>Clontech</td>
<td>—</td>
<td>—</td>
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<td>—</td>
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<tr>
<td>Sert1</td>
<td>37 y</td>
<td>From same tissue as CIS1</td>
<td>80</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Sert2</td>
<td>30 y</td>
<td>From same tissue as CIS2</td>
<td>90</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Sert3</td>
<td>26 y</td>
<td>From same tissue as CIS3</td>
<td>90</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
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</table>

Abbreviations: Oo, microdissected oogonia; FetO, fetal ovary; Gon, microdissected gonocytes; FetT, fetal testis; -Ampli, not amplified; NT, normal testis; Sert, microdissected Sertoli cells from CIS tubules; wg, weeks gestation; EC, embryonal carcinoma; YST, yolk sac tumor; TER, teratoma.
To investigate if previously described CIS markers were also identified in this data set, we performed a two-way SAM of microdissected CIS versus normal testis (Fig. 2A). Of the 26 most significant genes [false discovery rate (FDR), 0.1%], five genes had been identified as CIS markers in previous studies [PDPN (also known as M2A antigen; ref. 29), TFCP2L1, IL22RA1, UPP1, and MYCL1 (previously MYCL; ref. 7, 13)] and one (ADORA2B) had been reported in undifferentiated testicular tumors and cell lines (13). By comparison, similar two-way SAM analyses (with the same FDR of 0.1%) on CIST versus normal testis and CIST + CIS versus normal testis resulted in zero significant genes specific for CIS.

MYCL1 seemed to be highly expressed in CIS cells, and immunohistochemistry confirmed its presence in CIS cells (Fig. 2B). The heatmap of genes highly expressed in CIS included five tentative human consensus (THC) sequences that seemed to be largely restricted to CIS cells (Fig. 2A). This was confirmed by RT-PCR for three of them, whereas one (THC2341283) did not give any bands and one (THC2378933) resulted in multiple bands (not shown). In situ hybridization with a probe for THC2340734 confirmed its high expression in CIS cells (Fig. 2C). However, all three THC transcripts only included a single exon,\(^7\) and none seemed to encode open reading frames.

Among the genes very high in CIS compared with normal testis was a Sertoli cell marker, KRT16 (immunohistochemistry not shown), indicating a severe contamination of CIS samples with the neighboring Sertoli cells. However, a blast search at ENSEMBL revealed that the KRT16 oligo was only 95% identical to KRT16 (three mismatches), but had a 100% match to two other transcripts that seemed to be nonfunctional KRT16 pseudogenes. To avoid similar mistakes for other genes, we checked all the oligo probes shown in the heatmaps and found that only 77% of the oligo probes

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\(^7\) ensemble.org
for annotated genes were unique for their intended targets (indicated by asterisks). However, because the results in this study are not based on expression of single genes but on the overall profile, the lack of specificity for a subset of the oligos does not affect the results.

The CIS samples clearly included material from Sertoli cells, and therefore we determined the expression profiles of microdissected Sertoli cells from CIS tubules and tested various methods for subtracting Sertoli genes from the CIS data. However, irrespective of the method, this also subtracted a large number of genes expressed in both Sertoli and CIS cells. Instead, we decided to include the expression profile of microdissected Sertoli cells in the heatmap to facilitate a visual comparison (Fig. 2A).

**CIS cells are very similar to gonocytes.** A principal component analysis of the entire data set (Fig. 3) showed that the expression profiles generally grouped by cell type. Normal testis, ESC, and Sertoli were the most distant groups, and oogonia and gonocytes grouped closely together. The gene expression profiles for CIS were clearly distinct from the CIST samples, whose profiles resembled normal testis whereas the microdissected CIS samples grouped with fetal samples and Sertoli cells. This confirmed that the microdissection was successful in eliminating normal germ cells from the CIS gene expression profile. Interestingly, the content of CIS in CIST samples was reflected in the plot; CIST2, which contained only 15% CIS tubules, was closer related to normal testis, whereas CIST1, containing 95% CIS, was much closer related to the fetal samples and microdissected CIS. The overlap between CIS and Sertoli cells was probably due to different levels of contamination with Sertoli cells. Sample clustering of the 500 most differentially expressed genes from a six-way SAM (FDR, 0%; $\delta = 0.36$; Supplementary Fig. S1) gave similar results: The gene expression profile of CIS cells was very similar to that of gonocytes and also related to the

