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Embryonic Stem Cell-Like Features of Testicular Carcinoma *in Situ* Revealed by Genome-Wide Gene Expression Profiling

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ABSTRACT

Carcinoma *in situ* (CIS) is the common precursor of histologically heterogeneous testicular germ cell tumors (TGCTs), which in recent decades have markedly increased and now are the most common malignancy of young men. Using genome-wide gene expression profiling, we identified >200 genes highly expressed in testicular CIS, including many never reported in testicular neoplasms. Expression was further verified by semiquantitative reverse transcription-PCR and *in situ* hybridization. Among the highest expressed genes were *NANOG* and *POU5F1*, and reverse transcription-PCR revealed possible changes in their stoichiometry on progression into embryonic carcinoma. We compared the CIS expression profile with patterns reported in embryonic stem cells (ESCs), which revealed a substantial overlap that may be as high as 50%. We also demonstrated an over-representation of expressed genes in regions of 17q and 12, reported as unstable in cultured ESCs. The close similarity between CIS and ESCs explains the pluripotency of CIS. Moreover, the findings are consistent with an early prenatal origin of TGCTs and thus suggest that etiologic factors operating *in utero* are of primary importance for the incidence trends of TGCTs. Finally, some of the highly expressed genes identified in this study are promising candidates for new diagnostic markers for CIS and/or TGCTs.

INTRODUCTION

Testicular carcinoma *in situ* (CIS) is the common precursor of nearly all of the testicular germ cell tumors (TGCTs) that occur in young adults (1). The incidence of TGCTs has increased markedly during the past several decades, and these tumors now are the most common type of cancer in young men (2). CIS cells may transform into either a seminoma or a nonseminoma. The seminoma retains a germ cell-like phenotype, whereas the nonseminoma retains embryonic stem cell (ESC) features and comprises embryonal carcinoma (EC) and various mixtures of differentiated teratomatous tissue components (3). The most commonly used marker for CIS in clinical practice is placental-like alkaline phosphatase (ALPP or PLAP), which also is a known marker of primordial germ cells (PGCs; Ref. 4). Epidemiologic evidence and evidence based on immunohistochemical comparative studies of proteins expressed in CIS and fetal germ cells indicate that CIS is an inborn lesion, probably originating from gonocytes in early fetal life that progress to an overt TGCT after puberty (5). The precise nature of the molecular events underlying the initiation of malignant transformation from the gonocyte to the CIS cell and the following progression into overt tumors remains largely unknown.

To shed more light on the CIS cell biology and to identify new markers for early detection of CIS, we decided to analyze gene expression in a more systematic manner. In our previous study that used a differential display methodology, we identified several known and several novel and previously unknown genes highly expressed in CIS cells (6). The advent of the microarray technology prompted us to continue the studies of CIS in a more efficient way using a cDNA microarray covering the entire human transcriptome. To circumvent detecting changes of gene expression not related to CIS (because CIS cells constitute only a small percentage of cells in a typical tissue sample), we used testis tissues containing different amounts of tubules with CIS and searched for genes regulated in a manner proportional to the content of CIS in the sample. Selected promising candidates for CIS markers then were verified by semiquantitative reverse transcription-PCR, and the expressing cell type was determined by *in situ* hybridization. The results revealed a striking resemblance between CIS cells and ESCs.

MATERIALS AND METHODS

Tissue Samples. Testicular tissue samples were obtained directly after orchidectomy and macroscopic pathologic evaluation. The Regional Committee for Medical Research Ethics in Denmark approved the use of the orchidectomy samples. Samples of homogeneous overt tumors (seminoma or EC) and testicular parenchyma preserved in the vicinity of a tumor were excised. Each testicular sample was divided into several tissue fragments: two or three fragments were snap frozen at −80°C for nucleic acid extraction; several adjacent fragments were fixed overnight at 4°C in Stieve fluid or paraformaldehyde and embedded in paraffin. Fixed sections subsequently were stained with H&E or with an antibody against ALPP (PLAP) for histologic evaluation (7). Most samples of testicular parenchyma contained variable numbers of tubules with CIS cells. Samples in which no tubules with CIS were found and complete spermatogenesis was present, which are rare in patients with TGCTs, were used as a normal reference. To limit the uncertainty about cellular differences between fragments from the same patient, we also made a direct print of the frozen fragment that was used specifically for RNA isolation. After staining with ALPP (PLAP) antibody, we could roughly confirm the approximate proportion of tubules with CIS as established by histology in adjacent tissue fragments. One sample from tissues containing ~50%, ~75%, and ~100% tubules with CIS cells, respectively, together with fragments with no neoplastic changes and preserved normal spermatogenesis (serving as the common reference) were chosen for microarray analysis.

Isolation and Labeling of Testicular RNA. Total RNA was purified using the Macherey & Nagel kit (Dueren, Germany), DNase digested, and the RNA quality evaluated using the Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Palo Alto, CA); 5 μg of total RNA were linearly amplified using the RiboAmp RNA amplification kit (Arcturus GmbH, Moerfelden-Walldorf, Germany) yielding a 400–700-fold amplification of the mRNA in the samples. The amplified mRNA was reevaluated on the Bioanalyzer 2100, and 5 μg then were subjected to indirect labeling with Cy3 and Cy5 (Amersham Biosciences, Piscataway, NJ) using the Atlas Glass Fluorescent labeling kit (BD Biosciences Clontech, Palo Alto, CA).

In addition, total RNA (unamplified) from a series of testicular specimens with 100% CIS, overt tumors (seminoma, EC, and teratoma), and normal spermatogenesis was isolated for reverse transcription-PCR analysis.

Hybridization and Processing of Microarrays. The labeled RNA probes were combined and hybridized at 42°C overnight to a 52,000-element cDNA

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microarray representing the complete Unigene database. The microarray consists of two slides, which are hybridized on top of each other with the labeled probe in between. Production and handling of the microarray are described elsewhere.[3] Hybridized slides were washed at 42°C for 5 min in 2× SSC and 0.1% SDS and for 10 min in 0.2× SSC and 0.1% SDS, rinsed, dipped in isopropanol, and dried.

