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Novel germ cell markers characterize testicular seminoma and fetal testis

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Seminomas are characterized by expression of several stem cell markers, supporting their origin from germ cells. The current study focuses on novel germ cell markers in normal testes compared to those in fetal testes and different progression stages of seminomas. Microarray data were followed by RT–PCRs and immunohistochemistry on pure seminomas (pT1 to pT3) compared to adult and fetal testis. An upregulation of known germ cell markers, KIT, OCT4 and NANOG, was confirmed in seminoma specimens. We also identified novel germ cell markers such as BOB1 (POU2AF1, OBF1) and prominin 1 (PROM1, CD133), which were significantly upregulated in seminoma specimens, compared to normal testes. Furthermore, two Sertoli cell markers, SCGF (SCF) and the newly identified neuronal stem cell factor, MCFD2 (SDNSF), were expressed in seminoma cells. While BOB1 was expressed in fetal testis of second and third trimester of gestation, MCFD2 and PROM1 were only present in gonocytes up to the second trimester. All marker genes investigated were not further regulated in progressing tumour stages between pT1 and pT3. In conclusion, the germ cell markers described here provide evidence for the origin of seminoma cells, which could be from the developmental stage of early gonocytes or from spermatogonia re-expressing markers of the developing germ cells.

Keywords: germ cell markers; pure seminoma; fetal testis; gene arrays; seminoma tumorigenesis

Introduction

The incidence of testicular germ cell tumours has doubled in the past 40 years (Horwich *et al.*, 2006). This increase suggests that critical changes in environmental factors are contributing to the development of these tumours (Horwich *et al.*, 2006). Extensive karyotypic and genomic studies on seminoma lead to two discrete hypotheses for the onset of tumour formation as reviewed recently (Houldsworth *et al.*, 2006). One model suggests aberrant recombination in a spermatocyte (4n) resulting in 12p-gain and formation of seminoma (Chaganti and Houldsworth, 1998). The second, currently widely accepted model for the origin of testicular germ cell tumours proposes a very early block in maturation during fetal development (Skakkebaek *et al.*, 1987; Rajpert-De Meyts, *et al.* 1998; Honecker *et al.*, 2004a,b; Horwich *et al.*, 2006). It has been thought that primary germ cells may develop into an intratubular germ cell neoplasia (ITGCN, carcinoma *in situ*) and remain at this stage until adulthood. ITGCN cells show similarities to embryonic germ cells, such as their positiveness for placental-like alkaline phosphatase (Jacobsen and Norgaard-Pedersen, 1984), the stem cell factor receptor KIT, OCT4 (Honecker *et al.*, 2004a,b) and their glycogen content (Dieckmann and Skakkebaek, 1999). However, the underlying molecular mechanisms as well as the time point of germ cell transformation into ITGCN or fully developed seminoma are poorly understood.

Recently, several microarray studies have been performed to identify new diagnostic markers (Okada *et al.*, 2003; Yamada *et al.*, 2004) and to investigate the origin, (Sperger *et al.*, 2003; Almstrup *et al.*, 2004; Korkola *et al.*, 2006) as well as genes characterizing progression, of seminoma (Gashaw *et al.*, 2005). These investigations have led to new insights into the pathogenesis of this tumour entity by describing their stem cell-like character (Sperger *et al.*, 2003; Almstrup *et al.*, 2004). Applying supervised analyses, we have described additional germ cell marker genes for seminoma such as MCFD2, BOB1 and PROM1, by focusing on their expression pattern.

These germ cell markers may be important for both tumorigenesis and the maintenance of the tumour. In this study, we specifically examined those transcripts known to be important for early differentiation processes and which play a role during migration, self-renewal, pluripotency or survival of cells. We aimed to identify the initial time point of transformation to seminoma by investigating the temporal expression patterns of these factors during testes development as well as in different tumour stages.

Materials and methods

Tissue samples

As already described (Gashaw *et al.*, 2005), we performed microarray analyses of 43 testicular tissue samples out of 72 specimens, including nine normal testes and 63 tumour specimens. The distribution of the different stages of pure seminoma was: stage pT1, $n = 40$; pT2, $n = 19$; and pT3, $n = 4$. The clinicopathological data of the patients have been described in the reference Gashaw *et al.* (2005).

Here, an additional 12 fetal testes were examined including eight testes from second trimester (14th, 16th, 21st, 22nd, 23rd and 26th weeks of pregnancy) as well as four testes from third trimester (30th, 32nd and 37th weeks of pregnancy). All fetal tissues were obtained for diagnostic or documentary reasons because of fetal or stillborn deaths (amnion infection or premature rupture, congenital heart failure). One prepubertal testicular tissue biopsy was obtained from a five year-old boy because of 5α-reductase deficiency.

