Gene Expression Profiling Identifies New Biological Markers of Neoplastic Germ Cells

KATHARINA BIERMANN[1*], LUKAS CARL HEUKAMP[1*], KLAUS STEGER[2], HUI ZHOU[1], FOLKER ERNST FRANKE[3], INES GUETGEMANN[1], VIOLETTA SONNACK[2], RALPH BREHM[4], JOHANNES BERG[5], PATRICK JAN BASTIAN[6], STEFAN CAJETAN MÜLLER[6], LIHUA WANG-ECKERT[7], HUBERT SCHORLE[8] and REINHARD BÜTTNER[1]

[1]*Institute of Pathology,* [6]*Department of Urology, and*
[8]*Department of Developmental Pathology, Institute of Pathology, University of Bonn;*
[2]*Department of Urology and Pediatric Urology;*
[3]*Institute of Pathology, and*
[4]*Institute of Veterinary-Anatomy, -Histology and -Embryology, University of Giessen;*
[5]*Institute of Theoretical Physics, University of Cologne;*
[7]*Laboratory for Molecular Neurobiology, Life and Brain Center, Bonn, Germany*

Abstract. *Background: There is only limited knowledge about gene expression in human testicular germ cell tumors of adolescents and adults (TGCTs), and, in particular in its pre-invasive stage intratubular germ cell neoplasia unclassified (IGCNU). Materials and Methods: Global gene expression was studied in 10 invasive human testicular germ cell tumors (TGCTs), 7 intratubular germ cell neoplasia unclassified (IGCNU) and 3 normal testes. The pattern of expression of several genes was studied by immunohistochemistry on tissue microarrays containing 126 TGCTs, IGCNU, normal testes and in 5 fetal testes. Results: RAS-related genes (KRAS2, RALA, RAB39B) and various core markers of embryonic stem cells were overexpressed in IGCNU compared to normal testes. CD9, PODXL and centromere-specific histone-H3-like protein CENPA were specifically identified in IGCNU, seminomas, embryonal carcinomas and in fetal gonocytes. Embryonic stem cell regulator SOX2 and downstream targets of the Nodal pathway were up-regulated in embryonal carcinoma only but not in IGCNU/seminoma. Preliminary data revealed that the expression profile of IGCNU is dependent on the histology of the adjacent invasive tumor. Conclusion: Our study determined the genes involved in early pathogenetic events of neoplastic germ cell formation, provided new insights into genetic pathways driving the transition of embryonal carcinoma and seminoma from IGCNU and identified new biomarkers of neoplastic germ cells such as CD9, CENPA and PODXL.*

The incidence of testicular germ cell tumors (TGCT) reveals a consistent increase (between 6 to 11 per 100,000 in Western populations) making TGCT the most frequent malignancy among young men (1, 2). This type of cancer is proposed to represent part of the so-called testicular dysgenesis syndrome (TDS), which includes also cryptorchidism and testicular atrophy (3). As first described by Skakkebaek, intratubular germ cell neoplasia (IGCNU, carcinoma *in situ*) is the common precursor lesion of all TGCT except spermatocytic seminomas (4, 5). According to the current hypothesis, delayed or compromised maturation of fetal germ cells, *i.e.* primordial germ cells/gonocytes, leads to the persistence of immature germ cells which subsequently (probably with the onset of puberty) may undergo malignant transformation (6). Our recent study identified different differentiation stages of fetal germ cells and indicated that the process of normal maturation must be interrupted at a distinct period of fetal development prior to neoplastic transformation (7). The precise nature of transformation of premature germ cells to IGCNU is not known, but is likely related to the deregulated expression of core transcription factors, such as OCT3/4, NANOG, AP-2γ and its proposed target gene, the receptor tyrosine kinase *KIT* (8). Both, IGCNU and seminomas, may give rise to non-seminomatous tumors but the genetic processes involved in this process remain largely unknown (9).

*Both authors contributed equally to this work.

Correspondence to: Katharina Biermann, MD, Institute of Pathology, University Hospital Bonn, Sigmund-Freud-Strasse 25, 53127 Bonn, Germany. Tel: +49 22828719245, Fax: +49 22828715030, e-mail: katharina.biermann@ukb.uni-bonn.de

Key Words: Testicular, germ, seminoma, embryonal, carcinoma, IGCNU, CIS, neoplastic, genome.

Table I. *Characterization of antibodies used in immunohistochemistry analysis.*

Antigen	Origin	Application		Code	Dilution	Source
		Frozen tissue	Paraffin-embedded tissue			
Kit	Rabbit		X	A4502	1:100	DAKO, Hamburg, Germany
POU5F1 (OCT3/4)	Goat		X	sc-8629	1:1.000	Santa Cruz, Biotechnology, CA, USA
MAGE-A4	Mouse		X	MAGE-A4	1:100	Professor Spagnoli, Institute for Surgical Research, Department of Surgery, Basel, Switzerland
D2-40	Mouse		X	D2-40	1:200	Abcam, Cambrigde, UK
Podoplanin	Mouse		X	18H5	1:200	Acris, Hiddenhausen, Germany
CENPA	Mouse		X	Ab13939	1:100	Abcam, Cambrigde, UK
p-SMAD2/3	Rabbit		X	sc-11769	1:100	Santa Cruz, Biotechnology, CA, USA
PODXL	Mouse		X	Anti-PODXL	1:100	Professor Chen, Department of Biochemistry, Stanford University School of Medicine, Stanford, California, USA
Cytokeratin 18	Mouse		X	DC10	1:10	DAKO, Hamburg, Germany
CD30	Mouse		X	Ber-H2	1:10	DAKO, Hamburg, Germany
Nanog	Goat		X	Anti-Nanog	1 :10	R&D Systems, Wiesbaden, Germany
CD9	Mouse	X		ALB 6	1:50	Immunotech, Marcelle, France
SOX2	Rabbit		X	Anti-Sox2	1:200	R&D Systems, Minnneapolis, USA

Gene expression array studies on invasive TGCT and their precursor lesions, the IGCNU, are limited in number and little is known about the molecular events in non-invasive or invasive neoplastic germ cells (10-13). Recent studies with few IGCNU samples showed a substantial overlap in the expression pattern of IGCNU and embryonic stem cells (14). However, to date it is not known whether IGCNU, precursors of seminomas and nonseminomas, display different expression profiles. Thus, to identify pathways that are involved in establishment of IGCNU and different histological types of TGCT, we analyzed gene expression in IGCNU and adjacent overt TGCT by cDNA microarrays covering the entire human transcriptome. Gene expression of a selection of genes detected by microarray analysis was further studied by immunohistochemistry in neoplastic and normal fetal and adult testes.