Data Evaluation. Slides were scanned on a GenePix 4000B scanner (Axon Instruments Inc., Union City, CA) using settings in which overall intensity of each channel was equal. The generated images then were analyzed in ChipSkipper[4] and quantified using histogram segmentation. Flagged spots and controls were taken out, and quantified spots were normalized using a framed median ratio centering (frame = 200 genes). The average of dye swaps was used, and spots that did not dye swap or showed a large SD were sorted out. Data then were imported into Genesis software (8) in which hierarchical clustering (9) was done using average linkage and Euclidian distances.

The microarray data comply with Minimum Information About a Microarray Experiment standards and have been submitted to MIAMExpress[5] at the European Bioinformatics Institute and acquired accession number E-EMBL-2.

Verification by Reverse Transcription-PCR. The expression of a selected set of the regulated genes identified in the microarray analysis was verified with reverse transcription-PCR as described previously (6). The samples used for reverse transcription-PCR included cDNA from biopsies with 100% of the tubules containing CIS, biopsies showing normal spermatogenesis, and biopsies from testes with seminoma and EC. Briefly, cDNA was synthesized using a dT_{24} primer. Specific primers targeting each mRNA were designed spanning intron-exon areas to avoid amplification of genomic DNA. PCR was performed in 30 μl of (final concentrations) 12 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.9 mM $MgCl_2$, 0.1% Triton X-100, 0.005% gelatin, 250 μM dNTP, and 30 pmol of each primer. H_2O was used as a negative control, and β-actin was used as an internal control in all of the PCR reactions, resulting in an ~800-bp actin fragment. Cycle conditions were 1 cycle of 2 min at 95°C; 30–40 cycles (depending on the intensity of bands) of 30 s at 95°C, 1 min at 62°C, and 1 min at 72°C; and finally 1 cycle of 5 min at 72°C. PCR products were resolved on 1.5% agarose gels and visualized by ethidium bromide staining. Digital images of the agarose gels were quantified using STORM phosphor imager software (ImageQuant; Amersham Biosciences). In a few of the reverse transcription-PCR analyses of less abundant transcripts, no bands were detectable after PCR, and nested primers were designed. One μl from the first PCR reaction was transferred to a new reaction containing the nested primers and analyzed as described previously.

Preparation of Biotin-Labeled Probes and *in Situ* Hybridization. *In situ* hybridization was performed to confirm that the identified transcripts were expressed in CIS cells. Probes for *in situ* hybridization were prepared by reamplification of the PCR products using nested primers specific to the individual products and with an added T7 or T3 promotor sequence. PCR conditions were 5 min at 95°C; 5 cycles of 30 s at 95°C, 1 min at 45°C, and 1 min at 72°C; and 20 cycles of 30 s at 95°C, 1 min at 65°C, 1 min at 72°C, and finally 5 min at 72°C. The resulting PCR product was purified on a 2% low melting point agarose gel and sequenced from both ends using Cy5-labeled primers complementary to the added T3 and T7 tags. Aliquots of ~200 ng were used for *in vitro* transcription labeling using the MEGAscript-T3 (sense) or MEGAscript-T7 (antisense) kits as described by the manufacturer (Ambion, Houston, TX). The composition of the 10×-nucleotide mix was 7.5 mM ATP, GTP, and CTP; 3.75 mM UTP; and 1.5 mM biotin-labeled UTP. To estimate quantity and labeling efficiencies, aliquots of the labeled RNA product were analyzed by agarose gel electrophoresis and dotted onto nitrocellulose filters and developed as described below.

In situ hybridization was performed essentially as described previously (10), except for one additional step needed to remove mercury from sections fixed in Stieve fixative. After deparaffination, mercury was removed by 15-min treatment with potassium iodide (KI:I ratio, 2:1), followed by three washes in diethyl pyrocarbonate water. The iodine subsequently was removed by incubation in sodium thiosulfate pentahydrate (5% w/v, 10 min), followed by washing (4× diethyl pyrocarbonate water). Subsequently, the procedure was, in brief, as follows: sections were refixed in 4% paraformaldehyde, treated with proteinase K (P-2308; Sigma, St. Louis, MO; 1.0–5.0 μg/ml), postfixed in paraformaldehyde, prehybridized for 1 h at 50°C, and hybridized overnight at 50°C with biotinylated antisense and sense control probes. Excess probe was removed with 0.1× SSC (60°C) 3 × 33 min. Visualization was performed with streptavidin conjugated with alkaline phosphatase (1:1000; cat. no. 1093266; Roche Diagnostics GmbH, Mannheim, Germany), followed by a development with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (10).

RESULTS

Identification of Genes Highly Expressed in CIS *versus* Normal Testicular Parenchyma. By analyzing testicular samples containing increasing amounts of tubules with CIS, we identified differentially expressed genes as compared with the normal reference with complete spermatogenesis and no CIS cells present.

From normalized scatter plots of gene expression in CIS-containing tissue *versus* normal testis tissue, we observed that the majority of differentially expressed genes were down-regulated and that fewer genes (200–300) were significantly up-regulated in CIS-containing tissues. In the group of down-regulated genes, we identified mainly genes related to spermatid maturation, such as the protamines and transition proteins (data not shown). This indicates that the down-regulation of these genes was related to impaired spermatogenesis in the patients with CIS. As we primarily were interested in genes expressed in CIS cells, data were filtered so that only genes that were up-regulated in the CIS samples and had low SD were considered for additional analysis.