All specimens were collected in accordance with the guidelines approved by the local ethics committees.

mRNA expression analyses

RNA preparation, cDNA synthesis and microarray analyses have been described previously (Gashaw *et al.*, 2005). Briefly, all biopsies of the tumour specimens were carefully investigated before further processing by staining one frozen section with toluidin blue and only those containing pure seminoma tissues, as classified by a pathologist, were used for RNA extraction. The RNA was extracted as described and only samples of high quality RNA were further processed and finally hybridized to a HG-U95Av2 chip (Gashaw *et al.*, 2005). Global scaling was applied to allow comparison of gene signals across multiple microarrays. Annotations of the probe sets were taken from material provided on the Affymetrix homepage (Liu *et al.*, 2003).

Semi-quantitative competitive RT–PCRs were performed in triplicates for chosen markers, such as KIT, SCGF, OCT4, NANOG, BOB1, MCFD2 and PROM1, on samples from the above cohort of patients including a total of 23 specimens analysed previously by microarrays (2 × NT, 11 × pT1, 6 × pT2 and 4 × pT3) and 11 additional tissues (4 × NT, 6 × pT1 and 1 × pT2) for which the RNA was either of poor quality or the quantity was not sufficient for microarray analysis. Primer sequences used in the PCRs are available in Table 1. Since most of the frequently used housekeeping genes were differentially expressed in seminoma (Neuvians *et al.*, 2005), the constantly expressed UBB (Ubiquitin B) mRNA was co-amplified as an internal standard in each experiment. In the densitometric analyses (Scion Image software, Scion Corporation, Frederick, MD, USA), the signal intensity of the target gene was related to the signal intensity of the UBB amplicon.

Immunohistochemical analyses

Formalin-fixed, paraffin-embedded sections from seven patients with seminoma (pT1) were randomly selected from the group described above. In addition, 12 prenatal, one prepubertal and three adult testicular biopsies were used for immunohistochemical analyses.

Immunostaining procedures were performed for SCGF, MCFD2, BOB1 and PROM1 using an adequate Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. All sections were incubated over night at 4°C with the primary antibody. Goat anti-human MCFD2 antibody (R&D Systems, Wiesbaden, Germany) and rabbit anti-human BOB1 (sc-955, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were specific for the respective protein (Marafioti *et al.*, 2003; Nyfeler *et al.*, 2006) and were used at 10 μg/ml. For SCGF and PROM1 localization, the sections were subjected to antigen retrieval in 0.01 M sodium citrate (pH 6.0) followed by incubation with polyclonal goat anti-SCGF antibody (1:50; sc-1302, Santa Cruz Biotechnology Inc.) or monoclonal murine anti-PROM1 antibody (25 μg/ml, ABGENT, San Diego, CA, USA), respectively. Control sections were stained according to the same protocol with buffer instead of the primary antibody. Colour visualization was performed using DAB (DakoCytomation, Hamburg, Germany) as chromogen substrate. Sections were counterstained with haematoxylin, and examined with an Axiophot microscope (Carl Zeiss, Jena, Germany) equipped with a Nikon DS-U1 camera and LUCIA Image Analysis software (Version 5.0, Nikon, Düsseldorf, Germany).

Statistical analysis

Statistical analyses performed in course of microarray studies were precisely described previously (Gashaw *et al.*, 2005). Exploratory data analysis, the nonparametric Kruskal–Wallis test as well as the Mann–Whitney test for the nonparametric independent two-group comparisons and Pearson's correlation were performed with the program SPSS 14 for Windows (SPSS Inc, Chicago, IL, USA). Differences with $P \leq 0.05$ were regarded as statistically significant.

Results

Germ cell markers are regulated in seminoma

The microarray data, which have been deposited in the National Centre for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/; accession no: GSE8607), revealed 1490 differentially regulated genes in 40 seminoma of different stages compared to three normal testicular specimens (Gashaw *et al.*, 2005). According to the aim of the study, annotations of all probe sets were scanned for the term: 'stem cells'. To set a cutoff for regulated stem cell genes, only transcripts expressed in at least 30 out of 43 tissue samples (70%) were further investigated. At least 14 transcripts fulfilled the criteria described above (Table 2) and 10 of those transcripts were significantly regulated in seminoma. Of these, eight genes were significantly upregulated: OCT4, ALPL, BOB1, KIT, CD9, THY1, PUM2 and PROM1, with more than four-fold increase in expression when compared to normal testis (for further characterization of the genes, see Table 2). Transcripts of MCFD2 and PIWIL1 were downregulated (Table 2). The probes of the other well-known stem cells markers such as NANOG, STELLAR and GDF3 were absent from the HG-U95Av2 chip. Five of the regulated transcripts (OCT4, ALPL, KIT, PROM1 and PIWIL1) were already described for their presence in seminoma. In addition, two novel interesting genes, BOB1 and MCFD2, have been identified in this study. We further focused on those genes, which were significantly differentially regulated between normal testis and seminoma and verified the microarray data by applying semi-quantitative RT–PCR and immunohistochemistry.