Materials and Methods

Testicular tissues. All fresh testicular tissue samples used for the expression profiling were obtained immediately after orchiectomy. Permission to use the tissue for scientific purposes was obtained from the patients and was approved by the institutional Regional Committee for Ethics. All samples were excised, snap-frozen and stored at –80˚C. Normal testicular specimens were obtained from three patients with prostate carcinomas. The tumor samples used for the expression analysis were obtained from 10 patients and contained pure seminoma (n=4), embryonal carcinomas (n=3) and mixed nonseminoma, composed of embryonal carcinoma and immature teratoma (n=3). All tumors were unilateral at the time of clinical examination and were classified according to the WHO classification of tumors based on their histology as pT1 stage by two independent pathologists (15). Of three patients with seminoma and three with pure embryonal carcinomas, testicular parenchyma samples adjacent to tumor containing IGCNU were available for RNA extraction and expression profiling. One IGCNU sample of an additional patient with embryonal carcinoma was also included (no invasive tumor was available for RNA preparation in this case). Prior to RNA extraction, samples were examined by frozen section to assure a significant tumor cellularity. Thorough preparation of IGCNU samples and immunohistochemical staining with an antibody to AP-2γ ensured that these samples were composed of IGCNU (100% of the tubules composed of IGCNU) only without any normal testicular tubules or invasive tumors.

Tissue array preparation from testicular tissues. Tissue arrays were constructed from paraffin-embedded tissue using a Tissue Arrayer (Beecher Instruments, Silver Springs, MD, USA) by obtaining 1.2 mm cores from each paraffin block and their placement in predrilled slots of the recipient block as previously described elsewhere (7). The array included 126 testicular tissue cores from 72 individuals including 10 normal testicular biopsies, 66 IGCNU (from 30 seminomas and 36 non-seminomas), 30 pure seminomas and 20 embryonal carcinomas.

RNA preparation and whole genome gene expression array analysis. Total RNA was extracted with Trizol (Invitrogen, Karlsruhe, Germany) from normal testes (n=3), seminomas (n=4) and adjacent IGCNU of the same patients (n=3), embryonal carcinomas (n=3) and adjacent IGCNU of the same patients (n=3), teratocarcinomas (n=3) and IGCNU of a patient with embryonal carcinoma. All samples had RIN (RNA integrity number) values >8. Applied Biosystems (ABI, Darmstadt, Germany) Human Genome Survey Arrays 2.0 were used to analyze the transcriptional profiles of the

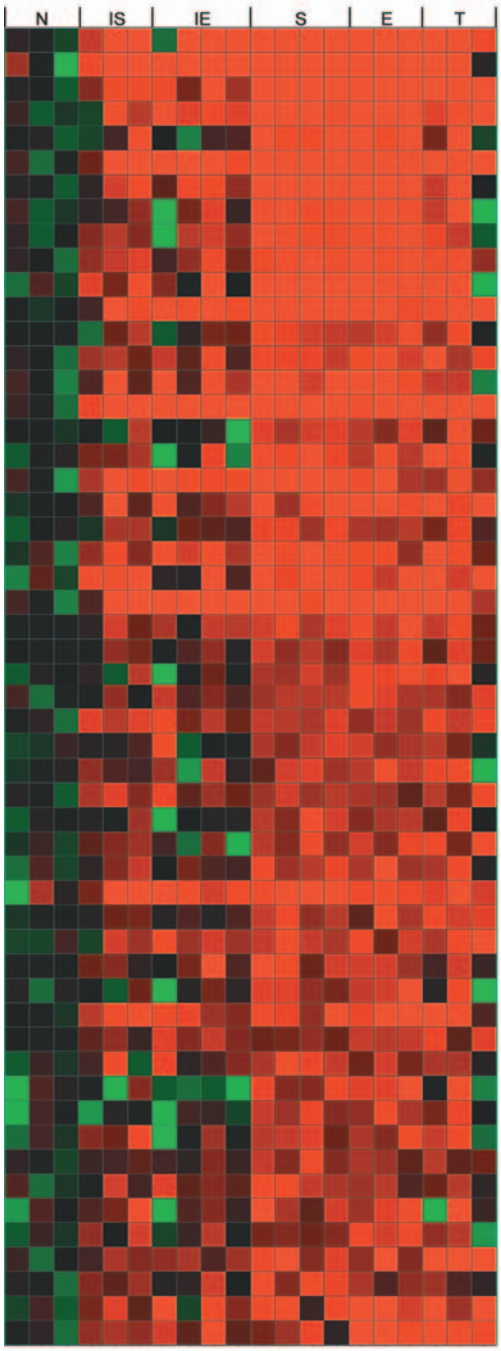
Table II. *Stem cell genes and major developmental signalling pathway-related genes in seminomas (S) and embryonal carcinomas (E). Genes are categorized by each signalling pathway. The means of fold changes (FC) from at least three independent microarray hybridisations calculated from comparison with the values in normal testes are shown.*

Gene symbol/pathway	Gene name	Fold change	
		E (n=3)	S (n=4)
Wnt pathway			
FRAT2	Frequently rearranged in advanced T-cell lymphomas 2	8.53	4.83
FRAT1	Frequently rearranged in advanced T-cell lymphomas	3.46	4.94
FZD8	Frizzled homolog 8 (Drosophila)	15.55	13.58
FZD10	Frizzled homolog 10 (Drosophila)	4.50	5.70
FZD7	Frizzled homolog 7 (Drosophila)	3.75	1.24
LRP6	Low density lipoprotein receptor-related protein 6	8.26	11.75
KREMEN2	Kringle containing transmembrane protein 2	8.10	1.17
WNT3A	Wingless-type MMTV integration site family, member 3A	2.90	4.95
WNT5B	Wingless-type MMTV integration site family, member 5B	19.26	3.10
WNT2B	Wingless-type MMTV integration site family, member 2B	0.23	4.24
WNT3	Wingless-type MMTV integration site family, member 3	5.08	2.08
JAG1	Jagged 1 (Alagille syndrome)	3.97	1.86
DLL3	Delta-like 3 (Drosophila)	8.20	9.17
Nodal/Activin			
ACVRL1	Activin A receptor, type II-like 1	4.29	2.08
ACVR1	Activin A receptor, type I	3.02	3.25
ACVR2B	Activin A receptor, type IIB	2.94	3.68
NODAL	Nodal homolog (mouse)	41.00	4.18
INHBA	Inhibin. beta A (activin A, activin AB alpha polypeptide)	6.00	0.52
LEFTB	Left-right determination, factor B	98.24	8.85
Notch pathway			
NOTCH4	Notch homolog 4 (Drosophila)	3.26	1.85
KIAA1977	KIAA1977 protein	3.74	1.17
ANK1	Ankyrin 1, erythrocytic	6.06	3.14
CRB2	Crumbs homolog 2 (Drosophila)	2.65	3.64
NOTCH3	Notch homolog 3 (Drosophila)	3.87	2.49
NOTCH1	Notch homolog 1, translocation-associated (Drosophila)	4.90	1.82
FBN3	Fibrillin 3	7.26	15.96
EGFL6	EGF-like-domain, multiple 6	6.07	0.79
MTPN	Myotrophin	2.38	3.00
ANKRD3	Ankyrin repeat domain 3	6.92	8.61
BMP pathway			
BMP10	Bone morphogenetic protein 10	1.78	3.33
BMP4	Bone morphogenetic protein 4	3.25	0.52
BAMBI	BMP and activin membrane-bound inhibitor homolog (*Xenopus laevis*)	3.14	8.14

different entities. The Human Genome Survey Microarray V contains 32,878 60-mer oligonucleotide probes representing a set of 29,098 individual genes and more than 1,000 control probes. Probe preparation, hybridization, image generation and analysis were carried out according to the manufacturer's guidelines for the AB1700 Microarray system. Autogridding, basic quality control, feature extraction, background correction and spot and spatial normalization were performed with the ABI 1700 Chemiluminescent Microarray Analyzer according to the manufacturer's instructions. Based on our experience and published data (16), extremely small variation was found between technical replicates on the AB1700 platform and in the light of limited resources one array per sample was hybridized and biological rather than technical replicates performed.