The up-regulated genes were a heterogeneous group, including transcription factors such as *TFAP2C* (*ERF-1*), *LBP-9*, *TEAD4* (*RTEF-1*), *NFE2L3* (*NRF3*), and *POU5F1*; solute carriers such as *SLC25A16* and *SLC7A3*; oncogenes such as *RAB-15*, *TCL1B* (*TML1*), *MYBL2* (*B-MYB*), and *PIM2*; and many more genes, which are listed in Fig. 1 and in the Supplementary Data. Some of these genes were described previously as CIS markers, for example, *KIT* (*c-KIT*; Ref. 11), *POV1* (*PB39*) and *MYCN* (*N-MYC*; Ref. 12), and *POU5F1* (*OCT-3/4*; Refs. 13, 14), but the majority were novel not previously characterized genes.

A hierarchical cluster analysis with average linkage of the top most up-regulated genes (minimum fourfold increase in the sample with CIS in 100% of the tubules and more than twofold in the other samples) is shown in Fig. 1. For most of the genes, a gradual increase in expression was observed with increase in the percent of tubules with CIS. This strongly indicates that expression of these genes is linked to the CIS phenotype and to the actual amount of tubules with CIS in the testis. To verify, we reanalyzed the expression of selected genes by reverse transcription-PCR in a panel of samples covering a broad spectrum of different testicular neoplasms and normal testicular tissue (Fig. 2*A*). Quantitating the density of PCR products (normalized to actin) allowed us to investigate the relative abundance of studied transcripts and to assess the approximate ratio between *POU5F1* and *NANOG* (Fig. 2*B*). We also investigated the expression of the nontissue-specific alkaline phosphatase (*ALPL*) simultaneously with the previously known CIS marker *ALPP* or *PLAP* and found that they were expressed in a similar manner (Fig. 2*A*). More reverse transcription-PCR verifications can be found in the Supplementary Data (S2).

The microarray and the reverse transcription-PCR analysis indicated that the observed up-regulation of genes in the samples with CIS were linked to the CIS phenotype and thus to CIS cells. However, it cannot be excluded that the high expression of some genes (*e.g.*, *SIAT1*, *TCL1A*, and *TCL1B*) may be caused by the presence of infiltrating lymphocytes, which are commonly seen in patients with

[3] Internet address: http://embl-h3r.embl.de/.
[4] Internet address: http://chipskipper.embl.de/.
[5] Internet address: http://www.ebi.ac.uk/miamexpress/.

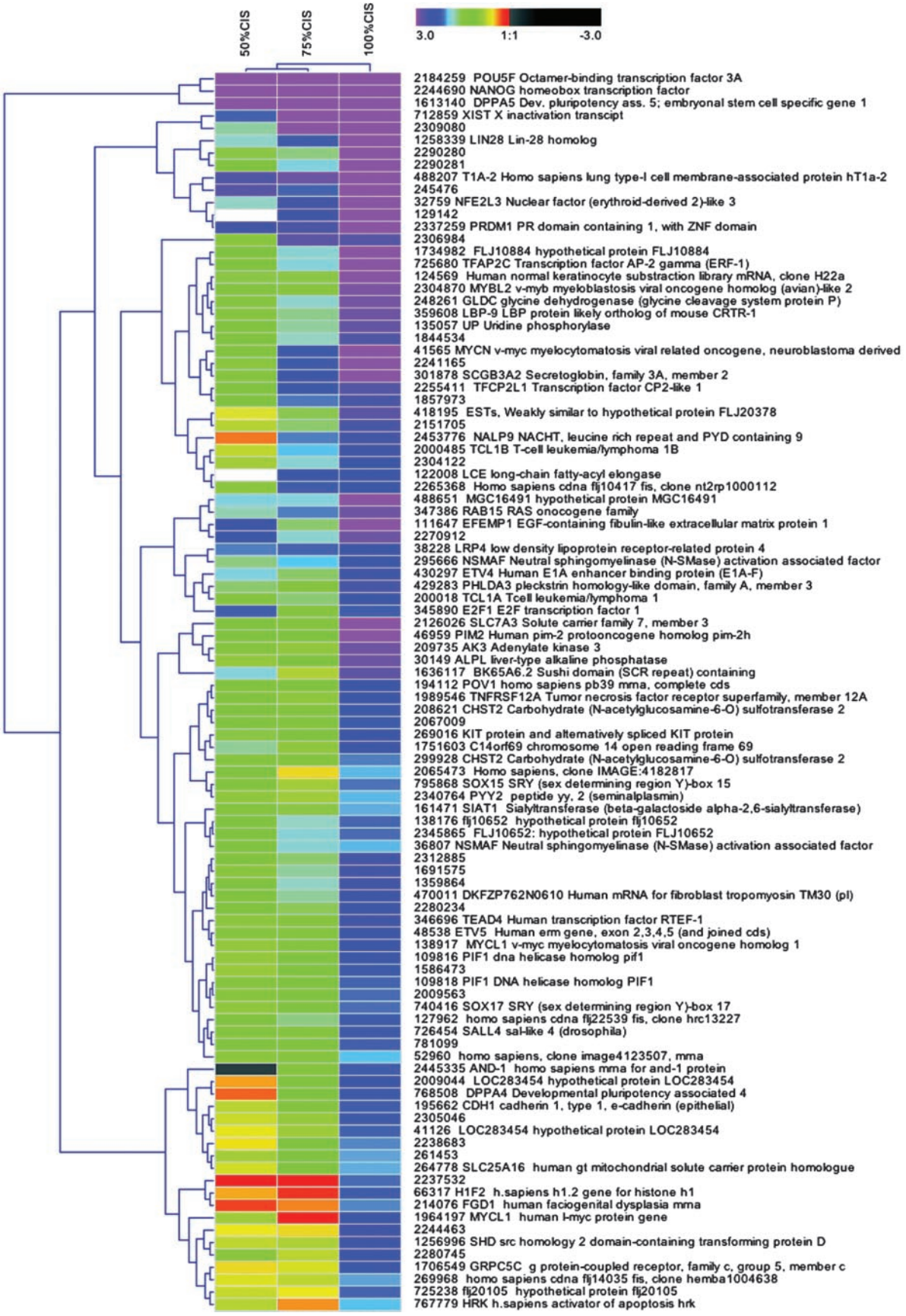

Fig. 1. Hierarchical clustering of genes found by filtering data so only genes fourfold or more up-regulated in the sample with carcinoma *in situ* (CIS) in 100% of the tubules and twofold in the samples with 50% and 75% tubules with CIS. Hierarchical clustering was done using Euclidean distances and average linkage. Some redundancy between entries is seen and implies consistency in the results. The image clone number (unique ID on the array) is reported in front of the gene names and can be used to search the German Resource Center for Genome Research for additional sequence information.[6] A more detailed list can be found in the Supplementary Data (S1).