Table 1: Oligonucleotide primers used for the semi-quantitative competitive RT–PCR

Gene (GenBank No.)	Forward primers (position)	Reverse primers (position)	PCR (annealing) conditions
UBB (NM_018955)	GGCTTTGTTGGGTGAGCTTG (53–72)	CTGGGCTCCACCTCCAGAGT (653–634)	–
KIT (NM_000222)	TCATGGTCGGATCACAAAGA (2424–2443)	AGGGGCTGCTTCCTAAAGAG (2629–2610)	60°C, 30 cycles
SCGF (NM_002975)	GAATCTCCCTTCCCTTCCTG (1223–1242)	CTGCCCCTCAAGTCAGATTC (1403–1384)	60°C, 30 cycles
OCT4 (NM_002701)	GTACTCCTCGGTCCCTTTCC (1055–1074)	CAAAAACCCTGGCACAAACT (1222–1203)	60°C, 26 cycles
NANOG (AB093576)	GATTTGTGGGCCTGAAGAAA (297–316)	AAGTGGGTTGTTTGCCTTTG (451–432)	60°C, 30 cycles
BOB1 (NM_006235)	CCATGGGCTTTCATTTCTGT (1771–1790)	CCTTGGCTGACTTTCTCAGG (1938–1919)	60°C, 28 cycles
PROM1 (NM_006017)	CACTCTTCACCTGCAGAACAG (1750–1771)	GGGCTTGTCATAACAGGATTG (2622–2602)	60°C, 26 cycles
MCFD2 (NM_139279)	CCCTGTGGCTTTGTAGGGTA (1528–1547)	GCAGAAGGGAAATGTGGTGT (1737–1718)	60°C, 28 cycles

We confirmed the expression of KIT, OCT4 and NANOG mRNA in seminoma specimens (Fig. 1) as already described by Honecker *et al.* (2004a,b) and Ezeh *et al.* (2005). However, our data showed that these stem cell markers (KIT, OCT4, NANOG), although significantly upregulated in all tumour tissues compared to normal tissue, revealed no obvious regulation associated with the progressing tumour stages from pT1 to pT3 (Table 2, Fig. 1).

In contrast to other investigators (Strohmeyer *et al.*, 1995; Bokemeyer *et al.*, 1996), we identified the KIT ligand SCGF in seminoma specimens at the mRNA level (Table 2, Fig. 1B). The transcripts were expressed in all tumours without a significant regulation compared to normal testes (Fig. 1B). SCGF immunoreactivity was localized in the cytoplasm of intratubular tumour cells as well as in nearly all seminoma cells (Fig. 2A and B).

MCFD2 is present in Sertoli cells and in seminoma cells

MCFD2 was significantly downregulated in seminoma specimens in the microarray analysis (Table 2). However interestingly, the corresponding RT–PCR analysis from 16 samples (10 analysed already by microarrays and six additional; 2 × NT, 3 × pT1, 1 × pT2) showed a constant transcript expression in all tumour specimens compared to normal testes (Fig. 1F). When investigating testis with regular spermatogenesis, we observed the presence of immunoreactive MCFD2 protein in the cytoplasm of Sertoli cells (Fig. 2C) but all spermatogonia were negative for MCFD2 (Fig. 2C). Moreover, we localized the MCFD2 protein to intratubular tumour cells and remaining Sertoli cells (Fig. 2D). As presented in Fig. 2D, not all intratubular tumour cells were positive for MCFD2 but nearly all seminoma cells were. Thus, the immunostaining procedures confirmed the results of the semi-quantitative RT–PCR, validating constantly expressed MCFD2 in seminoma.

By investigating fetal testis, the MCFD2 protein was identified in gonocytes and Sertoli cells from the second trimester of gestation (Fig. 2E), whereas in testes of the third trimester, the MCFD2 protein staining had disappeared as presented in Fig. 2F. All investigations confirmed that MCFD2 is expressed by gonocytes and Sertoli cells in second trimester, but is restricted later to Sertoli cells of adult testis. Thus, MCFD2 is maintained in some cells of ITGCN and in all seminoma cells.

BOB1 is expressed in testicular germ cells and highly upregulated in seminoma

The microarray data revealed an average 22-fold increased expression of BOB1 in all seminoma specimens compared to normal testes (Table 2). This could be confirmed by RT–PCR. Among the seminoma investigated, the transcripts of BOB1 mRNA revealed high inter-individual differences in expression levels (Fig. 1E) with a mean 30-fold upregulation in tumours when compared to controls (range: 2.3–85.3-fold). Similar to the expression pattern of the above described stem cell markers KIT, OCT4 and NANOG, expression levels of BOB1 mRNA remained steadily high in the different tumour stages investigated.