Bioinformatic data processing and analysis. All probe sets flagged as bad spots by the AB1700 software (FLAG >5000) were removed and samples with more than 50% missing values were excluded from analysis. Remaining missing values were replaced with average signals from replicate arrays within the same subgroup, quantile normalized and transformed to logarithmic 2 scale, using Bioconducter (http://www.bioconductor.org/docs/faq/).

Immunohistochemistry. For immunohistochemistry on paraffin-embedded tissue, dewaxed, 4-μm thick tissue sections were microwave-pretreated in citrate-buffer. Primary antibodies to the following proteins were used for detection on paraffin-embedded tissues: NANOG, SOX2, POU5F1 (OCT3/4), CENPA, pSMAD2/3, PODXL and CK18. CD9 immunohistochemistry was



Gene Symbol	Cytoband	Gene name	Score (d)	FC
POU5F1	6p21.31	POU domain, class 5, transcription factor 1	11.40	225.5
NANOG	12p13.31	Nanog homeobox	6.98	121.6
BASP1	5p15.1-p14	brain abundant, membrane attached signal protein 1	6.97	32.7
CENPA	2p24-p21	centromere protein A, 17kDa	6.48	20.3
SOX15	17p13	SRY (sex determining region Y)-box 15	6.44	15.0
STELLAR	14q13.2;12p13.31	germ and embryonic stem cell enriched protein STELLA	6.35	622.1
ALPL	1p36.1-p34	alkaline phosphatase, liver/bone/kidney	6.34	30.3
SOX4	6p22.3	SRY (sex determining region Y)-box 4	6.09	14.8
FZD8	10p11.22	frizzled homolog 8 (Drosophila)	5.99	13.8
TEAD4	12p13.2-p13.3	TEA domain family member 4	5.94	15.2
CDW52	1p36	CDW52 antigen (CAMPATH-1 antigen)	5.42	71.7
SCGB3A2	5q32	secretoglobin, family 3A, member 2	5.36	136.1
PIM1	6p21.2	pim-1 oncogene	4.69	8.1
SMAD7	18q21.1	SMAD, mothers against DPP homolog 7 (Drosophila)	4.69	8.7
DPPA4	3q13.13	developmental pluripotency associated 4	4.52	26.0
T1A-2	1p36	lung type-I cell membrane-associated glycoprotein	4.51	71.3
JUP	17q21	junction plakoglobin	4.36	6.1
ERBB3	12q13	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	4.27	10.4
REPRIMO	2q24.1	candidate mediator of the p53-dependent G2 arrest	4.06	25.4
KRAS2	12p12.1	v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog	4.03	23.0
TIMELESS	12q12-q13	timeless homolog (Drosophila)	3.96	6.0
CCND2	12p13	cyclin D2	3.89	11.3
RARRES3	11q23	retinoic acid receptor responder (tazarotene induced) 3	3.88	12.4
GDF3	12p13.1	growth differentiation factor 3	3.85	955.9
HOXB2	17q21-q22	homeo box B2	3.77	11.5
MYCN	2p24.1	v-myc myelocytomatosis viral related oncogene	3.75	5.8
REA	12p13	repressor of estrogen receptor activity	3.66	7.0
RALA	7p15-p13	v-ral simian leukemia viral oncogene homolog A (ras related)	3.66	5.5
SMAD3	15q21-q22	SMAD, mothers against DPP homolog 3 (Drosophila)	3.61	4.7
C6orf108	6p21.1	chromosome 6 open reading frame 108	3.60	5.0
TRO	Xp11.22-p11.21	trophinin	3.37	6.6
MLLT2	4q21	myeloid/lymphoid or mixed-lineage leukemia	3.26	4.3
ANP32D	12q13.12	acidic (leucine-rich) nuclear phosphoprotein 32 family, member D	3.22	5.7
LYN	8q13	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	3.19	5.3
SALL2	14q11.1-q12	sal-like 2 (Drosophila)	3.15	6.6
ELOVL6	4q25	ELOVL family member 6	3.14	7.8
EPS8L1	19q13.42	EPS8-like 1	3.14	6.5
PROM1	4p15.33	prominin 1	3.12	6.6
RAB39B	Xq28	RAB39B, member RAS oncogene family	3.10	8.6
FRAT2	10q24.1	frequently rearranged in advanced T-cell lymphomas 2	3.10	6.7
ALPPL2;ALPP	2q37	alkaline phosphatase, placental-like 2	2.95	41.6
TES	7q31.2	testis derived transcript (3 LIM domains)	2.94	4.0
MYB	6q22-q23	v-myb myeloblastosis viral oncogene homolog (avian)	2.92	6.2
SALL4	20q13.13-q13.2	sal-like 4 (Drosophila)	2.84	6.9
MKI67	10q25-qter	antigen identified by monoclonal antibody Ki-67	2.81	5.1
RHOG	11p15.5-p15.4	ras homolog gene family, member G (rho G)	2.80	5.1
TBC1D2	9q31.1	TBC1 domain family, member 2	2.67	4.9
TEAD2	19q13.3	TEA domain family member 2	2.64	5.7
PIM2	Xp11.23	pim-2 oncogene	2.64	6.9
CCND3	6p21	cyclin D3	2.61	4.8
RAB25	1q22	RAB25, member RAS oncogene family	2.60	11.2
HCST	19q13.1	hematopoietic cell signal transducer	2.59	6.1
STYK1	12p13.31	protein kinase STYK1	2.56	21.8
PODXL	7q32-q33	podocalyxin-like	2.22	10.9

Figure 1. *Comparison of TGCT and IGCNU with normal testicular tissue by significance analysis of microarrays. Expression data are presented as a heat map showing fold change change (FC) over the mean expression of the normal tissue samples for improved visualization. The genes are ordered by the score (d). Genes highlighted in gray were verified by immunohistochemistry (POU5F1, NANOG, CENPA, SMAD2/3, PODXL) and in situ hybridization (STELLAR, GDF3), or known to be expressed in neoplastic germ cells (T1A-2). N: normal testis; IS: IGCNU of seminomas; IE: IGCNU of embryonal carcinomas; S: seminoma; E: embryonal carcinoma; T: teratocarcinoma.*