[6] Internet address: http://www.rzpd.de/.

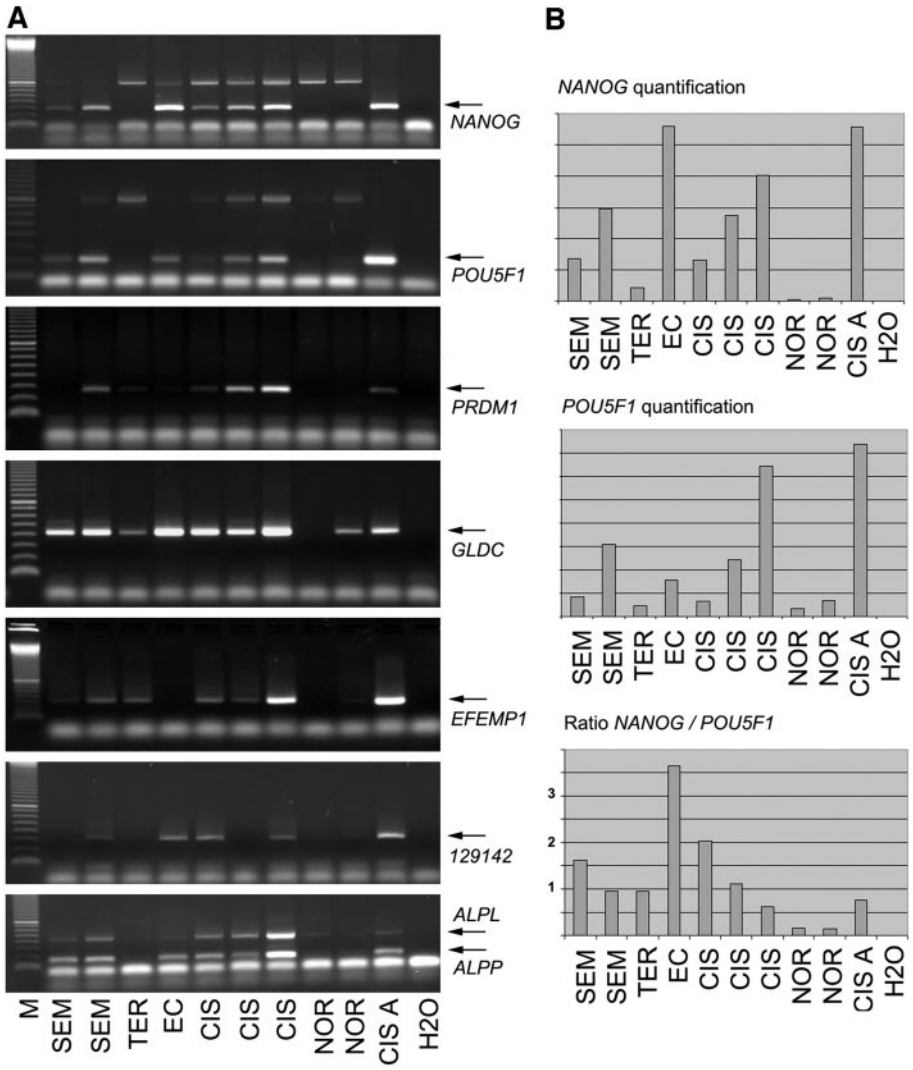

Fig. 2. Further analysis of selected genes that in microarray study were found up-regulated in carcinoma *in situ* (*CIS*). *A*, examples of the semiquantitative reverse transcription-PCR on a spectrum of testicular tissue samples. The gene name is on the right side of the panes. In the gels of *NANOG* and *POU5F1*, the β-actin marker band of 800 bp is visible. Representative DNA fragments from all of the reverse transcription-PCR amplifications were excised, cloned, and sequence verified. Interestingly, we found that *ALPL* was expressed in a similar way to *ALPP* (*PLAP*), which is the most used CIS marker today; 129142 is an Image clone number. *B*, corresponding quantifications of *NANOG* and *POU5F1* and the ratio between the two. Quantification was done by digitalizing the image of the agarose gel. *M*, 100-bp marker; *SE*, seminoma; *TER*, teratoma; *EC*, embryonal carcinoma; *CIS*, testicular tissue showing 100% tubules with carcinoma *in situ*; *NOR*, normal testicular tissue; *CIS A*, CIS amplified for microarray analysis; and H_2O, control. The CIS sample (*right*) is the same sample used in the microarray analysis.

CIS but not present in the normal testis (15). In addition, some transcripts up-regulated in the tissues with CIS may originate from immature Sertoli cells, which are sometimes present in testes with CIS (16).

To confirm the cellular localization, we performed *in situ* hybridization for some of the highest expressed genes in tissues with CIS. Representative examples of the results are shown in Fig. 3. All of the transcripts shown (*NANOG*, *ALPL*, *TFAP2C*, and *SLC7A3*) were localized to CIS cells. In most instances, the reaction was most intense around nuclei and at the cell membrane, and the cytoplasm appeared "empty." This empty space normally is filled in CIS cells with stored glycogen, which is washed out during fixation and paraffin embedding; thus, the cytoplasm is concentrated around the nuclei. We also observed in some cases variable staining of Sertoli cell nuclei, which was most probably unspecific because similar but lighter staining was observed for the control sense probe. However, in all of the cases, the strongest signal was from CIS cells.