Figure 3 illustrates the localization of BOB1 protein in testicular tissues. Testis with regular spermatogenesis showed strong positive

Table 2: Expression data (mRNA) for germ cell markers present on the array in at least 70% of 43 tissue samples including different tumour stages[a]

Gene	Probe set ID	NCBI ID	Gene description	P-call (%)	Factor I	*P**	NT		pT1		pT2		pT3	
							Mean	SD	Mean	SD	Mean	SD	Mean	SD
OCT4	39626_s_at	NM_002701	POU domain, class 5, transcription factor 1	98	143.5	0.000	160	19	19847	5474	22368	4994	26729	4890
ALPL	36623_at	AB011406	Alkaline phosphatase, liver/bone/kidney	93	79.0	0.000	85	39	6373	3768	6434	2557	8719	5525
BOB1	36239_at	NM_006235	POU domain, class 2, associating factor 1	93	21.5	0.000	58	31	1967	1704	1204	469	577	350
CD9	39389_at	NM_001769	CD9 molecule	100	7.8	0.005	1227	223	9587	3591	9489	3850	10177	8991
KIT	1888_s_at	NM_000222	Human homologue of the protooncogene c-kit	74	7.0	0.000	232	37	2173	4005	1451	1271	1240	1276
THY1	39395_at	AA704137	Thy-1 cell surface antigen	81	5.5	0.000	252	221	1269	484	1262	751	2473	932
PROM1	41470_at	NM_006017	Prominin 1	100	4.3	0.001	1253	315	5768	2477	5792	2478	4444	4340
SCGF	37147_at	NM_002975	C-type lectin domain family 11, member A, stem cell growth factor	100	1.6	0.000	600	53	763	266	787	212	1300	764
PUM2	35359_at	NM_015317	Pumilio homologue 2 (Drosophila)	100	1.4	0.184	2230	102	3632	585	3032	433	3009	393
PUM1	40048_at	NM_014676	Pumilio homologue 1 (Drosophila)	100	1.3	0.218	1735	358	2152	582	2594	884	1970	980
NGFR	1673_at	M14764	Nerve growth factor receptor (TNFR superfamily, member 16)	77	1.1	1.000	376	63	399	144	383	184	549	260
LIF	38441_s_at	NM_002389	Membrane cofactor protein (CD46, trophoblast-lymphocyte cross-reactive antigen)	100	0.9	0.736	2156	222	2498	980	2050	835	1270	398
MCFD2	38727_at	NM_139279	Multiple coagulation factor deficiency 2	98	0.5	0.000	1156	211	636	163	631	236	399	120
PIWIL1	35901_at	NM_004764	piwi-like 1 (Drosophila)	77	0.2	0.000	2425	595	321	310	392	298	529	335

[a]The P-call is an indicator of the presence of a positive hybridization signal for a specific gene on an array and describes the number of positive samples (as a percentage). Factor 'I' denotes the intensity of the upregulation resulting from an increase in signal intensity on the array.
*The *P*-value was estimated applying the Mann–Whitney test.