![Figure 2. Verification of microarray data, refining CIS genes. A, two-way SAM on CIS versus normal testis ($\delta = 0.84$; FDR, 0.1%) showing genes highly expressed in CIS; note that IL22RA1 is detected with two different oligo probes. Genes marked with red were previously described in CIS or undifferentiated testicular tumors and cell lines. The annotated genes with confirmed probe specificity are marked by asterisks. The color key at the top shows the relative expression levels from 4 to 16. A more extensive gene list (FDR <1%) can be found in Supplementary Table S2. B, expression of MYCL1 in CIS tests. C, expression of the THC2340734 transcript in CIS tests. Arrows, CIS cells; arrowheads, Sertoli cells. Note that MYCL1 is also expressed in interstitial Leydig cells. NT, normal testis; Sert, microdissected Sertoli cells next to CIS (the rest of abbreviations are as in the legend to Fig. 1).](cancerres.aacrjournals.org/article-figures/2.jpg)
oogonial expression profile, whereas CIST and normal testis clustered together, and ESCs were more distantly related to these tissues (Supplementary Fig. S1). The similarity between CIS and gonocytes was further supported by analysis of uncorrelated shrunken centroids (30). Leaving Sertoli samples out of the uncorrelated shrunken centroid analysis, all the CIS samples were classified as gonocytes, and all CIST samples as normal testis. Thus, based on gene expression profiles, CIS cells are most similar to gonocytes.

We made additional analyses to characterize the data set: Supplementary Fig. S1 shows a heatmap of the 500 most differentially expressed genes from a six-way SAM; Supplementary Fig. S2 shows the most frequent profiles among these genes; and Supplementary Fig. S3 shows a heatmap of genes specific for the respective cell types characterized in this study.

Identification of gene clusters possibly involved in the origin of CIS. Figure 4 shows biologically interesting clusters that may provide additional knowledge on how CIS arise.

When CIS and gonocytes were compared, only five transcripts came out as differentially expressed (FDR <1%). Two genes were up-regulated in CIS: \textit{DEFB119}, encoding an antimicrobial peptide, regulated by androgens and specifically expressed in the testes (31); and \textit{NMNAT2} (nicotinamide mononucleotide adenylyl-transferase-2), a central enzyme of the NAD biosynthetic pathway (32). Three genes were down-regulated in CIS versus gonocytes: \textit{PTPRZ1}, a protein tyrosine phosphatase receptor, which has been described in several cancer types (33); the predicted cancer associated gene \textit{ASXL3}, a human homologue of the \textit{Drosophila} additional sex combs (asx) gene (34); and one unannotated gene (AF318333).

An interesting finding was a large cluster of genes that were expressed at a lower level in CIS compared with the other germ cell types. This has not previously been described because it requires pure CIS preparations. These genes may give important clues to the mechanisms of the neoplastic transformation to CIS because their down-regulation may be linked to this process.

To further study the relationships between CIS, fetal gonocytes, and fetal oogonia, we performed a three-way SAM and selected the 62 most significant genes for correspondence analysis and partition clustering (Fig. 5). Comparing the three profiles of genes up-regulated in CIS, gonocytes, and oogonia, it was obvious that the genes representing the gonocyte and oogonia profiles were expressed at a lower level in all other cell types, whereas the genes in the CIS profile were also highly expressed in normal adult testis and in Sertoli cells. A relatively large cluster contained genes up-regulated in CIS and gonocytes and down-regulated in oogonia. Not surprisingly, among the 13 genes in this cluster, 6 were located on the Y-chromosome, consistent with previous observations (35) that CIS arises only in individuals with some Y chromosome material present.