Analysis of Expression of ESC Marker Genes in CIS Cells. Among the genes highly expressed in CIS, we noticed a range of genes known to be expressed primarily in ESCs and/or PGCs, such as *POU5F1* (17, 18), *DPPA4* (19), *DPPA5* (20), and *NANOG* (21, 22). Therefore, we performed a systematic comparison of genes highly expressed in CIS with genes recently reported in the literature to be highly expressed in human and mouse ESCs (23–25). As shown in Fig. 4, many of the genes found in ESCs also were highly expressed in our CIS samples. Among the 100 highest expressed CIS marker genes (listed in Fig. 1), 34 genes were reported in ESCs. The overlap may even be greater because many of the genes included on our array are yet uncharacterized and thus may be differently annotated on the microarrays used to expression profile ESCs. Excluding genes with no functional description (gene name) leads to an ~47% overlap. Furthermore, in the studies in which a relative level of expression in ESCs compared with a reference was reported (23, 24), the ranking of the expression level seemed to correlate between CIS and ESCs, although with some exceptions (results not shown). In addition, the studies we compare with all have used different reference samples to explore ESC-specific genes and therefore not always agree on the expression in ESCs (Fig. 4).

Chromosomal Distribution of Gene Expressed in CIS Cells. To find out whether the overexpression of genes in CIS may be associated with a nonrandom genomic amplification in certain "hot spot" areas, we created a map of the chromosomal distribution of the genes highly expressed in CIS. Filtering the data, so that only genes that were more than twofold up-regulated with a low SD in the sample with 100% CIS tubules, resulted in a list containing ~900 genes. We did not filter on the expression in the other samples because proper amounts of entries are needed for a chromosomal distribution analysis. Searching for the chromosomal localization of these genes and

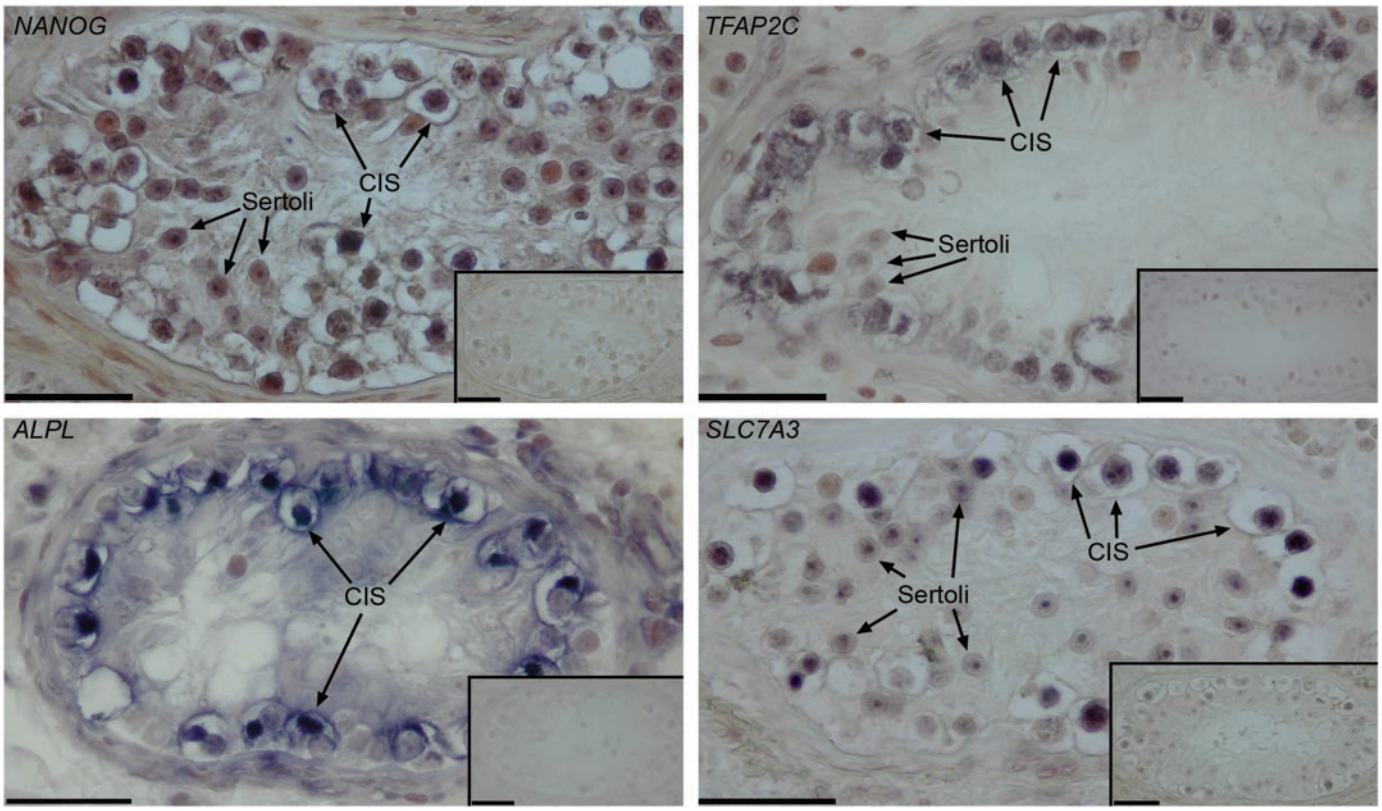

Fig. 3. Analysis of the cellular expression in tubules with carcinoma *in situ* (CIS) by *in situ* hybridization of *NANOG*, *SLC7A3*, *TFAP2C*, and *ALPL* showing that the expression was confined to CIS cells (*arrows*). CIS cells are seen as a stained ring with a stained nucleus in the center, whereas the rest appears empty because of large amounts of glycogen washed out during the tissue processing. Staining also was seen in Sertoli cells nuclei, probably because of overstaining necessary to visualize low abundance transcripts (*arrows*) because this also was seen with the sense probe. The sense control is from neighboring sections of the same tubule as the antisense and inserted in the lower right corner; *bar*, 50 μm.

normalizing with the length of each chromosome revealed that there was an over-representation of up-regulated genes on chromosomes 7, 12, 14, 15, and especially chromosome 17 (Fig. 5*A*). More or less the same picture was seen when we normalized with the number of UniGene clusters (Build 34, version 3) on each chromosome except that it also suggested genes on the X chromosome were over-represented. Plotting the density of up-regulated genes in each chromosomal band allowed us to narrow down hot spots to smaller regions, with 17q as the most pronounced region (Fig. 5*B*). Other regions worth mentioning were 15q, 12p, and 12q (Fig. 5*B*). A detailed map of the chromosomal distribution can be found in the Supplementary Data (S3).