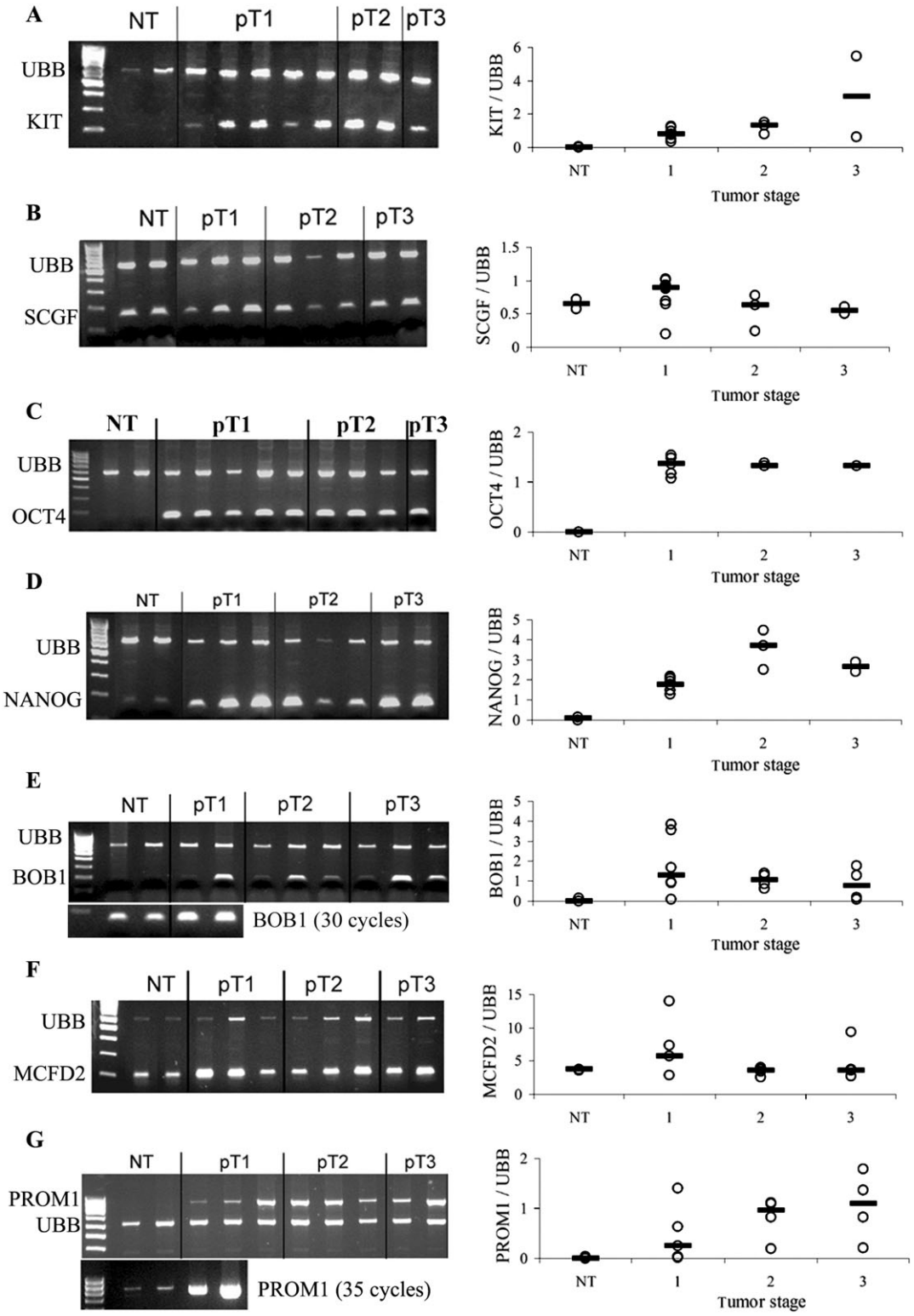

Figure 1: Gene expression data for stem and progenitor cell markers KIT (**A**), SCGF (**B**), OCT4 (**C**), NANOG (**D**), BOB1 (**E**), MCFD2 (**F**) and PROM1 (**G**) as revealed by semi-quantitative competitive RT–PCR

Representative RT–PCRs from normal and seminoma tissues as well as results from densitometric analyses of three independent reactions for all individuals are illustrated for each transcript. Circles represent the ratio of signal intensity of the target gene to the signal intensity for the UBB mRNA in each testicular sample examined. Horizontal bars represent the median of each group

nuclear staining for BOB1 protein within the germ cell epithelial layer from spermatogonia up to spermatids (Fig. 3A). The nuclei of most spermatogonia and of all Sertoli cells were immunoreactive to BOB1 (Fig. 3A). Cells of ITGCN and of the tumour revealed very strong, predominantly nuclear staining for BOB1 as illustrated in Fig. 3B and C, respectively. Infiltrating leukocytes were negative for BOB1 (Fig. 3C). All controls revealed no unspecific binding as presented in Fig. 3D. The gonocytes of second and third trimester of