Discussion

With this study, we, for the first time, performed gene expression analysis on isolated CIS and fetal germ cells, allowing direct comparison of gene expression profiles of CIS cells, gonocytes, and oogonia. The results were clear: No matter how the data were analyzed, CIS cells always grouped with gonocytes. This study supports the proposed fetal origin of CIS and provides the basis for a more detailed understanding of CIS.

Numerous previous studies of single genes clearly showed that CIS cells in many aspects resemble gonocytes (6, 15, 29, 36–41) and
that both CIS and gonocytes express a number of genes normally only seen in ESCs (23, 39, 40). This was confirmed in earlier microarray studies wherein gene expression in whole testes with CIS was compared with normal testes (7, 12, 13). In the study by Almstrup and colleagues (7), we analyzed three testis samples with increasing amounts of CIS tubules and sorted the data according to the percentage of CIS cells. Among the 100 genes most highly expressed in CIS compared with normal testis, 34 genes were also reported in ESCs. However, according to the present data set, CIS cells are much more similar to gonocytes than to ESCs. Although we cannot exclude that the in vitro adaptation of ESCs and contamination with Sertoli cells may add to the difference between CIS and ESCs, we believe that the previously reported similarity between these cells was caused by the study design (7, 12, 13), which led to a subtraction of genes expressed in both normal testis and CIS. This resulted in an overrepresentation of pluripotency genes and, thus, a more pronounced similarity to ESCs.

By isolating the different cell types, we found that gonocytes and CIS cells were very similar and that both were closely related to oogonia, which have only recently diverged from the gonocytes in the sex differentiation. However, according to the correspondence analysis on genes differentially expressed between CIS, oogonia, and gonocytes (Fig. 5), no genes distinguished CIS and oogonia from gonocytes, indicating that CIS do not originate from cells with oogonia characteristics, whereas several genes were highly expressed in CIS and gonocytes, but not in oogonia. Only five genes significantly distinguished CIS from gonocytes (Fig. 4), and interestingly, among these five genes, two cancer-associated genes had low expression levels in CIS. Moreover, among the genes highly expressed in CIS, we found no overrepresentation of oncogenes, and among the genes with low expression in CIS, we found no

**Figure 4.** Biologically interesting clusters. A two-way SAM of interesting cell combinations. CIST was excluded in all analyses. From the top: CIS + gonocytes versus others (\(d = 1.21; \text{FDR}, 0.31\%\)), CIS + ESC versus others (\(d = 1.24; \text{FDR}, 0.96\%\)), CIS + oogonia versus others (\(d = 1.33; \text{FDR}, 0\%\)), CIS versus others (\(d = 3.27; \text{FDR}, 0\%\)), and CIS versus gonocytes (\(d = 3.90; \text{FDR}, 0\%\)). Annotated genes with confirmed probe specificity are marked by asterisks. The color key at the top shows the relative expression levels from 4 to 16. Abbreviations are as in the legends to previous figures. Only the most differentially expressed genes are shown in Fig. 4; more extensive analyses are available in Supplementary Table S3.
overrepresentation of tumor suppressor genes. This indicates that CIS is not a malignant cancer cell in a classic sense but rather an arrested gonocyte.

There have been other proposals for the origin of CIS. Clark (42) suggested that CIS may originate from a multipotent spermatogonial stem cell. However, human spermatogonia do not express the multipotency genes characteristic of CIS, and although multipotent stem cells have been derived \textit{in vitro} from adult human testis (43), the expression profile of these cells does not correspond to the profile of CIS cells. Most noteworthy, the CIS markers POU5F1, NANOG, and CDH1 were only expressed at a low level, and KLF4 and STAT3, which are expressed at a very low level in CIS according to the present data set, were highly expressed in the spermatogonial cell population and adult germ line stem cells. Thus, this origin is rather unlikely.