DISCUSSION

We have used one of the largest microarrays currently available to describe the genome-wide gene expression profile of human testicular CIS, a common precursor to the majority of tumors occurring in the testis. A few published studies analyzed gene expression profiles in overt germ cell tumors, with focus on genes on chromosomes 17 and 12, because of frequent rearrangements of these chromosomes in human cancers (26–28). Except for our own previous study using differential display methodology (6), this study is the first global analysis of the gene expression in testicular CIS. This analysis enabled us to identify genes highly expressed in CIS, some known previously but most never before reported in germ cell neoplasia.

The most interesting finding of our study was the identification of a high abundance of genes linked to the stem cell phenotype in CIS cells. Approximately 35% of genes overexpressed in CIS were detected previously in ESC-derived cell lines. The overlap may even be as high as 50%, taking into account uncertainties, such as different gene annotations in different arrays and RNA references (Fig. 4).

We and others have noted previously that CIS cells share some similarities in gene expression with ESCs (*e.g.*, both express *TRA-1–60* and *POU5F1*; Refs. 29, 30). The expression of *POU5F1* has been linked to the stem cell phenotype and identified as required to maintain pluripotency, which is a hallmark of stem cells (17). In this study, we added a number of genes found previously in ESCs to the list of CIS marker genes, thus providing important information on the biology of early germ cell neoplasia and especially the stem cell-like properties of CIS.

Among the genes in this group, we detected in CIS a high expression of *NANOG*, recently identified as the only gene that, in contrast to *POU5F1*, is able to maintain the pluripotency and self-renewal of ESCs independently of leukemia inhibitory factor/signal transducers and activators of transcription 3 (21, 22). *NANOG* is not expressed in any somatic tissue, neither is it present in the adult testis or in non-ESCs (21). However, it also has been reported that *NANOG* is expressed in PGC, ECs, and embryonic germ cells, to which cell types CIS has consistently displayed similarities (21). At implantation stage, *NANOG* and subsequently *POU5F1* are down-regulated, leading to differentiation of the inner cell mass (21, 22). NANOG-deficient inner cell mass loses pluripotency and fails to form epiblast and thus PGCs. Likewise, inactivation of *POU5F1* also results in loss of pluripotency, and the actual stoichiometry between *POU5F1* and *NANOG* determines the fate of the ESCs (21, 31). Overexpression of *POU5F1*

[7] Internet address: http://www.ensembl.org/.
[8] Internet address: http://www.ncbi.nlm.nih.gov/.