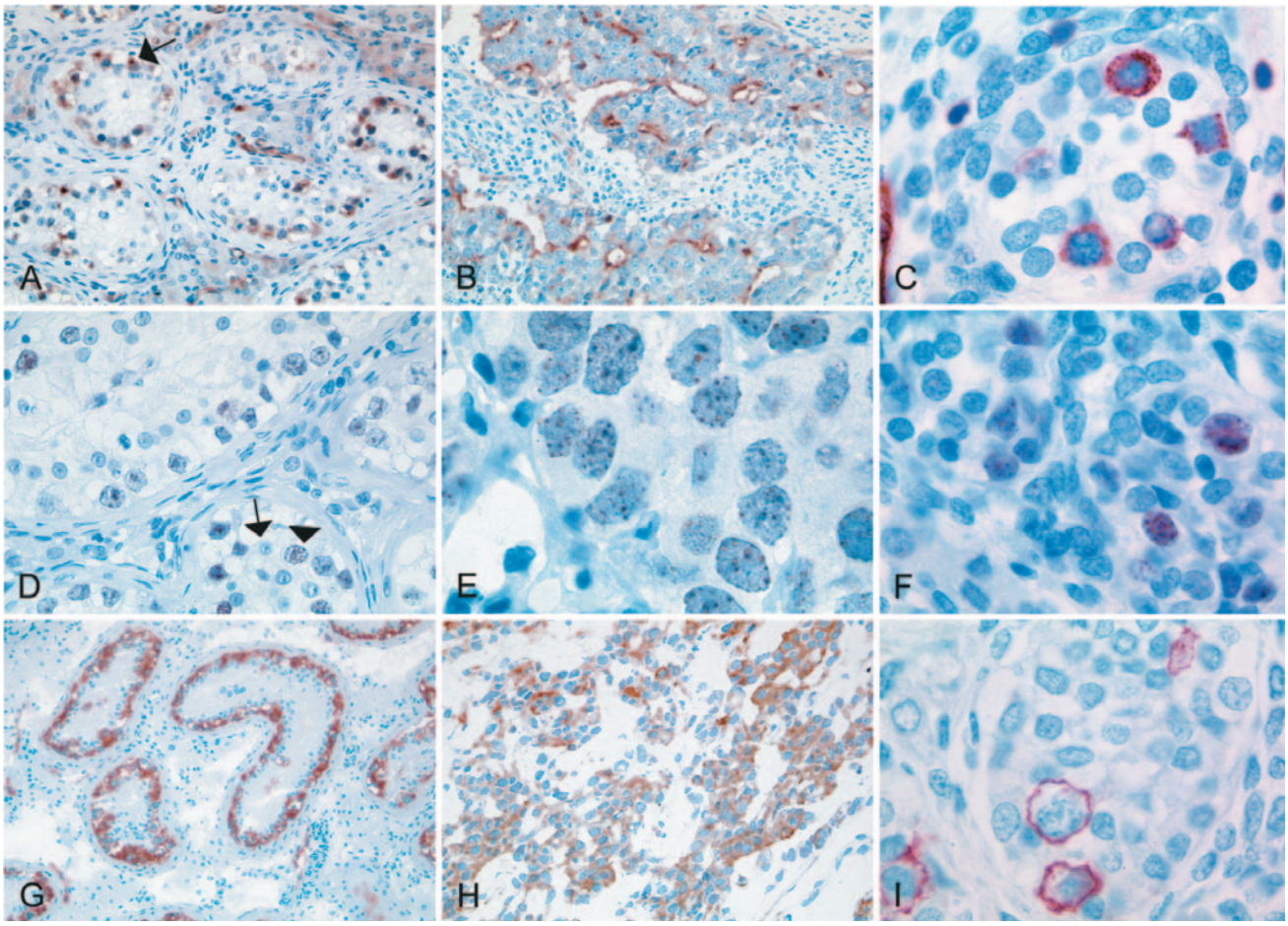


Figure 2. *Cellular expression of podocalyxin, CENPA and CD9 by immunohistochemistry in IGCNU (A, D, G), embryonal carcinomas (B), seminomas (E, H) and fetal testis (C, F, I). Notice a dot-like and membranous expression of podocalyxin in IGCNU (A, arrow) and fetal gonocytes (C), and its epithelial-like expression pattern in embryonal carcinomas (B). CENPA is overexpressed in IGCNU (D, arrowhead) but not in Sertoli cells (D, arrow), and is also expressed in seminomas (E) and gonocytes (F). CD9 is specifically expressed in neoplastic germ cells of IGCNU (G), seminomas (H) and in gonocytes (I).*

carried out on native 5-μm tissue sections of frozen testicular samples of 10 seminomas, 10 embryonal carcinomas, 10 testicular parenchyma samples with IGCNU and 3 normal testes. Details of antibodies and of their dilutions are given in Table I. Immunohistochemistry was performed using the DAKO EnVision-AEC Kit and manufacturer's protocol (DAKO, Hamburg, Germany). Briefly, endogenous peroxidase was blocked for 5 min in 0.03% hydrogen peroxid (diluted in distilled water). Sections were washed in Tris-buffered saline (TBS; 0.05 M tris and 0.85% NaCl, pH 7.6) and incubated with primary antibodies overnight at 4˚C. Thereafter, an HRP-labeled polymer conjugated with a secondary antibody was applied (DAKO EnVision-AEC KIT). Because the expression of CD9, pSMAD2/3 and SOX2 has not been studied in IGCNU and TGCT in detail before, we further examined the expression of these proteins in whole tissue slides of 10 seminomas and 10 mixed non-seminomas and adjacent IGCNU.

Results

Genes up-regulated in IGCNU and TGCT compared with normal testicular parenchyma. The generally accepted non-malignant counterpart of IGCNU is a primordial germ cell/gonocyte. However, because of the lack of sufficient availability and expression profile data, we used normal testicular parenchyma as control. Although with limitations (see Discussion), this analysis is of interest. To prevent over-interpretation of results, we only studied genes that are concordantly up-regulated in IGCNU, seminomas and non-seminomas compared with normal testicular tissues. For this survey, we first used Panther (http://www.pantherdb.org/) and the Gene ontology (GO) consortium database to remove immune response-related genes contributed by the lympho-