Some CIS cells are hypertriploid (44, 45), and this feature was suggested as an argument for their origin from spermatocytes, which duplicate their genome in preparation for meiosis (46). Alternatively, CIS cells could originate from fetal gonocytes that, in analogy to female oogonia, attempted to enter meiosis in fetal life because of insufficiently virilized microenvironment in dysgenetic testis (11). The polyploidization followed by selective gene losses and gains have been proposed by several studies as the earliest abnormality in CIS cells (10, 47, 48) and has been detected even in pre-CIS, an abnormal germ cell in severely dysgenetic subjects with disorders of sex differentiation (49). However, the hypertriploidy of CIS cells does not need to be related to meiosis because most other cancers are also aneuploid. Accordingly, we found no meiosis-related genes among the genes specific for CIS. Moreover, when we selected genes highly expressed in CIS and normal adult testis but with low expression in gonocytes (Supplementary Table S4), we did not find any genes specific for meiosis, which strongly suggest that the cell of origin is not a meiotic cell.

\textbf{Pitfalls in the microdissection technology.} Several challenges with the microdissection technology must be taken into consideration. Especially, it is necessary to verify the enrichment of RNA from target cells by RT-PCR before expression profiling. The preliminary verification in this study was encouraging, but also showed that it is impossible to obtain completely pure cell populations by laser microdissection of tissue sections because inclusion of material from neighboring cells is unavoidable and cellular material from cells cut open by the microtome will inevitably leak to the surrounding tissue. Nevertheless, both RT-PCR and subsequent microarray analysis showed a substantial

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5.png}
\caption{Correspondence analysis of genes characterizing CIS, gonocytes, and oogonia. Correspondence analysis for the 62 most significant genes from a three-way SAM on the classes CIS, gonocytes, and oogonia. The 62 genes were clustered into nine distinct gene groups (visualized by coloring), and the corresponding mean cluster profiles are displayed as legend. The positions of the cluster names (CIS, CISoO, etc.) represent the ideal profiles for genes expressed only in the particular cell types. O, gene located on the Y chromosome. E, ESCs; O, oogonia; G, gonocytes; C, carcinoma in situ; T, testis tissue containing CIS; N, normal testis; S, Sertoli cells next to CIS; CISoO, CIS and oogonia; GonOo, gonocytes and oogonia; CISGon, CIS and gonocytes.}
\end{figure}
enrichment of RNA from target cells, and expression profiling clearly showed different profiles for different cell types. We also observed specificity problems with the Agilent oligos. Many probes either matched multiple distinct transcripts or recognized opposite strands or introns in their designated targets. This underlines the importance of checking that the oligos match their targets, and further emphasizes that microarray results should always be verified. However, in this study, we do not focus on expression of single genes; instead, we compare global expression profiles, which leads to quite robust results that are not affected by uncertainty about the identity of a few genes.

Origin of CIS. Although this study confirms previous candidate gene–based studies and clearly shows that CIS cells are very similar to gonocytes, it still remains to be determined why these gonocyte-like cells do not differentiate to spermatogonia but persist in postnatal testes. We know many risk factors for testis cancer, and virtually all are related to a poor embryonic and fetal development of the testes. Animal studies suggest that this may be related to reduced testosterone levels causing a maldevelopment of somatic cells of the testis (50). The poor function of the somatic cells affects the germ cells, which fail to differentiate properly without the appropriate paracrine signals from Sertoli and peritubular myoid cells (4). Thus, we suggest that CIS originates from gonocytes that failed to differentiate to pre-spermatogonia due to the under-masculinized somatic cells that (a) fail to stimulate the germ cells sufficiently and (b) constitute a microenvironment that allow fetal gonocytes to survive in the postnatal testes (Fig. 6). Further investigation of the somatic compartment and its role in the progression from gonocyte to pre-spermatogonium will aid in the understanding of CIS.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis of Gene Expression Profiles of Microdissected Cell Populations Indicates that Testicular Carcinoma *In situ* Is an Arrested Gonocyte

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