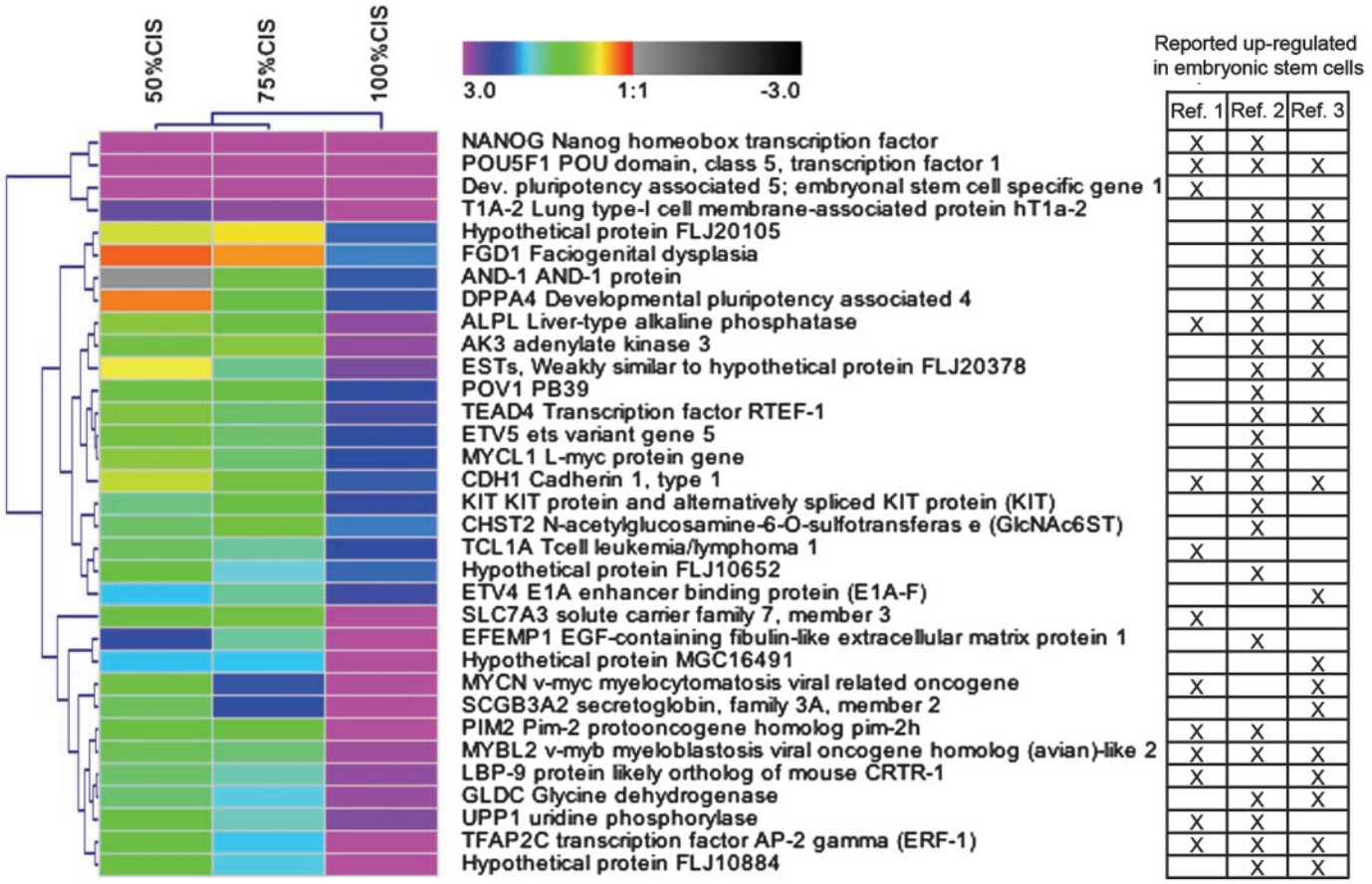

Fig. 4. Genes from Fig. 1 reported to be up-regulated in embryonic stem cells (ESCs). Data on mouse ESCs (Ref. 1 on Fig. 4) are from Ramalho-Santos *et al.* (24), whereas data on human ESCs are from (Ref. 2 on Fig. 4) Sato *et al.* (25) and (Ref. 3 on Fig. 4) Sperger *et al.* (23). The overlap between genes reported in these studies and genes identified in carcinoma *in situ* (*CIS*) (Fig. 1) amount to roughly 35% but may be even greater because of the differences in gene annotation and RNA references used.

commits ESCs to differentiation, and overexpression of *NANOG* prevents differentiation. In a recent study of *POU5F1* (*OCT3/4*) in TGCTs, the authors suggested that the high abundance of *POU5F1* might be solely responsible for the malignant behavior of these tumors and proposed that the targeted inactivation of *POU5F1* might be a valid therapeutic approach (14). We think that the situation is more complex, and the ratio between *POUF51* and *NANOG* may be more important. We hypothesize that CIS progression may involve a shift in the *NANOG*:*POU5F1* ratio. Our results from the microarray analysis and from the reverse transcription-PCR analysis indicate that in CIS cells, *POU5F1* is more highly expressed than *NANOG* (Fig. 2, *A* and *B*). From the microarray analysis, which is more quantitative than reverse transcription-PCR, we established the ratio *NANOG*:*POU5F1* to be ~0.5. Using semiquantitative reverse transcription-PCR, the actual ratio between *NANOG* and *POU5F1* is not meaningful, but the change in the ratio across samples should be valid. Interestingly, in the reverse transcription-PCR analysis, the *NANOG*:*POU5F1* ratio seemed to increase when CIS cells were compared with ECs (Fig. 2*B*). This observation has to be confirmed on a larger number of samples but may indicate that when CIS cells transform into an EC, *NANOG* is further up-regulated, thus maintaining or even increasing the ESC-like pluripotency. Conversely, when CIS cells progress into a seminoma, the relative expression of *NANOG* seems to remain unchanged, thus "freezing" seminoma in a gonocyte-like (CIS-like) phenotype. Alternatively, CIS cells may be heterogeneous with regard to the *NANOG*:*POU5F1* ratio, and those with a relatively high *NANOG* expression, perhaps originating from PGCs, retain the stem cell-like behavior and transform into ECs and teratomas, whereas the CIS cells with a low *NANOG* expression, perhaps the progeny of more differentiated gonocytes, can only transform into a seminoma. To solve this problem, additional studies on microdissected CIS cells will be necessary.

Not surprisingly, our subtractive microarray analysis did not detect some antigens previously reported as highly abundant in CIS cells (*e.g.*, *VASA*, *MAGE-A4*, and *CHK2*) because these antigens also are expressed in normal adult germ cells (29). However, among genes of interest identified in CIS in this study, we briefly highlight here *TCL1A*, *PIM2*, and *TFAP2C* because their functions suggest a possible involvement on the malignant transformation. T-cell leukemia/lymphoma 1A (TCL1A) is expressed in the early embryo and interacts with Akt by enhancing its kinase activity and thus attenuates the phosphatidylinositol 3′-kinase pathway (32). PIM2 is a member of the PIM family of serine/threonine protein kinases and may act in a similar manner to Akt but probably not through Akt (33). Transcription factors AP-2α, AP-2β, and AP-2γ (TFAP2C or ERF-1) comprise the OB2–1 transcription factor. OB2–1 up-regulates ERBB-2 (NEU), which interacts with the phosphatidylinositol 3′-kinase pathway. The three genes interact (directly or indirectly) and may lead to disruption of the Akt/phosphatidylinositol 3′-kinase pathway. Interestingly, two possible mouse models for humanlike testicular germ cell neoplasms, the *PTEN* knock-out mouse (34) and the 29.MOLF-Chr 19 chromosome substitution strain (35), involve disruption of the phosphatidylinositol 3′-kinase pathway.

Another conceptually important question we asked concerned the mechanism of the differential expression of so many genes in CIS. The over-representation of transcripts may be a result of a true