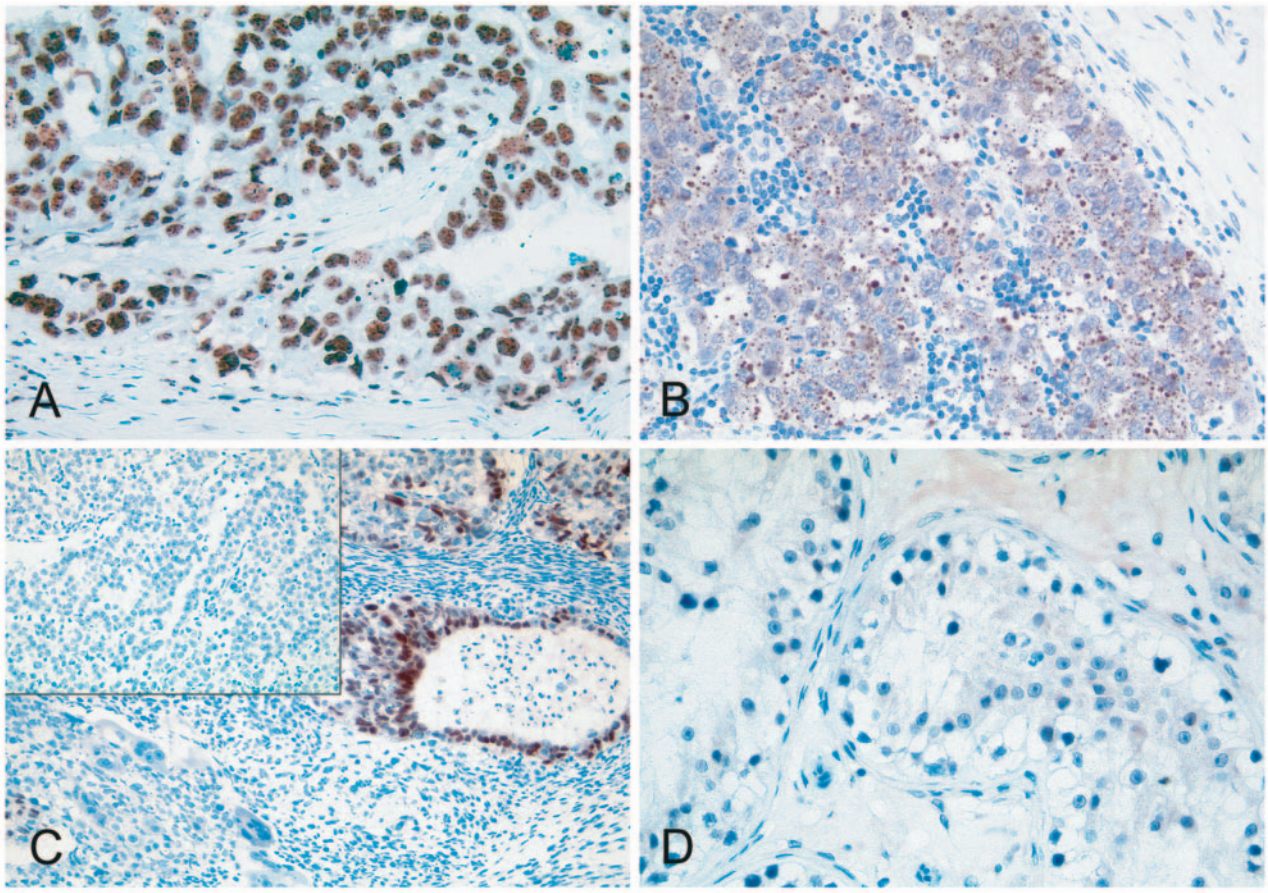


Figure 3. *Representative staining of pSMAD2/3 in embryonal carcinomas (A) in contrast to cytoplasmic staining in seminomas (B). Notice intense nuclear staining in embryonal carcinoma and cytoplasmic staining in seminoma. Representative staining of SOX2 in embryonal carcinoma component in a teratocarcinoma (C, main picture) but not in seminomas (C, inset) or adjacent IGCNU (D). Strong homogeneous CK18 expression in a pure embryonal carcinoma (E) and embryonal carcinoma component TGCT composed of a seminoma and non-seminoma (F).*

plasmacellular infiltrate in seminomas (immunoglobulins and cytokines). Thereafter, significance analysis of microarrays (SAM 2.21, Applied Biosystems, Darmstadt, Germany) was performed, resulting in 2896 up-regulated genes overexpressed in all IGCNU and TGCTs (delta value =1.261; fold change >2; false discovery rate (F)=0.069; data not shown).

After removal of unclassified genes, the 603 most up-regulated genes were analyzed (full data are available on request), of which a selection of 53 genes that were either previously published or validated as part of this study is given in Figure 1 as a heat map showing fold change over the mean expression of normal tissue samples for improved visualization. The genes are ordered by the score (d) derived from the SAM analysis which has been shown to correlate more closely with expression as validated by Northern Blot or immunohistochemical staining than simple fold change alone (17); fold change (FC) values are also indicated. These genes include the known embryonic stem cell (ES) genes (*CD133, PODXL*), as well as previously published genes (*FLZ8, SCGB3A2, MYCN, PIM2*) (10, 14). Highest score d-values were attributed to *POU5F1* (*OCT3/4*) and *NANOG*, as expected based on previous publications (11, 12). This is also true for the 12p13 locus related genes *STELLAR, TEAD4, CCND2, REA, GDF3,* and *STYK1, CD9*, RAS-related genes (*KRAS2, RALA, RAB39B, RAB25, RHEBL1,RHOG, RALA, RIS1, RAC2*) as well as several proto-oncogenes, such as *MYB, LYN*, *MYCN, ERBB3, PIM1, PIM2* which were found to be significantly up-regulated (Figure 1). Out of the identified 603 genes, 169 were previously detected in ES cells or are known to be expressed during early development (10).

In addition, we compared the overall expression of genes involved in differentiation-related pathways including Nodal, WNT, Notch and BMP in embryonal carcinomas and seminomas relative to the expression of normal testes (Table II). Interestingly, genes related to the Nodal