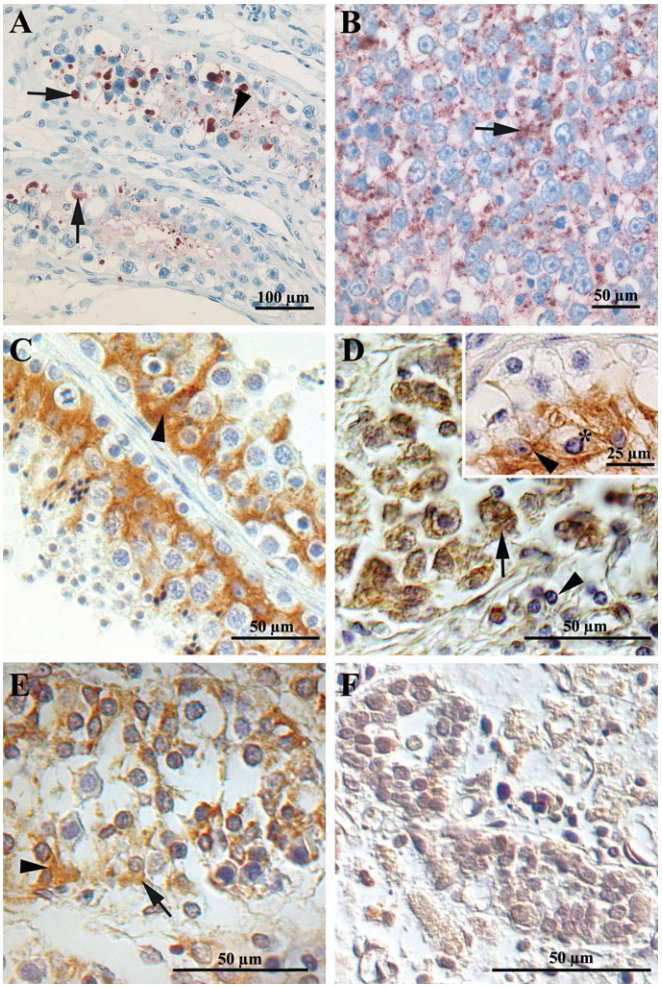

Figure 2: Localization of SCGF and MCFD2 proteins in testicular tissues (**A**) Representative staining showing cytoplasmic localization of SCGF protein in intratubular tumour cells (arrows). Note the weak positive staining of Sertoli cells (arrowhead). (**B**) Immunoreactive SCGF was present in the cytoplasmic region of seminoma cells (arrow). (**C**) Immunohistochemical localization of MCFD2 protein to Sertoli cells (arrowhead) in testis with regular spermatogenesis. Note the MCFD2 negative spermatogonia. (**D**) MCFD2 was present in cells of intratubular tumour cells (inset, marked by an asterisk), in Sertoli cells (inset, arrowhead) and in seminoma cells (arrow). The MCFD2 protein was absent from leukocytes (arrowhead). (**E**) At 21st week of gestation, MCFD2 was present in gonocytes as marked by an arrow. Note the MCFD2 positive Sertoli cells (arrowhead). (**F**) MCFD2 was absent from testicular tissue of third trimester (37th week of gestation)

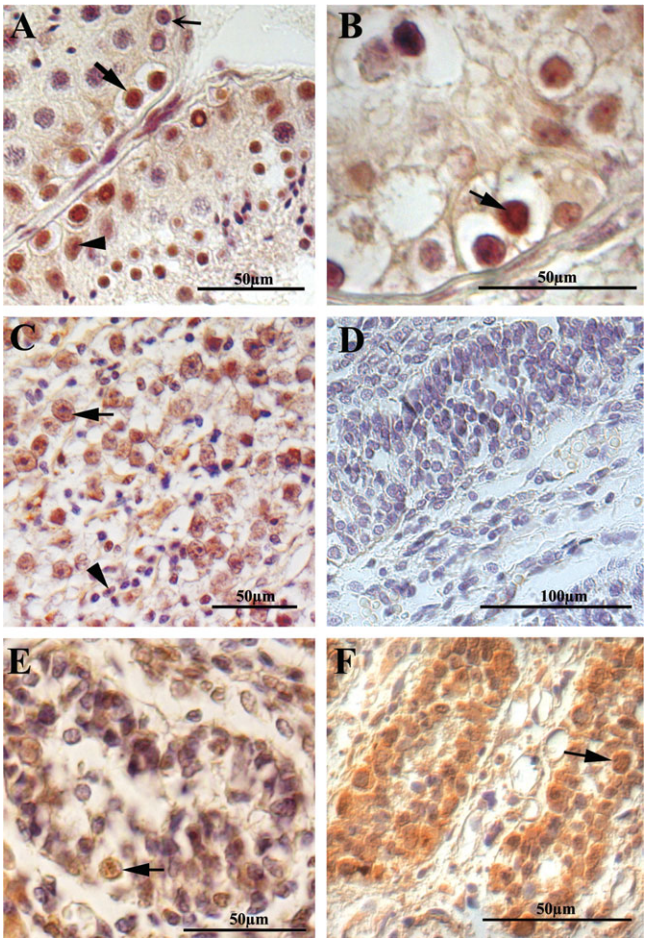

Figure 3: Localization of BOB1 protein in testicular tissues (**A**) Representative staining of cellular localization of BOB1 protein in a testis with regular spermatogenesis. The big arrow marks the localization of the protein to a spermatogonia and the arrowhead to a nucleus of a Sertoli cell. Note the unstained spermatogonia (small arrow). (**B**) Immunohistochemical localization of BOB1 to nuclei of preinvasive tumour cells (arrow). (**C**) Black arrow marks the positive staining of seminoma cells for BOB1. Leukocytes are negative for BOB1 (arrowhead). (**D**) No positive signal was visible in negative control experiments as shown for a section of a fetal testis from the 16th week of gestation (corresponding to E). (**E**) Gonocytes of a fetal testis from 16th week of gestation are positive for BOB1 as indicated by the arrow. Note the BOB1-negative gonocytes in the tubulus. (**F**) Representative staining of BOB1 in testicular tissue from third trimester (37th week of gestation). The arrow points to a BOB1-positive pre-spermatogonia

gestation were positive for BOB1 as illustrated in Fig. 3E and F, respectively. Similar to the situation in adult testis, BOB1 negative gonocytes and some positive Sertoli cells were present in the tubules of fetal testes.