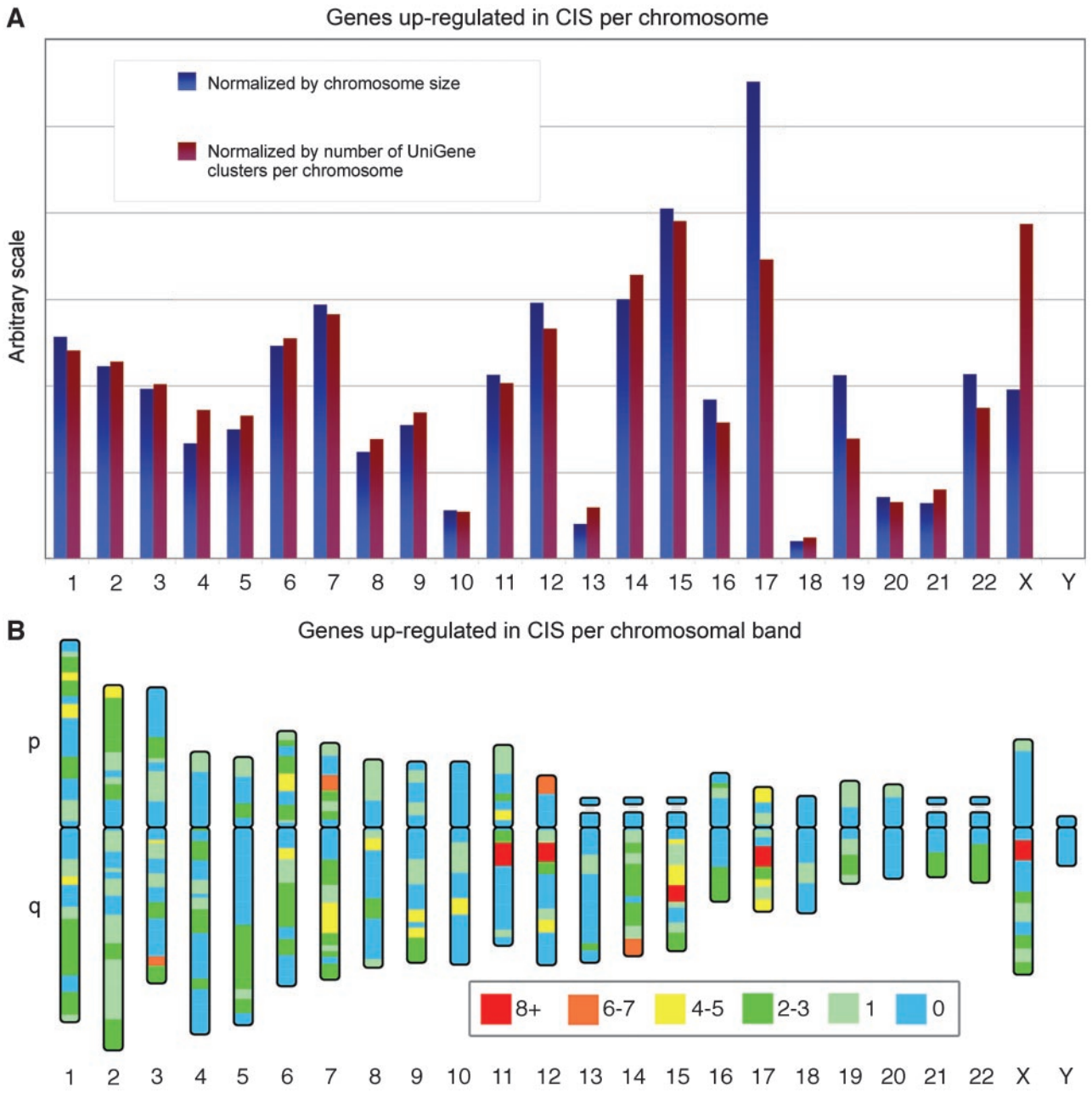

Fig. 5. Chromosomal distribution of genes more than twofold up-regulated in the sample with 100% tubules having carcinoma *in situ* (CIS). *A*, distribution per chromosome and normalized by the size of the chromosomes (*blue bars*) or by the number of UniGene clusters on each chromosome (*red bars*). Chromosomal size was acquired from Ensemble[7] and number of UniGene clusters (Build 34, version 3) from NCBI.[8] *B*, detailed distribution of the number of up-regulated genes in each chromosomal band. The color coding is outlined in the inserted box.

functional up-regulation but also may reflect a gene-dosage effect caused by structural amplification (*e.g.*, duplication of parts of chromosomal regions), especially if the over-represented genes appear in clusters. The chromosomal distribution of genes up-regulated in our sample with 100% CIS tubules revealed that it was not random. Two of the over-represented chromosomal regions, 12p13 and 17q, also were often reported in TGCTs (36). Interestingly, recurrent genomic gain of chromosomes 17q and 12 recently was discovered in cultured human ESCs (37, 38). It was postulated that cultured ESC might gain extra chromosome material in these areas after freezing and thawing, which could inhibit apoptosis and increase their self-renewal in culture (37). Gain of extra chromosomal material in these areas in CIS cells may likewise improve their survival while maintaining their pluripotency, ultimately leading to uncontrolled cell division and tumor formation. Genomic amplification of 12p, frequently associated with a formation of the isochromosome i(12)p, is the most consistent genetic abnormality of TGCTs (36, 39, 40). The presence of this aberration in CIS cells has been a contentious issue. Most studies have not detected i(12)p in CIS and suggested that the gain of 12p occurs when the CIS cells progress to overt tumors (41, 42). Our recent investigation documented the presence of a genomic gain of 12p in CIS cells in vicinity of overt tumors but not in CIS cells before the tumor has developed (43). Because our microarray analysis in this study was done in CIS adjacent to overt tumors, the significant over-expression of genes mapped to chromosome 12p (*e.g.*, *NANOG* and *CCND2*) may be a gene dosage effect. Conversely, many genes highly expressed in CIS (*e.g.*, *POU5F1*, which is located on 6p21) are not associated with chromosomal gains. Therefore, we think that the

expression pattern observed in CIS probably is a combined result of genomic amplification and increased transcriptional activation (or the absence of epigenetic silencing of transcription).

The findings of this study provide additional strong support for our long-standing hypothesis of early fetal origin of CIS cells. This has profound implications for our understanding of the etiology and the incidence trends of this disease, linking it with factors operating *in utero*. The revealed stem cell-like phenotype of CIS cells explains its pluripotency and opens possibilities to study basic mechanisms of cell differentiation using CIS cells as a model. Finally, some of the highly expressed genes identified in this study are promising candidates for new diagnostic markers for CIS/TGCTs and will be investigated in future clinical studies.

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