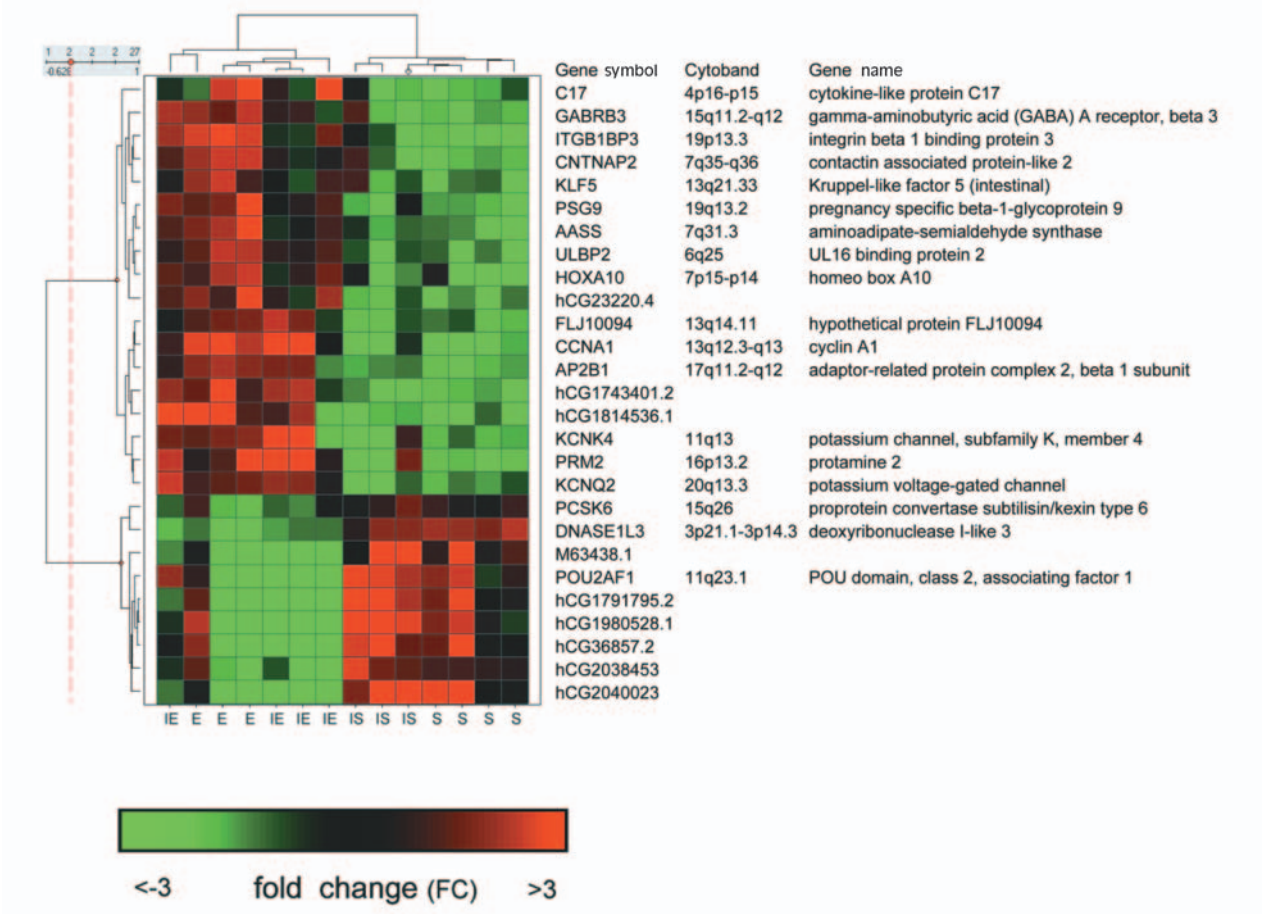


Figure 4. *Hierarchical clustering of genes differentially expressed in seminomas (S) and embryonal carcinomas (E), and their corresponding precursor lesions adjacent to embryonal carcinomas (IE) and seminomas (IS).*

pathway, including NODAL and LEFTB, were found up-regulated in embryonal carcinoma compared to seminoma (Table II). Nodal signaling in human ES cells requires activation of pSMAD2/3, followed by translocation of active protein to the nucleus (18). To investigate whether this up-regulation is associated with activation of SMAD2/3, we examined the expression and cellular localization of phosphorylated SMAD2/3 in seminomas and embryonal carcinomas (see below).

Verification of overexpressed genes in IGCNU and TGCT. A number of novel genes possibly involved in TGCT-development were selected for follow-up studies, including *CD9, CENPA, pSMAD2/3* and *PODXL* (podocalyxin). To confirm data of the applied techniques, *i.e.* immunohisto-chemistry and mRNA *in situ* hybridization, a number of known targets were included (NANOG, OCT3/4, and GDF3, STELLAR), which revealed the expected pattern (data not shown). CENPA, CD9 and podocalyxin have not been studied before in invasive or pre-invasive TGCTs. Podocalyxin was present in IGCNU at the cell membrane and cytoplasm and as a dot-like staining in seminoma (Figure 2A). In embryonal carcinomas its expression pattern was preferentially membranous (Figure 2B) Podocalyxin was expressed in the cytoplasm of fetal gonocytes (Figure 2C) and down-regulated in pre-spermatogonia. CENPA showed a strong nuclear granular presence in IGCNU (Figure 2D), seminomas (Figure 2E) and embryonal carcinomas (not shown). While no expression of CENPA was detected in Sertoli cells or Leydig cells in normal and neoplastic testicular tissues, it was detected at a lower level in spermatogonia in normal adult testes and in fetal gonocytes (Figure 2F). CD9 was detected consistently and homogeneously in IGCNU (Figure 2G), in most of the seminomas (Figure 2H), in fetal gonocytes (Figure 2I) and embryonal carcinoma cells (not shown). No expression of CD9 was detected in adult or fetal spermatogonia (not shown).

Furthermore, different expression patterns in seminomas and embryonal carcinomas was observed for pSMAD2/3, a

well-known regulator of genes closely related to self-renewable capacities in ES cells including NODAL, LEFTY-A and LEFTY-B (18). Phosphorylated SMAD2/3 was present in the cytoplasm of IGCNU and seminoma cells, while embryonal carcinoma cells showed distinct nuclear staining in most samples (Figure 3 A and B).

Comparison of gene expression in IGCNU of seminomas and embryonal carcinomas. To date, it is unknown whether the gene expression patterns of IGCNU reflect the histology of the adjacent invasive TGCTs, whether seminoma or nonseminoma. In particular, the analysis of differentially expressed genes in IGCNU/seminoma and embryonal carcinoma is of relevance in this context. Cytokeratin 18 (CK18) is known to be expressed in embryonal carcinomas but was not detected in IGCNU, neither in embryonal carcinomas nor in seminomas (not shown). The same pattern was detected for transcription factor SOX2, which was expressed in embryonal carcinomas (Figure 3 C) only but not in any seminomas (Figure 3 C inset) or IGCNU, neither of seminomas nor of non-seminomas (Figure 3 D).

To identify additional genes that were differentially expressed in IGCNU adjacent to seminomas and nonseminomas, the IGCNU-derived data were re-investigated by comparison of two groups. The first group included 4 IGCNU adjacent to embryonal carcinomas and the second group included 3 IGCNU adjacent to seminoma. In total, 15,399 out of 32,878 transcripts (46.8%) showed the same directional fold change in IGCNU and the corresponding histological TGCT type. Genes which were up-regulated in seminoma (S) and adjacent IGCNU (IS) compared to embryonal carcinoma (E) and adjacent IGCNU (IE), and vice versa were extracted by using the following filter: mean (S)–mean(E)/mean (IS)–mean (IE)>0. Moreover, SAM was performed to select genes that were differentially expressed in seminomas and embryonal carcinomas (delta=0.559; FC>2; FDR<0.1); there were 181. A subset of these genes was identified using SAM (delta=0.278 and a FDR<0.45). Because the IGCNU samples used were significantly intermingled with stromal cells (including Sertoli and Leydig cells), a relatively non-stringent parameter set was applied. A hierarchical cluster of the resulting list is shown in Figure 4. A cluster of 18 genes was significantly up-regulated in embryonal carcinomas and their IGCNU compared to seminomas and their precursors. These included the cell cycle regulator CCNA1, the transcription factor HOXA10 and a member of the carcinoembryonic antigen family PSG9. Nine transcripts were differentially up-regulated in seminomas and their IGCNU including a large number of unknown transcripts.