Upregulation of PROM1 is an early event in the pathogenesis of seminoma

Microarray analyses showed a significant 4.3-fold upregulation of PROM1 transcripts in tumour tissues (Table 2). Semi-quantitative RT–PCR analyses confirmed the upregulation with a mean 64-fold factor of increase (Fig. 1G) indicating a very strong upregulation of PROM1 mRNA expression in seminoma. Normal testicular tissue showed no signal at 26 cycles and only a weak signal at 35 cycles, when the cDNA from tumour specimens reached already the saturation of amplification (Fig. 1G). PROM1 mRNA expression was maintained at high levels in all tumour stages investigated (Fig. 1G). Though PROM1 was strongly expressed in the apical region of the epididymal epithelium, which was chosen as a positive control (Fig. 4A), only sporadic specific immunostaining could be detected in spermatogonia of tissues with regular spermatogenesis (Fig. 4B). However, the protein was localized at the cell membranes of preinvasive tumour cells (Fig. 4C). Interestingly, the strong prominin expression in seminoma cells was not only restricted to the membranes but was also present in the cytoplasm of tumour cells (Fig. 4D). In addition, prominin was also detected in the stromal compartment including lymphocytes (Fig. 4D), a fact which could contribute to high mRNA levels of PROM1 seen in tumour tissues. Thus, the immunohistochemical analysis (Fig. 4) confirmed the transcript expression pattern for PROM1 (Fig. 1), regarding its weak expression in normal testis and a high upregulation in seminoma. Moreover, the upregulation of PROM1 seems to be a very early event in the pathogenesis of germ cell tumours because of its strong staining in the ITGCN (Fig. 4C).

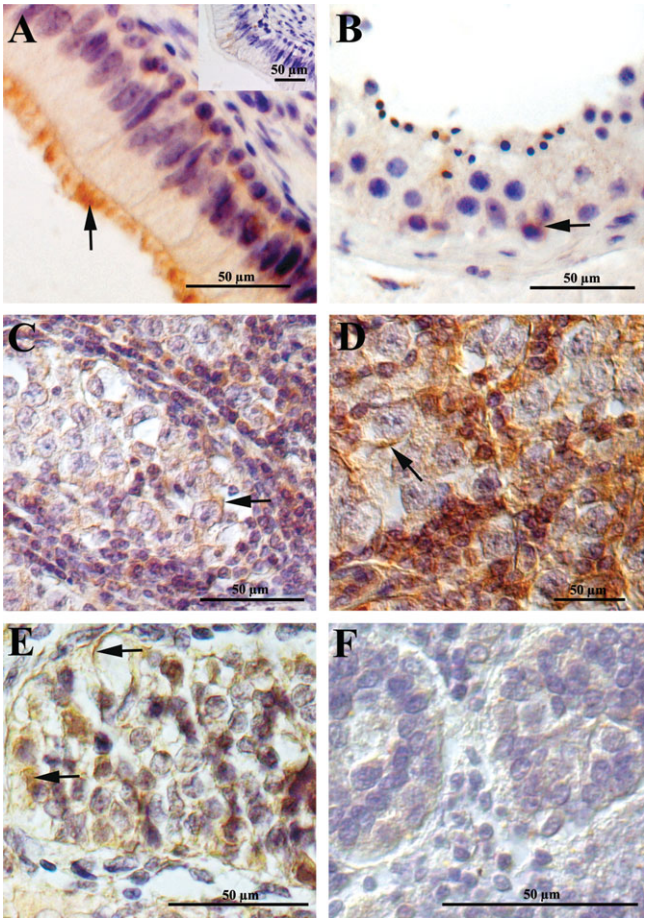

Figure 4: Localization of PROM1 protein in testicular tissues (**A**) Positive staining of PROM1 in the stereocilia (arrow) of epididymal duct in an adult. The inset presents a corresponding negative control. (**B**) Sporadic expression of PROM1 protein was visible in testis with regular spermatogenesis. The arrow points to a spermatogonia with a slightly stained cytoplasm. (**C**) PROM1 was present in membranous compartment of intratubular tumour cells as marked by the arrow. (**D**) Solid arrow marks the positive, membranous staining of seminoma cells for PROM1. Note the cytoplasmatic staining in several cells and the positive signal in stromal compartment including leukocytes. (**E**) Representative staining of PROM1 in testicular tissues from the 22nd week of gestation. The arrows mark the localization of PROM1 in gonocytes to a membrane and cytoplasmatic region. (**F**) No distinct PROM1 signal could be observed in third trimester of gestation (here, 30th week of gestation is presented)

PROM1 positive germ cells were found in the seminiferous tubules of second trimester fetal testes as illustrated in Fig. 4E. Similar to the expression pattern seen in seminoma cells, germ cells from second trimester showed membranous and cytoplasmatic staining for the PROM1 protein. The latest expression of PROM1 observed was in a testis from the 26th week of gestation. The protein was absent from testicular tissues in third trimester as shown in Fig. 4F.