Discussion

By analysis of the whole genome gene expression in precursor lesions and TGCT, we aimed to identify genes that are up-regulated at a high level in IGCNU and invasive germ cell tumors and thus are likely to be involved in the formation of neoplastic germ cells. We found that a large proportion of genes up-regulated in IGCNU are known to be core markers of pluripotent ES cells (*TDGF1, POU5F1, NANOG, CD133, PODXL, CD9*) (10). This result corroborates the close relationship between IGCNU and embryonic stem/progenitor cells which was proposed by recent studies using few IGCNU samples and is in agreement with the general concept of IGCNU formation through deregulated development of stem/progenitor cells (6, 14). Aberrant RAS expression and up-regulated expression of genes from the 12p13 locus are thought to be causative for initiation or progression of TGCT (19). Here, we have shown that 12p13.31 genes and the RAS gene family including *KRAS2, RALA, RAB39B, RAB25* are up-regulated both in neoplastic precursor cells, IGCNU and in invasive TGCT. This finding strongly supports the functional relevance of the 12p13.31 locus and oncogenic RAS for the establishment of IGCNU.

Expression of one of the best characterized ES cell markers, tetraspanin receptor CD9 (20), was, to our knowledge, not studied previously in testes. Here, we have shown that CD9 is selectively expressed in neoplastic germ cells, including IGCNU. The protein encoded by the *CD9* gene is a member of the transmembrane 4 superfamily. These proteins mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility (20). Recent results indicate that CD9 is regulated during embryo implantation (21) and is involved in the maintenance of undifferentiated ES cells (22). Thus, constant expression of CD9 in IGCNU indicates its potential role for the expression of stem cell genes and maintenance of the undifferentiated state in IGCNU.

Another important gene that might be involved in the malignant transformation of neoplastic germ cells is that for the centromere protein CENPA. CENPA, also known as centromere-specific histone-H3-like variant, is crucial for normal centromere function and structure, and thus is a central element in the epigenetic maintenance of centromere identity (23). Its up-regulation is believed to stimulate chromosomal aneuploidy in colorectal adenocarcinomas (24). A consistent presence of CENPA protein was identified in IGCNU and invasive TGCTs. This finding suggests a functional relevance of CENPA for hypertriploidy or tetraploidy formation, which is a well-known phenomenon in TGCTs, including its pre-invasive stage (25).

The third novel gene which was explored immunohisto-chemically in the present study is podocalyxin, a heavily sialated and sulfated member of the CD34 family of integral

membrane proteins. Podocalyxin, originally identified as an important component of glomerular podocytes, functions as an antiadhesin in non-malignant cells and was also found in embryonal stem cells, hematopoietic progenitors and vascular endothelia (26). It has been proposed that podocalyxin expression is an independent predictor of breast cancer progression through advanced anti-adhesive properties of tumor cells (27). Podocalyxin was present in gonocytes, IGCNU and invasive TGCT, although in different patterns. IGCNU and seminoma showed membranous and dot-like staining, while it was strongly accentuated at the cell membrane in epithelial-like structures of embryonal carcinomas. Whether this membranous expression has functional implications for anti-adhesive features of embryonal carcinomas remains to be elucidated. However, podocalyxin as well as CD9 and CENPA were expressed in fetal gonocytes but not in differentiated fetal germ cells, the prespermatogonia. This finding further corroborates the close relationship between gonocytes and IGCNU and is in line with the currently preferred idea of gonocytes as cells of origin for IGCNU (6).

Reprogramming of a IGCNU/seminoma cell to an embryonal carcinoma cell goes along with a significant change of the gene expression pattern. This includes expression of DNA methyltransferases (DNMTs) and core embryonic stem cell genes in embryonal carcinomas (11, 25). Here we found that genes responsible for the maintenance of pluripotency in human ES cells, including those involved in the Nodal/Activin pathway and the core regulator of pluripotency SOX2, were differentially up-regulated in embryonal carcinomas but not expressed in seminomas or IGCNU. Functionally relevant activation of the Nodal pathway was substantiated by detection of nuclear pSMAD2/3 in embryonal carcinomas, as translocation of pSMAD2/3 into the nucleus occurs upon activation of Nodal signaling (18). In contrast, staining of phosphorylated SMAD2/3 was located mostly in the cytoplasm in seminomas and IGCNU (Figure 3) and correlated with low expression of *NODAL* and *LEFTY*. Thus, our data strongly favor a functional involvement of the Nodal pathway and SOX2 in the establishment of embryonal carcinoma phenotype.

Currently is it not possible to predict whether a specific IGCNU will develop into a seminoma or an embryonal carcinoma. Representative genes of embryonal carcinomas including *SOX2* and *CK18* were not expressed in IGCNU samples. However, it is likely that IGCNU display differences in their expression profile which is related to the adjacent TGCT phenotype. By the examination and analysis of IGCNU of embryonal carcinomas compared to those of seminomas, we here identified a gene cluster of 27 differentially expressed genes (Figure 4). Several well-known genes including cyclin A1 (*CCNA1*), pregnancy-specific glycoprotein 9 (*PSG9*) and transcription factor *HOXA10* were found to be differentially expressed in IGCNU of embryonal carcinoma patients. *PSG9* is a member of the carcinoembryonic antigen (CEA)/PSG family and is expressed at high levels during pregnancy, mainly by syncytiotrophoblasts. Cyclin A1 is involved in the G1/S phase progression in somatic cells and forms a complex with CDC2, whose activity peaks during the G2/M transition and whose up-regulation has an oncogenic effect (28). According to previous studies, cyclin A1 is expressed in embryonal carcinomas and teratocarcinomas but not in seminomas (29). Thus, our finding of overexpression of *CCNA1* at the stage of IGCNU strongly suggests that deregulation of *CCNA1* might be an early molecular event associated with the development of embryonal carcinomas.

Conclusion

The present study represents the largest analysis of whole gene expression profiling of IGCNU to date and broadens our knowledge on the pathogenesis of this disease. The finding that the 12p13 locus and RAS family are up-regulated at the precursor stage of TGCT suggests that these genes are involved in IGCNU formation. Three novel functionally important genes including *CENPA*, *CD9* and podocalyxin were shown to be up-regulated in neoplastic germ cells. The close relationship of the core pluripotency gene *SOX2* and the Nodal pathway to the embryonal carcinoma phenotype gives rise to the question of how these genes contribute to the complex regulatory network responsible for the pluripotency in embryonal carcinomas.

Acknowledgements

The technical assistance of W. Jeske, U. Rommerscheidt, B. Reddeman, S. Steiner, C. Esch and Gerrit Klemm is gratefully acknowledged. This study was supported from a BONFOR research grant to K.B. and L.C.H.

References

1 McKiernan JM, Goluboff ET, Liberson GL, Golden R and Fisch H: Rising risk of testicular cancer by birth cohort in the United States from 1973 to 1995. J Urol *162*: 361-363, 1999.

2 Adami HO, Bergstrom R, Mohner M, Zatonski W, Storm H, Ekbom A, Tretli S, Teppo L, Ziegler H, Rahu M *et al*: Testicular cancer in nine northern European countries. Int J Cancer *59*: 33-38, 1994.

3 Skakkebaek NE, Holm M, Hoei-Hansen C, Jorgensen N and Rajpert-De Meyts E: Association between testicular dysgenesis syndrome (TDS) and testicular neoplasia: evidence from 20 adult patients with signs of maldevelopment of the testis. APMIS *111*: 1-9, 2003.

4 Skakkebaek NE: Possible carcinoma *in situ* of the testis. Lancet *2*: 516-517, 1972.
5 Skakkebaek NE: Carcinoma *in situ* of the testis: frequency and relationship to invasive germ cell tumours in infertile men. Histopathology *2*: 157-170, 1978.
6 Oosterhuis JW and Looijenga LH: Testicular germ-cell tumours in a broader perspective. Nat Rev Cancer *5*: 210-222, 2005.
7 Pauls K, Schorle H, Jeske W, Brehm R, Steger K, Wernert N, Buttner R and Zhou H: Spatial expression of germ cell markers during maturation of human fetal male gonads: an immunohistochemical study. Hum Reproduction *21*: 397-404, 2006.
8 Pauls K, Jager R, Weber S, Wardelmann E, Koch A, Buttner R and Schorle H: Transcription factor AP-2gamma, a novel marker of gonocytes and seminomatous germ cell tumors. Int J Cancer *115*: 470-477, 2005.
9 Looijenga LH and Oosterhuis JW: Pathogenesis of testicular germ cell tumours. Rev Reprod *4*: 90-100, 1999.
10 Sperger JM, Chen X, Draper JS, Antosiewicz JE, Chon CH, Jones SB, Brooks JD, Andrews PW, Brown PO and Thomson JA: Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. Proc Nat Acad Sci USA *100*: 13350-13355, 2003.
11 Korkola JE, Houldsworth J, Chadalavada RS, Olshen AB, Dobrzynski D, Reuter VE, Bosl GJ and Chaganti RS: Down-regulation of stem cell genes, including those in a 200-kb gene cluster at 12p13.31, is associated with *in vivo* differentiation of human male germ cell tumors. Cancer Res *66*: 820-827, 2006.
12 Korkola JE, Houldsworth J, Dobrzynski D, Olshen AB, Reuter VE, Bosl GJ and Chaganti RS: Gene expression-based classification of nonseminomatous male germ cell tumors. Oncogene *24*: 5101-5107, 2005.
13 Skotheim RI, Lind GE, Monni O, Nesland JM, Abeler VM, Fossa SD, Duale N, Brunborg G, Kallioniemi O, Andrews PW and Lothe RA: Differentiation of human embryonal carcinomas *in vitro* and *in vivo* reveals expression profiles relevant to normal development. Cancer Res *65*: 5588-5598, 2005.
14 Almstrup K, Hoei-Hansen CE, Wirkner U, Blake J, Schwager C, Ansorge W, Nielsen JE, Skakkebaek NE, Rajpert-De Meyts E and Leffers H: Embryonic stem cell-like features of testicular carcinoma *in situ* revealed by genome-wide gene expression profiling. Cancer Res *64*: 4736-4743, 2004.
15 Mostofi FK and Sesterhenn IA: Histological typing of testis tumours. Berlin: Springer, p. 132, 1998.
16 Walker SJ, Wang Y, Grant KA, Chan F and Hellmann GM: Long *versus* short oligonucleotide microarrays for the study of gene expression in nonhuman primates. J Neurosci Methods *152*: 179-189, 2005.
17 Tusher VG, Tibshirani R and Chu G: Significance analysis of microarrays applied to the ionizing radiation response. Proc Nat Acad Sci USA *98*: 5116-5121, 2001.
18 Besser D: Expression of nodal, lefty-a, and lefty-B in undifferentiated human embryonic stem cells requires activation of Smad2/3. J Biol Chem *279*: 45076-45084, 2004.
19 Zafarana G, Grygalewicz B, Gillis AJ, Vissers LE, van de Vliet W, van Gurp RJ, Stoop H, Debiec-Rychter M, Oosterhuis JW, van Kessel AG, Schoenmakers EF and Looijenga LH: 12p-Amplicon structure analysis in testicular germ cell tumors of adolescents and adults by array CGH. Oncogene *22*: 7695-7701, 2003.
20 Maecker HT, Todd SC and Levy S: The tetraspanin superfamily: molecular facilitators. FASEB J *11*: 428-442, 1997.
21 Liu WM, Cao YJ, Yang YJ, Li J, Hu Z and Duan EK: Tetraspanin CD9 regulates invasion during mouse embryo implantation. J Mol Endocrinol *36*: 121-130, 2006.
22 Oka M, Tagoku K, Russell TL, Nakano Y, Hamazaki T, Meyer EM, Yokota T and Terada N: CD9 is associated with leukemia inhibitory factor-mediated maintenance of embryonic stem cells. Mol Biol Cell *13*: 1274-1281, 2002.
23 Sullivan KF: A solid foundation: functional specialization of centromeric chromatin. Current Opi Gen Dev *11*: 182-188, 2001.
24 el-Naggar AK, Ro JY, McLemore D, Ayala AG and Batsakis JG: DNA ploidy in testicular germ cell neoplasms. Histogenetic and clinical implications. Am J Surg Pathol *16*: 611-618, 1992.
25 Almstrup K, Ottesen AM, Sonne SB, Hoei-Hansen CE, Leffers H, Rajpert-De Meyts E and Skakkebaek NE: Genomic and gene expression signature of the pre-invasive testicular carcinoma *in situ*. Cell Tissue Res *322*: 159-165, 2005.
26 Takeda T, Go WY, Orlando RA and Farquhar MG: Expression of podocalyxin inhibits cell-cell adhesion and modifies junctional properties in Madin-Darby canine kidney cells. Mol Biol Cell *11*: 3219-3232, 2000.
27 Somasiri A, Nielsen JS, Makretsov N, McCoy ML, Prentice L, Gilks CB, Chia SK, Gelmon KA, Kershaw DB, Huntsman DG, McNagny KM and Roskelley CD: Overexpression of the anti-adhesin podocalyxin is an independent predictor of breast cancer progression. Cancer Res *64*: 5068-5073, 2004.
28 Ji P, Agrawal S, Diederichs S, Baumer N, Becker A, Cauvet T, Kowski S, Beger C, Welte K, Berdel WE, Serve H and Muller-Tidow C: Cyclin A1, the alternative A-type cyclin, contributes to G1/S cell cycle progression in somatic cells. Oncogene *24*: 2739-2744, 2005.
29 Muller-Tidow C, Diederichs S, Schrader MG, Vogt U, Miller K, Berdel WE and Serve H: Cyclin A1 is highly expressed in aggressive testicular germ cell tumors. Cancer letters *190*: 89-95, 2003.

Received March 16, 2007
Revised June 22, 2007
Accepted July 24, 2007