Discussion

Numerous data exist for expression of different stem cell markers such as KIT, OCT4, NANOG and PIWIL1 in seminoma (Qiao *et al.*, 2002; Honecker *et al.*, 2004a,b; Ezeh *et al.*, 2005). Re-investigating our microarray data (Gashaw *et al.*, 2005), we confirmed the expression pattern of five known markers in the tumour. Remarkably, we could identify three novel germ cell factors, BOB1, MCFD2 and PROM1, elevated in seminoma compared to the adult testis. For the first time, we demonstrated that all germ cell markers investigated revealed no further regulation in low (pT1) versus advanced seminoma stages (pT3). These constantly high levels further confirm that seminoma maintain their germ cell characters during tumour progression.

In contrast to the literature, which describes that testicular seminomas express KIT (Honecker *et al.*, 2004a,b) but do not express its ligand SCGF (Strohmeyer *et al.*, 1995; Bokemeyer *et al.*, 1996), we showed a constitutive expression of SCGF in seminoma cells on mRNA and protein level. The other stem cell marker, MCFD2 was not known to be expressed in seminoma as well as in testicular tissues before. The MCFD2 gene (multiple coagulation factor deficiency 2 protein, SDNSF) encodes the neural stem cell derived protein and is discussed as a factor maintaining stem cell potential in adult central nervous system (Toda *et al.*, 2003). In contrast to previously described stem cell markers, such as KIT and hiwi which have been localized to germ cells and later on to spermatogonia in adult testis (Rajpert-De Meyts, 2006), we localized MCFD2 protein to testicular Sertoli cells.

Interestingly, MCFD2 was significantly downregulated in seminoma specimens in microarray analyses but not in RT–PCR and immunohistochemistry. We traced back this discrepancy to the probesets used for the hybridization on the array, which anneal to the 3′ untranslated region, whereas the PCR primers were placed into two exons and are more appropriate for interpretation of data. In addition, the detection of high protein levels in tumour tissues confirmed the RT–PCR data.

Among the germ cell markers upregulated in seminoma, we found PROM1 (CD133). Prominin 1 was originally found on neuroepithelial stem cells in mice (Shmelkov *et al.*, 2005) and is also expressed in embryonal carcinoma cells (Chadalavada *et al.*, 2007). Prominin has been reported to be responsible for initiation of new brain tumours (Singh *et al.*, 2004). Recently, PROM1 was identified via microarray analyses in seminoma (Looijenga *et al.*, 2006), but without further characterization. We present herewith for the first time the expression pattern of PROM1 mRNA and protein in human testicular tissues. The levels of PROM1 mRNA were very weak in normal testicular tissue and only sporadic spermatogonial immunostaining could be observed. The murine homologue of PROM1 has been detected previously on the tail of developing spermatozoa (Fargeas *et al.*, 2004), a finding we failed to confirm for human testicular tissues, although the same antibody showed a strong positive signal on epididymal tissues used as a control and confirmed the results found in the mouse (Fargeas *et al.*, 2004). Intratubular tumour cells as well as seminoma specimens revealed strong signals for PROM1. Like MCFD2, PROM1 was only detected in fetal testis of the second, and not third, trimester.

BOB1 (POU2AF1, OBF1) is more a germ cell factor than a stem cell marker. BOB1 represents a transcription factor predominantly expressed in human tissues of lymphoid origin and is required for germinal centre formation (Teitell, 2003). In our study, BOB1 protein present in germ cells of adult testis was highly upregulated in seminoma and was expressed in fetal testis up to the third trimester. Thus, BOB1 is a novel testicular germ cell marker probably involved in the pathogenesis of seminoma.

All stem cells markers previously described are expressed in gonocytes and many of them in germ cells of adult testis (Rajpert-De Meyts, 2006). Germ cells that are predominantly found before 25th week of development co-express OCT3/4, KIT and AP-2γ (Pauls *et al.*, 2006). These authors stated that after 25th week, most germ cells have lost their pluripotent potential and acquired a spermatogonial phenotype (Pauls *et al.*, 2006). Since MCFD2 and PROM1 are expressed in gonocytes of fetal testis only until the second trimester, the origin of seminoma cells could derive latest from a second trimester gonocyte or from a spermatogonia or spermatocyte (Chaganti and Houldsworth, 1998) re-expressing markers of an early gonocyte.

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